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New microbiological approaches to improve white wines winemaking

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Abstract

During the recent years winemaking had significant changes in relation to the need to move towards increasingly sustainable production, to meet the objectives of the 2030 Agenda for Sustainable Development. It is a program of actions for people, the planet and prosperity signed in September 2015 by the governments of the 193 member countries of the United Nations (UN). It includes 17 Objectives for Sustainable Development - Sustainable Development Goals, SDGs on a set of important issues for development: the fight against poverty, the eradication of hunger and the fight against climate change, among the others. In this contest, it is important to find solutions to reduce the *input* during winemaking, such as use of SO_2 and use of energy. For this reason it becomes important the manage of alcoholic fermentation using microbiological tools, such as non-*Saccharomyces yeasts*, enzymes and use of optimized microbial nutrition during the acclimatation of the yeasts to avoid stuck fermentations and improve fermentation kinetics.

In this thesis different aspects were considered:

Part A) Different strains of *Lachancea thermotolerans* were evaluated for biological acidification of Glera.

Part B) The bio-protection and natural acidification of *Lachancea thermotolerans* and *Metschnikovia pulcherrima* on Sauvignon Blanc

Part C) The impact on yield and quality of must using pectolytic enzyme's (enzyme Lallzyme™)

Part D) The effect of the type of yeast's acclimatation on the organoleptic characteristics of Sauvignon Blanc and Friulano grapes.

The results confirmed that the acidification power of *Lachancea thermotolerans* is strictly linked to the strain and the permanence in the must before *Saccharomyces cerevisiae* inoculum. Instead, the bio-protection effect was not effective, probably these yeasts required more time to highlight the bioprotection capabilities.

As far as the pectolytic enzyme is concerning, Lallzyme™ increased the yield of must by 6.2% considering the sum of free run must and the pressed one.

During acclimatation sterols, micronutrient and vitamin have an important role on the organoleptic impact of the final product, and this was confirmed by the different impact of GoFerm Protect Evolution™ and GoFerm Sterol Flash™.

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1. INTRODUCTION

Wine production is responsible for approximately 0.3% of annual global greenhouse gas emission from anthropic activities. This is the main industry in agriculture linked to sustainability problems, initially correlated with vineyard management, even so, it is important to consider the environmental impact of the entire process of winemaking from vineyard practises to shipment processes (Trioli et al., 2015).
Nowadays, is becoming even more important to discover different techniques to produce sustainable wines, because recent analyses of consumer preference showed that they are willing to pay more for wines that are produced using sustainable processes (Pomarici et al., 2019).
For these reasons, it is important to implement new tools to manage the wine production to obtain high quality wines according to costumers' expectation (Tedesco et al., 2022).
New microbiological tools that will be discussed in the following chapters are:
Bio-protection and bio-acidification using sequential inoculum with non-*Saccharomyces* yeasts, for example *Lachancea thermotolerans* is a valid option to increase the acidity and to reduce the pH of wine from warm viticultural areas (Petruzzi et al., 2017; Benito et al., 2018, Vilela et al., 2019; Ferreira et al., 2020), and *Metschnikovia pulcherrima*, used for its interesting antimicrobial properties (Vicente et al., 2020). The use of enzymes during clarification can reduce the time of this process step, reducing the input of energy consumed, while the best rehydration technique of yeast before inoculation can reduce the use of additives (such as SO_2) to control the indigenous microbiota, thanks to a fast start of a vigorous fermentation. Moreover, it can reduce the duration of fermentations, leading to a reduction of frigories spent during the process, reducing the impact on the production of CO_2 and the costs of production.

1.1 Non-*Saccharomyces* yeast

If not properly controlled, non-*Saccharomyces* yeast have to be considered as deleterious for the wine production, however, some specific species have peculiar characters that could be particularly appreciated for enhancing wines quality. Always considering the type and the final characteristics of the expected wine, non-*Saccharomyces* strains could be used to improve acidity, colour stability, clarification, polysaccharides content and aroma complexity (Castrillo et al., 2019; Lin et al., 2020).
These yeasts can be used in different stages of the winemaking process, starting from the pre-fermentative stages up to the wastewater treatments (Nardi et al., 2020).

Lachancea termotolerans

Previously known as *Kluyveromyces thermotolerans, L. thermotolerans* genome was sequenced in 2009 by Genelovures Consortium (Belloch et al., 1997) (Souciet et. al, 2009). Nowadays only three industries produces seven different commercial strains in the market (Roudil et al., 2020; Vejarano et al., 2021). In addition, there is a eighth product "Melody™", that is a multi-starter containing three different yeast species (Vicente et al., 2021), among which *L. thermotolerans* is present.

Commentato [AC2]: Tirare via tutti i . Prima della citazione

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Commentato [AC4]: Prima volta che nomini il microrganismo sempre per esteso, dopo puoi abbreviarlo

Commentato [AC5]: Qualcosa non torna nella frase

Commentato [AC6]: Uniforma tutti i distanziamenti tra paragrafi

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Di seguito vengono elencate alcune delle principali specie di non saccha di interesse enologico.

E iniziare un elenco puntato delle specie togliendole dai titoli che figurano nel sommario

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Per fortuna cercando la frase su google è uscito subito l'articolo da cui è stato fatto un copia e incolla diretto https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8625220/

Lachancea thermotolerans was found in very different habitats worldwide (Hranilovic,et al, 2017) and is often a constituent of grape/wine microbiota (Mora et al., 1990; Jolly et al., 2013), for this reason oenological environments are the best source for isolation (Porter et al., 2019).

This non-*Saccharomyces* yeast is often used in mixed or sequential fermentations (Shekhawat et al., 2019). Studying the proteomic profile of *S. cerevisiae* in mixed fermentation stands out that *Saccharomyces* showed different signals of stress in presence of *L. thermotolerans* (Peng et al., 2019).

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The fermentative power of *L. thermotolerans* is valuated from 4.24% v/v to 10.6% v/v (Benito et al., 2018; Hranilovic et al., 2018; Binati et al., 2019), although recent studies observed that *L. thermotolerans* can ferment wines up to 13.6% v/v (Hranilovic et al., 2018), and for this reason it is possible that in the future specific strains could complete wines' fermentation without using mixed fermentation (Vicente et al., 2021).

L. thermotolerans are selected considering a wide range of parameters (Figure 1), the scientific interest on *L. thermotolerans,* is focused on increasing the acidity of wines and reducing their content of ethanol Benito et al., 2019; Blanco et al., 2020).

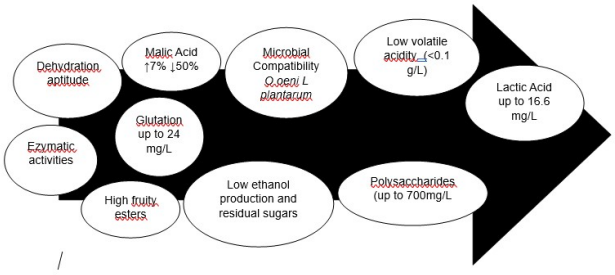

Figure 1: Summaize of parameters selections *of L. thermotolerans*

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L. thermotolerans decrease ethanol percentage in a range from 0.1% and 1.6% v/v (Sgouros et al., 2020; Korenika et al., 2021); or 3% v/v in combined fermentation with *Saccharomyces pombe* (Romani et al., 2020). Talking about commercial strains for example Laktia™ commercialized by Lallemand, it reduces ethanol percentage by about 0.9% v/v (Hranilovic et al., 2021).

Commentato [AC12]: Bibliografia

Lactic acid production is extremely variable between the different strains (Benito et al., 2018; Hranilovic et al., 2018; Snyder et al., 2021), the highest amount that had been observed is around 16.6 g/L (Banilas et al., 2016). The production is linked to a group of three genes coding for various lactate dehydrogenases, *ldh1*, *ldh2* and *ldh3*. *Ldh1* is located in chromosome D and the other two genes in chromosome G. Measuring the expression of these three genes during fermentation process by RT-qPCR it has been showed that it is *ldh2* that is responsible for the variability of production among the strains (Sgouros et al., 2020). The expression of this genes is correlated to a lack in the expression of alcohol dehydrogenase (Shekhawat et al., 2020).

Metschnikovia pulcherrima

Metschnikovia pulcherrima has cells that are spherical to ellipsoid or can be pyriform, cylindroid, or lunate. In the determination of the asexual reproduction, it is seen that budding is multilateral (Kurtzman et al. 2011).

This non-*Saccharomyces* yeast has a low fermentative power, for this reason is often used in sequential fermentation with *S. cerevisiae* (Combina et al., 2005; Escott et al., 2018; Zhang 2018; Grazia et al., 2019; Morata et al., 2019a).During winemaking is often employed in bioprotection as an antifungal, for example to inibith *Botrytis cinerea* (Sipiczki et al., 2006). Moreover, *M. pulcherrima* can reduce ochratoxin A up to 80% at 30 ◦C in 15 days (Spadaro et al., 2017; Benito et al. 2019).

M. pulcherrima show a high strain variability, in fact selection processes focused on different oenological parameters (Vicente et al.,2020), these can be summarized as follow (Figure 2):

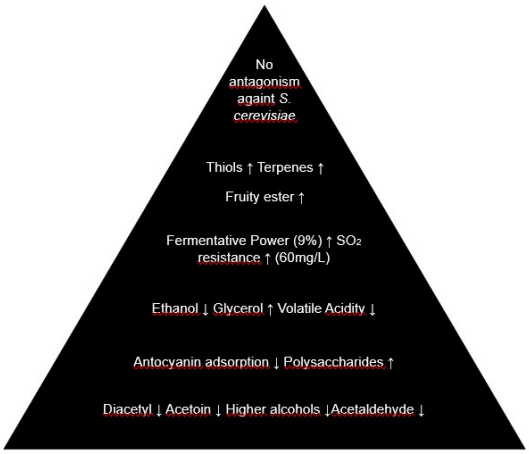

Figure 2: Summarize of selection parameters for *Metschnikovia pulcherrima*

1.2 Enzymes

Enzymes are composed by long chain of amino acid bounded by peptide bounds, they are biological catalyst (Espejo et al., 2020).

They are commonly used in different food industries (Sarrouh et al., 2012) for their minimal side effects and low costs and are obtained from selected and optimized microorganism (Espejo et al., 2020).

Their use during winemaking is associated to different goals in different stages of the process (Figure 3)

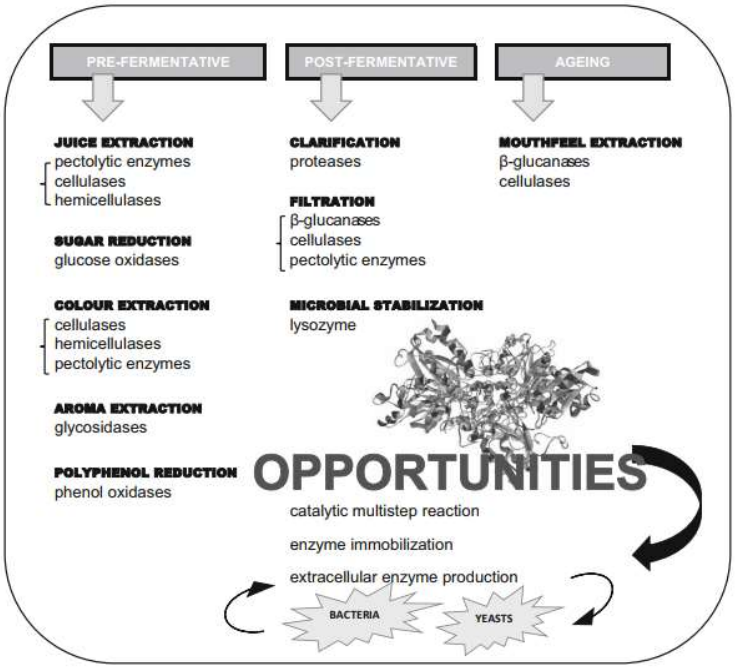

Figure 3: Use of enzymes during winemaking stages (Source Espejo et al., 2020)

During winemaking some different problem occur, such as loss of aroma and colour during filtration before bottling, long clarification processes, turbidity, low juice yield (Viegas et al., 1989; Kim et al., 2017; Guo et al.,2018; Jiang et al., 2020) , and haze formation during storage (De Souza Bezerra et al., 2015; Narnoliya et al., 2019).

The clarification process often takes place by using different chemicals products such as bentonite and poly (vinylpolypyrrolidone), the disadvantage of using bentonite is a decrease of many important aromatic compounds and in addition can cause oxidation of some phenolic compounds (Verma et al, 2018). Enzymes can increase yield and maximize quality of wines (Toy et al., 2020)

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Pectinases

Pectinases enzyme is one of the most used enzymes in winemaking process to obtain higher yields and to improve aroma profile (Toy et al., 2020), and constitute 25% of the global enzyme market (Oumer et al., 2017).

The commercial application of pectinases was observed for the first time in 1930s

(Ribeiro et al., 2010; Suneetha et al., 2011)

The substrate of these enzymes are pectins, that are complex polysaccharides composed by a linear homogalacturonan chain, bonded with α-1,4-linkages, and rhamnogalacturonan side chains.

Pectinase operate primarily by degradation of pectic polymers, by converting them into galacturonic acid (Figure 4).

These enzymes are produced by fungal sources such as *Aspergillus niger, Aspergillus awamori, Pennicilium restrictum, Trichoderma viride, Mucor piriformis* and *Yarrowia lipolytica* (Toy et al., 2020; Haile et al., 2022).

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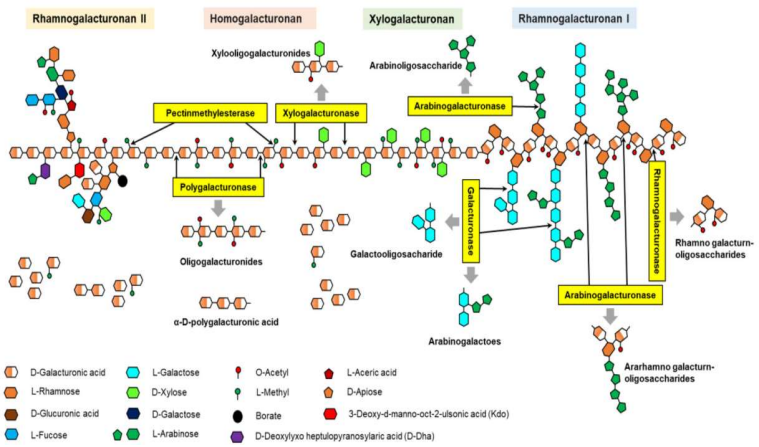

Figure 4: Reaction mechanisms of pectinase on pectin (Source: Toy et al., 2020)

1.3 Rehydration techniques

Must is an inhospitable medium, very different from the optimal growth condition of yeasts. For this reason, despite of the achievements during last years, winemaking must be optimized (Ferreira et al., 2017).

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The lag phase is defined as the period when the microbial population adapts to the new medium (Madawar et al., 2003; Swinenn et al., 2004).

Active dry yeasts

Active dry yeasts (ADY) were introduced as an oenological practice in 1960s (Bartowsky et al., 2009), but previously in 1890s Müller-Thurgau introduced the concept of inoculation (Pretorius, 2000). ADY, as the *piè de cuvè*, are primarily used to reduce to the minimum the lag phase, then to avoid fermentative blocks and to standardize the production during vintages (Vaudano et al., 2014). Nowadays, some

winemakers are trying to reduce the use of starter cultures to increase the complexity of wine by using native yeast, anyway this can cause uncontrolled fermentation (Malfeito-Ferreira, 2011). During winemaking yeast is subjected to different stresses such as osmotic pressure, ethanol concentration, availability of nutrients, vitamins and lipids, acidic pH and accumulation of toxic components (Bely et al., 1990; Casalta et al., 2012).

Sterols

Yeasts are able to assimilate sterols from must (Casalta et al., 2012; Jacquier et al., 2012; Ruggiero et al., 2013) ,the addition of these to the must or during the adaptation process is often implemented (Casalta et al, 2018).

The function of sterols is to regulate membrane permeability and fluidity and to other essential processes (Rosenfeld et al, 2003; Caspeta et al., 2014; Aguilar et al., 2010).The addition of exogenous phytosterols to the must has a positive impact on maximum cell population and viability and can also impact the aroma of wines influencing some compound such as esters (Basson et al., 1986; Miziorko et al., 2011; Rollero et al., 2015; Rollero et al., 2016)higher alcohols (Fairbairn et al., 2019; Guittin et al, 2021)and thiols (Saharan et al., 2010; Roland et al., 2011)

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Importance of micronutrients and vitamins

Sugar metabolism is highly influenced by the ratio of mineral components in the must (Jones et al., 1984; Walker et al., 1994). The inclusion of micronutrients during yeast rehydration impact on the quality of the final products.

As previously observed micronutrient supplies, such as magnesium, zinc and manganese, as well as vitamins such as thiamine, biotin and pantothenate during yeast acclimatation impact on yeast viability, stimulate cell growth and limit acetic acid production (Ournac, 1970; Lodolo et al., 1995; Julien et Delau, 2002).

Magnesium influences a very high number of yeast metabolic pathways, such as in growth and division (Walker, 1986), and is a principal cofactor of above 300 enzymes (Birch et al., 2014). It can improve the ethanol production of the yeast and play an important role in tolerance to stresses (Dombek et Ingram, 1986; Walker, 1998; Birch et Walker, 2000), stimulates fatty acids synthesis and activates the membrane ATPasi, responsible for active transport system (Jones et Greenfield, 1984). High Mg^{2+} concentration in the must is related to a higher alcohol tolerance (Dasari et al., 1990), in contrast lower ratio of Mg^{2+} in the must show an increase of volatile acidity production (Julien et Dulau, 2002).

As reported by Birch et al. (2014) the acclimatation of yeast with higher ratio of magnesium have a beneficial effect on the fermentation process. In addition, pre-conditioning with magnesium generally show more balanced wine in terms of sensory analyses.

Manganese in adequate concentrations allows the increase of the synthesis of proteins and vitamin B1 and, as a consequence, also the increase of biomass. In addition, it has been showed a significantly higher production of alcohol dehydrogenase (Stehlik-Tomas et al., 2004)

Zinc is a cofactor of many enzymes. Low amount of this ion affects negatively cell growth and fermenting activity (Bromberg et al, 1997).

Pantothenic acid, called vitamin B5, affect *S. cerevisae* metabolism in anaerobic and aerobic conditions, and is involved in fatty acids and ammino acids synthesis (Julien et Dulau, 2002).

Thiamine, called vitamin N1, is an important cofactor of pyruvate decarboxylase (Diefenbach et Duggleby, 1991), plays an important role in isoleucine and valine synthesis. Moreover, limits the production of pyruvate, acetaldehyde and acetic acid at the end of fermentation (Oumarc, 1970).

2. SCOPE OF THE WORK

The scope of this thesis was to investigate different innovations in the microbiology field of white winemaking. Trials were made on harvest 2021 and 2022, using different grapes from cultivars Glera, Ribolla gialla, Sauvignon Blanc and Friulano

In particular, the aim of this study was to evaluate:

1. Biological acidification of Glera using different strains of *Lachancea thermotolerans*
2. Natural acidification and bio-protection and with *Lachancea thermotolerans* and *Metschnikovia pulcherrima* on Sauvignon Blanc
3. Pectolytic enzyme's (enzyme Lallzyme™) impact on yield and quality of must
4. The effect of the type of yeast's acclimatation on the organoleptic characteristics of Sauvignon Blanc and Friulano grapes.

Commentato [AC17]: Ancora sbagliato il nome

3. MATERIALS AND METHODS

3.1 Vinification protocol

Part 1) Biological acidification of Glera using different strains of Lachancea thermotolerans

Glera must was sourced from "Viticoltori Ponte Winery", located in Ponte di Piave (TV).
The grapes were crushed, destemmed and sulphited without exceeding 40 mg/L of total SO_2 and 15 mg/L of free SO_2. The must, added with Lallzyme HC 2 g/hL in order to facilitate the clarification process, underwent a cold static clarification process at 6 °C for 48 h.
The mass was then divided into 8 theses that were treated and inoculated using the different yeast strains as reported in Table 1.

Table 1: Outline of the different thesis considered and strains used in Harvest 2021 for Glera grapes

Commentato [AC18]: Uniformare tutte le tabelle, mettendo il testo centrato e non giustificato

	Thesis	During filling (day 0)	Inoculation of the 1st yeast (day 0)	After 48 h inoculation of the 2nd yeast (day 2)	After 72 h inoculation of the 2nd yeast (day 3)
1	LA48		***L. thermotolerans* A 25 g/hL**	*S. cerevisiae* **Lalvin Sensy** 25 g/hL	
2	LA72		***L. thermotolerans* A 25 g/hL**		*S. cerevisiae* **Lalvin Sensy** 25 g/hL
3	LB		***L. thermotolerans* B 55 g/hL**	*S. cerevisiae* **Lalvin Sensy** 25 g/hL	
4	LC		***L. thermotolerans* C 20 g/hL**	*S. cerevisiae* **Lalvin Sensy** 25 g/hL	
5	LD		***L. thermotolerans* D 30 g/hL**	*S. cerevisiae* **Lalvin Sensy** 25 g/hL	
6	CTRL		*S. cerevisiae* **Lalvin Sensy** 25 g/hL		
7	Tar	Tartaric acid 150 g/hL	*S. cerevisiae* **Lalvin Sensy** 25 g/hL		
8	Bac	Bactiless 30 g/hL after clarification during tank filling	*S. cerevisiae* **Lalvin Sensy** 25 g/hL		

During the filling of tanks, theses 7 – Tar and 8 - Bac were added with tartaric acid 150 g/hL and Bactiless™ 30 g/hL, respectively. Bactiless™ is a product composed by Chitosan and Chitin-glucan of fungal origin Aspergillus Niger.
Fermentation temperature was 18 °C.
At the end of the alcoholic fermentation, all tanks were racked to remove the lees in suspension. Molecular SO_2 and free SO_2 have been maintained from the end of fermentation at around 1 mg/L and 35 mg/L, respectively.

Part 2) ***Bioprotection and natural acidification with Lachancea thermotolerans and Metschnikovia pulcherrima on Sauvignon Blanc***

Sauvignon blanc must was sourced from "Livio Felluga Winery", located in Cormons (GO). Grapes were crushed, destemmed, pressed and sulphited with 20 mg/L of SO_2. The must equally divided in 5 tanks, was added with Lallzyme C-Max 2 g/hL in order to facilitate the clarification process, and underwent to a cold static clarification process at 10 °C for 24 h.

The five theses were treated and inoculated using the different yeast strains as reported in Table 2.

Table 2:Outline of the different thesis considered and strains used in Harvest 2021 for Sauvignon Blanc grapes

	Thesis	During filling (day 0)	End of clarification	After 48h from clarification
1	Bac	**Bactiless** 40 g/hL	*S. cerevisiae* **Lalvin Sensy** 25 g/hL	
2	LA	***L. thermotolerans* A 25 g/hL**		*S. cerevisiae* **Lalvin Sensy** 25 g/hL
3	LC	***L. thermotolerans* C 20 g/hL**		*S. cerevisiae* **Lalvin Sensy** 25 g/hL
4	CTRL			*S. cerevisiae* **Lalvin Sensy** 25 g/hL
5	MX	***M. pulcherrima* X 10 g/hL**		*S. cerevisiae* **Lalvin Sensy** 25 g/hL

During filling of tanks, thesis 1 - BAC was added with Bactiless™ 30 g/hL.

Fermentation temperature was 18 °C.

At the end of the alcoholic fermentation, all tanks were racked to remove the lees in suspension. Molecular SO_2 and free SO_2 have been maintained from the end of fermentation at around 1 mg/L and 35 mg/L, respectively.

Part 3) Pectolytic enzyme's (enzyme Lallzyme™) impact on yield and quality of must

Ribolla grapes were sourced from "Livio Felluga Winery" (UD), and harvested at commercial ripeness, average value of 19.3 °Brix. This cultivar was chosen for its intrinsic characteristics of extraction difficulty. The lot was destemmed and crushed, and divided in 4 homogeneous fractions of 400 kg each. Two fractions were subjected to enzymatic treatment using Lallzyme™ at a dosage of 4.0 mL/hL. The enzyme, stored at 4 °C until the usage, was diluted 1:10 with water and then dispersed on the grapes being moved directly inside the pneumatic press (PA8, Siprem International). The remaining 2 fractions were used as controls, and underwent the same operative protocol but without being subjected to enzymatic treatment.

The pressing cycle lasted 2 h. The evaluations were performed on both the free running juice and the pressed must in duplicate.

Part 4) Organoleptic impact of yeast rehydration on Sauvignon Blanc and Friulano grapes

Friulano must was sourced from "La Tunella Winery", located in Premariacco (UD) and Sauvignon blanc was provided by "Cantine di Casarsa Winery". The grapes were crushed, destemmed and sulphited without exceeding 50 mg/L of total SO_2 and 15 mg/L of free SO_2. The must, added with Lallzyme HC 2 g/hL in order to facilitate the clarification process, underwent a cold static clarification process at 6 °C for 48 h. The must was added with saccharose to reach the right amount of potential alcohol. At the beginning of AF 20 g/hL of lisozyme were also added.
Fermentation temperature was 18 °C.
At the end of the AF three transfers were performed. Then, friulano theses were added with yeast cell walls and, after two weeks, they were filtered and bottled.

Table 3: Outline of the different theses considered and rehydration techniques used for Friulano grapes, Harvest 2022

Organoleptic impact of different yeasts' rehydration techniques on Friulano grapes						
Thesis			Rehydration	Inocolum	AF Beginning	1/3 AF
1	GFSF	50 mg/L SO2 on destammed grapes	GFSF 30 g/hL	25 g/hL *S.cerevisiae* Y	30 g/hL Fermaid O	20 g/hL Fermaid E Blanc
2	GFPE$_{(Y)}$		GFPE 30 g/hL	25 g/hL *S.cerevisiae* Y		
3	DIRECT			25 g/hL *S.cerevisiae* Y		
4	GFPE$_{(SPF52)}$		GFPE 30 g/hL	25 g/hL *S.cerevisiae* SPF52		

Table 4: Outline of the different theses considered and rehydration techniques used for Sauvignon Blanc grapes, Harvest 2022

Organoleptic impact of rehydration on Sauvignon Blanc						
	Thesis		Rehyration	Inocolum	AF Beggining	1/3 AF
1	DIRECT (H3)	50 mg/L SO2 on destemmed grapes	Direct	25 g/hL *S.cerevisiae* H3	40 g/hL SSB	20 g/hL Fermaid E Blanc
2	DIRECT (S1)		Direct	25 g/hL *S.cerevisiae* S1		
3	GFPE (Q1)		30 g/hL GFPE	25 g/hL *S.cerevisiae* Q1		
4	GFSF (Q1)		30 g/hL GFSF	25 g/hL *S.cerevisiae* Q1		
5	GFPE (SPF52)		30 g/hL GFPE	25 g/hL *S.cerevisiae* SPF52		

At 2/3 of AF thesis 5 was added by Nutrient Vit End 30 g/hL.
Fermentation temperature was 18 °C.

3.2 Protocol of yeast inoculation

- Harvest 2021

The Yeast *S. cerevisiae* Lalvin Sensy was inoculated as reported below:
GoFerm Protect evolution (30 g/hL calculated on the final volume) was dissolved in a quantity of water equal to 10 times the weight of the yeast (25 g/hL) at T between 38-40 °C. After temperature decreased to 35-38 °C, the yeast (25 g/hL) was added to the GoFerm Protect evolution suspension. The mixture was left to rehydrate for 20 minutes and then mixed gently. To avoid thermal shock: the yeast was acclimated to the temperature by adding progressively equivalent volumes of must. The difference in temperature between yeast and must at the moment of the inoculum was <10 °C. The yeast was inoculated homogeneously in the tank.
Lachancea thermotolerans yeast (theses 2,3 Sauvignon 2021 and thesis 1-6 Glera 2021) and M. pulcherrima X ware rehydrated in 10 parts of water at T of 25-30 °C. The suspension was left to stand for 15 minutes for *L. thermotolerans* and 20 minutes for *M. pulcherrima*, then mixed gently. The difference of temperature between the rehydration medium and the must didn't exceed 10 °C. If necessary, the yeast was acclimatized to the temperature by adding an equivalent volume of must. In any case, the total duration of rehydration didn't exceed 45 minutes.

- Harvest 2022

S. cerevisiae Y (thesis 3) was added during the filling of the tank *or* rehydrated *(thesis 2,4)* with 30 g/hL of GFPE (Go Ferm Protect Evolution™, an additive rich in vitamins and microelements in particular of Magnesium) and were dissolved in 10 parts of water amounting to the yeast dose at 38°C. The adequate dose of yeast (see Table 1) was rehydrated in 10 parts of water at 37 °C. The suspension was left to stand for 20 minutes, then mixed gently. The difference of temperature between the rehydration medium and the must did not exceed 10 °C. In any case, the total duration of rehydration didn't exceed 30 minutes.
S. cerevisiae S1 was rehydrated with 30g/hL of GFSF (Go Ferm Sterol Flash™, an additive with a high concentration and bioavailability of ergosterol) and were dissolved in 10 parts of water amounting to the yeast dose at room temperature (>15°C). The adequate dose of yeast (see Table 1) was rehydrated in 10 parts of water at 21 °C. The suspension was gently mixed and was left to stand for 15 minutes. The difference of temperature between the rehydration medium and the must did not exceed 10 °C. Then, it was added to the tank and homogenized.

Table 5: Initial must parameters

Initial must parameters					
Parameters	**Glera 2021**	**Sauvignon 2021**	**Sauvignon 2022**	**Friulano 2022**	**Measure unit**
pH	3.58	3.39	3.17	3.7	pH units
YAN	122	122	160	140	mg/L
Total sugars	158.8	205.3	246.3	240.6	g/L
Potential alcohol	9.6%	12.4%	14.8%	14.4%	%v/V
Volatile acidity	0.1	0.13	0.11	0.11	g/L
Total acidity	5.8	7.4	7.05	3.1	g/L
Free SO2	6	8	6	5	mg/L
Total SO_2	29	34	25	42	mg/L
Malic acid	2.98	3.39	1,14	0,73	g/L
Lactic acid	0.05	0.08	<0,05	<0,05	g/L

3.3 Analyses

Microbiological analyses

Saccharomyces spp. and non-*Saccharomyces* were counted on WLN + chloramphenicol (total yeast count), WLD agar medium (Oxoid, Italy) and agar Lysine + chloramphenicol (no-*Saccharomyces* count) by spread plating 0.1 mL of each decimal dilution obtained from the wine samples. *Metschnikowia* spp. and *Hanseniaspora* spp. appear as pink-red and small dark green colonies, respectively. Sabouraud + chloramphenicol + methylene blue incubated at 30 °C was used to differentiate *L. thermotolerans* (blue colonies) and *S. cerevisiae* (white colonies). One (1) mL of each sample was enumerated on a double layer of DeMan, Rogosa, and Sharpe (MRS) agar (Oxoid, Italy) with the addition of DELVOCID®INSTANTANT (DSM Food Specialities, Netherland) 0.01 mg/mL for the counts of LAB. Plates were incubated at 30 ºC in an anaerobic atmosphere (Anaerogen, Oxoid, Italy) for 2-5 days. The counts were performed on plates containing from 30 to 300 colonies. Acetic acid bacteria were counted on GYC medium (10% glucose, 1.0 % yeast extract, 2.0 % calcium carbonate, 1.5 % agar, pH 6.8) by spread-plating 0.1 mL of each decimal dilution obtained from the samples.

Chemical analyses

Chemical analyses were performed as follow:

- Ethanol concentration

Ethanol content trend was monitored using an Anton Paar Alcolyzer Plus (Anton Paar GmbH, Austria).

- pH

For all the collected samples, pH was measured using the Basic20 pH instrument (Crison Instruments S.A., Spain).

- Acetic acid

The Acetic Acid was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Italy) using a commercial kit (K-ACET Megazyme, Ireland) and following the manufacturer's instructions.

- L-malic acid concentration

The concentration of L-malic acid was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Rodano, Italy) using the enzymatic kit L-malic acid (K-LMAL Megazyme, Ireland) and following the manufacturer's instructions.

- L-lactic acid concentration

The concentration of L-lactic acid was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Rodano, Italy) using the enzymatic kit L-lactic acid (K-LATE Megazyme, Ireland) and following the manufacturer's instructions.

- Total acidity

Total acidity was assessed by the official method OIV-MA-AS313-01.

- Total and free SO_2

The concentration of free and total SO_2 was assessed with the methods OIV-MA-A323-04A1 and OIV-MA-A323-04A2.

- Total Sugars

The sugar content was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Italy) using the enzymatic kit D-glucose/D-fructose (K-FRUGL Megazyme, Ireland) and following the manufacturer's instructions.

- YAN

The content of yeast assimilable nitrogenous compounds (YAN) was assessed with the derivatization of amino acids with o-phtaldialdehyde and their quantification by spectrophotometry (Dukes and Butzke, 1998. Am J Enol Vitic. 49: 125-134).

- Determination of yield of extraction

Four homogeneous fractions of destemmed grapes (400 kg each) were subjected to pressing process. Both the free running must and the pressed must were harvested and weighted. The yield of extraction of must was calculated from the following equation: Yield % = (W1 × 100)/W2 where W1 was the weight of the extracted must and W2 was the weight of destemmed grape mass.

- Catechins index

Catechins index was spectrophotometrically determined. 0,5 mL of wine sample were added with 2,5 mL of a solution composed of 0,1 g of p-dimethylaminocinnamaldehyde, 75 mL of ethanol, and 25 mL of 37 % v/v of HCl. After 5 min the absorbance at 640 nm was determined.

- Total polyphenols index (ipt)

Total polyphenols were spectrophotometrically determined. Samples were diluted 1:10 with Milli-Q water, and absorbances at 280 nm were determined. The obtained values were multiplied by a 10 factor.

- Pom test (oxidizability)

The oxidizability test (POM-Test) was conducted with 5 mL of wine added with 25 µL of 3% v/v of H2O2, stored at 65°C for 1h. The POM-Test was determined by the equation: $POM{-}Test$= Abs 420 nm $(H2O2)$−Abs 420 $nmAbs$ 420 nmx100

Abs 420 nm = Wine absorbance at 420 nm

Abs 420 nm $(H2O2)$ = Wine added with H2O2 absorbance at 420 nm

The percentage of colour oxidation (OX%) produced was estimated. The percentage increase in absorbance at 420 nm was considered in the case of white wine.

- Pectin index (polysaccharides and acidic polysaccharides)

Polysaccharides were determined by SE-HPLC after ethanol precipitation. Five (5) mL of wine were added to 5 volumes of ethanol (96% v/v), then stored at 0-4°C for 24 hours. The precipitated pellet was washed twice with ethanol, redissolved in 5 mL of MilliQ water and filtered on 0,22 µm cellulose acetate filter membranes before injection. SE-HPLC separation was performed with a binary pump (Model LC 250 Perkin-Elmer, Waltham, MA, USA), a manual injection valve (type 7125 NS Rheodyne, Rohnert Park, CA, USA), and a refractive index detector (RID-10A (Shimadzu, Kyoto, Japan). The column was an Ultrahydrogel 250 (6 µm, 300 x 7,8 mm, Waters); the mobile phase consisted of MilliQ water, and the separation was performed in isocratic mode, with a flow rate of 0,7 mL min-1 and an injection volume of 20 µL. Total polysaccharides were quantified by a calibration curve prepared with mannan (10-1000 mg L-1) and the results were expressed in mg/L.

- Dissolved O_2

  Dissolved O2 was measured using OxyGuard Handy Polaris 2.

- K^+ concentration

  K^+ concentration was spectrophotometrically measured using enzymatic kit (Steroglass, Italy) as described by the technical manual.

<u>Aromatic compounds analysis</u>

The aromatic profile was evaluated with SPME-GC-MS analysis.

Samples of 10 mL of wine were introduced in 20 mL glass Vial, after were added:

- 3 g NaCl
- Internal standards: Ethyl heptanoate (50 µL ethanol stock solution at 0,400 mg/mL) and 1-heptanol (100 µL ethanol stock solution at 0,310 mg/mL).

The Vials were sealed with silicon/PTFE septa and stored at 0/+4 ºC until analysis.

Solid phase micro extraction (SPME) was carried out under the following conditions: the fibre used was 2 cm 50/30 µm of divinylbenzene/carboxy/polydimethylsiloxane (Supelco, Bellefonte, PA, USA); sample pre-treatment (equilibration) was performed at 40 ºC for 15 minutes, under shaking (repeated cycles of 5 seconds at 500 revolutions/min, followed by a stop for 2 seconds).

Commentato [AC19]: O UK o US....lo abbiamo viso oggi...

The microextraction conditions were 15 minutes at 40 inches C. The injection was performed in split mode (split ratio 1:10); fibre desorption time in the GC injector: 5 min. The fibre was subjected to a cleaning procedure between successive injections, keeping it at 250 ºC for 5 minutes.

The GC analysis was carried out with the following conditions: the analysis was carried out by column DB-WAX (Agilent Technologies) of 60 m x 0.25 mm with 0.25 µm film thickness. Helium with a linear flow rate of 35 cm/s was used as carrier gas, maintaining the oven temperature at 40 ºC for 5 minutes, followed by a ramp of 40 ºC/min up to 240 ºC, maintained for 10 minutes. The temperatures of the injector and the line of the injector and the transfer line have been set to 250 ºC.

High resolution mass spectra were recorded at 70 eV; qualitative analysis was performed by comparing mass spectra with those reported in the MS NIST 14 library, comparing retention indices (calculated on the basis of the retention times of a mixture of n-alkanes) with those reported in the literature, as well as the retention time and mass spectra of commercial standards (if available).

The semiquantitative analysis was carried out on the basis of the relationship between the peak area of the individual compounds and those of the internal standards considering a response factor of 1.

To conduct the sensorial analysis, a panel was set up with women and men between 23 and 60 years with previous experience in sensorial analysis of wine. During the session the subjects were trained in the association of perceived intensity at a scale of values and, therefore, the ability to describe the perceived factors dividing them into visual, aromatic, gustatory and retro-olfactory perceptions. They were also trained to provide assessments using unstructured scales of increasing intensity from left (minimum intensity) to right (maximum intensity) until consensus was reached among panel members. Prior to the evaluation of wine samples, the panel was calibrated using a random sample from the various tests (using the median of the values provided by each judge). The purpose of the calibration was to evaluate, subsequently, the different samples of the thesis under examination in the most homogeneous way possible. All analyses were conducted in a dedicated room, using white light to a temperature of 20°.

The single sample of wine (30 mL) was presented at the same time to the various judges, who were asked to describe it in the most complete and accurate way possible. The result is an evaluation sheet (Annex 1) dividing the attributes into visual, olfactory, gustatory and retro-olfactory perceptions. The evaluation forms, thus drafted, were subsequently used in order to perform a quantitative sensorial analysis of the various characterizing factors of wine.

Each sample was administered to the judges using clear glass tulip glasses and presented in random order. The judge had the task of representing the intensity of the sensory factor by drawing a line of 118 mm without scales.

The panel was compound as follow:

- Glera and sauvignon 2021: 5 women and 7 men
- Friulano and Sauvignon 2022: 3 women and 7 men

Statistical analysis

The statistical analysis was performed with processing the scores obtained by measuring the distances of each point from the origin.
Individual judge scores have been standardised (Z-score) in order to eliminate the subjective use of valuation scales and related to those assigned by the entire group in order to identify the presence of judgment that deviated significantly, an index of possible errors of the individual judge (Senstools for Windows, version 2.3). The normalized data were analysed through analysis of factor variance (factors: type of starter and repetition of treatment) and Fisher's LSD (Least Significant Difference) test.
The variances were considered homogeneous according to the Levene and Bartlett tests. Analysis of the principal component analyses (PCA) was also carried out using the software Statistic for Windows, version 8).

Commentato [AC20]: Visti oggi

Timetable of Analysis

Chemical and microbiological analyses were performed as follow in Table 6 and 7:

Table 6: Timetable microbiological analyses

Time table Microbiological analysis- Harvest 2021		
Glera	Before adding L. thermotolerans on theses 1,2,3,4,5	
		Before adding S. cerevisiae to all the theses
		End of alcoholic fermentation: only lactic acid bacteria control
Sauvignon Blanc	Before adding L. thermotolerans and M. pulcherrima and after clarification on theses 2,3,4,5	
		Before adding S. cerevisiae to all the theses
		End of AF

Table 6: Timetable chemical analyses

Commentato [AC21]: Non si legge, mettete il testo verticale in alcuni punti per recuperare spazio e aumentate il carattere

Time table Chemical analysis

			Ethanol concentration	Total Sugars	pH	Acetic Acid	Total Acidity	Lactic and Malic Acid	Total and free SO_2	YAN	Catechin Index	Pectin Index	POM Test	Yield	Dissolved O_2	K*
Harvest 2021	Glera	Initial mass														
		before inoculation of S. cerevisiae on thesis 1, 2, 3, 4, 5														
		End of AF														
		Every day														
	Sauvignon Blanc	Initial mass														
		Before *S.cerevisiae* inocula (thesis 1,2,3,4)														
		After *L.thermotolerans* inocula (thesis 2,3)														
		End of AF														
		Every 2 days														
	Ribolla Gialla	Free must														
		Pressed must														
Harvest 2022	Sauvignon Blanc	Initial mass														
		End of AF														
		Every few days														
	Friulano	Initial mass														
		End of AF														
		Every few days														

4. RESULTS AND DISCUSSION

Part 1) Biological acidification of Glera using different strains of *L. thermotolerans*

Microbiological analyses

The must showed a consistent indigenous microflora, which was around 5 Log CFU/mL. Indigenous *S. cerevisiae* were 3.12 Log CFU/mL in the must, but increased quickly at day 2, ranging from 6.55 to 6.94 Log CFU/mL in trials 1, 2, 3, 4, and at day 3 in trial 5, before *S. cerevisiae* Lalvin Sensy inoculation (Figure 5). Even near the end of alcoholic fermentation (day 13), *S. cerevisiae* viable colonies were so far present at concentrations ranging from 4.11 and 4.95 Log CFU/mL. The highest values for *S. cerevisiae* counts at the end of fermentation were observed in the trials LA48, LB, and LD, whereas comparable results were detected in the remaining trials. Among non-*Saccharomyces* yeast, *Hanseniaspora* spp. was the main genera identified, showing counts above 5 Log UFC/mL before yeast inoculation at day 0, but they started decreasing while *S. cerevisiae* increased and the fermentation proceeded. *Metschnikowia* spp. was never detected (<10 CFU/mL) as well as acetic acid bacteria (<10 CFU/mL). Lactic acid bacteria (LAB) were limited, lining up at about 2.7 Log CFU/mL in the must, then they remained almost stable, but at the end of fermentation an increased concentration was found in trials LA48 and LC, which showed 3.84 and 3.69 Log CFU/mL, respectively.

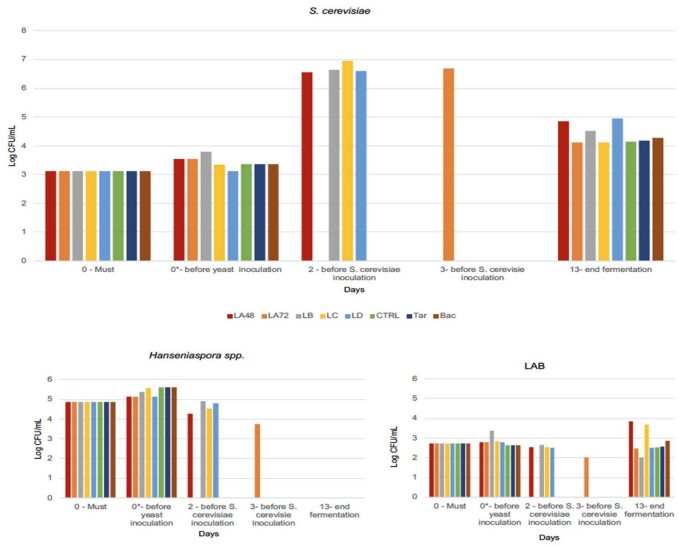


Figure 4: Results of the counts of indigenous microflora on the must and during fermentations before each step of yeast inoculation

Commentato [AC22]: Ingrandire e formattare

L. thermotolerans inoculated in trials LA48, L72, LB, LC, and LD before *S. cerevisiae* for biological acidification was able to grow of about 1 Log after 2 days in trials LA48 and LA72 (Figure 6). In trials LA72 and LC remained almost stable, whereas a slight decrease was seen in trial 5, for *L. thermotolerans* D. In the case of trial LA72, *L. thermotolerans* A showed the same concentration after 3 days of inoculation, but being the same strain inoculated in trial LA48, it is possible that after an initial increment, a decrement to the initial values of inoculation could have happened. At end fermentation *L. thermotolerans* resulted always below the detection limit of the method (< 10 CFU/mL).

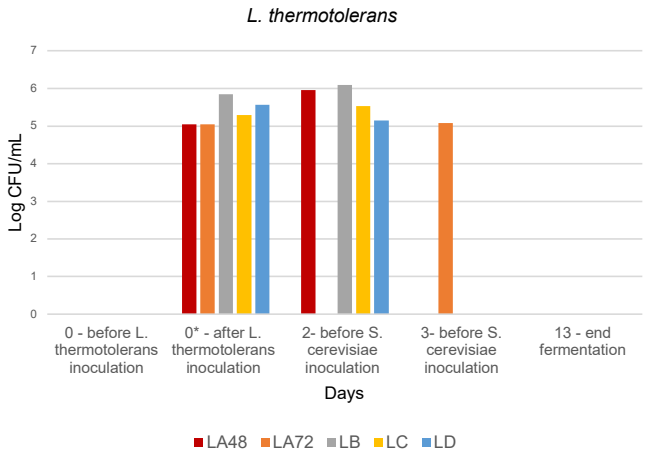


Figure 5: *L. thermotolerans* counts in the trials inoculated LA48, LA72, LB, LC, and LD.

Chemical analyses

Commentato [AC23]: È da scegliere una formattazione per tutti i titoli

As can be observed from Figure 7, the trials inoculated with *L. thermotolerans* suffered an almost instant depletion of YAN, especially for strain A, compared to strains B, C and, in particular, D.
However, attention should be paid to the behaviour of strain A, which when left to ferment for 48 h led to an almost total consumption of YAN, while in the case of thesis LA72, when left to ferment for 72 h, the YAN residue evaluated was greater than in thesis LA48.
At t2 and t3 thesis LA72 and LD show a higher ratio of YAN, this can be correlated to the higher amount of acid lactic production, in fact the transformation of ethanol in lactic acid of *Lachancea thermotolerans* is linked to nitrogen metabolism as reported in recent studies (Battjes et al. 2022; Sainz et al., 2022).
Considering the control trials, a final comparable YAN concentration was observed for thesis Bac, added of Bactiless™, whereas trial CTRL, and trial Tar showed higher YAN residual values of 69 and 46 mg/L, respectively.

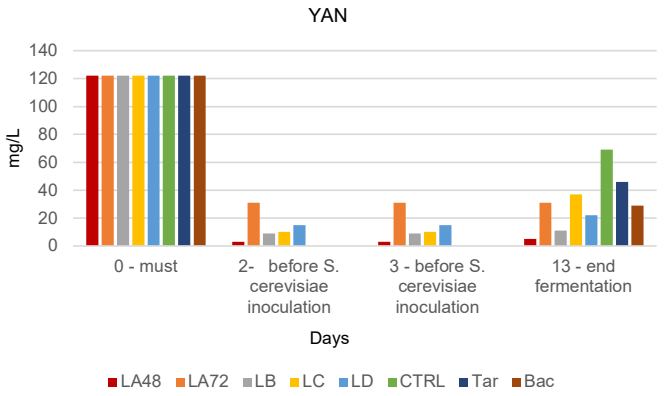


Figure 6: Yeast assimilable nitrogenous compounds (YAN) in the different trials during the first stages and at the end of fermentation

Despite the high consumption of YAN immediately after inoculation, the fermentation proceeded correctly for all theses, without running into slowdowns or fermentation blocks, leading to alcohol values close or equivalent to the potential degree of 9.6% v/v, calculated at the beginning on the basis of the sugar concentration (158.8 g/L), and leaving no residual sugar in the wine (Figure 8, panel A).

Thesis LA72 show the lower amount of ethanol, as reported in different *papers L. thermotolerans* can reduce the ethanol percentage in a range from 0,1% v/V and 1,6% (Sgouros et al., 2020; Korenika et al., 2021). This is explained by the weaker Crabtree effect show in *L. thermotolerans* than *S. cerevisiae* in addition additional factors that contribute at ethanol reduction are the production of lactic acid, as mentioned as follow, and the yeast biomass (Vicente et al., 2021)

The theses showed residual sugar values between 1 (LA48) and 0.1 g/L. The LA72 and LB theses had a similar behaviour in respect to the three controls (CTRL, Tar, Bac), showing an equivalent residual sugar content of 0.1 g/L. LC and LD displayed values of 0.4 and 0.2, respectively (Figure 8, panel B). The profiles of sugar depletion during the time clearly showed also that thesis LD had the slower fermentation activity at the beginning, however after *S. cerevisiae* inoculation, it increased the fermentative vigour leading to a development of 9.4 EtOH % v/v in the same time of the other theses. LA72 thesis was one of the fastest to ferment, although the one that developed the lowest alcohol content (9.1% v/v). However, the fastest was LC thesis, which also led to a development of EtOH equal to 9.5% v/v.

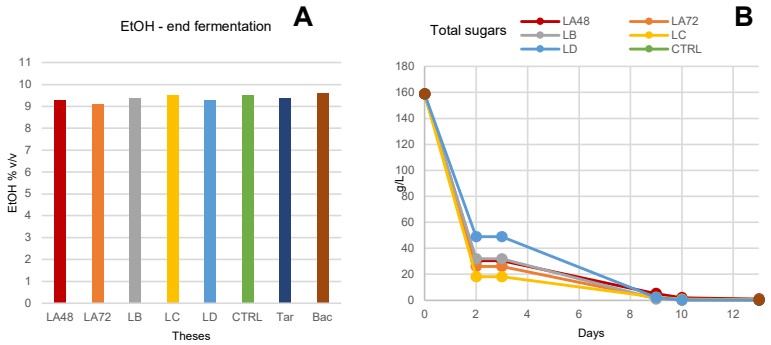


Figure 7: Final EtOH % v/v concentration (panel A) and fermentation profile as shown from the sugar depletion in the time (panel B).

The theses LA72 and LD, which developed the lowest alcohol content and showed the initial slowest fermentative activity, respectively, resulted the two theses with the highest amount of lactic acid produced (Figure 9, panel A). The production of lactic acid depends on the *L. thermotolerans* activity, as can be observed from the malic acid degradation. In fact, the lactic acid production is not stoichiometrically attributable to the conversion of malic acid (Figure 9, panel B). Taking into consideration that there was a degradation of malic acid of about 1 g/L with a practically overlapping trend in all the theses, including the controls, from Figure 8 it is possible to state that all the inoculated *L. thermotolerans* acidified. As previously mentioned, LA72 and LD were the most effective, leading to a production of 3 and 3.5 g/L of lactic acid, respectively.

But also, the others, albeit a smaller quantity, increased the quantity of lactic acid compared to the 3 controls, which showed values close to 0 g/L, with values of 0.76 for LA48 and about 0.5 for LB and LC. Different papers show different increase of lactic acid from 0,8 g/L to 5,8 g/L using commercial strains with sequential inoculum of *S. cerevisiae* (Binati et al. 2020; Vaquero et al., 2020; Hranilovic et al., 2021; Snyder et al., 2021; Zhang et al., 2021).

These results are confirmed for pH and total acidity too, in fact thesis LA72 and LD were the two in which the purpose of the work was achieved (Figure 10, panels A and B).

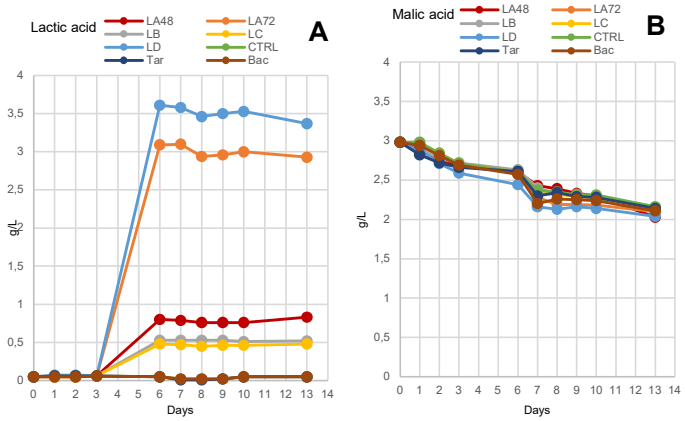


Figure 8: Lactic and malic acid (panel A and B, respectively) evolution during alcoholic fermentation.

Commentato [AC24]: Formattazione tutti i titoli

For thesis LD, pH values resulted very similar to those obtained in thesis Tar (3.41 vs 3.42, respectively), where acidification was obtained by adding tartaric acid (150 g/hL) (Figure 9, panel A).

It should be noted that looking at the evolution of the pH over time (Figure 9, panel B), it decreased in the early stages of fermentation, immediately after the inoculation of *Lachancea*, but this lowering did not remain stable and the pH curve goes back up. This behavior is evident for all theses, but with a greater emphasis on thesis LA72. However, this thesis showed a drop of 0.2 pH units compared to the CTRL and BAC control thesis. This rise in pH could be caused by the buffering effect of the wine. Thesis LD reduced the pH in respect to the controls CTRL and BAC of 0.25 pH units, according to many different studies that estimate an increase of pH from 0.13 to 0.29 pH units (Kapsopoulou et al., 2007 ; Comitini et al., 2011;Benito et al., 2015a; Benito et al., 2015b; Balikci et al., 2016; Benito et al., 2016; Benito et al., 2017; Chen et al., 2018; Benito et al., 2019; Blanco et al., 2019; Morata et al. 2019; Benito et al., 2020; Blanco et al., 2020; Romani et al., 2020**;** Sgouros et al., 2020; Hranilovic et al., 2021; Snyder et al., 2021)

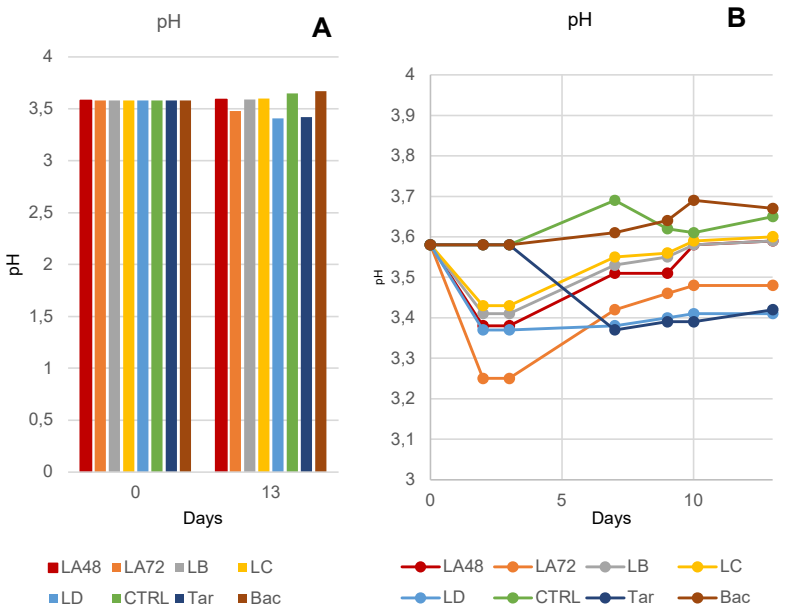


Figure 9: pH value at the end of fermentation in respect to the initial pH of the must (panel A); evolution of pH over the time in the different theses (panel B)

As expected, total acidity was also influenced by the greater production of lactic acid, as can be seen in Figure 10 panel A, which led to its increase in the theses LA72 and LD. All the other theses resulted comparable.

As far as volatile acidity is concerned, in this case all the theses inoculated with *L. thermotolerans* showed higher values in respect to the controls, exceeding the threshold of the perception value (200 mg/L) (Figure 10, panel B).

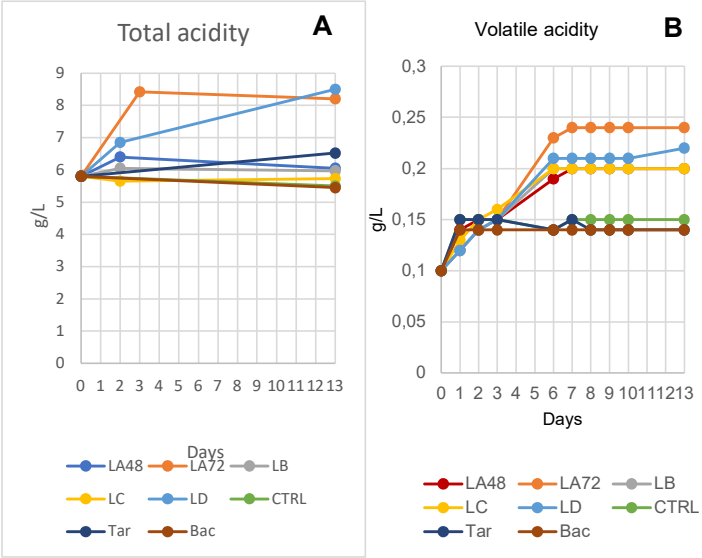


Figure 10:Total acidity (panel A) and Volatile acidity (panel B).

The free (Figure 11, panel A) and total (Figure 11, panel B) SO_2 content were also reported. The evaluation was done to add the exact amount of $K_2S_2O_5$ to obtain final concentrations as reported in the scheduled program of vinification for each trial. After evaluation at each sampling point, $K_2S_2O_5$ was added in a concentration sufficient to reach the values defined in the winemaking protocol.

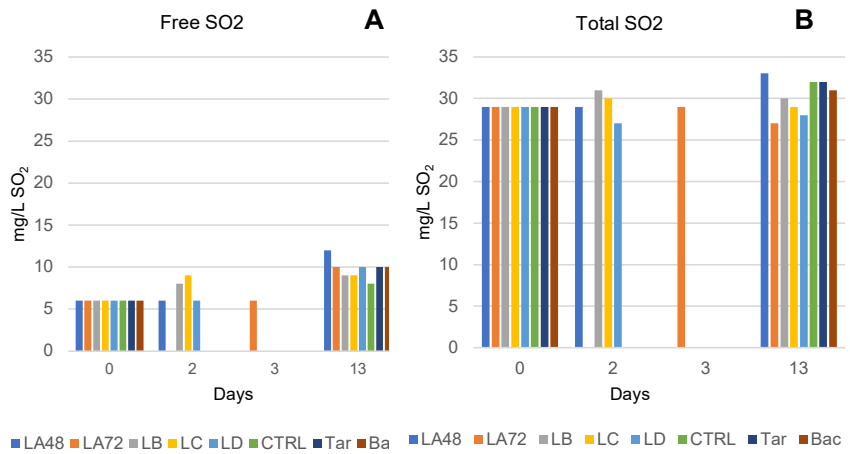


Figure 11: panel A, Free SO2 (mg/L); panel B, Total SO2 (mg/L).

Commentato [AC25]: Non sono gli stessi colori per ogni tesi in tutti i grafici??

Aromatic compound analysis

Higher alcohols

Tar and Bac theses had the higher concentration of higher alcohols (Figure 13).

In particular, this stands out for phenylethyl alcohol, thesis inoculated with *L. thermotolerans* show a lower concentration of this compound, some papers reported that in mixed fermentation the amount of phenylethyl alcohol increase but decrease in pure fermentation (Comitini et al., 2011; Gobbi et al., 2013; Morata et al., 2019).

The trend of higher alcohols production is different by compering different papers and different strains, in fact commercial strains of Concerto produce an higher concentration and Laktia™ produced by Lallemand show a lower concentration about 13% (Hranilovic et al., 2021), other papers report that the same commercial strains produce similar final amounts of higher alcohols in pure fermentation whereas S. *cerevisiae* control produce 31% less (Vaquero et al., 2020)

LC show a lower value of higher alcohols.

Thesis LD and LA48 had a similar concentration of higher alcohols as thesis Tar e Bac., these data shown that that the permanence of the strain *L. thermotolerans* A 48 h, had not increased the concentration of superior alcohols. In particular, for thesis LA72, the permanence was of 72h and the higher alcohols content was significantly decreased.

In thesis BAC, as already mention, was added 30 g/hL of Bactiless™ . This thesis showed a lower amount of higher alcohols, different reports confirm the decrease of higher alcohols caused by chitosan products (Scansani et al., 2020; Cosme et al., 2021)

Considering 3-methyl-1-butanol, this was the most abundant higher alcohols (Hrillanovic et al., 2021) in thesis inoculated with *L. thermotolerans*. Thesis LA48, had the higher concentration of this compound. Thesis LA72 and LB showed a lower amount, but substantially comparable to LD, CTRL and Tar. Thesis LC and Bac showed the lowest concentration.

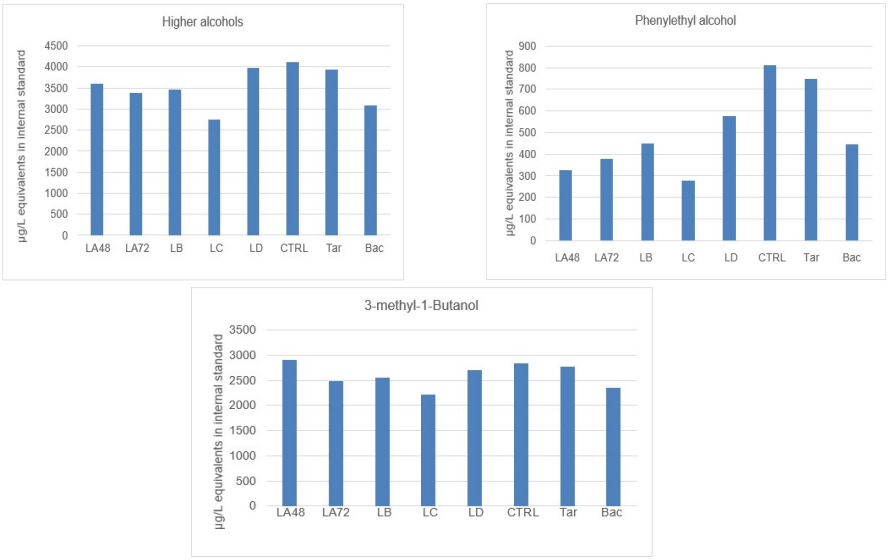


Figure 12: Higher alcohols

Medium chain fatty acids and their ethyl esters

Thesis LA48 and Thesis LA72 had the lowest content of medium chain fatty acids (MCFA) except for thesis BAC, it should be considered the possibility of evaluating the lower "toxic" effect of this yeast on high alcohol content wines with less risk of slowing down or stopping fermentation, this is shown in different papers too (Gobbi et al., 2013; Comitini et al., 2011; Sgouros et al., 2020; Hranilovic et al., 2021) but in contrast with other studies (Binati et al., 2019; Nisiotoue et al., 2019)

Thesis LB showed a higher amount of these compounds compared to thesis CTRL and Tar, these latter showed a concentration of medium fatty acids like LC and LD.

The ethyl esters were variable but in line with the control thesis. Thesis LA72 showed a lower concentration than thesis LA48, this is in line with what reported in Hranilovic et al. 2021 for the thesis with sequential inoculum.

The acetic esters with floral and fruity aromas were more concentrated in thesis without *L. thermotolerans* this is confirmed by Hranilovic et al. 2021, less than about 50% these esters contribute significantly to the sensory profile of young wines (Figure 13)

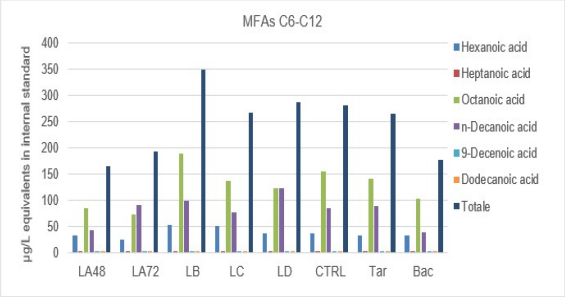


Figure 13: MCFAs, ethyl esters and acetic esters

Monoterpens and norisoprenoids

Thesis CTRL, Tar and Bac showed similar concentration of monoterpenes, despite thesis Bac that had a lower ratio of linalool and citronellol.

On the other hand, thesis added with *L. thermotolerans* showed variable concentrations of these compounds.

Thesis LC showed the lowest concentration, while LD showed the higher, also in comparison with the thesis inoculated only with *Saccharomyces cerevisiae.*

Also, in this case in thesis LA72, the monoterpenes decreased, leading this thesis to have lower concentrations than all the others.

The concentration of β-damascenone was significantly higher in thesis LC, but in all the theses inoculated with L. *thermotolerans* ad exception of thesis LA72.

This molecule is particularly important because remind the small of exotic flowers, cooked apples and tea. It has a very low threshold of perception about 0,05 ppb (Figure 14)

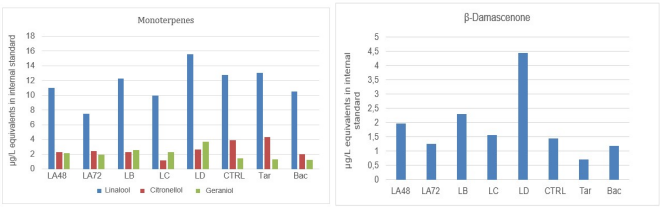
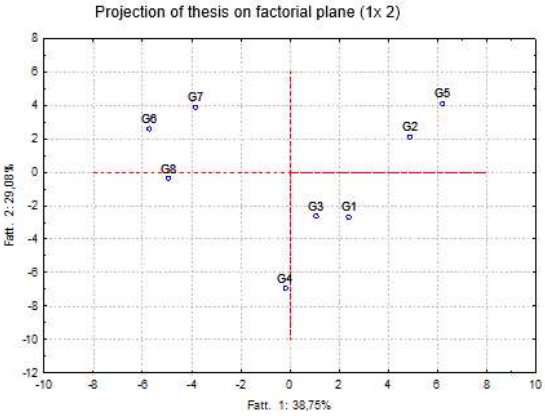
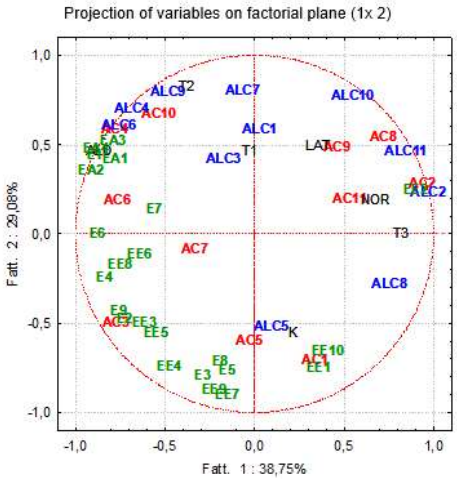


Figure 14: Terpenes and norisoprenoids

Sensory evaluation

PCA analyses showed that thesis CTRL, Tar and Bac, inoculated only with *S. cerevisiae*, had a different profile aroma compared to other inoculated with *L. thermotolerans*.
(Figure 15, 16, 17).
Theses without *L. thermotolerans* were characterized by a higher ratio of acetic esters, and amyl alcohols. Linked to the perception of grassy but also fruity and spicy notes.
Thesis LC was characterized by ethyl esters, complex esters (isobutyl hexanoate, isoamyl decanoate, esters of fatty acids and alcohols other than ethanol. Linked to the perception of spices, greater floral notes, greater frankness, and fewer defects.
Hower this trial had the higher concentration of acetoin, this means a higher ratio of diacetyl over the time and consequently it involves a grater body in the wine, these also applies to thesis LA48 e LB.





COMPOSTO	CODIC
Acetic acid	AC1
Propanoic acid, 2-methyl-	AC2
Butanoic acid	AC3
Butanoic acid, 3-methyl-	AC4
Hexanoic acid	AC5
Heptanoic acid	AC6
Octanoic acid	AC7
lactic acid	AC8
n-Decanoic acid	AC9
9-Decenoic acid	AC10
Dodecanoic acid	AC11
1-Butanol, 3-methyl-	ALC1
1-Hexanol	ALC2
1-Hexanol, 2-ethyl-	ALC3
2,3-Butanediol	ALC4/(
1-Octanol	ALC5
1-Propanol, 3-(methylthio)-	ALC7
1-Decanol	ALC8
Phenylethyl Alcohol	ALC9
Glycerin	ALC1C
3-Hexen-1-ol, (Z)-	ALC11
Benzaldehyde	ALD
Acetic acid, hexyl ester	E1
Octanoic acid, methyl ester	E2
Isopentyl hexanoate	E3
Isoamyl lactate	E4
Decanoic acid, methyl ester	E5
Octanoic acid, 3-methylbutyl ester	E6
Decanoic acid, propyl ester	E7
n-Capric acid isobutyl ester	E8
decanoic acid, 3-methylbutyl ester	E9
1-Butanol, 3-methyl-, acetate	EA1
3-Hexen-1-ol, acetate, (Z)-	EA2
Acetate	EA3
Acetic acid, 2-phenylethyl ester	EA4
Hexanoic acid, ethyl ester	EE1
lactic acid, ethyl ester	EE2
Octanoic acid, ethyl ester	EE3
Nonanoic acid, ethyl ester	EE4
Decanoic acid, ethyl ester	EE5
Ethyl 9-decenoate	EE6
Ethyl dodecanoate	EE7
Tetradecanoic acid, ethyl ester	EE8
Butanedioic acid, diethyl ester	EE9
Ethyl hydrogen succinate	EE10
Acetoin	K
Butyrolactone	LAT
β-Damascenone	NOR
Linalool	T1
Citronellol	T2
Geraniol	T3

Figure 15: PCA the aromatic component that distinguishes the different theses. Legend: G1=LA48, G2,=LA72, G3=LB, G4=LC, G5=LD, G6=CTRL, G7=Tar, G8=Bac

However, the latter thesis had a lower amount of ethyl esters. This was confirmed during the sensorial analyses in fact in this thesis the floral notes result less evident.

Thesis LA72 and LD were more characterized by a good acidity (caused by the higher amount of lactic acid) and less aromatic. Between the two, LD thesis was more characterized by a good sapidity. Both were close to the area where β-damascenone and monoterpenes were present, the main compounds were citronellol and linalool.

The spider charts do not highlight significant differences at olfattive level, while at the mouth level there are significant differences for all indicators, except for the sapidity; while considering the retrolfactive characters there are significant differences for the indicators spicy, persistence and floral.

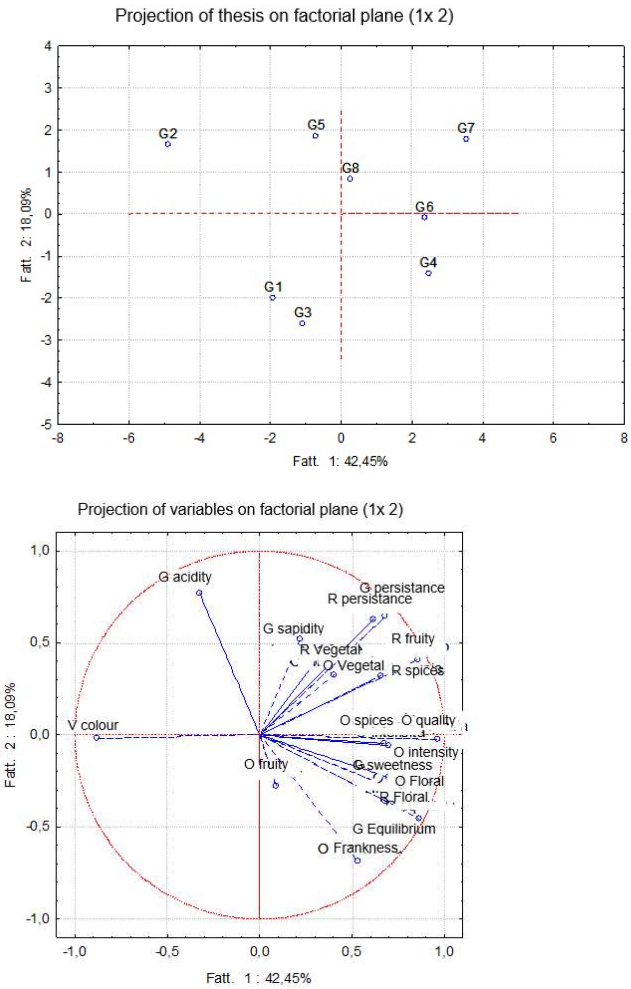


Figure 16: PCA relating to the sensory notes that distinguish the different theses, Legend: G1=LA48, G2,=LA72, G3=LB, G4=LC, G5=LD, G6=CTRL, G7=Tar, G8=Bac

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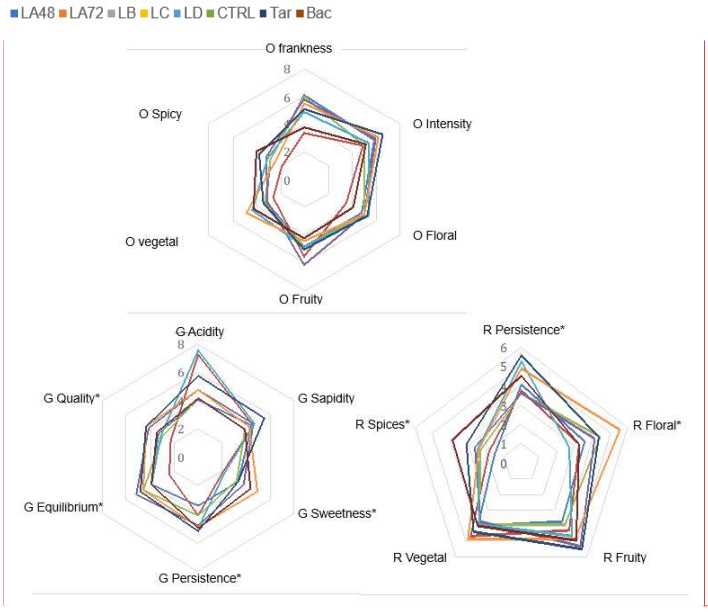


Thesis	G acidity	G swetness	G persistance	G equilibrium	G quality	R persistence	R Floral	R Spices
LA48	4,16a	3,37bcd	3,27a	3,98b	3,11ab	3,90abc	3,51ab	1,17a
LA72	7,18c	1,98a	4,14ab	2,36a	2,36a	3,43a	2,83a	1,46ab
LB	4,12a	2,90abc	4,02ab	4,46bc	2,91a	3,42a	4,25abc	2,17abc
LC	4,50a	3,81cde	4,81bc	5,43c	4,41c	3,66ab	4,30abc	2,80cd
LD	7,45c	2,30ab	5,04bc	3,76b	3,05ab	5,24cd	2,76a	2,38abc
CTRL	4,67a	5,01e	4,77bc	4,50bc	4,16bc	4,96bcd	5,65c	2,52bc
Tar	5,91b	3,46bcd	5,47c	4,73bc	4,36c	5,97d	4,45bc	3,25cd
Bac	4,04a	4,29de	4,85bc	3,89b	3,51abc	4,65abcd	2,96ab	4,10d

Figure 17: Graphics associated at sensory analyses and test LSD Fisher

Commentato [AC26]: Scale non uguali

Part 2) Natural acidification and bioprotection with *L. thermotolerans and M. pulcherrima* on Sauvignon blanc

Microbiological analyses

The indigenous microflora of the must showed a low microbial load of *Saccharomyces* yeasts, settling between 2.2 and 2.7 Log CFU/mL, which remained stable for the period of clarification. During the next 2 days a slight growth was seen for all theses, but in particular for the control CTRL thesis, which showed an increase of 1.3 Log CFU/mL. At the end of alcoholic fermentation all the theses showed comparable *S. cerevisiae* concentrations ranging from 4.6 to 4.8 Log CFU/mL (Figure 18, panel A).

As far as non-*Saccharomyces* yeasts are concerned (Figure 18, panel B), particular attention is paid to apiculate yeasts, responsible for the increase in volatile acidity and therefore for the lowering of the quality of the wine itself. The contamination of these yeasts was quite high, with values in the must equal to 4.6 Log CFU/mL. These concentrations changed during the first stages of subsequent processing. In particular, the addition of Bactiless allowed greater containment of the development of *Hanseniaspora* spp. up to the clarification step. In fact the Bac thesis had a concentration of 1 Log less compared to the CTRL thesis (not treated) and the MX thesis (addition of bioprotective strain *Metschnikowia* X). The theses added with *Lachancea thermotolerans* also allowed a slowdown in growth, which stopped at a reduction of 0.5 Log compared to the CTRL control thesis. This containment remained substantially stable for the following 48 h in the case of both the LA/LC thesis and the MX thesis, which required more time to highlight the bioprotection capabilities. Viceversa, Bactiless lost its effect, in fact, the apiculate yeasts have grown, reaching values similar to those with bioprotective yeasts. In all cases the final reduction with respect to the control, 3 days after inoculation, was from 0.3 to 0.6 Log. At the end of fermentation, no non-*Saccharmyces* yeasts were detected.

The load of lactic acid bacteria was also relatively high in the must (4.8 Log CFU/mL), but in all theses there was a reduction over time comparable with the CTRL control and in some cases (LC and MX) even better in the control. This allows us to state that the inoculated strains do not appear to have had a significant effect on LAB containment (Figure 18, panel C).

Acetic acid bacteria were always under the detection limit for all the samples considered (<10 CFU/mL).

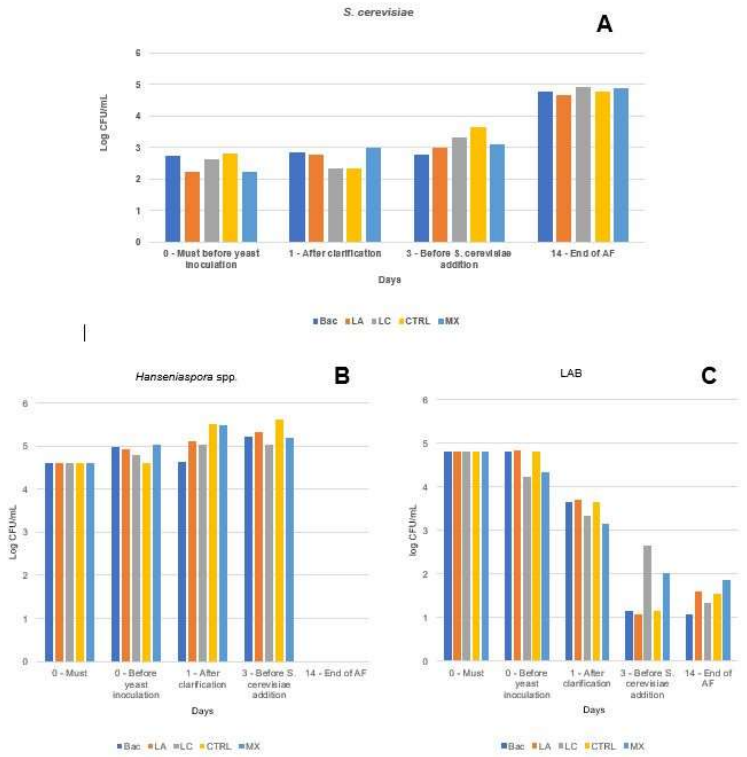


Figure 18: Results of the counts of indigenous microflora on the must and during fermentations before each step of yeast inoculation and at the end of alcoholic fermentation (AF). Panel A, Saccharomyces cerevisiae; panel B, apiculate yeast, Hanseniaspora spp.; panel C

The bioprotective and acidifying strains inoculated in the must and kept for a total period of 3 days before the inoculation of *S. cerevisiae*, were monitored in the theses considered to verify the concentration of the inoculum and their effective permanence in the must in the post clarification step. As can be seen in Figure 19, panels A, B and C, all strains inoculated as bioprotection/acidification were present at a concentration close to 6 Log CFU/mL. The clarification process only slightly lowered the microbial load (about 0.5 Log), which then remained stable until the end of the period considered (before the inoculation of *Saccharomyces* yeasts).

Neither *Lachancea thermotolerans* nor indigenous *Metschnikowia* spp. were detected in the draining must. This evaluation is fundamental, because the clarification process could retain a more or less significant amount of inoculated microorganisms in the sediment, but this seems to be avoided by the results.

Commentato [AC27]: ?? Vedo solo lettere sotto

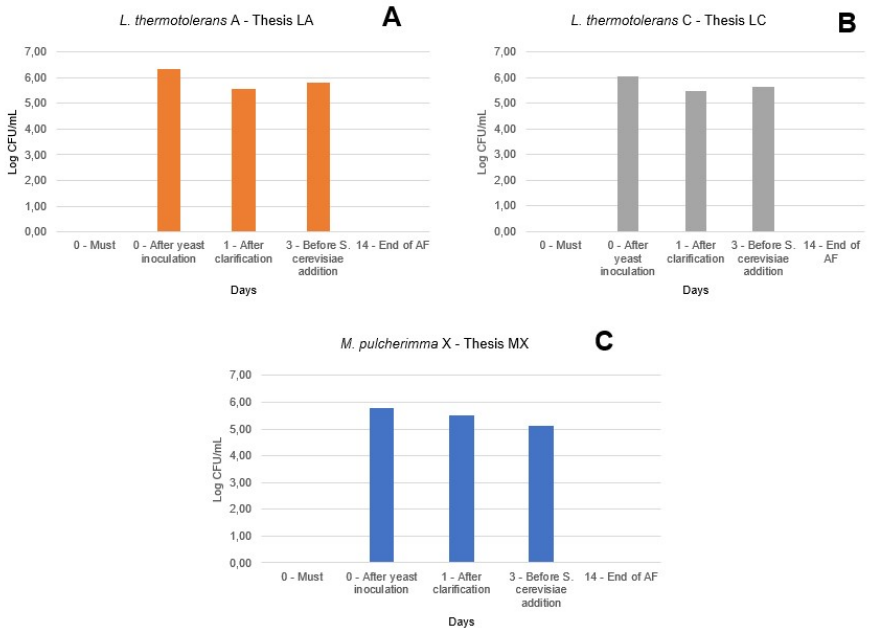


Figure 19: Bioprotect and acidifying inoculated yeast counts in the trials inoculated LA, panel A, LC,panel B and, MX panel C.

Chemical analyses

Alcoholic fermentation proceeded regularly, ending between 8 and 9 days for all theses. Sugar consumption was complete, leaving a sugar residue < 1.3 g/L in all theses, with values even of 0.4 and <0.1 g/L for LC and Bac, respectively. The LA and BAC theses started slightly faster than the others, but all of them ended concurrently. All theses reached the expected alcoholic potential (12.6 % v/v for Bac, LC, CTRL; and 12.5 % v/v for MX and 12.2 % v/v for LA). There were no significant differences either in the fermentation trend or in the quantity of alcohol developed (Figure 20, panel A and B). The fermentation trend just described was possible despite Bac and LA theses showed a very rapid consumption of N, compared to the other theses. These two theses were indeed the ones that showed the greatest vigor. CTRL and MX had a superimposable consumption of N (Figure 20, panel C).

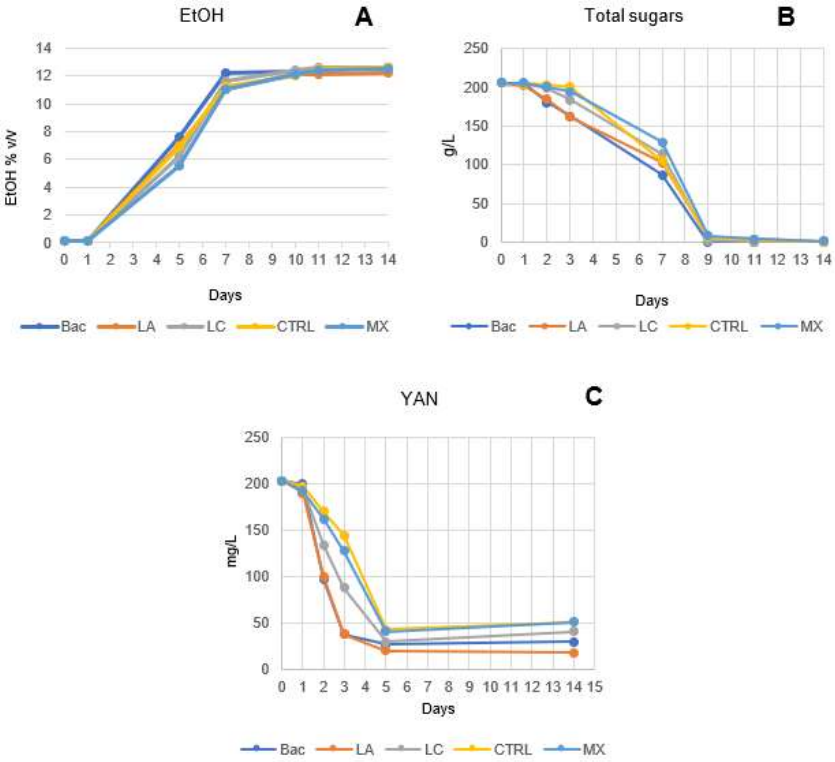


Figure 20: Fermentation profile as shown from the alcohol development (panel A), sugar depletion in the time (panel B) and N consumption (panel C)

The malolactic fermentation did not take place, and this can also be explained by the concentration of lactic acid bacteria present in the must and in the various monitored phases of winemaking. The concentrations detected, which stood at values <2 Log CFU/mL starting from post-clarification, were incompatible with the start of malolactic fermentation. In the LA thesis, however, the production of about 1 g/L of lactic acid is highlighted, presumably attributable to *L. thermotolerans* A as reported in previous studies (Binati et al. 2020 ; Vaquero et al. 2020; Hranilovic et al. 2021; Snyder et al. 2021; Zhang et al. 2021), comparing with Glera study the production of lactic acid that is produce is lower, this is probably associated to the clarification process that in Glera was about 48 h not 24h and to the S. *cerevisiae* addition that in Glera was added after 48h for thesis LA48 and after 72 h in thesis LA72. (Fig. 21)

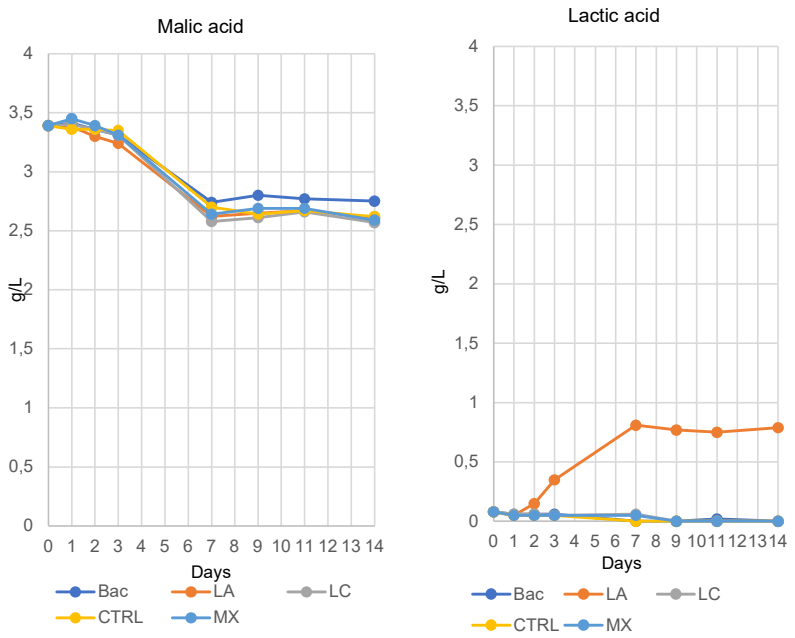


Figure 21: Lactic and malic evolution during AF

The total acidity was different only for trial LA, which had about 1 g/L more total acidity in respect to the control CTRL, this is correlated to the production of lactic acid showed in the previous paragraph.
CTRL trial resulted the lowest in total acidity, even if the differences cannot be considered significant (Figure 22, panel A). The volatile acidity was above the perception threshold (200 mg/L) for white wines, but the values, which were found in trials resulted all in volatile acidity lower than the CTRL control, for this reason the displayed values could be considered acceptable (Figure 22, panel B). *L. thermotolerans*

generally produce a lower amount of acetic acid than Saccharomyces genus (Benito et al. 2018; Vilela et al. 2018), altough *L. thermotolerans* possed a strain variability of about 50% in acetic acid production (Benito et al. 2018; Escribano et al. 2018). Compared to commercial strains used in sequential inoculom *L. thermotolerans* strains produce an higher amount of volatile acidity, about 0,50 g/L of acetic acid contrary to the control that produce only 0,15 g/L (Hralinovic et al. 2021)

According to the production of lactic acid by LA strain the pH observed is 0.15 unit of pH lower than the CTRL, this is also reflected in the higher total acidity, according to many other studies (Kapsopoulou et al., 2007 ; Comitini et al., 2011;Benito et al., 2015a; Benito et al., 2015b; Balikci et al., 2016; Benito et al., 2016; Benito et al., 2017; Chen et al., 2018; Benito et al., 2019; Blanco et al., 2019; Morata et al. 2019; Benito et al., 2020; Blanco et al., 2020; Romani et al., 2020**;** Sgouros et al., 2020; Hranilovic et al., 2021; Snyder et al., 2021) and to the results obtained in Glera study.

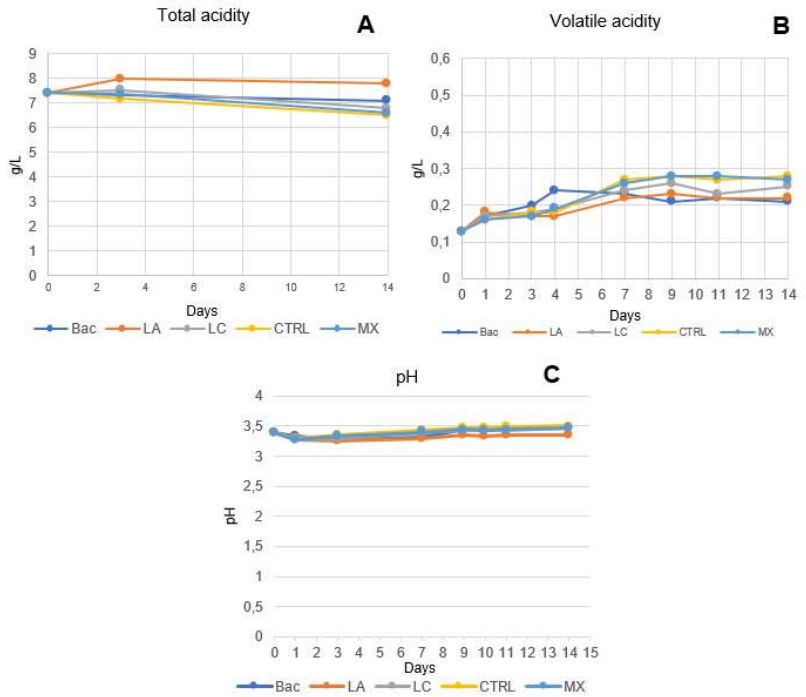


Figure 22: Total acidity (panel A), Volatile acidity (panel B), and pH (panel C).

As regard MX thesis, inoculated with *M. pulcherrima*, studies report different results, according to our study malic acid decrease during sequential inoculum inoculum (Du Plessis et al., 2017; Escribiano-

Viana et al., 2018). In the same papers the pH increase from no significant values as 0.1 pH units (Benito et al., 2015; Chen et al., 2018) or significant values as 0.80 pH units (Comitini et al. ,2011; Dutraive et al., 2019) in contrast with our data that show a non-significant decrease in comparison with the CTRL Regarding volatile acidity the MX thesis has an equal amount of acetic acid as the CTRL (Benito et al.; 2015; Ruiz et al.,2018)

Total and free SO_2 were monitored to add the exact amounts to obtain the defined concentration in the protocol (Figure 23, panel A and B).

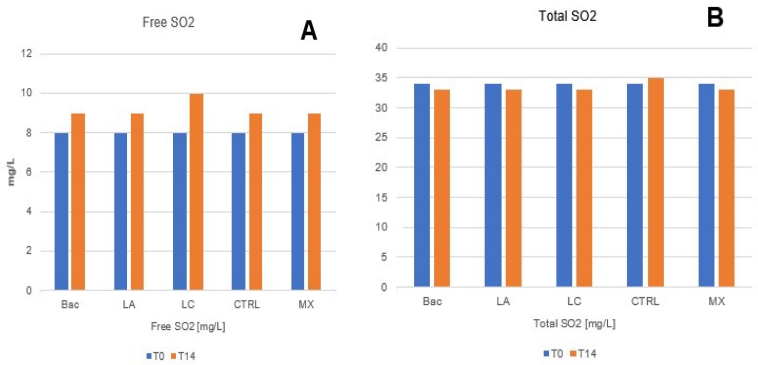


Figure 23: panel A, Free SO2 (mg/L); panel B, Total SO2 (mg/L).

Aromatic compounds analysis

Higher alcohols

Commentato [AC28]: Rendere titoli tutti questi titoli

Thesis LA showed a higher content of total higher alcohols, in particular, of 3-methyl-1-butanol compared to all other theses; this is ascribable to the different yeast strain as already described from different authors (Ciani et al. 2006; Kapsopoulou et al. 2007; Ciani et Comitini 2010b; Bueso et. al., 2016). Vice versa thesis LC showed the lower content of higher alcohols, in particular for phenyl alcohol.

Compared with Glera's higher alcohol content, thesis inoculated with *L. thermotolerans* A showed a lower amount of total higher alcohol in contrast with Sauvignon wines, this is imputable to the permanence of the yeast as already mentioned for thesis LA72 Glera. However, 3-methyl-1-butanol increased in LA48 and LA72 too, in contrast with the phenyl alcohol's trend. Strain C show the same trend in both varieties, in fact it was lower than in other thesis.

Thesis MX, inoculated with *M. pulcherrima*, had a higher alcohols content similar to BAC and CTRL (Figure 24).

Thesis BAC showed the lowest amount of organic acids, thesis LA showed a bit higher amount of these compounds. Otherwise, other thesis had double concentrations of higher alcohols. This trend was similar for MFAs (Figure 25).

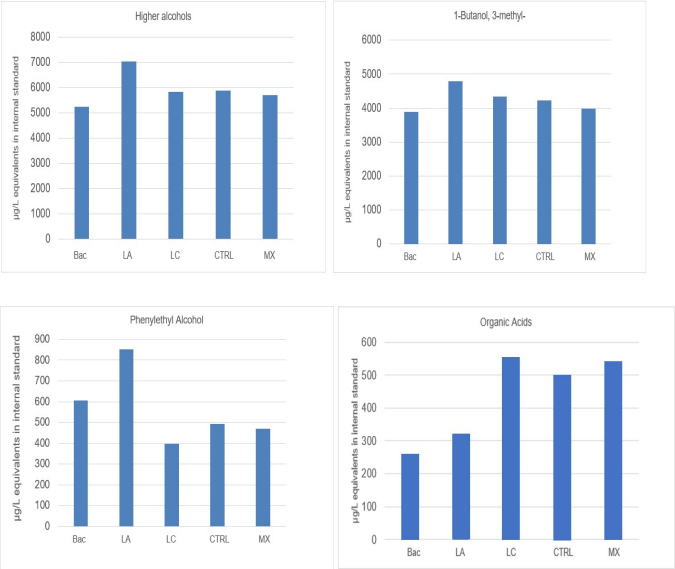


Figure 24: Higher alcohols and Organic Acids

Medium chain fatty acids and Esters

As concern for esters trial MX, CTRL and Bac had a similar content of this compounds (Figure 25). Thesis LA and LC showed an opposite trend. Trial LA that showed the higher phenyl alcohol amount, responsible for floral aroma, in contrast showed the lowest ratio of esters. In an opposite way LC showed a lower ratio of phenyl alcohol reveal the higher content of ester.

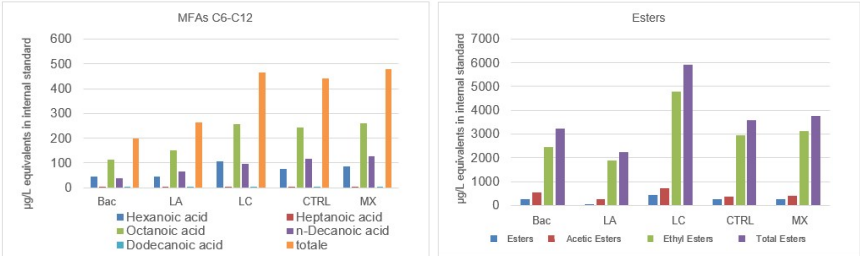


Figure 25: MFAs and related esters

Norisoprenoids

α-ionone and β-damascenone were identified. Thesis LA was rich in α-ionone, had a double concentration of this compound compared to other thesis. β-damascenone is higher concentrated in trials Bac, LA and LC with a similar trend on Glera (Figure 26).

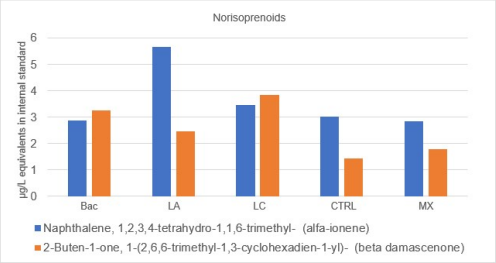


Figure 26: Norisoprenoids

Terpenes

Linalool represents the main terpen component (Figure 27). Thesis CTRL, MX and BAC showed similar ratio of this compound. Thesis LA show the lower ratio on the other hand LC showed the higher. On Glera wines linalool showed a different trend: LA48 and LC had a similar amount of this and LA72 had the lower.

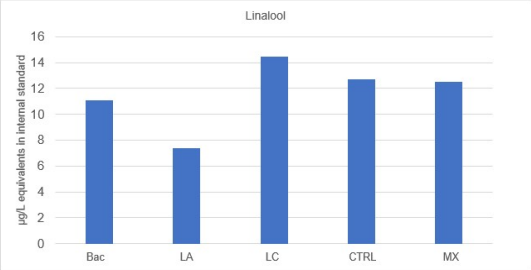
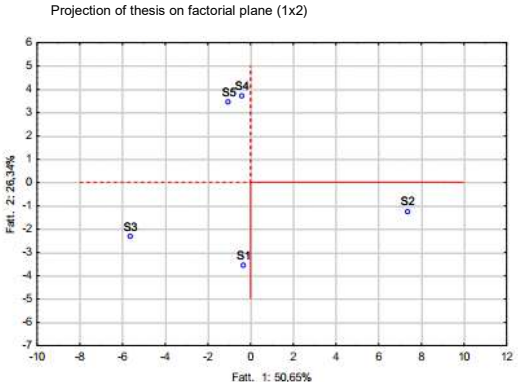
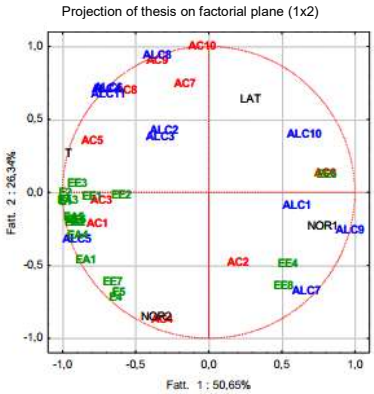


Figure 27: Linalool





CODICE	COMPOSTO
AC1	acido acetico
AC2	Propanoic acid, 2-methyl-
AC3	Butanoic acid
AC4	Butanoic acid, 3-methyl-
AC5	Hexanoic acid
AC6	Heptanoic acid
AC7	2-Hexenoic acid
AC8	Octanoic acid
AC9	n-Decanoic acid
AC10	Dodecanoic acid
ALC1	1-Butanol, 3-methyl-
ALC2	1-Hexanol
ALC3	2-Nonanol
ALC4	2,3-Butanediol, [R-(R*,R*)]-
ALC5	1-Octanol
ALC6	2,3-Butanediol, [R-(R*,R*)]-
ALC7	1-Propanol, 3-(methylthio)-
ALC8	1-Decanol
ALC9	Phenylethyl Alcohol
ALC10	Glycerin
ALC11	*cis* 3-Hexen-1-ol
E1	Acetic acid, hexyl ester
E2	Octanoic acid, methyl ester
E3	Acetic acid, octyl ester
E4	Octanoic acid, 3-methylbutyl ester
E5	decanoic acid, 3-methylbutyl ester
EA1	1-Butanol, 3-methyl-, acetate
EA2	*trans* 3-Hexen-1-ol, acetate
EA3	*cis* 3-Hexen-1-ol, acetate
EA4	Acetate ?
EA5	Acetic acid, 2-phenylethyl ester
EE1	Hexanoic acid, ethyl ester
EE2	2-Hexenoic acid, ethyl ester
EE3	Octanoic acid, ethyl ester
EE4	Nonanoic acid, ethyl ester
EE5	Decanoic acid, ethyl ester
EE6	Ethyl 9-decenoate
EE7	Dodecanoic acid, ethyl ester
EE8	Butanedioic acid, diethyl ester
LAT	Butyrolactone
NOR1	alfa-ionene
NOR2	beta damascenone
T	Linalool

Figure 28: PCA. Legend: S1, Bac; S2, LA; S3, LC; S4, CTRL; S5, MX.

Sensory evaluation

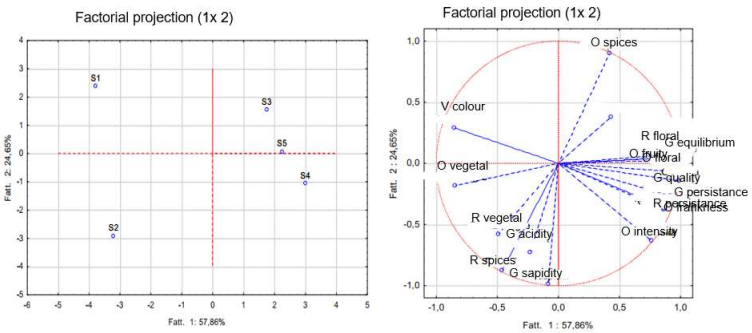
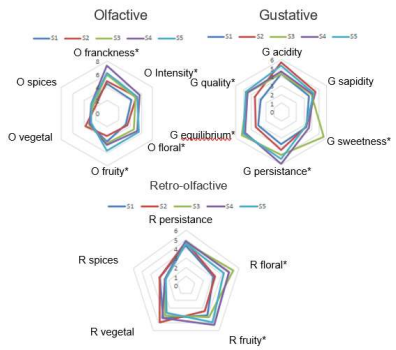


Figure 29: PCA. Legend: S1, Bac; S2, LA; S3, LC; S4, CTRL; S5, MX.

In the following picture (Figure 30) we can observed the spider graphs related to the individual components of the analyses which olfactive, retro-olfactive and gustatory.
Thesis MX had higher floral and fruity notes. Thesis LC showed a higher sweetness. Thesis CTRL and MX had a higher frankness and persistence.



Thesis	V colour	O frankness	O intensity	O floral	O fruity	G sweetness	G persistance	G equilibrium	G quality	R floral	R fruity
BAC	8,15d	4,25a	3,68a	3,55a	3,95a	3,55a	3,40a	2,99a	2,52a	3,07a	3,65a
LA	6,06c	5,29ab	5,20b	3,55a	3,87a	3,34a	3,98ab	3,38a	3,14ab	3,31a	3,38a
LC	4,85ab	5,97bc	5,18b	4,87b	4,70ab	5,86b	4,81bc	5,26b	4,41bc	5,42b	4,33ab
CTRL	5,05bc	7,17c	5,88b	5,22b	5,22ab	4,22a	6,42d	4,92b	4,75c	5,26b	6,02c
MX	3,80a	5,78abc	5,36b	5,67b	5,89b	3,25a	5,25cd	4,63b	4,52bc	4,34ab	5,25bc

Figure 30: Sensory Analyses

PART 3) Pectolytic enzymes's impact on yield and quality of must

The yield of free run must be not significantly different between the enzymatically treated trial (EfrM) and the control (frM).

Results changed considering the pressed must: differences between the enzymatically treated destemmed grapes and the control were significantly different, showing an increment of 6.49% of yield of extraction after the treatment with Lallzyme™ for 2h. A total increment of 6.2% was obtained considering the sum of free run must and the pressed one. Also in this case the difference was significant between the trials, showing a total yield of 74.65% and 68.45 for the EfrM and frM, respectively (Figure 31).

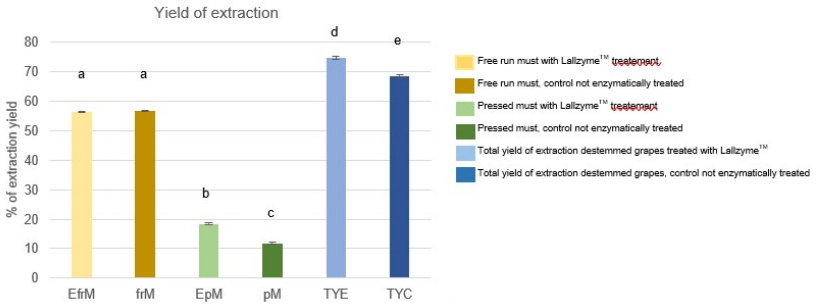


Figure 31: Yield of extraction expressed as the average value of the trials. Different letters above bars indicate significant differences ($p<0.05$), statistical analysis performed using T student test (95% significance level). Analysis was performed considering enzymatically treated samples versus the not enzymatically treated (controls).

Looking at the results of the chemical parameters, such as total acidity, pH, YAN, and sugars, no significant differences were observed among the different trials, except for the total acidity, which was significantly lower in the control frM (5.74 g/L) in respect to the EfrM (6.19 g/L) (Figure 32). Although the differences are not significant, the YAN value has a lower average value in the EfrM thesis than frM, but this may be due to the fact that the enzymatically treated sample had a much faster sedimentation, which probably led to a clearer must resulting in a loss, albeit statistically not significant, of YAN. The effect on the sedimentation aid is absolutely positive, because it allows to reduce the clarification times and to obtain a stable must.

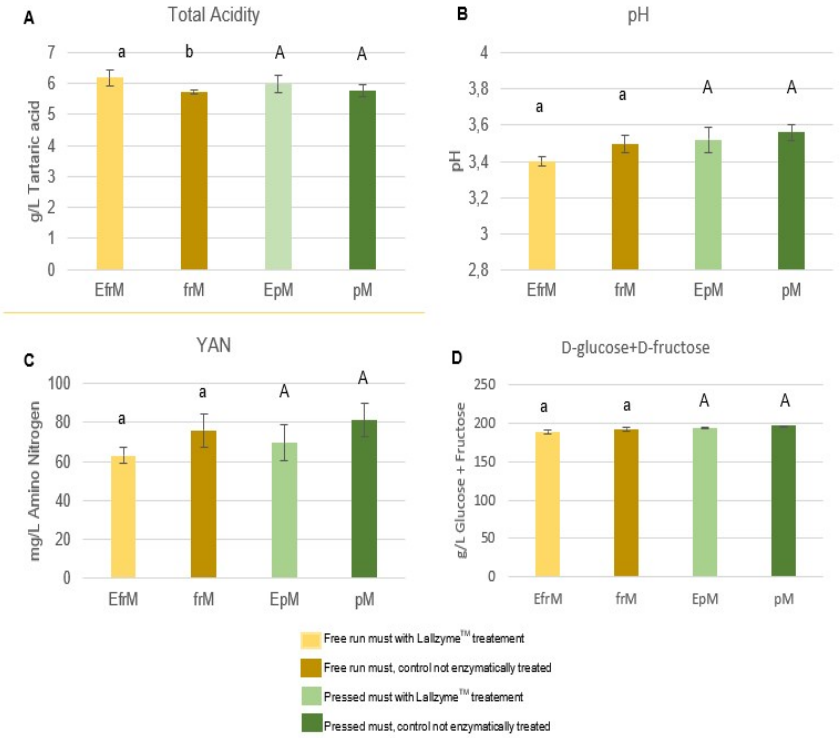


Figure 32: Total acidity (panel A), pH (panel B), YAN (panel C) and sugars (panel D) expressed as the average value of the trials. Different letters above bars indicate significant differences ($p<0.05$), statistical analysis performed using T student test (95% significance level). Analysis was performed considering enzymatically treated samples versus the not enzymatically treated (controls) (EfrM vs frM; EpM vs pM).

In Figure 33 can be observed this evident sedimentation effect on the two samples.

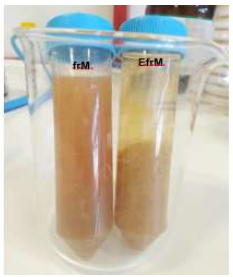


Figure 33: Differences in sedimentation in the samples treated with LallzymeTM EfrM, in respect to the not treated frM.

Considering the catechins content (Figure 34) the higher the value of the catechins, the less oxidized the wine. In both the free running must and pressed must treated with Lallzyme ™ the average value of catechins showed an higher value than the not enzymatically treated controls. However, only the values found in the free running musts resulted significantly different ($p<0.05$).

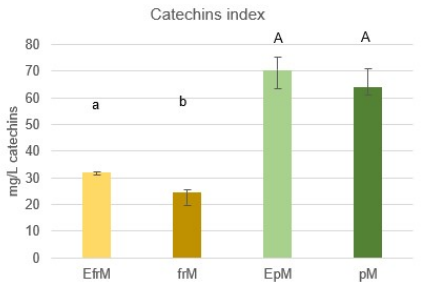


Figure 34: Catechins index expressed as the average value of the trials. Different letters above bars indicate significant differences ($p<0.05$), statistical analysis performed using T student test (95% significance level). Analysis was performed considering enzymatically treated samples versus the not enzymatically treated (control).

The total polyphenol index resulted significantly lower in the enzymatically treated samples than in the controls (Figure 35). The reduction was comparable between the free running must and the pressed must trials. One reason that has been proposed as the cause of a lower phenolic extraction in the case of pectolytic enzyme treatment, even when maceration enzymes are used, is the interaction between the extracted phenolic compounds and the skin and pulp cell walls suspended in the must. The components of the cell walls show high affinity for phenolic compounds and adsorb them into their structure (Bindon et al., 2010a, Bindon et al., 2010b; Bautista-Ortín et al., 2014; Osete-Alcaraz et al., 2020; Osete-Alcaraz et al., 2021; Osete-Alcaraz et al., 2022), those of the structural polysaccharides with the highest affinity for phenolic compounds being pectins, hemicellulose, and to a lesser extent, cellulose (Bautista-Ortín et al., 2016). Therefore, after grape crushing, phenolic compounds are extracted but also a great amount of suspended cell wall material is generated. Once these binding have occurred, these interactions are difficult to reverse and they may reduce the phenolic concentration in the must and, consequently, in the wine. This effect can be positive, in particular for wines with natural high polyphenols content, where a too high concentration of polyphenols can have a negative impact on wine taste and character, as they are responsible for the bitter and stringent features. However, it should be emphasized that the protein component in white wines contributes to the value obtained from the spectrophotometric reading, as the proteins are also detected at a wavelength of 280 nm. Therefore, in the non-enzymated samples, which as shown, did not undergo sedimentation, the proteins left in solution could have falsified the analytical data obtained, increasing the total concentration value of the polyphenols.

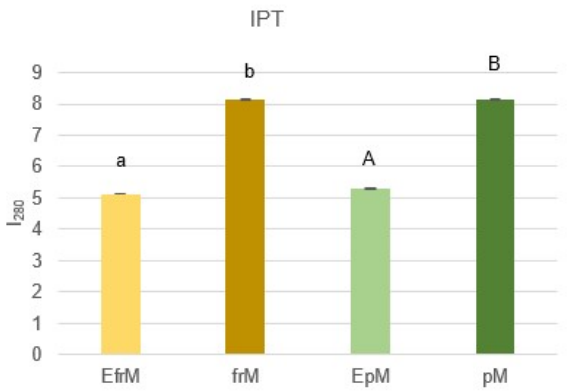


Figure 35: Total plyphenols index expressed as the average value of the trials. Different letters above bars indicate significant differences ($p<0.05$) statistical analyses performed using T student test (95% significance level). Analyses was preformed considering enzymatically treated sample versus the not enzymatically treated (controls).

Oxidizability (Figure 36) confirms the data obtained by the cathechins index previously described. Catechins are the most reactive components to oxidation and in fact the enzymatically treated samples resulted those with the highest POM index.

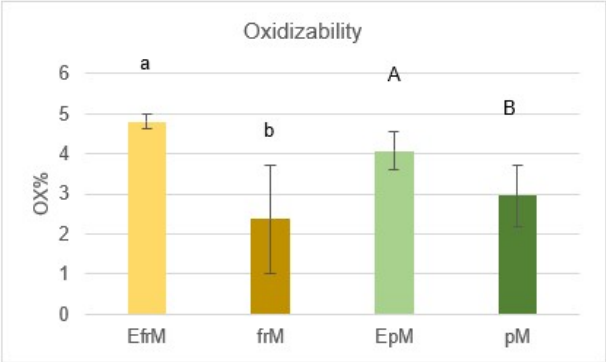


Figure 36: Oxidizability expressed as the average value of the trials conducted in duplicate. Different letters above bars indicate significant differences ($p<0.05$), statistical analysis performed using T student test (95% significance level). Analysis was performed considering enzymatically treated samples versus the not enzymatically treated (controls).

Polysaccharides and acidic polysaccharides resulted present only in EpM and pM samples (Figure 37).

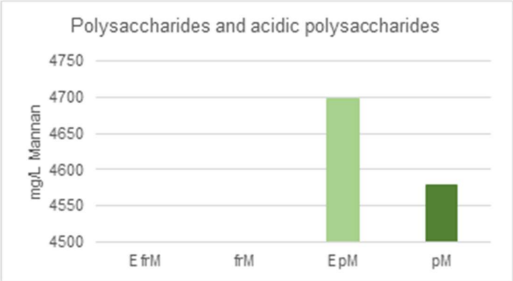


Figure 37: Polysaccharides and acidic polysaccharides expressed as the average value of the trials conducted in duplicate. Different letters above bars indicate significant differences ($p<0.05$), statistical analysis performed using T student test (95% significance level). Analysis was performed considering enzymatically treated samples versus the not enzymatically treated (controls).

The dissolved Oxygen showed not significant differences among the trials (Figure 38).

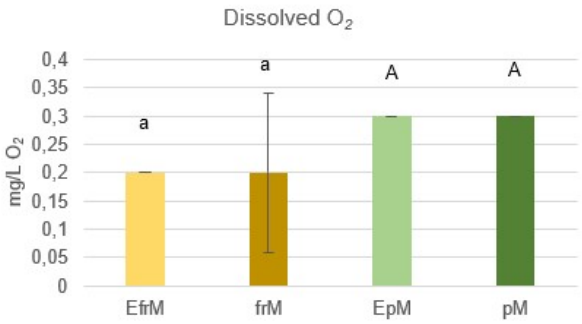


Figure 38: Dissolved O_2 expressed as the average value of the trials conducted in duplicate. Different letters above bars indicate significant differences ($p<0.05$), statistical analysis performed using T student test (95% significance level). Analysis was performed considering enzymatically treated samples versus the not enzymatically treated (controls).

The content of K was significantly affected by the enzymatic treatment. In fact, both the trials treated with LallzymeTM showed significantly higher K values than the corresponding control trials. Low concentrations of K in must can lead to wines with low total acidity. In this case, the enzymatic treatment led to an increment in the extraction of K^+, that had a directly proportional effect on the increment of the total acidity of EfrM and EpM samples (Figure 39).

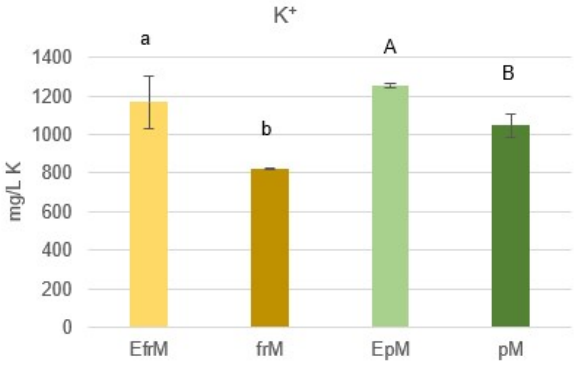


Figure 39: K concentration expressed as the average value of the trials conducted in duplicate. Different letters above bars indicate significant differences ($p<0.05$), statistical analysis performed using T student test (95% significance level). Analysis was performed considering enzymatically treated samples versus the not enzymatically treated (controls).

Part 4a) Organoleptic impact of yeast rehydration on Sauvignon Blanc

Chemical analyses

Sauvignon blanc must was the fermentation that had the longest duration, in fact it ended after 22 days (Figure 40). Although with slight differences in the starting point, in which the DIRECT(S1), DIRECT(H3), and GFPE(Q1) theses stood out, it was the DIRECT(H3) thesis that had the fastest fermentation progress. After the first 6 days of fermentation, the DIRECT(S1) thesis had a slowdown, which allowed the GFSF(Q1) thesis to reach it, even if it started more slowly. The two theses after 15 days of fermentation were then superimposable. The GFPE(SPF52) thesis is, among all, the one that has had the slowest fermentation trend, and it is also the only one that has not completely exhausted the sugar present, leaving a residual sugar content of 8.12 g/L and reaching the lowest alcohol content (13.5% EtOH v/v), approx. 1 degree of alcohol less than the other theses, which settle at the end of fermentation at 14.4-14.6% EtOH v/v. Residual sugars in the other theses were 0.2, 0.07, 0.5, and 0.7 g/L for DIRECT(H3), DIRECT(S1), GFPE(Q1), and GSFS(Q1), respectively.
At the end of fermentation YAN content was < 5 g/L in all the theses. Malolactic fermentation didn't occur, malic acid content was comparable in all the theses remaining more or less at the same initial value found at day 0. In fact, at day 0 the initial malic acid concentration was 1.14 g/L, and the values after 22 days of fermentation were 0.96, 1.06, 1.06, 1.06, and 1.1 for DIRECT(H3), DIRECT(S1), GFPE(Q1), GSFS(Q1), and GFPE(SPF52), respectively.

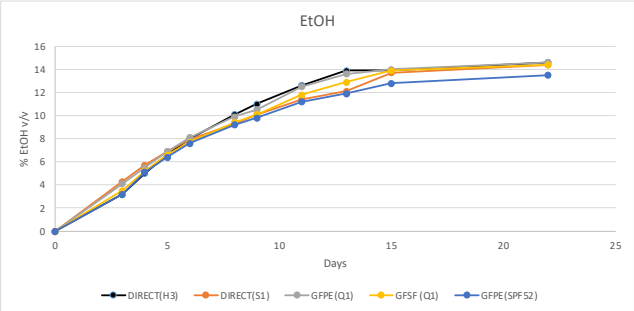


Figure 40: development of Ethanol during the time in different theses

The volatile acidity (Figure 41) of this trial 5 in general is the one that has undergone a minor increase compared to the trials with Friulano grapes , with an increase that didn't exceed 0.2 g/L from the beginning to the end of the fermentation, even if, as we have seen, it is the one that lasted the longest. The final values are < 0.35 g/L, well below the perception and organoleptic limits, therefore these values have no impact on the quality of the final wine. Among the theses, the one that differs the most was the DIRECT(S1) thesis, whose volatile acidity values remained constant from the beginning to the end of the fermentation, settling at 0.16 g/L. Conversely, the thesis that showed the greatest increase was the GFPE(SPF52), with a final value of 0.35 g/L. This is also the thesis, which, as previously described, had the slowest fermentation progress and did not completely exhaust the sugar present, confirming that the yeast could have undergone greater stress than the other theses. However, the values found in the thesis are optimal for the quality of the wine.

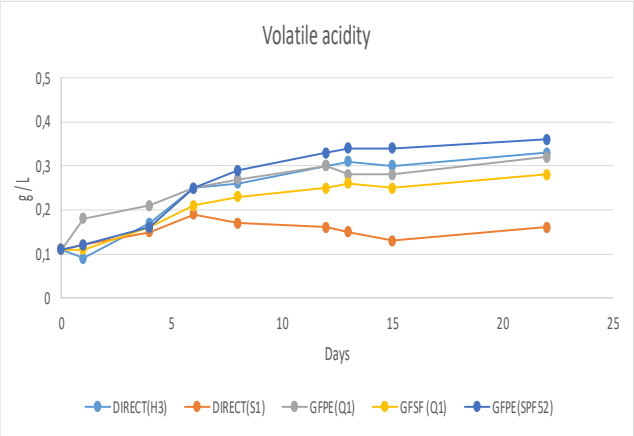
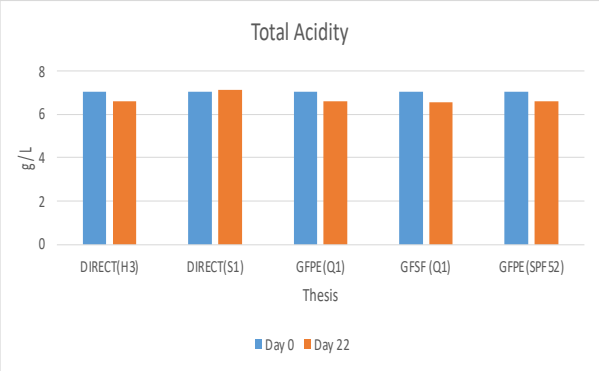


Figure 41: Trend of volatile acidity during alcoholic fermentation in the different theses.

Also, for the parameters of volatile acidity and pH no significant differences were observed, neither between the theses, nor between day 0 and the end of alcoholic fermentation. Total acidity varied from 7.05 at day 0 to 6.7±0.24, whereas pH changed from 3.17 at day 0 to 3.2±0.04 (Figure 43).



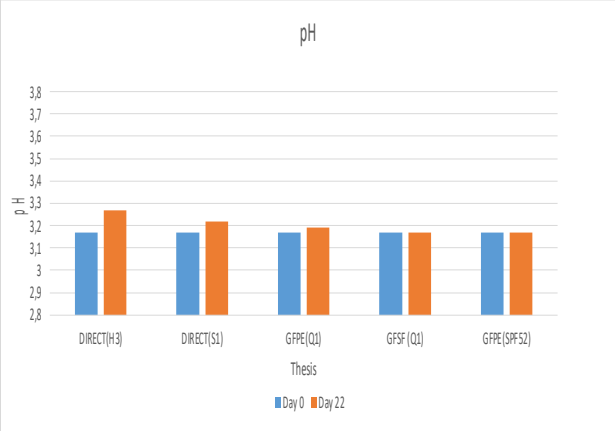


Figure 42: Total acidity and pH at day 0 (must) and at the end of alcoholic fermentation (day 22).

As for the trial on Friulano, also in this case there was an increment in free and total SO_2 during fermentation, in all the theses. Significant differences among the theses per each time of monitoring were not observed (Figure 43). Considering that also in this case no addition was made after yeast starter addition and during alcoholic fermentation, the role of yeast in the production must be taken into consideration.

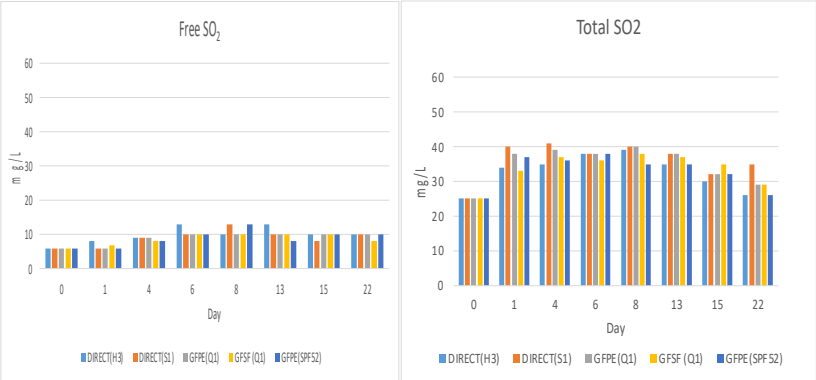


Figure 43: Free and total SO2 concentration during the fermentation

Aromatic compounds analysis

Aromatic profiles and sensory analyses were not performed for thesis GFPE(SPF52)

Higher alcohols

The isoamyl alcohol was the most present in each thesis (Rankine,1967).
Thesis DIRECT(H3),GFPE(Q1) and GFSF(Q1) didn't show differences regarding higher alcohols despite of the various yeast that have been used and the different rehydration techniques (Figure 44).
Thesis DIRECT(S1) had a larger amount of higher alcohols in particular as regards phenylethyl alcohol, this is ascribable to an higher production. The organoleptic impact of higher alcohols depend on their concentration, in fact, when the concentration exceed 400mg/L have a negative impact on the aroma (Lee et al. 2019).

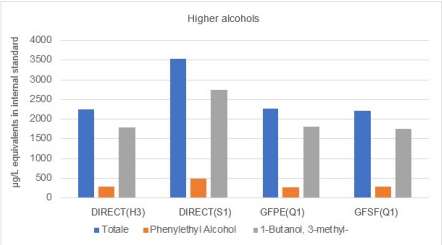


Figure 44: Higher alcohols

MFAs and related esters

As far as MCFAs and esters are concerned (Figure 45), thesis GFPE(Q1) had a higher concentration of esters and an intermediate concentration of MCFAs, this concentration was increased from the use of GFPE additive.
DIRECT(H3) higher percentage of MCFAs, whereas thesis DIRECT(S1) has the lower concentration of esters in particular of ethyl esters

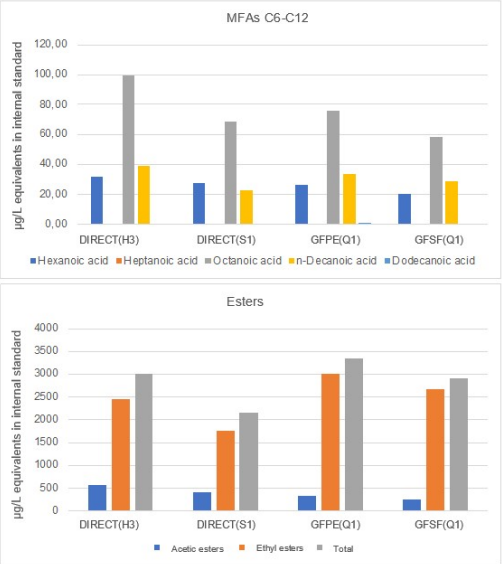


Figure45: MCFAs and their related esters

Terpenes

Thesis GFPE(Q1) and GFSF(Q1) had the higher concentration of terpenes, thesis DIRECT(H3) showed the lower concentration (Figure 46).
Given the importance of the strain on the specific enzymatic activity, in this case the increase in activity observed in thesis GFPE(Q1) and GFSF(Q1) depends on the specific capacities of the strain used.

Commentato [LI29]: Ma queste due tesi non sono state fermentate con lo stesso lievito? Se è così è assodato che l'effetto sugli aromi

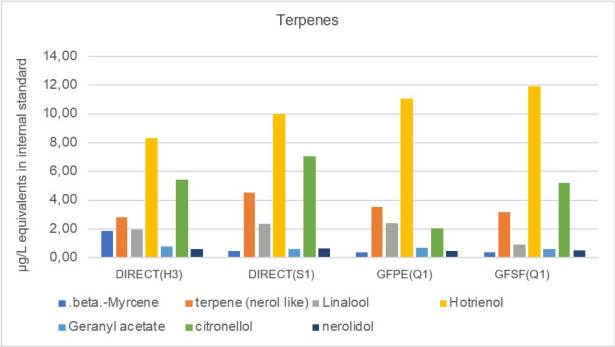
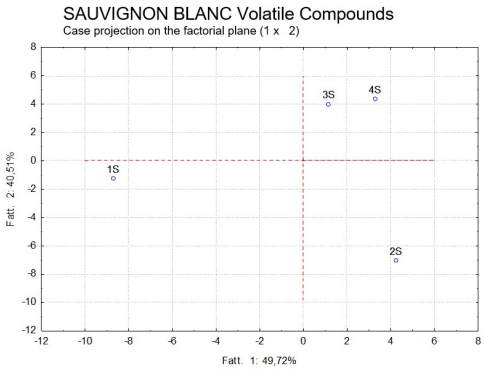
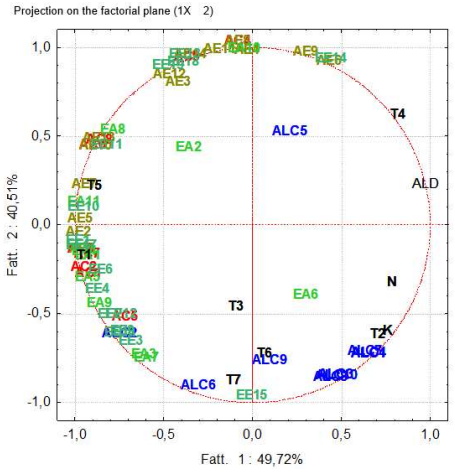


Figure 46: Terpenes

Looking at the results of the PCA analysis (Figure 47), yeast Q1, regardless of the type of additive used in rehydration showed similar profiles, but different from the others, in fact theses GFPE(Q1) and GFSF(Q1) are positioned in the same quadrant and very close to each other, in an area characterized by low aromatic variability, composed of *o*-trienol, few esters and esters of fatty acids. DIRECT(H3) was significantly different from the others and particularly rich in aromatic compounds, this was mainly attributable to the yeast strain, considering that depends on a prevalence of fermentative aromas.

Commentato [LI30]: Nome tesi





Propanoic acid	AC1
Propanoic acid, 2-methyl-	AC2
Butanoic acid	AC3
Butanoic acid, 2 and 3-methyl-	AC4
Hexanoic acid	AC5
Heptanoic acid	AC6
Octanoic acid	AC7
n-Decanoic acid	AC8
Dodecanoic acid	AC9
Acetic acid, pentyl ester	AE1
Hexanoic acid, propyl ester	AE2
Octanoic acid, methyl ester	AE3
Isoamyl hexanoate	AE4
Propyl octanoate	AE5
n-Caprylic acid isobutyl ester	AE6
Decanoic acid, methyl ester	AE7
Octanoic acid, 3-methylbutyl ester	AE8
Undecanoic acid, methyl ester	AE9
Decanoic acid, propyl ester	AE10
n-Capric acid isobutyl ester	AE11
decanoic acid, 3-methylbutyl ester	AE12
Decanoic acid, hexyl ester	AE13
Isoamyl laurate	AE14
1-Propanol, 2-methyl-	ALC1
1-Butanol	ALC2
1-Butanol, 3-methyl-	ALC3
1-Hexanol	ALC4
3-Hexen-1-ol, (E)-	ALC5
1-Propanol, 3-ethoxy-	ALC6
3-Hexen-1-ol, (Z)-	ALC7
1-Propanol, 3-(methylthio)-	ALC8
1-nonanol	ALC9
Phenylethyl Alcohol	ALC10
Dodecanal	ALD
Isobutyl acetate	EA1
Acetic acid, butyl ester	EA2
1-Butanol, 3-methyl-, acetate	EA3
Acetic acid, hexyl ester	EA4
3-Hexen-1-ol, acetate, (E)-	EA5
3-Hexen-1-ol, acetate, (Z)-	EA6
Acetic acid, heptyl ester	EA7
Acetic acid, octyl ester	EA8
Acetic acid, 2-phenylethyl ester	EA9
undecyl acetate	EA10
Lauryl acetate	EA11
Ethyl Acetate	EE1
Butanoic acid, ethyl ester	EE2
Butanoic acid, 2-methyl-, ethyl ester	EE3
Butanoic acid, 3-methyl-, ethyl ester	EE4
2-Butenoic acid, ethyl ester, (E)-	EE5
Hexanoic acid, ethyl ester	EE6
Hex-4-enoic acid, ethyl ester	EE8
3-Hexenoic acid, ethyl ester, (E)-	EE9
3-Hexenoic acid, ethyl ester, (Z)-	EE10
Octanoic acid, ethyl ester	EE11
7-Octenoic acid, ethyl ester	EE12
Nonanoic acid, ethyl ester	EE13
Decanoic acid, ethyl ester	EE14
Butanedioic acid, diethyl ester	EE15
Ethyl 9-decenoate	EE16
Undecanoic acid, ethyl ester	EE17
Dodecanoic acid, ethyl ester	EE18
Tetradecanoic acid, ethyl ester	EE19
2-nonanone	K
β-Damascenone	N
beta-Myrcene	T1
terpene (carylike)	T2
Linalool	T3
Geraniol	T4
Geranyl acetate	T5
citronellol	T6
nerolidol	T7

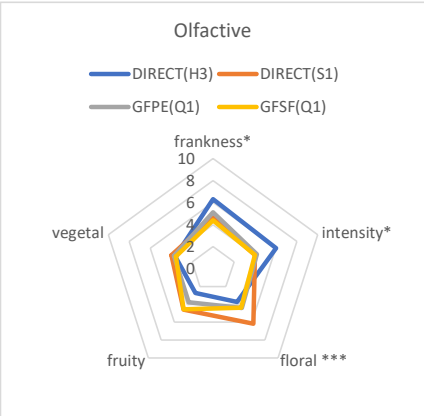
Figure 47: PCA. Legend: 1S, DIRECT(H3); 2S, DIRECT(S1); 3S, GFPE(Q1); 4S, GFSF(Q1).

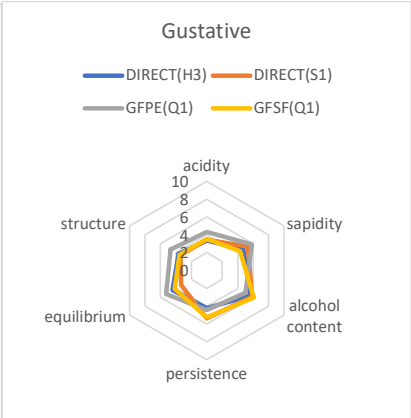
Sensory evaluation

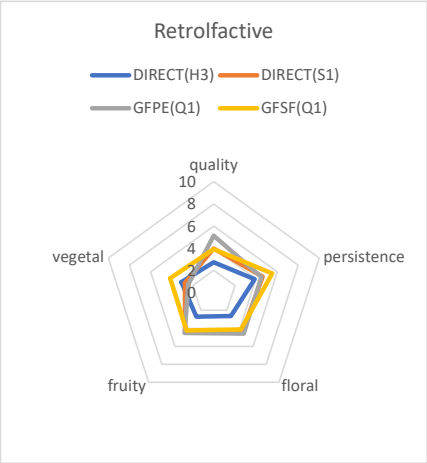
In the olfactive analysis, the thesis DIRECT(H3) showed a greater frankness and intensity than the other theses, however, stand out that had the lower fruity and floral values. Thesis DIRECT(S1) had the highest floral values.

In the gustatory analysis, the values are quite similar in all theses, the thesis three has slightly higher values in balance, structure and acidity.

In the retrolfactive analysis, thesis GFPE(Q1) had the highest quality value. Thesis GFSF had the higher values in persistence and vegetal.
Thesis DIRECT(H3) showed the lowest values (Figure 48).







Trial	V clearness	V color
DIRECT(H3)	8,32a	5,08
DIRECT(S1)	6,12bc	4,99
GFPE(Q1)	7,25ab	4,87
GFSF(Q1)	4,94c	5,87

Trial	O frankness	O intensity	O floral	O fruity	O vegetal
DIRECT(H3)	6,29	6,01	3,73b	2,74	3,61
DIRECT(S1)	4,66	3,99	6,19a	4,58	3,98
GFPE(Q1)	5,12	4,19	4,43b	3,77	3,72
GFSF(Q1)	4,39	3,99	4,38b	4,55	3,53

Trial	R quality	R persistance	R floral	R fruity	R vegetal
DIRECT(H3)	2,72	3,86	2,63	2,7	3,09
DIRECT(S1)	3,97	4,62	4,39	4,43	2,74
GFPE(Q1)	5,13	4,49	4,59	4,53	2,42
GFSF(Q1)	3,9	5,53	4,12	4,22	4,15

Trial	G acidity	G sapidity	G alcohol content	G persistence	G equilibrium	G structure
DIRECT(H3)	3,34	4,75	5,65	4,21	4,41	3,65
DIRECT(S1)	3,45	5,31	5,88	5,36	3,31	3,4
GFPE(Q1)	4,31	5,83	5,02	4,45	5,26	4,7
GFSF(Q1)	3,54	4,31	6,07	5,27	4,13	3,49

Figure 48: “Statistical analysis (anova) is reported next to the descriptor name (* $p<0.05$, ** $p<0.01$ *** $p<0.001$).”

PART 4b) Organoleptic impact of yeast rehydration on Friulano

Chemical analyses

The fermentation trend (Figure 49) was exactly the same in all 4 theses considered. No significant changes in the start of fermentation or variations in its speed over time were found. All the theses dried the sugar present, leaving a sugar residue equal to 0.2 g/L in the GFSF, GFPE(Y), and DIRECT theses, as well as 0.4 g/L in the GFPE(SPF52) thesis. Fermentation was monitored until day 16, but it could have been considered concluded after 12 days. The development of alcohol was in line with what was expected, establishing final values of 14.3 % v/v for the GFSF and Direct theses, and 14.1 % v/v for the GFPE(Y) and GFPE(SPF52) theses.

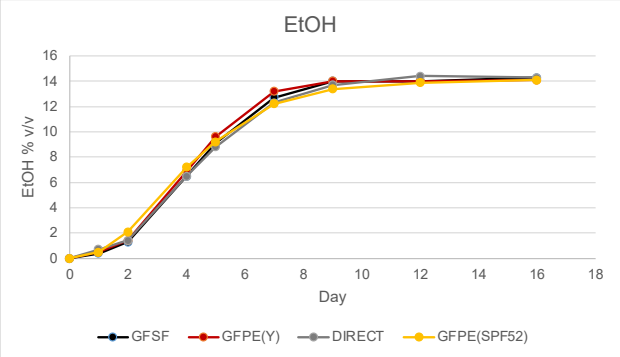


Figure 49: Development of EtOH during the time in the different theses.

No differences were also observed in residual YAN at the end of alcoholic fermentation. Data ranged from 19 mg/L in GFPE(SPF52) and 24 mg/L in GFSF (Figure 50).

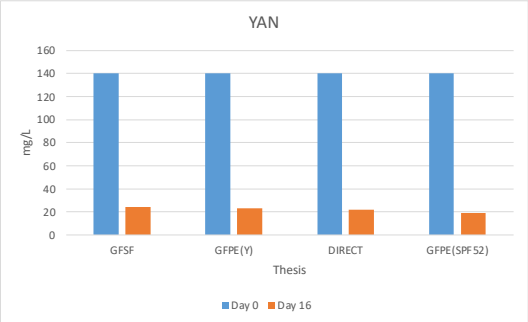


Figure 50: YAN values at day 0 and at the end of alcoholic fermentation (16 days).

Malolactic fermentation didn't occur (Figure 51). Malic acid was 0.73 g/L at day 0 and decreased of values < 0,1 mg/L in the various theses at the end of fermentation. Lactic acid was <0.05 g/L at day 0 and remained under the detection limit also at the end of alcoholic fermentation. Only in thesis GFSF it was revealed at 0.05 g/L.

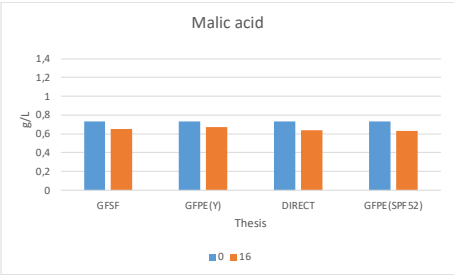


Figure 51: Malic acid values at day 0 and at the end of alcoholic fermentation (16 days).

Volatile acidity (Figure 52, panel A) was found to be similar and lower in the GFSF and GFPE (Y) theses, which in addition to showing superimposable trends during fermentation, also showed similar values at its conclusion, with values of 0.39 and 0.36 g/L, respectively. Micronutrients supplies generally decreased volatile acidity production (Kontkanen, 2004)The same can be said for the DIRECT and GFPE(SPF52) theses, since they have very similar trends too and reach comparable values of 0.45 and 0.43 g/L, respectively. However, the differences found in volatile acidity remain minimal, and in all cases the values reached were below the sensory detectability limit, therefore not influencing the organoleptic characteristics of the wine. Looking at the final values of total acidity (panel B), there are no differences, not even minimal ones, between the different wines obtained from the 4 different rehydrations. The differences found between the theses are in fact not significant, the maximum difference was 0.21 g/L. Compared to the initial total acidity value (3.1 g/L), at the end of fermentation there was a maximum increase for the GFPE (Y) and DIRECT theses of 1.49 g/L, which led to a final value of 4.59 g/L of total acidity. the GFSF thesis showed a value of 4.55 g/L, while the GFPE(SPF52) thesis was the one with the lowest total acidity (4.38 g/L).

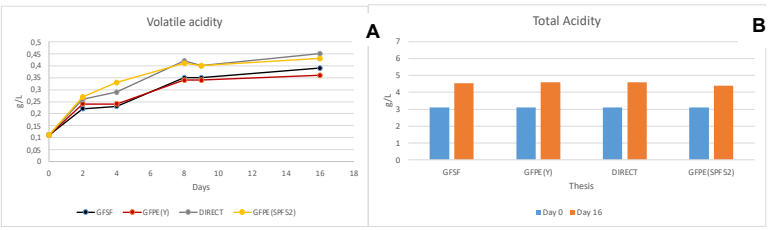


Figure 52: Trend of volatile acidity (A) during alcoholic fermentation in the different theses. In panel B the total acidity at day 0 and at the end of fermentation (16 days) was shown.

According to the results described, also the pH didn't show any significant difference among the theses. Considering the variability of the samples, no differences can also be attributed to the initial pH value of the must and the final pH of the abtained wines. pH remained at values of 3.76±0.1.

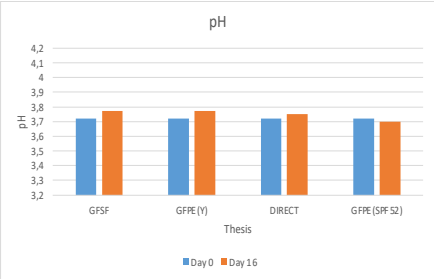


Figure 53: pH values at day 0 and at the end of alcoholic fermentation (16 days).

Looking at the monitoring of free SO_2 and total SO_2 (Figure 54), it can be observed that in all theses there was an increase during fermentation compared to day 0. Since there were no intermediate additions, it is possible to attribute the production of SO_2 to the metabolic activity of the inoculated yeast to conduct alcoholic fermentation.

Figure 54:Free and total SO2 concentration during the fermentation

Aromatic compounds analyses

Higher alcohols, MCFAs and related esters

Higher alcohols (Figure 55) appear to be similarly affected by the addition of the two different rehydration additives, in fact thesis GFPE(Y) had a higher amount of this compounds.
The use of GFPE produce a similar effect on the different yeast this was true for the MCFAs and esters. Thesis GFPE(Y) and GFPE(SPF52) had a similar organoleptic profile. Thesis DIRECT, which was the thesis with direct inoculum, had a higher amount of ethyl esters and MFAs (Figure 56).

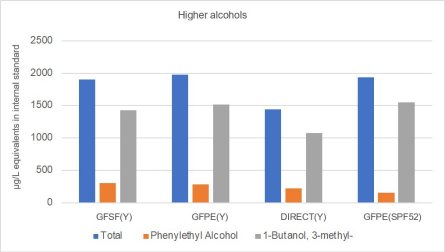


Figure 55: Higher alcohols and focus on the major representants.

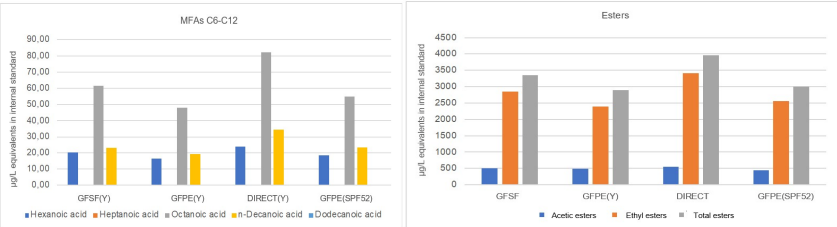


Figure 56: MCFAs and related esters

Terpenes

Although thesis GFPE(Y) and thesis DIRECT were fermented by the same yeast, so the same enzymatic activity, we can observe that the different rehydration technique influences the amount of free terpen. DIRECT was characterized by higher level of β-myrcene considering thesis GFSF and GFPE(Y) (Figure 57). The latter showed higher amount of *o*-trienol and geranyl acetate respectively responsible for linden and fruity aroma. Thesis GFPE(Y) and GFPE(SPF52), added by GFPE, showed the major increase of terpenes.

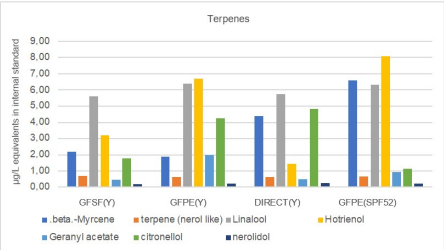


Figure 57: Terpenes composition

Considering PCA analyses (Figure 58), thesis GFPE(Y) and GFPE(SPF52) turned out to the same axis, highlighting that, despite of the different yeast, the same type of rehydration can lead to similar results. Vice versa, strain Y used in direct inoculum showed a more complex and intense aromatic profile in respect to the other types of rehydration, although in the other thesis a major terpenes component emerged.

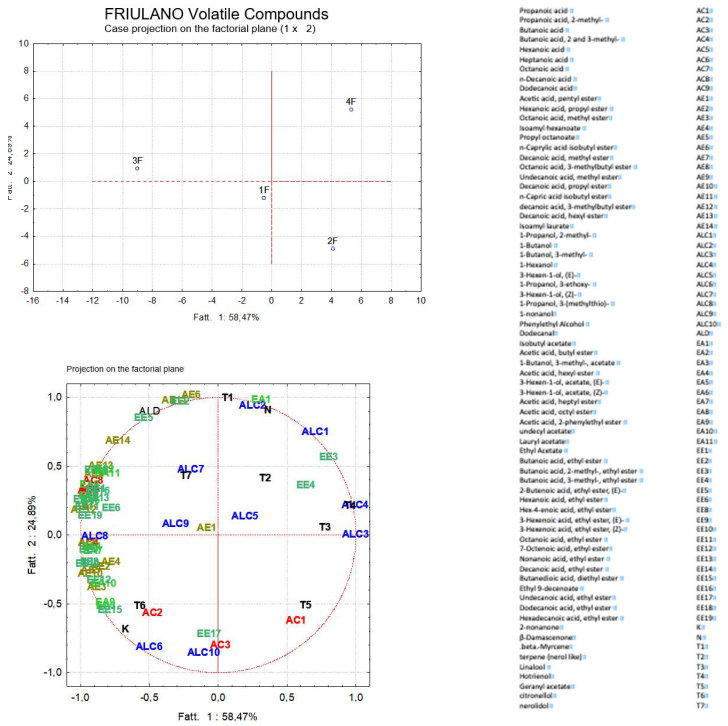
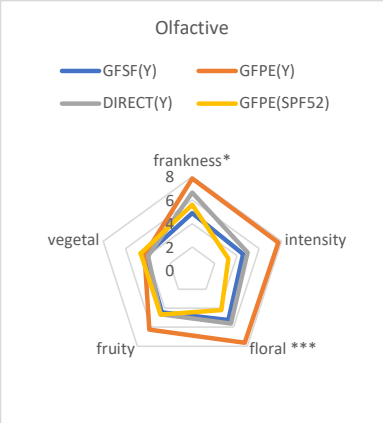
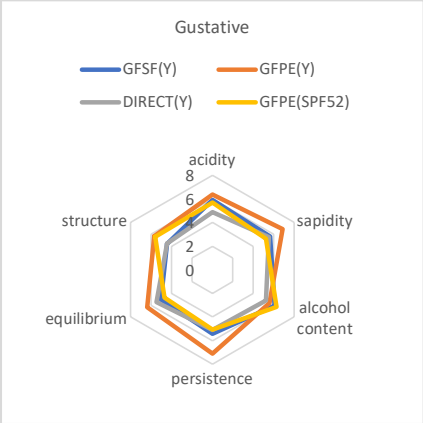


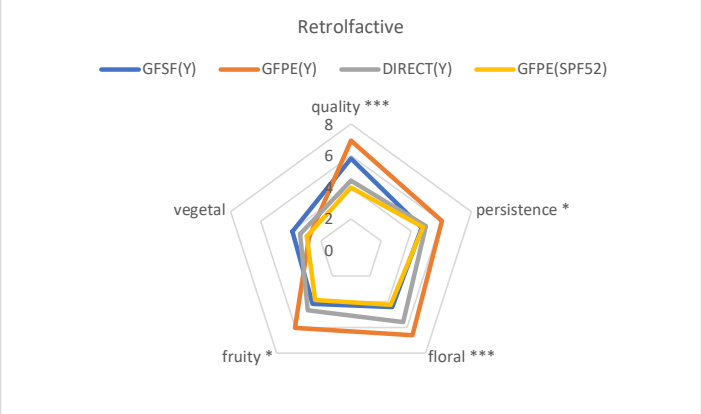
Figure 58: PCA. Legend: 1F, GFSF(Y); 2F, GFPE(Y); 3F, DIRECT(Y); 4F, GFPE(SPF52)

Sensory evaluation

The olfactive analysis showed that thesis GFPE(Y) had higher values of frankness, intensity, floral, fruity. Theses GFSF(Y) and DIRECT(Y) had similar values for all descriptors except for the frankness. Thesis GFPE(SPF52) has lower values of intensity, floral, fruity than the others (Figure 60).
For the gustatory aspect the highest values were observed in thesis number GFPE(Y) with values of acidity, sapidity, persistence, balance higher than the other thesis.
Regarding the retrolfa perception, the thesis GFPE(Y) had the following parameters higher than all the other theses: quality, persistence, floral, fruity. GFSF(Y) showed higher vegetal values. Comparing with Sauvignon Blanc wines, the results were quite different, probably linked with the different strains that were used. However, the spider graphs show that GFPE additive give more sapidity, structure and quality to the wines.


Olfactive
GFSF(Y)
GFPE(Y)
DIRECT(Y)
GFPE(SPF52)
frankness*
intensity
floral ***
fruity
vegetal
8
6
4
2
0


Gustative
GFSF(Y)
GFPE(Y)
DIRECT(Y)
GFPE(SPF52)
acidity
sapidity
alcohol content
persistence
equilibrium
structure
8
6
4
2
0


Retrolfactive
GFSF(Y)
GFPE(Y)
DIRECT(Y)
GFPE(SPF52)
quality ***
persistence *
floral ***
fruity *
vegetal
8
6
4
2
0

Trial	v clearness	v color
GFSF(Y)	7,541456	2,95b
GFPE(Y)	7,965871	5,24ab
DIRECT(Y)	7,176067	6,47a
GFPE(SPF52)	7,67078	5,01ab

Trial	O frankness	O intensity	O floral	O fruity	O vegetal
GFSF(Y)	4,90b	4,56bc	5,23b	4,447856	4,171771
GFPE(Y)	7,84a	7,71a	7,63a	6,244647	4,295327
DIRECT(Y)	6,65ab	4,95b	5,62b	4,650214	3,982732
GFPE(SPF52)	5,60ab	3,24c	4,19b	4,654774	4,64293

Trial	R quality	R persistence	R floral	R fruity	R vegetal
GFSF(Y)	5,92044	5,667302	6,103728	5,42ab	5,025639
GFPE(Y)	6,365106	6,864733	5,606356	7,11a	6,374821
DIRECT(Y)	4,885057	5,534053	5,223372	5,11ab	5,458339
GFPE(SPF52)	5,690227	5,249876	6,284462	5,07b	4,701087

Trial	G acidity	G sapidity	G alcohol content	G persistence	G equilibrium	G structure
GFSF(Y)	4,433712	5,82ab	4,70ab	4,41b	4,17ab	3,893644
GFPE(Y)	5,693803	6,95a	6,04a	6,60a	6,04a	2,803918
DIRECT(Y)	4,447154	4,41bc	4,97ab	5,59ab	4,65ab	3,378415
GFPE(SPF52)	5,583742	3,98c	4,76b	4,23b	3,85b	2,939084

Figure 59: “Statistical analysis (anova) is reported next to the descriptor name (* $p<0.05$, ** $p<0.01$ *** $p<0.001$).

CONCLUSIONS

The use of these microbiological approaches during winemaking to improve wine quality is becaming even more important due to the awareness of costumers, that gaives more importance to the sustainability of the industries. For these reasons these results obtained with the present work can be summarized as follow:

(1) biological acidification of Glera using different strains of *Lachancea thermotolerans*, thesis LA72 and LD were the most effective. Thesis LA72 showed the lowest amount of ethanol, it showed a decreased of 0.4 % v/v compared to the control. In addition, thesis LA72 and LD produced the highest concentration of lactic acid 3 g/L and 3.5 g/L respectively, this is also confirmed in the sensorial analyses. However, thesis LA48, LB and LC showed show a lower production from 0.5 g/L to 0.76 g/L. The production of the high production of lactic acid in thesis LA72 and LD it's reflected on a lower pH and a higher total acidity. Thesis inoculated with *L. thermotolerans* showed lower amounts of higher alcohols, stend out that the permanence of the strain *L. thermotolerans* A 48 h, had not increased the concentration of superior alcohols. In addition, generally thesis inoculated with *L. thermotolerans* had an higher amount of β-damascenone, expect for thesis LA72, strain LD show the highest amount of terpenes.

(2) Sauvignon Blanc natural acidification and bio-protection with *L. thermotolerans* and *Metschnikovia pulcherrima*, respectively that LA thesis had a significant production of lactic acid despite of less abundant then in Glera trials, the pH is lower than CTRL about 0.15 pH units. This confirm that biological acidification is extremely variable not only between the strains but is also related to the grapes and the permanence of the yeast. For this reason, yeast selection process is necessary to menage winemaking and to reach similar results as using chemical products such as tartaric acid. As concern MX thesis showed a decrease of malic acid according to other papers. As concern for sensorial analyses this thesis showed higher fruity and floral notes. Organic acids were higher in thesis LA.

(3) impact on yield and quality of must using pectolytic enzyme's (enzyme Lallzyme™): the effect on the sedimentation aid was positive, because it allowed to reduce the clarification times and to obtain a stable must. The yield increased in the pressed must above 6.49%, and the total increment considering also the free run must it's above 6.2%.

The catechin content was lower in thesis treated enzymatically this is linked to the less oxidizability o. As concern for chemical parameters only the total acidity showed significant differences, this is linked to the higher extraction of potassium in thesis treated with enzymes. The total polyphenol index resulted significantly lower $p<0.05$ in the enzymatically treated samples.

(4) Organoleptic impact of yeast's acclimatation on Sauvignon Blanc and Friulano grapes. MFAs and ethyl esters were influenced by GFPE addition in Sauvignon Blanc grapes. In addition, the additives increased the terpenes concentration in Sauvignon Blanc. Thesis DIRECT(H3) had more aromatic compounds, this is attributable to the yeast strain, and was also verified to floral component during sensorial analyses. Despite the higher ratio of aromatic compound in DIRECT(H3) the use of the additives during acclimatation, in particular GFPE, showed better quality, frankness, sapidity, equilibrium and

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persistence. As concern for Friulano grapes the use of GFPE decreased the volatile acidity production. Moreover, the addition of GFPE influenced the terpen liberation, according to the Sauvignon thesis. Despite of the higher complexity of aroma founded during the evaluation of aromatic profiles of DIRECT(Y) compared to GFPE(Y) the sensorial analyses showed a different trend

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