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Chemical and sensory evaluation of Merlot wines fermented by mixed cultures of *Lachancea thermotolerans* and *Saccharomyces cerevisiae*

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Summary

Topic position & objectives: Excessive ethanol and insufficient acidity are the key challenges in winemaking considering the trends in increasing sugar and decreasing acidity in grapes/musts due to global warming. The species *Lachancea thermotolerans* yeast has been researched recently due to its high yields of lactic acid and low yields of ethanol. The present work tested the performance of five strains of *L. thermotoleran*s in mixed fermentation with *S. cerevisiae.*

Methods: *Vitis vinifera* L. cv. Merlot grapes were fermented with mixed cultures of *L. thermotolerans* and *S. cerevisiae* in sequential and co-inoculation regimes, alongside a pure culture *S. cerevisiae* and an un-inoculated treatment set as controls. Yeast growth was monitored by plate counts, fermentation metabolites were analyzed by High Performance Liquid Cromatography (HPLC), colorimetric properties by CIELab coordinates and sensory analysis was conducted by RATA.

Results: Tested yeast treatments affected wine profiles. pH dropped drastically, especially in the sequential inoculations. Ethanol content was lower in sequential inoculations compared to pure culture *S. cerevisiae*. Sequential inoculations also had significantly higher titratable acidity, explained by high level of lactic acid. Low levels of acetic acid (mean: 0.35 g/L) were confirmed in all the cases. CIELab showed visible differences ($\Delta E^* > 2$) between all the treatments, and sensory analysis confirmed the differences observed in the chemical profiles of wines, in terms of acidity and alcohol.

Main conclusions: *L. thermotolerans* yeast has enological advantages and may be used in mixed cultures with *S. cerevisiae*. The five different strains showed different behavior regarding both the fermentation kinetic and the yield of metabolites produced. Furthermore, the *S. cerevisiae* inoculation timing influenced these aspects. The appropriate strains and inoculation regimes can therefore be targeted by winemakers to manage the characteristics of wines.

Keywords: *Lachancea thermotolerans*, sequential inoculation, non-*Saccharomyce*s, mixed fermentation, lactic acid

Resumo

Posição do tópico e objetivos: O etanol excessivo e a acidez insuficiente são os principais desafios na vinificação, considerando as tendências no aumento do açúcar e na diminuição da acidez nas uvas devido ao aquecimento global. A espécie *Lachancea thermotolerans* tem sido pesquisada recentemente devido à sua alta produção de ácido láctico e baixos rendimentos de etanol. O presente trabalho testou o desempenho de cinco estirpes de *L. thermotoleran*s em fermentação mista com *S. cerevisiae.*

Métodos: *Vitis vinifera* L. cv. Merlot foram fermentadas com culturas mistas de *L. thermotolerans* e *S. cerevisiae* em regimes sequenciais e de co-inoculação, juntamente com uma cultura pura *S. cerevisiae* e um tratamento não inoculado definido como controle. O crescimento da levedura foi monitorado pela contagem de placas, os metabolitos de fermentação foram analisados pela HPLC, as propriedades colorimétricas pelas coordenadas CIELab e análise sensorial foram realizadas pela RATA.

Resultados: Os tratamentos testados do fermento afetaram perfis do vinho. O pH deixou caiu drasticamente, em especial nas inoculações seqüenciais. O teor de etanol foi menor em inoculações sequenciais em comparação com a cultura pura *S. cerevisiae*. As inoculações sequenciais também apresentaram acidez titulável significativamente maior, explicada pelo alto nível de ácido láctico. Baixos níveis de ácido acético (média: 0,35 g/L) foram confirmados em todos os casos. O CIELab mostrou diferenças visíveis ($\Delta E^* > 2$) entre todos os tratamentos e a análise sensorial confirmou as diferenças observadas nos perfis químicos dos vinhos, em termos de acidez e álcool.

Principais conclusões: *L. Thermotolerans* tem vantagens enológicas e pode ser usada em culturas mistas com *S. cerevisiae*. As cinco estirpes diferentes apresentaram comportamento diferente em relação à fermentação cinética e ao rendimento dos metabolitos produzidos. Além disso, o timing de inoculação *de S. cerevisiae* influenciou esses aspectos. Os regimes adequados de estirpes e inoculação podem, por conseguinte, ser alvo de produtores de vinho para gerir as características dos vinhos.

Palavras-chave: *Lachancea thermotolerans*, inoculação sequencial,*não-Saccharomyce*s, fermentação mista, ácido láctico

Resumo estendido

Os principais desafios na produção de vinho em regiões de vinho temperado quente são o alto nível de pH combinado com uma concentração excessiva de açúcares nas parcelas; um problema exacerbado no contexto das alterações climáticas.
Entre as várias soluções que podem ser usadas, cada vez mais o interesse está na abordagem microbiológica. Em outras palavras, o uso de microrganismos, para a inoculação da fermentação alcoólica, que têm menor produção de etanol e maior em termos de acidez total.
Várias leveduras *não-Saccharomyces* são consideradas candidatas promissoras para esse fim.
O uso combinado de *Saccharomyces* e não-*Saccharomyces*, em fermentações mistas, é um tema extremamente interessante no mundo do vinho de hoje. O maneio da fermentação mista está principalmente preocupado com modos de inoculação e condições de crescimento.
A este respeito, nos últimos anos, *Lachancea termotolerans* (anteriormente *Kluyveromyces termotolerans)* tem sido reconhecida como um fermento não-*Saccharomyces* relevante para a produção de vinho. Sua característica mais notável é a alta produção de ácido láctico a partir de piruvato, uma reação catalisada pela enzima lactato desidrogenase.
Este estudo avaliou o desempenho e a influência, em vinhos acabados, de cinco estirpes (três comerciais e duas experimentais) de *Lachancea thermotolerans* em fermentações mistas com *Saccharomyces cerevisiae.* Os testes foram comparados com uma fermentação pura de Saccharomyces *cerevisia* e com fermentação espontânea. A variabilidade entre as estirpes e a comparação com os controles foram testadas tanto no regime simultâneo quanto na inoculação sequencial, totalizando doze tratamentos diferentes.
A vinificação em tinto foi feita a partir de *Vitis vinifera cv* Merlot em pequena escala (2,5 L); Todo o experimento foi realizado na Hickinbotham Roseworthy Experimental Cellar (HRWSL) no Departamento de Pesquisa em Viticultura e Enologia da Universidade de Adelaide, Austrália.
A análise estatística dos principais parâmetros químicos nos vinhos revelou diferenças significativas ($p< 0.\ 0001$) nas concentrações de todos os metabolitos medidos. No geral, o controle levou a níveis mais elevados de etanol, menor glicerol, maior pH, acidez total e menor ácido láctico do que os tratamentos de cultura mista. Este último teve influência sobre os vinhos acabados, em que uma queda acentuada no pH (até -0,4 pontos) e aumento da acidez total (máximo: 6,06 g/L), explicada pela alta produção de ácido láctico característico do fermento. Como resultado, o teor de etanol também mudou, particularmente em inoculações sequenciais em comparação com a cultura de controle puro (média: -0,84% v/v). Baixos níveis de ácido

acético (média: 0,35 g/L) foram confirmados em todos os casos, exceto na fermentação espontânea.

A cinética da fermentação do álcool foi mais lenta em fermentações sequenciais do que em fermentações simultâneas e cultura de controle puro. A maior densidade populacional (10 cfu/ml) foi observada no cultivo puro de *S.cerevisiae.*

As cores dos vinhos acabados foram analisadas tanto no pH padronizado quanto no pH original dos vinhos, em ambos os casos, os tratamentos influenciaram a intensidade e a coloração, e os dados do CIELab mostraram diferenças visíveis ($\Delta E^*> 2$) de todas as provas.

A análise sensorial, realizada pelo método RATA, confirmou as diferenças observadas nos perfis químicos do vinho, particularmente em termos de acidez total, volátil e álcool.

L. thermtolerans tem vantagens e pode ser usado em culturas mistas com *S. cerevisiae.* As cinco estirpes diferentes apresentaram comportamento diferente tanto na cinética de fermentação quanto no rendimento dos metabólitos produzidos. Além disso, o seu impacto no perfil químico e sensorial do vinho dependeu muito do momento da inoculação de *S. cerevisiae.* Na verdade, os resultados mostram uma maior concentração de metabolitos característicos de *L. thermotolerans* numa inoculação sequencial. Condições e regimes de inoculação adequados podem então ser considerados pelos produtores de vinho para gerir as características finais dos vinhos.

São necessários mais estudos e avaliações analíticas para aprofundar ainda mais a influência da dinâmica da fermentação do açucar em ácido láctico e no desenvolvimento de componentes voláteis. Também seria interessante expandir a pesquisa no contexto das fermentações de vinho espumante.

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Abbreviations

LAB	lactic acid bacteria
MLF	malo lactic fermentation
AF	alcoholic fermentation
LT, *L.thermotolerans*	*Lachancea thermotolerans*
g/L	grams per liter
mg/L	milligrams per liter
SC, *S.cerevisiae*	*Saccharomyces cerevisiae*
YAN	*yeast assimilable nitrogen*
DAP	diammonium phosphate
cv.	cultivar
CFU	colony forming units
ppm	parts per millions
%v/v	alcohol content by volume

1. Introduction

Wine is a result of complex microbiological interactions**.** It is produced from grape fermented by either indigenous (i.e. already present in the vineyard and/or in the winery) or externally added microorganisms. However, the whole wine microbiota contributes to the winemaking and yeasts and lactic acid bacteria (LAB) play the main role (Capozzi *et al*., 2015), each involved in a winemaking phase: the alcoholic fermentation (AF) and the malolactic fermentation respectively (MLF; Petruzzi *et al.,* 2017).

AF is conducted by yeasts, predominantly of the *Saccharomyces cerevisiae* species. During AF, the pool of enzymes of the yeast transforms the pyruvic acid formed during glycolysis into acetaldehyde. This aldehyde is then reduced to ethanol (Fig.1), liberating CO_2. Glycolysis and the subsequent AF enables an energy gain for yeasts metabolism (Ribéreau-Gayon *et al.,* 2004) and along with ethanol and CO_2, a wide range of secondary metabolites impacting wine bouquet is also formed during AF. *S. cerevisiae* is the main 'wine' yeast due to the better fermentation performance developed during millennials of natural selection. Contrarily, the presence of non-*Saccharomyces* yeasts in fermentations has traditionally been associated with problematic fermentation and high levels of acetic acid and other off-flavours. Nowadays, researchers and winemakers are considering non-*Saccharomyces* species as an alternative to *S. cerevisiae* to manage current problems in wine industry, i.e. high ethanol, low acidity and aromatic uniformity.

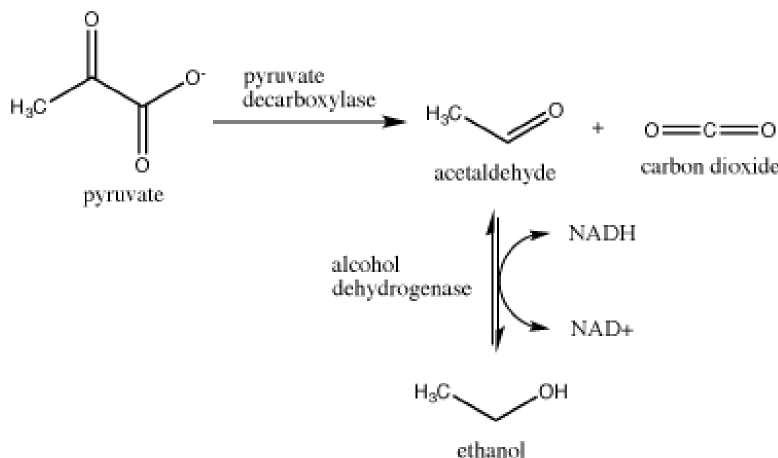

Figure 1. Alcoholic fermentation

The combined use of *Saccharomyces* and non-*Saccharomyces* yeasts in mixed fermentations is an extremely interesting topic in the current wine world. The management of the mixed fermentations concerns the evaluation of how they can be carried out, especially regarding the inoculation modes and the growth conditions. For instance, the level of nutrients presents in the matrix consisting mainly of sugars, YAN and vitamins (Medina *et al*., 2012). *Saccharomyces* has a faster consumption of carbon and nitrogen than non-*Saccharomyces* yeasts (Albegaria *et al*, 2003), and is more efficient in producing biomass with the same nutrients (Andorrà *et al*, 2012; Medina *et al*., 2012). Temperature and oxygenation are other important variables which need monitoring mainly in relation to the toxicity of ethanol (Ribéreau-Gayon, 2006). Different works with non- *Saccharomyce*s in mixed and mono-cultures have shown that *S.cerevisiae* is more resilient with high temperature and it grows faster than non-*Saccharomyces* at 25° Celsius, but under 15°C with both of them the fermentation had a slowdown (Wang *et al.*, 2014). *S. cerevisiae* strains can grow rapidly in anaerobic conditions, but it is not the same for non-*Saccharomyces* yeasts such as *Hanseniaspora, Kloeckera* and *Torulaspora* (Vissers *et al*., 1990). Finally, other factors to consider are the killer factors produced (Medina *et al.*, 2012).

1.1 Climate change and winemaking

Over the last decades, grapevine physiology and, consequently, organoleptic characteristics of wines have been shifted due to climate change. The major effects of climate change are clear and has led to an increase of temperatures, a decrease on average rainfalls, and extreme weather events (van Leeuwen *et al*., 2016). These factors are critical throughout the grapevine biological cycle and they affect the ability to achieve the optimal ripeness of grapes for winemaking, in terms of an adequate balance between sugar and acid, the wine flavours of wine or colour. For example, the lack of low temperature during dormancy season can cause inhomogeneity of budburst. Heat waves during blooming can cause an early veraison, the abortion of the berries and the failure to ripen, while an excessive heat or extended sun exposure during maturation can compromise the balance between technological and phenolic maturity (Jones *et al*., 2005).

Indeed, climate modelling predict for the middle of the century an increase of temperatures ranged between 2°C to 4°C °C in the wine-making region of Bordeaux (www.ipcc.ch). Likewise, in California the sugar content in wine grapes at harvest meanly increased from 21.4 degrees Brix in 1980 to 23.3 degrees Brix in 2008 (Alston *et al*., 2011). As hot weather

accelerates the ripening of grapes, an early harvest could irreversibly alter the iconic flavour of the most famous wines worldwide.

Most notable examples of climate change effect are increased accumulation of sugars in berries, and faster degradation of organic acids (Jones *et al*., 2005). As a consequence, levels of ethanol and pH in wines have been progressively rising (Benito *et al*., 2016, Van Leewen *et al.,* 2016). Therefore, the higher the temperature, the more the accumulation of sugar and, concomitantly, the faster degradation of organic acids. Under these circumstances, the winemaking process is impacted hugely, and the chemical composition of musts can increase the risk of failed fermentations and microbiological stability. In fact, high sugar and ethanol can cause stuck or sluggish AF and MLF and low acidity can promote proliferation of spoilage organisms. Because of that, chemical end sensory parameters are compromised by a potential increase of acetic acid or ethyl acetate level, that together make up the volatile acidity, a major aroma fault of wines. The strategies proposed so far to contain these problems in the wineries can be unaffordable or too expensive. Finally, is also important consider the consumer acceptance, which is being increasingly difficult to achieve (Jones *et al.*, 2005; Van Leeuwen *et al*., 2016).

1.2 Mitigation strategies for high sugar level and low acidity

Different strategies are used to overcome a high sugar level and a low acidity of must. Firstly, the viticultural approach aims to limit the effects of climate change directly on the metabolic processes of grapevines. It includes an optimization of varieties/clones which accumulate less sugars and/or better retain acidity (Soar *et al*., 2006; Tramontini *et al*., 2013), a shift to cooler grape-growing regions (Bonfante *et al.,* 2018), an early pick of grapes, the implementation of altered canopy management (Baiano *et al.,* 2015, Silvestroni *et al*., 2019), such as application of nets and clay-based products (e.g. kaolin; Shellie *et al*., 2015), or the optimization of the pruning regimes in terms of period and winter and spring load in order to determine a higher competition between carbon sinks and sources on vines (Bravetti *et al*., 2012).

An oenological approach can be also applied to counteract the effects of climate change in wines. In relation to wine acidity and pH, the most common practice is the acidification of wines, normally achieved by the addition of tartaric acid, but is not the sole and other more sophisticated techniques are also used for wine acidification, such as electrodialysis (El Rayess *et al.,* 2016) or cation exchangers. The decrease in sugars (and consequently ethanol) can be

achieved by dilution with water or earlier harvest wines (Schelezki *et al.*, 2018) or the post-fermentative dealcoholisation (Varavuth *et al.,* Diban *et al.*, 2008). For the last, three main techniques are proposed: vacuum distillation, spinning cone column and membranes (dialysis, pervaporation, reverse osmosis, nanofiltration; Garcìa-Martìn *et al.*, 2012; Ferrarini *et al.,* 2013).

A further winemaking approach for of overly high sugars and pH relies on microorganisms. The microbiological approach aims to attain a lower ethanol level and a higher acidity by the inoculation of microorganisms that yield less ethanol and more acids. Promising candidates for this purpose are different non-*Saccharomyces* yeasts.

1.3 Non-*Saccharomyces* yeasts

In the past, non-*Saccharomyces* yeasts were considered either inconsequential or adverse for wine quality. Nowadays, selected strains of non-*Saccharomyces* yeast are commonly used as a starter in wine industry (Padilla *et al.*, 2016).

Until now, about 40 non-*Saccharomyces* yeast species have been cultured from grape juice/must, including those belonging to genera *Candida, Debaryomyces, Hanseniaspora, Hansenula, Metschnikowia, Pichia, Schizosaccharomyces, Torulaspora and Zygosaccharomyce*s (Jolly *et al*., 2014; Tab.1). These species generally predominate during the onset of fermentation in wine must, and when alcoholic content exceeds 5–7% (v⁄v), *Saccharomyce*s species become predominant not only due to their higher ethanol tolerance but also thanks to a range of other traits that helps them outcompete other microbes present in the must (Esteve-Zarzoso *et al*., 1998; Romancino *et al*., 2008).

Indigenous species are found mostly on the grapes however their presence in the cellar equipment have been also reported, albeit in lower quantities (Martini *et al*., 1996). Non-*Saccharomyces* yeasts coming from the grapes can contribute to fermentation and the wine overall quality. In particular, some studies demonstrated that that the veraison-damaged grapes exhibit higher total yeast counts and a much greater diversity of species (Barata *et al*., 2008, 2012).

The selected strains can be used as a starter culture in two different inoculation modalities: co-inoculation or sequential inoculation. The co-inoculation of non-*Saccharomyces* strains foresees the simultaneous addition of a *Saccharomyces spp*, thus creating an important competitivity among species that leads to a lower development of the non-Saccharomyces. The

addition of *Saccharmoyces* is deferred to a later step in sequential inoculation. thus, allowing the settlement of non-*Saccharomyces* inoculum and more evident effect on the chemical and sensory profile of wines (Gobbi *et al*., 2013).

1.4 Influence of non-*Saccharomyces* yeasts on ethanol level

Several studies reports a lower content of ethanol in mixed fermentations conducted with some non-*Saccharomyces* yeasts (Ciani and Maccarelli, 1998; Ciani and Comitini, 2011; Magyar *et al.,* 2011; Bely *et al.,* 2013; Jolly *et al.,* 2014), determining a difference on the alcohol in wines that ranges 0.2-2% (v/v) (Soden *et al.,* 2000; Jolly *et al.,* 2014; Contreras *et al.* 2014). The lower production of ethanol is caused by an increase of secondary metabolites like glycerol, succinic acid, acetic acid and lactic acid. The concentrations of those metabolites vary between different genera and species of non- *Saccharomyces* yeasts and the effect on wine quality is variable not only depending on the viticultural and oenological management, but also on microbiological choices (Jolly *et al.,* 2014).

1.5 Influence of non- *Saccharomyces* on acidity

Each species of non-*Saccharomyces* yeast has its own metabolic pathways; therefore, they can have an acidification or deacidification activity more or less marked depending on the yeast specie used for the fermentation. For examples *L. thermotolerans* is known to produce high concentration of lactic acid and *C. zamplinina* yields high succinic acid, both contributing to increased titrable acidity (Ciani & Ferraro, 2008; Su *et al*., 2014). thers (e.g. *Schizosaccharomyces* spp) can instead degrade organic acids (malic acid or gluconic acid), thereby deacidifying wine during the fermentation (Thornton *et al.,* 1996). Volatile acidity is also an important component modulated by yeasts during fermentation. Non-*Saccharomyces,* in particular *T. delbruekii* and *C. zamplinina* produce lower acetic acid (Bely *et al*., 2008; Soden *et al.,* 2000), in contrast to some *Hanseniaspora spp* (apiculate) and *Zygosaccharomyces* spp that are reported to produce high amounts (Ciani & Picciotti, 1995; Loureiro & Malfeito-Ferreira, 2003; Jolly *et al.,* 2014).

Table1 Comparison of metabolic activity of several oenologically relevant non- Saccharomyces species (higher activity, ↓: lower activity, // similar activity; compared to S. cerevisiae performance)

SPECIES	ETHANOL	ACIDITY	FLAVOUR	REFERENCES
Torulaspora delbrueckii	↓ ethanol	↓ volatile acidity	↑ Succinic acid ↑ Linalool	Moreno *et al.*, 1991; Renault et al., 2009; Belda *et al*., 2015
Metschnikowia pulcherrima	↓ ethanol	↑ acidification -volatile acidity: strains dependent	↑ Terpenes ↑ Varietal thiols ↑ esters	Jolly *et al.*, 2003; Sadoudi *et al.*, 2012 Contreras *et al.*, 2014
Candida zemplinina	↓ ethanol	↓ volatile acidity	↑ Glycerol ↑ Succinic acid	Ciani & Maccarelli, 1998; Soden *et al*., 2000
Hanseniaspora species	↓ ethanol	↑ volatile acidity (apiculate)	↑ 2-phenyl-acetate ↑ Heavy sulphur compounds ↑ Ethyl esters ↑ Medium chain fatty acid	Moreira *et al.*, 2008; Viana *et al.*, 2009
Zygosaccharomyces species	//	↑ volatile acidity	↑ Polysaccharides ↓ higher alcohols -Often involved in spoilage	Romano *et al.*, 1993; Loureiro& Malfeito-Ferreira, 2003; Domizio *et al.*, 2011
Schizosaccharomyces species	↓ ethanol	↑ deacidification ↓ L-malic acid	↑ Acetaldehyde, Propanol, 2,3-butanediol ↑ polysaccharides	Peinado *et al.*, 2004; Benito *et al.*, 2013
Lachancea thermotolerans	↓ ethanol	↑acidity ↑ L-lactic acid	↑ Glycerol ↑ Terpenes ↑ Ethyl esters	Kapsopoulou *et al.*, 2007; Gobbi *et al.*, 2013; Jolly *et al.*, 2014; Hranilovic *et al.*, 2017; 2018.

1.6 Influence of non-*Saccharomyces* on wine aroma/flavour and mouthfeel

Wine aroma compounds can be grouped on the basis of their origin: primary aromas are derived from grapes, secondary aromas are developed during the fermentation and tertiary aromas appear during wine ageing (Swiegers *et al*., 2005). Therefore, different yeast species and strains can influence primary and secondary aromas. In the case of primary aromas (terpenes and thiols the most important examples), some non-Saccharomyces yeasts have strong β-glycosidase and β-lyase activities, which allow their release from non-volatile precursors. (Swangkeaw *et al*., 2011). Thus, mixed fermentations can produce wines with a higher concentration either of terpenols (Garcia *et al*., 2002; Sadoudi *et al*., 2012) or volatile

thiols (Zott *et al*., 2011). Certain non-Saccharomyces yeasts are considered as“flavour-producing species” (H. anomala, H. uvarum), as they can increase concentrations of a number of flavour-active metabolites like esters(Van Zyl *et al*., 1963; Moureira *et al*., 2008; Sadoudi *et al*., 2012). Higher alcohols are also important metabolites for wine secondary flavours; different strains can produce different amount of high alcohols. Generally, at lower concentration, of higher alcohols in wines confer complexity to wine aroma, however at higher amounts are considered to cause off-flavours (Ribéreau-Gayon *et al*., 2004; Romano *et al*., 2003; Moreira *et al*., 2005).

Several non-Saccharomyces yeasts can produce high glycerol concentrations during fermentation, potentially contributing to smooth mouthfeeling (Ciani & Maccarelli, 1998; Soden *et al*., 2000). However, high concentrations of glycerol in S. cerevisiae can be strongly connected to an increase in acetic acid and volatile acidity (Prior *et al*., 2000). This problem can be avoided by using mixed fermentations with S. cerevisiae. Some non-Saccharomyces can also produce succinic acid, which can contribute to total acidity levels, and potentially also boost flavour complexity by increasing the “saltiness” (Ferraro *et al*., 2000). Other metabolites also impacted by the use of non-Saccharomyces species are sulfur compounds and polysaccharides, they can give respectively off-flavours (Moreira *et al*., 2008) and positive mouthfeel (Vidal *et al*., 2004) About the sensory influence of yeast metabolites on wine it is important to note that chemical concentrations of flavours compunds may be affected but may not be recognized/ rated by testers in a sensory panel, for this reason it is important have either trained panelists or a large number of participants (Danner *et al*., 2017).

1.7 *Lachancea thermotolerans*

Over the recent years, *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) has been recognized as a non-*Saccharomyces* yeast relevant for oenological practices. Taxonomically, it belongs to the genus *Lachancea*, which currently contains twelve other species (Lachance and Kutzman, 2011). Its relevance is highlighted in the current context of climate change as *L. thermotolerans* abundantly produces lactic acid from sugars via lactate dehydrogenase (Fig.2, 3) with a consequent decrease in ethanol level, an increase in titratable acidity and a decrease in pH (Kapsopoulou *et al.,* 2007; Gobbi *et al*., 2013; Jolly *et al*., 2014; Hranilovic *et al.,* 2018).

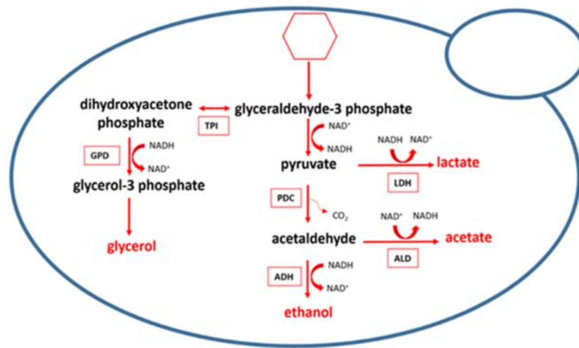

Figure 2. Overview of sugar catabolism in yeast cells

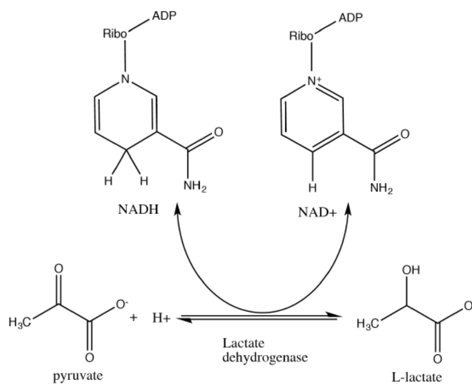

Figure 3. Lactic acid fermentation: conversion of pyruvate to lactic acid by lactate dehydrogenase

In the oenological environments, *L. thermotolerans* is a glucophilic yeast and a relatively strong fermenter. *L. thermotolerans* has similar needs of nitrogen as *S. cerevisiae:* to not stop or retard the fermentation, at least 150-200 mg./L of yeast assimilable nitrogen (YAN) (Kemsawasd *et al.,* 2015). However, as typical for *non-Saccharomyces* yeasts, alcoholic fermentation conducted by pure *L. thermotolerans* culture cannot reach completion without co-inoculation or sequential inoculation of *S. cerevisiae* (Jolly *et al.,* 2014). Previous studies have shown that mixed fermentations with *L. thermotolerans* increase titratable acidity, glycerol and a range of flavour-active compounds (e.g. 2-phenylethanol), while pH, ethanol and volatile acidity decrease. (Comitini *et al.,* 2011, Gobbi *et al.,* 2013; Jolly *et al*., 2014 Hranilovic *et al*., 2018). In general, in sequential inoculations the initial absence of *S. cerevisiae* allows for the maximised proliferation and metabolic involvement of the non-*Saccharomyces* strains (Ciani *et al*. 2016).

Compared to *S. cerevisiae*, *L. thermotolerans* is a lower ethanol, glycerol, succinic acid and acetic acid producer and on the other hand, it is a great producer of lactic acid. According to

previous works, *S. cerevisiae* ethanol production could reach 15-16% (v/v), also producing about 8-10 g/L of glycerol, up to 1 g/L of acetic acid and only 0.5 g/L of lactic acid (Ribéreau-Gayon *et al.,* 2006). As is showed in tab 2, *L. thermotolerans* ethanol concentration maximum tolerance is 10.6% (v/v), the mean of glycerol produced is 5.4 g/L, acetic acid is about 0.20 g/L, but is important to point out the high level of lactic acid, which can reach 12 g/L. Nevertheless, all the main metabolites showed in the table 2, are subjected to a significant strain effect (Hranilovic *et al.,* 2018).

Table 2. Analytical properties of Chardonnay wines (initial sugars 236 g/L and 3.5 pH) fermented by 94 different L. thermotolerans strains (Adapted from Hranilovic et al., 2018).

Parameter	Minimum	Maximum	Mean
Consumed sugar (g/L)	161.6 ± 1.4	223.4 ± 2.9	199.6 ± 11.4
Glucose (g/L)	3.7 ± 0.5	36.2 ± 0.7	13.6 ± 6.2
Fructose (g/L)	8.9 ± 1.3	39.1 ± 1.6	23.2 ± 6.0
Ethanol (% v/v)	7.3 ± 0.7	10.6 ± 0.7	9.3 ± 0.9
Ethanol yield (g/g)	0.34 ± 0.04	0.40 ± 0.03	0.37 ± 0.03
Glycerol (g/L)	3.9 ± 0.1	8.0 ± 0.2	5.4 ± 0.6
Glycerol yield (g/g)	0.0205 ± 0.0004	0.0478 ± 0.0002	0.0274 ± 0.0039
Lactic acid (g/L)	1.8 ± 0.2	12.0 ± 0.2	5.8 ± 2.3
Lactate yield (g/g)	0.0086 ± 0.0011	0.0658 ± 0.0018	0.0291 ± 0.0119
Acetic acid (g/L)	0.06 ± 0.01	0.32 ± 0.01	0.20 ± 0.05
Acetate yield (mg/g)	0.30 ± 0.05	1.53 ± 0.03	0.98 ± 0.24
Pyruvic acid (mg/L)	13 ± 1	78 ± 3	44 ± 14
Malic acid (g/L)	3.0 ± 0.1	4.1 ± 0.2	3.6 ± 0.3
pH	3.16 ± 0.03	3.81 ± 0.13	3.44 ± 0.03

Recent studies focused on intra-specific diversity of *L. thermotolerans* (Hranilovic *et al,* 2017; 2018) have evaluated a large number of different isolates (about 200) from different geographical origin and ecological niche. Out of 136 unique genotypes (Hranilovic *et al*. 2017), 94 strains were evaluated in Chardonnay fermentations (Hranilovic *et al.* 2018) and twenty of them were selected, from enological environments, based on their interesting winemaking properties (ethanol yield, acidity and growth parameters). Until now, some strains seem to have more relevance for alcoholic fermentation. Some strains are already available on the market (e.g. Levulia Alcomeno® by AEB group, IT; Concerto™ by Chr. Hansen, TR and Laktia™by Lallemand, FR). Besides, there are two isolates from University of Bordeaux and University of Foggia, and they seem to have superior performance in AF, considering short lag-phase and pH decrease (Hranilovic *et al*., 2017; 2018; Tab.3).

In general, former studies, mixed fermentations with *L.thermotolerans* yeast strains affects positively the overall quality of the wine produced from musts with low acidity (Benito *et al.,* 2016). Indeed, the contribution of *L.thermotolerans* is either on fermentation analysis or on sensory analysis; thus the sensory evaluation of samples from different fermentations has

shown a better aroma quality and overall impression in wines fermented with strains of *L.thermotolerans* (Benito *et al.,* 2016). However, little is known on the effect of different *L.thermotolerans* strains on malolactic fermentation.

Table 3. Main winemaking characteristics of some commercial L.thermotolerans strains.

STRAIN	WINEMAKING APPLICATIONS	INOCULATION TIME	REFERENCES
Levulia Alcomeno®	↓ ethanol ↑ total acidity, ↑ lactic acid ↓ volatile acidity Alcohol tolerance:7.2-10.5 % (v/v) N demand: medium	• Sequential inoculation (24-72 hours before *S. cerevisiae)*	www.aebgroup.com
Concerto™	↑ flavour complexity and intensity (esters) ↓ ethanol Recommended for reds Alcohol tolerance 10% (v/v) N demand: medium	• Co-inoculation • Sequential inoculation -24 hours before *S. cerevisiae* inoculation (high temp) - 48 hours before *S. cerevisiae* inoculation (low temp.)	www.chr-hansen.com
Vinilfora® Melody™	blend of 3 yeast strains (*Saccharomyces.cerevisiae, Lachancea thermotolerans, Torulaspora delbrueckii*) ↑ fruity flavours (thiols, esters) ↓H_2S ↓volatile acidity Alcohol tolerance 17% (v/v) N demand: medium	dry mixed cultures, standard inoculation	www.chr-hansen.com
Laktia™	↑ aromatic complexity ↑ total acidity, ↑ lactic acid ↓ volatile acidity Recommended for reds Alcohol tolerance: 10% (v/v) High tolerance high temperature	Sequential inoculation (24 hours before *S. cerevisiae)*	www.lallemandwine.com

2. Materials and methods

2.1 Vineyard and winemaking

The experimental *Vitis vinifera* L. Merlot clone D3V14 vineyard is located within the perimeter of the Waite Agricultural Research Institute (Urrbrae, SA 5064. Coordinates: 34°58'03.1"S 138°37'59.9"E). South Australia is a moderate climate region with hot/dry summer, the average annual daily temperature and rainfall are is 18 -20 °C and 600-800 mm.

The soil is managed by spontaneous cover crops maintained by irrigation, mowing operations (twice per year) and one herbicides (glyphosate) treatment per year under the plants. All the vines are on their own roots, the pruning system used is cordon spur with two nodes per spurn and 30-40 nodes per vine. The plant density is 1,8 m between vines and 3 m of row space. A rotation system of sulfur-based and systemic products in rotation is used against powdery mildew.

Grapes were hand-harvested on March 7th, 2019 to be de-stemmed and crushed in the University of Adelaide's Hickinbotham Roseworthy Wine Science Laboratory (HRWSL) with the addition of 50 ppm of SO_2 as potassium metabisulfite (PMS). Juice was then separated from the skins using a basket press and diluted from 16 to 14.5 °Beaumé using distilled water. To ensure proper homogenization of the initial matrix with a consistent solids-to-liquid ratio, each fermenter (5 L buckets with lids) was filled with 80% solids and 20% juice.

The buckets were placed in a temperature-controlled room at 20°C temperature and inoculated with yeasts preapared as described below. Daily cap management involved gentle punch downs. After 14 days of maceration wines were pressed of the skins into 2 L bottles to complete the fermentation. Wines were stabilized at 0 degrees, hand-bottled and stored until further analysis.

2.2 Grape must and additions

The initial parameters of the must were: 14.5 ° Beaumé and pH 3.9. The final sugars level was diluted with distilled water. The initial yeast assimilable nitrogen (YAN) level was measured using enzymatic kits (Primary Ammino Nitrogen Assay (K-PANOPA) and Ammonia Assay K-AMIAR) Megazyme International, Wicklow, Ireland) Total YAN concentration was calculated by the sum of ammonium (mg/L) and the concentration of the primary amino nitrogen (PAN; mg/L). The initial YAN level was increased from 80 mg/L to 180 mg/L with diammonium phosphate (DAP 10% solution). Further nitrogen additions were carried out after

120 hours from the first inoculation, in form of 50 mg/L DAP and 30 mg/L of NUTRISTART® AROM (Laffort, FR) based on yeast auto lysates, inactive yeast, DAP and thiamine. (Arrows in fig.6).

2.3 Yeasts treatments and inoculation procedure

All strains used in the trial were grown from glycerol stocks stored at -80 °C. This includes both experimental and commercial strains. Prior to the study, the latter were rehydrated according to the manufacturer's instructions to be streaked out for single colonies which were stored in glycerol. The strains were revived on a YPD plates (1% yeast extract, 2% bacto-peptone, 2% glucose and 2% agar) incubated at 24 °C for 3 days. Single colonies were then grown overnight in YPD broth (24° C) with shaking. The cultures were transferred into a diluted grape juice (45 % water, 5 % YPD) at 10^7 cells/mL for an overnight incubation. The achieved cell densities were determined with flow cytometer (Guava® easyCyte™ 12HT, Merck, NJ, USA) to achieve the inoculation densities reported below (Tab 4).

Table 4. Experimental details of the fermentation with 12 different yeast treatments and different inoculation

TREATMENT NAME	SPECIES	INOCULATION MODALITY	INOCULATION DENSITY	INOCULATION TIME	COLOUR CODE
SC	*S.cerevisiae*	pure culture| control	$2* 10^6$	Day 1	Black
LT_E1X	*L.thermotoleras+S.cerevisiae*	Co-inoculation	$3*10^6+10^6$	Day 1	Pink
LT_E1…	*L.thermotoleras+S.cerevisiae*	Sequential inoculation	$2*10^6+10^6$	Day 1… Day 2	Pink
LT_E2X	*L.thermotoleras+S.cerevisiae*	Co-inoculation	$3*10^6+10^6$	Day 1	Brown
LT_E2…	*L.thermotoleras+S.cerevisiae*	Sequential inoculation	$2*10^6+10^6$	Day 1… Day 2	Brown
LT_C1X	*L.thermotoleras+S.cerevisiae*	Co-inoculation	$3*10^6+10^6$	Day 1	Yellow
LT_C1…	*L.thermotoleras+S.cerevisiae*	Sequential inoculation	$2*10^6+10^6$	Day 1… Day 2	Yellow
LT_C2X	*L.thermotoleras+S.cerevisiae*	Co-inoculation	$3*10^6+10^6$	Day 1	Light blue
LT_C2…	*L.thermotoleras+S.cerevisiae*	Sequential inoculation	$2*10^6+10^6$	Day 1… Day 2	Light blue
LT_C3X	*L.thermotoleras+S.cerevisiae*	Co-inoculation	$3*10^6+10^6$	Day 1	Green
LT_C3…	*L.thermotoleras+S.cerevisiae*	Sequential inoculation	$2*10^6+10^6$	Day 1… Day 2	Green
UN-INOC	-	Un-inoculated			Gray

Five *L.thermotolerans* were trialled in co-inoculation and sequential inoculation with *S. cerevisiae*.. the mixed cultures were compared with the control treatment (pure *S. cerevisiae* inoculum) and an Un-inoculated treatment. Each treatment was tested in triplicate, for a total of 12 treatments and 36 different ferments.

Fermentations were done in triplicate in 5 L pails with 2 L of juice and 0.5 Kg of solids. After crushing, the must was separated from the solids, in order to have in each bucket the same liquid/ solid percentage (80%/20%). The alcoholic fermentation was conducted in 20 °C room. Sugars, pH and temperature were checked once a day after a light punch down.

The inoculation scheme can be explained as follow: *S.cerevisiae,* in the control trial, was inoculated in a concentration of $2x10^6$ cfu/mL. Five *L. thermotolerans* strains were tested in two modalities: co-inoculation and sequential inoculation with *S. cerevisiae*. For the co-inoculum, *L.thermotolerans* was inoculated at concentration of $3x10^6$ CFU/mL and, at the same time, the *S. cerevisiae* was inoculated at10^6 CFU/mL. Meanwhile, for the sequential inoculation trial, *L.thermotolerans was* inoculated at concentration of $2x10^6$ cfu/mL and, after 44 hours, the inoculum was completed with 10^6 CFU/mL of *S. cerevisiae*(Tab.4). The first implantation of the yeasts was evaluated on day 3 as described below.

2.4 Mcrobiological analysis

Microbiological analysis was performed under aseptic condition on day 0, 2, 7, 14, 21, 28 by plating the diluted samples onto theYPD agar (1% yeast extract, 2% bacto-peptone, 2% glucose and 2% agar) coloured with an indicator which enables the visual differentiation of *S. cerevisiae* and *L. thermotolerans* colonies. Samples were diluted within a range of 10^{-3} and 10^{-5} using sterile 0.9 % NaCl solution, and the volume used for spead plating was 100 µL. were used. The colonies were counted after 5 - 7 days of incubation at 24 °C.

Microscopic observation was also performed (Fig.4). *L. thermotolerans* strains have spherical to ellipsoidal cells that are slightly smaller than those of *S. cerevisiae*, with dimensions of approximately 3– 6×6–8 µm (Benito *et al.,* 2018).

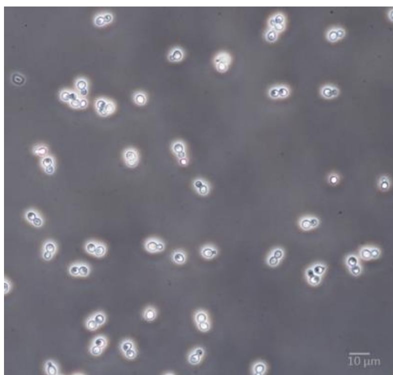

Figure 4. Microscopic observation of Lachancea thermotolerans cells

2.5 Chemical analysis

The alcoholic fermentation progress was monitored daily by the automatic density meter DMA 35 (Anton Paar, Graz, Austria). During the last phase of the alcoholic fermentation the residual total sugars were checked by an enzymatic kit (K-FRUGL enzymatic kit, Megazyme, Ireland) performed using a 96-well plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland).

At the end of the alcoholic fermentation, the final ethanol concentrations were determined with an alcolyzer (Anton Paar, Graz, Austria). pH and titratable acidity (TA) were determined by pH meter (CyberScan 1100, Eutech instruments, Thermo Fischer Scientific, MA, USA) and auto titrator (Mettler Toledo T50, Ohio, USA), respectively. Total and free SO_2 were measured by an aspiration/titration method (VA1/SO2 -4 places, GlassChem, Stellenbosch), according to the official method (Type II) proposed by the International Organization of Vine and Wine (OIV, 2012).HPLC was used to determine the final concentration of glucose, fructose, ethanol, glycerol and organic acids (lactic, acetic and, malic). An Agilent 1100 (Santa Clara, CA, USA) instrument was equipped with an HPX-87H column (300 mm × 7.8 mm, BioRad, Hercules, CA, USA). The eluent was 2.5 mM H_2SO_4 with a flow rate of 0.5 mL/min and the column temperature was 60 °C for a 35-minute run time. The injection volume was 20 µL and signals were detected using an Agilent G1315B diode array detector (DAD, organic acids; 210 nm) and G1362A refractive index detector (RID, hexoses and alcohols) from injections. Analytes were quantified using the external calibration curves ($R2 > 0.99$) in ChemStation software (version B.01.03).

The phenolic analysis was performed using modified Somers method (Mercurio *et al.*, 2007) in 96-well plates and UV-VIS spectroscopy technique (Infinite 200 PRO, Tecan, Männedorf, Switzerland).

The following parameters are evaluated using this method: wine colour density, wine hue (shift from red to brown), concentration of total anthocyanins, degree of ionisation (percentage of total anthocyanins present in coloured forms), SO_2 resistant pigments and total phenolics.

2.6 CIELab analysis

According to the OIV method (OIV, 2006), the chromatic characteristics of the wines were defined by CIELab coordinates: clarity (L*), red/green colour component (a*), and blue/yellow colour component (b*); and by its derived magnitudes: chroma (C*), tone (H*) and chromacity [(a*, b*) or (C*, H*)]. (OIV,2006).

The spectrophotometric measureaments were carried out at a wavelength of 300 and 800 nm, with illuminant D65 and observer at 10º in glass cuvettes with 10 mm of optical thickness. Distilled water in the same type of cuvettes was used to establish the base line. All the calculations were made by Microsoft Excel (Microsoft Office 365, en-us Version 16.0.10730.20348).

2.7 Sensorial analysis

Rate-All-That-Apply (RATA) method was used to conduct the sensorial analysis on the obtained wines. This method is a variation of the more commonly used CATA question format. For a pre-specified list of attributes, panellists indicate whether they apply to the given wine, and if they do so, to rate their intensity on a point intensity scale(Meyners *et al.*, 2016).Prior to RATA, an expert panel tasted the wines to confirm the absence of fault in wines and the consistency between the replicates, as well as to define the appropriate list of attributes.

A set of 12 samples was evaluated by a panel of 47 wine consumers. The age range was 18-47, 62% of panelists were females, and 88% consumes red wine at least once per fortnight. Moreover, l but 2% were enrolled/completed wine-related degree and/or had wine-industry experience. All the panelists were recruited by e-mail in the University of Adelaide environment and gave a written informed consent prior to the tasting. Wine samples (30 mL) were served in a randomized order in black 215 mL ISO tasting glasses at room temperature. Prior to serving, triplicates of each treatment were blended so that panelists were presented one sample of each treatment. Distilled water and crackers were provided as palate cleaners for panelists during a 60 second break between each sample. Data were collected using Red Jade software (2016, Redwood City, USA). The panelists used with a 7-point intensity scale, from “extremely low” to “extremely high”, to rate 43 attributes in Merlot wines (Tab.5). During the RATA test. we added a separate section concerning the acidity descriptors. In this part. it was required to describe the acidity feelings choosing between four given attributes (i.e. flat/flabby. bright/crisp. sour/tart. harsh/acrid).

Table 5. List of attributes for RATA analysis

Aroma	**Flavour**	**Taste**
dark fruit	dark fruit	sweetness
red fruit	red fruit	acidity
dried fruit	dried fruit	bitterness
jammy	jammy	hotness
confectionery	confectionery	body
chocolate	chocolate	astringency
cooked vegetables	cooked vegetables	balance
earthy/dusty	earthy/dusty	length of fruit flavours
floral/perfume	floral/perfume	length of non-fruit flavours
herbaceous	herbaceous	length of acidity perception
medicinal/rubbery	medicinal/rubbery	**Acidity descriptors**
pepper	pepper	flat/crisp/tart/harsh
savoury	savoury	
spices	spices	
oxidation, acetaldehyde	oxidation, acetaldehyde	
volatile acidity	volatile acidity	

2.8 Statistical analysis

Data was subjected to analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) post-hoc test in XLSTAT (version 2019.2.1.58999; Addinsoft, Paris, France). For RATA, panelists were selected as a random factor. All statistical analyses were performed at 5% level of significance (p value < 0.05). Principal component analysis (PCA) was performed using Rstudio, Version 1.2.1335 (RStudio Inc, Boston MA, USA).

3. Results

3.1 Yeast growth

The five strains of *L. thermotolerans* were used for Merlot fermentation both in sequential inoculations and co-inoculations with *S. cerevisiae* strain ZYMAFLORE®SPARK and their fermentation performance were compared with control fermentations inoculated with the sole *S. cerevisiae* strain. The population dynamics were regularly monitored throughout fermentation for quantification of both *L. thermotolerans* and *S. cerevisiae* (Fig. 5).

In most co-inoculations, *L. thermotolerans* showed a rapid population decline. Conversely, the same strains in sequential inoculations had a slower growth, but a longer lifespan. During the first two days, *S. cerevisiae* control population had a faster growth rate, after 7 days we could

clearly see the growth spurt in all the strains. In the co-inoculated ferments, *L. thermotolerans* population was undetectable at the end of the fermentation. In sequential inoculations, *L. thermotolerans* population was at day 21 maintained at around 10^6 CFU/mL, corresponding to slightly slower fermentation rate and population decline. The proliferation of *L. thermotolerans* strains had an impact on the *S. cerevisiae* growth in mixed-treatments, which, in fact, was lower than in the control monoculture, possibly due to competition for nutrients.

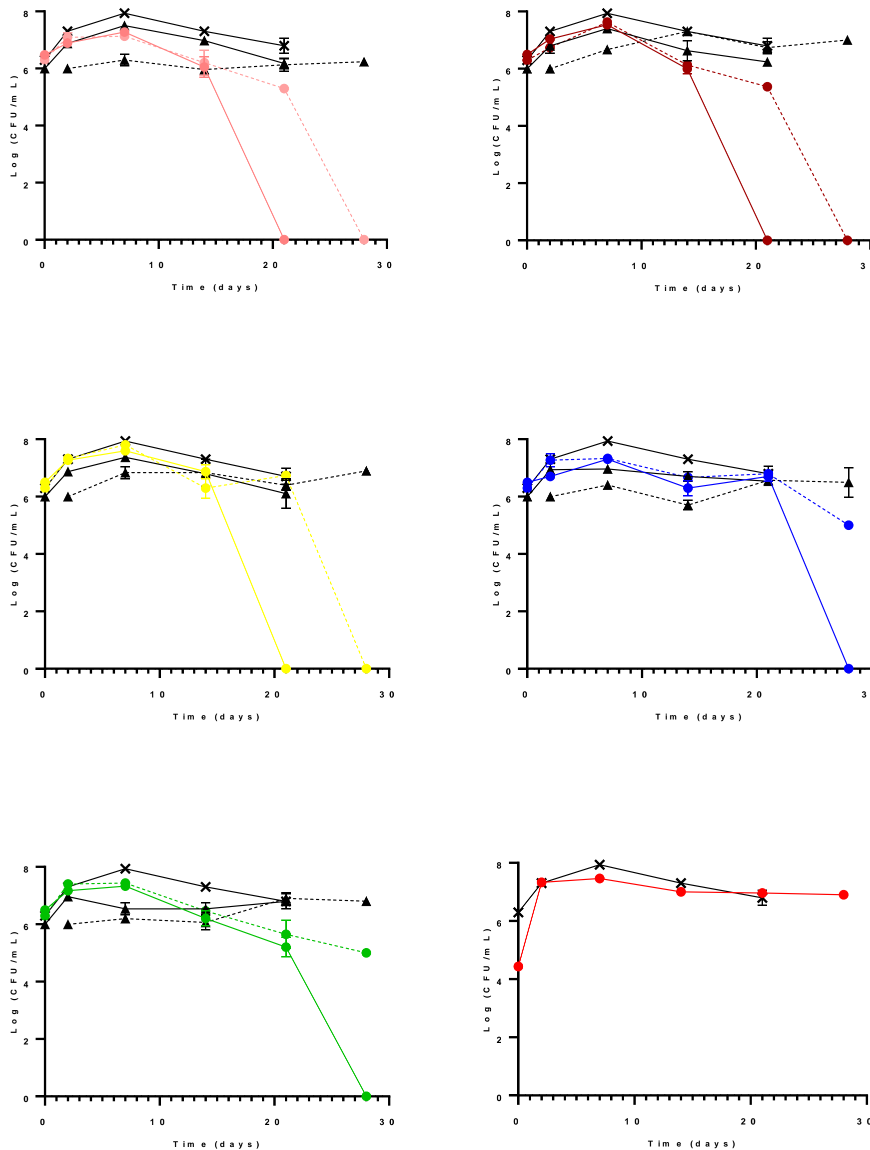

*Figure 5. Population dynamics for five L. thermotolerans strains in sequential and co- inoculation regimes and a S. cerevisiae (SC) control. Population dynamics in uninoculated trial (last graph). The initial growth of the cultures was monitored via flow cytometry and then by plate counting. Each graph represents one strain in mixed culture, L. thermotolerans is highlighted by different colours: LT_E1(●), LT_E2 (●), LT_C1 (●), LT_C2 (●), LT_C3 (●). S.cerevisiae was monitored in monoculture (**x**), in sequential inoculation (dottet black line) and co-inoculation (solid black line) with L. thermotolerans: In the last graph: spontaneous yeasts are compared with the S.cerevisiae control (**x**).*

3.2 Fermentation kinetics

Despite the high initial total sugars (glucose and fructose) level (~260 g/L), 10 out of 12 yeast treatments were able to catabolize all the sugars and complete the alcoholic fermentation. Nevertheless, the fermentation kinetics differed markedly between the treatments.

Depletion of sugars in *S. cerevisiae* control was quite fast and comparable with the co-inoculations (Figure 6). In fact, the 6 treatments depleted sugars within 12 to 13 days. Contrary, the sugar consumption kinetic in the sequential culture was sluggish. A slowdown in fermentation dynamics is noticeable after approximately 60% of sugars consumption (Fig. 6, Tab 7). Within the sequential inoculations group, LT_C1 and LT_E2 finish the alcoholic fermentation after 28 days and LT_C3 after 35 days. Fermentation that did not complete in sequential inoculations with LT_E1 and LT_C2 which after 60 days, showed significant concentrations of residual sugars (respectively 8.2 g/L and 3.7 g/L; Tab.6). As expected, the un-inoculated must showed the slowest fermentation rate even if the sugars were completely exhausted. After 22 days, most of the sequential cultures still had not finished the AF. Enzymatic method was therefore used to more precisely monitor the depletion of sugars during the last days of AF (Tab.6).

Table 6. Residual sugars (glucose+fructose; g/L) after 23 days of AF in sequential inoculation trials.

DAYS	LT_E1…	LT_E2…	LT_C1…	LT_C2…	LT_C3…	UN-INOCULATED
23	15.1±4.3	13.7±1.2	4.1±0	26.1±4.7	24±5.2	8.2±5.4
28	11.3±2.5	2.9±0.5	2.4±0.2	11±2.3	8.8±2.2	5.3±1.1
32	10.5±2.6	0±0	0±0	5.5±1.2	5.6±2.5	3.1±1.3
35	8.7±2.3			4.8±1.3	3.5±1.1	1.7±0.5
60	8.2±2.1			3.7±1.3	0±0	0±0

Overall, sugar consumption rates presented two trends: one displayed by pure *S.cerevisiae* culture and co-inoculations (solid lines) and the other displayed by sequential inoculations (dotted lines), the latter being slower for all the *L. thermotolerans* strains. This is in accordance with previous studies (Kapsopoulou *et al*., 2005).

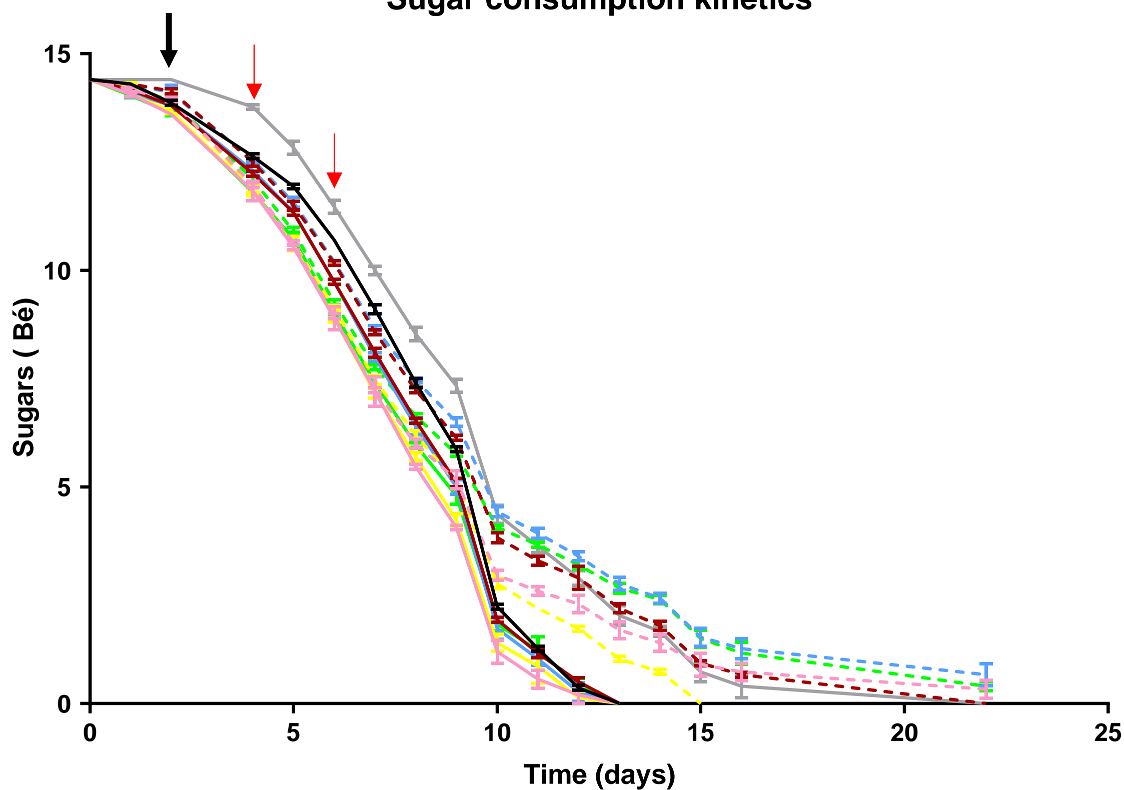

Figure 6. Sugar (Beaumé) consumption kinetics in twelve treatments of five L. thermotolerans strains, S. cerevisiae control, and uninoculated must. In sequential (dotted lines) and co-inoculation (solid lines) cultures. Error bars represent standard deviations (SD) of analysed triplicates. The black arrow represents the sequential inoculation time and the first DAP addition (after 2 days from the L. thermotolerans inoculum). The red arrows represent further additions of nutrients.

Table 7. Percentage of sugars depletion in the 12 different trials. Calculated based on Beaumé data.

DAYS	SC	E1X	E1…	E2X	E2…	C1X	C1…	C2X	C2…	C3X	C3…	UN
0	0	0	0	0	0	0	0	0	0	0	0	0
1	1	2	2	2	1	2	1	2	1	3	2	2
2	4	6	3	4	2	5	4	4	2	5	3	3
4	12	18	17	15	13	18	17	15	13	18	16	5
5	17	27	26	21	20	27	26	21	20	26	24	11
6	26	38	38	32	29	38	37	32	29	37	36	20
7	37	50	50	44	41	50	48	45	40	49	46	31
8	49	62	58	55	50	60	57	56	48	59	54	41
9	59	72	64	65	57	71	65	65	55	67	60	49
10	85	92	79	87	73	90	81	88	69	87	72	70
11	91	96	82	92	77	94	85	93	73	91	75	75
12	97	99	84	97	80	99	88	98	76	97	78	80
13	100	100	88	100	85	100	93	100	81	100	82	86
14	100	100	90	100	88	100	95	100	83	100	83	88
15	100	100	94	100	94	100	100	100	89	100	90	95

3.3 Metabolites

Statistical analysis of the main chemical parameters in wines revealed significant differences ($p < 0.0001$) in concentrations of all measured metabolites (Tab. 8). In general, *S.cerevisiae* control resulted in higher levels of ethanol, lower levels of glycerol, higher pH and lower TA and lactic acid compared to the mixed-culture treatments. In most *L. thermotolerans* wines a marked drop in pH and an increase in TA was observed (e.g.- 0.49 and +6.05 g/L comparing *S.cerevisiae* control with LT_E2 strain). The results of the mixed culture fermentations show that both the *L. thermotolerans* strain, and the inoculation timing, strongly affect the final wine profile. Generally, the sequential inoculations had more trouble to end the AF (e.g. 8.20 g/L of residual sugars in LT_E1 strain), but a more noticeable drop of pH, production of lactic acid with a consequent increase of titratable acidity compared to the co-inoculations.

Table 8. Analytical profiles of final wines fermented by mixed cultures of different strains of L.thermotolerans and S.cerevisiae.

TREATMENT	Residual sugars (g/L)	Ethanol (% v/v)	pH	TA (g/L)	Lactic acid (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Malic acid (g/L)	SO_2 total(ppm)
SC	0.12±0.05a	16.48±0.05e	3.86±0.01d	5.03±0.10ab	0.42±0.02a	8.30±0.04a	0.15±0.01a	2.38±0.07f	13.33±3.33d
LT_E1X	0.26±0.12a	16±0.05c	3.50±0.04b	8.90±0.51d	5.41±1.31de	9.25±0.24bcd	0.21±0.03a	1.68±0.08cde	2.38±3.70 abc
LT_E1…	8.20±2.11c	15.04±0.24a	3.37±0.03a	10.95±0.58e	7.61±0.58fg	9.57±0.26de	0.29±0.08ab	1.20±0.09b	2.13±3.70ab
LT_E2X	0.41±0.08a	16.12±0.02cd	3.49±0.01b	8.15±0.17cd	3.69±0.71cd	8.82±0.02abc	0.22±0.04a	1.9±0.04de	8.53±0.92bcd
LT_E2…	1.36±0.18a	15.65±0.06b	3.36±0.02a	11.09±0.11e	8.14±0.16g	10.24±0.09f	0.54±0.02cd	1.22±0.08b	9.07±0.92cd
LT_C1X	0.13±0.03a	16.39±0.01de	3.85±0.01d	5.15±0.03ab	0.64±0.03a	8.75±0.19ab	0.29±0.05ab	2.07±0.06ef	3.73±3.33abc
LT_C1…	1.37±0.22a	16.21±0.02cde	3.90±0.00d	5.13±0.05ab	0.95±0.06a	9.92±0.09ef	0.47±0.03cd	1.06±0.05b	0.53±0.92a
LT_C2X	0.21±0.04a	16.26±0.02cde	3.71±0.03c	6.23±0.13b	1.79±0.06ab	9.36±0.18cd	0.17±0.03a	1.99±0.06ef	6.40±2.77abc
LT_C2…	3.77±1.36b	15.56±0.18b	3.58±0.01b	8.07±0.08cd	3.42±0.14bc	11.65±0.05g	0.49±0.03cd	1.29±0.08bc	5.87±1.85abc
LT_C3X	0.30±0.06a	16.08±0.02c	3.55±0.01b	7.58±0.1cd	3.64±0.08bcd	9.18±0.10bcd	0.29±0.05ab	1.87±0.02de	5.33±0.92abc
LT_C3…	2.15±0.98ab	15.71±0.07b	3.51±0.05b	9.12±0.34d	5.83±1.09ef	10.09±0.19ef	0.45±0.01bc	1.47±0.03bcd	4.80±0abc
UN-INOCULATED	1.27±0.35a	16.23±0.06cde	3.89±0.06d	4.71±0.01ab	1.66±0.03abc	10.22±0.2f	0.67±0.04d	0±0a	3.73±1.85abc
mean	1.63	15.98	3.63	7.51	3.60	9.61	0.35	1.51	5.60
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.004

Values are means of three replicates; lower case letters denote significant differences (Tukey's HSD).

Lactic acid production is the most remarkable trait of the *L. thermotolerans* utilized for winemaking (Gobbi *et al*., 2013, Jolly *et al*., 2014; Benito *et al*., 2018). However, great differences in lactic acid production reported are influenced by both the strain and the inoculation regime (Gobbi *et al*. 2013; Hranilovic *et al*., 2018.). In this study, the LT_C1 strain did not show any significant decrease in pH or increase in lactic acid or titratable acidity compared to the *S.cerevisiae* control, regardless of the inoculation regime (Fig. 7). Concerning all the other tested strains, there was an increase in lactic acid ranging from 0.4 g/L up to 7.6 g/L for co-inoculation and up to 8.1 g/L for sequential ferments. In fact, in the mixed cultures, the titratable acidity level increased in most treatments (e.g. 8.9 g/L and 11.1 g/L in LT_E1 co-inoculation and LT_E2 sequential inoculation, respectively; Tab. 8, Fig.8). Among the strains, LT_E2 is the most performant both in co-inoculation and in terms of lactic acid production. Lactic acid production caused an increase in titratable acidity. No significant differences in LT_C1 and UN-inoculated treatments could be observed.

In mixed co-cultures, the final pH was decreased by 0.4 point in the trials with both the experimental strains, while, with the commercial ones, the pH decrease activity was different between the three strains. The variation was from 3.90 pH points to 3.85 with LT_C1, to 3.71 with LT_C2 and 3.55 with LT_C3. In all the cases, there was a stronger pH drop effect with the sequential inoculation treatments. The pH levels dropped from 3.9 to 3.37 and 3.36 with the experimental strains. In this case, with the first commercial strains no significant differences could be noted, while in the other commercials the pH level was 3.58 with LT_C2 and 3.51 with LT_C3, in sequential inoculation. A small drop of pH was observed in pH value after AF in the control wine (from an initial value of the must of 3.90 to 3.86; Fig.9).

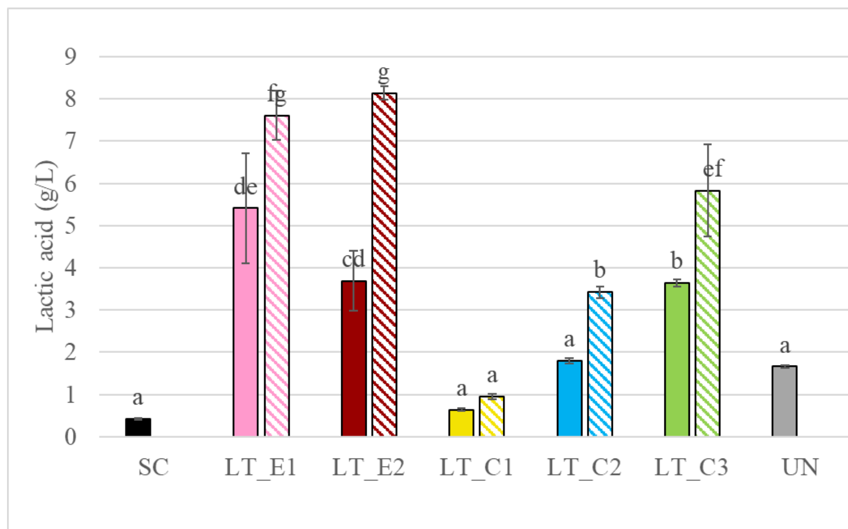

Figure 7. Lactic acid production in sequential (\\\) and co- inoculations → (■) of L. thermotolerans and S. cerevisiae

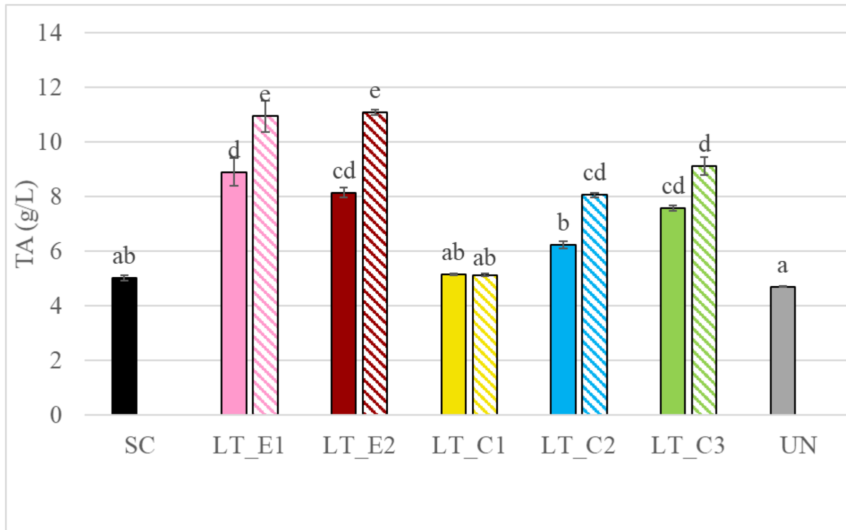

Figure 8. Titratable acidity level (g/L) in sequential (\\\) and co-inoculations (■) of L.thermotolerans and S.cerevisiae

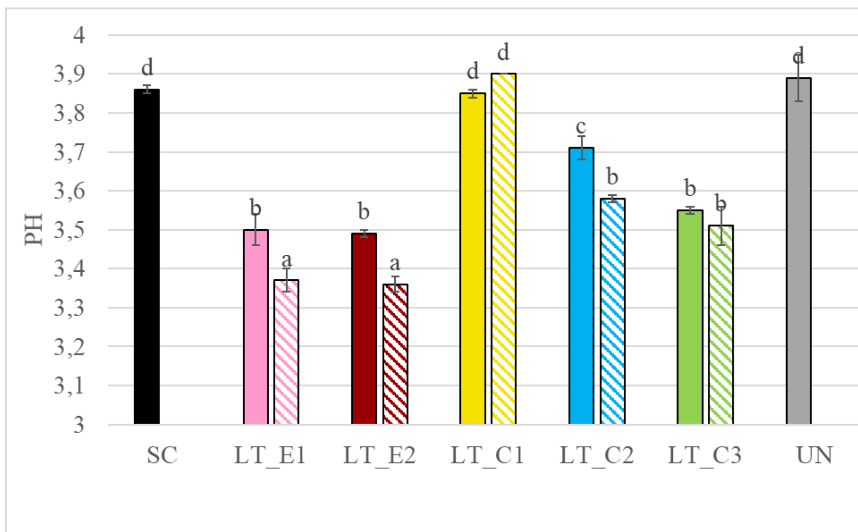

Figure 9. pH level in sequential (\\\) and co- inoculations (■) of L.thermotolerans and S.cerevisiae

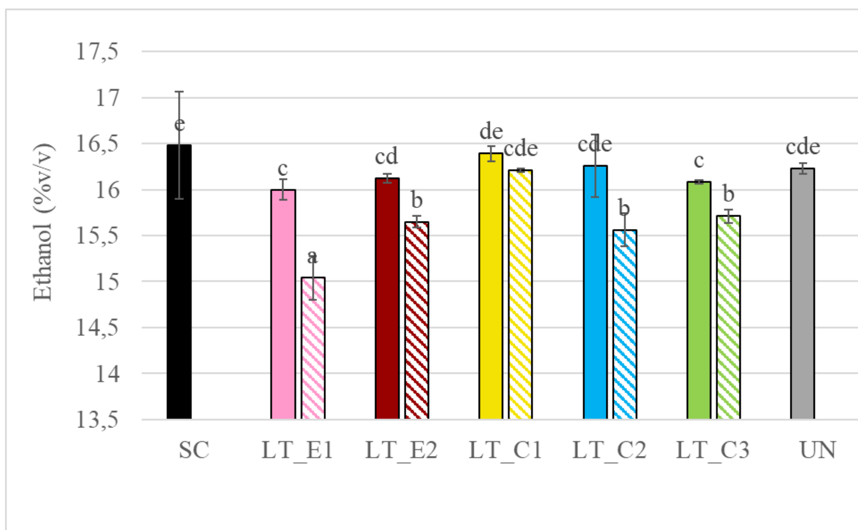

Figure 10. Ethanol production in sequential (\\\) and co-inoculations (■) of L.thermotolerans and S.cerevisiae

The ethanol yields of *L.thermotolerans* are seemingly lower than those of *S. cerevisiae*, i.e. about 0.37 and 0.47 grams of ethanol per gram of consumed sugar, respectively (Hranilovic *et al*., 2018). However, *L. thermotolerans* monocultures cannot deplete all grape sugars, and thus are not recommended as a single inoculum for alcoholic fermentation. Considering that the purpose of this study was to evaluate the performance of *L. thermotolerans* performance in the context of wine industry application, the strains were tested in mixed cultures with *S. cerevisiae*.

The *S.cerevisiae* control wine resulted in highest concentration of ethanol (16.48 % v/v), comparable to those in the uninoculated treatment, LT_C1 in both inoculation modalities and LT_C2 co-inoculation. Co-inoculations with LT_E1, LT_E2 and LT_E3 had less ethanol than *S.cerevisiae* control. Sequential inoculations with all LT strains except LT_C1 resulted in significant decrease in ethanol compared to the *S.cerevisiae* control. However, in sequentially inoculated LT_E1 wine, the decrease of 1.44 % v/v was partially related to the residual sugar (8.2 g/L). Sequential inoculation treatment with LT_E1 had the lowest ethanol content, i.e. 0.84 % less than *S.cerevisiae*, amongst the dry wines (<2 g/L RS).

Scatter plot in figure 11 shows a negative correlation between ethanol and lactic acid concentrations; indeed, higher levels of lactic acid correspond to lower level of ethanol and vice versa. As we expected, the *S.cerevisiae* monoculture has the highest concentration of ethanol (16.48 %v/v) and lowest concentration of lactic acid (0.4 g/L). On the other hand, all the mixed cultures show lower level of ethanol and higher of lactic acid.

Figure 11. Lactic acid and ethanol concentrations inversely related

In literature, mixed cultures of *L. thermotolerans* with *S. cerevisiae* have been reported as greater producers of glycerol (Gobbi *et al*., 2013; Benito *et al*., 2015). In this study, the concentrations of glycerol were generally higher in mixed cultures with sequential inoculum (e.g. 8.3 g/L in *S.cerevisiae* control and the maximum of 11.6 g/L in LT_C2 mixed cultures; Tab.8).

In the present study there are some significant differences in concentration of acetic acid between *S.cerevisiae* pure culture and mixed cultures, but all of them remain in the acceptable level (about 0.2-0.4 g/L). Compared to the co-inoculated cultures, a higher concentration in the sequential inoculation trials (up to 0.54 g/L), was observed; however, LT_E1 has the lowest (0.29 g/L) concentration of acetic acid amongst the sequential inoculations even if it had some issues in the end of AF. As expected, the uninoculated trial has the highest concentration of acetic acid (0.67 g/L).

In general, a higher degradation of malate was observed in mixed fermentations. This phenomenon can be explained by demalication activity of the yeast (Vilela *et al.,* 2017; Kunicka-Styczyńska *et al.,* 2017). Besides, *L. thermotolerans* in pure and mixed cultures is often associated with partial malate degradation (Hranilovic *et al*., 2018). In this study the maximum malate value was found in the control wine (2.38 g/L), while 1. 06 g/L was the lowest value amongst the inoculated trials, and it was founded in LT_C1 sequential inoculation treatment. In fact, malate levels were lower in sequential inoculation treatments compared to the others. We can hypothesize that indigenous lactic acid bacteria metabolized malic acid in these wines. However, given the partial malate degradation by *L.thermotolerans* and antibacterial properties of lactic acid which are widely recognized (De Vuyst *et al.,* 1994), this is unlikely. No malic acid was found in the uninoculated trial, probably because MLF occurred.

Concentrations of total sulfur dioxide were very low in all yeast treatments (mean value5 ppm, Tab. 8). Nevertheless, significant differences can still be noticed, in particular, a higher value for *S. cerevisiae* control (13.33ppm).

3.4 Phenolics and colour

3.4.1 Phenolics from Modified Somers analysis

In this study, the phenolics component of the wines was analysed by the modified Somers method (Mercurio *et al*., 2007). Significant difference was observed for all the measured parameters (Tab. 7).

Table 9 Phenolic compounds measured by the modified Somers' method

TREATMENT	Total anthocyanins (mg/L)	Degree of Ionization (%)	Colour density (au)	Hue	Total phenolics (au)	SO_2 resistant pigments (au):
SC	139.00±13.40abc	9±0.01a	2.87±0.33a	0.84±0.01fg	11.7.0±0.42bcd	0.96±0.14b
LT_E1X	176.38±5.78f	12±0.02ab	3.22±0.16abc	0.73±0.02bc	12.49±0.70cd	0.79±0.09a
LT_E1…	138.57±9.33ab	20±0.03c	3.85±0.56c	0.69±0.01a	10.14±0.29a	0.86±0.08ab
LT_E2X	159.13±2.86cdef	9±0.00a	2.73±0.08a	0.80±0.01de	11.82±0.3bcd	0.76±0.02a
LT_E2…	137.76±5.81ab	14±0.00b	3.06±0.10ab	0.73±0.01bc	11.68±0.33bcd	0.82±0.02ab
LT_C1X	160.04±1.19def	9±0.00a	2.7±0.02a	0.82±0.0efg	12.11±0.26cd	0.76±0.01a
LT_C1…	150.58±5.33abcdef	13±0.01ab	3.17±0.16ab	0.81±0.01ef	11.72±0.41bcd	0.80±0.03ab
LT_C2X	161.98±5.12def	11±00ab	2.95±0.01a	0.8±0.02e	12.07±0.34bcd	0.74±0.02a
LT_C2…	143.89±10.17abcd	12±00ab	2.89±0.05a	0.75±0.00c	11.34±0.59abc	0.81±0.04ab
LT_C3X	166.75±5.03ef	12±0.02ab	3.03±0.26ab	0.76±0.01cd	12.43±0.37cd	0.72±0.04a
LT_C3…	135.03±3.60a	19±0.01c	3.67±0.18bc	0.72±0.01ab	10.86±0.22abc	0.83±0.03ab
UN-INOCULATED	157.59±5.27bcdef	12±0.01ab	3.19±0.05ab	0.85±0.0g	12.88±0.48d	0.75±0.04a
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.004

The mean value of total anthocyanins was 152.26 mg/L (range 135.03 - 176.38; Tab. 7). In general, the concentrations of total anthocyanins in co-inoculations were higher than in sequential inoculations (mean values 164.86 mg/L and 141.17 mg/L, respectively). In addition, concentrations of total anthocyanins in the *S.cerevisiae* control were more similar to those in sequential cultures, while the un-inoculated treatment was more similar to the co-inoculations.

Some recent studies (Benito *et al*., 2017; Hranilovic *et al*., 2017) observed higher levels of anthocyanins in final wines co-fermented with non-*Saccharomyces* yeasts compared with the *S.cerevisiae* control, with increases from 8 to 12 %. Accordingly, the levels of total anthocyanins in co-inoculations in this study increased from 15 to 26%. Conversely, sequential

inoculation treatments did not lead to a significant increase in the concentrations of total anthocyanins (Table 9, Fig 12).

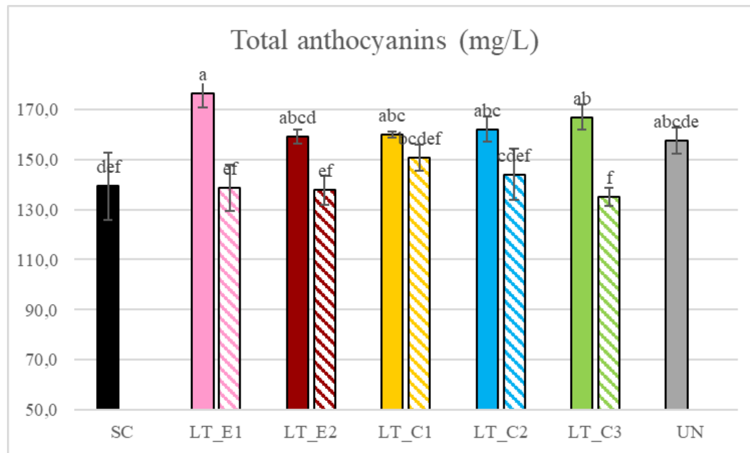

Figure 12. Total anthocyanins concentration measured by Modified Somers method

Concentrations of total anthocyanins were quite low mostly due to small skin/juice ratio during maceration. For the same reason, the value of total phenolics was also low with a mean value of 11,77 au (Tab.7). However, significant differences ($p<0.0001$) among all the treatments were observed.

Total phenolics followed the same trend as the total anthocyanins, i.e. higher values were obtained in co-inoculations (mean value: 12.19 au) than in the sequential ones (mean value: 11.15 au). The *S. cerevisiae* control and the un-inoculated treatment were, again, similar to sequential inoculations and co-inoculations, respectively (Tab. 9).

An increase in colour density in sequential inoculation treatments compared with the pure *S. cerevisiae* treatment of up to up to 34% was observed, in agreement with previous studies (Benito *et al*., 2015; 2017; 2018). Within the same strains the sequential inoculation has resulted an approximately 20% of difference from the co-inoculations in all the cases the sequential inoculations ferments were observed with higher colour density (Fig. 13). Previously, an increase of colour density of about 10% in the sequential fermentations with L. thermotolerans was reported by Benito *et al*. (2015).

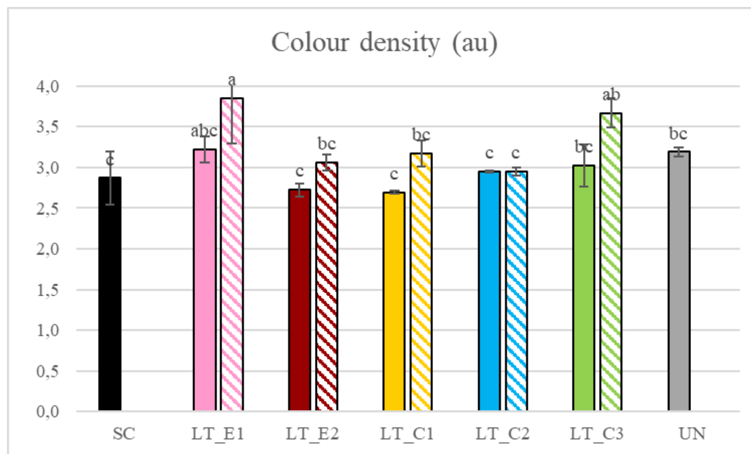

Figure 13. Colour density measured by Modified Somers method

The hue or tint of the wine indicates the ratio between yellow and red tonalities, thus the higher the value the more the colour of the wine is shifted from purple red to brick red (Somers, 1977). The highest hue values were recorded in the monoculture control (0.84) and the un-inoculated treatment (0.85; Tab. 9). With the use of the same *L. thermotolerans* strain, significantly lower hue values are observed in sequential cultures than in co-inoculations.

The mean of degree of ionisation of anthocyanins is 12.66% and it is within the average of young Australian wines (2-34%; Somers and Evans 1974). As mentioned above, the degree of ionisation of anthocyanins represent the percentage of anthocyanins present in coloured or flavylium form. The total anthocyanins value was higher in the co-inoculation treatments, however the coloured forms are more present in the sequential cultures. As a confirmation of that, the correlation analysis in fig.8 shows a positive correlation between degree of ionisation of anthocyanins and colour density.

While, on the other hand, there is a negative correlation between degree of ionization and Hue, as reported in Somers and Evans in 1974 (Fig.14).

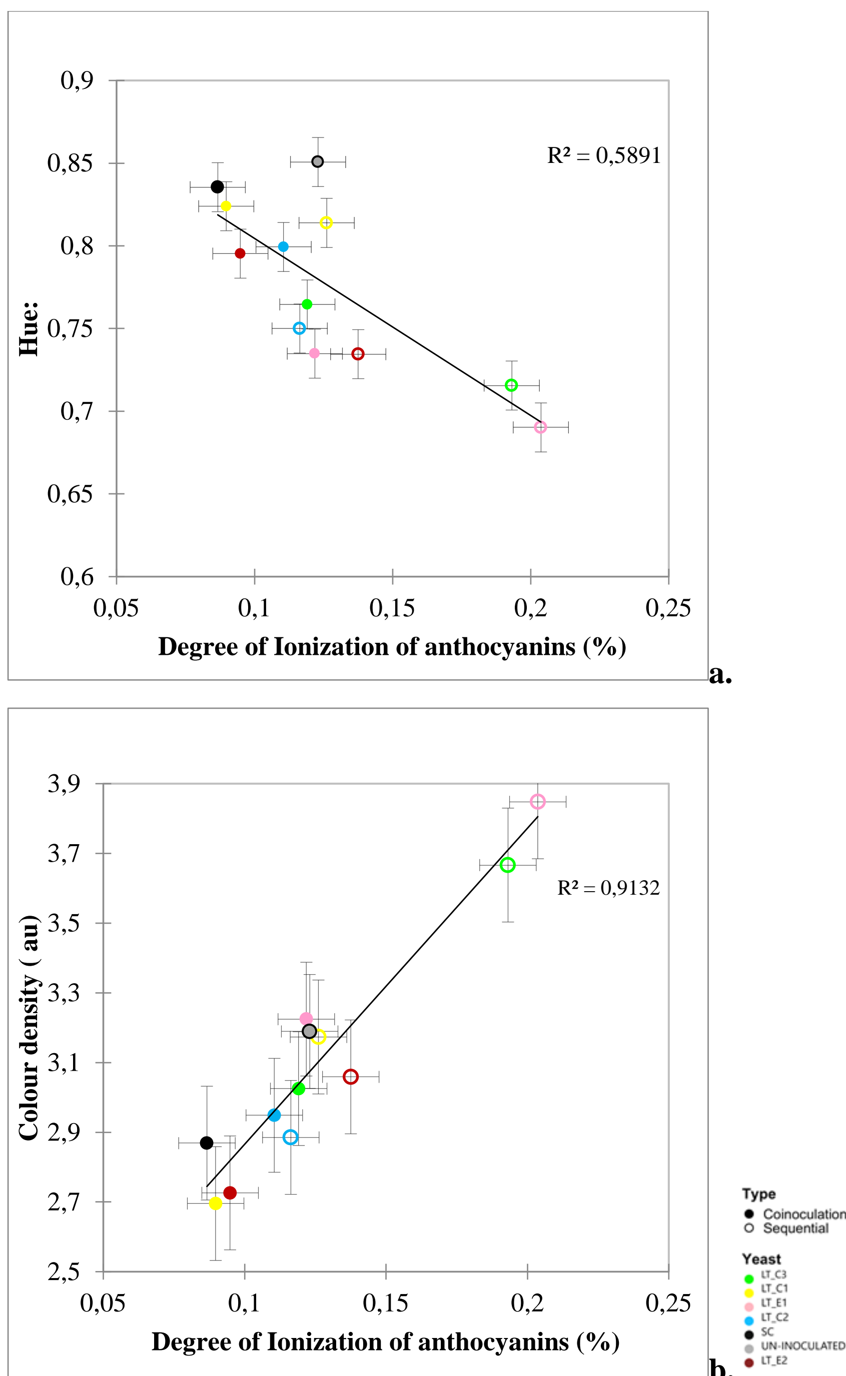

Figure 14 Relation between degree of ionisation of anthocyanins and hue (a) or colour density (b) in all the different treatments studied.

A multivariate analysis of the statistically significant chemical parameters was undertaken with the principal component coordinate analysis (PCA; Fig. 15). The first two PCs accounted for 70.1% of the total variation in the samples. Ethanol, total acidity, lactic acid and pH were the variables that contributed to the PC1 (54.7% of the variance explained). Along the PC1 we can observe a clear separation between the sequential inoculations and the co-inoculations. One exception is the LT_C2, positioned amongst the co-inoculated treatments and the *S.cerevisiae*

control and UN-inoculated. About the phenolics data, the degree of ionization and hue are the variable contributing to separate the wines on PC1. Acetic acid and malic acid gave the major contribution to the PC2, which represented 15.4% of variance. For this reason, we can easily see a clear separation of the un-inoculated wine which had the highest levels acetic acid and the lowest levels of malic acid, having very possibly completed the malolactic fermentation.

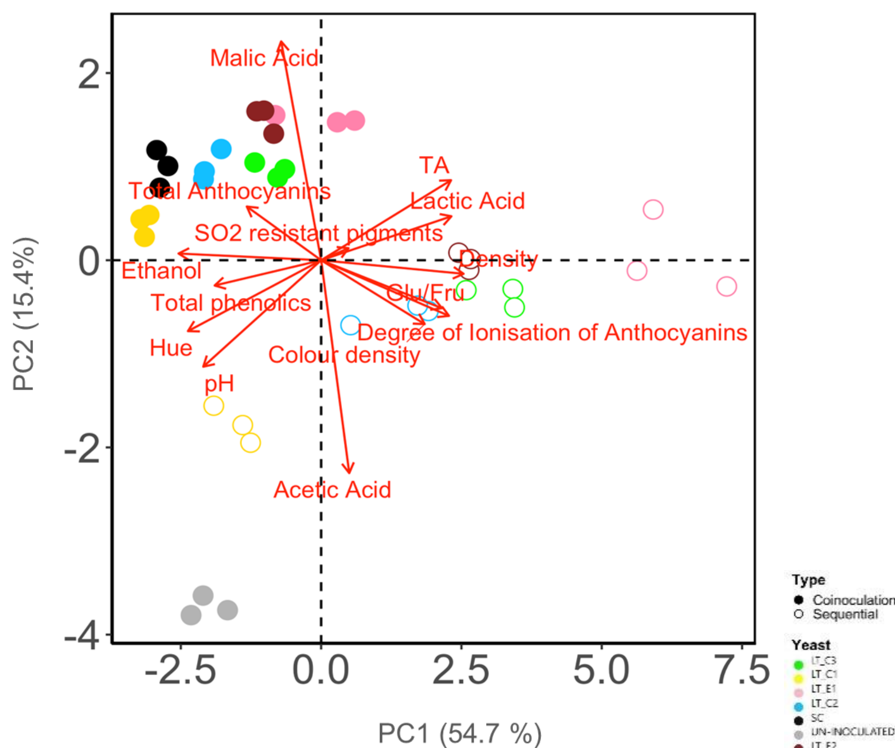

Figure 15. Principal component analysis (PCA) biplot of chemical data

3.4.2 CIELab coordinates

The current International Organization of Vine and Wine (OIV) method uses CIELab coordinates as parameters for defining the colour of wines.

The parameters used in the CIELab method are L* (lightness), a* (from green to red), b* (from blue to yellow), C* (chroma or saturation), and h (hue angle). Using these coordinates, the total colour difference (E*) between two samples can be obtained using the following expression: $\Delta E^*=\sqrt{}(\Delta L^*)^2 +(\Delta a^*)^2+(\Delta b^*)^2$, in CIELAB units (www.oiv.int).

In the current study, CIELAB units were used to measure the differences in colours between treatments and highlight whether they could be considered detectable by eye, i.e. $\Delta E^*\geq 1$

(Gonnet *et al*., 1998; Pérez Margarino *et al.,* 2003); ΔE*≥2.7 (Martinez *et al*., 2001). However, if the observations are made through glass, the discrimination is lower and ΔE* values should exceed the value of 5 (Negueruela *et al*., 1995b). In general, the visible difference between colours is directly proportional to the increase of ΔE*.

Calculated CIELab units ΔE*;(Tab.10) show undoubtedly discriminant differences between the samples. The results are surely influenced by pH level, and confirm overall colorimetric difference, which are detectable by the observers. The largest ΔE* (24.5) values which suggest the biggest observable differences were obtained for LT_C1X and LT_E1 samples. Conversely, lowest ΔE*(1.4) values which indicate the smaller observable differences were obtained for LT_E1X and LT_E2X comparing the treatments to the control, an average colour differences of 8 were found in co-inoculation treatments and 13,2 in sequential ones.

Table10. Matrix of colorimetric difference (ΔE units) between all the wines studied*

	SC	LT_E1X	LT_E1…	LT_E2X	LT_E2…	LT_C1X	LT_C1…	LT_C2X	LT_C2…	LT_C3X	LT_C3…	UNINOC
SC	0	11.41	21.02	9.99	14.34	4.66	3.06	5.58	11.66	8.68	16.34	3.14
LT_E1X	11.41	0	12.51	1.48	4.57	13.68	10.58	10.75	2.80	3.50	7.28	13.62
LT_E1…	21.02	12.51	0	13.59	8.01	24.54	20.51	22.43	11.65	15.66	5.38	23.55
LT_E2X	9.99	1.48	13.59	0	5.66	12.27	9.25	9.41	3.41	2.29	8.37	12.21
LT_E2…	14.34	4.57	8.01	5.66	0	17.39	13.78	14.88	4.61	7.84	3.11	16.85
LT_C1X	4.66	13.68	24.54	12.27	17.39	0	4.36	3.67	13.94	10.32	19.51	2.53
LT_C1…	3.06	10.58	20.51	9.25	13.78	4.36	0	4.43	10.32	7.60	15.58	3.39
LT_C2X	5.58	10.75	22.43	9.41	14.88	3.67	4.43	0	11.28	7.27	17.19	5.28
LT_C2…	11.66	2.80	11.65	3.41	4.61	13.94	10.32	11.28	0	4.56	6.28	13.62
LT_C3X	8.68	3.50	15.66	2.29	7.84	10.32	7.60	7.27	4.56	0	10.34	10.53
LT_C3…	16.34	7.28	5.38	8.37	3.11	19.51	15.58	17.19	6.28	10.34	0	18.74
UNINOC	3.14	13.62	23.55	12.21	16.85	2.53	3.39	5.28	13.62	10.53	18.74	0

Chroma (C*) values which indicate the relative saturation (intensity) of the colour were lower in the *S.cerevisiae* control, UN-inoculated treatment, both LT_C1 treatments, and the co-inoculations trials for the other strains, compared to the sequential treatments. Based on this parameter, the colour of the LT_E1… was the most 'intense'. In the mixed cultures, the treatments that had the lowest Chroma (i.e. co-cultures), had the highest hue (H*) value, which implies a further shift from red towards yellow colour. Conversely, *S.cerevisiae* control, UN-inoculated and LT_C1… had lower chroma, but high hue. In general, all sequential inoculation treatments as well as the *S.cerevisiae* and UN-inoculated treatment, had higher hue values compared to the sequential inoculation treatments (Fig. 16).

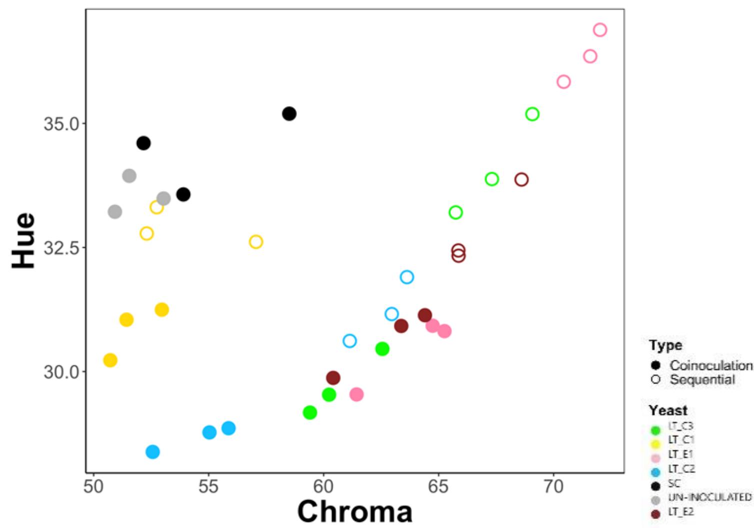

Figure 16. Relation between Chroma (C) and Hue (h*) from CIELab calculation.*

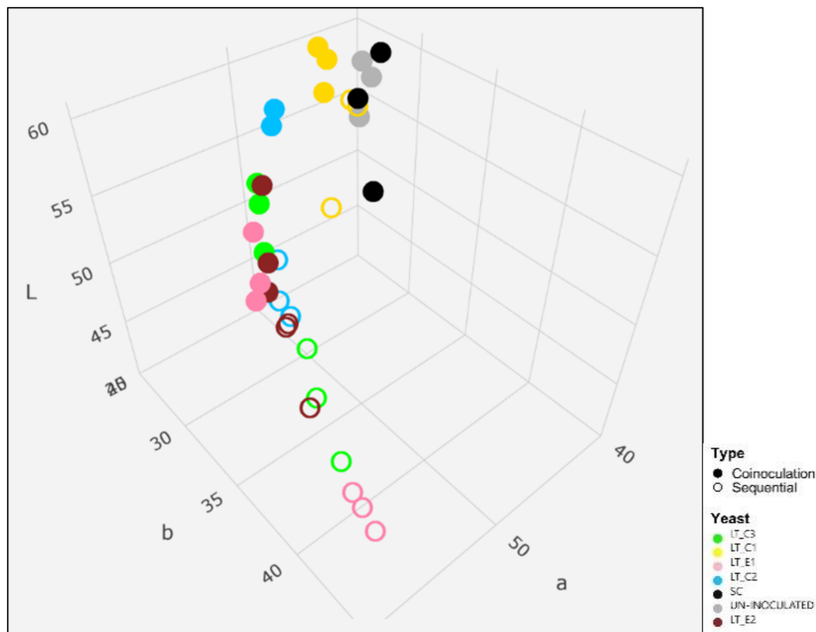

Figure 17. CIELab coordinates for twelve different treatments

Spatial representation of wine colours obtained with the CIELab coordinates (Fig 17) displays the differences in colours, especially between the sequential and co-cultures. This can be also explained mainly by the pH difference.

3.5 Sensory analysis

Sensory analysis of final wines highlighted differences in wines produced by 12 yeast treatments in terms of aroma, flavour and mouthfeel. Twenty two out of 43 attributes used in RATA were reported as significantly different ($p \leq 0.05$).

Regarding the aroma profile, 7 significant attributes (Tab 11) highlighted substantial differences both between different strains and different inoculation regimes, as seen with the other wine compositional parameters.

Table11. Aroma attributes significantly different (($p \leq 0.05$)

	dark fruit	red fruit	cooked vegetables	Floral perfume	Medicinal rubbery	oxidation	VA(volatile acidy)
SC	2.8 e	3.4 ab	0.9 c	2.3 ab	1.2 bc	1.7 cde	1.6 b
LT_E1X	3.6 abc	3.4 ab	0.6 c	2.0 abcd	0.9 c	1.2 e	1.4 bc
LT_E1…	3.3 bcde	3.0 ab	1.1 bc	2.0 abcd	1.3 bc	3.0 a	1.6 bc
LT_E2X	3.5 abcd	3.1 ab	0.8 c	2.0 abcd	1.3 abc	1.5 de	1.3 bc
LT_E2…	3.7 abc	3.1 ab	0.8 c	1.9 abcd	1.0 bc	1.4 de	1.5 bc
LT_C1X	3.3 bcde	3.1 ab	0.7 c	2.1 abc	1.1 bc	1.2 e	1.3 bc
LT_C1…	4.0 a	3.0 ab	0.9 c	2.0 abcd	0.8 c	1.2 e	1.1 c
LT_C2X	3.8 ab	2.8 bc	0.9 c	1.5 cd	1.6 ab	1.4 de	1.1 bc
LT_C2…	3.5 ab	2.9 bc	1.5 c	1.4 cd	1.3 ab	2.3 de	1.6 bc
LT_C3X	3.1 cde	3.1 ab	1.1 bc	1.6 bcd	0.9 c	1.3 e	1.3 bc
LT_C3…	2.7 e	3.5 a	0.8 c	2.3 a	1.2 bc	2.0 cd	1.3 bc
UN-INOC	3.0 de	2.1 c	1.9 a	1.4 d	1.9 a	2.7 ab	2.3 a
p-value	0,001	0,009	< 0.0001	0,041	0,025	< 0.0001	0,004

The LT_E1 strain was fruitier and less floral compared to the *S.cerevisiae* control with an important effect of the inoculation timing. In fact, particularly in LT_E1, LT_E2 and LT_C3 the attributes were rated with significantly different scores (Tab 23). This can be seen also from the spider plot (Fig. 18), where co-inoculations and sequential inoculations generally showed different profiles. This variance can be observed with LT_C2 and LT_C3 strains. Bigger correspondence between the co-inoculation and the sequential inoculation treatment is displayed with the LT_E2 strain with differences in “dark fruit” and “medicinal/rubbery” attributes only. LT_C1 strain had a different outline from the other LT strains and was more similar to the *S.cerevisiae* control. In fact, there are no significant differences from the control except from the “dark fruit” attribute which was scored higher in the sequential LT_C1 mixed culture.

Regarding the flavour (Tab. 12; Fig.19), 7 attributes differ significantly through the treatments. Same quantity as the previous attributes, but three different descriptors (i.e. dried fruit, chocolate and herbaceous). Generally, the behaviour of the five strains is comparable, but not

the same as the aroma profile. Indeed, also in this case LT_E1 strain showed the highest score for the fruity attributes, but in this case was "red fuit". A different spider plot shape between co-inoculation and sequential inoculation treatments was observed also for the flavour. In contrast to the aroma profile, this characteristic was observed also in LT_C2 and LT_C3 strains. LT_C1 differed from the *S.cerevisiae* control and was more similar to the LT_C3 co-inoculated wine profile. As seen before, for the aroma, in LT_E2 sequential inoculation and co-inoculation gave approximately the same flavour profile except from the "herbaceous". Even though there are significant differences between all five *L. thermotolerans* strains, the sensory profile of the UN-inoculated wine and the *S. cerevisiae* monoculture (control) further highlighted the impact of L. *thermotolerans* strains on the sensory profile of the final wines. Furthermore, the UN-inoculated wine is somewhat an"outlier" in terms of its sensory profile with the aroma/flavour characteristics which can be typical of 'spontaneously fermented wines (i.e. oxidative, bitterness, medicinal and volatile acidity).

It is also important to consider how the alcoholic fermentation dynamics,which is impacted by a yeast treatment,can also influence the final wine aroma/flavour. Indeed. The highest rate of oxidative descriptor (i.e. "oxidation" and volatile acidity (VA)) in flavour and aroma corresponds in every case to the sluggish fermentation samples (e.g. LT_E1…. LT_C3… or UN-inoculated). In all these cases the alcoholic fermentation was completed more than one week later compared to the control; in this time frame the samples were more exposed to air contact.

Table 12 Flavour attributes significantly different ((p≤0.05)

	red fruit	**dried fruit**	**chocolate**	**herbaceous**	**medicinal/rubbery**	**oxidation**	**VA (volatile acidity)**
SC	3.0 cd	2.1 ab	1.1 abc	1.4 bcd	1.2 b	1.5 cde	1.3 bcd
LT_E1X	3.6 a	1.8 b	0.9 cde	1.1 cd	0.7 cd	0.9 e	1.9 abc
LT_E1…	3.4 abc	2.2 ab	0.8 cde	1.3 bcd	0.8 bcd	2.4 ab	1.9 abc
LT_E2X	3.2 abc	2.1 b	0.9 cde	1.9 a	0.8 bcd	1.4 de	2.0 a
LT_E2…	3.3 abc	1.8 b	0.6 e	1.2 bcd	0.6 cd	1.4 de	1.9 ab
LT_C1X	3.0 bc	2.3 ab	1.4 a	1.1 cd	0.9 bcd	1.0 e	0.8 d
LT_C1…	2.9 cd	2.7 a	1.3 ab	1.2 bcd	1.0 bcd	1.3 de	0.8 d
LT_C2X	3.1 abc	2.2 ab	1.0 abcd	1.7 ab	1.0 bcd	0.9 e	1.0 d
LT_C2…	3.6 ab	2.2 ab	0.9 cde	1.6 abc	1.0 bc	2.0 bc	1.9 ab
LT_C3X	3.2 abc	2.1 b	0.7 de	1.6 abc	0.9 bcd	1.3 de	1.3 cd
LT_C3…	3.4 abc	1.9 b	0.7 de	1.6 abc	0.5 d	1.7 cd	2.0 a
UN-INOC	2.4 d	2.7 a	1.0 bcde	0.9 d	2.0 a	2.8 a	2.2 a
p-value	0.005	0.044	0.002	0.007	< 0.0001	< 0.0001	< 0.0001

In terms of mouthfeel. 8 out of the 10 attributes were significantly different (Tab.13). Interestingly, an increased perception of “hotness” corresponded to higher ethanol levels (Tab. 7). For example, the mean score of “hotness” for the *S.cerevisiae* control wine v/v was 4 with a correspondent 16.5 % v/v ethanol level. Equally, wines made with LT_E2 in sequential with 15.6% v/v of ethanol, were actually perceived less “hot” (i.e. 3.4 scores; Tab.13).
Besides, observing the mouthfeel spider plot (Fig. 20) we can notice as the strain-effect is the discriminant to draw different profile. As we can read in the table 13, in fact, the most significant differences rated are between different strains.
The majority of *L. thermotolerans* treatments significantly influenced the acidity perception: LT_E1 and LT_E2. in agreement with the analytical (Tab. 8). played an important role in the increase in acidity. Indeed, the highest scores of “acidity” and “length of acidity” (approximately 5-5.5) were given to wines fermented byLT_E1 and LT_E2 followed by LT_C3 and LT_C2 strains. Wines fermented by LT_C1 showed a mouthfeel profile similar to the control (Fig.20). The sequential inoculation trials,in all the cases, follow the co-inoculation trend. However, the descriptors were rated higher in the sequential inoculation (e.g. LT_E2 “acidity” attribute was rated 5 in the co-inoculation trial and 5.6 in the sequential inoculation one).
Conversely. the “sweetness” descriptor sometimes does not match with the residual sugars (g/L) in the chemical data table (Tab. 8). Indeed. some samples were rated as sweeter than others even if the residual sugars concentration was the same or even higher. The SC control was perceived sweeter than the LT_E1. even if the latter wine contained quite high concentrations of residual sugars (SC 0.12 g/L; LT_E1 8.2 g/L), for example. This phenomenon could be explained by balance in perceptions of sweetness and acidity. In fact, for example,TA in LT_E1 wine. was double than TA in control wine (.10.95 and 5.03 g/L. respectively).
Regarding the other attributes. “bitterness” and “body” showed a consistent trend in all the sequential-inoculation trials; sequential inoculations were in all the cases rated as less bitter and lower body than the co-inoculation trials and the control.

Table 13 Mouthfeel attributes significantly different ((p≤0.05)

	sweetness	**acidity**	**bitterness**	**hotness**	**body**	**balance**	**length of non-fruit flavours**	**length of acidity perception**
SC	3.5 ab	3.4 f	3.4 a	4.0 ab	3.7 a	2.9 abcd	3.9 ab	3.3 d
LT_E1X	2.8 d	5.0 bc	2.872 def	3.9 abc	3.5 abcd	3.2 ab	3.5 bc	4.8 a
LT_E1…	3.0 cd	5.4 a	2.7 f	3.4 d	3.2 d	2.9 bcd	3.6 bc	4.9 a
LT_E2X	2.7 d	5.0 bc	2.9 cdef	3.9 abc	3.5 abc	3.0 abcd	3.8 b	4.7 ab
LT_E2…	2.7 d	5.6 a	2.8 ef	3.4 d	3.3 cd	2.7 d	3.3 c	5.1 a

LT_C1X	3.3 bc	3.6 f	3.3 abcd	4.1 a	3.7 a	2.9 abcd	3.8 b	3.4 d
LT_C1…	3.6 ab	3.6 f	3.1 abcde	4.0 ab	3.7 a	3.2 ab	3.6 bc	3.4 d
LT_C2X	2.9 cd	4.1 e	3.3 abc	4.1 a	3.6 ab	3.3 a	3.7 bc	4.0 c
LT_C2…	3.5 ab	4.4 de	2.7 f	3.7 bcd	3.7 a	3.2 ab	3.9 ab	4.3 bc
LT_C3X	2.9 cd	4.7 cd	2.9 bcdef	3.9 abc	3.6 abc	3.1 abc	3.6 bc	4.3 bc
LT_C3…	2.9 cd	5.2 ab	2.6 f	3.6 cd	3.4 bcd	2.8 cd	3.6 bc	4.7 ab
UN-INOC	3.8 a	3.5 f	3.4 ab	3.9 abc	3.7 ab	3.0 abcd	4.3 a	3.3 d
p-value	< 0.0001	< 0.0001	< 0.0001	0.000	0.003	0.045	0.005	< 0.0001

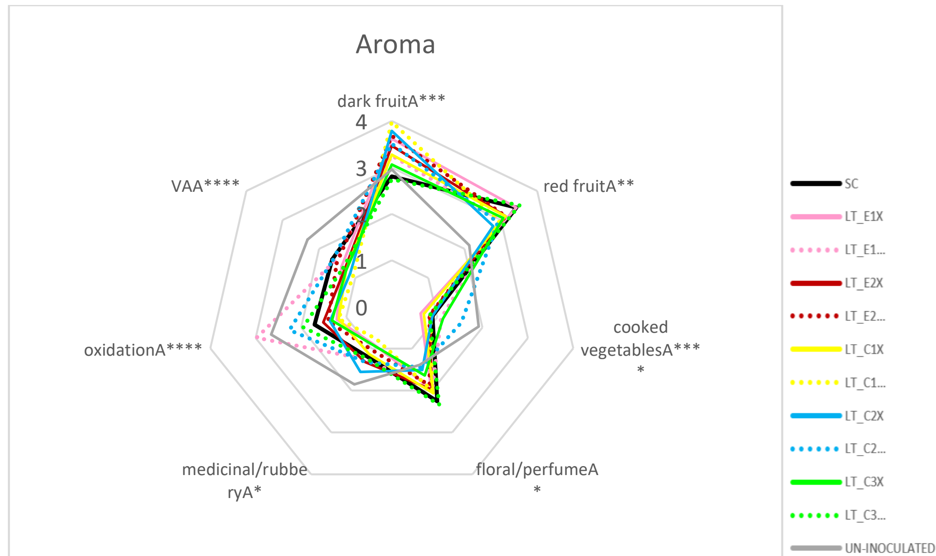

*Figure 18. Results of sensory aroma analysis of bottled wines from different fermentation treatments with mixed cultures of L.thermotolerans. p-value are summarized with asterisks: *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.*

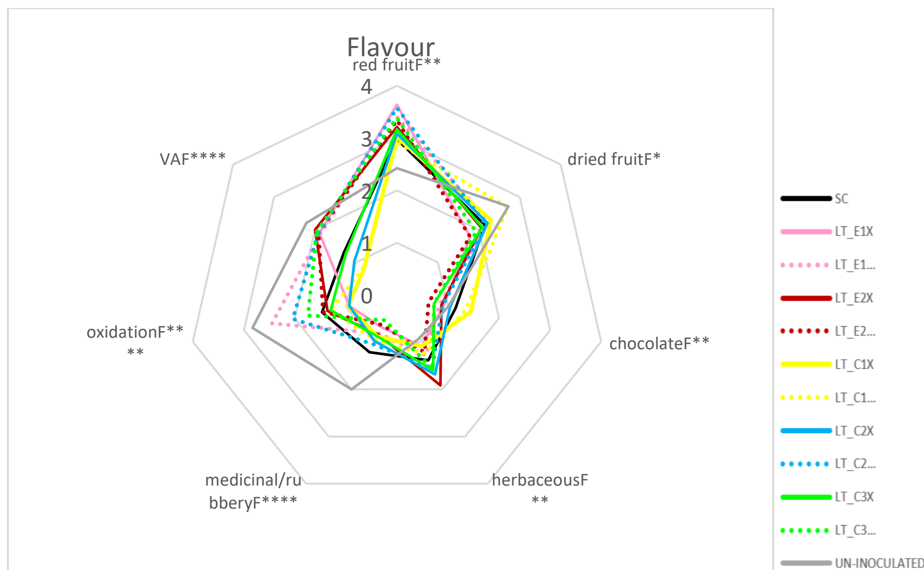

*Figure 19. Results of sensory flavour analysis of bottled wines from different fermentation treatments with mixed cultures of L.thermotolerans. p-value are summarized with asterisks: *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.*

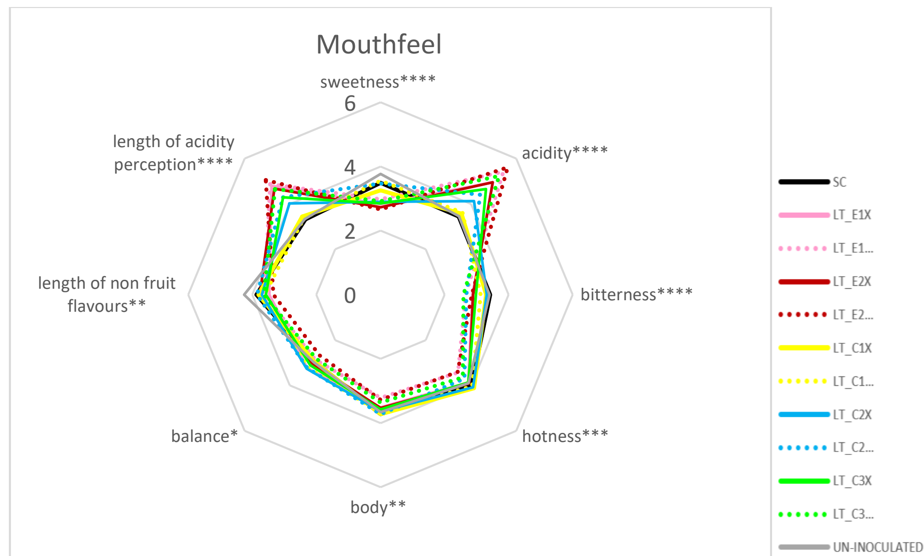

*Figure 20. Results of sensory taste/mouthfeel analysis of bottled wines from different fermentation treatments with mixed cultures of L.thermotolerans. p-value are summarized with asterisks: *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.*

Since bioacidification is a fundamental characteristic of *L. thermotolerans* metabolism; sensory analysis was particularly focused on the acidity aspect. For this reason. during the RATA test. a separate section concerning the acidity descriptors was added. In this part. it was required to describe the acidity feelings choosing between four given attributes (i.e. flat/flabby. bright/crisp. sour/tart. harsh/acrid). As illustrated in the graph (Fig. 21). Wines from *S. cerevisiae* control. UN-inoculated treatment and LT_C1 strain were described by 50% of the panellists as "flat/flabby". The LT_E1. LT_E2 and LT_C3 wines were considered more "sour/tart". while 20% of the responses of the responses for LT_C2 were "bright/crisp" and 20% "sour/tart". This treatment was also perceived as less "acid" and more "balanced". as seen in the spider plots (Fig 20). Interesting how More than 40% of the panellist described the acidity in the LT_E2… wine as "harsh", which matches with the analytical data in terms of titratable acidity (Tab.8).

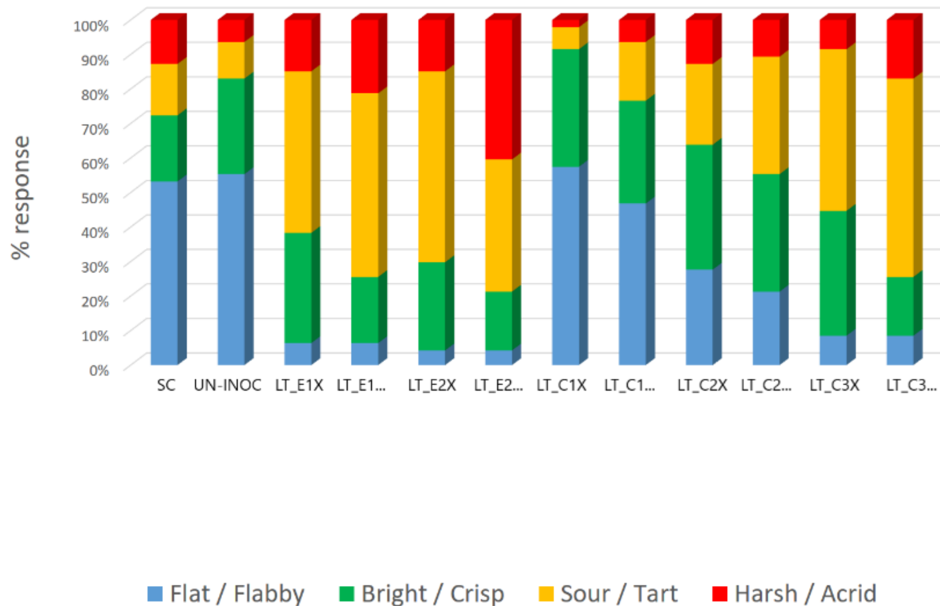

Figure 21. Results from sensory analysis: description of the acidity perception for 12 fermentation treatments with L.thermotolerans strains.

4. Discussion

Decreasing acidity and increasing potential alcohol levels in grapes exacerbated by climate change in viticultural areas, as well as consumers' preference of fresher, lower-alcohol wines, are pushing the wine researches to find methods to produce wines with lower ethanol content and alternative acidifying solutions. One of the last trends to overcome the latest challenges in oenology whilst making high quality wines is to harness the microbial diversity. Wine fermentation from grape must is a complex process which exceeds the simple conversion of sugars to ethanol and CO_2 as it also impacts the wine volatile profile and overall chemical composition. Indeed, the selection of yeast, or a mix of yeasts, to be used in fermentation is known to have an impact on the olfactory and flavour attributes of the finished wines (Ciani and Maccarelli, 1997; Jolly *et al*., 2003; Comitini *et al.,* 2011), which needs to be understood when monitoring the alcoholic fermentation.

The mixed culture fermentations with *S. cerevisiae* and non-*Saccharomyces* yeasts, generally, display slower fermentation kinetics compared to the *S.cerevisiae* monocultures. The yeast population dynamics, and in turn, the duration of the alcoholic fermentation is strongly affected by the time of inoculation. In one of the first studies on the performance of *L.thermotolerans* in co-cultures *S.cerevisiae* (Kapsopoulou *et al*., 2007), the *S. cerevisiae* strain strongly antagonized the *L. thermotolerans* strain, which reached lower cell concentrations and

completely died off after 7 days, 20 days before the *S.cerevisiae* strain. In the present work, similar phenomenon could also be observed; the *L. thermotolerans* population in the co-inoculations died off somewhat earlier than in the sequential cultures. Fermentation kinetics of the co-inoculation treatments was comparable to that of the *S. cerevisiae* monoculture (Fig.6). Conversely, the duration of the alcoholic fermentation in the sequentially inoculated cultures was longer (e.g. after 13 days, all co-inoculation completed AF, while sequential ones, on average, reached 85% AF completion, Tab 7). Interestingly, the delay in fermentation completion corresponds to the prolonged proliferation of *L.thermotolerans* cells in sequential inoculation treatments. The prolonged fermentation duration was also reported in the previous studies (Morata *et al*., 2019; Benito *et al*., 2016; Kapsopoulou *et al*., 2005). This is potentially due to lower availability of nutrients with delayed inoculation of *S. cerevisiae* (Gobbi *et al*., 2013), as well other stressors such as lower pH. Non-*Saccharomyces* yeasts differ from *S. cerevisiae* in their yields of ethanol and organic acids, as reported for *Starmenella bacillaris* (Ciani 2014; Comitini *et al*., 2011), *Metschnikowia pulcherrima* (Morales *et al*., 2015; Contreras *et al*., 2014), *Hanseniaspora species* (Moreira *et al*., 2008; Wang *et al*., 2015) and *Lachancea thermotolerans* (Kapsopoulou *et al.,* 2007; Gobbi *et al*., 2013; Morales *et al*., 2019).

In agreement with previous studies (Kapsopoulou *et al.,* 2005, 2007; Cominiti *et al.,* 2011, Gobbi *et al*., 2013; Balikci *et al.,* 2016), fermentations with *L.thermotolerans* resulted in increased levels of total acidity compared to the *S.cerevisiae* pure culture. The increase in titratable acidity is stronger in sequential inoculations compared to the co-inoculations. The increase in titratable acidity is related to the metabolic particularity of *L. thermotolerans*: L-lactic acid production, which is driving the decrease in pH level (Mora *et al*., 1990; Kapsopoulou *et al*., 2007, Hranilovic *et al.,* 2018). (Hranilovic *et al.,* 2018). LT can therefore be used as a biological acidifying agent in winemaking. In current study, the use of an *L. thermotolerans* LT_E2 strain in sequential inoculation lead to a pH decrease of 0.4 units compared to the *S.cerevisiae* control. In the traditional winemaking practice this operation would need about 4 g/L of tartaric acid addition (Ribéreau Gayon *et al*., 2006). However, not all LT strains showed an acidifying character. In particular, LT_C1 strain did not cause a pH drop/TA increase compared to the *S.cerevisiae* control. This further highlights large diversity in the metabolic behavior of different *L.thermotolerans* strains, in agreement with previous studies (Hranilovic *et al.,* 2018, not sure who else).

The impact of *L.thermotolerans* strains on wine profile is strongly dependent on the *S. cerevisiae* inoculation timing. In fact, the results show higher concentration of metabolites typical for *L.thermotolerans* with a delayed inoculation of *S.cerevisiae* strain. This is linked to the opportunity of non-*saccharomyces* yeasts to better develop in absence of *S.cerevisiae*. Accordingly, in other studies (Morata *et al*., 2019; Gobbi *et al.,* 2013; Comitini *et al*., 2011) pH reduction occurs at the beginning of the fermentation, when *L. thermotolerans* can still be competitive with *S. cerevisiae* population. The reduction of the ethanol concentration matches with the increase of the total acidity (Morata *et al.,* 2019). Other authors have reported a decrease in the alcoholic degree in wines produced by *L.thermotolerans* (Kapsopoulou *et al*., 2007; Gobbi *et al*., 2013; Benito *et al*., 2016, 2018; Morata *et al*., 2019).

The highest concentration of malic acid was in the *S.cerevisiae* control. Additionally, in accordance with Morata *et al*., 2019, malic acid levels were higher in the co-inoculations than in the sequential ones. However, some studies did not observe this phenomenon (Benito *et al*., 2015; Escribano *et al*., 2018). Nevertheless, in most *L.thermotolerans* treatments, an increase in the total acidity and a decrease in the final pH occurred, because the influence of lactic acid formation was stronger than the partial degradation of malic acid.

In line with other studies (Kapsopoulou *et al.*, 2007; Gobbi *et al*., 2013; Benito *et al*., 2016; Hranilovic *et al.,* 2018) the results showed higher glycerol levels in mixed cultures than in the control. Furthermore, glycerol production is increased in sequential inoculations compared to the co-inoculations. However, it remains uncertain whether the increased amounts of glycerol were produced by the *L.thermotolerans* strains, or whether this is attributable to the sequentially inoculated *S.cerevisiae* control.

Previous studies reported reduced concentrations of acetic acid in mixed cultures with *L.thermotolerans* (Comitini *et al*., 2011; Gobbi *et al*., 2013; Ribereau Gayon *et al*., 2006; Vilela, 2018; Morales *et al*., 2019). When comparing mixed co- and sequential inoculations, in accordance with other studies (Gobbi *et al*., 2013; Kapsopoulou *et al*., 2007) the concentrations of acetic acid were lower in co-inoculations, possibly due to the slower alcoholic fermentation process in the sequential fermentations. Control wine has the lowest acetic acid concentration and as expected,it was over the perceivable threshold in the Un-inoculated wine.

L. thermotolerans mixed fermentations showed lower final concentrations of total SO_2 than fermentations with *S. cerevisiae*, in accordance with Benito *et al* (2015). A possible lower sulfur metabolism could explain those results, besides, that kind of metabolism can be due to

the low SO_2 tolerance which characterizes *L.thermotolerans* compared to *S.cerevisiae* and to several strains of non-*Saccharomyces* (Comitini *et al*., 2011).

Phenolics are important to both red and white wines. In red wines, those substances contribute to the astringency, bitterness, and structure, as well as to the wine's red colour. The grape anthocyanins are extracted from grape skins to the must, and they are the major pigments contributing to the colour of young red wines. Anthocyanins are short-lived because of their high reactivity; in fact their concentrations into the wine decrease quickly after the formation of new pigments, resulting from the reaction of anthocyanins with other wine constituents such as yeast metabolites (Hayasaka *et al*., 2007) and proanthocyanidins (Somers 1971). One possibility for managing phenolics in wine is the choice of yeast strain. Infact, wine colour is influenced by direct yeast interaction with phenolics (Morata *et al*., 2005; Medina *et al.*, 2005) and by reaction between phenolics compounds and yeast metabolites or by products of fermentation. Indeed, the composition and porosity of the cell walls of the yeasts can cause losses of colour via the adsorption of pigment flavonoids (e.g., anthocyanins; De Nobel *et al.*, 1990; Vasserot *et al*., 1997; Morata *et al*., 2005).

In this study the total monomeric anthocyanins level was lower in the control, this phenomenon may be explained by a stronger adsorption effect from *S.cerevisiae* than *L.thermotolerans* yeast as reported in Benito *et al* (2017). However, in the sequential inoculations the concentration of total monomeric anthocyanins was significantly inferior compared to co-cultures, but the colour density and the degree of coloured form of anthocyanins was higher. This may be explained by co-pigmentation or most probably by a possible formation of polymeric pigments with consequent hyperchromic and hypsochromic effect (Morata *et al*., 2003).

The concentrations of phenolics in red wine is affected by numerous factors including both genetic factors such as variety fingerprint, environmental factors like soil, climate, canopy management, as well as winemaking techniques (e.g. skin contact duration or fermentation temperature; Jackson and Lombard, 1993). In the present study, the overall values of total phenolics and anthocyanins are quite low, mainly because of the low ratio skin/juice during fermentations. Nonetheless, these results suggest that different yeast strains adsorb anthocyanin derivatives to different degrees, and different inoculation time affected the level of phenolic compounds. In fact, usually, red wines have a range of phenolics from 23 to 100 AU, with an average of 54 AU (Waterhouse A., 2002). In summary, the appropriate strains and inoculation modalities should be selected from winemakers to manage the colour of wines.

Besides, lower levers of total phenolics were obtained in sequential inoculations cultures compared to the co-inoculations. This data may be explained also considering that, in general, phenolics extraction increase with production of ethanol during alcoholic fermentation with maceration (Ribéreau-Gayon *et al*., 2006), and in the sequential cultures after 8-10 days of alcoholic fermentation was developed only 75% of the alcohol potential, compared with 89% in the co-cultures.

Unlike the analysis of phenolic compounds with the modified Somers method through which the levels of pH and ethanol in wine samples are standardised via a dilution in a buffer (Mercurio *et al.,* 2007), CIELab analysis was purposely conducted without pH corrections. The rationale was to obtain data analogous to a sensory analysis made, i.e. tasters observing the wine through a glass. We obtained quite high ΔE*, hence, accordingly to Gonnet (1998) and Pérez Margarino (2002), we are able to say the wines were perceivably different in terms of colour, and that may be explained by significant differences in pH, besides the dissimilar anthocyanin's extraction and SO_2 level (Somers, 1977; Ribéreau- Gayon, 2006).

The main influence from *L. thermotolerans* on the sensory profile in wine is clearly the perception of acidity. As reported in previous studies (Benito *et al*.,2015; 2018), the change in the acidity perception was manifested via the increase in titratable acidity (by + 3 g/L on average) and a decrease in pH (by – 0,3 point on average). As confirmed from previous studies, the perception of 'sweetness' and 'body' (often associated with glycerol level; Ciani and Maccarelli, 1998) was affected by the acidity level (Benito *et al*., 2016). Moreover, the co-inoculation treatments were rated as sweeter and less acidic than the sequential treatments, as seen previously (Gobbi *et al*., 2013; Benito *et al*., 2016). In same cases, Co-inoculated treatments were less fruity than the sequential ones. In general, the mixed culturesshowed increased perception of 'dark fruit' aroma and 'red fruit' flavour compared to the *S.cerevisiae* control. Further work (notably analysis of volatile compounds) is required to understand whether this is related to differences in flavour-active volatile compounds such as higher alcohol (e.g. 2-phenylethanol) and some esters (e.g. ethyl lactate, isoamyl acetate, ethyl hexanoate). Indeed, previous work (e.g. Gobbi *et al*., 2013; Benito *et al.,*2016; Morales *et al.,* 2018) reported increased concentrations of certain aroma compounds in wines co-fermented with *L. thermotolerans* compared with pure cultures of *S.cerevisiae*. Thus, it might be said that the bioacidification and *L. thermotolerans* metabolism, has also a central position on sensorial aspect. Besides, as acids have different taste and sensorial perception (Amerine *et al*., 1965) Supplementary studies are required to examine the differences on palate perception between

bioacidification by lactic acid produced by *L. thermotolerans* and common winemaking acidification by tartaric acid addition.

Regarding the strains, the most performant in terms of fermentation dynamics was LT_C1, both in sequential and co-inoculation treatments. The other strains caused protracted fermentation dynamics in sequential cultures, but less so in co-inoculated cultures.

Another important aspect to be considered is the influence of this yeast species on malolactic fermentation. MLF is an enzymatic degradation (catalyzed by malolactic enzyme) where L-malic acid is decarboxylated into L-lactic acid (Seifert, 1901; Fig.2). This bioconversion improves the microbiological stability of wines, acidity perception and aroma complexity (Davis *et al.,* 1985). The microorganisms responsible for MLF are lactic acid bacteria. Lactic acid bacteria mainly belong to the genera *Lactobacillus, Pediococcus*, and *Oenococcus* (Fleet *et al.,* 1984). *Oenococcus oeni* is the main species in charge for MLF. The interaction between non- *Saccharomyces* yeast and lactic acid bacteria have received little attention. However, the increasing number of non- *Saccharomyces* yeast in winemaking environment, guides to better understanding of this interaction (Nardi *et al*., 2018).

In particular *L. thermotolerans* can have an important impact on MLF because of its metabolism. In fact, low level of pH can cause difficulties in malolactic fermentation, mainly because of the higher levels of molecular SO_2 at lower pH levels (Ribéreau-Gayon *et al*., 2006; Spano and Massa, 2006). Besides, the high production of lactic acid can reduce the viability and proliferation of lactic acid bacteria because of its antimicrobical properties (De Vuyst *et al.,* 1994). Nevertheless, some studies revealed that the dynamics of AF and MLF were not affected, even in the case of complex mixed situations, likely due to plentiful nutrient supplementation (du Plessis *et al*., 2016; Nardi *et al.,* 2018).

5. Conclusion

One of the most important features of non-*Saccharomyces* yeast strains in mixed culture fermentations is their ability to positively impact the wine quality, even in musts with sub-optimal characteristics. In particular, the metabolism of *L. thermotolerans* seems perfectly suitable to achieve the winemakers' goals and the customers' demand for less alcoholic, 'fresh' and well-balanced wines (Benito, 2018; Hranilovic *et al*., 2018). However, it is important to

consider the differences at the strain level, as well as the effect of oenological practices such as inoculation timing or dosage which largely affect the final result (Gobbi *et al*., 2013).

The aim of this study was to further understand the potential of *L. thermotolerans* yeasts in modulating final wine characteristics. This yeast has several metabolic traits requested in oenology, especially in the context of climate change. In fact, it abundantly produces lactic acid, increasing the total acidity level and can also lead to decreased ethanol content and modulated sensory aspects in wines (Jolly *et al.,* 2014). The objective for this small-scale winemaking trial was to compare a commercial *S. cerevisiae* strain to three commercial *L. thermotolerans* strains and two experiential *L.thermotolerans* strains. The winemaking was carried out with two different modalities of inoculating mixed cultures: co-inoculation and sequential inoculation. All the tested strains were able to proliferate and catabolize sugars, however the rate of sugar consumption was lower for two treatments. Upon fermentation completion, analysis of main metabolites, total phenolics, CIELab coordinates and sensory RATA analysis were undertaken. From this multi-level comparative study, we obtained significant differences.

The main finding of this study can be summarized as follows:

- Twelve yeast treatments significantly affected the chemical and sensory parameters of wines with a stronger L. thermotolerans effect in sequential inoculations than in co-inoculations, in terms of the chemical, colorimetric and sensorial profiles of the wines.

- The modulation in L. thermotolerans mixed cultures is depended on the L.thermotolerans strain and inoculation modality.

- The L. thermotolerans strains and inoculation modalities differently affect wine fermentation dynamics. Compared to the S.cerevisiae control, the dynamic can range from linear process (e.g. LT_C1), slower fermentation (e.g. LT_E1X, LT_C1X), sluggish fermentation (e.g.LT_C3…) to stuck fermentation (LT_E1…)

- In terms of AF performance, in all the cases the mixed cultures were faster than the control to reach 50% of the fermentation. After the 80% there was a slowdown of the kinetics in all the sequential trial compared both to the control and to the co-inoculated treatments. The Un-inoculated trial was the slowest until the AF reached 85%.

- Compared to the S.cerevisiae control monoculture, the drop in pH and the increase in TA ranged up, on average, to - 0,3 points and +3 g/L. Low pH shift the SO_2 equilibrium to the molecular form, with a consequent higher preservative action.

- Bioacidification was not achieved by LT_C1 strain regardless of the inoculation modality. In contrast the most significant bioacidification effect was performed by LT_E2.

- Mixed cultures in sequential inoculation compared to both S.cerevisiae control monoculture and co-inoculation trials had a lower level of total anthocyanins and total phenolics, but they resulted more colored in the CIELab evaluation; most probably because of the pH effect.

- In the sensory evaluation, compared to the S.cerevisiae monoculture, L. thermotolerans mixed cultures were perceived a bit fruitier and fresher. But some of the sequential trials were perceived more oxidated and unbalance on the palate.

While there are many advantages for use L. *thermotolerans* in winemaking, there are some disadvantages, such as delayed fermentation completion that increases in undesirable molecules (e.g. succinic acid, or acetoin) under some circumstances (Benito *et al.,* 2018). For this reason, further study needs to be conducted.

Besides limited knowledge, another potential limitation for their wider use in wine sector is their higher price (e.g. ~140 USD/kg; aebgroup.com). This is costlier than generally used S. *cerevisiae* inocula, which also need to be sourced to conduct mixed culture fermentations. However, chemical acidification and fine-tuning of ethanol levels also impose costs on winemakers. Besides, observing future perspectives, the change in customers taste and climatic conditions, microbiological acidification can play an essential role in satisfying the wine market demand of low ethanol, crisp and high-quality wines. Another important aspect is stability of lactic acid, i.e. it is not degraded by microorganisms and it does not precipitate out.This is important for red wines that mature in oak barrels and/or bottles before consumption (Vivas *et al*., 1995; Ribereau-Gayon *et al*., 1998), and is thus an interesting perspective to make age-worthy wines.

Appendix:

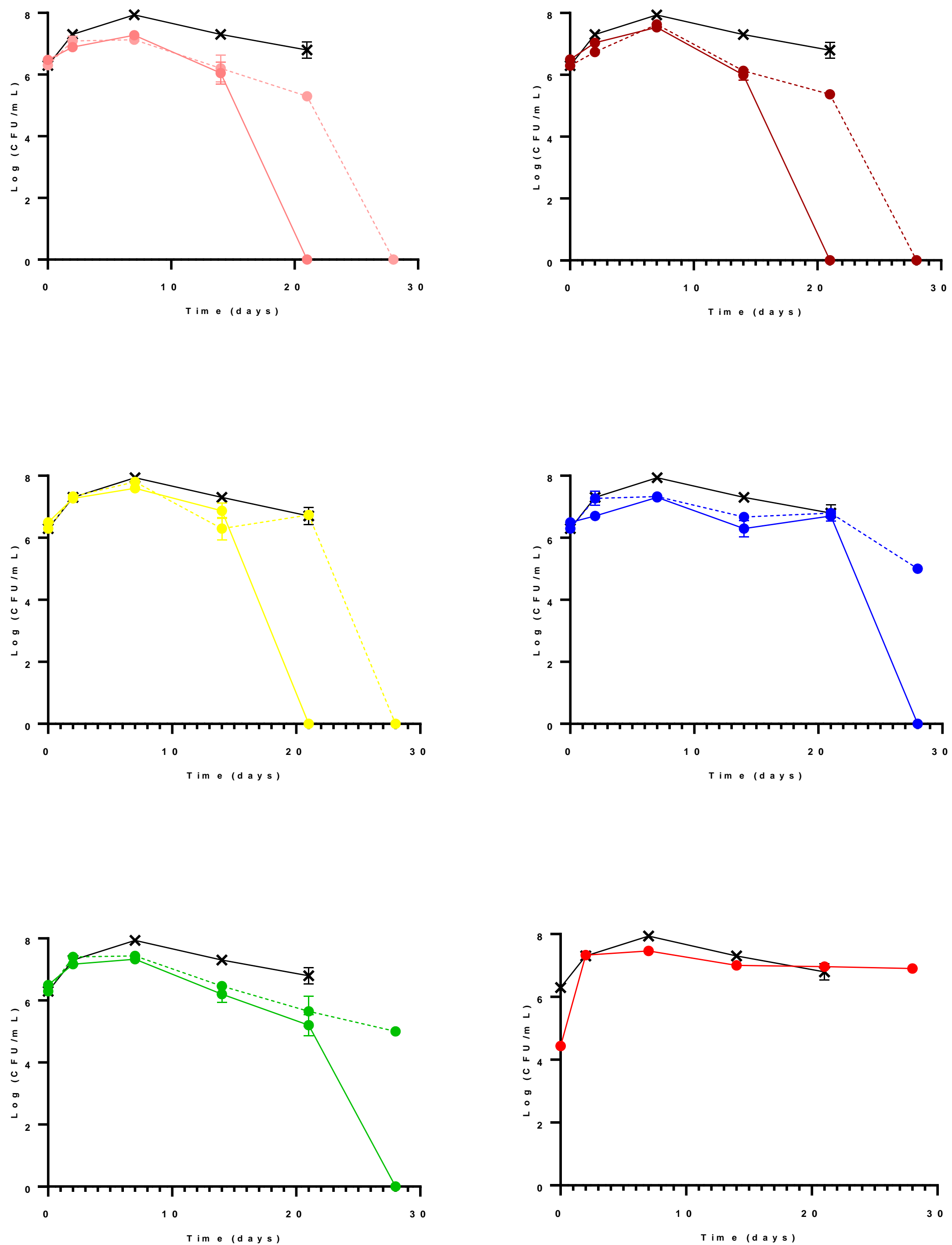

Figure 22. Population dynamics of five L. thermotolerans strains in sequential (dotted lines) and co- inoculation (solid lines) regimes compared to S. cerevisiae (SC) control (black). Population dynamics in uninoculated trial (last graph). Different colours correspond to different strain: LT_E1(●), LT_E2 (●), LT_C1 (●), LT_C2 (●), LT_C3 (●).

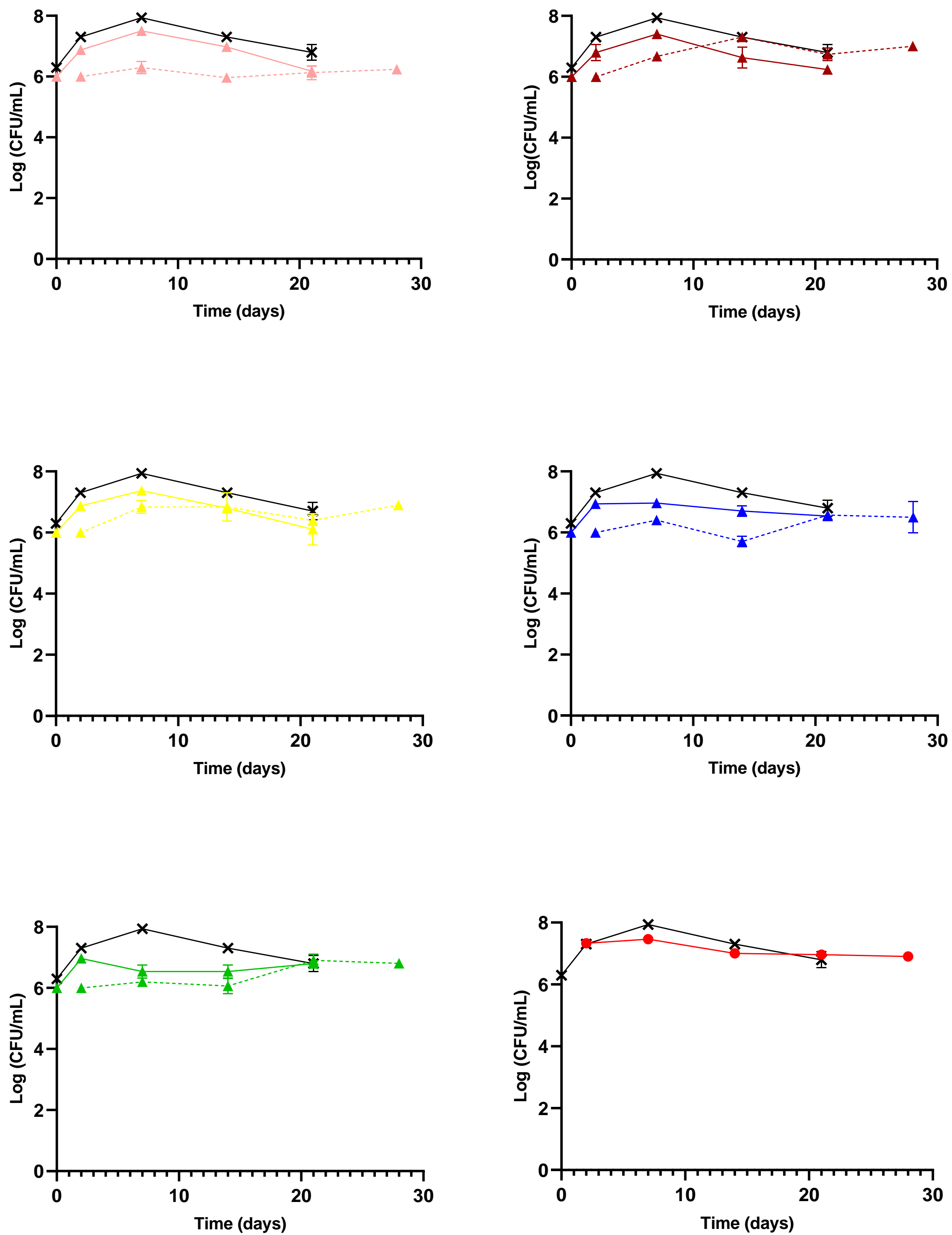

Figure 23. Population dynamics of only S.cerevisiae yeast in sequential (dotted lines) and co- inoculation (solid lines) regimes compared to S. cerevisiae monoculture (SC) control (black). Population dynamics in uninoculated trial (last graph). Different colours indicate the different strain in mixed culture with S.cerevisiae: LT_E1(●), LT_E2 (●), LT_C1 (●), LT_C2 (●), LT_C3 (●).

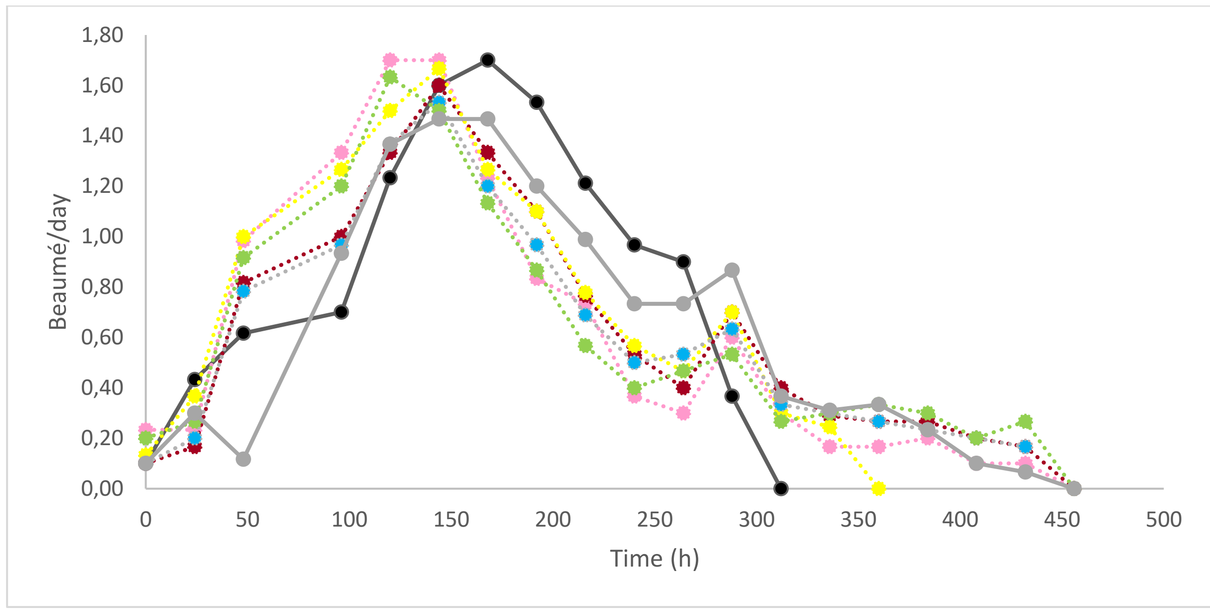

Figure 24. Sugar consumption rate per day of five L.thermotolerans strain in sequential trial compared to S.cerevisiae control (black) and Un-inoculated trial (grey). Colour code: LT_E1(●), LT_E2 (●), LT_C1 (●), LT_C2 (●), LT_C3 (●).

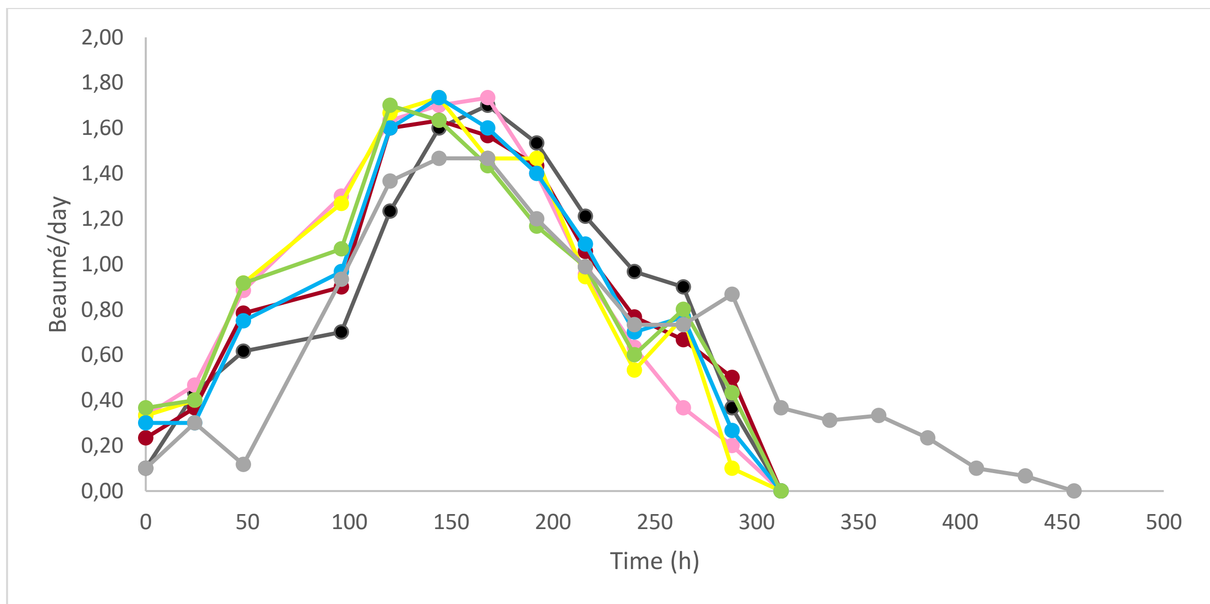

Figure 25. Sugar consumption rate per day of five L.thermotolerans strain in co-inoculated trial compared to S.cerevisiae control (black) and Un-inoculated trial (grey). Colour code: LT_E1(●), LT_E2 (●), LT_C1 (●), LT_C2 (●), LT_C3 (●).

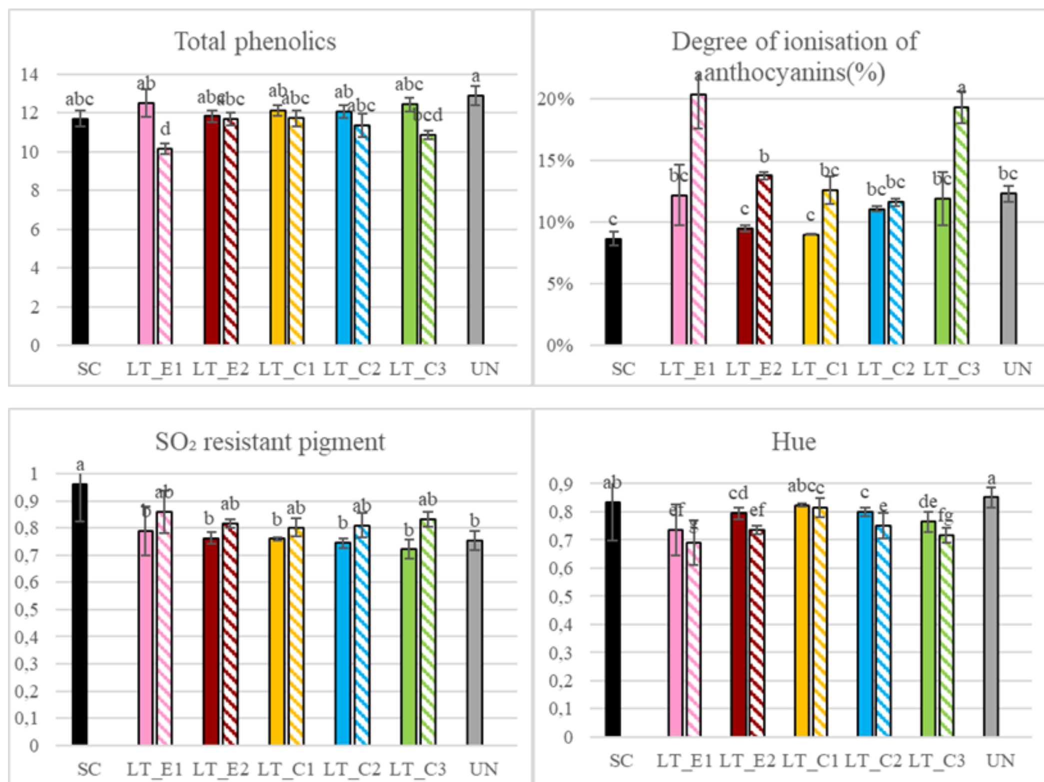

Figure 26. Total phenolics, SO_2 resistant pigment, degree of ionisation and hue of 12 Merlot wines fermented in mixed cultures with 5 strains of L.thermotolerans, a monoculture of S.cerevisiae (control, black) and an UN-inoculated trial (grey). Co-inoculations (xSC) are represented with full bars and sequential inoculations (...SC) with patterned bars, respectively. The values are means of triplicates ± SD, letters denote significance levels differences (ANOVA, Tukey's HSD, $p<0.05$). Colour code of mixed cultures: LT_E1(●), LT_E2 (●), LT_C1 (●), LT_C2 (●), LT_C3 (●).

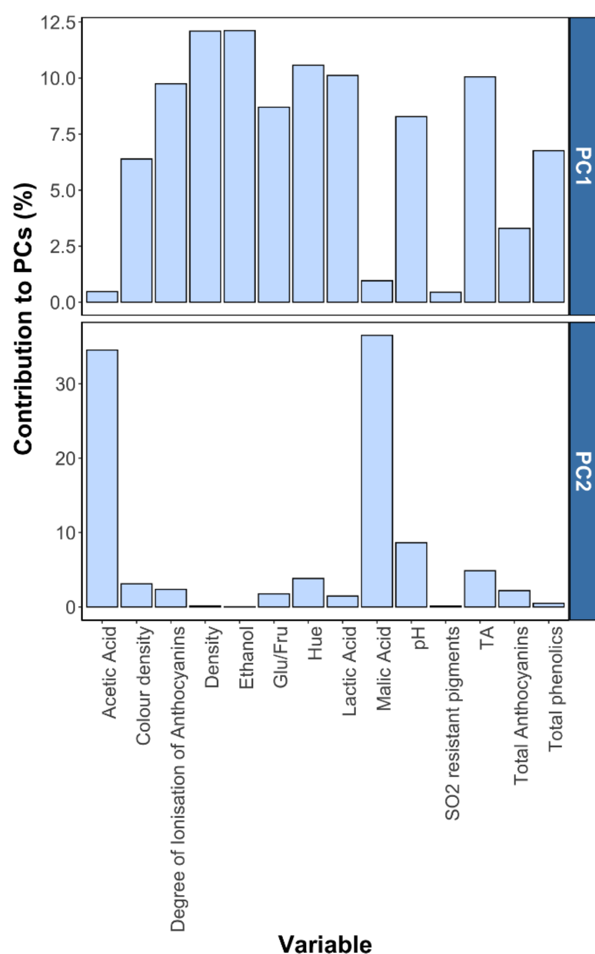

Figure 27. Contribution to PCs of 14 variables.

Table 14. CIELab coordinates of 12 bottled Merlot wines.

TREATMENT	L*	a*	b*	C*	H*
SC	58,10367	45,222	31,04967	54,8592	34,45555
LT_E1X	52,37267	55,01	32,32633	63,80887	30,42493
LT_E1…	45,23033	57,456	42,30133	71,35038	36,35916
LT_E2X	53,36167	53,96267	31,98367	62,73183	30,64132
LT_E2…	50,304	56,067	36,26233	66,77679	32,88094
LT_C1X	58,733	44,391	26,50967	51,70571	30,83914
LT_C1…	55,56733	45,37333	29,34633	54,03726	32,90211
LT_C2X	57,43067	47,807	26,146	54,49001	28,67009
LT_C2…	50,01267	53,50233	32,44433	62,57368	31,22564
LT_C3X	53,855	52,73567	30,11867	60,73315	29,7208
LT_C3…	47,60667	55,78467	37,77767	67,37969	34,09064
UN-INOCULATED	58,07233	43,20233	28,64867	51,83879	33,54974

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