Selection and characterization of novel malolactic starters from Rioja Alavesa region: in the pursuit of free-histamine wine

Iñaki Diez Ozaeta

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Universidad del País Vasco

Euskal Herriko Unibertsitatea

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Directores:

Dr. Félix Amárita Vega

Dr. Francisco José Pérez Elortondo

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Quien quiera ser feliz, lo sea.

Del mañana no hay certeza.

Lorenzo de Medici

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Resumen

Las aminas biógenas (AB) son un asunto de gran relevancia para la industria del vino en términos de calidad y seguridad. Estos compuestos pueden llegar a causar múltiples síntomas adversos, los cuales se agudizan en individuos sensibles. La inoculación de cultivos malolácticos seguros es una estrategia para prevenir la acumulación de estos compuestos en el vino. Así pues, el objetivo principal de este trabajo fue identificar y caracterizar cepas autóctonas de bacterias ácido-lácticas (BAL) de la Rioja Alavesa que careciesen la capacidad de producir AB y poseyeran propiedades tecnológicas y sensoriales óptimas.

En primer lugar, tras el análisis de 70 vinos tintos se obtuvo una instantánea de la situación actual de los niveles de AB en Rioja Alavesa. La más abundante fue la putrescina (14,85 ± 8,9 mg/L) seguida de la histamina (4,43 ± 2,8 mg/L), tiramina (3,29 ± 3,28 mg/L) y cadaverina (2,14 ± 1,58 mg/L). La ausencia de diferencias significativas entre los diferentes tipos de vinos analizados (vinos jóvenes, crianzas y reservas) indicó que la producción de AB en estos vinos ocurre durante los procesos de fermentación y no durante el período de envejecimiento. Las correlaciones positivas entre casi todas las AB mostraron que la producción de un compuesto implicaba la producción de casi el resto. Si bien la situación no se consideró alarmante, debido a las recomendaciones establecidas por diferentes países europeos sobre los niveles de histamina, se observó que era necesario seguir trabajando para reducir al mínimo los niveles de AB. Además de las aminas consideradas toxicológicas, como la histamina y la tiramina, se prestó especial atención a la concentración de putrescina, la cual se detectó en niveles excesivos. Así pues, considerando la demanda del sector vitivinícola

de reducir los niveles de AB al mínimo, y teniendo en cuenta la relación entre la acumulación de AB y las poblaciones de bacterias lácticas (BAL) presentes en el vino, se inició la búsqueda de nuevos cultivos malolácticos seguros como estrategia para prevenir la aparición de estos compuestos en el vino.

Se comenzó con el análisis de las poblaciones de BAL de dos bodegas de la Rioja Alavesa durante todo el proceso de vinificación. De cerca de 300 aislamientos, se identificaron 27 genotipos de BAL pertenecientes a las especies *Pediococcus parvulus* (3), *Lactobacillus plantarum* (1), *Lactobacillus mali* (3), *Lactobacillus hilgardii* (3) y *Oenococcus oeni* (17). Aunque durante el proceso de vinificación la evolución de las especies de BAL fue diferente en ambas bodegas, *O. oeni* se convirtió en la especie predominante en ambas bodegas una vez comenzada la fermentación maloláctica (FML). Cada bodega mostró un ecosistema exclusivo con una microbiota propia, puesto que se detectaron pocos genotipos coincidentes en las dos bodegas. Tanto por métodos fenotípicos como moleculares se detectó una baja incidencia de cepas de BAL productoras de AB, ya que exclusivamente las cepas correspondientes a la especie *Lactobacillus hilgardii* fueron positivas para la producción de putrescina. También se identificaron otros géneros de bacterias que rara vez se encuentran en el entorno del vino, como *Staphylococcus* y *Paenibacillus*. De hecho, este es el primer trabajo en el que esas especies han sido reportadas como productoras de AB en vino. Estos resultados enfatizaron la posible aplicación de las cepas de BAL para minimizar la formación de AB durante todo el proceso de vinificación. Sin embargo, para dilucidar las características tecnológicas y sensoriales de las cepas identificadas fue necesario un análisis más detallado.

De esta forma, en primer lugar, se realizó la caracterización tecnológica de 22 cepas de BAL pertenecientes a las especies *Oenococcus oeni*, *Lactobacillus mali* y *Lactobacillus plantarum.* Tras analizar su comportamiento frente a las duras condiciones del vino, así como su vigor fermentativo, se confirmó la mejor idoneidad de las cepas de *O. oeni* sobre el resto de las especies. Además, sobre las cepas de *O. oeni*, se llevó a cabo el análisis de diversas actividades enzimáticas. Mediante ensayos fenotípicos y moleculares, se confirmó que todas las cepas eran capaces de metabolizar el citrato. También se realizó la cuantificación de actividades glicosidasa (α-glucosidasa, β-glucosidasa, β-xilosidasa y α-arabinosidasa) y esterasa bajo diferentes combinaciones de pH y concentraciones de etanol. Cabe destacar que todas las cepas exhibieron actividad α-glucosidasa, β-glucosidasa y esterasa. Por el contrario, sólo unas pocas cepas mostraron actividad β-xilosidasa y α-arabinosidasa. Se observó un efecto sinérgico negativo del pH y el etanol sobre la actividad enzimática en las condiciones más extremas, de esa forma, cuando la concentración de etanol era más agresiva, una pequeña disminución del pH del medio se traducía en una disminución significativa de la actividad enzimática. Sin embargo, bajo las condiciones más restrictivas muchas cepas conservaban aún actividades detectables. Estos resultados supusieron un avance importante para considerar el uso potencial de muchas de las cepas autóctonas de *O. oeni* como una estrategia eficaz para realizar una FML fiable, así como para mejorar la complejidad del aroma del vino en la región de la Rioja Alavesa. En este sentido, y en función de sus mejores características, se seleccionaron las cepas P2A, P3A, P3G, P5A, P5C y P7B de *O. oeni* para proseguir con su caracterización. En consecuencia, el siguiente paso en el proceso de selección de

nuevos cultivos malolácticos fue determinar su influencia en la modificación del aroma del vino.

Así, se examinó la eficacia de las cepas seleccionadas para llevar a cabo fermentaciones en vino real. Las vinificaciones realizadas a escala de laboratorio dieron una idea de que cepas eran las más vigorosas: las cepas P2A y P3A pudieron concluir la fermentación maloláctica (FML) en menos de 15 días. Las cepas restantes mostraron buena viabilidad y pudieron terminar con éxito la FML en el tiempo establecido de análisis, a excepción de la cepa P5A, cuya viabilidad se perdió totalmente después de la inoculación. La fermentación espontánea tampoco llegó a iniciarse. No se observó el aumento de AB durante el proceso de vinificación; sin embargo, tras la FML realizada por la cepa P5C, se observó un aumento significativo de la concentración de ácidos hidroxicinámicos, compuestos precursores de fenoles volátiles. La evolución de compuestos aromáticos mostró que los principales cambios después de la FML se produjeron tanto para los ésteres de etilo como de acetato; sin embargo, también se observó un aumento significativo de compuestos aromáticos clave, como alcoholes, terpenos o ácidos. El análisis de componentes principales clasificó las cepas en dos grupos distintos, cada uno correlacionado con diferentes compuestos volátiles clave. Las cepas P2A, P3A, P3G y P5C se unieron principalmente a ésteres de acetato y ésteres de etilo, mientras que la cepa P7B y la cepa comercial Viniflora OENOS mostraron mayor relación para el ácido hexanoico, β-damascenona, linalol o 2-feniletanol. Estos resultados confirmaron el impacto específico que cada cepa tenía en el perfil aromático del vino, lo que podría conducir a la producción de vinos con características individuales, en los que la fiabilidad y seguridad de la FML estaban garantizadas.

El último paso en el proceso de selección de nuevos cultivos malolácticos pretendía, por un lado, dilucidar la influencia que las cepas seleccionadas tenían en la percepción sensorial de los vinos, y, por otro lado, determinar la idoneidad de cualquiera de las cepas para llevar a cabo fermentaciones a gran escala en bodega. Las cuatro cepas de *O. oeni* más prometedoras (P2A, P3A, P3G y P7B), según sus propiedades tecnológicas y sensoriales, junto con la cepa comercial Viniflora OENOS fueron sometidas a diferentes procesos de vinificación donde se analizaron diferentes estrategias de inoculación. Es decir, la inoculación de las cepas se realizó por coinoculación (24 h después de la inoculación de la levadura) e inoculación secuencial. La coinoculación condujo a la rápida consecución de la FML, destacando especialmente el comportamiento de las cepas P2A y Viniflora OENOS, que finalizaron la FML junto con la fermentación alcohólica (FA). En general, cuando se realizó la inoculación secuencial, el proceso de vinificación necesito de entre 20 y 30 días más para concluir. La estrategia de inoculación también influyó en el perfil volátil de los vinos. Los vinos coinoculados mostraron significativamente menor concentración de compuestos volátiles, un hecho especialmente reseñable en aquellos vinos donde el proceso fermentativo concluyó rápidamente (P2A y Viniflora OENOS). La principal reducción se detectó en alcoholes superiores y ácidos y, por lo tanto, en la concentración de ésteres. Hay que destacar que la menor concentración tanto de ácidos como de alcoholes superiores puede prevenir el enmascaramiento de los atributos aromáticos deseados. De hecho, en los vinos coinoculados la percepción del aroma de fruta madura destacó sobre los demás atributos, y se percibió ampliamente en comparación con los respectivos vinos inoculados secuencialmente. Así, se constató

la influencia específica de cada cepa en la modulación del perfil sensorial del vino, la cual también se percibió a nivel sensorial.

Finalmente, se analizó la eficacia de la cepa P2A para trabajar a mayor escala en bodega. Se volvieron a analizar ambas estrategias de inoculación, comparándolas con la fermentación espontanea llevada a cabo por la bodega de manera habitual. Se volvió a observar la idoneidad de la coinoculación para una rápida FML, concluyendo el proceso un mes y 15 días antes en comparación con la fermentación espontánea y la estrategia de inoculación secuencial, respectivamente. Así pues, se confirmó la idoneidad de la cepa P2A para trabajar en fermentaciones a gran escala en bodega, resultando una alternativa ventajosa para reducir significativamente el tiempo total de vinificación, así como para controlar mejor el proceso fermentativo. En este sentido, esta cepa reúne todas las características que debe cumplir un nuevo cultivo iniciador. Es una cepa segura, con una capacidad de implantación rápida y total, que también está indicada para realizar la FML mediante coinoculación. Si bien pueden ser necesarios futuros trabajos para confirmar plenamente la idoneidad de esta cepa como cultivo maloláctico, con el presente trabajo se abre un nuevo campo en la selección de cultivos malolácticos autóctonos de la Rioja Alavesa. En este sentido, la caracterización y selección de cultivos novedosos, y combinaciones de los mismos, con las características deseadas, pueden representar una prometedora línea de investigación para potenciar la calidad de los vinos de Rioja Alavesa.

Summary

Biogenic amines (BAs) are considered a high priority issue for wine industry in terms of product quality and safety. They may cause several adverse symptoms which are enhance in susceptible individuals. The inoculation of safe malolactic starters is one possible strategy to prevent the accumulation of these compounds in wine. In this sense, the main objective of this work was to identify and characterize autochthonous lactic acid bacteria (LAB) strains from Rioja Alavesa region lacking the ability to produce BAs and owning desired technological and sensorial properties. Firstly, after the analysis of 70 red wines it was obtained a snapshot of the current situation of BAs levels in Rioja Alavesa region. The most abundant was putrescine (14,85 ± 8,9 mg/L) followed by histamine (4,43 ± 2,8 mg/L), tyramine (3,29 ± 3,28 mg/L) and cadaverine (2,14 ± 1,58 mg/L). Considering wine sectors demand to reduce BAs levels to minimum, the pursue of novel malolactic starters was initiated. From near 300 isolates, 27 LAB genotypes belonging to *Pediococcus parvulus* (3), *Lactobacillus plantarum* (1), *Lactobacillus mali* (3), *Lactobacillus hilgardii* (3) and *Oenococcus oeni* (17) species were identified. In this regard, it was confirmed the great predominance of *O. oeni*. Among LAB species, only *L. hilgardii* strains were able to produce putrescine via the agmatine deiminase pathway. The technological characterization of LAB strains elucidated the great suitability of *O. oeni* species against typical harsh conditions found in wine as well as their better performance at conducting the malolactic fermentation (MLF) over the rest of species. Further characterization over *O. oeni* strains elucidated their ability to retain different glycosidase (α-glucosidase, β-glucosidase, β-xylosidase and α-arabinosidase) and esterase activities under winemaking conditions. The prospective use of *O. oeni* strains as malolactic starters

was first examined at laboratory scale microvinifications. No production of BAs was detected, and the evolution of aromatic compounds showed that main changes after MLF occurred for both ethyl and acetate esters. Principal component analysis classified the strains in two distinct groups, highlighting the specific impact of each strain on wine aroma profile. Most promising five *O. oeni* strains were submitted to co-inoculation and sequential inoculation fermentation processes. Co-inoculation led to the prompt consecution of winemaking process and no production of BAs was detected during any MLF. In co-inoculated wines the perception of ripe fruit aroma was extensively perceived in comparison with their respective sequentially inoculated wines. Finally, it was elucidated the suitability of the strain P2A to work in large scale fermentations at winery, resulting an advantageous alternative to significantly reduced the overall winemaking time as well as to better control the fermentative process.

Laburpena

Amina biogenoak (AB) garrantzi handiko gaia dira ardoaren industriarentzat kalitateari eta segurtasunari dagokionez. Konposatu horiek sintoma kaltegarri ugari sor ditzakete, pertsona sentikorrengan areagotzen direnak. Ardoan konposatu horien pilaketa ekiditeko estrategia gisa, kultibo malolaktikoen inokulazioa proposatu da. Horrela, lan honen helburu nagusia Arabako Errioxako azido laktiko bakterio (ALB) autoktonoak identifikatzea eta ezaugarritzea izan zen, AB ekoizteko gaitasuna ez zutenak eta propietate teknologiko eta sentsorial optimoak zituztenak. Lehenik eta behin, 70 ardo beltz aztertu ondoren, Arabako Errioxako AB-en egungo egoera aztertu zen. Ugariena putreszina (14,85 ± 8,9 mg / L) izan zen, ondoren histamina (4,43 ± 2,8 mg/L), tiramina (3,29 ± 3,28 mg/L) eta kadaberina. (2,14 ± 1,58 mg L) detektatu ziren. Ardo-sektoreak AB-en maila murrizteko duen eskaera kontuan hartuta, kultibo malolaktiko berrien bilaketa hasi zen. Ia 300 isolatuetatik, *Pediococcus parvulus* (3), *Lactobacillus plantarum* (1), *Lactobacillus mali* (3), *Lactobacillus hilgardii* (3) eta *Oenococcus oeni* (17) espezieetako 27 genotipo identifikatu ziren. Horrela, *O. oeni*-ren nagusitasun handia baieztatu zen. ALB espezieen artean, *L. hilgardii* anduiek bakarrik izan zuten putreszina ekoizteko gaitasuna agmatina deiminasa bidearen bidez. ALB anduien karakterizazio teknologikoak, *O. oeni*-k bai ardoaren baldintza gogorrei aurre egiteko bai hartzidura malolaktikoa (HM) burutzeko zuen egokitasuna egiaztatu zuen. *O. oeni* anduiek ardogintza baldintzetan glikosidasa (α-glukosidasa, β-glukosidasa, β-xilosidasa eta α-arabinosidasa) eta esterasa aktibitateak mantentzeko gaitasuna ere aurkeztu zuten. *O. oeni* anduiek kultibo malolaktiko gisa izan zezaketen erabilera ardoztatze txikien bitartez aztertu zen laborategi mailan. Ez zen AB-en ekoizpenik detektatu, eta konposatu aromatikoen eboluzioak HM-aren ondorengo aldaketa

nagusiak etil eta azetato esterretan gertatu zirela erakutsi zuen. Osagai nagusien analisiak, anduiak bi talde desberdinetan sailkatu zituen, andui bakoitzak ardoaren profil aromatikoan izan zezakeen eragina nabarmenduz. Itxaropen handiena zuten *O. oeni*-ren bost anduiak inokulazio estrategia ezberdinak (ko-inokulazioa eta sekuentziala) jasango zituzten hartzidura prozesu ezberdinetan murgildu ziren. Nabarmentzekoa da, HM bakoitzaren ostean ez zela AB-en ekoizpenik atzeman, baita ko-inokulazio estrategiak ardozte prozesua azkar amaitzea eragin zuela ere. Ko-inokulatutako ardoetan, sekuentzialki inokulatutako ardoetan baino fruta helduaren usainaren pertzepzioa altuagoa atzeman zen. Azkenik, P2A anduiak upategian hartzidura handietan lan egiteko duen egokitasuna berretsi zen, eta ondorioz, ardogintza denbora nabarmen murrizteko eta hartzidura prozesua hobeto kontrolatzeko alternatiba abantailatsua kontsideratu daiteke.

INDEX

Abbreviations

ACN	acetonitrile	**L**	liters
ad	arginine deiminase	**LAB**	lactic acid bacteria
adc	arginine decarboxylase	**LDC**	lysine decarboxylase
ADH	alcohol dehydrogenase	**NAD**	nicotinamide adenine dinucleotide
ADP	adenosine diphosphate	**NMT**	N-methyltransferase
AGDI	agmatine deiminase	**MAO**	monoamine oxidase
ALDH-2	acetaldehyde dehydrogenase-2	**maeP**	citrate permease
α-ARA	α-arabinosidasa	**MCFA**	medium chained fatty acids
α-GLU	α-glucosidase	**MDA**	decarboxylase medium
ANOVA	analysis of variance	**μm**	microgram
AF	alcoholic fermentation	**μL**	microliter
ATP	adenosine triphosphate	**mg**	miligram
BA	biogenic amine	**min**	minute
β-GLU	β-glucosidase	**mL**	mililiter
β-XYL	β-xylosidase	**mleA**	malolactic enzyme
CFU	colony forming unit	**MLF**	malolactic fermentation
citD	α-subunit of citrate lyase	**n.d.**	not detected
citE	β-subunit of citrate lyase	***odc***	ornithine decarboxylase
citF	γ-subunit of citrate lyase	**OIV**	international organization of vine and wine
CO_2	carbon dioxide	**OPA**	o-phthaldehyde
DAO	diamine oxidase	***pad***	phenolic acid decarboxylase
DNA	deoxyribonucleic acid	**PCA**	principal component analysis
DNTP	deoxyribonucleotide triphosphate	**PCR**	polymerase chain reaction
EFSA	european food safety agency	**ppm**	parts per milion
FAD	flavin adenine dinucleotide	**PMF**	proton motive force
FAO	food and agricultura organization of UN		

g	grams
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GMP	good manufacturing practices
HACCP	hazard analysis of critical control points
HCA	hidroxycinnamic acids
hdc	histidin decarboxylase
HNMT	histamine-N-methyltransferase
HS-SPME-GC-MS	headspace solid-phase microextraction-gas chromatography-mass spectrometry
IgE	inmunoglobulin-E
kg	kilograms
RAPD-PCR	random amplification of polymorphic DNA-PCR
RNA	ribonucleic acid
RP-HPLC	reverse-phase high performance liquid chromatography
SNP	single nucleotide polymosphism
SO_2	sulphur dioxide
SPONT	spontaneous fermentation
TDC	tyrosine decarboxysale
WAO	world allergy organization
YAN	yeast-assimilable nitrogen

List of Tables and Figures

RESULTS & DISCUSSION

Study 1

Study 2

Study 3

1. INTRODUCTION

1.1. Wine intolerance

Food intolerance has become one of the main health issues in first world societies. In that way several foods, including wine, can promote different adverse symptoms in consumer´s health. It is estimated that 10% of the population is susceptible to suffer somehow alcoholic beverage-linked hypersensitivity reactions (Wüthrich, 2018). According to the World Allergy Organization (WAO), "hypersensitivity reactions cause reproductible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal subjects" (Johansson et al., *2003*). Among these adverse reactions it must be discerned between immunologic, mainly IgE-mediated, reactions and intolerance reactions, in which no allergen-specific reaction mechanism is detected (**Figure 1**).

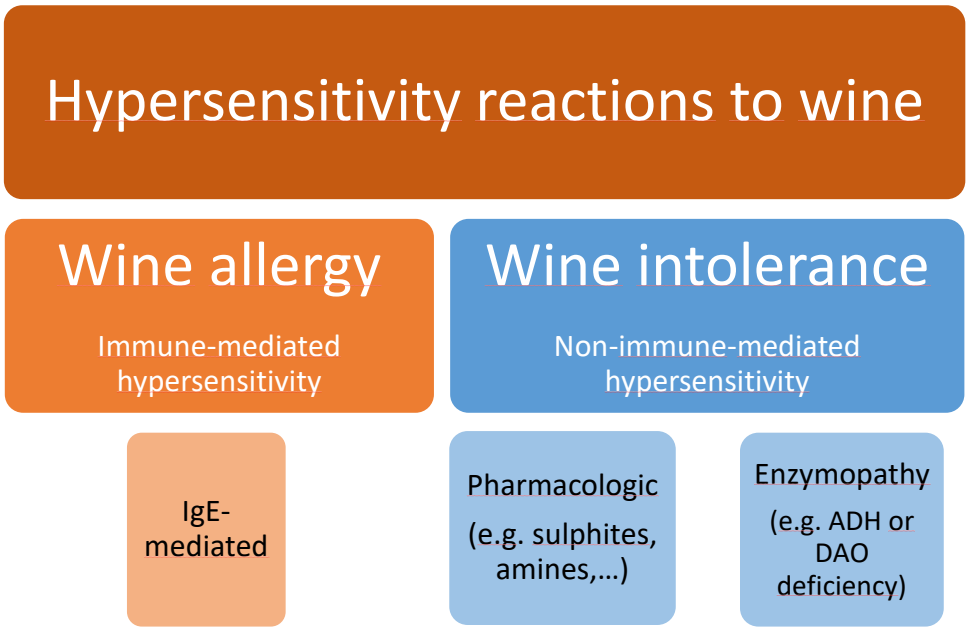


Figure 1. General classification of adverse reactions to wine. Most common reactions are non-immune-mediated driven by an enzymopathy.

In the context of wine, proteins, mainly the lipid transfer protein Vit v1 (found in grapes), fining agents (such as gelatine, isinglass, casein or ovalbumin) or enzymes (cellulase, glucanase, pectinase, glucosidase) used throughout the winemaking process, could be responsible, among others, for wine allergy (Pastorello et al., 2003; Kirschner et al., 2009). Other compounds, as sulfites, ethanol, acetaldehyde, flavonoids and biogenic amines, particularly histamine, are considered the main cause of wine intolerance reactions (Konakovsky et al 2011; Nakagawa et al., 2006; Panconesi et al., 2008; Vally et al., 2001).

Most wine intolerance reactions are due to an enzymopathy (Wüthrich, 2018). For example, alcohol flush syndrome (alcohol hypersensitivity), is determined by a high activity of the enzyme alcohol dehydrogenase (ADH), which catalyses the conversion of ethanol to acetaldehyde in the liver. In that way, high concentration of toxic acetaldehyde is accumulated. A second enzyme disorder, as the deficit of the enzyme acetaldehyde dehydrogenase 2 (ALDH-2) which catalyses the conversion of acetaldehyde to acetate, prevents acetaldehyde detoxification and leads to symptoms of intoxication (Harada et al., 1981; Wigand et al., 2012). Mutated ALDH-2 is typically frequent in eastern countries, indeed, 38% of Japanese and 37% of Chinese people are affected by ALDH-2 polymorphism (Brooks et al., 2009). Wine polyphenols are also thought to be responsible of adverse symptoms. Patients with low activity of the enzyme phenol sulfotransferase are unable to detoxicate certain phenols. As a result, these compounds could pass from the bloodstream to the brain causing migraine-like symptoms (Pergolizzi et al., 2019). Another enzymopathy which causes several problems among wine consumers is diamine oxidase (DAO) deficiency. This enzyme, together with monoamine oxidases and histamine methyl-transferase, leads to the

metabolization of biogenic amines (BAs) (Komericki et al., 2011; EFSA, 2011). In this sense, histamine intolerance derives from the disequilibrium of accumulated histamine and the ability for its degradation (Maintz and Novak, 2007). BAs, mainly histamine, are recognized among the main cause of wine intolerance among genetically susceptible individuals, leading to several symptoms that mimic a food allergy as headaches, flushing, palpitations, nausea or increasing blood pressure (Stockley & Johnson, 2015; Ladero et al., 2010). Due to the multiple symptoms that histamine can cause, the existence of histamine intolerance is frequently underestimated,. Clinical symptoms and their provocation by certain foods and beverages appear similar in different diseases, such as food allergy and intolerance of sulphites, histamine, or other biogenic amines (e.g, tyramine) and thus its symptoms are often misinterpreted. The International Society of DAO Deficiency estimates that 10% of the global population shows some kind of DNA polymorphism that reduces DAO activity. Indeed, several single nucleotide polymorphisms (SNPs) have been identified and associated with reduced enzyme activity (Petersen et al., 2005). Furthermore, the ingestion of widely consumed drugs, as analgesics, antidepressants or tranquilizers, as well as the consumption of alcohol are involved in the deficiency or low activity of DAO enzyme, increasing in that way the population susceptible to having adverse symptoms beyond genetically sensitive individuals (Maintz and Novak, 2007). Furthermore, among patients suffering migraine or with inflammatory gastrointestinal disorders, a great percentage of individuals show DAO deficiency, which highlights the genetic predisposition of a group of patients for histamine intolerance (Maintz et al., 2011; Manzotti et al., 2016 Petersen et al., 2005). All these

reasons contribute to arise the risk of the presence of histamine in foodstuff, and specifically in this case in wine.

1.2. Biogenic amines

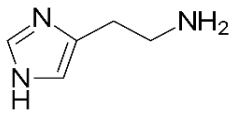
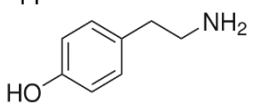
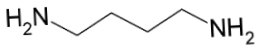
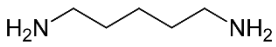
1.2.1. Biogenic amines in wine: a definition

Biogenic amines (BAs) are nitrogenous compounds of low molecular weight which are present in several living organisms playing a key role in multiple biological functions, such as regulators of cell growth and development or mediators in neuronal and inflammatory processes (Galgano et al., 2009). However, high concentrations of these compounds in foodstuff may represent a health risk through direct or indirect toxicity (Maintz & Novak, 2007; Frascarelli et al., 2008). Foods likely to contain high amounts of biogenic amines are fish, fish-derived products and above all, fermented foods (meat, dairy, vegetables, beer, wine, etc) (EFSA, 2011). In foods and beverages their presence mainly derived from the decarboxylation of their precursor amino acids through the activity of microorganisms responsible for the fermentation process or the presence of spoilage microorganisms (Coton et al., 2010; Linares et al., 2012). BAs can be classified according to their chemical structure, as aliphatic (putrescine, cadaverine, spermine, spermidine, agmatine), aromatic (tyramine, 2-phenylethylamine) and heterocyclic (histamine, tryptamine) and according to the number of amine groups into monoamines (tyramine and 2-phenylethylamine) and diamines (histamine, putrescine and cadaverine) (Guo et al., 2015).

In wine, BAs, together with ethanol and sulphites, are considered the main reason for wine intolerance (Konakovsky et al., 2011). The most representative BAs found in wine are histamine, tyramine, putrescine and cadaverine, which derived from the

decarboxylation of histidine, tyrosine, ornithine and lysine, respectively (**Table 1**). Putrescine can also be formed from the deamination of agmatine (Lopez et al., 2012).

Table 1. Chemical properties of main biogenic amines found in wine

Name	Molecular formula	Structure formula	Precursor
Histamine	$C_5H_9N_3$		Histidine
Tyramine	$C_8H_{11}NO$		Tyrosine
Putrescine	$C_4H_{12}N_2$		Agmatine Ornithine
Cadaverine	$C_5H_{14}N_2$		Lysine

The presence of these compounds in wines will mainly depend on the concentration of precursor amino acids and the presence of decarboxylase positive microorganisms (Anli & Bayram, 2009). BAs may be present as normal constituents of grapes, as polyamines, which have seen to mediate cell growth and development processes (Koukourikou et al., 2015). Other factors, such as grape variety, maturity degree, fertilisation, irrigation, climatic conditions and other agronomic factors may influence BAs level and above all, the concentration of precursor amino acids (García-Villar et al. 2007). Vinification techniques such as extended grape skin maceration or ageing of wine on lees (yeast autolysis) can also lead to an increase of precursor amino acids and thus, potential BA formation (Smit and du Toit, 2011). Although viticultural and winemaking practices will limit the concentration of precursor amino acids, the

presence of decarboxylase positive microorganisms do have the main contribution to BA formation. Indeed, mould infections of grapes display significant impacts on the initial content of BAs in grape must (Grossmann et al., 2007). In that way, special emphasis should be paid on the sanitary status of grapes as well as to the hygiene care during all winemaking and ageing period (Leitao et al., 2005). Fermentation conditions, as pH, temperature, and ethanol and sulphur dioxide concentrations, should be handle also with special care since they will limit the development of potential spoilage microorganisms. In last years, in order to meet consumers demand, prolonged grape maturity has led to wines with lower acidity and higher pH. As pH increases, usually above 3.6, the diversity and number of microorganisms increases, thereby promoting the formation of BAs (Lopez et al., 2012 and Wang et al., 2014).

1.2.2. Toxicity of biogenic amines

Under normal conditions, BA removal takes place in the gut lumen through the action of monoamine (MAO) and diamine (DAO) oxidases and specific N-methyltransferases (NMT) (Tofalo et al. 2016). In that way, biogenic amines can be metabolized by oxidative deamination or by ring methylation (**Figure 2**). Whether histamine is catabolized by DAO or HNMT depends on its localization. DAO protein is stored in epithelial cells and is released into the circulation by stimulation (Schwelberger et al., 1997; Schwelberger et al., 1998). Thus, DAO may be responsible for degrading extracellular histamine (e.g. after the ingestion of histamine-rich foodstuff). On the contrary, HNMT, is a cytosolic protein, which can only convert histamine only intracellularly (Klocker et al., 2005). If the intake of these compounds is too high (specially histamine) or genetical disorders are present, as low or no expression of BA

metabolising enzymes, a food intoxication or intolerance may arise (EFSA, 2011). In addition, alcohol and several medicaments can inhibit the action of amino oxidases and enhance potential toxic effects (Spano et al. 2010). Histamine and tyramine are responsible for typical food intoxications related to BAs. Histamine is a potent mediator of numerous biologic reactions, that is why in humans it is synthesized by many cells (mast cells, basophils, platelets, histaminergic neurons, and enterochromaffin cells) where it is stored intracellularly and released on stimulation. Histamine exerts its effects by binding to different receptors on target cells in various tissues. Thus, when ingested in high quantities it initiates a cascade of reactions that can cause muscle cell contraction, vasodilatation, increased vascular permeability and mucus secretion, tachycardia, alterations of blood pressure, arrhythmias, stimulation of gastric acid secretion, etc. In addition, histamine has been known to play various roles in neurotransmission, immunomodulation, hematopoiesis, wound healing, day-night rhythm, and intestinal ischemia (Maintz, 2007; Ladero et al., 2010).

Recently performed studies around BA toxicity have elucidated the cytotoxic effect as well as the synergistic negative effect of tyramine and histamine over intestinal cell cultures (del Rio et al., 2017). Surprisingly, tyramine had a stronger and more rapid cytotoxic effect than histamine, and whereas tyramine caused cell necrosis, histamine induced cell apoptosis over intestinal epithelium cells (Linares et al., 2016; del Rio et al., 2017). They elucidated their toxicity at concentrations commonly found in BA-rich foodstuff, around 400 mg/kg and 300 mg/kg of histamine and tyramine, respectively. Although fish-derived products, fermented meat products and cheese are likely to have higher concentrations, in wine these concentrations are rare to occur (EFSA, 2011). However, minimal concentrations might also cause adverse symptoms over

sensitive individuals. People with genetic deficiencies and weak BA-detoxification systems, suffering from gastrointestinal diseases, or ingesting mono- or diamine oxidase inhibitor drugs or other potentiating factors, as ethanol, (Maintz & Novak, 2007), might be at greater risk. In this sense, the Food and Agriculture Organization of the United Nations (FAO) (Food and Agriculture Organization of the United Nations, 2014) defined the presence of BAs in foodstuff as a biological hazard. However, legislation on BA maximum levels in foodstuff is still insufficient. Actually, it is a complex task to establish a uniform maximum limit for ingested BAs as their toxicity depends on the type of BA in question, the presence of modulating compounds, and on the efficiency of each person's detoxification system. The only BA for which maximum limits have been legally set by the EFSA is histamine, and only in scombroid-like fish (200 mg/kg) and fish products (400mg/kg) (European Comission, 2005).

Generally, the toxic dose in alcoholic beverages is considered to be between 8 and 20 mg/L for histamine, 25 and 40 mg/L for tyramine, although little consensus exists in this regard (Smit et al., 2008). In susceptible individuals, histamine intolerance was triggered by the intake of 4 mg histamine due to consumption of 0.2 l of sparkling wine containing 20 mg/l (Menne et al., 2001). Furthermore, polyamines such as putrescine and cadaverine may have indirect toxic effects enhancing the toxicity of other BAs, as histamine or tyramine. Polyamines may act as competitive substrates for tyramine and histamine metabolising enzymes, perpetuating in that way their toxic effects (Smit et al., 2008). Via the competitive inhibition of MAO and DAO enzymes, it is facilitated the passage of histamine and tyramine across the small intestine, increasing their levels in the blood stream and leading to the specific union to different cellular receptors which results in a cascade of adverse symptoms (Chu et al., 1982; del Rio et

al., 2019; Jung & Bieldane, 1979). In addition, it has been recently demonstrated the cytotoxic effect of both putrescine and cadaverine, with both compounds causing cell necrosis in intestinal cells (del Rio et al., 2019). In that way, the absence of these compounds may represent a good indicator of product quality and safety, based on good viticultural practices, careful handling of grapes and special control along all winemaking and ageing process.

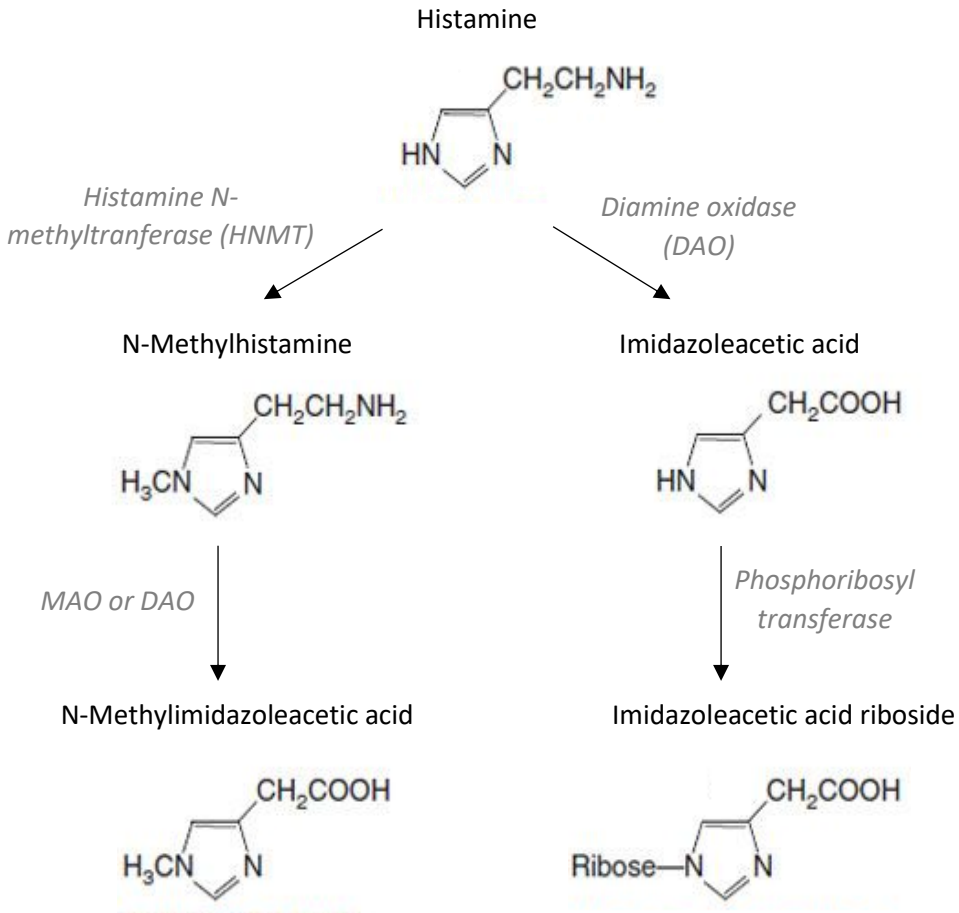


Figure 2. Summary of the histamine metabolism. Histamine can be metabolized by extracellular oxidative deamination of the primary amino group by diamine oxidase (DAO) or intracellular methylation of the imidazole ring by histamine-N-methyltransferase (HNMT) (3). Therefore, insufficient enzyme activity caused by enzyme deficiency or inhibition may lead to accumulation of histamine. N-Methylhistamine is oxidatively deaminated to N-methyl-imidazoleacetic acid by monoamine oxidase B (MAO B) or by DAO.

1.2.3. Who is responsible for BA accumulation in wine?

BAs are produced by the activity of several microorganisms in different stages of winemaking and ageing process. Yeast and lactic acid bacteria (LAB), main microorganism found in wine, are all considered possible BA producers. (Caruso et al., 2002; Smit et al., 2013). Main yeast species found in wine, as *Saccharomyces cerevisiae, Kloeckera apiculata, Candida krusei, Metschnikowia pulcherrima* and *Brettanomyces bruxellensis,* have been described as BA producers in wine (Caruso et al., 2002; Del Prete et al., 2009). However, the contribution of yeast to the overall BA accumulation is not clear, as few studies support this statement. It is widely stablished that BA production by yeast is negligible in wine, as low or non-production of BA have been linked with wine yeast and alcoholic fermentation (Torrea and Ancín, 2001; Landete et al., 2007; Marcobal et al., 2005; Smit et al., 2013; Henríquez-Aedo et al., 2016). In that way, and due to an extensive bibliographical support, it is considered that LAB and malolactic fermentation (MLF) are the main factors that determine BA accumulation in wine.

Oenococcus, *Leuconostoc*, *Lactobacillus* or *Pediococcus* species have been all described as BA producers in wine (Moreno-Arribas et al., 2003; Coton et al., 2010). Regarding *O. oeni*, this is the main species associated with MLF due to its ability to develop under wine harsh conditions (low pH and high ethanol and sulphur dioxide concentrations) (Ribérau-Gayon et al., 2006). In that way, if BA production is associated with MLF, *O. oeni* would be expected to be the main responsible for BA accumulation. However, the ability of *O. oeni* for BA formation is a matter of debate. While many authors have confirmed its role in BA formation (Coton et al., 2010; Landete et al., 2005), others have questioned *O. oeni* BA, especially histamine,

producing ability (Garai et al., 2007; Garcia-Moruno *et al*., 2012; Moreno-Arribas et al., 2003). Recently, Berbegal et al. (2017) reported the ability of indigenous *O. oeni* strains to produce tyramine, putrescine and specially histamine at high concentrations during fermentation and ageing period. *Lactobacillus* and *Pediococcus* species, mainly *L. brevis*, *L. mali*, *L. hilgardii*, *L. buchneri*, *P. parvulus* and *P. damnosus*, have been widely associated with BA production and spoilage in wine (Landete et al., 2007; Moreno-Arribas et al., 2000; Sebastian et al., 2011). Moreno-Arribas *et al*. (2003), after studying 78 strains isolated from grape and wine samples, reported the ability of *Leuconostoc spp*., *L. brevis* and *L. hilgardii* strains for tyramine production. Costantini *et al*. (2006), after the evaluation of 133 LAB strains, confirmed the ability of *L. brevis* and *L. hilgardii* strains for tyramine and putrescine production, respectively. In the same way, Landete et al. (2007) found that *P. parvulus*, *L. mali* and *L. mesenteroides* produced histamine, while all *L. brevis* strains were able to produce tyramine and phenylethylamine. Sebastien et al. (2011) confirmed the production of histamine by *P. parvulus*, *P. damnosus* and *L. casei* strains. Recently, *Lactobacillus rhamnosus*, which was identified as the predominant species in five different wineries during spontaneous MLF in Chilean Cabernet Sauvignon wines, showed the highest BAs and histamine forming capacity among the different species identified (Henríquez-Aedo et al., 2016).

However, BA production ability varies significantly among strains and several works in which no BA production was detected (Ruiz et al., 2010a; Pramateftaki et al., 2012; Costantini et al., 2006), counteract other studies where LAB aminobiogenic capability was confirmed (Coton et al., 2010; Landete et al., 2007). Although in some species, as *L. brevis*, BA producing capability seemed to be widespread (Lucas et al., 2007;

Romano et al., 2014), it is well known that the ability of LAB to produce BA is a strain dependent characteristic (Ladero et al., 2012). The great variability among strains may be explained by the fact that genes coding for amino acid decarboxylases are located in genomic islands or in unstable plasmids, which enables horizontal gene transfer between different BA producing organisms (Lucas et al., 2005; Spano et al., 2010).

The synthesis of BA is generally composed of a transport protein, which facilitates the uptake of the precursor amino acid and secretion of the corresponding BA, and a decarboxylase which enables the formation of the BA from the amino acid (Guo et al., 2015). For putrescine formation, two biosynthetic pathways are possible, in which multiple enzymes are required. It can be formed through ornithine decarboxylase or agmatine deiminase pathways, and both ornithine and agmatine may be present in grapes or in turn they may derive from arginine metabolization (Galgano et al., 2009) (**Figure 3**). BA synthesis by microorganisms may be related to defense mechanisms used against acidic conditions (Lee et al., 2007, Spano et al., 2010). The coupled reactions of amino acid decarboxylation and amino acid/biogenic amine antiporter lead to both pH homeostasis and energy generation. Amino acid decarboxylation reaction consumes an intracellular proton, giving rise to the corresponding BA and carbon dioxide. The BA (which has increased its charge +1) is exported via amino acid/biogenic amine antiport. In that way protons are pumped out, increasing intracellular pH as well as inside negative membrane potential. Thus, amino acid decarboxylation system not only ensures a response against acidic environment but also generates secondary metabolic energy through proton motive force (PMF).

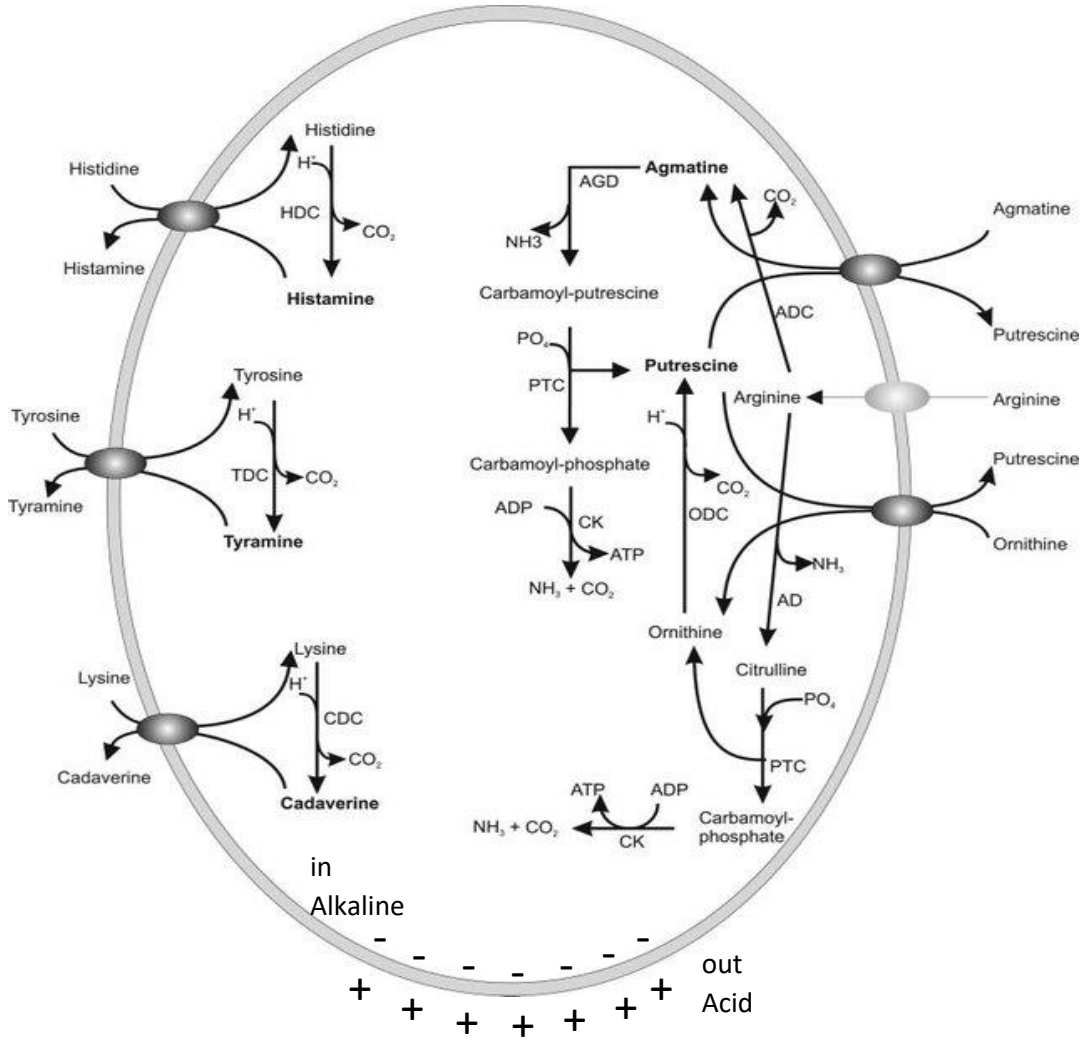


Figure 3. Biogenic amine biosynthesis pathways. Arginine decarboxylase (ADC), agmatine deiminase (AGD), arginine deiminase (AD), histidine decarboxylase (HDC), lysine decarboxylase (LDC), tyrosine decarboxylase (TDC), ornithine decarboxylase (ODC), carbamate kinase (CK), and putrescine carbamoyl transferase (PTC) (adapted from Linares et al., 2011).

1.2.4. Factors influencing biogenic amine production in wine

Although the majority of reported positive strains are not involved in MLF, the identification of positive strains that lead spontaneous MLF makes the control of MLF

of great relevance. Taking into account that the main premise for BA accumulation is the presence of BA forming strains, different factors can promote their appearance/growth as well as enhance their activity and increase the concentration of BA in wine. Different factors promoting the accumulation of BA in wine have been described in the las decade (**Table 2**). Wine quality is closely linked to the quality of grapes and consequently to all winemaking practices. Grape variety, degree of maturity, integrity and sanitary status of grapes, soil type, agricultural practices (irrigation, fertilization), climatic conditions, maceration time, degree of autolysis, fermentation conditions (pH, temperature, alcohol and sulphur dioxide concentrations), ageing time, etc. will determine the final concentration of BA in wine (Binner et al., 2013). Many of them will limit the concentration of precursor amino acids, and others, such as the sanitary status of grapes and hygienic care during all winemaking process, will stablish the microbial load (potentially contaminating or not) that will be present in the fermentation process (Smit et al., 2008; Ancín-Azpilicueta et al., 2008).

In the last years, in order to meet consumers demand, grape maturity is prolonged as far as possible to raise phenolic and aroma compounds, thus contributing to free precursor amino acids accumulation (Martinez-Pinilla et al., 2013). Winemaking techniques, such as prolonged maceration, can also lead to a potential increase in BAs. It has been observed how a prolonged contact time of the wine with the skins leads to an increase in precursor amino acids and BAs (Marques et al., 2008; Smit & du Toit, 2013). Performing MLF and ageing on lees can also promote BA accumulation through an increase in precursor amino acids due to yeast autolysis (Pérez-Serradilla et al., 2008). In this way, BA appearance has been observed after months of ageing,

following a slow release of precursor amino acids in the presence of residual microbial activity (Polo et al., 2010; Berbegal et al., 2017). Among the vinification conditions, the relevance of the pH stands out. Due to consumers demand, wines tend to be less acidic, which can lead to an increase of pH. As the pH value rises, microbial diversity increases, favouring the growth of potential spoilage microorganisms. It is stablished that a pH below 3,6 limits the growth of contaminants, in fact, Lopez et al. (2012) and Wang et al. (2014) found that all wines being above pH 3.7 contained relatively large levels of BAs. For all these reasons BA formation is mainly associated to red wines. Indeed, white wines do not normally undergo MLF, and lower fermentation temperatures, lower pH and higher levels of SO_2 in order to prevent oxidation inhibits the appearance of potential microorganism. In addition, they do not typically undergo skin maceration neither long ageing processes which increase BAs formation potential (Restuccia et al., 2018).

Table 2. Different factors reported to have an influence on BA accumulation in wine

Factors affecting BA accumulation in wines	References
Grape maturation degree	Herbert et al., 2005; Del Prete et al., 2009; Wang et al., 2014
SO_2 concentration	García-Marino et al., 2010
pH	López et al., 2012; Wang et al., 2014
Nitrogen supplementation	Batch et al., 2011; Bordiga et al., 2020
Yeast metabolism	Bordiga et al., 2020; Restuccia et al., 2018
LAB metabolism	Berbegal et al., 2017; Guerrini et al., 2002, Henriquez-Aedo et al., 2012
Ageing and storage conditions	Polo et al., 2010; Smit et al., 2013; Hernandez-Orte et al., 2008

However, despite the foregoing, the key factor affecting BA formation is the microbial population present during the winemaking process. Microorganisms can be present naturally in the grape surface or can be introduced accidentally by contamination or deliberately by adding starter cultures. Nowadays, in most wineries, MLF takes place spontaneously where the microbial population is diverse and the responsible for leading MLF is unknown. This fact makes MLF difficult to predict and there is not an exhaustive control over the process. In this sense, a significant increase in the concentration of BA has been observed after spontaneous malolactic fermentations (Izquierdo et al., 2008; Martuscelli et al., 2013; Patrignani et al., 2012; Berbegal et al., 2017). Lack of control over MLF can not only lead to a significant increase in BAs, but also to the appearance of off-flavours and organoleptic deterioration of the wine. In addition, spontaneous MLF could lead to stuck or sluggish fermentation that may be protracted for months. Indeed, it has been demonstrated that residual nutrients, as malic acid, together with low amounts of SO_2 after AF, may boost the appearance of potential deleterious organisms, depreciating in that way wine quality (Gerbaux et al., 2009; Sumby et al., 2019).

1.2.5. Presence of biogenic amines in worldwide wines

The European Union has not set limits for BA concentration in the wine industry. However, different countries have recommended different upper limits for histamine content. For instance, Australia and Switzerland recommend an upper limit for histamine of 10 mg/L, 8 mg/L in France, 6 mg/L in Belgium, 3.5 mg/L in Netherlands and 2 mg/L in Germany (Guo et al., 2015; Martuscelli et al., 2013). Switzerland was the only country that set an official maximum limit for histamine of 10 mg/L, however, this legal limit for imported wines was removed in 2011. Anyway, the presence of

metabolites of safety concern could be a limiting factor for wine commerce. The International Organization of Vine and Wine (OIV) published the "OIV code of good vitivinicultural practices to minimize the presence of BAs in vine-based products" (OIV, 2011), arising the importance of that subject in the wine industry. The suggested actions concerned both viticultural and cellar practices (hygiene care, inoculation of safe yeast and LAB, etc). Since then, different screening studies have evaluated the current situation of BAs in wine (**Table 3**). In 2011 the EFSA performed an extensive study of the occurrence of BAs in foodstuff. The report showed that after evaluating 300 red wines, 90% contained histamine, with a mean concentration of 3,6 mg/L, while when evaluating 225 white wines an average concentration of 0,8mg/L was detected in 78% of wines. For tyramine, in red wines an average concentration of 2,8 mg/L was detected in the 78% of the samples, while in white wines a mean concentration of 1,1 mg/L was found in the 83% of wines. The study of Konakovsky et al. (2011), performed through 100 wines categorized as high-quality wines, highlighted that variable concentrations of histamine, tyramine and putrescine were detected in all wines. The study underlined that 34% of the wines exceeded the concentration of 10mg/L, the upper recommendation limit established by different European countries. Patrignani et al. (2012) after the evaluation of eight Italian wineries found that histamine concentration ranged between 1,49 mg/L and 16,34 mg/L, while tyramine ranged between 1,58 mg/L and 10,19 mg/L. Recently, Zurga et al. (2019) carried out a complete evaluation of Croatian wines. The levels detected by the authors ranged from undetectable to 25 mg/L histamine, 28 mg/L tyramine, 14 mg/L cadaverine and 55 mg/L putrescine. As seen, a wide range of concentrations can be observed in the different screening studies performed, from not detected up to

high concentrations. Generally, as shown in **Table 3**, putrescine is found in higher concentration in all the studies, followed by histamine, tyramine and cadaverine, which is usually found in trace quantities (Ancin-Azpilicueta et al., 2008). Other amines, as phenylethylamine, agmatine, tryptamine or ethylamine have been also described in wine (Anli et al., 2009).

Table 3. Mean concentration of main biogenic amines found in wines over the world. Minimum and maximum levels detected are shown in parentheses.

Wine (origin)	Wines (n)	Histamine	Tyramine	Putrescine	Cadaverine	
Croatia	60	2,14 (0,1-8,7)	1,42 (0,1-8,4)	5,38 (1-14,1)	0,89 (0,1-3)	Žurga et al., 2019
Italy	30	2,91 (0-10,8)	5,22 (0-18,8)	7,88 (2,4-31,8)	0,11 (0-1,1)	Martuscelli et al., 2013
Worldwide	300	3,7 (0-34,3)	2,9 (0-18,5)	4,8 (0-21,6)	0,5 (0-5)	EFSA, 2011
Austria	100	7,2 (0,52-27)	3,52 (1,07-10,7)	19,4 (2,93-122)	0,58 (0-3,27)	Konakovsky et al., 2011
Italy	73	3,2 (0,1-11,69)	2,83 (1,06-9,36)	6,24 (1,5-21,05)	2,87 (0,7-6,8)	Galgano et al., 2011
Grecee	45	0,31 (0-2,11)	0,43 (0-3,65)	1,17 (0-5,23)	0,52 (0-3,21)	Soufleros et al., 2007
Spain	224	4,46 (0-25)	3,13 (0-19)	6,05 (0-55)	2,02 (0-14)	Marcobal et al., 2006

1.2.6. Strategies to prevent the accumulation of BAs in wine

At the end of the winemaking process, different curative methods have been described to decrease the BA content, however, these methods often imply a modification of sensory quality of the wine and not always induce a decrease in BA content (Corzani et al., 2008). Clarification through physical methods (sedimentation, centrifugation and filtration) or fining agents (bentonite, casein, gelatin) (Mannino et al., 2006; Ribèreau-Gayon et al., 2006). In the last years, novel strategies as the

inoculation of amine oxidase positive LAB have been postulated (Callejon et al., 2016; Capozzi et al., 2012). However, according to general principles of hygiene in foodstuff, strategies to prevent the formation of BA content rather than methods based on BA elimination should be carry out (EFSA, 2011). In this sense, main aspects to take into account are hygiene care during all the vinification process, inoculation of safe malolactic starters and stabilization of wine after MLF. Overall, the control of BA accumulation in wine lays on two strategies, (i) assurance of hygienic care throughout the winemaking process (ii) inhibit or prevent the growth of BA-forming microorganisms. The application of GMP (Good Manufacturing Practices) and HACCP (Hazard Analysis of Critical Control Points) protocols to ensure hygiene care during all the process may help the prevention of BA formation by spoilage microorganisms (EFSA, 2011; OIV, 2011). However, taking into account the role of MLF in BA formation, the better control of MLF is the main strategy to reduce the presence of these metabolites. In that way, one possible strategy to inhibit the growth of potential spoilage bacteria and prevent the appearance of BA in wine, is the inoculation of safe malolactic starters (Polo et al., 2010; Lopez et al., 2011; Martuscelli et al., 2013, Henríquez-Aedo et al., 2016). In addition, beyond safety issues, the inoculation of autochthonous cultures of LAB already adapted to specific winemaking region conditions have been suggested not only to improve MLF reliability but also to preserve the singularity and biodiversity of specific wines (Sumby et al., 2019). It is also necessary to ensure their implantation and viability, in order to be able to displace existing populations and inhibit the growth of potential contaminants. The control of BAs in wine will significantly reduce consumers health risk, and thus, it will increase the competitiveness of local wineries.

1.3. Lactic acid bacteria and malolactic fermentation

1.3.1. Definition

Lactic acid bacteria (LAB) are generally described as Gram positive, oxidase and catalase negative, facultative anaerobes, non-spore forming rods and cocci (König & Fröhlich, 2009). Besides their microscopic morphology, the homo- or heterofermentative metabolism of sugars constitutes a decisive criterion for their classification (**Figure 4**). In homofermentative LAB, hexoses are fermented via Embden-Meyerhof pathway, where two moles of lactic acid are obtained from each mol of metabolized hexose. These species are unable to utilise pentoses as carbon source. Heterofermentative LAB, on the contrary, produce carbon dioxide, ethanol, acetic acid and lactic acid from both pentoses and hexoses. Facultative heterofermentative LAB follow the homofermentative pathway to ferment hexoses, obtaining only lactic acid, while they use the heterofermentative way to metabolize pentoses and gluconate, obtaining acetic or ethanol, CO_2 and lactic acid. LAB species that only have this way to ferment sugars are called strict heterofermentative. This is carried out through the 6-phosphogluconate pathway and from each mol of hexose consumed, 1 mol of CO_2, 1 mol of ethanol (or acetic acid) and 1 mol of lactic acid are obtained. The yield of homofermentation (2 mol ATP/mol glucose) is higher than heterofermentation (1 mole ATP/mol glucose). *Oenococcus oeni*, *Lactobacillus hilgardii* and *Lactobacillus brevis* are classified as strict heterofermentatives, while *Lactobacillus plantarum* and *Pediococcus spp.* are facultative heterofermentative and homofermentative species, respectively (Khalid, 2011; Ribereau-Gayon et al., 2006).

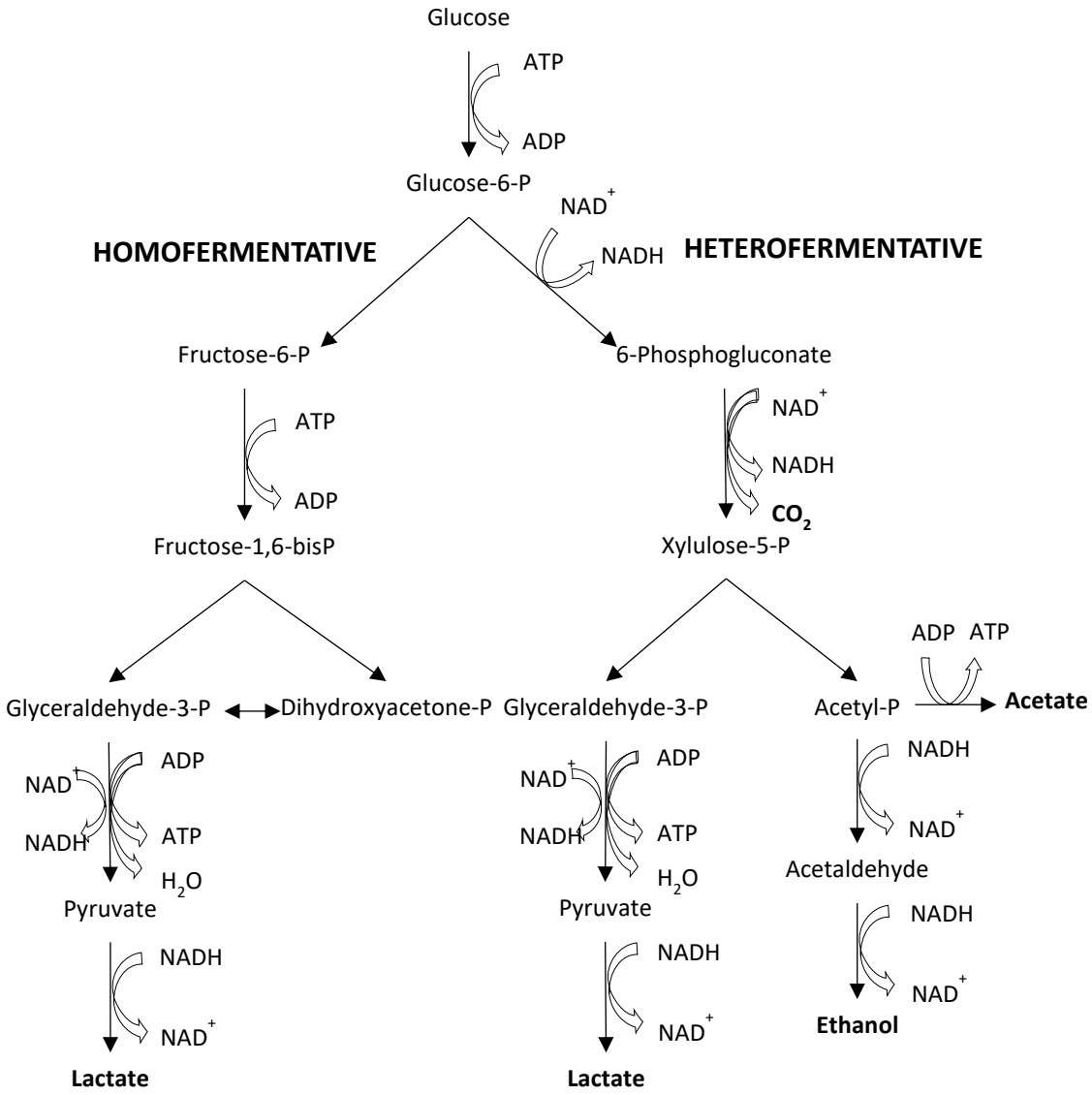


Figure 4. Metabolic pathways of homofermentative and heterofermentative bacteria.

1.3.2. Lactic acid bacteria in wine

Wine is a complex matrix resulted from multiple biochemical and biological reactions where microbial ecosystem plays a critical role influencing wine quality and safety. Grapes will determine at first instance the microbial load that enters the winery.

Typically, yeast, LAB, acetic acid bacteria and filamentous fungi are part of the natural microbiota of grapes (Barata et al., 2012). Winery environment is the second source of hundreds of microorganisms belonging to multiple families and species. In that way, both vineyard and winery environment will determine the specific microbiota of each winery (Garijo et al., 2009; González-Arenzana et al., 2012). In the vineyard, the diversity and density of LAB population is very limited compared with the population of yeasts found in grapes. Main LAB species found during the vinification process are displayed in **Table 4**.

Table 4. Main LAB species found during the winemaking process.

Morphology	Metabolism	Species
Lactobacilli	Facultative heterofermenters	*Lactobacillus casei*
		Lactobacillus plantarum
	Strict heterofermenters	*Lactobacillus brevis*
		Lactobacillus hilgardii
Cocci	Homofermenters	*Pediococcus damnosus*
		Pediococcus pentosaceus
		Pediococcus parvulus
	Heterofermenters	*Oenococcus oeni*
		Leuconostoc mesenteroides

The population density depends largely on the sanitary status of grapes and typically *Lactobacillus*, *Pediococcus* and *Leuconostoc* species are more frequently found in grape surfaces than *O. oeni* (Mesas et al., 2011; Lonvaud-Funel, 1999). When crushing and before the start of AF, LAB population ranged in 10^3-10^4 CFU/mL, being *L. plantarum*, *L. mali*, *L. hilgardii*, *P. parvulus* or *P. pentosaceous* predominant species

over *O. oeni*. However, most of these species decline through AF and finally disappear. This decrease is attributed to the explosive growth of yeast and thus to the increase of ethanol concentration, depletion of nutrients and the competitive yeast-bacteria interactions (Riberau-Gayon et al., 2006). After AF and bacterial lag phase, surviving LAB strains, commonly *O. oeni* species, start to multiply. Finally, when reaching populations over 10^6 CFU/mL malolactic fermentation (MLF) is induced (Riberau-Gayon et al., 2006).

MLF is a decarboxylation reaction, in which L-malic acid is converted into L-lactic acid through the malolactic enzyme of LAB in the presence Mn^{2+} and NAD^+ as cofactors. The conversion generates energy in the form of ATP by means of membrane proton motive force (Salema et al., 1996). L-malic enters the bacteria through malate permease and then it is decarboxylated intracellularly by means of the malolactic enzyme, generating L-lactic acid and CO2. For every lactic acid molecule that leaves the cell, one proton is also translocated outside the cell. This establishes a gradient across the cell membrane between the cytoplasm and the surrounding medium. This gradient combined with a specific ATPase in the cell membrane facilitates the generation of energy in the form of ATP (**Figure 5**) (Konings et al, 2002). This decarboxylation reaction leads to a decrease in the total acidity of wine and a slight increase in the pH. In addition, due to the consumption of the remaining nutrients after AF by LAB, a microbial stability of wine is achieved as well as an increase in the sensory complexity of wine through the secondary metabolism of LAB (Lerm et al., 2010). Although different species have been described in wine environment, as *Pediococcus*, *Lactobacillus*, *Leuconostoc* and *Oenococcus*, the principal agent involved in the MLF is *Oenococcus oeni* due to it better adaptability to wine harsh conditions

(Garofalo et al., 2015). Nowadays, MLF is a crucial step in the production of most red wines, and in the last decade it is gaining more relevance in the elaboration of white and sparkling wines in order to reduce acidity and enhance sensory complexity. Indeed, MLF is much more than a deadification process, it also implies wine aroma profile modification (Sumby et al., 2019).

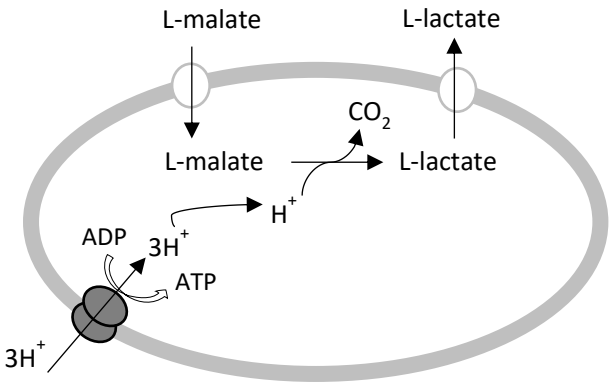


Figure 5. Generation of a proton motive force via malolactic fermentation. The decarboxylation and fixation of a proton on lactic acid with subsequent translocation of the lactic acid out of the cell leads to the generation of a proton motive force across the plasma membrane that can be used in the generation of ATP.

1.3.3. Factors influencing the success of MLF

Different factors can influence LAB growth and viability, and thus, the success of MLF (**Table 5**). These factors include pH, ethanol and SO_2 concentration, temperature and yeast-derived metabolites, among others. It must be stated that not only individual factors must be considered but also the synergistic effects must be taken into account (Cinquanta et al., 2018; Guzzon et al., 2009).

At first instance, the interaction between yeast and LAB will influence the evolution of MLF. These interactions may range from stimulatory, to neutral and inhibitory and will

depend on (i) must composition, (ii) uptake and release of nutrients by yeasts and (iii) the release of yeast-derived metabolites that affect LAB growth (Du Plessis et al., 2017). Nutrient exhaustion by yeast as well as the production and release of inhibiting compounds, as SO_2 and medium chained fatty acids (MCFA), may represent a threat for LAB development. Considering the complex nutrient requirements of LAB, its growth will firmly depend on remaining nutrients after AF. In that way, yeast presenting high nutritional demand would show a relevant antagonistic relationship with LAB (Ivey et al., 2013). In addition, during AF yeast may produce different compounds that can limit LAB growth. MCFA (C_8-C_{14}) have been shown to inhibit the ATPase of LAB and thus, reduce the ability to maintain the intracellular pH and transmembrane proton gradient which is essential for transport purposes and energy production (Carreté et al., 2002). However, these compounds are typically release in low quantities, far from the inhibitory concentrations reported (Nehme et al., 2008). Besides MCFAs, yeast-derived proteins and bioactive peptides have been observed to inhibit LAB growth (Osborne & Edwards, 2006; Nehme et al., 2010). More recently, Branco et al. (2014) and Rizk et al. (2018) identified protein fractions produced by S. cerevisiae which showed activity against LAB. In both cases, they were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein fractions, which inhibited the malolactic reaction and thus, bacterial growth was compromised. However, other yeast by-products, as pyruvic acid, citric acid and amino acids, can stimulate LAB growth. Nitrogenated compounds, such as amino acids, are mainly released through yeast autolysis, stimulating LAB growth and MLF performance (Diez et al., 2010). Other autolysis by-products, as glucans and mannoproteins, can stimulate LAB growth (Diez et al., 2010). Mannoproteins seem to be of high importance, as they can absorb

MCFAs, detoxifying in that way wine medium, and they can also enhance the nutritional content of wine since they can be hydrolysed by LAB enzymes (Jamal et al., 2013).

Table 5. Main inhibitors of MLF in wines

Main inhibitors	Optimal for MLF	Wine conditions	Action mechanism of inhibitor
Ethanol	>5% favors growth	12-15% (v/v)	Affects cell wall structure
Low pH	4,8-5,5	2,5-3,5	Slows down or inhibits the bacterial growth and metabolic acitivities
Low temperature	25ºC	12-20ºC	Increases lag phase affecting growth rate
SO2	0 mg/L	10-210 mg/L	Decreases ATPase specific activity and produces a loss of cell viability

Adapted from Bettridge et al., 2015

Although yeast-bacteria interactions may define the success of MLF, the most relevant factors are the winemaking conditions in which takes place the MLF. Among the main factors that inhibit the development of LAB strains in wine environment, the most relevant are low pH, high ethanol and high SO_2 concentrations (Romero et al., 2018). Under high ethanol concentrations (10-14%) LAB cell membrane fluidity and integrity are seriously compromised, leading to a decrease of cell viability (Olguín et al., 2015). The pH of wine will determine the success as well as the length of MLF. Although high values (> 3,6) will contribute to rapid MLF, they will also lead to greater microbiological instability. Lower values (3,4-3,5) will be safer, although below 3.2 the MLF can be compromised. A pH of 3,5 tends to favour the growth of *O. oeni*, while values over 3,6 promote the growth of *Lactobacillus* and *Pediococcus* species. In that way, when higher values are reached, the risk of microbial spoilage as well as the formation of undesirable compounds (e.g. biogenic amines or volatile phenols) is higher (Cinquanta

et al. 2018; Lerm et al., 2010). Finally, SO_2 is considered the main restrictive factor for LAB growth and survival. Addition of SO_2 at crushing and in different winemaking stages is the common practice for the inhibition and control of microbial communities. SO_2 can be present in different chemical forms, including bound and free SO_2. The equilibrium of the different forms depends on the pH of wine. At low pH, free forms of SO_2 predominate, as bisulphite and sulphite ions, and molecular SO_2. Molecular SO_2 is considered the most inhibitory form, since is the only form that can cross bacterial cells via diffusion. The action mechanism of SO_2 include the rupture of disulphide bridges in proteins, the inhibition of ATPase activity as well as the reaction with NAD+ and FAD cofactors (Carreté et al., 2002), limiting in that way LAB viability. It is stablished that a concentration of 0,5-0,8 mg/L of molecular SO_2 are sufficient for wine stabilization, which under typical wine pH values (3,4-3,6) correspond to 30-40 mg/L of free SO2 (Lerm et al., 2010). It must be taken into account that yeasts are also able to produce these compounds as a by-product of their metabolism. Typical concentrations of SO_2 produced by *S. cerevisiae* strains is less than 30 mg/L, although some strains can produce more than 100 mg/L (Wells & Osborne, 2011). In that way, in order to promote the initiation of MLF it is important to choose a yeast strain that does not produce excessive SO_2.

Due to all above mentioned inhibiting factors spontaneous MLF may lead to stuck or sluggish fermentation in which MLF can be protracted for months. As seen, winemaking conditions, as low pH, high ethanol and SO2 concentrations and the low nutrients available could difficult the achievement of MLF. Spontaneous MLF does not ensure consistent outcomes in terms of MLF completion, organoleptic profile or resulting wine quality. That is why the main strategy to overcome MLF difficulties and

perform a reliable and consistent process is the inoculation of selected malolactic starters (Sumby et al., 2019).

1.3.4. Trends in the selection of novel malolactic starters

The selection of novel malolactic cultures typically follows the scheme shown in **Figure 6**. Thus, at first instance it is necessary to certify the suitability of the strains in terms of safety, that is, it must be certificate that LAB strains are not able to produce metabolites of health concern (e.g. biogenic amines). This process may be followed by the technological characterization of selected strains. Resistance to wine conditions, vigorosity of MLF, strains viability throughout fermentation process or yeast-bacteria compatibility are different features that should be checked. Further characterization to elucidate strains influence on wine sensory evolution, may comprised the analysis of different key enzymes under winemaking conditions, the production of desired compounds and the absence of off-flavours (Torriani et al., 2011).

In the last years, the selection of novel malolactic cultures has been focused on the potential positive impacts of microbial resources in terms of safety and sensorial properties as well as in the potential of microbial-based strategies allowing the reduction of overall winemaking time (Berbegal et al., 2017). In this sense, the role of starter cultures as biocontrol agents, together with their influence on wine sensory complexity and novel inoculation strategies, are the main issues studied nowadays.

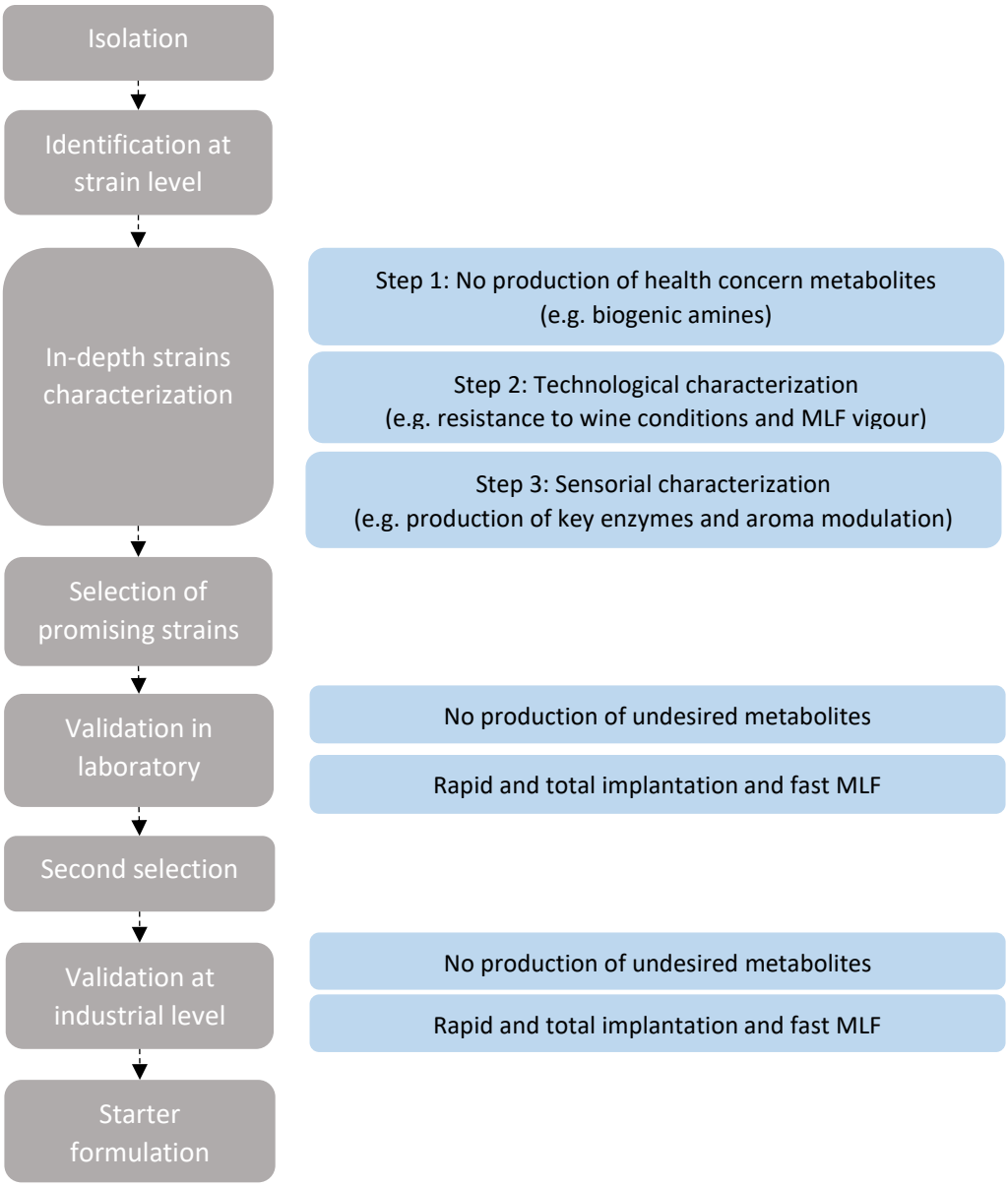


Figure 6. Typical scheme followed in the selection of novel malolactic starter cultures.

1.3.4.1. Starter cultures as biocontrol agents

Metabolism of microorganisms related to winemaking, from grapes to fermentation processes, define the success in terms of safety and quality of the wine. Many microorganisms can lead to the production of undesired compounds as metabolites of health concern or volatile compounds that impair negative flavours. Indeed, the presence of spoilage microorganisms could result on important economic losses (Berbegal et al., 2018).

In this sense, malolactic starter cultures have been described as a useful strategy to reduce or prevent BA accumulation during winemaking process (Berbegal et al., 2017; López et al., 2012; Patrignani et al., 2011; Smit et al., 2013). These findings underline the relevance of selecting malolactic cultures lacking the genetic determinants to produce BAs. Malolactic cultures have been also described as useful biocontrol agents against *Brettanomyces* proliferation (Berbegal et al., 2018). This species is the main responsible of wine deterioration through the production of unpleasant volatile phenols from the corresponding hydroxycinnamic acids (HCAs) (**Figure 7**). Typically, its growth is detected in the lapse time between the consecution of AF and the beginning of MLF, when there are still nutrients available and SO_2 concentrations are not restrictive (Chescheir et al., 2015). Thus, the rapid implantation of LAB starter and the fast launch of MLF have been described as a useful strategy to prevent the growth of *Brettanomyces*. Furthermore, in this species no cinnamoyl esterase has been yet described, so the selection of LAB strains without the ability to release HCAs from their esterified forms, means that *Brettanomyces* does not have these precursors for the production of the respective vinyl and ethyl phenols (Berbegal et al., 2018; Chescheir et al., 2015; Schopp et al.,2013) (**Figure 7**). That is, the use of selected malolactic

cultures can prevent both the production of these unwanted compounds as well as the proliferation of *Brettanomyces*.

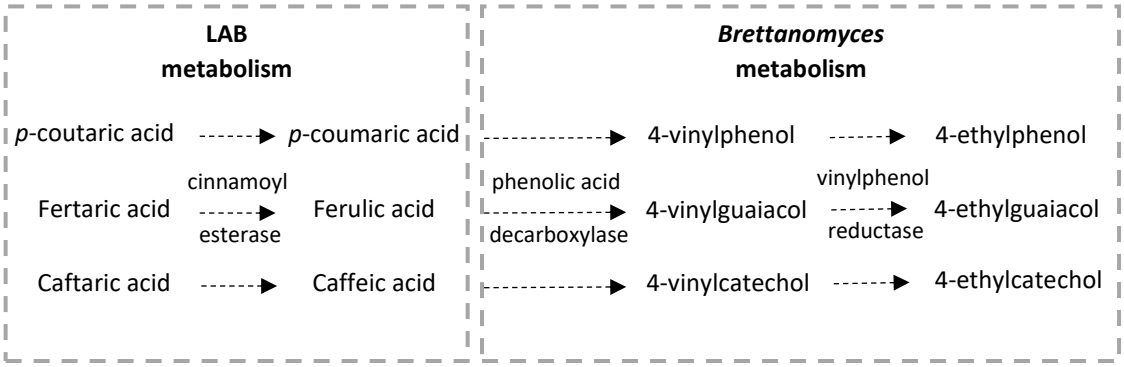


Figure 7. Formation of volatile phenols from their precursor hydroxycinnamic acids

1.3.4.2. Timing of inoculation

In addition to the influence of bacterial strain selection on the outcome of MLF, the inoculation strategy used for MLF could also influence LAB metabolism, and thus, impact the organoleptic profile of wine. The induction of MLF can mainly occur at two main stages during winemaking. The most common scenarios are simultaneous (or 24 h difference) inoculation for AF and MLF (co-inoculation) or inoculation after AF completion (sequential inoculation). Other strategies, as the inoculation before AF, for low malic acid content wines, as well as the inoculation during AF have been also proved to successfully complete MLF (Abrahamse and Bartowsky, 2012; Bartowsky et al., 2015). Timing of inoculation is an important factor determining the success of MLF, and many studies have been performed to elucidate the effect of inoculation time on fermentation kinetics, chemical composition and aromatic profiles of wine (Antalick et al., 2013; Izquierdo-Cañas et al., 2012; Knoll et al., 2012).

Most winemakers opt for sequential inoculation seeking for the absence of negative interactions between yeast and bacteria as well as to prevent an increase of volatile acidity derived from LAB metabolism (Costello et al., 2006). In addition, in sequential inoculation, yeast can promote the growth of LAB culture, releasing nutrients after its autolysis. However, many risks must be considered when performing sequential inoculation, as the loss of viability of the malolactic culture due to the low nutrient content of wine and high ethanol concentration. The release of antimicrobial compounds by yeast could also limit LAB growth when inoculated sequentially (Balmaseda et al., 2018). The selection of sequential inoculation over co-inoculation may also be due to the antagonistic effect attributed to yeast, based on nutritional competition or the presence of medium chain fatty acids, which can compromise the viability of malolactic bacteria (Larsen et al., 2003). Furthermore, the heterofermentative metabolism of *O. oeni* could lead, under the co-metabolism of citric acid and sugars, to produce wines with elevated volatile acidity due to higher production of acetic acid (Costello et al., 2006). Another feature concerning the use of co-inoculation is the potential negative effect of LAB on yeast growth and viability, leading to stuck or sluggish AF (Muñoz et al., 2014). In that way, yeast-bacteria compatibility may be also considered when selecting starter cultures in order to ensure a successful vinification. All these considerations have made sequential inoculation the most common practice for wineries.

In the last years, however, a special trend for co-inoculation has gained special attention. It is thought that simultaneous inoculation may boost the growth of LAB, since there is greater availability of nutrients and there is less alcohol and other potential yeast-derived inhibitors, thereby improving MLF performance (Zapparoli et

al. 2009, Azzolini et al. 2010). It has been also demonstrated that no significant increase on volatile acidity happens when following this strategy (Pan et al. 2011, Abrahamse and Bartowsky 2012). Important advantages of co-inoculation include a reduction in total fermentation time and better control over MLF, due to early implantation and dominance of the inoculated strains keeping out other undesirable bacteria (Azzolini et al., 2010; Zapparoli et al., 2009; Garofalo et al., 2015; Brizuela et al., 2018). The length of the MLF itself has been reported shorter when following co-inoculation strategy, and no evidence of negative impact on the final wine parameters has been found (Izquierdo-Cañas et al., 2012; Knoll et al., 2011). Indeed, co-inoculation strategies have been found to benefit production of Shiraz (Abrahamse and Bartowsky 2012), Cabernet Sauvignon (Guzzon et al. 2013), Merlot (Izquierdo-Cañas et al. 2012, Antalick et al. 2013), Cabernet Franc (Izquierdo-Cañas et al. 2015), Tempranillo (Izquierdo-Cañas et al. 2012), Riesling (Knoll et al. 2011), Teroldego and Marzemino (Guzzon et al. 2013), and Nero di Troia wines (Garofalo et al. 2015), among others. Regarding technical aspects, these wines, after successful co-inoculation, take benefit as they are ready for early stabilization (racking, fining, and SO2 addition), increasing in that way microbiological stability and processing efficiency.

Besides MLF efficiency improvement, the sensory profile can also vary between inoculation strategies. Massera et al., 2009 demonstrated that co-inoculation tends to retain more fruity descriptors and showed less astringency and bitterness. A sensory study of Shiraz wine showed that wines produced through co-inoculation showed more fruity compounds (Abrahamse & Bartowsky, 2012). The studies performed by Jussier et al. (2006) and Knoll et al. (2011) showed that more compounds contributing to the fruity character of wine were identified with co-inoculation when analysing

Chardonnay and Riesling wines, respectively. Although a trend for more fruity wines has been usually reported, in other studies no significant differences on aromatic profile have been detected (Antalick et al., 2013; Lombardi et al., 2020). The inconclusive effects of inoculation strategy on wine aromatic profile reflect the complex interactive effects of yeast and bacteria strains. However, it is clear that the timing of inoculation for MLF and yeast-bacteria compatibility play and important role in the success of MLF.

1.3.4.3. LAB and MLF influence on wine aroma development

Wine is the outcome of a complex mixture of chemical and biological interactions, in which microorganisms play a critical role. All these interactions contribute to enhance the complexity of the volatile compounds responsible for wine aroma (Ribéreau-Gayon et al., 2006; Antalick et al., 2013). Wine aroma is considered the major contributor to the global flavour perception, and depending on the origin of the aromatic compounds it can be divided into: varietal aroma (volatile compounds present in grapes), fermentative aroma (volatile compounds originating by yeast and bacteria during alcoholic and malolactic fermentations) and ageing aroma (volatile compounds developed during ageing/storage period after physicochemical and residual enzyme reactions) (Ferreira et al., 2000). Among them, fermentation compounds represent a critical aspect on the overall wine aroma and flavour perception, since they constitute the largest concentration of aromatic compounds. Typically, most of the fermentative volatile compounds have high aromatic perception thresholds, and thus, they individually contribute slightly to wine aroma complexity (Belda et al., 2017). However, the combination of compounds that build different chemical families, such as esters, alcohols or acids, and which present similar sensorial

properties could synergically contribute to different aroma intensities. In addition, interactions between compounds, as aroma inhibitions or enhancements and synergistic effects, must also be considered when defining wine complexity (Ferreira et al., 2007). On the other hand, compounds described as impact odorants are generally present in low concentrations, but since they show very low perception thresholds (ng/L), they have a major impact on the overall wine aroma complexity. Although much more attention has been paid on yeast (both *Saccharomyces* and non-*Saccharomyces* species) influence on wine aroma modulation, it has been also proved the influence of LAB and MLF on wine aroma enhancement (Cappello et al., 2017). MLF not only drives a deacidification process but it also influences the organoleptic complexity of wine by modifying grape and yeast-derived compounds and producing aroma-active compounds (Bartowsky 2005). Different pathways have been described for aroma profile modification by LAB, as amino acid metabolism, citrate metabolism, hydrolysis of glycosides, synthesis and hydrolysis of esters, metabolism of polyols and degradation of phenolic acids, among others (Swiegers et al., 2005; Liu, 2015, Matthews et al., 2004; Lerm *et al*., 2010). In the next lines, main aromatic compounds associated with MLF as well as their production mechanisms will be explained. The group of compounds include, organic acids, higher alcohols, esters, glucoside-derive aroma compounds, volatile phenols, volatile sulphur compounds, volatile fatty acids and aldehydes.

Organic acids

Acidity plays a key role in many aspects of the winemaking process, as influences taste and mouthfeel perception, solubility of proteins and wine colour intensity (Mendes-Ferreira & Mendes-Faia, 2020). Grape must mainly consists of water, around 80%, and

many dissolved solids. Next to sugars, organic acids are the second largest group accounting for the 1% of solids present in grape must. L-tartaric acid and L-malic acid account for the 90% of total acids, whereas citric acid and ascorbic acid represent less than 10% (Mendes-Ferreira & Mendes-Faia, 2020). Among them, citric acid plays an important role on wine aroma complexity. Citric acid metabolism in LAB leads to the production of diacetyl, acetoin, 2,3-butanediol and acetic acid, which are important for wine aroma enhancement (Olguín et al., 2009). Among them, diacetyl, which confers a buttery character to the wine, is the most important compounds due to its low aromatic threshold (Bartowsky & Henschke, 2004). Its detection threshold varies among different wines, while a concentration between 1-4 mg/L confers a positive character, concentrations exceeding 5-7 mg/L are considered detrimental (Swiegers et al., 2005). The genes coding for citrate metabolization are organized in a gene cluster, in which *maeP* gene encodes for citrate transporter which enables the uptake of citrate into the cell, and citrate lyase complex (comprising three subunits: α-subunit (encoded by *citD*), β-subunit (encoded by *citE*) and γ-subunit (encoded by *citF*)) drives the conversion of citrate into acetate and oxalacetate (Mills *et al*., 2005). Oxaloacetate is then decarboxylated to pyruvate by oxaloacetate decarboxylase. Most of the pyruvate is reduced to lactate in the presence of NADH, however, it can follow a decarboxylation to α-acetolactate through acetolactate decarboxylase which finally may lead to acetoin, diacetyl and 2,3-butanediol production (**Figure 8**). The final concentration of diacetyl depends also in different factors as the selected LAB strain, sulphur dioxide and oxygen concentrations, or the period that wine is in contact with lees. While oxygen favours the oxidation of α-acetolactate to diacetyl, SO_2 content binds diacetyl minimizing its sensory effects. In addition, prolonged contact with lees

also reduces diacetyl content of wine (Belda et al., 2017). All these factors provide a tool for manipulating the desired influence of this compound on the final wine. In addition, citrate metabolism is considered sequential to malic acid degradation in LAB (Bartowsky & Henschke, 2004). In that way, an immediate sulphitation after malic acid exhaustion, a common criterion for MLF completion, would result in incomplete citrate degradation, disrupting the formation of key carbonyl flavour compounds, as diacetyl, and minimizing LAB influence on aroma and flavour development.

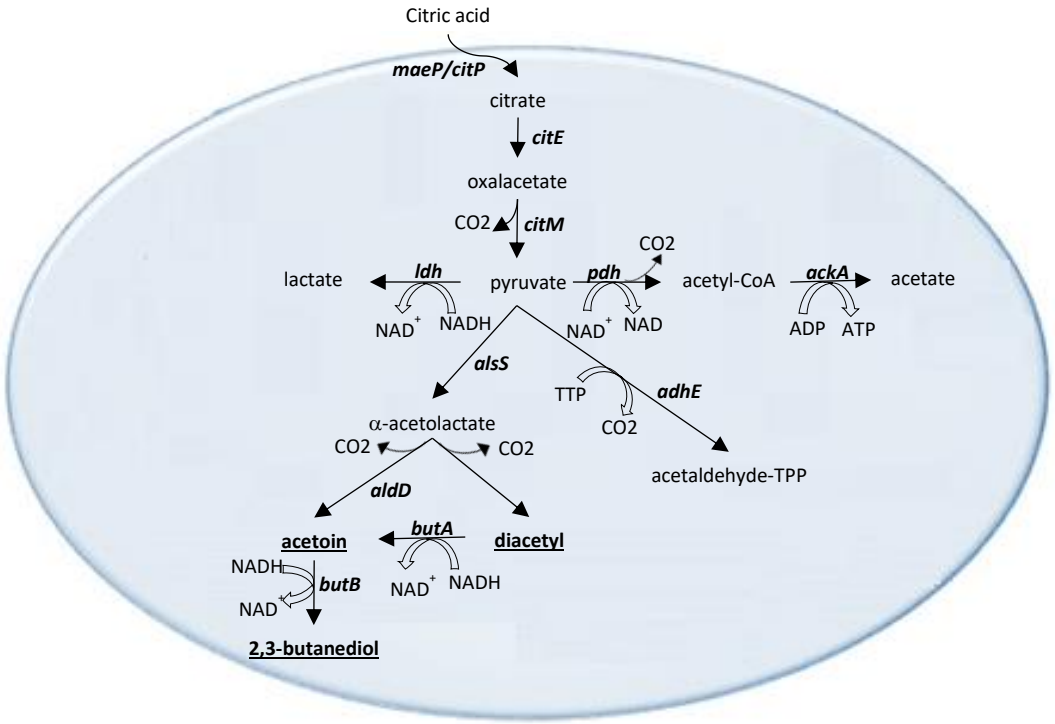


Figure 8. Schematic representation of citrate metabolism in LAB. Reactions are carried out by: *citP*/*maeP* (citrate permease), *citE* (citrate lyase), *citM* (oxaloacetade decarboxylase), *ldh* (lactate dehydrogenase), *pdh* (pyruvate dehydrogenase), *ackA* (acetate kinase), *alsS* (α-acetolactate synthase), *alsD* (α-acetolactate decarboxylase), *adhE* (acetaldehyde dehydrogenase), *butA* (acetoin dehydrogenase), *butB* (2,3-butanediol dehydrogenase); TPP (thiamine PPi)

Acetic acid is described as a pungent, vinegar-like aroma when it is above its perception threshold of 0,7 g/L (Francis & Newton, 2005). At lower levels it is

considered to enhance wine aroma complexity. Acetic acid production could happen via different pathways: (i) the heterofermentative metabolism (Swiegers *et al.*, 2005), and (ii) citrate metabolism of LAB (Bartowsky & Henschke, 2004).

Higher alcohols

MLF is often accompanied with the formation of aliphatic and aromatic alcohols known as fusel or higher alcohols. While at higher concentrations are considered to impart off-flavours, at low levels they positively contribute to the basic matrix of aromas in wine. They are synthesized via amino acid metabolism through the Ehrlich pathway (Smid & Kleerebezem 2014), and when present in concentrations below 300 mg/L they contribute to the complexity and fruity aroma of wine. However, higher concentrations could add solvent-like, spiritous character (Swiegers *et al*., 2005; Tao et al., 2008). Main higher alcohols include isoamyl alcohol (whiskey/malt aroma) which derived from the metabolism of the amino acid leucine, isobutanol (solvent-like aroma) which derived from valine, 3-methylbutanol (herbaceous/spiritous) which derived from leucine and 2-phenylethanol (rose) which derived from phenylalanine (**Figure 9**). Although yeast influence on higher alcohol production is well-known, the role of MLF is still inconclusive (Belda et al., 2017). While many studies have detected no changes on higher alcohols concentrations after MLF (Hernández-Orte et al., 2012, Izquierdo-Cañas et al., 2008), others have reported significant changes during MLF (Pozo-Bayón et al., 2005, Brizuela et al., 2018). All in all, higher alcohols have a direct impact on the organoleptic quality of wine and they are often the base in the formation of another important family of compounds, the esters.

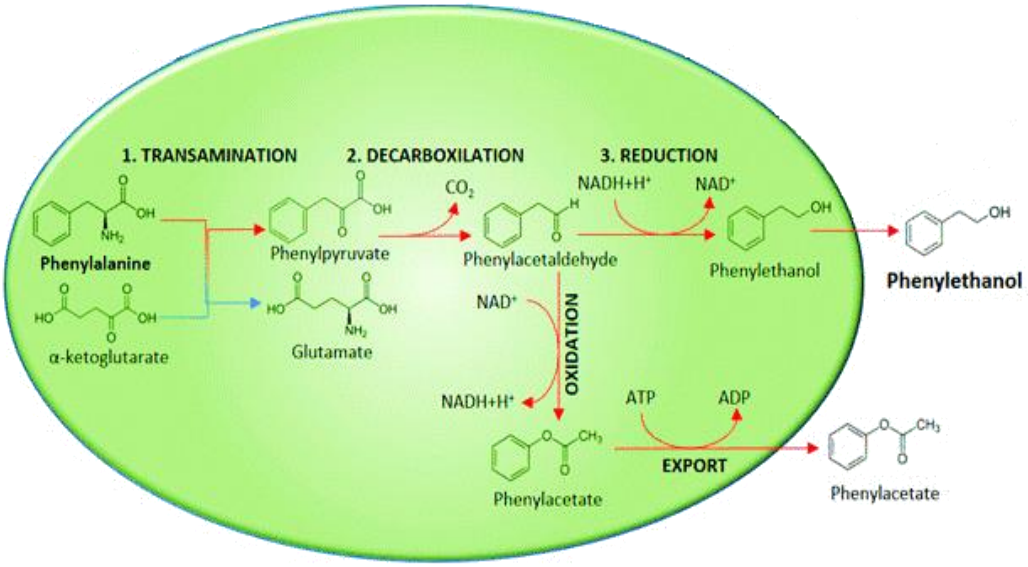


Figure 9. Schematic representation of Ehrlich pathway. Example of phenylalanine metabolism leading to phenylethanol and phenylacetate production (adapted from Belda et al., 2017).

Esters

Esters constitute one of the most relevant groups of aromatic compounds, and as a chemical family, they tend to act collectively having an additive effect on wine aroma (Sáenz-Navajas et al., 2016). They are responsible for the fruity aroma of young wines and their concentration will be defined as the result of the balance between synthesis and hydrolysis reactions carried out by esterases and synthesis reactions performed by alcohol acetyltranferases (Matthews et al., 2007). They are formed by the esterification of an alcohol and carboxylic acid, thus, in wine typical esters are ethyl esters (formed by ethanol and volatile fatty acids or organic acids) and acetate esters (build by acetil Coa and higher alcohols) (Sumby et al., 2009) (**Figure 10**). It has been demonstrated that wine LAB possess an extensive collection of enzyme activities that can increase the content of esters in wine, highlighting their influence on wine aroma

modification (Sumby et al., 2013; Pérez-Martín et al., 2013). LAB strains are found to have higher activity towards short-chained esters (C_2-C_8), conferring desirable fruity character, compared to long-chained esters (C_{10}-C_{18}), which are responsible for waxy, soap-like aromas (Matthews et al., 2007; Sumby et al., 2009). Generally, *O. oeni* species has been found to show higher esterase activities under winemaking conditions compared to *Lactobacillus* and *Pediococcus* species. In that way, the selection of proper malolactic starters would contribute to modulate the overall fruitiness of wine (Matthews et al., 2007). The most important esters typically associated with MLF are ethyl lactate (fruity/milky), ethyl acetate (fruity), isoamyl acetate (banana), diethyl succinate (fruity/apricot), ethyl hexanoate (green apple), 2-phenylethyl acetate (flowery, rose), hexyl acetate (pear, pineapple), ethyl octanoate (waxy/fruity) and ethyl decanoate (waxy/fruity) (Costello et xal., 2012; Antalick et al., 2012; Brizuela et al., 2018; Izquierdo-Cañas et al., 2016).

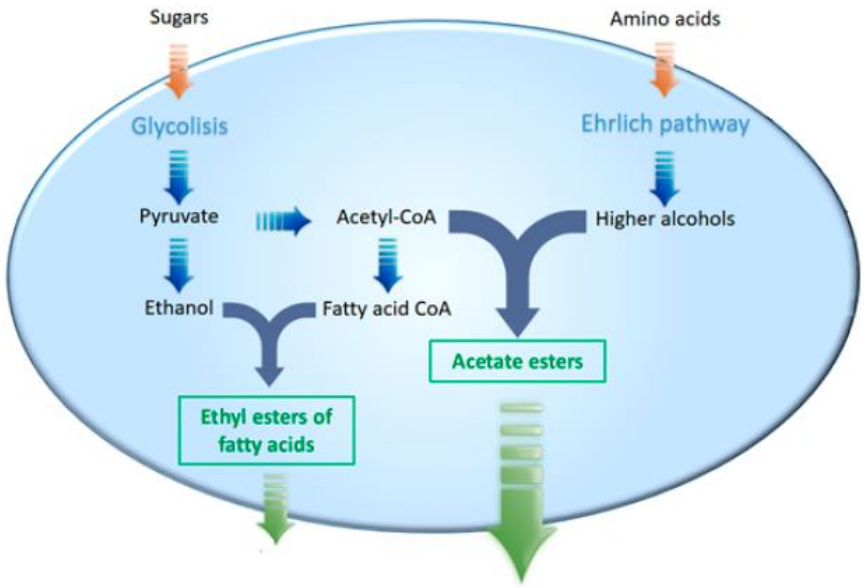


Figure 10. Schematic representation of ethyl esters and acetate esters formation (adapted from Belda et al., 2017)

Glycosides-derived compounds

Glycosylated aroma precursors are considered the main reserve of active compounds in grapes, and thus, they may also have a considerable impact on flavour characteristics of wine. These active and sensorially relevant compounds responsible for floral and fruity aromas include monoterpenes, C13-norisoprenoids, benzene derivatives and aliphatic alcohols (Liu et al., 2017; Hernández-Orte et al., 2009). In fact, these compounds are generally characterized by low perception thresholds and potent sensory properties. Glycosylated precursors are mainly present as mono- or diglucosydes, and thus the action of different glycosidases is essential to aroma compound (aglycon) release (Liu et al., 2017).

The aglycon moiety in monoglycosides is always linked to a β-D-glucopyranose, thus, the enzymatic hydrolysis is driven by β-D-glucosidase (βGlu). Disaccharides, incorporating other sugar than glucose require the sequential action of specific enzymes, as β-D-xylosidase α-L-arabinosidase α-L-rhamnosidase or β-apiofuranosidase to hydrolyse the intersugar linkage before the hydrolysis of aglycone-glucose linkage by βGlu (D´Incecco et al., 2004) (**Figure 11**). Different potential glycosidases have been identified in the genome of *O. oeni* (Borneman et al., 2010), being the study carried out by Olguín et al. (2011) the first attempt to study the expression of *O. oeni* βGlu gene under winemaking conditions. This study highlighted the great influence of ethanol, pH and the selected *O. oeni* strain on enzyme activity. Different studies have elucidated the role of different glycosidases to contribute to aroma profile modification during MLF. Activity towards glycosides extracted from Muscat wines (Ugliano *et al*., 2003), or Verdejo, Chardonnay, Garnacha and

Tempranillo grapes (Hernandez-Orte *et al*., 2009), all confirm the glycosidase activity of *O. oeni*. Ugliano et al. (2003) described the increase in monoterpenes such as linalool (floral/citrus), α-terpineol (pine/floral), nerol (rose-like/citrus) and geraniol (rose) after the hydrolysis of grape-derived extracts. Hernández-Orte et al. (2009) reported the ability of different *O. oeni* strains to release different amount of benzenic compounds, terpenes and norisoprenoids when supplementing glycosidic-precursor grape extract in synthetic wine. Antalick et al. (2012) found an increase of C13-norisoprenoids, whereas Michlmayr et al. (2012) reported that *O. oeni* glycosidase activity led to higher linalool, citronellol and nerol concentrations. Not only the hydrolysis of grape-derived compounds by LAB glycosidases but also the release of wood-related compounds has been documented. The importance of LAB interaction with wood during MLF has demonstrated the release of oak active compounds such as vanillin (vanilla) or whiskey lactone (coconut) (Bloem et al., 2008; Gagné et al., 2011). In that way, when MLF is conducted in oak barrels it has been proved to augment the oak character of wine.

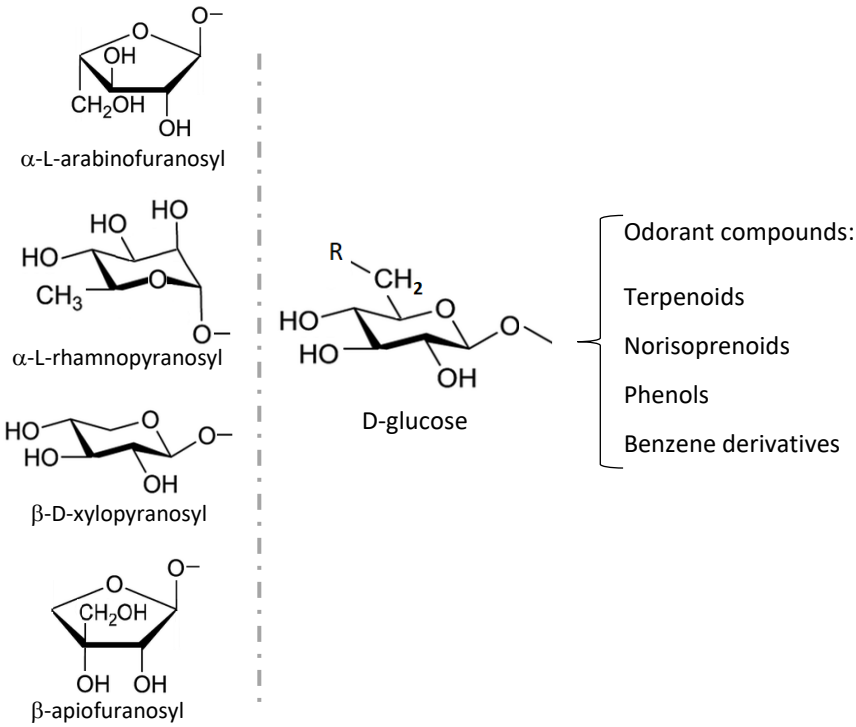


Figure 11. In grapes, glucosides may be present as monoglucosides, in which the aroma compound is linked to glucose, or they may form complex structures, in which glucose moiety is linked to other sugars.

Volatile phenols

Hydroxycinnamic acids (HCAs) are known as volatile phenol precursors and they conform an important group of non-flavonoid phenolic compounds that are naturally present in wine. They are commonly esterified with tartaric acid, and during winemaking process these esters may be hydrolysed, releasing free HCAs through cinnamoyl esterase enzymes (Santamaría et al., 2018). Main HCAs found in wine are ferulic, *p*-coumaric and caffeic acids which could be microbially metabolized to produce the corresponding vinyl- and ethyl-derivatives by the sequential action of phenolic acid decarboxylases and vinyl phenol reductases and (Chescheir et al., 2015). Although it has been documented the ability of certain LAB species, as *Lactobacillus*

plantarum, to produce volatile phenols during winemaking (Santamaría et al., 2018), the production of volatile phenols is mainly associated to *Brettanomyces* species.

These compounds are responsible for the depreciation of the organoleptic quality of wines conferring medicinal, horse-sweat like, smoky character. Schopp et al. (2013) reported that *Brettanomyces* lack the ability to metabolize esterified HCAs and only could convert free HCAs to volatile phenols. Thus, the inoculation of cinnamoyl esterase negative MLF starters as biocontrol agents has been described as a useful strategy to prevent both growth of this species and the appearance of these off-flavours (Berbegal et al., 2018; Gerbaux et al., 2009).

Volatile sulphur compounds

Due to their low perception thresholds volatile sulphur compounds make an important contribution to the overall wine sensorial profile. They are often referred as reductive aromas, such as rotten eggs, onion, garlic and cabbage aromas (Moreira et al., 2002). However, in small quantities they can add a beneficial character. Pripis-Nicolau *et al.* (2004) provided the first evidence regarding the methionine metabolising ability of wine LAB during MLF. LAB have been found to metabolize this amino acid, resulting in the formation of characteristic aroma compounds, as methanethiol, dimethyl disulphide, methionol (3-(methylsulphanyl)propan-1-ol) and 3-(methylsulphanyl) propionic acid. Among them, methionol is considered the most relevant sulphur-volatile compound in wines (Cappello et al., 2017).

Volatile fatty acids

In spite of the evidence supporting the low lipase activity of wine LAB, different studies have reported changes in the evolution of fatty acids after MLF (Matthews et al., 2004;

Pozo-Bayón et al., 2005; Costello et al., 2012). Wine consists of both straight chain and branched chain fatty acids and because of their low aroma threshold, their presence may significantly contribute to wine sensorial complexity. However, excessive quantities may negatively affect wine quality by conferring cheesy/rancid attributes (Francis & Newton, 2005). Significant increases are reported for hexanoic, octanoic and decanoic acids after MLF (Pozo-Bayón et al., 2005; Costello et al., 2012)

Aldehydes

Acetaldehyde is quantitatively the most important aldehyde found in wine. When present around its odour threshold (0,5 mg/L) it contributes to fruity, nutty aroma to wine; however, at higher concentrations it imparts a green, oxidative, apple-like aroma (Ferreira *et al*., 2000). The metabolism of acetaldehyde in wine LAB is not well understood, however, it has been shown their ability to release acetaldehyde by degrading SO2-bound acetaldehyde (Burns and Osborne, 2015). Other aldehydes such as (*E)*-2-nonenal, octanal, nonanal, decanal or (*E*,*Z)*-2,6-nonadienal, are important odorants responsible for a sawdust off-flavour, while the herbaceous odour in wine is often associated with hexanal, (*E*)-2-hexenal, (*E*)-2-heptenal, octanal and (*E*)-2-octenal (Mozzon et al., 2016).

1.3. Rioja Alavesa region characteristics

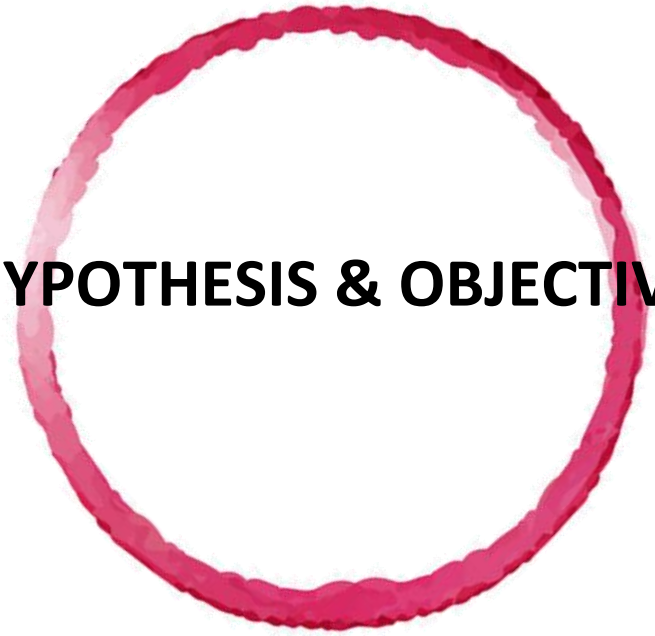
Rioja Alavesa is the northernmost of the three sub-regions which constitute the Qualified Designation of Origin Rioja (DOP Rioja). The vineyard of Rioja Alavesa region is an exceptional model of continuity of a living cultural tradition that begins in Roman times and has a special development in the Middle Ages, until becoming nowadays the dominant element in the landscape. It has based its development on a balanced

coexistence between tradition, development and innovation. The culture of wine and its landscape has become the fundamental element of the local identity. The coincidence of singular geographical and climatic characteristics has allowed a special development of viticulture. The vineyard is distributed in small plots (the average size barely exceeds half a hectare), comprising more than 13000 ha in total, which are located along 18 different municipalities. In 2019, a total of 65.211.423 L of wine were produced, from which 57.853.171 L belong to red wine (DOC Rioja Annual Memory, 2019). The sector is based on a high number of small producers. Nowadays, with more than 600 wineries, this primary sector represents the 20% of the economic activity of the region. Indeed, this region is considered the richest region of Basque Country, with a PIB per capita of 62.120 € (Eustat, 2019) and doubling the median of Basque Country, although it is not uniformly distributed along the region.

The geographical features of the region, as many vineyards oriented to the south and a climate with Atlantic and Mediterranean influence ensure a good grape maturation and a signature freshness and good acidity in the wines (Etaio et al., 2009). Sierra Cantabria mountains, extending in a west-east direction, greatly protects this area from the Atlantic climate, protecting the region from cold and humid winds from the northwest. In addition, its location between the slopes of Sierra Cantabria, in the north, and the Ebro river, in the south, makes the vineyard to be oriented to the south, which means that the insolation is higher, and the ripening of the grape is favored (Etaio et al., 2009). The vineyard is mainly settled clay-calcareous soils, which has a beneficial effect on the regulation of the hydration of the vine and, consequently, of the grape. In fat, clay-calcareous soils have traditionally been related to high quality grapes and wines. Tempranillo varietal is the utmost grape variety used (>90% of

cultivated ha), and although Tempranillo wine may differ among the different regions, in general Rioja Alavesa young wine has a mature fruit, floral and balsamic (licorice) character (Etaio et al., 2007). Other typical red grape varieties included in the DOP Rioja are Garnacha, Mazuelo and Graciano, and among white varieties, Viura, Tempranillo, Verdejo and Malvasía. Despite making some monovarietal wines with some of the varieties mentioned, the red wines of Rioja Alavesa, and especially the young, are necessarily associated with the variety Tempranillo, although a minority presence of other authorized red and/or white varieties could happen.

In Rioja Alavesa, although most wineries inoculate yeast strains to overcome AF, few inoculate for MLF induction. As already stated, spontaneous MLF could lead to protracted fermentation period in which spoilage microorganisms and different metabolites of health and sensory concern may arise. To face this situation, in the last few years a trend for the selection of authocthonous strains, which are already adapted to regional winemaking conditions, is gaining special attention (Franquès et al., 2017; Petruzzi et al., 2017). Regional branding is an effective tool of producing higher returns for wine companies and a mean of differentiation in the high-competing wine industry. Premium wines are importantly associated with the region they come from. Thus, each winemaking area has its own wine characteristics, determined by the grape cultivar, climate, geology, winemaking practices, etc. And in the last years, the contribution of indigenous bacterial ecology to the specific wine´s terroir has been evidenced (Gilbert et al., 2014; Zarraonaindia et al., 2015). In that way, the contribution of autochthonous microbial strains and their potential application in winemaking is an interesting strategy to pursue in order to enhance the regionality of wine through MLF.



2. HYPOTHESIS & OBJECTIVES

In the pursuit of the production of high-quality wines the control of every step of the vinification process is highly relevant. In order to ensure that fermentative processes lead to the production of consistent, safe and high-quality products, the selection of starter cultures from winery environment has become a suitable strategy. Regarding malolactic fermentation (MLF), the inoculation of autochthonous strains already adapted to a specific regional area will enhance the specific sensorial characteristic and maintain the biodiversity of the region. Furthermore, it will prevent or decrease the presence of potential spoilage microorganisms.

In that way, in-depth characterization of indigenous lactic acid bacteria (LAB) strains unable to produce compounds of health concern, as biogenic amines (BAs), and presenting suitable technological and sensorial characteristics was the primary objective of this research. In addition, the lack of an indigenous starter culture from Rioja Alavesa region for conducting the MLF was another motivation in order to build another tool for the wine industry of that region. Indeed, Rioja Alavesa is well-known worldwide due to the quality of their wines. Thus, the inoculation of autochthonous starters for the production of wines with a low concentration of BAs as well as with different organoleptic nuances may represent a competitive commercial advantage for wineries.

In general, a wine without BAs could be considered a safer, healthier and higher quality product than the wines currently available on the international market. Additionally, the achievement of an original or differentiated sensory profile would provide added value to these wines, improving consumer confidence and opening new market opportunities for wineries. This project will strongly contribute to responding the needs of consumers for safe, healthy and high-quality food. Specifically, it will address the search for solutions to avoid or reduce some health disorders that are closely related to the diet and the foods we consume. The strategies and end-products that will derive from the achievement of this project may represent an innovative solution that will allow an improvement in the health and well-being of a part of the population that is especially sensitive to the toxic action of BAs.

Furthermore, it should be noted that some countries, such as Austria, Belgium, Finland, the Netherlands and Germany, use histamine as a marker of safety and

quality of wines, and they have imposed recommendations on the maximum concentration allowed in wines. This fact could directly affect the export of wine to these countries, which could be paralyzed in the future, turning the presence of BAs into a potential economic threat for the wine sector.

In this sense, the hypothesis of this doctoral thesis was: **The inoculation of safe malolactic starters unable to produce metabolites of health concern (e.g. biogenic amines) and presenting suitable technological and sensorial characteristics, is a useful strategy to produce biogenic amines free high-quality wines.** The principal aim to demonstrate this hypothesis was to develop new wines that do not present biogenic amines through the inoculation of novel autochthonous LAB strains.

To achieve this general aim different partial objectives were stablished:

- To know the situation of BAs in commercial red wines from Rioja Alavesa region (Study 1)
- To isolate, identify and evaluate the genetic diversity of LAB strains from Rioja Alavesa as well as to identify indigenous LAB strains unable to produce BAs (Study 2)
- To identify and select LAB strains according to their technological and sensorial characteristics (Study 3)
- To elucidate the ability of LAB strains to modify wine aromatic profile (Study 4)
- To evaluate the effect of different inoculation strategies on wine aroma development and sensorial perception to select the most appropriate LAB strain to be used as malolactic starter (Study 5)

3. METHODOLOGY

3.1. Material and Methods

3.1.1. Chemicals

All culture media were from Oxoid (Hampshire, UK). All chemicals were at least of analytical grade and obtained from Sigma-Aldrich (Steinheim, Germany), or Merck (Darmstadt, Germany), unless otherwise stated.

3.1.2. Lactic acid bacteria reference strains

LAB reference strains, known for the biogenic amine production ability, were used as positive controls in biochemical and molecular assays. These strains were *Lactobacillus brevis* IOEB 9809 (tyrosine decarboxylase (tyrdc) and agmatine deiminase (agdi) positive), *Lactobacillus reuteri* CECT 925 (histidine decarboxylase (hdc) positive) and *Lactobacilllus* 30a ATCC 33222 (ornithine decarboxylase (odc) and hdc positive). Also, two of the most used starters in the region (according to the main regional dealer), corresponding to the *Oenococcus oeni* strains Viniflora® OENOS and Viniflora® CH16 (Chr, Hansen, Hoersholm, Denmark), were used in fingerprinting and characterisation analysis as comparative strains.

3.1.3. Bacterial isolation, characterization and typification

3.1.3.1. Samples

A total of 31 samples of Tempranillo wine were collected during the 2016 vintage from two wineries located in Rioja Alavesa region, part of the Qualified Denomination of Origin Rioja (Spain). Samples were taken during all the vinification process: must, tumultuous alcoholic fermentation (AF) (density < 1,075 g/L), end of AF (reducing sugars < 2 g/L), beginning of malolactic fermentation (MLF) (when 10% of the initial malic acid is consumed), tumultuous MLF (60% of the initial malic acid is consumed)

and the end of MLF (malic acid content < 0,5 g/L). In all cases MLF undergone spontaneously.

3.1.3.2. Bacterial isolation and growth conditions

Bacterial isolation was carried out in three different culture media: MRS (De Man, Rogosa & Sharpe, 1960) supplemented with cysteine (0,5% w/v), MRS-A supplemented with cysteine (0,5% w/v) and apple juice (20% v/v), and M17 (Terzaghi & Sandine, 1975) supplemented with glucose (0,5% w/v). Cycloheximide (4g/L) was added to all media to inhibit yeast and fungal growth. Serial dilutions were plated in duplicate onto the different media and incubated for 5-7 days at 30 ºC under anaerobic conditions (Anaerogen, Oxoid). After incubation, colonies were counted (CFU/mL) and 5-10 colonies were randomly selected from each plate and transferred to the same media to obtain pure cultures.

3.1.3.3. Bacterial characterization by phenotypic methods

Isolates were phenotypically characterized by macroscopic and microscopic morphology determination (i.e. shape, size, edge, opacity, colour, elevation, surface and consistency). Gram staining and Gram staining confirmation with potassium hydroxide (3% v/v), as well as catalase activity determination with hydrogen peroxide (3% v/v) and oxidase test strip analysis (Microplate, Oxoid) were carried out. Colonies presenting typical LAB profile (Gram positive, catalase negative and oxidase negative) were selected; colonies showing catalase positive reaction were also selected. After characterization, presumptive LABs were transferred to the same isolation media broth and maintained at -80 ºC in glycerol (20% v/v).

3.1.3.4. Bacterial DNA extraction and identification by 16S rDNA sequencing

Cultures of each presumptive LAB were transferred to fresh growth medium and incubated 24 h at 30 ºC under anaerobic conditions. These cultures were used for DNA extraction using PreSeq Extraction Kit (Promega, Madison, WI, USA) following the instructions of the manufacturer. 16S rDNA genes were amplified by conventional PCR. PCR amplification was performed with an Eppendorf® Mastercycler (Eppendorf, Hamburg, Germany) by using a Master mix (Taq DNA polymerase Master mix red 2X, Ampliqon A/S, Odense, Denmark), 1 µM of each primer and 30 ng of DNA template, and the following cycling parameters. DNA denaturation was performed at 94 ºC for 1 min, followed by annealing at 64 ºC for 1 min and extension at 72 ºC for 80 s. This cycle was performed 20 times and followed by a second cycle consisting on a DNA denaturation at 94 ºC for 1 min, followed by annealing at 57 ºC for 1 min and an extension at 72 ºC for 80 s. This second cycle was performed for 15 times. Reaction was followed by a final extension at 72 ºC for 10 min. The oligonucleotide primers used in the study were 27Fc (Tanasupawat et al., 2000) and PUBr (table 1). They were synthesized by Isogen (Utrecht, Netherlands). PCR products were run on a 0,8% agarose gel with 1X TAE (40 mM Tris-acetate-EDTA buffer, pH 8) buffer, and photographed under UV light using a BioDoc-It™ Imaging System (Biorad, Marnes La Coquette, France). 16S rDNA PCR products were purified with Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). 16S rDNA Sanger sequencing was performed and DNA homology searches were carried out in the GenBank database of the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool, BLAST (Altschul, et al., 1990)

3.1.3.5. Typification by RAPD-PCR

RAPD-PCR typing was used to distinguish culture at strain level. Primers M13 (Zapparoli et al., 2000) and 1254 (Akopyanz et al., 1992) were used. Two commercial malolactic starters were also typified for comparative analysis (Viniflora® OENOS and ViniFlora® CH16, from Christian Hansen, Denmark). RAPD-PCR reaction was performed with an Eppendorf® Mastercycler (Eppendorf) by using a Master mix (Taq DNA polymerase Master mix red 2X, Ampliqon A/S), 1 µM of primer (M13 or 1254, table 1) and 30 ng of DNA template, and the following cycling parameters. M13 reaction was carried out as follows. The DNA denaturation was performed at 94 ºC for 1 min, followed by annealing at 40 ºC for 20 s and extension at 72 ºC for 2 min. This cycle was performed 35 times. Reaction was followed by a final extension at 72 ºC for 10 min. 1254 reaction was carried out as follows. The DNA denaturation was performed at 94 ºC for 2 min, followed by annealing at 45 ºC for 20 s and extension at 72 ºC for 2 min. This cycle was performed 30 times. Reaction was followed by a final extension at 72 ºC for 7 min. PCR products were separated by gel electrophoresis on 1,5% agarose gel with 1X TAE (40 mM Tris-acetate-EDTA buffer, pH 8) buffer and photographed as described earlier.

After RAPD-PCR each strain showed their own amplicon pattern. From these amplicon patterns a binary matrix was created based on the presence/absence of each pattern bands. Genetic diversity among each possible pair of the sampling group was calculated with the formula $S= 2\ b_{ij}/ (b_i+b_j)$ (Nei & Li, 1979), where b_{ij} is the number of shared bands by the pair i and j, and b_i and b_j are total number of bands of i and j, respectively. Genetic distance was defined as 1-S. A dendrogram was built with these data, applying the Unweighted Pair Group Method using Arithmetic Averages

(UPGMA) (Vauterin & Vauterin, 1992) method with DendroUPGMA (Department of Biochemistry and Biotechnology**,** University of Rovira i Virgili, Spain) and Phylodendron (Department of Biology, University of Indiana, USA) software.

A reproducibility study for each primer was carried out in order to determine the minimum percentage of similarity necessary for strain discrimination. In that way, 10% of the strains were selected and DNA of each strain was extracted in duplicate. Three independent amplification reactions were carried out with each duplicate (following the methodology described above). Patterns were analyzed as already described, and a limit of discrimination was established for discerning different strain profiles.

3.1.4. Suitability of LAB strains to be used as malolactic starters

3.1.4.1. Technological characterization

Biogenic amine production ability through phenotypical methods

All identified LAB strains (**Table 6**) were characterized for their ability to produce biogenic amines. Histamine, agmatine, tyramine, putrescine (via ornithine decarboxylase or agmatine deiminase) and cadaverine production, as well as arginine degradation, were assessed in decarboxylase medium (DM) broth (Bover-Cid & Holzapfel, 1999), containing the corresponding precursor amino acid: L-histidine monohydrochloride, L-ornithine monohydrochloride, L-arginine monohydrochloride and L-lysine monohydrochloride (all of them at 0,5% (w/v)), 0.25% L-tyrosine and 0.1% agmatine sulfate salt. Pyridoxal-5-phosphate (0.005% w/v) was added as cofactor for the decarboxylation reaction, and purple bromocresol was added as pH indicator. pH was adjusted to 5.3 and the medium was sterilized.

Table 6. Identified LAB strains which were used for the different characterization studies.

Oenococcus oeni		*Lactobacillus hilgardii*	*Lactobacillus mali*	*Pediococcus parvulus*	*Lactobacillus plantarum*
P1A	P3F	LH1	LM1	PP1	LP1
P1B	P3G	LH2	LM2	PP2	
P1C	P5A	LH4	LM3		
P1D	P5B				
P2A	P5C				
P3A	P5D				
P3B	P7A				
P3C	P7B				
Viniflora	Viniflora				
OENOS	CH16				

For amino acid decarboxylase induction, strains were previously grown in MRS broth supplemented with 0.01% (w/v) of each precursor amino acid and 0.005% (w/v) of pyridoxal-5-phosphate for 48 h at 30 ºC. Sterile 96-well polystyrene microtiter plates were fulfilled with 250 μL of DM broth and inoculated with 10% (v/v) of induced culture in MRS broth. Incubation was carried out for 14 days at 30 ºC under anaerobic conditions by overlaying with paraffin. Positive and weakly positive strains induced a colour change of the medium from yellow to purple. Positive strains were selected and inoculated again into MRS broth until growth was noticeable. From this MRS broth, strains were re-inoculated again in DM broth for confirmation. After incubation under anaerobic conditions for 7 days at 30 ºC, 2 ml of broth were collected and centrifuged (16,200 *g* for 5 min). The cell-free supernatant was collected and stored at –20 ºC. Phenotypical positive results from the biogenic amine tests were confirmed

by reverse-phase high-performance liquid chromatography (RP-HPLC), following the method described by the OIV (OIV, 2009) with slight modifications (*the followed RP-HPLC method is available in **2.6.1. section***). To confirm arginine degradation, ammonia production was quantified through an enzymatic kit (*K-AMIAR, Megazyme,* Bray, Ireland).

Growth performance under different limiting conditions (pH, ethanol and SO_2)

LAB strains were subjected to different growth conditions usually found in wine environment. MRS broth (De Man, Rogosa & Sharpe, 1960) was used as base culture medium. Different pH values, ethanol and SO_2 concentrations were implemented in the base broth. Sterile 96-well polystyrene microtiter plates were fulfilled with 300 μL of each culture media and subsequently, fresh MRS cultures in late exponential growth phase were inoculated at $1x10^7$ CFU/ml. Finally, plates were maintained at 30 ºC without shaking. Growth was monitored during 14 days by measuring optical density at 600 nm wavelength, using a Bioscreen C^{TM} Microbiological Growth Analyzer (Labsystems, Helsinki, Finland).

Effect of pH was analyzed by measuring growth in the range from 3.4 to 3.8, in 0.1 units steps. The pH value was adjusted with HCl 6M. Ethanol tolerance was evaluated in MRS broth (pH 3.6) supplemented with 10, 12 and 14% (v/v) ethanol. Finally, sulfite tolerance analysis was evaluated by growing each strain in MRS broth (pH 3.6) supplemented with 10, 30, 50, 75 and 100 ppm potassium metabisulfite. Total and free SO_2 were measured by titration following the Ripper method (OIV, 2009). Controls were carried out by comparing cultures that only differed in pH (set at 4.6). All assays were performed in duplicate.

Vigorousity of malolactic fermentation (MLF) in synthetic wine

Before performing MLF in synthetic wine, fresh MRS cultures in late exponential growth phase, grown at pH 4.6, were transferred to same volume of an acclimation medium (50 g/L MRS, 40 g/L D(-)-fructose, 20 g/L D(-)-glucose, 4 g/L L(-)-malic acid, 1 g/L Tween 80 and 10% v/v ethanol, pH 4.6) and incubated at 25 ºC for 48 h. After acclimation, bacterial cells were harvested by centrifugation at 10000 x g for 10 min, resuspended in sterile water and inoculated in 40 ml of modified synthetic wine (13.5% ethanol, pH 3.5) (Ugliano et al., 2003) to reach a final concentration of $1x10^7$ CFU/ml. Strains were incubated at 25 ºC for 40 days. L-malic acid evolution was monitored every five days using an enzymatic kit (K-LMALQR, *Megazyme,* Bray, Ireland) and bacterial growth was evaluated every week by plating on MRS, except *Oenococcus oeni* strains*,* that were plated on MRS supplemented with apple juice (20% v/v). These analyses were carried out in duplicate.

3.1.4.2. Sensorial characterization

Phenotypical characterization of citrate fermenting strains

Strains previously grown in MRS broth were spot inoculated in KMK agar medium (Kempler and McKay 1980) which allows the differentiation between citrate-fermenting and non-fermenting strains. Plates were incubated at 30 ºC for 5 days. The appearance of blue colonies indicated citrate consumption. Phenotypical assays were performed in triplicate.

Multi-enzymatic analysis

Oenococcus oeni strains were exclusively used for the analysis of different enzymatic activities using the API®-ZYM galleries (BioMerieux, Montalieu-Vercieu, France). This semiquantitative analysis allows the rapid study of 19 enzymatic reactions. Results

were recorded following manufacturer´s instructions, in that way a color change in each reaction was classified as positive, weak or negative result.

Quantification of glycosidase activities under winemaking conditions

Four glycosidase activities (α-glucosidase, β-glucosidase, β-xylosidase and α-arabinosidase) were analyzed following the method described by Grimaldi et al., (2005) with some modifications. The assays were performed in McIlvane buffer (0.1M citric acid and 0.2M K_2HPO_4) at different pH (3.4, 3.6, 3.8) and ethanol concentration (0, 10, 12, 14%) combinations. p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-xylopyranoside and p-nitrophenyl-α-L-arabinofuranoside were used as substrate for each reaction.

Sterile 96-well polystyrene microtiter plates were fulfilled with 40 μL of the corresponding buffer and 20 μL of bacterial suspension (previously grown on MRS at 30 ºC until late exponential growth phase, harvested by centrifugation at 10000 x g for 10 min, and resuspended in 145 mM NaCl) to reach a final concentration of $1x10^7$ CFU/ml. Substrate solutions (20 μL) were added to reach the following final concentrations: p-nitrophenyl-α-D-glucopyranoside (10 mM), p-nitrophenyl-β-D-glucopyranoside (10 mM), p-nitrophenyl-β-D-xylopyranoside (7.5 mM) and p-nitrophenyl-α-L-arabinofuranoside (7.5 mM). Control assays were carried out in McIlvane buffer (0.1M citric acid and 0.2M K_2HPO_4; pH 5). Blank samples were treated in the same way but they were not inoculated. Assays were incubated at 37 ºC during 1 h, then reaction was stopped by adding 160 μL of 0,5 M Na_2CO_3 and microplates were centrifuged (2500 x g, 18 min). Supernatants were transferred into another 96-well plate and the absorbance was determined at 400 nm with a Varioskan Flash

spectrophotometer (Thermo Scientific, Barcelona, Spain). From these measurements, the concentration of released p-nitrophenol (p-NP) was determined from a p-NP calibration curve. Enzyme activity was expressed as nanomole of released p-NP per min per mg of cell (dry weight). Culture dry weight was obtained from 15 mL cultures which had been grown for 48 h. Assays were performed in duplicate.

Quantification of esterase activity under winemaking conditions

Esterase activity determination was based on the enzymatic split down of p-NP-substrates, as described for glycosidase activities, with some modifications. The assays were performed in McIlvane buffer (0.1M citric acid and 0.2M K_2HPO_4) at different pH values (3.4, 3.6, 3.8) and ethanol concentrations (0, 10, 12, 14%). Both p-nitrophenyl acetate (C2) and p-nitrophenyl octanoate (C8) were used as substrates for each reaction.

Sterile 96-well polystyrene microtiter plates were fulfilled with 215 μL of the corresponding buffer and 25 μL of bacterial suspension (previously grown on MRS at30 ºC until late exponential growth phase were reached, harvested by centrifugation at 10000 x g for 10 min, and resuspended in 145 mM NaCl) to reach a final concentration of $1x10^7$ CFU/ml. Substrate solutions (10 μL) were added to reach a final concentration of 1mM for both substrates. Control and blank samples were prepared as previously described. Assays were incubated at 37 ºC during 2 h, then reaction was stopped by adding 75 μL of 0,5 M Na_2CO_3 and microplates were centrifuged (2500 x g, 18 min). Samples were treated and measurements were performed as previously described. Assays were performed in triplicate.

3.1.4.3. Molecular characterization of LAB strains

Multiplex PCR for detection of biogenic amine-forming LAB

Multiplex PCR was performed to detect simultaneously the presence of four genes, histamine decarboxylase (*hdc*), tyramine decarboxylase (*tyrdc*), ornithine decarboxylase (*odc*) and agmatine deiminase (*agdi*). *Lactobacillus brevis IOEB 9809* strain (*tyrdc* and *agdi* positive), *Lactobacillus reuteri* CECT 925 (*hdc* positive) and *Lactobacillus* 30a ATCC 33222 (*hdc* and *odc* positive) were used as positive standards. PCR was carried out following the method described by Coton *et al*. (2010), with some modifications. Experiments were carried out with 1,25 U of Ex Taq DNA Polymerase (Takara Clontech). Primers used are listed in **Table 7** and amplification program is shown in **Table 8**. PCR products were visualized on a 0.8% agarose gel with 1X TAE (40 mM Tris-acetate-EDTA buffer, pH 8) buffer, and photographed under UV light using a BioDoc-It™ Imaging System.

Molecular characterization of citrate fermenting strains

LAB strains were analyzed for genes encoding citrate permease (*maeP*) and citrate lyase complex (*citF*, *citE* and *citD*). PCR reactions were performed following the method described by Mtshali et al., (2010) with some modifications. Primers used are listed in **Table 7** and amplification program is shown in **Table 8**. A Master mix was used throughout the study (Taq DNA polymerase Master mix red 2X, Ampliqon A/S). PCR were run in an Eppendorf® Mastercycler (Eppendorf). Amplified products were analyzed by agarose gel electrophoresis, run on 0,8% agarose gels with 1X TAE buffer (40 mM Tris-acetate-EDTA buffer, pH 8), and photographed under UV light using a BioDoc-ItTM Imaging System (Biorad, Marnes La Coquette, France).

Amplification of phenolic acid decarboxylase (pad) gene

The presence of phenolic acid decarboxylase (*pad*) gene in *O. oeni* strains was analysed following the method described by Mtshali et al. (2010) with slight modifications. Primer used is listed in **Table 7** and amplification program is shown in **Table 8.** A Master mix was used throughout the study (Taq DNA polymerase Master mix red 2X, Ampliqon A/S). PCR were run in an Eppendorf® Mastercycler (Eppendorf). Finally, PCR products were analysed and phoyographed as described above.

Table 7. Primers used in the present study.

Name	Target gene	Sequence (5'- 3')	Product (bp)	Reference
27FC	16S rDNA	[b]F-AGTTTGATCCTGGCTCAG	1500	Tanasupawat *et al*., 2000
PUBr	16S rDNA	R-CCCGGGAACGTATTCAC	1500	Internal primer, unpublished
Hdc3	Histidine decarboxylase *(hdc)*	F-GATGGTATTGTTTCKTATGA	440	Coton *et al*., 2010
Hdc4	*hdc*	R-CCAAACACCAGCATCTTC	440	Coton *et al*., 2010
Td2	Tyrosine decarboxylase *(tdc)*	F-ACATAGTCAACCATRTTGAA	1100	Coton *et al*., 2010
Td5	*tdc*	R-CAAATGGAAGAAGAAGTAGG	1100	Coton *et al*., 2010
Odc1	Ornithine decarboxylase *(odc)*	F-NCAYAARCAACAAGYNGG	900	Coton *et al*., 2010
Odc2	*odc*	R-GRTANGGNTNNGCACCTTC	900	Coton *et al*., 2010
AgD1	Agmatine deiminase *(agdi)*	F-CAYGTNGAYGGHSAAGG	600	Coton *et al*., 2010
AgD2	*agdi*	R-TGTTGNGTRATRCAGTGAAT	600	Coton *et al*., 2010
BSF8	16S rRNA (internal control)[a]	F-AGAGTTTGATCCTGGCTCAG	1500	Edwards *et al*., 1989
BSR1541	16S rRNA (internal control)	R-AAGGAGGTGATCCAGCCGCA	1500	Edwards *et al*., 1989
M13	unspecific	F-GAGGGTGGCGGTTCT	unspecific	Zapparoli *et al*. 2000
1254	unspecific	R-CCGCAGCCAA	unspecific	Akopyanz *et al*. 1992
maeP-f	Citrate permease (*maeP*)	F-ATGGGTGTTTTTTGGACATCG	984	Mtshali et al., 2011
maeP-r	*maeP*	R-TCAAATAAAGTTGATGATACTCATTA	984	Mtshali et al., 2011
citD-f	Citrate lyase γ-subunit (*CitD*)	F-ATGGAAATTAARAMAACKGCAKTMGC	245	Mtshali et al., 2010
citD-r	*CitD*	R-GCYGCYGTAATRGTYGKYGCYTTWAT	245	Mtshali et al., 2010
citF-a	Citrate lyase α-subunit (*CitF*)	F-ATGGYATGACRATTTCWTTYCAYCAYCA	1331	Mtshali et al., 2010
citF-b	*CitF*	R-ATCAATVAHBSWRCCRTCRCGRTAYTC	1331	Mtshali et al., 2010
citE-1	Citrate lyase β-subunit (*CitE*)	F-TTACGBCGSACRATGATGTTTGT	897	Mtshali et al., 2010
citE-2	*CitE*	R-TATTTTTCAATGTAATTDCCCTCC	897	Mtshali et al., 2010
pad-1	Phenolic acid decarboxylase (*pad*)	F-AARAAYGAYCAYACYRTTGATTACC	210	Mtshali et al., 2010
pad-3	*pad*	R-TTCTTCWACCCAYTTHGGGAAGAA	210	Mtshali et al., 2010

[a]Internal control used in multiplex PCR
[b]F (forward); R (reverse)

Table 8. PCR conditions.

Target gene	Initial denaturing	Cycles	Denaturing	Annealing	Extension	Final extension
16S rRNA	95°C, 5 min	35	95°C, 1 min	53°C, 1 min 30 s	72°C, 1 min 30 s	72°C, 5 min
hdc	95°C, 5 min	35	95°C, 1 min	53°C, 1 min 30 s	72°C, 1 min 30 s	72°C, 5 min
tdc	95°C, 5 min	35	95°C, 1 min	53°C, 1 min 30 s	72°C, 1 min 30 s	72°C, 5 min
odc	95°C, 5 min	35	95°C, 1 min	53°C, 1 min 30 s	72°C, 1 min 30 s	72°C, 5 min
agdi	95°C, 5 min	35	95°C, 1 min	53°C, 1 min 30 s	72°C, 1 min 30 s	72°C, 5 min
maeP	95°C, 5 min	35	95°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
citD	95°C, 5 min	35	95°C, 45 s	54°C, 30 s	72°C, 1 min	72°C, 5 min
citE	95°C, 5 min	35	95°C, 30 s	54°C, 1 min	72°C, 1 min	72°C, 10 min
citF	95°C, 5 min	35	95°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
pad	95°C, 5 min	35	95°C, 40 s	50°C, 1 min	72°C, 30 s	72°C, 5 min

3.1.5. Vinification assays

3.1.5.1. Lab-scale microvinifications

Oenococcus strains P2A, P3A, P3G, P5A, P5C, P7B and the commercial strain Viniflora OENOS were used for microvinification assays. The must used in the vinification was obtained from Tempranillo grape variety from Rioja Alavesa region and belonged to the 2018 vintage. Must chemical characteristics were the following: pH 3,5, L-malic acid 3,17 g/L, L-lactic acid <0,1 g/L, total acidity 5,81 g/L tartaric acid, volatile acidity <0,1 g/L acetic acid and reducing sugars 229,75 g/L. The chemical analysis of must and wine after AF and MLFs were performed following the EC Official Methods (1999).

Alcoholic fermentation was conducted with the commercial strain Uvaferm VRB® (Lallemand, Blagnac, France), which inoculation was performed following dealer instructions. The process was carried out at 21ºC under constant agitation and reducing sugar content was measured every 2 days with the dinitrosalicylic method (Miller, 1959) until sugar content was less than 2g/L. The batch was then separated from the lees, filter-sterilized (0,22 µm, PVPF filter, Millipore) and divided in 100 ml batches. MLFs were carried out with the six selected strains and the commercial strain. Also, spontaneous fermentation (which was not filter-sterilized) was studied. Each MLF was performed in duplicate. Before performing MLF, fresh cultures in late exponential growth phase grown in MRS broth (De Man, Rogosa & Sharpe, 1960) at pH 4,6, were harvested after centrifugation at 10000 x g for 10 min. Then, they were resuspended in the same volume of an acclimation medium (50 g/L MRS, 40 g/L D(-)-fructose, 20 g/L D(-)-glucose, 4 g/L L(-)-malic acid, 1 g/L Tween 80 and 10% v/v ethanol; pH 4,6) and incubated at 25 ºC for 48 h. After acclimation, bacterial cells were harvested by centrifugation at 10000 x g for 10 min, resuspended in sterile water and

inoculated in 100 ml of wine to reach a final concentration of $3x10^7$ CFU/ml. Fermentations were performed at 21ºC without shaking. Fermentation evolution was periodically monitored through L-malic and L-lactic acid quantification through enzymatic kits (*Megazyme,* Bray, Ireland). Cell viability was evaluated every week by plating on MRS agar supplemented with apple juice (20% v/v). When malic acid content was less than 0,2 mg/L, wines were separated from cell debris and stabilized by adding SO_2 at a final concentration of 30 mg/L. They were kept under refrigeration temperature (<4ºC) until subsequent analysis were carried out.

3.1.5.2. Lab-scale vinifications (co-inoculation vs sequential inoculation) and strains implantation ability

O. oeni strains P2A, P3A, P3G, P7B and the commercial strain Viniflora OENOS were used for vinifications assays. Grapes of Tempranillo variety from Rioja Alavesa region belonging to the 2019 vintage were used. Grapes were manually crushed, and potassium metabisulphite was added to reach a final concentration of 50 mg/L free SO2. Yeast-assimilable nitrogen (YAN) (200mg/L) and total acidity (4,5 g/L), were adjusted through yeast extract and tartaric acid supplementation. The obtained must showed the following chemical characteristics: pH 3,45, L-malic acid 2,71 g/L, L-lactic acid <0,1 g/L, total acidity 4,5 g/L tartaric acid, volatile acidity <0,1 g/L acetic acid and reducing sugars 190,76 g/L. The chemical analysis of must and wine after AF and MLFs were performed following the EC Official Methods (1999).

A scheme of the followed winemaking processes is shown in **Figure 12**. Must was submitted to cold premaceration at 5ºC during 24 h. Then, must and grape skins were equally divided in ten batches of 1 L for co-inoculation performance. The remaining volume of must and skins (around 12 litres), which were used for sequential

inoculation strategy, were kept in the same fermentation vessel. After batch division, AF was induced through commercial yeast strain Uvaferm VRB® (Lallemand, Blagnac, France) which was inoculated following dealer instructions. The process was carried out at 21ºC and reducing sugar content was measured every 2 days with the dinitrosalicylic method (Miller, 1959) until sugar content was less than 2g/L. When one third of AF was performed, 10mg/L of yeast-extract were supplemented for a correct fermentation kinetic. Maceration with skins was performed for 7 days, skins were mixed daily twice with must with punch-down method. Then, skins and must were separated and skins were manually pressed.

Before performing MLF, fresh cultures in late exponential growth phase grown in MRS broth (De Man, Rogosa & Sharpe, 1960) at pH 4,6, were harvested after centrifugation at 10000 x g for 10 min and resuspended in the same volume of an acclimation medium (50 g/L MRS, 40 g/L D(-)-fructose, 20 g/L D(-)-glucose, 4 g/L L(-)-malic acid, 1 g/L Tween 80 and 10% v/v ethanol; pH 4,6) and incubated at 25 ºC for 48 h. After acclimation, bacterial cells were harvested by centrifugation at 10000 x g for 10 min, resuspended in sterile water and inoculated in 1L of wine to reach a final concentration of $1x10^7$ CFU/ml. Fermentations were performed at 21ºC. For co-inoculation strategy, strains were inoculated after 24 h of yeast inoculation. Sequential inoculation was performed once AF had concluded. Before sequential inoculation, wine was divided in 12 batches of 1 L (5 strains plus spontaneous fermentation, in duplicate). Fermentation evolution was periodically monitored through L-malic and L-lactic acid quantification through enzymatic kits (*Megazyme,* Bray, Ireland). Cell viability was evaluated every week by plating on MRS agar supplemented with apple juice (20% v/v). When malic acid content was exhausted,

wines were separated from cell debris and stabilized by adding SO_2 at a final concentration of 30 mg/L. They were kept under refrigeration temperature (<4ºC) for 7-14 days to allow debris to precipitate. Finally, they were racked, SO2 concentration was adjusted and they were bottled. Bottles were kept at 14ºC until subsequent analysis were carried out.

To confirm the implantation capacity of each strains during the winemaking process RAPD-PCR analysis was carried out. From each of the periodically performed bacterial platings (beginning, middle and end of MLF and after bottling), 10 colonies for each strain were randomly selected. The colony was picked and suspended in 20 μL of sterile milli-Q water (Millipore). From these suspensions, 1 μL was directly used for PCR assays. Primer M13 was used following the method described by Zapparoli et al. (2000). RAPD-PCR reaction was performed with an Eppendorf® Mastercycler. PCR products were separated by gel electrophoresis on 1,5% agarose gel with 1X TAE buffer and photographed as earlier described. After RAPD-PCR each strain showed its own amplicon pattern. From these amplicon patterns a binary matrix was created based on the presence/absence of each pattern bands. Genetic diversity among each possible pair of the sampling group was calculated following the method described by Nei and Li (1979). A dendrogram was built applying the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) (Vauterin & Vauterin, 1992) with Phylodendron (Department of Biology, University of Indiana, USA) software.

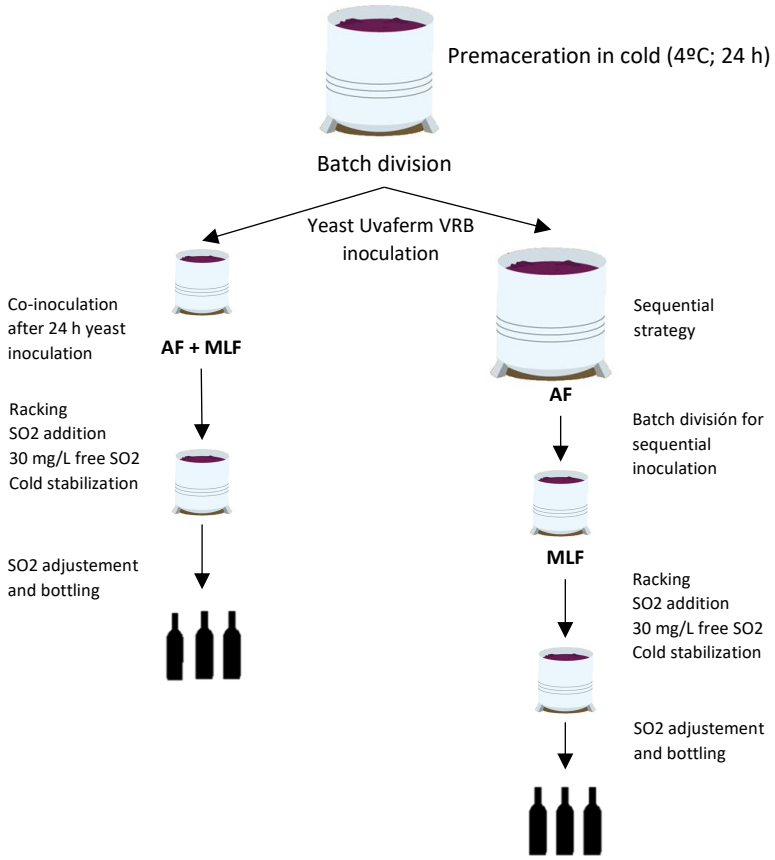


Figure 12. Scheme of the followed winemaking processes.

3.1.5.3. Pilot test in the winery

In winery three batches of 100 L of Tempranillo grape must were fermented in duplicate. First batch was inoculated with P2A strain through co-inoculation (bacteria was inoculated 24 h after yeast inoculation). Second batch was sequentially inoculated with P2A when AF had concluded, and the third batch followed MLF spontaneously. The obtained must showed the following chemical characteristics: pH 3,55, L-malic acid 4,34 g/L, L-lactic acid <0,1 g/L, total acidity 5,21 g/L tartaric acid, volatile acidity <0,1 g/L acetic acid and reducing sugars 224,94 g/L. Winemaking practices were

followed according to winery decisions (maceration, devatting, racking, stabilization, etc).

Culture of P2A strain was prepared in modified OPM broth medium (Berbegal et al., 2015) (550 mL/L white grape must, 400 m/L white wine, 23 mL/L apple juice, 5 g/L yeast extract, 3,5 g/L L-malic acid, 1 g/L Tween 80; adjusted to 6% v/v ethanol and pH 3,7) from 10 mL to 1 L in two-stage scale up process. This inoculum was inoculated to 100 L of wine to obtain a final density of 1 x 10^7 CFU/ml. Wine samples were taken for microbial and physicochemical analysis through all the fermentation process (24 h after inoculation, middle MLF and once MLF had finished). To elucidate implantation rate, at every sampled stage ten colonies were randomly selected, and corresponding RAPD-PCR analysis were performed. When MLF was concluded, wines were stabilized and finally bottled. Bottles remained at 14ºC for subsequent analysis.

3.1.6. Wine chemical analysis

3.1.6.1. Quantification of biogenic amines and amino acids by RP-HPLC

Biogenic amine analysis was performed by reverse-phase high-performance liquid chromatography (RP-HPLC), following the method described by the OIV (OIV, 2009) with slight modifications. RP-HPLC was performed using an Agilent 1200 Series chromatograph (Agilent Technologies, Madrid, Spain) equipped with an ALS autosampler (Agilent 1200 Series) and a G1321A fluorometric detector (Agilent 1200 Series). A NovaPak® C18 (4.6 x 250 mm, i.d. 4 μm) (Waters; Milford, MA, USA) column was used.

Briefly, samples were submitted to automatic precolumn derivatization with o-phthaldialdehyde (OPA). 10 μl of OPA were mixed automatically with 10 μl of sample.

A total amount of 10 µl of derivatized sample was injected at a constant temperature of 35ºC. Mobile phases were 25 mM potassium phosphate and acetonitrile (ACN). The gradient profile was as follows: 0-10 min, from 20 to 30% ACN; 10-15 min, from 30 to 40% ACN; 15-20 min, from 40 to 50% ACN; 20-27 min, from 50 to 65% ACN; 27-32 min, 65% ACN, 32-38 min, from 65 to 20% ACN. Identification of compounds was performed by comparison of their retention times with those of pure standards, and quantification was carried out by calibration, using external standards.

For amino acids analysis the method described by López et al. (2012), was followed with some modifications. The chromatographic system was the same as described above. The analyzed amino acids were histidine, tyrosine, arginine, ornithine and lysine. Each sample was derivatized as previously described. Mobile phases were 75mM sodium acetate, 0,018% triethylamine (pH 6,9) and 0,3% tetrahydrofuran (phase A); and water, acetonitrile and methanol (20:40:40, v/v/v) (phase B). The gradient was as follows: 0-16 min, from 90% to 65% phase A; 16-20 min, from 65% to 50% phase A; 20-30 min, from 50 to 40% phase A; 30-33 min, from 40 to 0% phase A; 33-34 min, 0% phase A, 34-35 min, 100% phase A). Compounds were identified by comparison of their retention times with those of pure standards, and quantification was carried out by calibration, using external standards.

3.1.6.2. Hydroxycinnamic acids analysis through RP-HPLC

For hydroxycinnamic acid (HCA) analysis the method described by Cabrita et al. (2008) was followed with some modifications. The chromatographic system was the same as previously described. Mobile phases were water:acetic acid (98:2 v/v) (A) and water:methanol:acetic acid (68:30:2 v/v). The gradient was as follows: 0-12 min, from 95% to 70% phase A ; 12-27 min, from 70% to 45% phase A; 27-33 min, from 45% to

23% phase A; 33-42 min 23% phase A; 42-47 min, from 23% to 5% phase A; 47-50 min, from 5% to 0% phase A; 50-55 min, 0 % phase A; 55-58 min, from 0% to 95% phase A. Flow was set at 1 mL/min. Wines were sampled in 20 μL volume and HCAs were detected at 320, 305 and 285 nm wavelengths. Compounds were identified according to the UV-Vis spectra and retention time of pure standards. Quantification was performed by calibration using external standards.

3.1.6.3. Citric acid quantification through RP-HPLC

For citric acid analysis the method described by Scherer et al. (2012) was followed. The chromatographic system was the same as described above. As mobile phase 10mM KH_2PO_4 (pH 2,6; adjusted with phosphoric acid) was used. The analysis was performed isocratically and the flow was set at 0,5 mL/min. Wines were sampled in 20 μL volume and citric acid were detected at 210 nm wavelength. Citric acid was identified according to the UV-Vis spectra and retention time of pure compound. Quantification was performed by calibration using external standards.

3.1.6.4. Aromatic compounds analysis through HS-SPME/GC-MS

Headspace solid phase microextraction (HS-SPME) was used for volatile compounds extraction. GC/MS was performed using an Agilent 7890A chromatograph (Agilent Technologies, Madrid, Spain) equipped with an Agilent 5975C inert MSD Triple-Axis Detector. Briefly, 10 ml of wine were place in a 20 ml headspace vial, together with the addition of 200 μl of 3,4-dimethylphenol internal standard (100 mg/L) and 3 g of NaCl. The extraction procedure was performed with a 2 cm CAR/DVB/PDMS 50/30 μm fibre (Sigma-Aldrich, Schnelldorf, Germany). Samples were pre-heated at 40ºC during 5 min with agitation at 500 rpm. Then, the fibre was inserted into the headspace for 30 min at 40ºC under agitation. Finally, the fibre was desorbed in the injector at 250ºC

during 10 min. Injections were carried out in splitless mode, using a 0,75 mm I.D. liner (Sigma-Aldrich, Schnelldorf, Germany).

For separation, a DB-WAX/UI (Agilent J&W, Folsom, CA) capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) was used with helium as carrier gas at 1 ml/min flow rate. Oven temperature was initially programmed at 40ºC for 5 min, then it was increased at 4ºC/min to 240ºC and maintained for 15 min. For the MS system, the temperatures of the transfer line, quadrupole and ionization source were 265, 150 and 230 °C respectively; electron impact mass spectra were recorded at 70 Ev. Acquisitions were carried out in scan mode, from 35 to 350 m/z. Peak identification was performed by comparison of mass spectra with those of the mass library (NIST 2.0), and with those from reference pure compounds. Quantitative data were obtained by calculating the relative peak area in relation to that of the internal standard. All analyses were performed in duplicate (one injection per sample vial). Analysed compounds and their retention times are displayed in **table 9**.

3.1.7. Sensorial analysis of wines

Wine samples were analysed by orthonasal evaluation to compare the different aromatic profiles obtained through the different bacteria inoculation strategies. Two replicates of samples of each wine were sensory analysed. The samples were presented randomly. This test was carried out in accordance with international standards. A list of five descriptors (ripe fruit, red fruit, vegetable/herbal, floral and dairy) were selected in order to simplify the analysis. Aromatic references for each attribute were designed following the method described by Etaio et al. (2007) with some modifications (**Table 10**). Before each sensorial session, these references were

presented together with wine samples. Panellists evaluated the presence/absence of these five attributes in each wine. The panel was composed by 20 panellists, where 56% were women. All the sessions were performed in a room equipped with individual booths with normalised glasses for wine sensorial analysis.

3.1.8. Statistical analysis

The statistical analysis was carried out using the Statgraphics® Centurion XVI program (StatPoint Technologies, Inc., Virginia, USA). For non-parametric data, the Mann-Whitney U-test, Kruskal-Wallis and Spearman´s correlation analysis were used. For parametric data, significant differences were evaluated with one-way ANOVA followed by Student-Newmans-Keuls test. Level of significance for all the statistical analysis was established as $p < 0,05$. Principal component analysis (PCA) was also performed to generate a comprehensible overview of possible correlations between aromatic compounds and *O. oeni* strains as well as to correlate aromatic compounds with sensorial attributes.

Table 9. Retention times of the identified compounds as well as their aroma description and odour threshold are displayed.

Retention time (t_R)	Compounds	Odour description	Odour threshold (mg/L)[1]
2,514	1-Ethoxy-1-methoxyethane	Liquorice, solvent	-
2,924	Ethyl acetate	Nail polish, fruity	12[d]
3,006	1,1,-Diethoxyethane	Liquorice, nutty, wood	1[o]
7,699	Hexanal	Green, grass	0,02[h]
8,891	Isobutanol	Fusel, spirituous	40[b]
9,177	Isoamyl acetate	Banana, fruity, sweet	0,03[e]
13,124	3-Methylbutanol	Whiskey, malt, nail polish	30[j]
13,373	Ethyl hexanoate	Green apple, pineapple	0,014[g]
14,801	Hexyl acetate	Pear, pineapple	0,67[d]
17,399	Ethyl lactate	Fruity, milky	154[f]
17,889	1-Hexanol	Green, grass	8[c]
18,857	3-Hexen-ol	Fresh grass	0,4[b]
20,396	Ethyl octanoate	Waxy, fruity, pear	0,58[d]
20,874	Acetic acid	Vinegar	280[e]
22,236	Methyl nonanoate	Sweet, fruity	-
22,937	Benzaldehyde	Almond, flagrant	2[d]
23,602	Ethyl nonanoate	Waxy, fruity	1,3[c]
23,846	2,3-Butanediol	Buttery	120[a]
24,057	Linalool	Floral, citrus	0,025[b]
24,401	1-Octanol	Sweet, floral	0,9[a]
24,553	Isobutyric acid	Buttery	2,3[b]
24,618	Isoamyl lactate	Fruity, milky	-
26,041	γ-Butyrolactone	Buttery, caramel, sweet	20[d]
26,309	Butanoic acid	Cheesy, rancid	0,173[h]
26,494	Phenylacetaldehyde	Floral, honey, sweet	0,001[i]
26,677	Ethyl decanoate	Waxy, fruity, grape	0,2[g]
27,543	3-Methyl butryric acid	Cheesy, rancid	0,033[b]
27,758	Diethyl succinate	Fruity	200[f]
28,146	Ethyl 9-decenoate	Waxy, fruity	0,1[a]
28,346	α-Terpineol	Pine-like, floral	0,25[m]
28,883	Methionol	Cauliflower	0,5[e]
30,336	Citronellol	Citrus, citronella	0,1[h]
31,468	Phenylethyl acetate	Flowery, rose, fruity	0,073[k]
31,593	β-Damascenone	Cooked apple, honey	0,00005[c]
32,328	Hexanoic acid	Fatty, cheese	0,42[b]

Table 9 continuation

Retention time (t_R)	Compounds	Odour description	Odour threshold (mg/L)[1]
32,498	Geraniol	Rose	0,02[b]
32,777	Isoamyl decanoate	Waxy, fruity	-
33,128	Benzyl alcohol	Almond-like	200[a]
34,043	2-Phenylethanol	Floral, rose, green	14[b]
37,275	Nerolidol	Rose-like, sweet, citrus	0,7[c]
37,686	Octanoic acid	Waxy, fatty, cheesy	10[d]
40,172	Nonanoic acid	Waxy, fatty, cheesy	3[l]
40,326	4-ethyl phenol	Barnyard, medicinal	0,44[i]
42,55	Decanoic acid	Leather, fatty, rancid	1[b]
43,445	Phenol, 2,4-tertbutyl	Phenolic	0,2[n]
44,831	Ethyl hydrogen succinate	Fruity	-
45,718	Benzoic acid	Balsamic	1[i]
47,003	Dodecanoic acid	Dry, metallic	1[a]

[1]Letters refer to references from which the odor threshold has been taken. [a]Tao et al. (2010), [b]Ferreira et al. (2000), [c]Peng et al. (2013), [d]Peinado et al. (2004), [e]Swiegers et al. (2005), [g]Kotseridis et al. (2000), [i]Campo et al. (2006), [j]Guth (1997), [k]Tat et al. (2007), [l]Fan et al. (2010), [m]Wang et al. (2017), [n]Gomez et al. (2007), [o]Moreno et al. (2005). These references calculated the corresponding thresholds in 10-14% v/v ethanol and pH 3,2-3,5 solutions. [f]Pineau et al. (2009) and [h]Etievant (1991) reported their odor thresholds in red wine.

Table 10. Construction of aromatic references. As base wine standard commercial wine (Don Simón) was used. * These aromas belong to the aroma kit set of Sosa Ingredients® (Barcelona, Spain)

Aroma	Mother solution (MS)	Reference preparation
Ripe fruit	150 μL of butyl acetate in a final volume of 15 mL of absolute ethanol	Add 90 μL of MS to 25 mL of base wine (BW)
Red fruit	25 μL of raspberry* aroma and 125 μl of blackberry aroma* in 15 mL of ethanol	Add 40 μl of MS to 25 mL of BW
Floral	150 μL of linalool and 150 μL of geraniol in 15 mL of ethanol	Add 15 μL of MS to 25 mL of BW
Dairy	150 μL of diacetyl in 15 mL of ethanol	Add 60 μL of MS to 25 mL of BW
Herbaceous	150 μL of herbal* aroma in 15 mL of ethanol	Add 40 μL of MS to 25 mL of BW

3.2. Experimental Design

Once the material and methods used throughout the whole work was explained, for each study the following experimental design was carried out:

Study 1: Current situation of biogenic amines in Rioja Alavesa red wines

It was analysed the current situation of BAs levels on commercial red wines from Rioja Alavesa region. It was determined the concentration of histamine, tyramine, putrescine and cadaverine in 70 wines through reverse-phase high performance liquid chromatography (RP-HPLC). Differences between wines submitted to different ageing time (young, "crianza" and "reserve" wines) as well as potential correlations among the different BAs were also analysed.

Study 2: Ecology of indigenous lactic acid bacteria from Rioja Alavesa red wines, focusing on biogenic amine production ability.

A collection of near 300 presumptive LAB isolates was isolated from must and wine samples belonging to different wineries from Rioja Alavesa. After bacterial identification by 16S rDNA sequencing, genetic diversity was analysed through RAPD-PCR (Random Amplification of Polymorphic DNA-PCR) method. The ability of LAB and non-LAB strains to produce BAs was analysed by both molecular and phenotypical analysis. A multiplex PCR was performed to elucidate the presence of the genes coding for the enzymes responsible for BA production. Furthermore, biogenic amines and precursor amino acids quantification through RP-HPLC was also performed in must and wine samples from which isolations had been carried out.

Study 3: Technological characterization of potential malolactic starters from Rioja Alavesa winemaking region.

The collection of 22 LAB strains isolated in the second study was technologically characterized. Their resistance and growth performance under different winemaking conditions of pH (from 3,4 to 3,8), ethanol (from 0 to 14% v/v) and SO_2 (from 5 to 50 ppm) as well as their fermentation vigour in synthetic wine formulation were analysed. In addition, further characterization of *Oenococcus oeni* strains was performed. Citrate fermenting strains were phenotypically characterized and the amplification of the genes coding for citrate permease and citrate lyase complex was also carried out. Furthermore, glycosidase (α-glucosidase, β-glucosidase, β-xylosidase and α-arabinosidase) and esterase activities under different pH (from 3,4 to 3,8) and ethanol concentration (from 0 to 14%) combinations were also quantified.

Study 4: Wine aroma profile modification by *Oenococcus oeni* strains from Rioja Alavesa region: selection of potential malolactic starters.

A group of six *O. oeni* strains selected according to the results obtained in the third study were further characterized. Strains viability and fermentation vigour in lab-scale vinifications were first analysed. The evolution of BAs throughout the winemaking process as well as strains ability to release free hydroxycinnamic acids (HCA) into the medium were studied. Amplification of phenolic acid decarboxylase (*pad*) gene was also evaluated. Furthermore, the aromatic profiles of obtained wines were analysed through headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) method. Fifty compounds comprising ethyl and

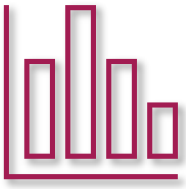
acetate esters, higher alcohols, acids and terpenoids, among others, were quantified along the fermentation process.

Study 5: Effect of inoculation strategy with autochthonous *Oenococcus oeni* strains on aroma development in Rioja Alavesa Tempranillo wines: within the framework of a novel starter selection.

The most promising four *O. oeni* strains, selected from the fourth study according to their better characteristics, were submitted to further analysis. Different inoculation strategies were evaluated in laboratory scale vinifications. In this sense, strains behaviour was assessed by both co-inoculation (bacteria was inoculated 24h after yeast inoculation) and sequential inoculation strategies. The evolution of BAs as well as wine aroma compounds was monitored during the winemaking process. Furthermore, a sensorial analysis was performed to compare the different aromatic profiles obtained through the different inoculation strategies. Potential correlations between aromatic attributes and aroma compounds were also analysed. Finally, the most promising strain was used in a pilot test in winery to confirm the ability to work on large scale fermentations.

4. RESULTS & DISCUSSION

4.1. Study 1



Current situation of biogenic amines in Rioja Alavesa red wines: a technical study

It is widely known that intolerance to certain foods is becoming a mass phenomenon in the industrialized societies of the first world. Likewise, various foods, including wine, can produce adverse effects of different kinds on consumers health. Biogenic amines (BAs), present under certain conditions in wine, have been frequently associated with the generation of adverse reactions on human's health (Capozzi et al., 2017; EFSA, 2011). Indeed, BAs, and specially histamine, are considered the main reason for wine intolerance (Konakovsky et al., 2011). Although they accomplish critical biological functions in living organisms, exogenous BAs can derive in direct toxicity (Ladero et al., 2010). They cause unpleasant symptoms which are enhanced in susceptible individuals lacking or underexpressing the enzymes responsible for BA degradation (EFSA, 2011). Particularly, in wine the main BAs are histamine, tyramine, putrescine and cadaverine (Restuccia et al., 2018), and due to their relevance for the wine sector, both the International Organization of Wine (OIV) and European Food Safety Agency (EFSA) have highlighted the importance of monitoring the concentration of these compounds through all the winemaking process as a parameter to qualify the quality of wines (EFSA, 2011; OIV, 2011).

In Rioja Alavesa, a worldwide recognized wine region, there is a lack of data about the situation of BAs in its wines. In that way, we identified the need to quantify the incidence of these compounds in Rioja Alavesa red wines to meet the real significance of BAs levels in this region, and subsequently, identify potential improvement opportunities. Thus, a total of 70 commercial red wines, belonging to different wine types of the Designation of Origin Rioja (young, "*crianza"* and "reserva" wines), were randomly selected and submitted to BA analysis through RP-HPLC. A list with the analyzed wines and their corresponding BA values are displayed in **Supplementary**

Table 1 (*Annex 1*). In this sense, mean concentrations found for histamine, tyramine, putrescine and cadaverine in the 70 analysed wines are displayed in **Table 11**. Putrescine was the most abundant BA, followed by histamine, tyramine and cadaverine, respectively. These results are consistent with previous studies in which similar tendency was found to occur (García-Villar et al., 2007; Landete et al., 2005; Martuscelli et al., 2013; Žurga et al., 2019).

Table 11. Mean concentration (mg/L) of the analysed BAs. Mean concentrations found in each type of wines are also displayed.

	Histamine	Tyramine	Putrescine	Cadaverine	Total BA
Mean concentration (min-max)	4,60 ± 2,60 (n.d. – 11,94)	3,19 ± 2,51 (n.d. – 9,66)	15,12 ± 8,31 (n.d. – 57,23)	2,19 ± 2,25 (n.d. – 6,71)	25,09 ± 12,36 (5,10 – 84,98)
Young	4,62 ± 2,74 (n.d. – 11,94)	3,71 ± 2,81 (n.d. – 9,66)	16,42 ± 11,69 (4,30 – 57,23)	2,05 ± 1,45 (n.d. – 6,71)	26,81 ± 16,73 (5,34 - 84,98)
Crianza	4,27 ± 2,74 (n.d. – 11,86)	2,63 ± 2,26 (n.d. – 8,42)	13,88 ± 6,20 (4,11 – 32,36)	2,16 ± 1,57 (n.d. – 5,24)	22,94 ± 9,52 (5,1 – 46,34)
Reserva	4,97 ± 2,51 (1,31 – 9,63)	3,33 ± 2,44 (n.d. – 7,88)	15,21 ± 5,91 (8,41 – 29,51)	2,36 ± 1,55 (n.d. – 5,38)	25,87 ± 10,30 (11,78 – 49,95)

In the same way, putrescine was detected in all samples, otherwise, histamine, tyramine and cadaverine were detected in 99%, 88% and 90% of wines, respectively. Putrescine is known to significantly contribute to total BAs content in wine (Del Prete et al., 2009; EFSA, 2011). Indeed, this polyamine, together with cadaverine which is usually found in trace levels, are commonly found in grape and must samples as they accomplish several biological functions in plant and berry development (Broquedis et al., 1989). The 20% of wines showed higher concentration than 20 mg/L of putrescine, actually, above this level putrescine can affect negatively wine sensorial quality (Arena

& Manca da Nadra, 2001). Furthermore, recently it has been also demonstrated, although in a concentration 20-fold higher than that found in wine, the cytotoxic effect of both putrescine and cadaverine, with both amines causing cell necrosis in intestinal epithelium (del Rio et al., 2019). This fact together with their ability to potentiate the toxicity of other amines (as histamine and tyramine) via the competitive inhibition of both DAO and MAO, makes the reduction of this amine essential (del Rio et al., 2019). In addition, above certain levels, those compounds can also cause a depreciation of wine aroma (Ladero et al., 2010; Maintz & Novak, 2007).

Regarding mean histamine level, similar values were observed by Marcobal et al. (2006) when analysing Spanish wines, however, lower concentrations were observed in the extensive study performed by the EFSA over 300 worldwide wines, with a mean concentration of 3,7 mg/L. Lower values were also observed in the studies performed by Martuscelli et al. (2013) and Zurga et al. (2019) in Italian and Croatian wines with a mean concentration of 2,9 and 2,1 mg/L, respectively. Konakovsky et al. (2011), however, after the analysis of 100 high-quality wines found a mean level of 8,5 mg/L and maximum concentrations up to 27 mg/L. Although no legal limits have been stablished for histamine (the legal histamine threshold of 10 mg/L stablished by Switzerland was removed in 2011) different European countries recommend different upper limits for this compound. For instance, Australia and Switzerland recommend an upper limit for histamine of 10 mg/L, 8 mg/L in France, 6 mg/L in Belgium, 3.5 mg/L in Netherlands and 2 mg/L in Germany (Guo et al., 2015). Applying the most restrictive recommendation of 2 mg/L, in the present study 99% of wines were above this threshold, and 40% showed more than 5 mg/L; however, less than 1% showed higher than 10 mg/L of histamine. Currently, histamine levels are only regulated in fish

products but no for wine or other foodstuff, and no limit at all exists for other toxicologic amines, as tyramine. Indeed, histamine and tyramine are the most toxic BAs found in fermented foodstuffs, causing vomiting, palpitation, headache and other symptoms that mimic a food allergy (Erdag et al., 2019). Although any kind of regulation by the corresponding authorities is felt necessary, the limited and contradictory information about the toxicologic levels of these compounds in wine makes this mission difficult. In wine, concentrations between 8 and 20 mg/L for histamine and between 25 and 40 mg/L for tyramine have been considered toxic (Broquedis et al.,1998; Menne et al., 2001), however for sensitive individuals a minimum concentration can be harmful (Comas-Basté et al., 2020). Furthermore, as ethanol may perpetuate the toxicity of these compounds by reducing MAO and DAO activities, the monitoring of these compounds is considered highly relevant for wine industry (Restuccia et al., 2018). In addition, certain gastrointestinal disorders and DAO-inhibiting drugs have been also identified as potential causes of histamine intolerance among population (Comas-Basté et al., 2020; Wöhrl et al., 2004). In this sense, all these factors make establishing a single toxicological level a complex task. In this regard, food labeling of BAs content could be helpful for consumers suffering from wine intolerance.

Wines were also classified depending on the elaboration type, in that way, these wines were differentiated into young wines, *crianza* wines (a minimum of 24 months of ageing, at least 12 of which spent in barrels) and *reserva* wines (a minimum of 36 months of ageing, at least 12 of which spent in barrels). The Kruskal-Wallis statistical test ($p<0.05$) did not report any significant difference between the different types of analyzed wines. No increase in the concentration of any of the amines was observed

for a longer ageing time. In other words, the BAs concentration is little altered once the vinification process has concluded. This suggests that the concentration of BAs in Rioja Alavesa red wines is mainly affected by the health status of the grape or the chemical changes throughout the different fermentation stages, both alcoholic and malolactic fermentations, prior to stabilization, ageing and bottling. Although moderate BAs levels can be found in grapes due to mould inflection (Grossmann et al., 2007), it reasonable to assume that nowadays special care is taken at grape harvest and selection. In the same way, it is widely stablished that BAs production by yeast is negligible in wine, as low or non-production of BAs have been linked with wine yeast and alcoholic fermentation (Henríquez-Aedo et al., 2016; Smit et al., 2013). In that way, and due to an extensive bibliographical support, it is considered that lactic acid bacteria (LAB) and MLF are the main factors that determine BAs accumulation in wine. In this sense, Hernández-Orte et al. (2008) and Izquierdo-Cañas et al. (2008) showed significant increases of BAs after spontaneous MLF. Other authors, although they also observed significant increases after spontaneous MLF, main increases took place after several months of ageing time (Berbegal et al., 2017; López et al., 2012; Polo et al., 2010). In these cases, an incorrect implantation of the starter culture due to the growth of indigenous BA-forming bacteria led to BA accumulation.

It was also analysed possible positive or negative correlations between the different BAs (**Table 12**). Actually, moderate positive correlations were observed between histamine and tyramine, and histamine and putrescine. In the same way, stronger positive correlations were evident between histamine and cadaverine, and tyramine and putrescine. In this sense, the production of any BA entailed the production of nearly the rest. Similar results were observed in previous works (Herbert et al., 2005;

Konakovsky et al., 2011; Meléndez et al., 2016). Thus, the correlation between almost all BAs suggests the same origin, possibly generated during MLF by indigenous LAB (; López et al., 2012; Moreno-Arribas et al., 2003). It must be also stated that, different variabilities, as grape integrity and sanitary status, soil type, agricultural practices (irrigation, fertilization), climatic conditions, maceration time, degree of autolysis, fermentation conditions (pH, temperature, ethanol and SO_2 concentrations), ageing time, etc. will determine the final concentration of BAs in wine (Binner et al., 2013; Martínez-Pinilla et al., 2013; Smit et al., 2013). Many of them will limit the concentration of precursor amino acids, and others, will stablish the microbial load (potentially contaminating or not) that will be present in the fermentation process.

Table 12. Spearman´s correlation test between the different BAs. Statistical level of significance was stablished as p<0,01.

	Histamine	Tyramine	Putrescine	Cadaverine
Histamine		**0,4209**	**0,4011**	**0,6697**
Tyramine	**0,4209**		**0,687**	0,1816
Putrescine	**0,4011**	**0,687**		0,171
Cadaverine	**0,6697**	0,1816	0,171	

Although the situation in Rioja Alavesa is not alarming, it has been observed slightly higher concentrations for all the BAs tested in comparison with other studies. In this way, there is an opportunity for improvement in order to reduce BAs levels to minimum and increase the competitiveness of these wines. In Rioja Alavesa region, most of the production is destined for export, thus, the reduction of histamine levels may suppose an opportunity to stand out in the market as a safe and quality product (Guo et al., 2015). Most of the wineries in this region follow the winemaking process

in a traditional way, in this sense, whereas yeast starter cultures are widely used by the wine industry, malolactic fermentation (MLF) is usually performed spontaneously. Spontaneous MLF may lead to stuck or sluggish process that can be delayed for months, arising the risk of the appearance of BA-forming spoilage microorganisms (Berbegal et al., 2017; Izquierdo-Cañas et al., 2008). Thus, the inoculation of safe malolactic starters may be considered as a possible strategy to minimize or prevent their formation (OIV, 2011; Sumby et al., 2014).

This study showed for the first time an overview of the BAs content in Rioja Alavesa red wines. It was seen that there is room for improvement to reduce the levels of BAs, not only those considered toxicological, as histamine and tyramine, but also special emphasis should be placed on reducing the levels of putrescine, which was detected in excessive levels. Due to the relationship between BA accumulation and MLF, one of the strategies to be pursued is the inoculation of safe autochthonous malolactic cultures, preventing in that way the appearance of spoilage microorganisms and maintaining the regional character of these wines. In this regard, the next step was the evaluation of the ecology of indigenous LAB strains from Rioja Alavesa with the aim of obtaining new malolactic cultures that meet the quality and safety requirements that the wine sector demands for the production of high-quality wines.

4.2. Study 2

Ecology of indigenous lactic acid bacteria from Rioja Alavesa red wines, focusing on biogenic amine production ability.

Indigenous microbiota can be constituted by both beneficial and potentially spoilage bacterial strains which will influence both fermentation and final product safety and quality (Nisitou et al., 2015; Pinto et al., 2015). Selection of strains lacking the ability to promote metabolites of safety concern, as biogenic amines (BA) or ethyl carbamate (EC), has been considered the first step to constitute a collection of LAB strains of potential application in wine industry. In that way, autochthonous strains already adapted to specific winemaking conditions have been suggested in order to minimize or avoid BA formation and improve malolactic fermentation reliability (Patrignani et al., 2011; Smit et al., 2013). To achieve that goal, in this study the ecology of indigenous LAB was screened by both phenotypical and molecular methods for their biogenic amine producing ability with the prospect of determine their oenological potential. In addition, the concentration of precursor amino acids and biogenic amines from which isolates were obtained was also monitored.

4.2.1. Bacterial identification and typification

For this study, a total of 31 samples of Tempranillo wine were collected during the 2016 vintage from two wineries located in the Rioja Alavesa subzone. Samples were taken during all the vinification process: must, tumultuous alcoholic fermentation (AF) (density < 1,075 g/L), end of AF (reducing sugars < 2 g/L), beginning of malolactic fermentation (MLF) (when 10% of the initial malic acid is consumed), tumultuous MLF (60% of the initial malic acid is consumed) and the end of MLF (malic acid content < 0,5 g/L).

In that way, microbial isolations were carried out throughout all winemaking process and after Gram, catalase and oxidase characterization, a total of 295 presumptive LAB

colonies were isolated, purified and subjected to 16S rDNA sequencing. **Table 13** displays the percentage of species identified in each winery at different stages of the fermentation process. Differences between wineries were clearly noticeable since *Oenococcus oeni* became the sole species isolated in winery B. Winery A showed a typical evolution of LAB species through the winemaking process. Common grape and must LAB, as *Lactobacillus plantarum*, *Lactobacillus mali* and *Pediococcus parvulus* (Godálová et al., 2016), were present in first stages of fermentation; however, their presence decreased once MLF started, allowing the rising of more adapted species like *Lactobacillus hilgardii* and *Oenococcus oeni*. Thus, *O. oeni* became the leading species responsible for conducting spontaneous MLF in both wineries, highlighting its major adaptation to wine strict conditions (Ruiz et al., 2008). The identification and characterization of LAB strains involve in MLF is considered of utmost importance, since MLF not only leads the biological deacidication of wine, but it also contributes to a higher microbial stability and increases sensorial complexity of wine throughout the secondary bacterial metabolism (Berbegal et al., 2017; Ribéreau-Gayon et al., 2006).

Besides LAB, species of *Staphylococcus* and *Paenibacillus* were also identified in winery A during MLF. These species, which were spread in a lesser extent, are rarely found in wine environment, although *S. epidermidis*, *S. warneri* and *P. polymyxa* have been recently found in grape and wine samples (Benavent-Gil et a., 2016; Von Cosmos et al., 2017). *S. epidermidis* and *S. warneri* are commonly found as inhabitants of human or animal skin (Nagase et al., 2002). Regarding *Paenibacillus* spp., in particular *P. polymyxa,* this species is well known as endophyte bacteria responsible for the production of antimicrobials, phytotoxins and siderophores (Lai *et al.*, 2012). In that

way, unselective harvest resulting in a poor sanitary condition of grapes, uncareful equipment maintenance or a lack of hygiene during the winemaking process could lead to the occurrence of these potential spoilage bacteria (Du Toit & Pretorius, 2000), and the associated potential problems arisen by BA-producing spoilage microorganisms (Bauer & Dicks, 2004). Finally, no bacterial growth was detected in any of the media during alcoholic fermentation.

Table 13. Percentage of the species identified in each winery in the different sampled stages (1: must; 2: intermediate MLF; 3: final MLF)*

Winery	A			B		
Stage*	1	2	3	1	2	3
Oenococcus oeni	-	52	93	100	100	100
Lactobacillus hilgardii	-	23	7	-	-	-
Lactobacillus mali	78	-	-	-	-	-
Pediococcus parvulus	11	-	-	-	-	-
Lactobacillus plantarum	11	-	-	-	-	-
Staphylococcus warneri	-	9	-	-	-	-
Staphylococcus epidermidis	-	6	-	-	-	-
Paenibacillus polymyxa	-	9	-	-	-	-
Paenibacillus taichungensis	-	1	-	-	-	-

For RAPD-PCR data analysis only primer M13 was used, as primer 1254 did not produce discriminatory patterns at strain level. RAPD-PCR analysis elucidated 36 different genotypic profiles out of 295 isolates (**Figure 13**). The reproducibility study established a cut-off level of similarity of 94%. *Oenococcus oeni* showed a total of 17 different genotypes, most of them appeared just in one stage of the MLF, only three strains appeared in more than one stage. Appearance frequencies are shown in **Table 14**. Winery A showed 6 distinct *O. oeni* genotypic patterns, while winery B, 14; in both

cases *O. oeni* diversity slightly increased during MLF (**Table 15**). As previously shown, winery A showed higher species diversity which share the same ecological niche, and therefore, this could lead to a minor *O. oeni* genotypic diversity. Only 3 out of 17 genotypes were shared by both wineries, however, genotype P3A which was present along the MLF, was the most frequent in both wineries, highlighting its great adaptation abilities. Nevertheless, as shown by the low number of shared genotypes, winemaking conditions of each winery may create a distinguish ecosystem in which different strains were the best adapted. In addition, most of the wine samples from which *O. oeni* was isolated (11/14) showed more than two genotypes and almost half of them (6/14) more than three. These results underline the ability of different wild *O. oeni* populations to share the same niche during MLF, suggesting that spontaneous MLF was led by a mix of *O. oeni* strains, as previously reported in other studies (Franques et al., 2018). In winery B, it was encountered a RAPD profile (P5E strain) that matched that of the commercial malolactic starter Viniflora®Oenos. It must be stated that this commercial strain had been used in previous vintages by winery B, but not by winery A. This fact indicates that strains that have been implanted in the past, are able to prevail over time. The adaptation of those strains, as well as the indigenous strains, to the changing winery conditions leads to the establishment of an endemic microbiota in each winery. In that way, the selection of the most predominant indigenous species should be a criterion to preserve the singularity and biodiversity of these wines.

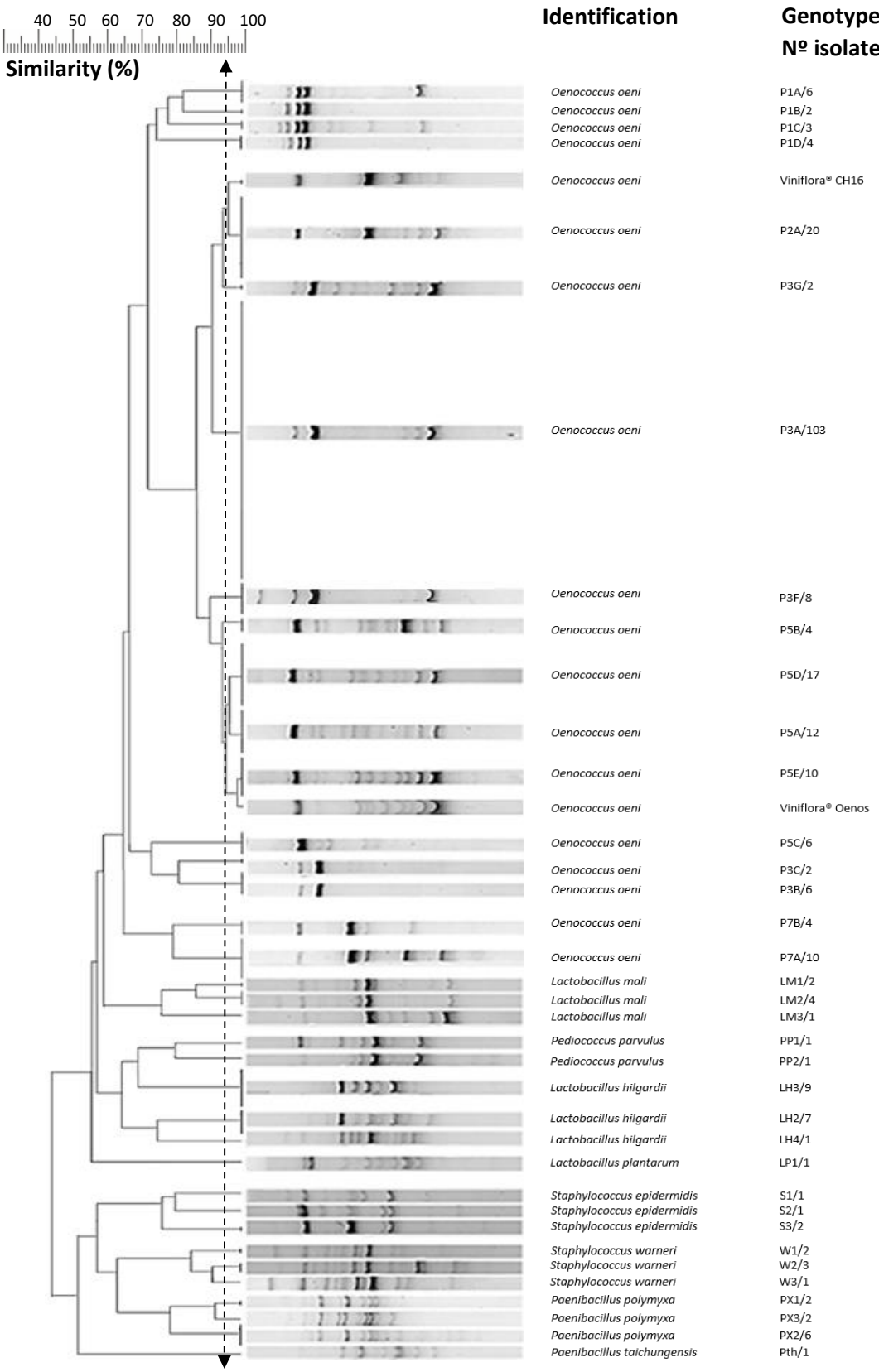


Figure 13. Dendrogram derived from RAPD-PCR with primer M13. The clustering analysis was carried out using the Unweighted Pair Group Method with Arithmetic Average (UPGMA). The vertical lane refers to the cut-off level of similarity of 94% stablished by the reproducibility study.

Table 14. *O. oeni* genotypes, fermentation stage and frequency[b] (%) in each winery

		Winery	
Stage[a]	Genotype	A	B
1-2	P1A	7,69[b]	1,19
2	P2A		11,9
2	P3G	3,85	
2	P5A	1,92	6,55
2-3	P3A	67,31	41,08
3	P1B		1,19
3	P1C		1,79
3	P1D		2,38
3	P3B		3,57
3	P3C	3,85	
3	P3F	15,38	
3	P5B		2,38
3	P5C		3,57
3	P5D		10,12
3	P5E		5,95
3	P7A		5,95
3	P7B		2,38

[a]1:must; 2: intermediate MLF; 3: final MLF

Table 15. *O. oeni* genotypes, isolates and diversity index at different stages of fermentation

Winery	A			B		
Stage[a]	1	2	3	1	2	3
Nº of total isolates	11	81	14	2	40	127
Nº of total *O. oeni* isolates	-	39	13	2	40	127
Nº of *O. oeni genotypes*	-	4	3	1	4	11
Diversity index (ID)[b]	-	0,4	0,63	-	0,67	0,72

[a]1:must; 2: intermediate MLF; 3: final MLF

[b]ID, Simpson´s diversity index

4.2.2. Biogenic amine production and arginine degradation ability

As already stated, LAB are considered the main drivers of BAs accumulation in wine, increasing their concentration from MLF to ageing period (Hernández-Orte et al., 2008). Different LAB species have been described as responsible for BA production (Coton et al., 2010; Marcobal et al., 2006); however, this reaction seems to be strain dependent (Coton & Coton, 2009).

Table 16 shows the results for histamine, tyramine, putrescine and cadaverine production, as well as arginine degradation for all the strains identified. Generally, comparable results were obtained by phenotypical and HPLC results, although some false positives were detected in the phenotypical assays in decarboxylase medium (MDA). The detection of false positive strains may appear as result of reactions that rise the pH of the culture media. In this way, a colour change did not always indicate the degradation of the precursor amino acid. Among LAB, the three strains belonging to *L. hilgardii* species showed putrescine production through the agmatine deiminase pathway. None of the other LAB strains gave a positive response in the phenotypical assay. After phenotypical and HPLC analysis, multiplex PCR were also carried out for the identification of LAB genes involved in BA production. Lysine decarboxylase gene was not analyzed due to the lack of positive control strains. Only those strains belonging to *L. hilgardii* species showed a positive response for agmatine deiminase gene (**Figure 14**), as the phenotypical analysis had shown. In the same way, the rest of LAB strains did not show any gene amplification, in contrast with what some authors have stated. Indeed, there is wide controversy about the incidence of BA-producing LAB in wine.

Some authors have questioned *O. oeni* histamine producing ability (Garcia-Moruno & Muñoz, 2012), and several works, in which no BA production was detected (Ruiz *et al*., 2010; Pramateftaki et al., 2012), counteract other studies where LAB aminobiogenic ability was confirmed (Coton et al., 2010; Landete et al., 2007). It must be stated that although in some species, as *L. brevis*, BA producing ability seemed to be widespread (Romano et al., 2014), it is well known that the ability of LAB to produce BA is a strain dependent characteristic (Ladero et al., 2012). Although LAB strains showed minor amino acid decarboxylase activity, among *Staphylococcus* and *Paenibacillus* strains a variable activity was detected. Except for *S. warneri* W2 and W3, *P. polymyxa* PX3 and *P. taichungensis,* remaining *Staphylococcus* and *Paenibacillus strains* produced simultaneously more than one BA (**Table 16**). However, this metabolic activity did not make a difference in the final concentration of BA in wine samples, as the prevalence of these strains during winemaking was really low. Benavent-Gil et al. (2016) reported for the first time a biogenic amine producing *S. epidermidis* strain in wine, and as far as we know, this is the first study in which BA producing *S. warneri* and *P. polymyxa* strains have been reported in wine as part of the indigenous microbiota.

Table 16 Biogenic amine production and arginine degradation ability of all the identified strains

		Histamine		Tyramine		Putrescine (via agmatine)		Putrescine (via ornithine)		Cadaverine		Arginine	
Species	Nº strains	MDA[a]	HPLC[b]	MDA	HPLC	MDA	HPLC	MDA	HPLC	MDA	HPLC	NH_3	Putrescine
O. oeni	17	-	-	4	n.d.	-	-	-	-	-	-	16 (0,35-1,54)	-
L. hilgardii	3	-	-	-	-	3	3 (0,04-0-05)	-	-	-	-	3 (0,47-1,07)	-
L. mali	3	-	-	1	n.d.	-	-	-	-	-	-	-	-
P. parvulus	2	-	-	-	-	-	-	-	-	-	-	-	-
L. plantarum	1	-	-	-	-	-	-	-	-	-	-	-	-
S. warneri	3	1	1 (0,11-0,32)[c]	1	n.d.	1	1 (0,09-0,21)	1	n.d.	1	1 (1,1-3,4)	1 (0,54-1,15)	-
S. epidermidis	3	2	2 (0,61-1,67)	2	n.d.	3	3 (0,02-1,2)	3	2 (0,22-3,2)	2	2 (0,19-0,41)	3 (0,35-1,21)	2 (0,35-039)
P. polymyxa	3	2	1 (0,17-0,41)	2	n.d.	2	2 (0,08-0,1)	2	2 (0,01-0.02)	2	2 (0,05-0.06)	-	-
P. taichungensis	1	-	-	-	-	-	-	-	-	-	-	-	-

[a]MDA, number of positive isolates in decarboxylase media (MDA)
[b]HPLC, number of positive isolates by HPLC
()[c], concentration in g/l
n.d., not detected

Besides BA production, LAB amino acid catabolism can also promote the formation of other metabolites of health concern, as ethyl carbamate (Mira de Orduña et al., 2000). In this way, ethyl carbamate (EC) precursors production through arginine degradation should be kept in mind as another strain selection criterion. *Oenococcus oeni* and *Lactobacillus hilgardii* showed arginine degradation (**Table 17**), whereas the rest of LAB strains were unable to metabolize it. *Staphylococcus* species degraded arginine, and genotypes S1 and S3 were also capable to produce putrescine from arginine. Finally, none of the strains belonging to *Paenibacillus* species degraded arginine. Arginine is mainly degraded by the arginine deiminase (ADI) pathway in most bacteria (Mira de Orduña et al., 2000), which involves the production of citrulline and carbamyl phosphate, both precursors of EC. Another metabolite of this pathway is ornithine, which in the presence of ornithine decarboxylase positive strains, as *S. epidermidis* S1 and S3, could lead to putrescine accumulation. This pathway also leads to ATP and ammonia formation, ensuring energy production and pH control in acidic environment for bacterial cells (Costantini et al., 2013). The ADI pathway has been described mainly in strict heterofementative LAB, as *O. oeni* and *L. hilgardii*, but not in homofermentative LAB (Mira de Orduña et al., 2000), as observed in this study. This metabolic strategy could explain the presence of *L. hilgardii* during MLF, not as resistant as oenococci to wine environment. Mangani et al., (2005), observed that arginine degradation was stimulated once malic acid was consumed. Thus, inhibiting bacterial growth by sulfites addition once MLF is finished could be a procedure to avoid arginine degradation (Mira de Orduña et al., 2001).

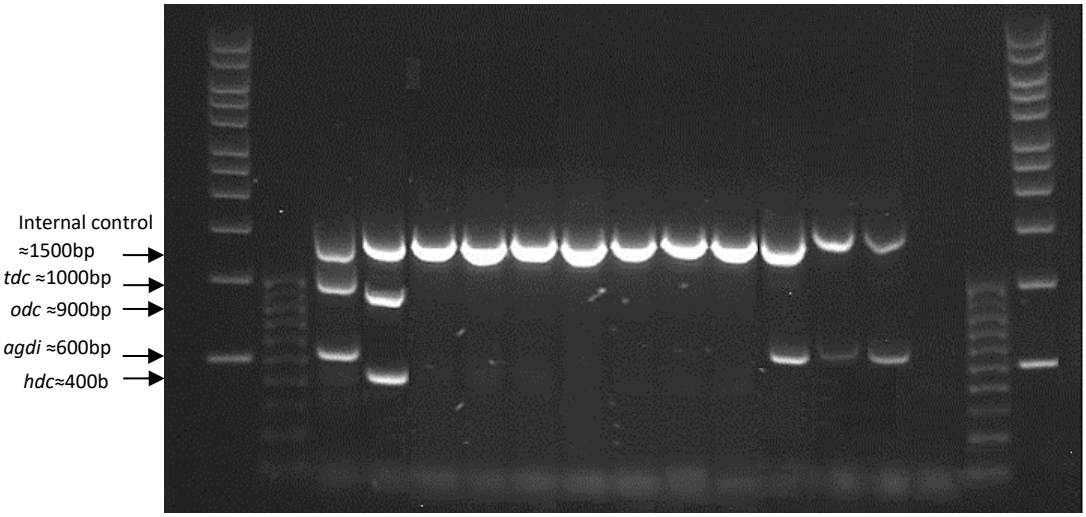


Figure 14 Multiplex PCR. Ladders of 1Kb (lanes 1 and 17) and 100 bp (lanes 2 and 16). 3. *Lactobacillus brevis* 9809 (*tdc* + and *agdi* +); 4. *Lactobacillus* 30a (*odc* + and *hdc* +); 5. *O. oeni* P1A; 6. *O.oeni* P3A; 7. *O. oeni* P7A; 8. *P. parvulus* PP1; 9. *L. mali* LM3; 10. *L. plantarum* LP1; 11. *L. hilgardii* LH1; 12. *L. hilgardii* LH2; 13. *L. hilgardii* LH4; 14; negative control

4.2.3. Amino acids and biogenic amines concentration in wine samples

Finally, it was also decided to track the evolution of precursor amino acids and biogenic amines from which isolates were obtained. Although both wineries used Tempranillo grape variety, differences in total and individual amino acids concentration were observed (**Table 18**). However, both wineries showed comparable amino acids and biogenic amines evolution, which revealed similar behaviour to that observed in other studies where Tempranillo wines were analyzed (López *et al*., 2012; Martínez-Pinilla, et al., 2013). Amino acid levels decreased or, in some cases, did not changed. Some authors have observed an increase in amino acid concentration during MLF, linked to yeast autolysis and LAB proteolytic activity (Pozo-Bayón *et al*., 2005).

Others, conversely, found decreasing amino acid concentrations (Soufleros et al., 2007). Actually, a simultaneous consumption and release of amino acids could happen, becoming a complex task the evaluation of amino acids profile during MLF (López *et al*., 2012). The Mann-Whitney statistical test ($p<0.05$) showed that in both wineries a significant reduction of histidine concentration ocurred between must and the end of MLF, mainly attributable to yeast metabolism (**Table 17**). In parallel, a significant increase in histamine was observed at the end of the MLF. Similar evolution occurred with tyramine in both wineries, which only appeared in latter stages of MLF. Although LAB strains are considered the main drivers of BAs accumulation in wine (Restuccia et al., 2018), no positive strains were detected in the present study.

Agmatine and putrescine were the most abundant BAs, indeed, they are known to significantly contribute to total BA content in wine (Del Prete et al., 2009). Those amines, together with cadaverine, were already present in must samples. Actually, they are commonly found in grapes as they are known to act as growth factors in plant and berry development (Broquedis et al., 1989). Putrescine and cadaverine levels did not vary during the whole process, whereas agmatine disappeared from must once fermentation processes began. Finally, the Spearman´s correlation test ($p<0.05$) showed a positive correlation between agmatine and arginine in both wineries ($r = 0.78$, $\alpha = 0.01$; winery A, and $r = 0.50$, $\alpha = 0.04$; winery B). However, only winery A showed positive correlation between ornithine and putrescine ($r = 0.68$, $\alpha = 0.05$) and arginine and putrescine ($r = 0.71$, $\alpha = 0.05$) pathways, highlighting the metabolic activity of the different pathways involved in putrescine production. No correlation was revealed between the rest of the amine/amino acid precursor pairs. The

correlation variability observed in both wineries between BAs and their precursor amino acids, agrees with other studies where no consensus was achieved about this regard (Izquierdo-Cañas et al., 2008; Martínez-Pinilla *et al*., 2013).

Table 17. Amino acids and biogenic amines concentration (mg/l) in each winery during all winemaking process (must; AF: after alcoholic fermentation; MLF: after malolactic fermentation)

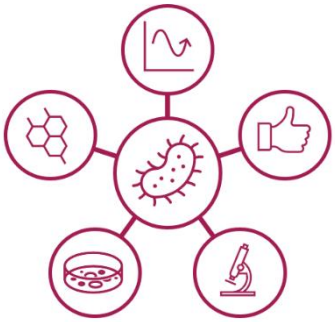
Winery	A			B		
Stage	Must	AF	MLF	Must	AF	MLF
Amino acids						
Histidine	5.80a ± 0.45	1.41b ± 0.39	0.38c ± 0.24	8.03a ± 3.50	2.32b ± 1.23	0.93b ± 0.34
Arginine	66.92a ± 12.69	8.26b ± 4.90	1.72c ± 0.86	96.80a ± 62.89	27.26a ± 24.91	1.67b ± 0.64
Tyrosine	3.59a ± 0.05	2.25ab ± 1.23	1.45bc ± 0.41	4.48a ± 2.50	2.06ab ± 1.35	1.42bc ± 0.29
Ornithine	1.86a ± 0.07	6.26a ± 4.88	2.38a ± 0.38	1.59a ± 0.87	2.27a ± 0.31	2.85a ± 0.47
Lysine	1.85a ± 0.27	3.31a ± 1.15	2.78a ± 0.68	2.18ab ± 0.54	1.23a ± 0.16	6.43b ± 1.55
Total	80.02 ± 11.63	21.49 ± 1.17	8.74 ± 0.37	113.07 ± 18.58	35.16 ± 5.06	13.30 ± 0.99
Biogenic amines						
Histamine	n.d.	0.15a ± 0.15	0.99b ± 0.23	0.27a ± 0.13	0.28a ± 0.28	1.12b ± 0.25
Agmatine	5.40a ± 0.09	0.28b ± 0.28	0.38b ± 0.22	4.37a ± 1.23	1.11b ± 0.79	0.83b ± 0.03
Tyramine	n.d.	n.d.	0.12 ± 0.12	n.d.	n.d.	0.46 ± 0.19
Putrescine	3.37a ± 0.35	2.74a ± 0.85	2.34a ± 0.36	3.66a ± 0.45	4.41a ± 0.29	4.96a ± 0.32
Cadaverine	2.11a ± 0.68	0.86a ± 0.25	0.98a ± 0.25	0.91a ± 0.23	1.15a ± 0.53	0.84a ± 0.08
Total	10.88 ± 0.71	4.02 ± 1.19	5.99 ± 1.15	9.21 ± 0.38	6.96 ± 0.23	8.82 ± 0.35

n.d. not detected. Mean values for each amino acid and biogenic amine with different letters are significantly different ($p < 0.05$).

Furthermore, no winery showed correlation between total amino acid and total biogenic amine concentrations, suggesting that the concentration of amino acids in must did not affect the concentration of biogenic amines after MLF. Other factors such as the indigenous microbiota, a lack of hygiene during winemaking or the sanitary conditions of grapes could have a major impact in the final content of BA (Marques et al., 2008). It is worth mentioning that both wineries showed low concentrations of BA, far from limits recommended for safety and quality principles (Arena & Manca de Nadra, 2001; Izquierdo-Cañas et al., 2008).

This study represented the first step in the selection process of novel malolactic starters from Rioja Alavesa region. After the identification and selection of non-BA-producing autochthonous LAB strains, subsequent characterization studies were based on the elucidation of their technological and sensorial prospects. The following studies constitute an in-depth analysis of the prospective use of indigenous LAB strains as novel cultures, not only to preserve the singularity and biodiversity of Rioja Alavesa wines, but also to minimize BA formation during MLF as well as to prevent the appearance of BA producing strains during wine ageing,

4.3. Study 3



Technological characterization of potential malolactic starters from Rioja Alavesa winemaking region

In the present study it was performed the technological characterization of previously identified indigenous LAB strains. Starter selection procedure must accomplish three main criteria: (i) no production of metabolites of health concern (e.g. biogenic amines), (ii) resistance to wine strict conditions as low pH and high ethanol and SO_2 concentrations and (iii) MLF vigour and contribution to wine aroma complexity (Torriani et al., 2011). In this sense, this study began with the evaluation of both growth behaviour under typical wine conditions and fermentation vigour of LAB strains. In addition, considering that MLF is much more complex than a simple deacidification process, and it could entail a modulation of sensorial complexity of wines (Cappello et al., 2017), different aspects that may affect the sensory quality of wines were evaluated. Thus, among others, the ability to metabolize citrate as well as the activity of multiple enzymes related to aroma compounds release were evaluated. Due to their significance, special emphasis was placed on the evaluation of different glycosidase and esterase activities under different vinification conditions. All in all, the main aim of this study was to evaluate the oenological potential of autochthonous LAB strains.

4.3.1. LAB strains growth at different limiting conditions

Among the main factors that inhibit the development of LAB strains in wine environment, the most relevant are low pH, high ethanol and high SO_2 concentrations (Romero et al., 2018). Accordingly, in the present study the growth performance of each strain under different growth limiting conditions (pH 3.4, 3.5, 3.6, 3.7 and 3,8; 0, 10, 12 and 14 % v/v ethanol and 5, 15, 30, 40 and 50 ppm total SO_2) was analysed. The analysed strains are displayed in **Table 18**. After monitoring the growth curves of each strain and condition by optical density measurement, the corresponding growth rates

(logCFU/ml/day) were quantified through ComBase software (USDA, Agricultural Research Service) **(Supplementary Table 2,** *Annex 2***)**. Finally, the obtained growth rates were submitted to one-way ANOVA statistical comparative analysis ($p<0.05$). In that way, when no significant differences were obtained among strains growth rates for each condition, strains were clustered and their mean growth rates were estimated (**Figure 15**).

Table 18. Bacterial strains used in this study

Oenococcus oeni		*Lactobacillus mali*	*Lactobacillus plantarum*
P1A	P3F	LM1	LP1
P1B	P3G	LM2	
P1C	P5A	LM3	
P1D	P5B		
P2A	P5C		
P3A	P5D		
P3B	P7A		
P3C	P7B		
Viniflora OENOS	Viniflora CH16		

Regarding the pH-related strains behaviour, it is remarkable the performance of *L. plantarum* LP1 (LP-pH), which showed by far the highest growth rate throughout the pH range studied (**Figure 15A**). Concerning *L. mali* strains, the three strains showed similar and homogeneous performances, synthesised in a single growth behaviour (LM-pH). In both cases, the growth rates profiles were linear-shaped. The behaviour of the 18 *O. oeni* strains was reduced to three different clusters (Oo1-pH, Oo2-pH and Oo3-pH). Cluster Oo1-pH gathered the behaviour of strains P1A, P1B, P1C, P1D, P3A, P5A and P7A; Oo2-pH collected strains P2A, P3C, P3F, P5C, P3G and CH16; and Oo3-

pH clustered strains P3B, P5B, P5D, P7B and OENOS. However, their growth rates were not as high as those of the abovementioned *Lactobacillus* ssp. strains, and linear fit was less evident. Anyway, as pH values below 3.6 must be ensured during MLF in order to inhibit potential spoilage species and their consequences (Lerm et al., 2010), the confirmed ability of certain strains to grow at such low pH would make them suitable for conducting a safe MLF.

Concerning ethanol influence, weak inter- and intra-species differences were found (**Figure 15B**). Main differences were detected in ethanol-free assays where each species showed its own growth rate. *L. plantarum* LP1 strain showed again the best performance (LP-OH). At 10% ethanol, growth rate of LP1 was moderately affected, but in the range 12-14% a significant decay was observed. *O. oeni* strains showed a similar behaviour, although they were affected by ethanol in a greater extent. These strains were grouped in two clusters: Oo1-OH, collecting the behaviour of most strains, and Oo2-OH, that explained the performance of P3B, P3F, P5A and P5B. *L. mali* strains, once again, converged in a single cluster (LM-OH). For both *L. mali* and *O. oeni* strains, growth rates were linear-shaped. In all cases ethanol was a growth inhibitor, but even at the highest ethanol concentration, bacterial growth was still detected.

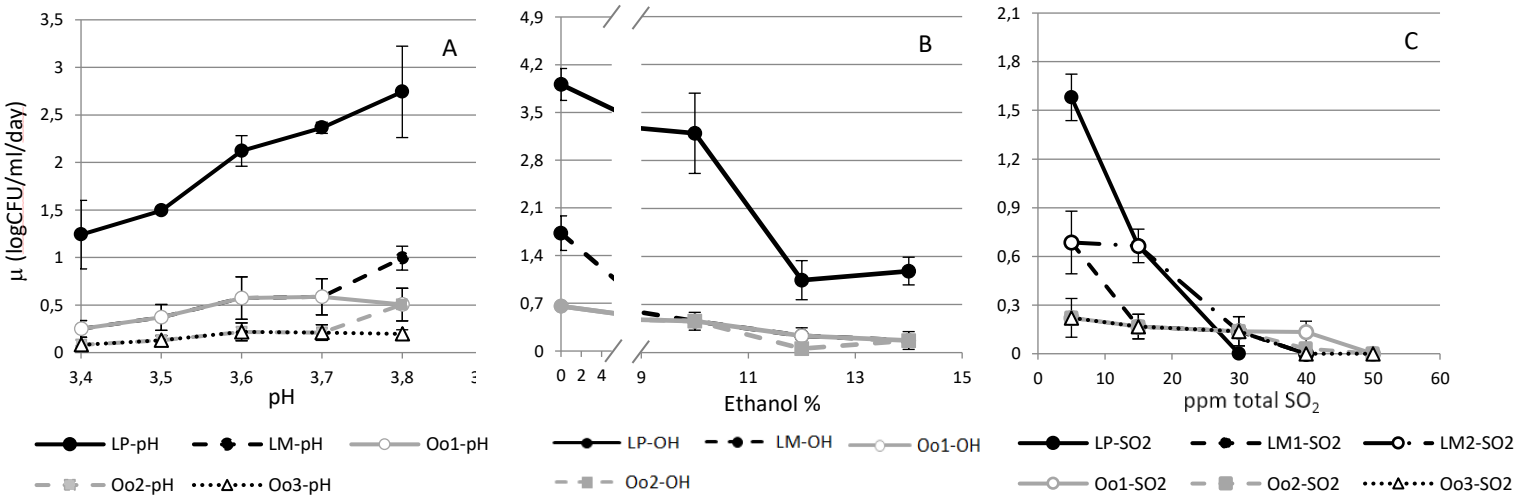


Figure 15. Growth behaviours obtained after one-way ANOVA statistical comparative analysis of strains growth rates (μ) at different pH values (A), ethanol (B) and SO2 (C) concentrations. Growth performances are identified with different letters, in that way, LP reflects the performance of *L. plantarum*, LM corresponds to *L. mali* strains and Oo explains the different behaviours of *O. oeni* strains. The different behaviours against the pH showed the following equations and R2 for their linear tendencies, **LP-pH**: y = 3,87x – 11,95, **R^2= 0,98**; **LM-pH**: y = 1,70x – 5,58, **R^2= 0,90**; **Oo1-pH**: y = 0,72x – 2,16, **R^2= 0,63**; **Oo2-pH**: y = 0,93x – 3,11, **R^2= 0,80**; **Oo3-pH**: y = 0,31x – 0,95, **R^2= 0,70**. For ethanol, **LP-OH**: y = -0,19x – 4,11, **R^2= 0,73**; **LM-OH**: y = -0,11x + 1,69, **R^2= 0,98**; **Oo1-OH**: y = -0,06x + 1,08, **R^2= 0,99**; **Oo2-OH**: y = -0,03x + 0,68, **R^2= 0,89**. And for SO_2, **LP-SO_2**: y = -0,06x + 1,77, **R^2= 0,96**; **LM1-SO_2**: y = -0,01x + 0,62, **R^2= 0,75**; **LM2-SO_2**: y = -0,02x + 0,86, **R^2= 0,93**; **Oo1-SO_2**: y = -0,004x + 0,24**, R^2= 0,81**; **Oo2-SO_2**: y = -0,005x + 0,25, **R^2= 0,94**; **Oo3-SO_2**: y = -0,005x + 0,25, **R^2= 0,90**.

Sulphur dioxide (SO_2) was the main restrictive factor for LAB growth and survival. It is stablished that a total SO_2 concentration of 30 ppm delays LAB growth, whereas more than 50 ppm completely inhibit growth (Lerm et al., 2010), which partially agrees to our results. In general, *O. oeni* strains endured the higher SO_2 concentration (**Figure 15C**): strains that comprised the clusters Oo1-SO_2 (P3G, P5B, P5C and P5D) and Oo2-SO_2 (P5A, P7A and P7B) were able to grow at 40 ppm of total SO_2. Remaining *O. oeni* strains (cluster Oo3-SO_2) tolerated a maximum concentration of 30 ppm. However, most of them suffered a growth delay of more than a week (**Supplementary Table 3**). *L. mali* strains were not able to grow with total SO_2 concentration over 30 ppm, while *L. plantarum* LP1 showed a threshold of 15 ppm. Growth-rate decay against SO_2 was seen to follow distinct tendencies among strains. While LP-SO_2, Oo2-SO_2 and Oo3-SO_2 clusters showed a linear shape, for the rest, this linear decay was less evident. Periods in which low SO_2 levels are found in wine, as the time lapse between alcoholic fermentation (AF) completion and the beginning of MLF, are critical due to potential microbial spoilage (Sumby et al., 2019). Hence, the pursuit of strains able to bear moderate concentrations of SO_2 may be considered as a goal to be attained. In that sense, although the non-oenococcal LAB may grew similarly (or better in the case of *L. plantarum* LP1) to *O. oeni* strains under the different pH and ethanol conditions, when SO_2 was analysed, it has been confirmed that *O. oeni* strains show greater stress tolerance. This observed behaviour agrees with previous results: non-oenococcal LAB species analysed in this work were all isolated from must samples, being absent after AF, as shown in the previous study, possibly due to a scarce nutritional composition of the medium and the presence of moderate SO_2 concentrations (Volschenk et al.,

2006). Contrarily, although *O. oeni* growth is slower during winemaking, it finally predominates in wine environment (Lonvaud-Funel, 1999).

4.3.2. Malolactic fermentation in synthetic wine

Three major groups of *O. oeni* strains were stablished based on malic acid consumption rate, malic acid consumption percentage and strain viability (**Table 19**): Strains belonging to the group A showed a malic acid consumption rate of 8-10 mg/L/h, consumed from 80 to 100% of malic acid and finished the fermentation process in less than 15 days (**Figure 16**). These strains, at the end of the 40-day trial, had low or non-existent counts, according to a rapid consumption of the nutrients and the adverse conditions of the environment after MLF (Lerm et al., 2011). Group B consumed from 65 to 80% of malic acid at a rate of 2,5-3 mg/L/h, and did not finish the fermentation process at the end of the trial. Although they were not able to exhaust malic acid within the established period, decreasing tendency was still noticeable at the end of analysis. Additionally, at this time, group-B strains showed high counts, which could indicate that, although at a lower rate, these strains could presumably finish the process on subsequent days. Finally, strains belonging to the group C showed poor cell viability during the process, which reflected a high malic acid concentration at the end of the analysis. The ability of strains to growth actively in the wine environment is critical for a successful MLF (Ong, 2010). It is widely stablished that MLF takes place when LAB population increases over 10^6 CFU/ml and sufficient biomass is achieved (Ribéreau-Gayon et al., 2006). However, for *O. oeni* strains, Brizuela et al. (2017), reported higher inoculum size (>10^8 CFU/ml) to ensure a reliable MLF. Accodingly, the inoculum size used in this study (≈ 3x10^7 CFU/ml), could be the reason why cell viability was compromised in some cases, and only a limited number

of strains finished MLF. Nevertheless, although a higher inoculum size could have boosted MLF completion, conditions used here provide an adequate insight of the most adaptive and resistant strains.

Table 19. Strains malic acid consumption percentage, consumption rate and viability after MLF in synthetic wine (n.d. not detected).

Strains	Group	Consumption rate (mg/L/h)	Consumption (%)	Log CFU/ml (after 40 days)
P5A	A	10,33 ± 0,2	99,93 ± 0,88	2,56 ± 0,11
P3G	A	8,82 ± 1,31	85,01 ± 15,71	n.d.
P3A	A	8,56 ± 0,15	84,44 ± 1,45	1,52 ± 1,52
P3C	A	8,37 ± 0,74	82,55 ± 7,34	n.d.
P3F	B	3,11 ± 0,33	81,86 ± 8,66	5,54 ± 0,12
P5C	B	3,07 ± 0,52	80,7 ± 13,59	5,92 ± 0,53
P5B	B	2,95 ± 0,45	77,59 ± 11,93	5,68 ± 0,64
P2A	B	2,89 ± 0,05	75,94 ± 1,4	4,06 ± 0,02
P5D	B	2,81 ± 0,26	73,86 ± 6,76	5,9 ± 0,23
OENOS	B	2,74 ± 0,48	72,04 ± 12,51	5,26 ± 0,42
P7B	B	2,72 ± 0,28	71,53 ± 7,38	4,99 ± 0,69
CH16	B	2,46 ± 0,33	64,59 ± 8,71	4,98 ± 0,21
P1A	C	2,8 ± 0,29	73,77 ± 7,52	n.d.
P3B	C	2,74 ± 0,31	71,98 ± 8,14	n.d.
P7A	C	2,65 ± 0,04	69,8 ± 0,98	1,8 ± 0,8
P1C	C	2,63 ± 0,03	69,08 ± 0,81	n.d.
P1D	C	2,55 ± 0,08	67,04 ± 2,16	n.d.
P1B	C	1,87 ± 0,56	49,29 ± 14,72	n.d.

In addition, many indigenous strains showed better fermentation rate than the commercial strains tested. For their part, non-oenococcal LAB strains were unable to consume malic acid, and their viability was lost after 5 days of analysis (data not shown). Although these species showed similar or better growth performance in MRS

at low pH and high ethanol concentration, in synthetic wine (pH 3,5 and 13,5% v/v ethanol) strains viability was rapidly lost. This difference may rely on synthetic wine nutrient deficiency; indeed, wine matrix may affect more significantly than both pH and ethanol content (Gockowak & Henschke, 2008). Due to their better performance over the rest of species, subsequent characterization analyses were only performed on *O. oeni* strains.

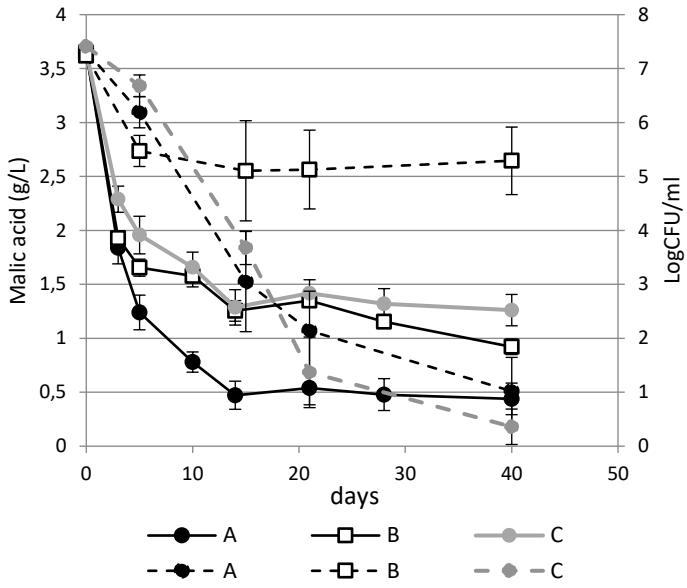


Figure 16. Evolution of malic acid consumption (straight lines) and cell count (dotted lines) of *O. oeni* strains. Group A represents those strains that finished MLF, group B reflects the behaviour of strains that did not finished MLF but still showed high viability, and finally, group C gathers those strains that did not finished MLF and showed poor viability

4.3.3. Citrate metabolism

During MLF, the by-products originated from citric acid catabolism, such as diacetyl, acetoin and 2,3-butanediol play an important role in the modification of wine aroma.

Specially diacetyl, due to its low aromatic threshold, is considered a key aroma contributor (Bartowsky & Henschke, 2004). All *O. oeni* strains harboured the genes responsible for citrate catabolism (citrate permease and citrate lyase complex) (**Figure 17**), and all of them gave a positive result in the phenotypical assay. This trait has been described as a strain-dependent characteristic in *O. oeni* (Lerm et al., 2011; Mtshali et al., 2010). Depending on target wine characteristics, the metabolic variability found among *O. oeni* strains would confer different sensorial properties to wines. Thus, this catabolic activity should be further characterized, as citrate metabolism is considered sequential to malic acid degradation in LAB (Bartowsky & Henschke, 2004). In that way, an immediate sulfitation after malic acid exhaustion, a common criterion for MLF completion, would result in incomplete citrate degradation, disrupting the formation of key carbonyl flavour compounds, as diacetyl, and minimizing LAB influence on aroma and flavour development.

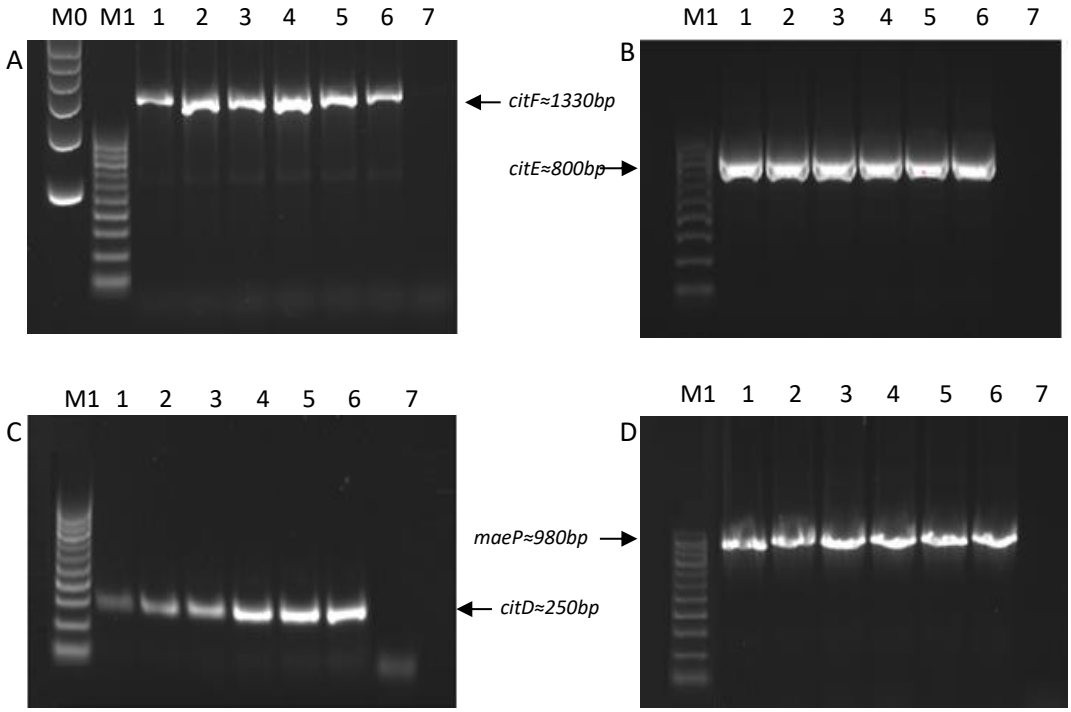


Figure 17. PCR amplifications showing the result for (A) citrate lyase subunit α (CitF), (B) citrate lyase subunit β (CitE), (C) citrate lyase subunit γ (CitD) and (D) citrate permease (maeP) genes. Ladders of 1 Kb (M0) and 100 bp (M1). 1. *O. oeni* P2A; 2. *O. oeni* P3A; 3. *O. oeni* P3B; 4. *O. oeni* P3G; 5. *O. oeni* P5C; 6. *O. oeni* P7B; 7. negative control.

4.3.4. Multi-enzymatic analysis

Autochthonous strains, already adapted to a specific winemaking region conditions, have been suggested not only to improve MLF reliability but also to enhance the singularity and complexity of specific wines (Sumby et al., 2019). In that way, among all the activities tested special attention was paid on those enzymes potentially correlated with aroma compounds release (**Table 20**). Indeed, current analysis has been found useful for a rapid identification of potential LAB starters (Iorizzo et al., 2016). All the strains showed clear aminopeptidase activity (valine and leucine arylamidase), which catalyses the hydrolysis of N-terminal amino acids from peptides. This activity is not only responsible for aroma precursors release but also is considered a bacterial strategy to increase nutrient intake and useful to reduce haze caused by large peptides (Trinh et al. 2010; Dizy & Bisson 2000). Leucine and valine (as other aminoacids) could derive in aroma compounds through the Erhlich pathway. The degradation of these aminoacids leads to the formation of different aromatic compounds such as fusel acids and fusel alcohols (Fairbairn et al., 2017; Santamaría et al., 2015). Regarding carbohydrate metabolism, although many activities were not observed, as α-manosidasa, α-fucosidasa or β-glucuronidasa, all the *O. oeni* strains exhibited strong α- and β-glucosidase and weak activity for α- and β-galactosidase. In the same way, weak esterase activity was detected for all the strains, although many exhibited strong response. Glycosidase and esterase activities were further analysed under different winemaking conditions in order to elucidate the oenological potential of *O. oeni* strains.

Table 20. API-ZYM multi-enzymatic analysis corresponding to *O. oeni* strains

	Strains																	
Enzymatic activities[a]	P1A	P1B	P1C	P1D	P2A	P3A	P3B	P3C	P3F	P3G	P5A	P5B	P5C	P5D	P7A	P7B	OENOS	CH16
Alkaline phosphatase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esterase (C4)	W	W	W	W	+	+	-	W	W	W	+	W	W	W	W	W	W	W
Esterase lipase (C8)	W	W	W	W	W	W	W	W	W	W	+	W	W	W	W	W	W	W
Lipasa (C14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Valine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cystine arylamidase	W	W	-	W	W	W	-	W	W	W	W	W	W	+	W	W	W	W
Trypsin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-chymotrypsin	W	+	+	+	-	W	-	W	-	W	W	-	-	-	W	-	-	-
Acid phosphatase	+	+	+	+	W	W	+	+	+	W	+	+	W	+	W	W	+	W
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-galactosidase	+	+	+	+	-	-	-	+	-	+	-	-	-	-	+	-	-	-
β-galactosidase	-	-	W	+	-	W	-	-	-	+	+	W	-	-	-	-	-	-
β-glucuronidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-glucosidase	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
β-glucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetil-β-glucosaminidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Positive (+), weakly positive (W) and negative (-)

4.3.5. Quantification of glycosidase activities

Although it has been already documented the presence of different LAB enzymes involved in wine aroma modification, information on the role of their activities under winemaking conditions is still limited (Romero et al., 2018). In that way, four different glycosidase activities were analysed under different pH and ethanol concentration combinations in order to evaluate the oenological potential of *O. oeni* strains. Glycosides, typically monoglucoside and diglucoside conjugates, are considered the main source of compounds from grapes contributing to wine aroma complexity (Liu et al., 2017). The aglycone moiety (aroma compound) in monoglucosides is always linked to a β-D-glucopyranose, thus, the enzymatic hydrolysis is driven by β-D-glucosidase (βGlu). Disaccharides, incorporating other sugar than glucose require the sequential action of specific enzymes, as β-D-xylosidase (βXyl) or α-L-arabinosidase (αAra), to hydrolyse the intersugar linkage before the hydrolysis of aglycone-glucose linkage by βGlu (D´Incecco et al., 2004; Liu et al., 2017).

Regarding βGlu, all strains presented βGlu activity under the different conditions tested, showing variable response in a strain-dependent manner, as previously seen in other studies (Bravo-Ferrada et al., 2016; Grimaldi et al., 2000). A summary of the behaviour of several representative strains including those which exhibited major βGlu activity is shown in **Figure 18.1**. The behaviour of all strains is displayed in **Supplementary Figure 1** (*Annex 2*). In ethanol-free assays, there were no significant variations at the different analysed pHs, in contrast to other studies, where βGlu activity of *O. oeni* was greatly altered by the pH (Grimaldi et al., 2005). By other hand, in general, ethanol seemed to slightly stimulate enzymatic activity in some strains,

with a maximum at 10% v/v, independently of the pH. Particularly, at 10%, as in ethanol-free assays, strain P2A showed the best performance. In addition, several strains (e.g. P2A, P3A and P3G) maintained similar activity when ethanol concentration increased to 12%. Increase of βGlu activity by ethanol has also been reported in other studies (Barbagallo et al., 2004; Grimaldi et al., 2000), where ethanol stimulation was seen until 8% ethanol. This trend would be due to the glycosyl-transferase activity of βGlu, where ethanol increases reaction rates as an acceptor of the glycosyl intermediate (Barbagallo et al., 2004). However, most of the strains suffered a dramatic activity decrease when 12-14% ethanol level was reached. Further, when a combination of 14% ethanol and pH 3.4 was analysed, most activities were reduced significantly ($p<0,05$), since relative activity against control was reduced more than 60% (**Supplementary Figure 2.1**). Nevertheless, at 14% ethanol and higher pH values (pH 3.8), noticeably the activity of some strains was similar to that observed in other conditions. This phenomenon was mainly observed in P2A, P3A, P3G and P3F strains, highlighting the activity of P2A and P3G, which showed a relative activity of >120% under this condition. Even though this exception, a shift to lower pH values resulted in a relevant enzyme activity decline also for these strains. In fact, when conditions became more aggressive, the synergistic negative effects of both ethanol and pH were more noticeable. In that way, at most restrictive conditions, when strains metabolic activity slows down, the activity of these key enzymes would be compromised. Hence, this combinative analysis of pH and ethanol could be a suitable strategy to discern the potentialities of microbial enzymes and thus, strains influence on wine development under oenological conditions.

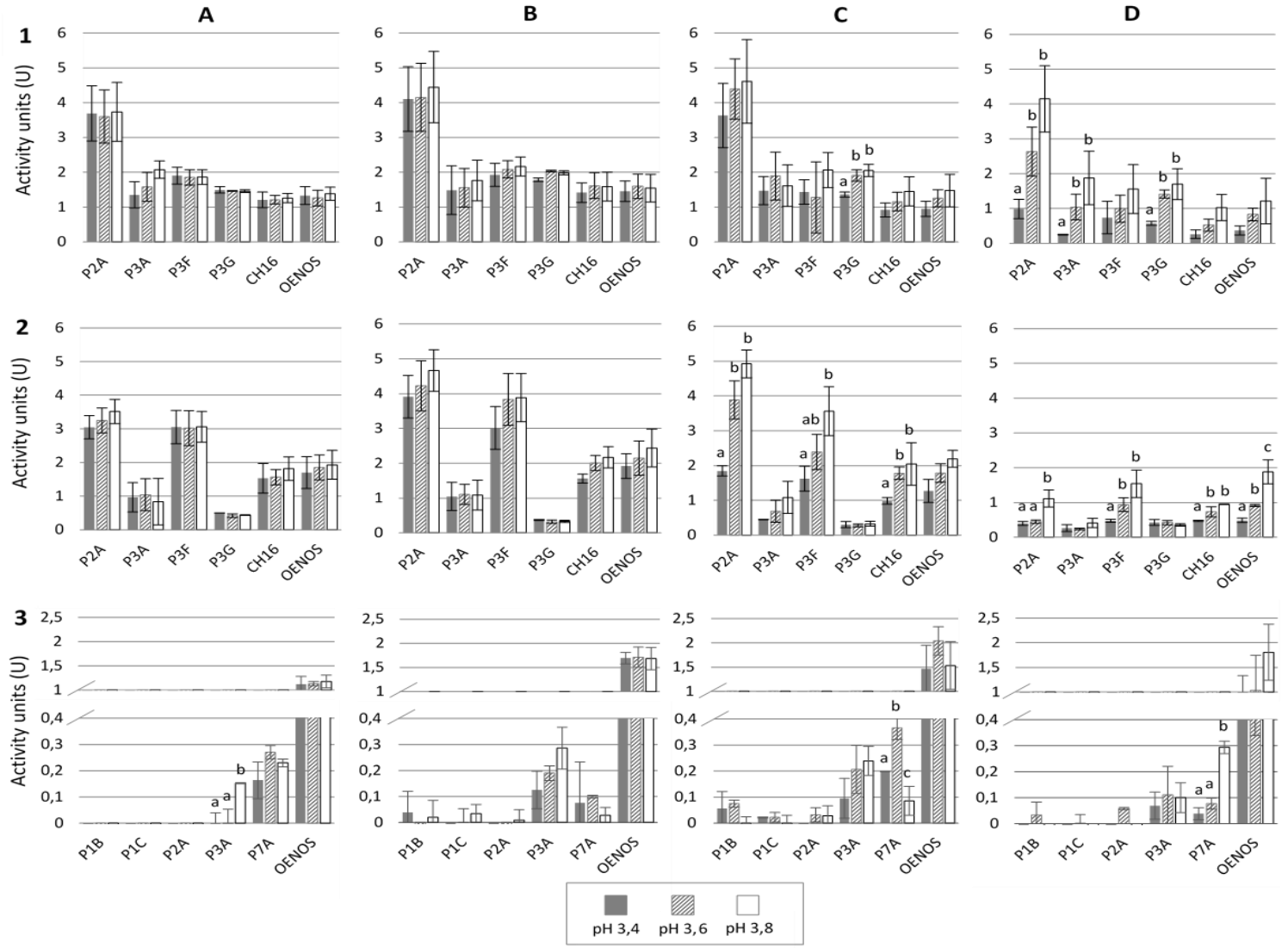


Figure 18. Glycosidase activities of representative strains. β-glucosidase (row 1), α-glucosidase (row 2) and β-xylosidase (row 3) activities under different pH and 0% (column A), 10% (B), 12% (C) and 14% v/v ethanol (D) combinations. Activity unit (U) refers to nmol of liberated p-nitrophenol per minute per milligram of cell dry weight. Only conditions in which significant differences are identified are marked, in that way different letters refer to significant differences ($p<0,05$)

All strains showed similar αGlu-activity evolution to that exhibited for βGlu, although, in overall, they were faster influenced by extreme pH and ethanol conditions (**Figure 18.2**). Even though ethanol at 10% did not affect enzyme activity, higher concentrations made activity rapidly decrease. A small decrease on the pH significantly reduced αGlu activity at 12% ethanol, while this effect was observed at 14% for βGlu. When comparing both enzymes, contradictory results have been found in the literature regarding their relative activity (Gagné et al., 2011; Grimaldi et al., 2005). In the present study, both activities were similar through each strain, more in agreement with Gagné et al. (2011), although at the harshest conditions, αGlu activity was dramatically reduced. As observed with βGlu activity, strain P2A showed the best performance under the different conditions. In addition, this strain, mainly together with P3F, showed higher βGlu and αGlu activity than the commercial strains in all conditions. Relevant αGlu and βGlu activities could positively influence the surviving capacity of these strains under wine limiting conditions, by providing an additional source of glucose, and thus, a strategy to survive under nutrient scarce environment (Gagné et al., 2011).

When disaccharide-glycosidases were studied, more than half of *O. oeni* strains did not show any activity under the different conditions (**Supplementary Figure 1.3**). **Figure 18.3** exhibits the responses of some of the few strains that showed βXyl activity; for αAra data are not shown due to the low number of positive strains and their low activity. Several strains maintained detectable βXyl activity, as P2A, P3A and P7A; however, it was at least 10-fold lower compared with reported αGlu and βGlu activities. This fact was also underlined in other studies (D´Incecco et al., 2004; Grimaldi et al., 2005), that highlighted the low specificity of *O. oeni* to lead the

cleavage of complex disaccharide compound. *O. oeni* disacchraride-glycosidase enzymes not only drive the release of grape derived aromatic compounds responsible for floral and fruity attributes such as monoterpenes, C13-norisoprenoids, benzene derivatives and aliphatic alcohols, but also play a key role in the liberation of oak-related aroma compounds, as vanillyn or whiskeylactone (Bloem et al., 2008). Consequently, this feature is highly desirable since MLF is often conducted in oak barrels to increase wine aroma complexity. In this sense, remarkably, the commercial strain Viniflora OENOS exhibited the best performance, maintaining similar activity under the different conditions, as well as comparable values to those observed for αGlu and βGlu enzymes.

4.3.6. Quantification of esterase activities

In wine, ester content is the result of both hydrolysis and synthesis reactions though the activity of several enzymes, as lipases, esterases and alcohol acyl transferases (Costello et al., 2013). Indeed, four putative esterase genes have been described in *O. oeni* species (Sumby et al., 2010). In the present study, esterase activity was measured at the same conditions than glycosidase activities. By the analysis of two different esterase substrates, p-nitrophenyl acetate (p-NAcetate) and p-nitrophenyl octanoate (p-NOctanoate), esterase specificity was also analysed. Results for all strains are shown in **Supplementary Figure 3**. Esterase activity was clearly influenced by ethanol concentration. This fact was clearly visible when p-NAcetate was used as substrate, as many strains showed a peak of activity at 12-14% ethanol (**Figure 19.1**). In this case, ethanol may have increased reaction rate by acting as nucleophile against the acylated enzyme and lead to the corresponding ethyl ester formation. Actually, esterases can act as both hydrolases and syntases depending on physicochemical conditions of the

medium (Sumby et al., 2010). Contrarily, when p-NOctanoate was used, half of the strains exhibited a peak of activity at 10% ethanol and the other half showed better results in free-ethanol assays (**Figure 19.2**). This variability was also found in other studies in which p-NOctanoate was used as substrate. Matthews et al. (2007) found an increasing esterase activity at different ethanol concentration ranging from 2 to 16%. Pérez-Martín et al. (2013), conversely, showed a marked activity decrease from 4 to 14% ethanol. This fact suggests that strains could possess more than one enzyme with different properties and different substrate affinities.

Overall, substrate specificity was significantly lean towards short chained substrate. In fact, all the *O. oeni* strains studied in this work showed about 10-fold higher activity against p-NAcetate. This specificity had been already reported (Matthews et al., 2007; Sumby et al., 2009). Indeed, Sumby et al. (2009) described increasing esterase Km value for increasing substrate chain length, confirming esterase affinity for short-chain esters. All in all, esters are considered the main contributors of fruity aroma complexity in wine (Costello et al., 2013). Short and medium-chained esters can contribute to fruity aroma at low concentrations, whereas, long-chained esters are contributors of soap-like waxy aroma (Díaz-Maroto et al., 2005). Therefore, as many strains retained esterase activity under most restrictive conditions, many of them (P2A, P3F or P7B) showing better performance than commercial strains, the selection of specific strains could be a promising strategy for modulate ester profile of wine during MLF.

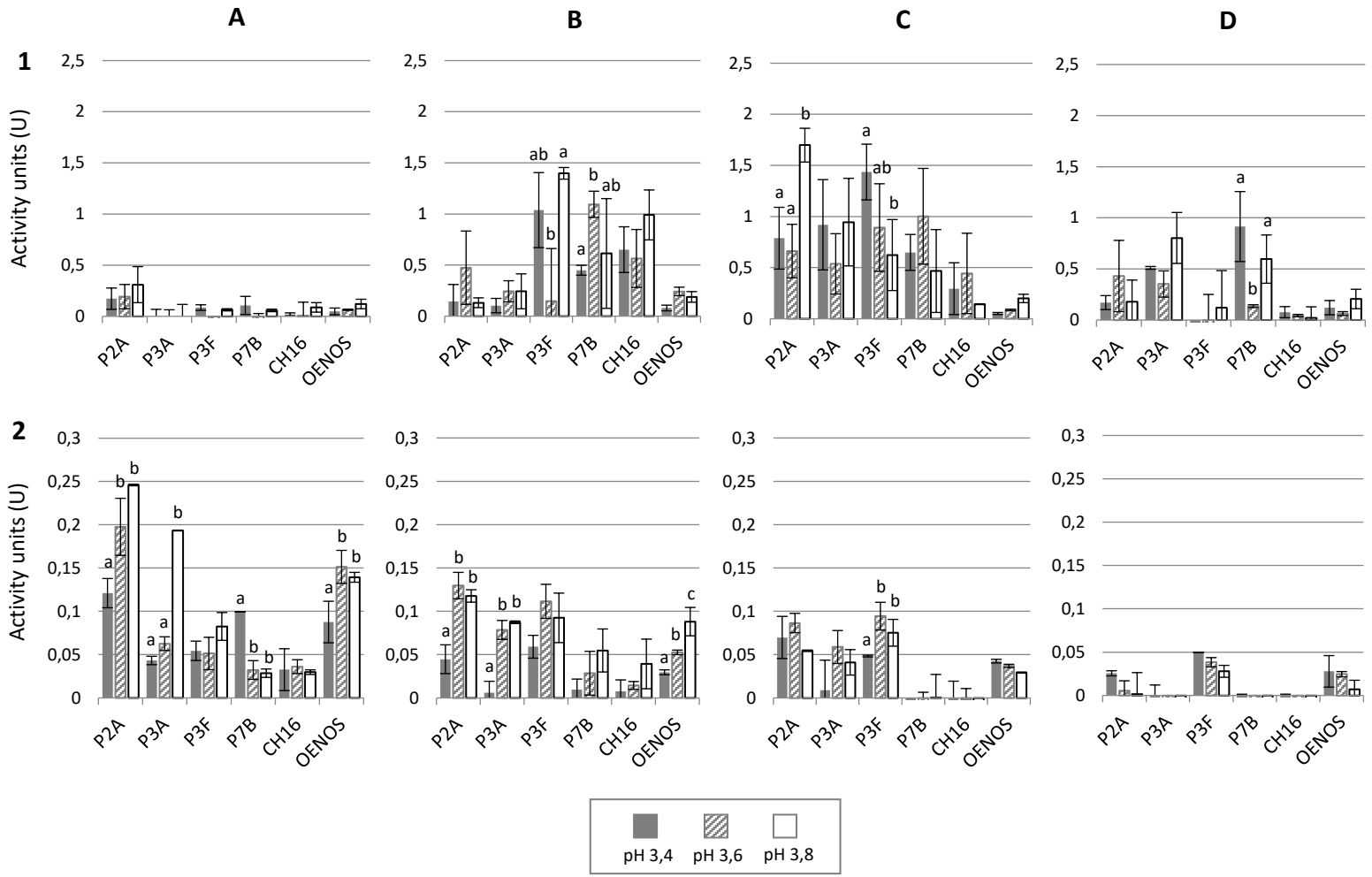
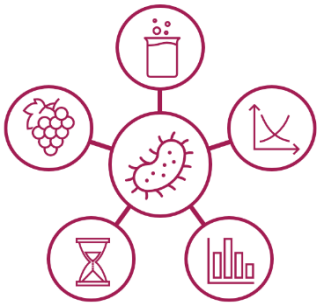


Figure 19. Esterase activity of representative strains. Activity against p-nitrophenyl-acetate (row 1) and p-nitrophenyl-octanoate (row 2) under different pH and 0% (column A), 10% (B), 12% (C) and 14% v/v ethanol (D) combinations. Only conditions in which significant differences are identified are marked, in that way different letters refer to significant differences ($p<0,05$)

In the last decade, in order to enhance wine aroma complexity, the use of commercial enzymes, usually of fungal origin, has been widespread. However, it has been observed that they are commonly inhibited under wine conditions. Also, unwanted reactions have been observed because of the use of enzyme cocktails that unbalance the product (Fia et al., 2014). Consequently, the screening of these enzymatic activities in *O. oeni* strains is highly relevant (Gagné et al., 2011). In Rioja Alavesa region, MLF commonly occurs spontaneously by autochthonous LAB strains, making it difficult to predict, and arising the risk of stuck or sluggish fermentation and the appearance of off-flavours (Garofalo et al., 2015). In that way, indigenous LAB inoculation should be considered as a suitable strategy, not only to ensure an efficient MLF, but also to preserve and enhance the aroma complexity of these wines.

In this work, several autochthonous strains (P2A, P3A or P3G, among others) were able to retain glycosidase and esterase activities under oenological conditions, and many exhibited higher enzyme activities than the commercial strains. Considering malic acid consumption rate as well as the different enzyme activities tested, strains P5A, P2A, P3A and P3G, showed the best potentialities to be extensively studied. Indeed, these strains showed better fermentation vigour as well as similar or better enzyme activity performances than commercial strains. Together with these strains, P5C and P7B strains, although they did not present special qualities, were also selected for further characterization studies. Results from this study encouraged the potential use of many of the characterized autochthonous *O. oeni* strains as an effective strategy to perform a reliable MLF and to enhance wine aroma complexity. In this way, subsequent studies, performed with real wine vinifications, tried to shed light on the potentialities as malolactic starters of selected O. oeni strains.

4.4. Study 4



Wine aroma profile modification by *Oenococcus oeni* strains from Rioja Alavesa region: selection of potential malolactic starters

Once the genetic typification and technological characterization of the strains had been carried out, in the present study the behaviour of selected strains in real wine vinifications was evaluated. The specific impact of each strain on the aromatic profile of the wine was also evaluated, which could lead to the production of wines with individual characteristics. Indeed, in the last decade the influence of LAB metabolism in wine has been exhaustively analysed, with special emphasis on modulating the aroma of wine (Berbegal et al., 2017; Brizuela et al., 2018). That is why MLF is considered a crucial step in red wines, and is increasingly performed on white and sparkling wines for aroma enhancement (Cappello et al., 2017). In the case of the Rioja Alavesa region, MLF commonly occurs spontaneously by indigenous LAB strains, which makes its prediction difficult and increases the risk of stuck or slow fermentation. In this sense, the six strains of *O. oeni* that had the best potential to be used as malolactic starters were subjected to further characterization in order to carry out a reliable, safe and sensorially promising MLF for the red wines of Rioja Alavesa. Thus, their fermentation vigour in lab-scale vinifications as well as their ability to modulate wine sensorial profile, among others, were evaluated.

4.4.1. Microvinifications at laboratory scale

Six *Oenococcus oeni* strains (P2A, P3A, P3G, P5A, P5C and P7B), which were characterized and selected as potential starter candidates as well as the commercial strain Viniflora OENOS (as comparative strain), were submitted to vinifications assays. After inoculation for MLF, during the first days of analysis all strains showed a significant viability decrease (**Figure 20A**). However, except for the strain P5A, after one week of analysis all the strains started recovering initial counts. This recovery was

likely possible thanks to the previous cell acclimation in synthetic medium (pH 4,5; 10% ethanol). Indeed, Brizuela et al. (2017) when compared previously acclimated and non-acclimated strains, reported no recovery of non-acclimated cells after the initial viability loss. It was noticed that the recovery of cell viability marked in some way the time needed to complete MLF. Thus, strains P2A and P3A, which had recovered a density of 10^7 CFU/ml after 10 days, were the first to complete the process. Both strains were able to exhaust malic acid in less than 15 days (**Figure 20B**). In fact, they conclude the fermentation process faster than the commercial strain Viniflora OENOS, which lasted 20 days. Strain P7B on one hand, and strains P5C and P3G on the other, successively conclude MLF, after 25 and 40 days, respectively. In all cases, strains were able to consume the 100% of initial malic acid. Differences between strains are common and could be explainable by small genetic differences. Olguín et al. (2010) evidenced that those *O. oeni* strains that performed MLF faster, showed higher expression of different stress response genes as well as an increased expression of the gene encoding malolactic enzyme (mleA). In addition, different survival strategies have been reported in *O. oeni,* as membrane fluidity adjustment for ethanol tolerance (Grandvalet et al., 2008), synthesis of stress proteins (Maitre et al., 2014) or biofilm formation capacity (Dimopoulou et al., 2015). Actually, its adaptation and resistance will define the success of the process. Furthermore, these strains that successfully finished the process showed no decrease in cell counts one week after MLF had concluded. This may be considered also as an important strain feature, since the ability of strains to implant and maintain in the wine is critical for successful MLF (Ong, 2010). By contrast, strain P5A, showed a total viability loss after 10 days of analysis, which resulted in no malic acid conversion (data not shown). Although this strain was

selected due to its potential as malolactic starter and presented good MLF performance in synthetic wine, when inoculated in real red wine its viability was totally lost despite previous acclimation. Actually, it has been already reported that some strains isolated from wine and grown in nutrient rich environment, when transferred then to nutrient scarce harsh environment they do have difficulties to survive (Ribéreau-Gayon et al., 2006). Different reasons, as the inhibitory effect of wine polyphenols over LAB and MLF or yeast and bacteria incompatibility through yeast-derived inhibitory metabolites, may be considered for P5A strain viability loss (Bartle et al., 2019; García-Ruiz et al., 2008). Regarding spontaneous fermentation, neither cell count nor malolactic activity were detected after the established time of analysis. Spontaneous MLF could lead to stuck or sluggish fermentation that may be protracted for months. Indeed, it has been demonstrated that residual nutrients, as malic acid, together with low amounts of SO_2 after AF, may boost the appearance of potential deleterious organisms, depreciating in that way wine quality (Gerbaux et al., 2009; Sumby et al., 2019).

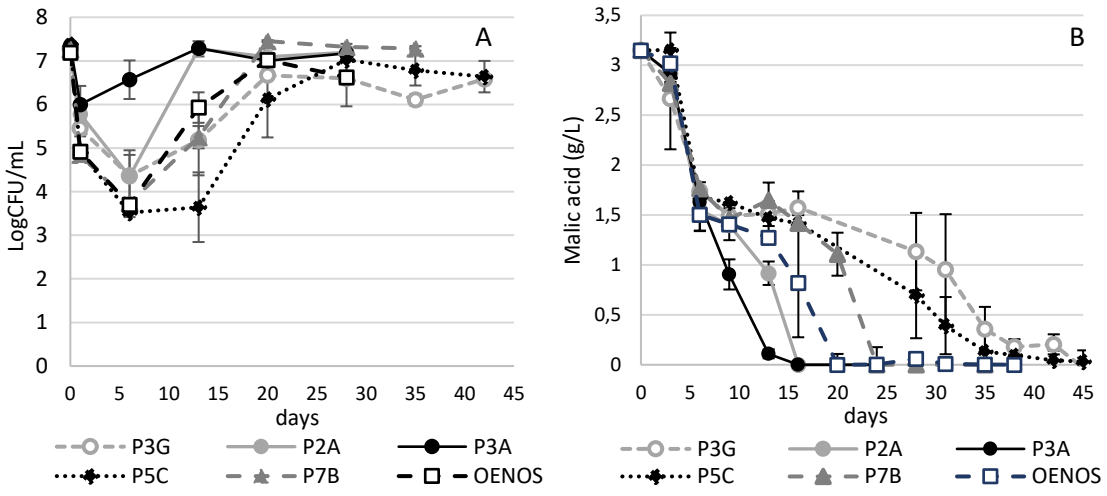


Figure 20. Malolactic fermentations evolution through strains viability (A) and malic acid consumption (B).

The comparison of the chemical analysis of wines after both successful and failed fermentations explained the main consequences of MLF, as the reduction of the total acidity and the subsequent pH increase of the resulting wine (**Table 21**). This fact shows why inoculation strategy with the proper starter is decidedly helpful to ensure an efficient and timely MLF, since these beneficial changes did not take place when failed fermentations (P5A and spontaneous fermentations) were analysed. In any case, it must be highlighted that in this case, none of the fermentations, including those unsuccessful, exceeded the sensory threshold for volatile acidity of 0,7 g/L (Swiegers et al., 2005). Additionally, after MLF it was also detected a reduction of colour intensity. It has been seen that the colour loss after MLF corresponds to polymeric pigments decrease (Burns & Osborne, 2015). Metabolites that remain after alcoholic fermentation, as acetaldehyde and pyruvic acid, have been seen to chemically react with anthocyanin compounds and form the corresponding polymeric pigments (de Freitas et al., 2017). Both pyruvic acid and acetaldehyde are typically catabolised by LAB during MLF, which prevents the formation of colour stabilizing pigments (Burns and Osborne, 2015). Devi et al. (2020) have recently underlined another possible reason for colour loss after MLF. They observed the absorption of anthocyanin compounds by *O. oeni* and *L. plantarum* strains after the breakdown of anthocyanin glucosides by these strains. In that way, colour loss was only detected after successful MLFs and not in failed fermentations.

Table 21. Chemical analysis of samples after alcoholic (AF) and malolactic fermentations (MLF).

		After MLF							
	AF	P2A	P3A	P3G	P5C	P7B	OENOS	P5A	SPONT
Ethanol (% v/v)	13,29± 0,21	-	-	-	-	-	-	-	-
pH	3,52 ± 0,01	3,76 ± 0,01	3,76 ± 0,01	3,73 ± 0	3,73 ± 0	3,74 ± 0,01	3,79 ± 0,01	3,63 ± 0,02	3,62 ± 0,01
L-malic acid (g/L)	3,14 ± 0,05	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	2,46 ± 0,06	2,71 ± 0,14
L-lactic acid (g/L)	<0,1	1,77 ± 0,04	1,81 ± 0,08	1,59 ± 0,14	1,65 ± 0,08	1,75 ± 0,02	1,69 ± 0,04	<0,1	<0,1
Volatile acidity (g/L)	0,15± 0,02	0,26± 0,01	0,27± 0,03	0,31± 0,02	0,29± 0,01	0,31± 0,03	0,29± 0,02	0,18± 0,03	0,21± 0,01
Total acidity (g/L)	5,51 ± 0,05	4,46 ± 0,06	4,23 ± 0,05	4,42 ± 0,10	4,5 ± 0	4,53 ± 0,06	4,38 ± 0,05	5,96 ± 0,05	5,88 ± 0,06
Reducing sugars (g/L)	0,36 ± 0	0,30 ± 0,49	0,33 ± 0,14	0,35 ± 0	0,36 ± 0,28	0,31 ± 0	0,31 ± 0,07	0,29 ± 0,07	0,32 ± 0,07
Total phenols (OD280)	4,25 ± 0,01	4,24 ± 0,02	4,25 ± 0,01	4,29 ± 0,01	4,30 ± 0,01	4,27 ± 0,03	4,28 ± 0,02	4,31 ± 0,03	4,33 ± 0,02
Colour intensity	0,57 ± 0	0,51 ± 0	0,53 ± 0	0,53 ± 0,01	0,52 ± 0,01	0,55 ± 0	0,52 ± 0,01	0,59 ± 0	0,63 ± 0

4.4.2. Biogenic amines (BA) and hydroxycinnamic acids (HCA) analysis

In wine, the main BAs are histamine, tyramine, putrescine and cadaverine (Konakovsky et al., 2011; Martínez-Pinilla et al., 2013). These compounds not only have health and commercial implications but also could impact wine organoleptic quality (Álvarez & Moreno-Arribas, 2014). The strains used in the present study had been previously screened by both phenotypical and molecular methods and none of them were identified as BA producer. However, in order to validate this attribute, in the present study the evolution of these compounds through all winemaking process was also tracked (**Table 22**). Putrescine and cadaverine were already present in must and remained unchanged throughout the process. Regarding agmatine, it disappeared after the AF, probably catabolised by the yeast itself. Putrescine is known to significantly contribute to total BA content in wine (Del Prete et al., 2009). Together with cadaverine and agmatine, these polyamines are commonly found in grapes as they are known to act as growth factors in plant and berry development (Broquedis et al., 1989). Nevertheless, despite this natural presence in must, it must be highlighted that the formation of any of the BAs studied was not observed, neither after AF nor MLF, confirming previous studies.

Additionally, off-flavour development is another threat that commonly arises when controlling the fermentation process. The most common compounds related to wine sensorial depreciation are volatile phenols (Chescheir et al., 2015). HCAs are known as volatile phenol precursors and they conform an important group of non-flavonoid phenolic compounds that are naturally present in wine. They are commonly esterified with tartaric acid, and during winemaking process these esters may be hydrolysed,

releasing free HCAs through cinnamoyl esterase enzymes (Santamaría et al., 2018). Thus, the evolution of free HCAs during winemaking was analysed in order to elucidate strains HCAs-release ability (**Table 23**). This strategy could be considered as an indirect measure to confirm strains cinnamoyl esterase activity. Among the analysed strains it must be underlined the performance of P5C. After MLF, this strain showed a significant increase on caffeic, coumaric and ferulic acid concentrations. Contrarily, when the rest of strains were analysed, no significant differences were detected after each MLF, since the main increase on these free HCAs took place after AF. Indeed, coumaric and ferulic acids were not detected in must, as previously reported (Ginjom et al., 2011), concluding that the main responsible in each trial for HCAs increase was the yeast itself. For its part, sinapic acid was only detected after the completion of the different MLF; however, no significant differences were detected between successful and failed MLF. It should be underlined that besides an enzymatic hydrolysis, the release of HCAs from tartaric acid is also promoted by chemical hydrolysis, a process that generally occurs slowly during winemaking and ageing period (Waterhouse et al., 2016).

Regarding resveratrol content, it must be highlighted a significant increase of this compound after AF, while similar resveratrol levels were observed after each MLF. Thus, although both chemical and enzymatic hydrolysis of resveratrol precursors (piceids) have been described (Roldán et al., 2010), in the present study, at the view of these results, yeast β-glucosidase activity may be considered to be the main responsible for such increase. Additionally, as previously stated, the release of free HCAs could lead to volatile phenols production in the presence of spoilage microorganisms, mainly *Brettanomyces*/*Dekkera* yeasts (Chescheir et al., 2015;

Santamaría et al., 2018). Increasing concentrations of coumaric, ferulic and caffeic acids, as reported for the strain P5C, could lead to the appearance of corresponding 4-vinyl and 4-ethyl derivatives. Schopp et al. (2013) reported that *Brettanomyces* lack the ability to metabolize esterified HCAs and that only can convert free HCAs to volatile phenols. Thus, the inoculation of cinnamoyl esterase negative LAB strains either after AF or in co-inoculation has proved to be a useful strategy to prevent the development of *Brettanomyces* and the volatile phenols they produce (Gerbaux et al., 2009).

Besides cinnamoyl esterase activity, among LAB species, strain-dependent activity for volatile phenol production has been also identified through the sequential expression of phenolic acid decarboxylases and vinyl phenol reductases (Santamaría et al., 2018; Silva et al., 2011). In that way, it was evaluated the presence of phenolic acid decarboxylase (pad) gene in order to confirm the inability of the selected strains to produce these non-desired compounds. None of the *O. oeni* selected strains showed amplification for pad gene (**Figure 21**), confirming in that way the results of other studies in which this pathway was rarely described in *O. oeni* (de las Rivas et al., 2009; Santamaría et al., 2018).

Table 22. Biogenic amine (mg/L) evolution through all winemaking process and after each MLF

			After MLF							
	Must	AF	P2A	P3A	P3G	P5C	P7B	OENOS	P5A	SPONT
Histamine	-	-	-	-	-	-	-	-	-	-
Agmatine	7,34 ± 0,49	-	-	-	-	-	-	-	-	-
Tyramine	-	-	-	-	-	-	-	-	-	-
Putrescine	1,82a ± 0,08	1,64ab ± 0,26	1,43b ± 0,08	1,48ab ± 0,21	1,58a ± 0,06	1,58a ± 0,07	1,55a ± 0,05	2ab ± 0,51	1,76ab ± 0,33	2,06ab ± 0,42
Cadaverine	0,12a ± 0,02	0,11a ± 0,00	0,10a ± 0,00	0,10a ± 0,10	0,10a ± 0,00	0,10a ± 0,00	0,10a ± 0,00	0,11a ± 0,02	0,1a ± 0,01	0,11a ± 0,01

Table 23. Hydroxycinnamic acids (HCAs) and resveratrol evolution (mg/L) through all winemaking process and after each MLF.

			After MLF							
	Must	AF	P2A	P3A	P3G	P5C	P7B	OENOS	P5A	SPONT
Caffeic acid	0,34a ± 0,00	0,55b* ± 0,02	0,55b ± 0,03	0,58bc ± 0,00	0,57bc ± 0,01	4,69e ± 1,30	0,57bc ± 0,00	0,58bc ± 0,00	0,63c ± 0,00	0,77d ± 0,03
Coumaric acid	-	0,42a ± 0,00	0,53b ± 0,04	0,64c ± 0,02	0,46ab ± 0,00	0,86d ± 0,02	0,46ab ± 0,02	0,46ab ± 0,01	0,47ab ± 0,06	0,47ab ± 0,01
Ferulic acid	-	0,16bc ± 0,01	0,17c ± 0,00	0,15abc ± 0,03	0,09a ± 0,01	0,52d ± 0,22	0,12ab ± 0,00	0,11a ± 0,00	0,13abc ± 0,02	0,18c ± 0,02
Sinapic acid	-	-	0,29a ± 0,01	0,29a ± 0,00	0,30a ± 0,01	0,32a ± 0,04	0,29a ± 0,01	0,28a ± 0,01	0,31a ± 0,014	0,31a ± 0,02
Resveratrol	0,11a ± 0,00	0,17bc ± 0,01	0,13ab ± 0,00	0,16bc ± 0,01	0,16bc ± 0,02	0,16bc ± 0,01	0,18c ± 0,01	0,15abc ± 0,02	0,17bc ± 0,02	0,11a ± 0,00

*Different letters among samples imply significant differences ($p<0,05$)

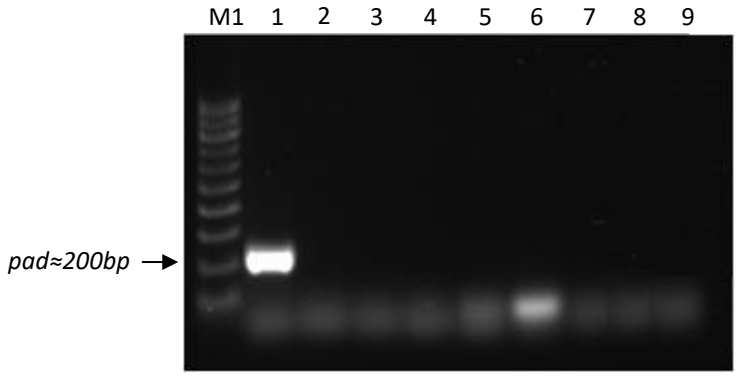


Figure 21. PCR amplifications showing the result for phenolic acid decarboxylase (pad). Ladder 100 bp (M1). 1. Positive control, internal strain *L. plantarum* LP1; 2. *O. oeni* P2A; 3. *O. oeni* P3A; 4. *O. oeni* P3G; 5. *O. oeni* P5A; 6. *O. oeni* P5C; 7. *O. oeni* P7B; 8. Viniflora OENOS; 9. negative control.

4.4.3. Analysis of volatile compounds

Finally, it was evaluated the contribution of each strain to wine aroma profile modification. Fifty volatile compounds, classified in eight main chemical families, were identified along the fermentation process. **Table 24** shows the evolution of individual compounds from must to each MLF. First differences were already identified between must and AF, and in turn between AF and each MLF. Among the compounds that were not detected in must, but appeared after AF and increased after MLF, stand out isobutanol (spiritous), 3-methylbutanol (cheese, sweet), isoamyl acetate (fruity, banana) and ethyl lactate (fruity, milky). The concentration of these compounds, as secondary aroma metabolites, depend on various factors, such as yeast and bacteria strains, the fermentation temperature, degree of aeration, and sugar and nitrogen content in the must (Cappello et al., 2017; Cortés-Diéguez et al., 2011). Conversely, several compounds that were present in must, as 1-hexanol, 3-hexen-1-ol or hexanal,

significantly decreased or disappeared after AF. These compounds are known as pre-fermentative volatiles, usually aldehydes and alcohols with six carbon atoms derived from grape fatty acids oxidation and they may infer an herbaceous character to wines (Mozzon et al., 2016). Other compounds that only appeared after the different MLFs and neither after AF nor stuck MLFs, were isoamyl lactate (fruity, milky) and α-terpineol (pine).

Among all analysed compounds, those presenting a concentration above their perception threshold are typically considered active aromatic compounds (Ferreira et al., 2019). This is the case of compounds highlighted in bold in **Table 24**. Many odorants were already present above their threshold in must, as ethyl hexanoate (green apple), ethyl decanoate (fruity, waxy), phenylacetaldehyde (floral, honey) and β-damascenone (cooked apples). These compounds, among others, have been described as aroma constituents of base wine (Ferreira et al., 2007), as they are typically found above their perception threshold in all wines. In addition, the concentration of these compounds significantly increased after AF and MLFs. Other compounds, as ethyl 9-decenoate (fruity, waxy), decanoic acid (fatty), linalool (floral), citronellol (citrus) and geraniol (rose), surpassed their odour threshold after MLF. These compounds could act as impact odorants, responsible for mainly fruity and floral descriptors. In fact, linalool and isoamyl acetate are considered part of the impact odorants of wine, since they are able to actively transmit their characteristic aroma notes to the wine (Ferreira et al., 2007). Although compounds that overcome the odour threshold are classically considered as key aroma components, it should be also underlined the capability of compounds or family of compounds, as esters and alcohols, that are present at subthreshold levels. In fact, these compounds may show

synergistic behaviour and overcome on the overall odour intensity of the mixture (de la Fuente-Blanco et al., 2020). In the same way, similar attention should be paid at threshold levels, when more interactions between odorants are expected (Lytra et al., 2013).

When volatile profile was compared between successful and stuck MLFs significant differences were elucidated (**Table 25**). Among the compounds that exhibited a significant increase after MLF, main differences were encountered when acetate esters (ethyl acetate, isoamyl acetate and phenylethyl acetates), ethyl esters (ethyl lactate, ethyl hexanoate, ethyl octanoate and ethyl decanoate), alcohols (3-methylbutanol and 2-phenylethanol), terpenoids (linalool and citronellol) and acids (hexanoic and octanoic acids) were analysed. In overall, strains P2A, P3A and P3G showed the highest increase in ethyl and acetate esters, responsible for fruity notes (Peinado et al., 2004; Tomasino et al., 2015), and conversely, the strain P7B and the commercial strain Viniflora OENOS showed higher scores for acids, alcohols and terpenoids, which are related with milky and floral aromas.

Regarding ester evolution through winemaking process, as in the present study, some authors relate MLF with an overall increase on ester concentration (Brizuela et al., 2018; Pozo-Bayon et al., 2005), while other studies report significant decreases (Gámbaro et al., 2001; Jeromel et al., 2008). Those differences may lay on strain-dependent esterase activity; indeed, as shown in the previous study these tested strains did retain esterase activity under winemaking conditions. Acetate esters, as isoamyl acetate (banana) or phenylethyl acetate (rose/fruity), responsible for characteristic fruity notes, significantly increased through MLF. Ethyl esters, as ethyl

hexanoate, ethyl octanoate or ethyl decanoate, have been also shown to increase after MLF in a strain-dependent manner, consistent with other studies (Antalick et al., 2012; Izquierdo-Cañas et al., 2016). Finally, diethyl succinate, isoamyl lactate and ethyl lactate, typical esters associated to MLF and considered the most relevant by-products of LAB metabolism (Cortés-Diéguez et al., 2015), also increased during MLF, mainly in the case of the strains P3A and P3G. Taking into account that esters are considered the main contributors of fruity aroma in wines (Lee et al., 2009), the ester profile was the most altered attribute by the different *O. oeni* strains, being ethyl esters the most relevant esters quantitatively. Although remarkable differences were not detected among strains, higher concentrations were observed for P2A and P3A strains.

In spite of the fact that several authors have failed to find any differences in higher alcohols and acids profile after MLF (Hernández-Orte et al., 2012; Izquierdo-Cañas et al., 2008), in the present study several changes in key aroma compounds were detected. Variations in total alcohols after MLF were mainly due to the contribution of 2-phenylethanol and 3-methylbutanol, largely the most abundant compounds. Their presence derives from amino acids metabolism, through phenylalanine and leucine catabolism, respectively (Smid & Kleerebezem 2014). The concentration of these compounds was significantly increased by all strains, highlighting the activity of the strain P7B, which significantly differ from the others. Both 2-phenylethanol and 3-methylbutanol have been described as relevant compounds in finished wines and responsible for aroma enhancement (Swiegers et al., 2005), conferring rose and herbaceous/spiritous character at low concentrations (Masson & Schneider, 2009;

Ugliano and Moio, 2005). However, at high concentrations they may be considered dentrimental, as they are able to mask the fruity aroma of wine (Ferreira et al., 2007).

Table 24. Evolution of volatile compounds (mg/L) through all winemaking process and after each MLF. Those compounds that after the winemaking process were detected above their odour threshold are highlighted in bold.

			After MLF							
	Must	AF	P2A	P3A	P3G	P5C	P7B	OENOS	P5A	SPONT
Alcohols										
Benzyl alcohol	0,011 ± 0,003	0,012 ± 0	0,025 ± 0,009	0,021 ± 0,002	0,028 ± 0,006	0,027 ± 0,005	0,028 ± 0,006	0,026 ± 0,008	0,068 ± 0,057	0,017 ± 0,001
2-phenylethanol	1,027 ± 0,561	17,022 ± 3,994	29,695 ± 1,003	29,003 ± 2,568	31,785 ± 1,554	29,544 ± 2,09	37,528 ± 0,273	31,881 ± 11,057	20,089 ± 4,995	27,819 ± 11,922
Isobutanol	-	0,592 ± 0,003	1,218 ± 0,085	1,053 ± 0,326	2,066 ± 0,702	1,525 ± 0,652	1,697 ± 0,23	1,185 ± 0,814	0,67 ± 0,14	0,954 ± 0,035
3-Methylbutanol	-	55,263 ± 10,298	89,359 ± 6,143	85,718 ± 18,758	102,929 ± 8,916	90,677 ± 6,76	139,487 ± 6,049	107,99 ± 35,465	102,732 ± 1,311	118,069 ± 10,8
1-Hexanol	8,588 ± 1,378	1,762 ± 0,278	2,418 ± 0,271	2,526 ± 0,305	3,004 ± 0,346	2,542 ± 0,074	3,296 ± 0,16	2,761 ± 0,319	2,183 ± 0,991	3,163 ± 0,683
3-Hexen-ol	0,275 ± 0,052	0,043 ± 0	0,082 ± 0,018	0,062 ± 0,005	0,073 ± 0,003	0,063 ± 0,005	0,1 ± 0,003	0,069 ± 0,016	0,058 ± 0,034	0,082 ± 0,028
1-Octanol	0,039 ± 0,003	0,039 ± 0,003	0,074 ± 0,009	0,07 ± 0,011	0,061 ± 0,006	0,076 ± 0,001	0,101 ± 0,017	0,078 ± 0,026	0,056 ± 0,017	0,052 ± 0,016
Acetate esters										
Ethyl acetate	0,083 ± 0,012	3,284 ± 0,79	4,849 ± 1,522	4,95 ± 0,067	5,706 ± 0,236	4,82 ± 0,129	4,218 ± 2,037	6,123 ± 0,279	1,282 ± 0,65	2,155 ± 0,08
Phenylethyl acetate	0,066 ± 0,031	0,686 ± 0,209	1,054 ± 0,021	1,078 ± 0,041	0,877 ± 0,13	0,969 ± 0,033	0,919 ± 0,15	0,879 ± 0,419	0,798 ± 0,015	0,744 ± 0,305
Isoamyl acetate	-	1,643 ± 0,381	4,498 ± 2,416	2,431 ± 0,172	2,504 ± 0,137	2,823 ± 0,246	4,351 ± 1,406	3,532 ± 2,06	1,961 ± 0,165	2,289 ± 0,448
Hexyl acetate	0,039 ± 0,009	0,156 ± 0,028	0,296 ± 0,029	0,222 ± 0,002	0,2 ± 0,044	0,176 ± 0,069	0,273 ± 0,032	0,274 ± 0,021	0,126 ± 0,087	0,179 ± 0,004
Ethyl esters										
Ethyl lactate	-	0,017 ± 0,001	0,505 ± 0,028	0,728 ± 0,169	1,045 ± 0,088	0,623 ± 0,129	0,825 ± 0,054	0,649 ± 0,06	0,079 ± 0,028	0,155 ± 0,041
Ethyl hexanoate	0,025 ± 0,005	3,793 ± 1,087	6,133 ± 1,311	7,022 ± 0,087	7,056 ± 0,254	5,844 ± 0,905	7,009 ± 2,095	5,385 ± 2,669	3,222 ± 0,344	5,951 ± 0,817
Ethyl octanoate	0,288 ± 0,162	5,343 ± 1,427	31,15 ± 4,942	24,99 ± 0,015	22,754 ± 1,842	25,914 ± 0,395	23,729 ± 3,733	28,152 ± 14,07	4,718 ± 1,95	6,908 ± 1,204
Ethyl nonanoate	-	0,02 ± 0,005	0,117 ± 0,006	0,093 ± 0,007	0,094 ± 0,027	0,102 ± 0,034	0,076 ± 0,013	0,077 ± 0,057	0,056 ± 0,05	-
Ethyl decanoate	0,692 ± 0,308	3,513 ± 0,936	14,565 ± 0,453	14,66 ± 0,328	8,909 ± 1,718	13,248 ± 0,568	9,764 ± 3,78	11,788 ± 9,662	7,337 ± 1,399	0,889 ± 0,269
Ethyl 9-decenoate	0,014 ± 0,007	0,101 ± 0,029	0,383 ± 0,002	0,398 ± 0,029	0,285 ± 0,063	0,39 ± 0,033	0,296 ± 0,099	0,317 ± 0,256	0,257 ± 0,027	0,028 ± 0,016
Ethyl hydrogen succinate	0,013 ± 0,008	0,028 ± 0,008	0,233 ± 0,081	0,633 ± 0,012	0,821 ± 0,29	0,228 ± 0,176	0,249 ± 0,186	0,09 ± 0,076	0,131 ± 0,014	0,164 ± 0,045
Diethyl succinate	0,044 ± 0,03	0,086 ± 0,008	0,468 ± 0,164	2,949 ± 0,104	4,034 ± 0,676	0,636 ± 0,124	0,604 ± 0,082	0,427 ± 0,198	0,946 ± 0,026	1,129 ± 0,189
Isoamyl lactate	-	-	0,041 ± 0,01	0,051 ± 0,01	0,075 ± 0,004	0,045 ± 0,007	0,054 ± 0,01	0,052 ± 0,014	-	-
Isoamyl decanoate	-	0,007 ± 0,002	0,02 ± 0,008	0,02 ± 0,004	0,013 ± 0,003	0,018 ± 0,003	0,012 ± 0,004	0,013 ± 0,01	-	-
Methyl nonanoate	-	0,041 ± 0,006	0,317 ± 0,319	0,09 ± 0,035	0,085 ± 0,02	0,095 ± 0,017	0,13 ± 0,041	0,117 ± 0,077	0,06 ± 0,036	0,082 ± 0,037

Table 24 continuation

			After MLF							
	Must	AF	P2A	P3A	P3G	P5C	P7B	OENOS	P5A	SPONT
Acids										
Acetic acid	0,031 ± 0,003	0,422 ± 0,1	1,236 ± 0,254	1,344 ± 0,146	1,18 ± 0,181	1,098 ± 0,115	1,521 ± 0,248	1,714 ± 0,475	0,759 ± 0,449	1,206 ± 0,08
Isobutyric acid	0,003 ± 0	0,103 ± 0,02	0,126 ± 0,024	0,142 ± 0,005	0,195 ± 0,069	0,139 ± 0,004	0,203 ± 0,037	0,171 ± 0,009	0,187 ± 0,026	0,198 ± 0,004
Butanoic acid	-	0,017 ± 0,003	0,028 ± 0,003	0,025 ± 0,006	0,034 ± 0,001	0,027 ± 0,006	0,048 ± 0,014	0,042 ± 0	0,026 ± 0,007	0,118 ± 0,01
3-Methyl butryric acid	0,004 ± 0,001	0,27 ± 0,057	0,379 ± 0,044	0,414 ± 0,034	0,492 ± 0,03	0,42 ± 0,001	0,634 ± 0,116	0,513 ± 0,072	0,475 ± 0	0,515 ± 0,033
Hexanoic acid	0,13 ± 0,034	0,502 ± 0,101	0,91 ± 0,019	0,872 ± 0,019	0,964 ± 0,093	0,92 ± 0,003	1,128 ± 0,052	0,959 ± 0,322	0,741 ± 0,205	1,004 ± 0,041
Octanoic acid	0,163 ± 0,076	1,985 ± 0,587	4,421 ± 0,429	3,861 ± 0,132	3,832 ± 0,008	4,096 ± 0,059	4,37 ± 0,16	3,827 ± 1,577	2,989 ± 1,133	3,422 ± 0,411
Nonanoic acid	0,017 ± 0	0,032 ± 0,002	0,065 ± 0,017	0,057 ± 0,002	0,059 ± 0,007	0,069 ± 0,017	0,065 ± 0,011	0,066 ± 0,04	0,063 ± 0,019	0,032 ± 0,006
Decanoic acid	0,118 ± 0,054	0,857 ± 0,305	2,622 ± 0,228	2,487 ± 0,084	1,491 ± 0,223	2,257 ± 0,071	1,893 ± 0,914	1,747 ± 1,317	1,604 ± 0,479	1,302 ± 0,211
Dodecanoic acid	0,003 ± 0	0,012 ± 0,006	0,051 ± 0,014	0,036 ± 0,001	0,021 ± 0,009	0,035 ± 0,007	0,027 ± 0,017	0,027 ± 0,026	0,025 ± 0,024	0,019 ± 0,001
Terpenes										
Linalool	0,003 ± 0	0,029 ± 0,001	0,068 ± 0,01	0,06 ± 0,003	0,086 ± 0,017	0,068 ± 0	0,095 ± 0,007	0,071 ± 0,03	0,051 ± 0,027	0,06 ± 0,009
α-Terpineol	-	-	0,011 ± 0,005	0,007 ± 0,002	0,011 ± 0,008	0,013 ± 0,009	0,017 ± 0,006	0,014 ± 0,002	0,024 ± 0,008	0,023 ± 0,002
Citronellol	-	0,073 ± 0,015	0,122 ± 0,009	0,116 ± 0,005	0,12 ± 0,002	0,11 ± 0,006	0,118 ± 0,028	0,115 ± 0,038	0,068 ± 0,045	0,055 ± 0,005
Geraniol	0,009 ± 0,002	0,013 ± 0,002	0,017 ± 0,005	0,014 ± 0,002	0,02 ± 0,001	0,017 ± 0,003	0,014 ± 0,003	0,016 ± 0,002	0,016 ± 0,007	0,007 ± 0,002
Nerolidol	0,002 ± 0,001	0,016 ± 0,005	0,09 ± 0,009	0,08 ± 0	0,074 ± 0,025	0,127 ± 0,018	0,12 ± 0,085	0,083 ± 0,074	0,082 ± 0,011	0,192 ± 0,017
Aldehydes										
Hexanal	2,569 ± 0,115	-	-	-	-	-	-	-	-	-
Benzaldehyde	0,057 ± 0,011	0,016 ± 0,006	0,062 ± 0,018	0,094 ± 0,005	0,058 ± 0,001	0,112 ± 0,031	0,05 ± 0,053	0,067 ± 0,018	0,094 ± 0,09	0,03 ± 0,004
Phenylacetaldehyde	0,018 ± 0,001	0,004 ± 0	0,008 ± 0,001	0,006 ± 0,001	0,014 ± 0,001	0,009 ± 0	0,01 ± 0,002	0,006 ± 0,001	0,007 ± 0,002	-
Phenols										
4-ethyl phenol	0,008 ± 0,001	0,005 ± 0	-	-	-	-	-	-	-	-
Phenol, 2,4-tertbutyl	0,039 ± 0,008	0,231 ± 0,063	0,555 ± 0,125	0,434 ± 0,008	0,251 ± 0,029	0,515 ± 0,019	0,452 ± 0,195	0,35 ± 0,32	0,238 ± 0,062	0,31 ± 0,131
Acetals										
1-Ethoxy-1-methoxyethane	-	0,024 ± 0,005	-	-	-	-	-	-	1,034 ± 0,955	4,868 ± 0,176
1,1-Diethoxyethane	-	-	-	-	-	-	-	-	2,242 ± 0,95	11,334 ± 1,204
Others										
β-damascenone	0,001 ± 0,001	0,033 ± 0,002	0,128 ± 0,005	0,121 ± 0,009	0,141 ± 0,003	0,15 ± 0,02	0,147 ± 0,007	0,143 ± 0,077	0,098 ± 0,073	-
2,3-Butanediol	0,003 ± 0,001	0,28 ± 0,077	0,377 ± 0,115	0,408 ± 0,035	0,555 ± 0,082	0,402 ± 0,031	0,606 ± 0,076	0,485 ± 0,037	0,373 ± 0,159	0,587 ± 0,022
γ-butyrolactone	-	0,038 ± 0,01	0,055 ± 0,015	0,07 ± 0,002	0,081 ± 0,04	0,063 ± 0,03	0,136 ± 0,053	0,099 ± 0,019	0,062 ± 0,007	0,101 ± 0,015
Methionol	0,008 ± 0,003	0,158 ± 0,039	0,247 ± 0,009	0,445 ± 0,073	0,259 ± 0,01	0,239 ± 0,012	0,319 ± 0,038	0,298 ± 0,063	0,247 ± 0,009	0,329 ± 0,02

Table 25. One-way ANOVA test showed significant differences for each compound during all winemaking process and each MLF performed. Different letters imply significant differences (p<0,05)

			After MLF							
	Must	AF	P2A	P3A	P3G	P5C	P7B	OENOS	P5A	SPONT
Alcohols										
Benzyl alcohol	a	a	a	a	a	a	a	a	a	a
2-phenylethanol	a	b	de	de	de	de	e	e	bc	cd
Isobutanol		a	abcd	abc	d	bcd	cd	abcd	ab	abc
1-hexanol	a	b	bc	bc	bc	bc	c	bc	bc	bc
3-hexen-ol	a	b	bc	bc	bc	bc	c	bc	bc	bc
1-octanol	a	a	bc	bc	b	bc	d	cd	ab	b
Acetate esters										
Ethyl acetate	a	bcd	cd	cd	d	cd	cd	d	ab	abc
Phenylethyl acetate	a	c	e	e	cde	de	cde	cde	cd	b
Isoamyl acetate		a	b	ab	ab	ab	b	ab	a	ab
Hexyl acetate	a	ab	b	ab	ab	ab	b	b	ab	ab
Ethyl esters										
Ethyl lactate	-	a	b	b	c	b	bc	b	a	a
Ethyl hexanoate	a	bc	d	d	d	cd	d	cd	b	cd
Ethyl octanoate	a	ab	d	cd	c	cd	c	d	ab	b
Ethyl nonanoate	-	a	b	b	b	b	b	b	-	-
Ethyl decanoate	a	ab	d	d	bcd	d	cd	d	abc	a
Ethyl 9-decenoate	a	ab	c	c	c	c	c	c	bc	a
Ethyl hydrogen succinate	a	a	a	b	b	a	a	a	a	a
Diethyl succinate	a	a	b	c	d	b	b	b	b	b
Isoamyl lactate	-	-	a	a	b	a	a	ab	-	-
Isoamyl decanoate	-	a	b	b	b	b	b	b	b	b
Methyl nonanoate	a	a	a	a	a	a	a	a	a	a
Acids										
Acetic acid	a	ab	d	d	cd	cd	de	e	bc	cd
Isobutyric acid	a	b	bc	bcd	cd	bcd	d	cd	cd	cd
Butanoic acid		a	a	a	ab	a	b	b	a	c
3-Methyl butryric acid	a	b	bc	bc	cd	bc	d	d	b	cd
Hexanoic acid	a	b	cd	cd	de	cd	e	de	c	cd

Table 25 continuation

			After MLF							
	Must	AF	P2A	P3A	P3G	P5C	P7B	OENOS	P5A	SPONT
Acids										
Octanoic acid	a	b	d	cd	cd	cd	d	cd	bc	b
Nonanoic acid	a	ab	c	bc	c	c	c	c	bc	ab
Decanoic acid	a	ab	e	e	bcd	de	cde	bcde	bc	bcd
Benzoic acid	a	a	b	b	b	b	b	b	b	b
Dodecanoic acid	a	ab	c	bc	ab	bc	abc	abc	abc	ab
Terpenes										
Linalool	a	b	cd	bcd	cd	cd	d	cd	bc	bc
α-terpineol	-	-	ab	a	ab	ab	ab	ab	b	b
Citronellol	-	a	b	b	b	b	b	b	a	a
Geraniol	a	a	a	a	a	a	a	a	a	a
Nerolidol	a	b	c	c	c	c	c	c	c	c
Aldehydes										
Hexanal	a	-	-	-	-	-	-	-	-	-
Benzaldehyde	a	a	a	a	a	a	a	a	a	a
Phenylacetaldehyde	a	b	bc	bc	d	c	c	bc	bc	-
Acetals										
1-Ethoxy-1-methoxyethane	a	-	-	-	-	-	-	-	b	c
1,1-Diethoxyethane	-	-	-	-	-	-	-	-	a	b
Others										
β-damascenone	a	a	b	b	b	b	b	b	b	b
2,3-Butanediol	a	b	bc	bcde	cde	bcd	d	cde	bc	cd
γ-butyrolactone		a	a	a	a	a	a	a	a	a
Methionol	a	b	bcd	e	bcd	bcd	d	d	bc	cd

Regarding the evolution of volatile fatty acids, due to the important sensory properties of these compounds (cheesy/rancid), their analysis in wines following MLF is highly relevant. Significant increases were reported for hexanoic, octanoic and decanoic acids, in line with other studies in which similar results were obtained when comparing wines following or not MLF (Costello et al., 2012; Pozo-Bayón et al., 2005). Because of their low aroma threshold, their presence may significantly contribute to wine sensorial complexity. However, as higher alcohols, excessive quantities may negatively affect wine quality (Ferreira et al., 2007). In the same way, when terpenes were analysed, significant differences were reported between stuck and successful MLFs. These compounds, which are considered key contributors for floral attributes, are present in grapes as both free and glycosylated forms, and they may increase through winemaking process due to yeast and bacteria glycosidase activities (Michlmayr et al., 2012). Among all the strains, it was observed a significant generalised increase of citronellol (citrus), and a particular significant increase of α-terpineol, linalool and geraniol in the case of the strains P3A, P7B and P3G, respectively.

Another compound which increased after MLF was methionol (3-(methylthio)-propan-1-ol), mainly when the strain P3A was analysed. Differences in sulphur-volatile compounds have already been described after MLF by *O. oeni* (Vallet et al., 2008). Actually, methionol is considered the most relevant sulphur-volatile compound in wines and it is known to contribute to wine aroma complexity, although at high concentrations may infer reductive flavour (Cappello et al., 2017). Regarding the spontaneous MLF test, unexpected volatile profiles were obtained. Although the process was not triggered, the obtained volatile profile did not fit those observed for

the must nor the one after AF. This aromatic profile and the obtained in the test with the P5A strain, unable to perform the MLF, showed characteristics halfway between that observed in the samples after AF and that observed in samples in which the MLF took place.

Generally, spontaneous fermentation showed higher volatile values compared to P5A. One possible explanation may be that this sample was not filter-sterilised and thus, residual yeast activity could have modified in some extent aroma profile. Also, protracted MLF could lead to oxidation processes in which non-desirable compounds may appear. It is the case of acetaldehyde acetals, detected in both spontaneous fermentation and P5A cases, but not in any of the accomplished MLFs. These compounds are commonly found in spirit or fortified wines due to a prolonged oxidation and ageing process. Their formation is catalysed by acid catalysis and they infer a liquorice/green character (Cheynier et al., 2010). In that way, it is arisen the importance of the protective role of a rapid and controlled MLF followed by a stabilization to prevent wine deterioration. Finally, it must be also underlined that the volatile phenols analysed (4-vinyl and 4-ethyl derivatives) were not detected after any MLF. These compounds, as previously stated, are responsible for the depreciation of the organoleptic quality of wines. They usually arise after the sequential bioconversion of the corresponding hydroxycinnamic acids through the activity of *Bretttanomyces bruxellensis* (Chescheir et al., 2015; Santamaría et al., 2018). In that way, the inoculation of tailored selected MLF starters as biocontrol agents has been described as a useful strategy to prevent both growth of this species and the appearance of these off-flavours (Berbegal et al., 2018; Gerbaux et al., 2009). In the present study, the inoculation of cinnamoyl esterase negative strains (P2A, P3A, P3G

and P7B) could have led to the non-detection of these compounds. In addition, the absence of *Brettanomyces,* since in the positive strain P5C the appearance of these compounds did not occur either, did prevent the formation of volatile phenols. Brettanomyces is mainly isolated after AF and during ageing process, thus, correct hygienic practices are highly relevant in both stages in order to reduce or prevent its appearance (Berbegal et al., 2018).

As already stated, not only aroma compounds showing concentrations above their odour threshold level must be considered. The presence of compounds that build different chemical families, such as esters, alcohols, lactones or acids, and which present similar sensorial properties could synergically contribute to different aroma intensities. In addition, interactions between compounds, as aroma inhibitions or enhancements and synergistic effects, must also be considered when defining wine complexity (Ferreira et al., 2007). Rioja Alavesa young wines are mainly characterized by tree fruit and red berry notes together with floral aromas. In the present study, it has been confirmed strains ability to significantly increase the concentration of compounds responsible for floral and fruity notes, as terpenoid compounds (linalool, citroneloll and geraniol), acetate esters (isoamyl and phenylethyl acetate) and ethyl esters (ethyl hexanoate, octanoate and decanoate), among others. Thus, these strains would be able to maintain and enhance the singularities of Rioja Alavesa red wines.

In order to elucidate a comprehensible overview of the potential relationship between the main aromatic compounds and the different strains, a Principal Component Analysis (PCA) was carried out. The first two principal components gathered the 75% of variance. The first component was correlated with linalool, β-damascenone,

hexanoic acid, 2-phenylethanol and octanoic acid, whereas the second component was characterized by 3-methylbutanol, ethyl nonanoate, isobutyric acid, butanoic acid, phenylacetaldehyde, ethyl decanoate, α-terpineol and isoamyl decanoate. Among the strains that successfully finished the MLF, strains were discriminated in two major groups along the PC2 (**Figure 22**). One group was positioned in right-up plot (P2A, P3A, P3G and P5C), while the other was stablished in right-bottom plot (P7B and OENOS). Both groups were clearly differentiated from unfinished MLFs and must and AF samples. When those compounds that exhibited major correlations were plotted along with the strains (**Figure 22**), esters (ethyl nonanoate, ethyl decanoate, diethyl succinate and ethyl lactate) showed the best scores for P2A, P3A, P3G and P5C strains. For P7B and Viniflora OENOS higher chemical diversity was found, with acids (hexanoic acid), C13-norisoprenoids (β-damascenona), terpenes (linalool) and alcohols (2-phenylethanol) showing the best correlation. The cluster formed by P2A, P3A, P3G and P5C is mainly characterized by compounds related to fruity notes, while the formed by P7B and the commercial strain Viniflora OENOS is related to floral, dairy and spiritous aroma compounds. In this sense, the inoculation of combined starters that present complementary characteristics (strains belonging to one cluster with the other) to obtain complex wines with novel characteristics may be another strategy to pursue.

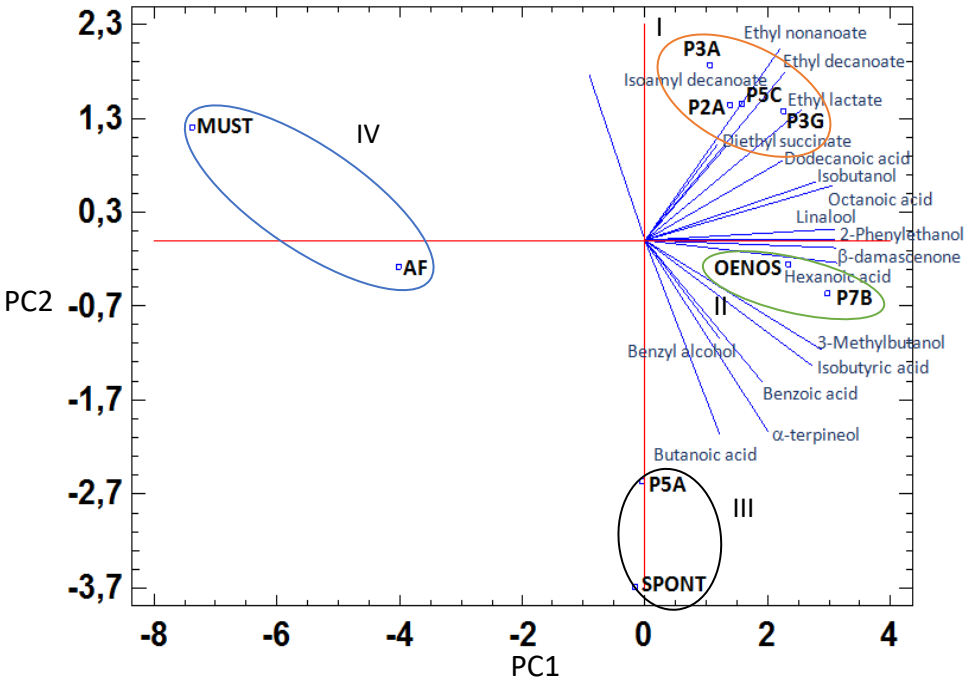


Figure 22. Principal component analysis (PCA) of volatiles profiles provided a differentiation between successful MLFs (I and II), failed MLFs (III) and previous winemaking stages (must and alcoholic fermentation (AF); (IV)). Corresponding loadings showed which aromatic compounds contribute to the differentiation of successful MLFs.

Rioja Alavesa region, due to its geographical features, ensures a good grape maturation and a signature freshness and good acidity in wines (Etaio et al., 2009). Tempranillo varietal is the utmost grape variety used, and although Tempranillo wine may differ among the different regions, in general, Rioja Alavesa young wine has a mature fruit, floral and balsamic (licorice) character (Etaio et al., 2007). In this sense, the proper selection of indigenous *O. oeni* strains with marked fruity character would enhance the typicity of floral/fruity wines from this region. Except for strains P5A, which was not able to accomplish the fermentation process, and P5C, that significantly

contribute to volatile phenol precursor release in wine, the remaining four strains showed promising oenological properties. The absence of biogenic amines in the resulting wines, together with the differential influence of each strain in wine sensory profile modulation, provide an insight of the prospective use of selected strains. However, it was still necessary to carry out a final study in order to correlate both wine aromatic profile and sensorial perception, and thus, firmly elucidate the potential of these strains as an additional strategy for high-quality wines production in Rioja Alavesa region.

4.5. Study 5

Effect of inoculation strategy with autochthonous *Oenococcus oeni* strains on aroma development in Rioja Alavesa Tempranillo wines: within the framework of a novel starter selection

The last step in the selection process of novel malolactic starters tried to elucidate the influence that the selected strains had on the sensorial perception of wines as well as the suitability of any of the strains to work in large scale fermentations at winery. In the last years, a trend for the selection of autochthonous strains, which are already adapted to regional winemaking conditions, is gaining special attention in order to perform a reliable MLF (Franquès et al., 2017; Petruzzi et al., 2017). Each winemaking area has its own wine characteristics, determined by the grape cultivar, climate, geology, winemaking practices and recently, the contribution of indigenous bacterial ecology to the specific wine´s terroir has also been evidenced (Gilbert et al., 2014; Zarraonaindia et al., 2015). Furthermore, few studies have investigated the influence of yeast and bacteria interactions on wine aroma profile as well as the implications of different inoculation strategies in real wine vinifications. In this sense, in this study different inoculation strategies were compared and, strains implantation capacity, as well as the chemical composition, biogenic amine evolution and the different aromatic profiles of wines and their sensorial perception were evaluated. Finally, after the entire selection process of potential malolactic starters, the strain that showed the best characteristics was tested in winery to confirm its suitability to perform a safe and reliable MLF.

4.5.1. Fermentation assays: co-inoculation vs sequential

MLF naturally takes place after AF, however, the success of MLF is often difficult to achieve due to wine harsh conditions. In that way, spontaneous MLF is not always ensured and the inoculation of commercial starters might offer an option to achieve a reliable MLF. In this sense, winemakers may follow traditional inoculation, once AF has finished, or simultaneous inoculation (generally 24 h after yeast inoculation)

(Izquierdo-Cañas et al., 2020). In the present study, four *O. oeni* strains (P2A, P3A, P3G and P7B), and the commercial strains Viniflora OENOS, which were previously characterized at laboratory scale microvinifications and selected as potential malolactic starters, were submitted to vinifications assays with different inoculation strategies.

During the onset of alcoholic fermentation (AF), in those batches that followed co-inoculation strategy it was perceived a slightly lower kinetics for AF (**Figure 23**). At day 6 of AF, in co-inoculated batches remained twice the concentration of reducing sugars in comparison with the conventional AF. However, this fact did not greatly influence the consecution of AF in co-inoculated batches, as they needed in average one day more to finish the AF (< 2g/L reducing sugars).

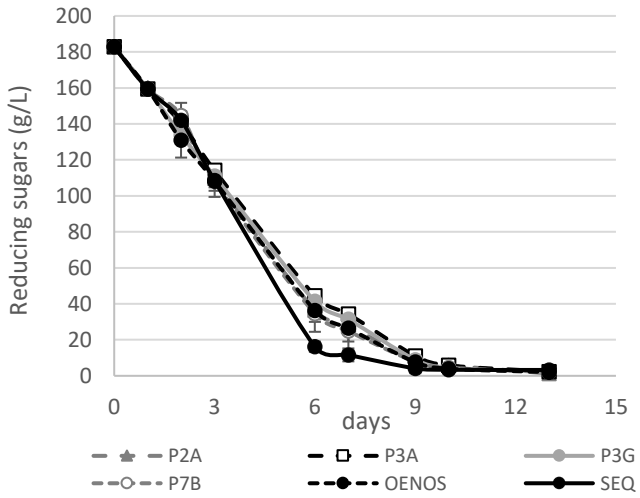


Figure 23. Evolution of alcoholic fermentation for the different co-inoculations and standard process (SEQ)

Thus, the presence of *O. oeni* strains did not compromise yeast viability and AF, as already reported by other authors (Izquierdo-Cañas et al., 2020; Mendoza et., 2011).

Major differences were encountered when all the vinification process (AF and MLF) was taken into account. It was observed that the co-inoculation strategy significantly led to an earlier achievement of the overall fermentation process. In the case of strains P2A and OENOS, MLF finished 12 days after being inoculated (**Figure 24A**). Taking into account that the AF fermentation took 11 days, it can be concluded that both AF and MLF finished at the same time. That is, in merely 13 days those wines could follow stabilization steps and bottling. The remaining strains needed more time to conclude MLF. In the case of P7B, it took 19 days to conclude, and the strains P3A and P3G strains needed 28 and 36 days, respectively (**Figure 24A**). Whereas P7B started MLF before AF had finished, P3A and P3G strains started the process once AF had ended. In these cases, the yeast-bacteria compatibility may prevent the correct development of *O. oeni* strains, which cannot compete for the nutrients, and require the depletion of the yeasts to successfully complete the process. Indeed, nutrient exhaustion by yeast as well as the production and release of inhibiting compounds, as SO_2, medium chained fatty acids (MCFA) and bioactive peptides may represent a threat for LAB development (Branco et al. 2014; Du Plessis et al., 2017; Rizk et al., 2018). However, all strains were able to successfully exhaust the malic acid present. In addition, co-inoculated strains did not only conclude the vinification process earlier, but also the MLF itself (the time needed for malic acid exhaustion after inoculation). When sequential inoculation was performed, initial reduction of strains viability led to slow MLF, a trend that has been frequently observed when inoculated sequentially (Brizuela et al., 2017; Cañas et al., 2012). In that way, P2A and OENOS strains needed 5 and 7 more days respectively to finish MLF, while P3A, P3G and P7B strains invested about 10 more days to complete the process (**Figure 24B**). That is, the low nutrient

content and the high ethanol concentration of the medium after AF, together with the potential presence of yeast-derived inhibiting metabolites, they all had a significant effect upon sequential inoculation success (Balmaseda et al., 2018). In the case of co-inoculation, a greater availability of nutrients as well as a progressive adaptation to the wine conditions could explain its better results. This fact is clearly reflected when cell viability was analysed. It was noticed that the recovery of cell viability marked in some way the time needed to complete MLF (**Figure 24C** and **24D**). In that way, MLF induction took place when strains recovered counts of 10^6 CFU/mL. In the case of co-inoculation, high counts were kept, except for P3G which therefore needed more time to conclude the process. In sequential inoculation, strains viability showed a marked decrease when inoculated. In these cases, the ability to recover high counts determined the induction and achievement of MLF. Previous acclimatization influenced this recovery, and although inoculation at 10^7 CFU/mL should be enough for malic acid exhaustion, inoculation in a greater density could be beneficial in the case of sequential inoculation (Brizuela et al., 2017). All in all, strains adaptation ability and resistance to wine harsh conditions will define the success of the process. Although most winemakers opt for sequential inoculation seeking for the absence of negative interactions between yeast and bacteria (Costello et al., 2006), in all fermentations trials, batches that undergo sequential inoculation took between 20-30 more days to conclude all the winemaking process. Although, co-inoculation does not guarantee that AF and MLF take place simultaneously, and MLF may be performed after AF, even in this case the overall fermentation time was reduced, probably due to an early adaptation of bacteria to the medium since the beginning of AF.

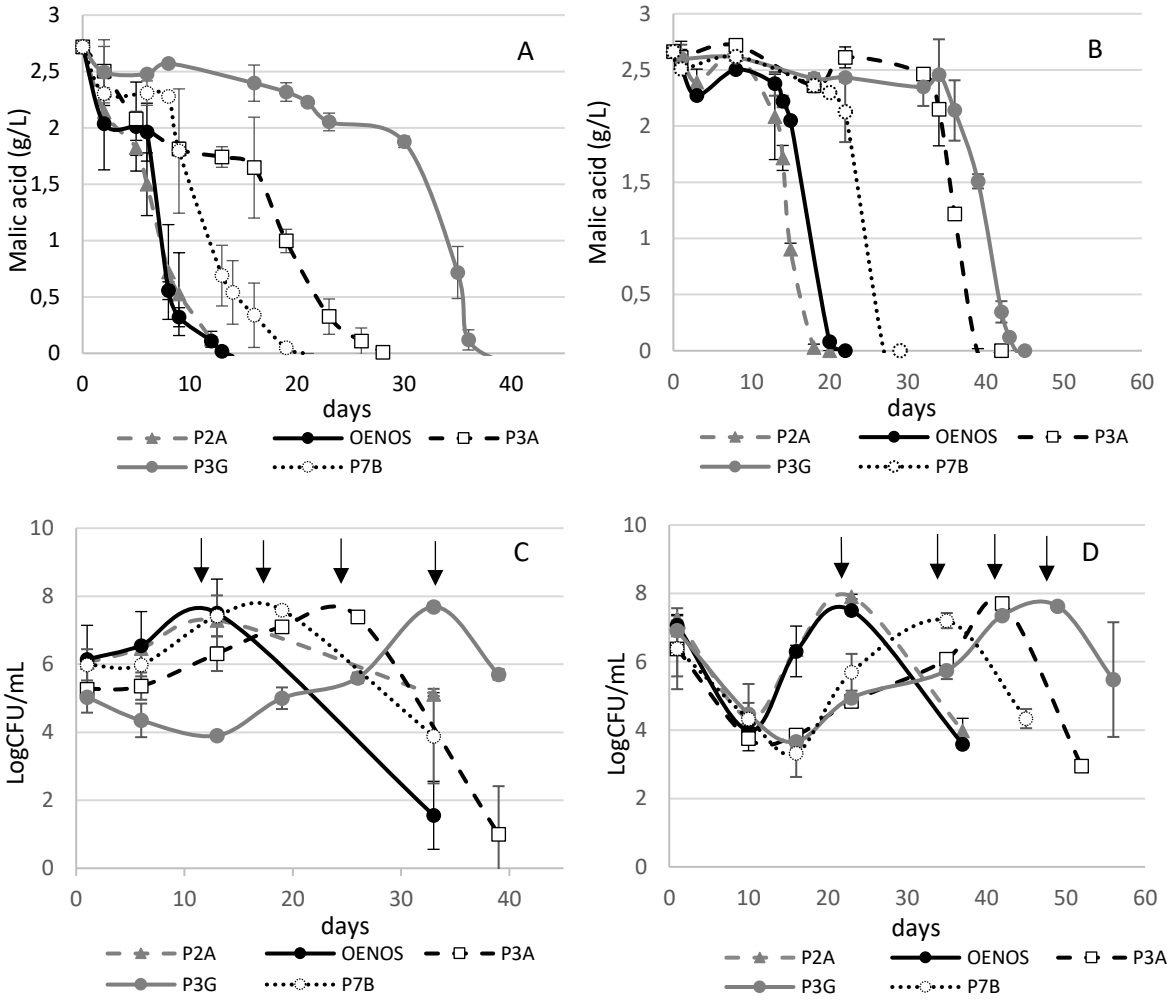


Figure 24. Evolution of malolactic fermentation and viability of *O. oeni* strains for both co-inoculation (A and C) and sequential (B y D) inoculation. Arrows indicate when MLF had conclude.

Advantages of co-inoculation include a reduction of total fermentation time and better control over MLF, which may lead to an early implantation and dominance of the inoculated strains keeping out other undesirable bacteria (Azzolini et al., 2010; Brizuela et al., 2018; Garofalo et al., 2015; Zapparoli et al., 2009). Furthermore, these wines, after successful co-inoculation, take benefit as they are ready for early stabilization (racking, fining, and SO2 addition), increasing in that way microbiological stability and processing efficiency. In addition, from a cost perspective, it could derive

in a lower consumption of energy resources as well as an earlier product launch which may have a positive effect on the costs of the winery. For all these reasons, in the last years the simultaneous inoculation of yeast and bacteria is gaining popularity (Versari et al., 2016).

Regarding spontaneous fermentation, neither cell count nor malolactic activity were detected in the established time of analysis. As seen, spontaneous MLF may lead to stuck or sluggish fermentation in which MLF can be protracted for months. Winemaking conditions, as low pH, high ethanol and SO_2 concentrations and the low nutrients available could difficult the achievement of spontaneous MLF. In that way, it does not ensure consistent outcomes in terms of MLF completion, organoleptic profile or resulting wine quality (Sumby et al., 2019). In addition, the protracted induction of MLF may boost the appearance of potential deleterious organisms, as *Brettanomyces*, acetic acid bacteria or undesirable LAB (Bartowsky & Pretorius 2008; Gerbaux et al. 2009) which may lead to the appearance of off-flavours and health concern metabolites (e. g. biogenic amines) (Sumby et al., 2019). In this way, in order to know the potential presence of contaminants and elucidate the implantation capacity of *Oenococcus* strains, in both co-inoculation and sequential strategies, more than 400 isolates were taken during all the winemaking process. RAPD-PCR patterns were obtained at each stage for all the strains (**Figure 25**). The reproducibility study established a cut-off level of similarity of 90%. For all strains and both inoculation strategies the implantation was 100% during all the process. It must be stated that in spontaneous fermentation it was not detected the presence of any microorganisms and thus, the implantation of *O. oeni* strains was eased in both inoculation scenarios.

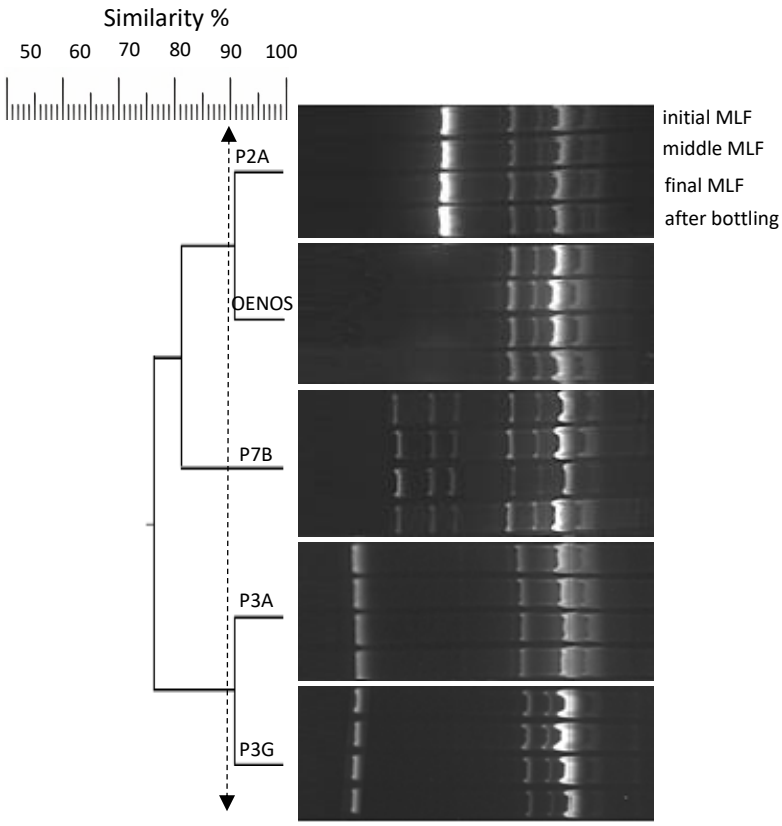


Figure 25. RAPD-PCR profiles obtained for each strain at each sampling steps shown. A single pattern per stage is shown as all the RAPD-PCR profiles elucidate the same pattern.

4.5.2. Chemical properties and biogenic amines evolution in the produced wines

Regarding chemical analysis of the different fermentation trials, main consequences of MLF were clearly observed (**Table 26**). A slight increase of the pH and volatile acidity together with a reduction of total acidity were perceived. These changes did not occur obviously when failed spontaneous fermentation was analysed. Volatile acidity was not higher in co-inoculated batches, as frequently thought with bacteria co-inoculation. However, it should be noted that the heterofermentative metabolism of *O. oeni* could lead, under the co-metabolism of citric acid and sugars, to produce wines

with elevated volatile acidity due to higher production of acetic acid (Costello et al., 2006). In this sense, this study confirmed that MLF could be performed in the presence of fermentable sugars without an increase in the concentration of the volatile acidity, as observed by Abrahamse and Bartowsky (2012) or Izquierdo-Cañas et al. (2020), and in contrast to other studies in which a significant increase was observed when simultaneous AF and MLF took place (Garofalo et al., 2015; Knoll et al., 2012). This contradictory data may rely on specific yeast and bacteria culture selection. Anyway, none of the fermentations exceeded the sensory threshold for volatile acidity of 0,7 g/L (Swiegers et al., 2005) and all of them showed typical values for Tempranillo wine (Izquierdo et al., 2008). Total acidity decrease was sharpened with sequential inoculation strategy, which may be due to the co-precipitation of tartaric acid after prolonged fermentation period. This fact could indicate that co-inoculated wines may keep more freshness and vivacity.

As expected, citric acid was significantly reduced after MLF (Olguín et al., 2009), however, inoculation timing did not affect its evolution. The similar reduction of citric acid among strains resulted in a similar increase of the volatile acidity. Regarding colour intensity, a differential behaviour was observed between co-inoculation and sequential strategies. In co-inoculated batches, it was observed a reduction of colour intensity after MLF, which corresponds to polymeric pigments decrease (Burns & Osborne, 2015). This may be due to the metabolization of compounds related with pigments stabilization, as pyruvic acid and acetaldehyde, since these compounds chemically react with anthocyanin compounds and form the corresponding polymeric pigments (Burns & Osborne, 2015; de Freitas et al., 2017). Reduction of colour intensity in co-inoculated wines was also observed in other studies in which both

inoculation strategies were compared. Abrahamse and Bartowsky (2012) and Cañas et al. (2015) perceived a loss in colour intensity, showing a reduction in the concentration of polymeric pigments. In sequential inoculation, in which MLF was prolonged, the stabilization/formation of these polymeric pigments may cause a higher colour intensity. In addition, prolonged oxidation processes also increase the level of polymeric pigments and their stability (de Freitas et al., 2017). Thus, it is in the spontaneous fermentation, where there was not microbial activity and therefore pyruvic acid and acetaldehyde had not been metabolized, and the oxidation process was significantly longer, where there was a relevant increase in wine colour intensity.

Although the inability to produce biogenic amines was previously confirmed for the present strains, it was decided to track the evolution of these compounds in order to elucidate the presence of potential spoilage microorganisms (**Table 27**). In wine, histamine, tyramine, putrescine and cadaverine are considered the main BAs (Martínez-Pinilla et al., 2013). Both health and commercial implications made the analysis of these compounds of utmost importance in wine (EFSA, 2011). Agmatine, histamine and tyramine were not detected neither in must nor through the winemaking process. Putrescine and cadaverine, however, were already present in must and maintained similar values during the process. They suffered a significant increase after alcoholic fermentation, probably due to extraction from grape skins during maceration and not due to yeast metabolism. Timing of inoculation did not affect BA concentration, although putrescine levels were reduced in sequential inoculation for P3G, P7B and OENOS strains. No increase was detected after 4 months of bottling, indeed, a slight decrease of putrescine and cadaverine was detected after this period. It could be attributable to a coprecipitation with the lees or to the

degradation of these compounds in others (Del Prete et al., 2009; Marques et al., 2008). All in all, the fact that biogenic amine concentration did not increase through all vinification process indicated the absence of potential spoilage microorganisms.

Table 26. Chemical analysis of wines following co-inoculation and sequential inoculation

			Co-inoculation MLF				
	Must	AF	P2A	P3A	P3G	P7B	OENOS
Ethanol (% v/v)	-	12,3 ± 0,01	-	-	-	-	-
pH	3,44 ± 0,02	3,46 ± 0,01	3,57 ± 0,01	3,57 ± 0	3,57 ± 0,01	3,66 ± 0,02	3,63 ± 0,01
Lactic acid (g/L)	<0,1	<0,1	1,27 ± 0,07	1,18 ± 0,09	1,14 ± 0,01	1,22 ± 0,05	1,25 ± 0,1
Malic acid (g/L)	2,72 ± 0,05	2,66 ± 0,11	<0,1	<0,1	<0,1	<0,1	<0,1
Citric acid (mg/L)	156,92 ± 3,47	148,72 ± 3,75	35,51 ± 3,36	27,8 ± 2,39	30,77 ± 2,95	30,06 ± 0,04	28,6 ± 0,36
Total acidity (g/L)	4,54 ± 0,05	4,73 ± 0,11	4,09 ± 0,05	4,16 ± 0,05	4,2 ± 0	4,2 ± 0,11	4,09 ± 0,16
Volatile acidity (g/L)	<0,1	0,27 ± 0,05	0,38 ± 0,05	0,35 ± 0,07	0,33 ± 0,05	0,41 ± 0,08	0,36 ± 0,09
Colour intensity	0,948 ± 0,015	0,828 ± 0,001	0,701 ± 0	0,767 ± 0,001	0,738 ± 0,002	0,75 ± 0	0,707 ± 0
Total polyphenols (OD280)	4,351 ± 0,005	4,52 ± 0,044	4,476 ± 0,024	4,507 ± 0,03	4,465 ± 0,038	4,531 ± 0,069	4,496 ± 0,062

	Sequential MLF					
	P2A	P3A	P3G	P7B	OENOS	SPONT
Ethanol (% v/v)	-	-	-	-	-	-
pH	3,64 ± 0,04	3,48 ± 0,01	3,5 ± 0,01	3,62 ± 0,02	3,6 ± 0	3,41 ± 0,01
Lactic acid (g/L)	1,29 ± 0,01	1,42 ± 0,07	1,31 ± 0,04	1,45 ± 0,01	1,24 ± 0,01	< 0,1
Malic acid (g/L)	<0,1	<0,1	<0,1	<0,1	<0,1	2,62 ± 0,03
Citric acid (mg/L)	27,44 ± 0,67	26,98 ± 0,64	27,51 ± 0,5	26 ± 0,82	37,98 ± 1,2	123,45 ± 5,65
Total acidity (g/L)	3,75 ± 0	3,56 ± 0,05	3,71 ± 0,05	3,79 ± 0,05	3,71 ± 0,05	5,18 ± 0,21
Volatile acidity (g/L)	0,34 ± 0,04	0,41 ± 0,07	0,39 ± 0,02	0,38 ± 0,10	0,32 ± 0,04	0,35 ± 0,08
Colour intensity	0,853 ± 0,002	0,96 ± 0	1,041 ± 0	0,958 ± 0,004	0,935 ± 0,003	3,92 ± 0,099
Total polyphenols (OD280)	4,554 ± 0,044	4,58 ± 0,01	4,59 ± 0,031	4,551 ± 0,017	4,574 ± 0,011	5,894 ± 0,091

Table 27. Biogenic amine evolution throughout all winemaking process and after bottling.

			Co-inoculation					Sequential					
	Must	AF	P2A	P3A	P3G	P7B	OENOS	P2A	P3A	P3G	P7B	OENOS	SPONT
Histamine	-	-	-	-	-	-	-	-	-	-	-	-	-
Agmatine	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyramine	-	-	-	-	-	-	-	-	-	-	-	-	-
Putrescine	2,02ab ± 0,01	2,73d ± 0,07	2,9d ± 0,15	2,76d ± 0,16	2,8d ± 0,06	2,48cd ± 0,21	2,89d ± 0,42	2,59d ± 0,08	2,4bcd ± 0,15	2,1abc ± 0,36	1,8a ± 0,04	1,97ab ± 0,22	2,86d ± 0,42
Cadaverine	0,38a ± 0,02	0,48b ± 0,01	0,45ab ± 0,01	0,46b ± 0,06	0,48ab ± 0	0,45ab ± 0,01	0,47b ± 0,26	0,5b ± 0	0,48b ± 0,01	0,49b ± 0,07	0,42b ± 0	0,44ab ± 0,22	0,46ab ± 0,02

After bottling	Co-inoculation					Sequential					
	P2A	P3A	P3G	P7B	OENOS	P2A	P3A	P3G	P7B	OENOS	SPONT
Histamine	-	-	-	-	-	-	-	-	-	-	-
Agmatine	-	-	-	-	-	-	-	-	-	-	-
Tyramine	-	-	-	-	-	-	-	-	-	-	-
Putrescine	2,36cd ± 0,12	2,43cd ± 0,18	2,58d ± 0,1	2,67d ± 0,09	2,77d ± 0,04	2,6d ± 0,11	2,4cd ± 0,12	2,41cd ± 0,03	2,36cd ± 0,18	2,32cd ± 0,24	2,51cd ± 0,31
Cadaverine	0,28a ± 0,02	0,31a ± 0	0,28a ± 0,08	0,25a ± 0,08	0,25a ± 0,06	0,27a ± 0,06	0,27a ± 0,05	0,28a ± 0,09	0,26a ± 0,07	0,25a ± 0,07	0,32a ± 0,02

4.5.3. Volatile compounds evolution and sensorial analysis

Besides grape varietal and ageing derived aroma compounds, metabolites derived from yeast and bacterial secondary metabolism usually dominate volatile profile of wines (Belda et al., 2017). In this sense, more than fifty volatile compounds, including ethyl and acetate esters, higher alcohols, acids and terpenoids, among others, were quantified throughout all winemaking process. **Table 28** shows the evolution of these compounds from must to each inoculation strategy. These results showed that the inoculation regime has an impact on the aromatic profile of wines.

Regarding higher alcohols, 3-methylbutanol and 2-phenyletanol were particularly relevant as they were detected in major quantities and around their odour threshold in all fermentations. In other cases, such as C6 alcohols, responsible for the green character of wines (Oliveira et al., 2006), the must had the highest concentrations and they significantly decreased along the winemaking process (**Table 29**). However, a significant increase in the total concentration of higher alcohols occurred after AF. When comparing the concentration of total alcohols in co-inoculations with the values obtained after standard AF, the reduction that occurred in the case of P2A and OENOS strains was particularly relevant. Although not significant, there was a reduction for nearly all compounds, clearer in the case of 3-methylbutanol (herbaceous/spiritous) and 2-phenyletanol (rose). The simultaneous performance of AF and MLF led to a lower production of higher alcohols. It must be stated that the presence of main alcohols derives from amino acids metabolism through Ehrlich pathway. In this sense isobutanol (solvent-like aroma) derives from valine, 3-methylbutanol from leucine and 2-phenylethanol from phenylalanine (Smid & Kleerebezem 2014). Thus, metabolic interactions of both P2A and OENOS with yeast and the competitiveness for the

nutrients present in the medium could have limited the production of amino acid metabolism derived volatile compounds. This fact could be beneficial due to the unpleasant aromas (solvent-like, fusel, nail polish) that higher alcohols show at higher concentrations and which could mask the aroma complexity of the mixture (Ferreira et al., 2007). For example, 3-methylbutanol, which after AF was detected above its odour threshold, in co-inoculations leaded by P2A and OENOS it was found below its threshold. Remaining co-inoculated strains presented different evolution for 3-methylbutanol and 2-phenylethanol, in fact they showed similar or higher concentration in comparison with standard AF. In the same way, all co-inoculated batches showed higher concentration for heptanol (green), 2-nonanol (waxy) and 2-ethyl-1-hexanol (citrus). When wines that followed sequential inoculation were analysed, they showed, except for the strain P7B, higher amounts of higher alcohols compared to their respective co-inoculations. These differences mainly derived from the increase of 3-methylbutanol, significant for strains P2A and P3A strains, and a generalised significant increase of 2-ethyl-1-hexanol, 2-nonanol and 1-octanol (sweet, floral). The concentration of these compounds was also higher than that found after AF. That is, sequential inoculation can lead to an increase in the concentration of higher alcohols. The role of MLF in higher alcohols evolution is still inconclusive (Belda et al., 2017), while many studies have shown no changes after MLF (Hernández-Orte et al., 2012, Izquierdo-Cañas et al., 2008), others have proved significant changes after MLF (Brizuela et al., 2018).

For acids, one of the main fears when carrying out co-inoculation is the possible increase in acetic acid due to the high availability of sugars by bacteria (Costello et al., 2006). In the case of the strain P7B, there was a slight increase of this compound, but

in both co-inoculation and sequential regimes. On the contrary, with remaining strains, the concentration was similar or lower than that obtained after standard AF. Thus, with the correct choice of the strain, the risk of increased volatile acidity is minimal. After AF there was a significant increase in mainly all acids, actually, hexanoic acid (fatty) and 3-methylbutyric acid (cheesy, rancid) were detected above their odour threshold after AF. Regarding co-inoculated batches a different evolution between short and long chain acids was observed. Whereas, they tended to present a higher (or equal) concentration of short chain acids, a generalized decrease in long chain acids (octanoic, nonanoic, decanoic and dodecanoic) was observed for all of them. It should be noted that the fermentations that concluded earlier, P2A and OENOS, showed a significant lower total acids concentration. Considering that fatty acid synthesis from acetyl CoA from both yeast and bacteria lead to the formation of medium and long-chain fatty acids, yeast-bacteria co-metabolism may have influenced the availability of acetyl CoA, by using pyruvate for other purposes. Yeast and bacteria co-metabolism could have also enabled the rapid utilization of fatty acids by bacteria for several purposes (e.g. cell membrane regeneration, homeostasis, energy storage) and thus, lead to a reduction in long-chained fatty acids. Wines that followed sequential inoculation did not elucidate significant changes after the achievement of AF, although a tendency towards a lower concentration of acids was generalized. In this way, and mainly derived from yeast metabolism, sequential strategy showed higher total acids concentration than co-inoculated wines. Similar results were obtained in other studies when both inoculation strategies were analysed (Hernández-Orte et al., 2012; Izquierdo-Cañas et al., 2020). The reduction in the concentration of acids, more acute in the case of co-inoculation can lead to more

complex wines, where impact aromas are not masked by large concentrations of acids (Miranda-Lopez et al., 1992). Although acids are considered to contribute to the balance of the overall aroma complexity in wine (Gammacurta et al., 2017), high concentration of volatile fatty acids conferring rancid and cheesy notes may mask the fruity character that gives personality to Tempranillo wines from Rioja Alavesa region.

Together with higher alcohols and acids, esters are quantitatively one of the most important compounds for modulating fruity character of red wines (Ugliano and Moio, 2005). It was detected a significant increase of acetate and ethyl esters after AF, mainly for ethyl acetate (nail polish, fruity), ethyl hexanoate (green apple), ethyl octanoate (waxy, pear) and ethyl decanoate (waxy), as well as for hexyl acetate (pear, pineapple), isoamyl acetate (banana) and phenylethyl acetate (rose, fruity), among others. Indeed, many of them, as ethyl hexanoate, ethyl octanoate, ethyl decanoate and hexyl acetate, surpassed their odour threshold after AF. In the same way, it must be discerned between major and odorant esters. Major esters present high odour thresholds, while odorant esters easily overcome their odour threshold (Gammacurta et al., 2017), as those mentioned earlier. When comparing the evolution of esters between the standard AF and co-inoculated batches, a generalized reduction of the esters is observed when co-inoculation was carried out. Esters belong mainly to two categories, acetate esters of higher alcohols and ethyl esters of fatty and organic acids (Belda et al., 2017). In this sense, a lower concentration of both higher alcohols and fatty acids in the medium will lead to a lower concentration of both acetate and ethyl esters. This fact was particularly clear when strains P2A and OENOS where analysed. These batches, which showed significant lower concentrations of higher alcohols and volatile fatty acids, presented a significantly lower total esters concentrations in

comparison with remaining strains. That is, yeast-bacteria interactions led to particular volatile profiles, where LAB may modify yeast metabolism, by directly altering yeast-derived metabolites or by altering the expression of yeast genes related to aroma compounds synthesis pathways (Rossouw et al., 2012). In addition, hydrolysis of esters by LAB esterases neither can be excluded (Sumby et al., 2009). Those co-inoculated strains that needed more time to conclude MLF, exhibited higher concentration of relevant esters, such as ethyl hexanoate, ethyl octanoate, isoamyl acetate or phenylethyl acetate in comparison with P2A and OENOS strains. It should be noted that the production of esters is not based solely on microbial action, but it can be also modulated chemically over time (Hernández-Orte et al., 2012). Either those strains that ended earlier or those that needed more time, they all showed a significant increase of ethyl lactate, isoamyl lactate, diethyl succinate and ethyl hydrogen succinate in comparison with standard AF. These esters impart fruity, milky and creamy notes to wines, contributing to pleasant mouthfeel (Izquierdo et al., 2008; Lerm et al., 2010). The appearance of these compounds elucidates that MLF has successfully taken place (Cortés-Diéguez et al., 2015).

When MLF took place sequentially, esters concentration did not vary significantly in comparison with that obtained after AF. Total acetate esters maintain similar values among strains, total ethyl esters, however, showed strain-dependent evolution. While strains P3A and P3G showed higher concentrations, strains P2A, P7B and OENOS showed a significant reduction of total ethyl esters. This reduction was mainly driven by ethyl acetate and ethyl decanoate. However, it was not as significant as that observed in the co-inoculated batches. As in the co-inoculation, a significant increase of representative esters of MLF, as ethyl lactate, isoamyl lactate, diethyl succinate and

ethyl hydrogen, was observed. In both sequential and co-inoculation strategies, strains showed the same behaviour, that is, strains P2A and OENOS presented less ester concentration, while P3A and P3G, the higher. By means of the sequential strategy, the profile of esters obtained after AF was maintained more stable, being quantitatively higher than that observed after co-inoculation with each strain. The decrease in total ester concentration that is observed after co-inoculation could derive into a loss of fruity character, or on the contrary, it could lead to a greater aroma balance. The high concentration of certain esters, as ethyl hexanoate, ethyl octanoate and ethyl decanoate, which far exceed their odour threshold could mask the impact of others, reducing in that way the complexity of the mixture; however, the synergy between the different compounds at subthreshold concentrations could lead to greater sensory complexity (Ferreira et al., 2007).

Regarding terpenoids, these group of aroma compounds is typically related to key varietal aromas (Ruiz et al., 2019). These compounds are found in grape as non-odorant precursors and after the metabolic activity of yeast and bacteria glycosidases they are released into the medium (Michlmayr et al., 2012). All these compounds significantly increased after AF and in the case of co-inoculation, except for P2A and OENOS, a significant increase was observed for linalool, as well as a tendency to increase for citronellol and nerolidol compared with standard AF. In all cases, after fermentation, citronellol and linalool were detected above their perception threshold. In sequential inoculations, the increase of these compounds was more noticeable. It is worth noting the significant increase that occurs for nerolidol and linalool. Thus is, the activity of *O. oeni* strains in both strategies stimulate the liberation of terpenoids. This fact is of great relevance since they are able to transform significantly wine

sensory perception (Ruiz et al., 2019). Among volatile phenols, ethyl phenols are particularly relevant due to their negative impact on final quality of wine (Chescheir et., 2015). It is important to note that although 4-ethyl phenol was detected, and it was already present in must, there was a minimal increase after both inoculation strategies, far from its odour threshold. Regarding metabolites derived from citrate metabolism, the presence of 2-butanedione was not detected in none of the fermentations, however, there is a difference between the two strategies in the case of 2,3-butanediol, which is significantly higher in co-inoculations. In wines following sequential inoculation, the prolonged contact with lees could have caused the degradation of this compound (Antalick et al., 2013). Also, it is noteworthy the evolution of β-damascenone, which after MLF was detected above its odour threshold. It was not detected after AF, but its release was stimulated with both co-inoculation and sequential strategies.

Table 28. Evolution of volatile compounds (mg/L) through the different winemaking and inoculation strategies. Those compounds that after the winemaking process were detected above their odour threshold are highlighted in bold.

			Co-inoculation MLF				
	Must	AF	P2A	P3A	P3G	P7B	OENOS
Higher alcohols							
Isobutanol	0,759 ± 0,01	0,574 ± 0,127	0,317 ± 0,097	0,39 ± 0,037	0,461 ± 0,052	0,497 ± 0,071	0,241 ± 0,058
3-Methylbutanol	0 ± 0	38,4 ± 11,037	30,987 ± 3,546	33,472 ± 1,902	41,346 ± 1,919	44,385 ± 6,729	21,317 ± 2,23
3-Methyl-1-pentanol	0 ± 0	0,069 ± 0,002	0,047 ± 0,009	0,0597 ± 0,009	0,08 ± 0,014	0,099 ± 0,024	0,02 ± 0,021
1-Hexanol	3,803 ± 0,095	1,168 ± 0,068	0,928 ± 0,035	1,185 ± 0,059	1,262 ± 0,103	1,347 ± 0,228	0,671 ± 0,082
3-Hexen-1-ol	0,307 ± 0,024	0,081 ± 0,052	0,047 ± 0,017	0,052 ± 0,007	0,053 ± 0,007	0,054 ± 0,005	0,041 ± 0,016
Heptanol	0,027 ± 0,001	0,048 ± 0,007	0,101 ± 0,026	0,168 ± 0,004	0,229 ± 0,055	0,147 ± 0,036	0,132 ± 0,091
2-Ethyl-1-hexanol	0,025 ± 0,004	0,078 ± 0,01	0,203 ± 0,032	0,194 ± 0,077	0,17 ± 0,066	0,149 ± 0,045	0,077 ± 0,072
2-Nonanol	0 ± 0	0,007 ± 0	0,023 ± 0,002	0,035 ± 0,004	0,039 ± 0,005	0,035 ± 0,004	0,021 ± 0,003
1-Octanol	0,077 ± 0,016	0,082 ± 0,008	0,043 ± 0,006	0,066 ± 0,001	0,068 ± 0,003	0,069 ± 0,01	0,043 ± 0,007
2-Phenylethanol	0,422 ± 0,121	12,31 ± 0,372	9,241 ± 1,17	10,582 ± 1,555	11,784 ± 0,704	12,64 ± 1,173	7,875 ± 2,789
Acids							
Acetic acid	0,062 ± 0,01	0,358 ± 0,001	0,333 ± 0,203	0,331 ± 0,17	0,429 ± 0,096	0,505 ± 0,065	0,309 ± 0,089
Isobutyric acid	0,007 ± 0,001	0,084 ± 0,006	0,092 ± 0,036	0,091 ± 0,031	0,11 ± 0,021	0,11 ± 0,007	0,06 ± 0,004
Butanoic acid	0,006 ± 0,001	0,049 ± 0,003	0,041 ± 0,012	0,046 ± 0,014	0,06 ± 0,01	0,06 ± 0,006	0,034 ± 0,009
3-Methyl butryric acid	0,016 ± 0,002	0,21 ± 0,002	0,241 ± 0,078	0,244 ± 0,09	0,304 ± 0,055	0,33 ± 0,003	0,177 ± 0,028
Hexanoic acid	0,018 ± 0,007	0,799 ± 0,019	0,486 ± 0,15	0,648 ± 0,265	0,73 ± 0,078	0,758 ± 0,035	0,485 ± 0,211
Heptanoic acid	0,026 ± 0,002	0,024 ± 0,003	0,044 ± 0,011	0,049 ± 0,005	0,049 ± 0,003	0,047 ± 0,002	0,037 ± 0,023
Octanoic acid	0,716 ± 0,144	3,86 ± 0,134	1,015 ± 0,143	1,575 ± 0,574	1,641 ± 0,092	1,673 ± 0,034	1,503 ± 0,365
Nonanoic acid	0,049 ± 0,004	0,096 ± 0,011	0,056 ± 0,014	0,076 ± 0,025	0,069 ± 0,008	0,085 ± 0,004	0,048 ± 0,006
Decanoic acid	0,045 ± 0,009	0,789 ± 0,024	0,106 ± 0,003	0,112 ± 0,047	0,09 ± 0,005	0,119 ± 0,011	0,186 ± 0,04
Dodecanoic acid	0,025 ± 0,003	0,16 ± 0,038	0,076 ± 0,017	0,107 ± 0,034	0,116 ± 0,017	0,121 ± 0,024	0,107 ± 0,038
Benzoic acid	0,006 ± 0	0,028 ± 0,006	0,015 ± 0	0,016 ± 0	0,015 ± 0,003	0,02 ± 0,004	0,008 ± 0
Acetate esters							
Ethyl acetate	0,434 ± 0,004	3,819 ± 0,036	0,848 ± 0,128	1,23 ± 0,216	1,133 ± 0,266	1,387 ± 0,209	0,635 ± 0,029
Isoamyl acetate	0 ± 0	8,892 ± 0,405	3,412 ± 0,704	4,892 ± 1,595	5,023 ± 0,026	6,279 ± 1,242	2,91 ± 0,419
Phenylethyl acetate	0,107 ± 0,023	2,066 ± 0,027	0,781 ± 0,076	0,976 ± 0,042	0,975 ± 0,05	1,075 ± 0,045	0,777 ± 0,307
Hexyl acetate	0,045 ± 0,013	1,405 ± 0,053	0,253 ± 0,023	0,269 ± 0,067	0,216 ± 0,025	0,368 ± 0,06	0,263 ± 0,071
3-Hexen-1-ol acetate	0,005 ± 0,001	0,047 ± 0,01	0,031 ± 0,001	0,028 ± 0,005	0,028 ± 0,001	0,03 ± 0,003	0,018 ± 0,006

Table 28 continuation

	Sequential MLF					
	P2A	P3A	P3G	P7B	OENOS	ESP
Higher alcohols						
Isobutanol	0,703 ± 0,154	0,46 ± 0,141	0,445 ± 0,087	0,477 ± 0,241	0,49 ± 0,061	0,61 ± 0,262
3-Methylbutanol	55,503 ± 4,933	38,105 ± 15,889	47,64 ± 2,775	22,537 ± 3,747	39,966 ± 6,325	42,372 ± 14,456
3-Methyl-1-pentanol	0,1 ± 0,037	0,108 ± 0,044	0,121 ± 0,013	0,092 ± 0,021	0,109 ± 0,02	0,017 ± 0,004
1-Hexanol	1,854 ± 0,189	1,185 ± 0,553	1,644 ± 0,172	1,617 ± 0,333	1,414 ± 0,335	1,382 ± 0,615
3-Hexen-1-ol	0,084 ± 0,018	0,152 ± 0,05	0,111 ± 0,027	0,075 ± 0,012	0,094 ± 0,032	0,147 ± 0,026
Heptanol	0,07 ± 0,008	0,064 ± 0,016	0,07 ± 0,003	0,062 ± 0,014	0,07 ± 0,022	0,067 ± 0,033
2-Ethyl-1-hexanol	0,274 ± 0,093	0,13 ± 0,059	0,228 ± 0,07	0,287 ± 0,076	0,091 ± 0,014	0,152 ± 0,034
2-Nonanol	0,031 ± 0,003	0,024 ± 0,006	0,036 ± 0	0,054 ± 0,011	0,019 ± 0,003	0,016 ± 0
1-Octanol	0,143 ± 0,021	0,129 ± 0,047	0,143 ± 0,004	0,152 ± 0,025	0,12 ± 0,015	0,101 ± 0,026
2-Phenylethanol	11,458 ± 0,018	10,508 ± 3,34	12,773 ± 0,479	11,796 ± 0,435	12,349 ± 1,122	11,861 ± 1,673
Acids						
Acetic acid	0,39 ± 0,021	0,475 ± 0,085	0,379 ± 0,013	0,499 ± 0,049	0,338 ± 0,062	0,337 ± 0,015
Isobutyric acid	0,068 ± 0,002	0,055 ± 0,027	0,076 ± 0,003	0,087 ± 0,016	0,063 ± 0,003	0,05 ± 0,017
Butanoic acid	0,045 ± 0,002	0,078 ± 0,021	0,073 ± 0,015	0,067 ± 0,005	0,05 ± 0,006	0,114 ± 0,02
3-Methyl butryric acid	0,173 ± 0,005	0,166 ± 0,071	0,195 ± 0,024	0,213 ± 0,034	0,191 ± 0,045	0,179 ± 0,043
Hexanoic acid	0,676 ± 0,036	0,769 ± 0,276	0,826 ± 0,087	0,705 ± 0,028	0,681 ± 0,113	0,81 ± 0,137
Heptanoic acid	0,024 ± 0,002	0,011 ± 0,002	0,019 ± 0,01	0,028 ± 0,002	0,025 ± 0,005	0,011 ± 0
Octanoic acid	2,564 ± 0,41	3,904 ± 0,858	3,324 ± 0,193	2,801 ± 0,048	2,857 ± 0,343	3,967 ± 0,411
Nonanoic acid	0,072 ± 0	0,05 ± 0,006	0,067 ± 0,007	0,068 ± 0,023	0,063 ± 0,004	0,088 ± 0,02
Decanoic acid	0,21 ± 0,006	0,21 ± 0,107	0,342 ± 0,057	0,24 ± 0,009	0,219 ± 0,037	1,139 ± 0,096
Dodecanoic acid	0,141 ± 0,004	0,255 ± 0,068	0,191 ± 0,049	0,127 ± 0,019	0,176 ± 0	0,258 ± 0,005
Benzoic acid	0,016 ± 0,002	0,01 ± 0,002	0,013 ± 0	0,013 ± 0,001	0,012 ± 0	0,012 ± 0
Acetate esters						
Ethyl acetate	2,215 ± 0,385	1,581 ± 1,057	1,415 ± 0,229	1,464 ± 0,473	1,622 ± 0,274	2,911 ± 1,263
Isoamyl acetate	7,954 ± 1,12	8,115 ± 3,376	7,795 ± 0,658	6,635 ± 1,039	7,194 ± 0,89	6,163 ± 1,444
Phenylethyl acetate	1,609 ± 0,138	1,823 ± 0,358	1,773 ± 0,114	1,705 ± 0,232	1,807 ± 0,107	1,414 ± 0,156
Hexyl acetate	0,648 ± 0,127	0,379 ± 0,126	0,579 ± 0,04	0,632 ± 0,068	0,541 ± 0,07	0,563 ± 0,086
3-Hexen-1-ol acetate	0,038 ± 0,004	0,035 ± 0,013	0,039 ± 0,006	0,047 ± 0,006	0,024 ± 0,004	0,02 ± 0,003

Table 28 continuation

			Co-inoculation MLF				
	Must	AF	P2A	P3A	P3G	P7B	OENOS
Ethyl esters and others							
Ethyl butyrate	0,019 ± 0,002	0,288 ± 0,032	0,21 ± 0,055	0,323 ± 0,095	0,346 ± 0,003	0,375 ± 0,081	0,174 ± 0,028
Ethyl hexanoate	0,169 ± 0,031	5,162 ± 2,358	1,457 ± 2,061	3,93 ± 1,599	5,499 ± 0,384	5,356 ± 0,44	2,685 ± 0,155
Ethyl heptanoate	-	0,022 ± 0,006	0,079 ± 0,005	0,113 ± 0,002	0,156 ± 0,005	0,128 ± 0,036	0,116 ± 0,027
Ethyl 2-hexenoate	-	0,014 ± 0	0,01 ± 0,003	0,01 ± 0,001	0,012 ± 0,001	0,009 ± 0,001	0,007 ± 0,001
Ethyl 3-hydroxy hexanoate	-	0,007 ± 0,001	0,006 ± 0,001	0,011 ± 0,002	0,014 ± 0,001	0,011 ± 0,001	0,011 ± 0,006
Ethyl octanoate	0,254 ± 0,032	24,941 ± 0,372	5,899 ± 0,336	11,251 ± 3,314	12,941 ± 0,284	12,652 ± 2,625	8,239 ± 2,013
Ethyl 7-octenoate	-	-	0,059 ± 0	0,09 ± 0,028	0,11 ± 0	0,082 ± 0,007	0,045 ± 0,013
Ethyl 9-decenoate	0,038 ± 0,021	1,886 ± 0,047	0,357 ± 0,025	0,661 ± 0,093	0,678 ± 0,029	0,602 ± 0,066	0,686 ± 0,173
Ethyl decanoate	0,111 ± 0,028	4,405 ± 0,019	0,458 ± 0,059	0,631 ± 0,1	0,506 ± 0,005	0,621 ± 0,063	1,065 ± 0,359
Ethyl dodecanoate	0,232 ± 0,041	0,059 ± 0,013	0,009 ± 0,001	0,017 ± 0,006	0,016 ± 0,002	0,013 ± 0,002	0,022 ± 0,007
Isoamyl octanoate	-	0,074 ± 0,003	0,012 ± 0	0,029 ± 0,004	0,032 ± 0,003	0,023 ± 0,003	0,033 ± 0,002
Diethyl succinate	0,003 ± 0,001	0,043 ± 0,009	0,256 ± 0,039	7,172 ± 0,581	1,132 ± 0,053	0,489 ± 0,082	0,281 ± 0,07
Ethyl hydrogen succinate	-	-	0,005 ± 0	0,191 ± 0,117	0,055 ± 0,002	0,027 ± 0,014	0,028 ± 0,005
Isoamyl lactate	-	-	0,042 ± 0	0,041 ± 0,004	0,043 ± 0,005	0,07 ± 0,012	0,034 ± 0,007
Ethyl lactate	-	-	0,44 ± 0,074	0,481 ± 0,032	0,503 ± 0,063	0,697 ± 0,174	0,312 ± 0,009
Terpenoids							
Linalol	0,01 ± 0,002	0,056 ± 0,009	0,067 ± 0,007	0,102 ± 0,018	0,119 ± 0,016	0,116 ± 0,021	0,07 ± 0
Citronellol	0,002 ± 0	0,074 ± 0,003	0,067 ± 0,006	0,099 ± 0,011	0,105 ± 0,003	0,101 ± 0,013	0,07 ± 0,034
Geraniol	-	0,03 ± 0	0,016 ± 0	0,02 ± 0,004	0,012 ± 0,001	0,016 ± 0,001	0,015 ± 0,005
Nerolidol	-	0,014 ± 0,001	0,024 ± 0,001	0,03 ± 0,002	0,029 ± 0,001	0,026 ± 0	0,052 ± 0,01
Phenols							
4-Ethyl phenol	0,006 ± 0	0,004 ± 0	0,006 ± 0	0,007 ± 0,001	0,007 ± 0	0,006 ± 0	0,008 ± 0,004
Phenol, 2,4-tertbutyl	0,002 ± 0	0,021 ± 0,002	0,023 ± 0,008	0,016 ± 0,007	0,023 ± 0,007	0,022 ± 0,005	0,029 ± 0,027
2,3-Dimethyl phenol	0,033 ± 0	0,025 ± 0,001	0,028 ± 0	0,028 ± 0,003	0,028 ± 0,001	0,026 ± 0,002	0,021 ± 0,001
Others							
Acetoin	-		0,047 ± 0,018	0,046 ± 0,001	0,045 ± 0	0,037 ± 0,002	0,057 ± 0,058
2,3-Butanediol	-	0,161 ± 0,007	0,172 ± 0,012	0,166 ± 0,041	0,189 ± 0,015	0,235 ± 0,009	0,092 ± 0,018
γ-Butyrolactone	0,008 ± 0,001	0,039 ± 0,014	0,03 ± 0,002	0,036 ± 0,002	0,036 ± 0,001	0,039 ± 0,006	0,017 ± 0,002
Methionol	-	0,093 ± 0,003	0,068 ± 0,006	0,174 ± 0,056	0,087 ± 0,006	0,096 ± 0,01	0,046 ± 0,009
β-Damascenone	-	-	0,069 ± 0,003	0,08 ± 0,005	0,076 ± 0,012	0,082 ± 0,007	0,077 ± 0,023

Table 28 continuation

	Sequential MLF					
	P2A	P3A	P3G	P7B	OENOS	ESP
Ethyl esters and others						
Ethyl butyrate	0,466 ± 0,037	0,4 ± 0,14	0,43 ± 0,027	0,378 ± 0,079	0,354 ± 0,056	0,426 ± 0,123
Ethyl hexanoate	6,718 ± 0,926	5,779 ± 2,175	4,855 ± 1,218	2,103 ± 0,381	4,966 ± 0,829	5,106 ± 1,24
Ethyl heptanoate	0,014 ± 0	-	0,015 ± 0,002	0,023 ± 0,015	-	0,113 ± 0,045
Ethyl 2-hexenoate	0,012 ± 0	0,016 ± 0,005	0,014 ± 0,005	0,018 ± 0,001	0,011 ± 0	-
Ethyl 3-hydroxy hexanoate	0,013 ± 0	0,022 ± 0,002	0,015 ± 0,003	0,014 ± 0,002	0,022 ± 0,001	0,021 ± 0,001
Ethyl octanoate	20,213 ± 4,054	31,024 ± 4,819	24,683 ± 0,779	23,641 ± 0,18	15,201 ± 1,605	31,588 ± 3,666
Ethyl 7-octenoate	0,016 ± 0,003	0,026 ± 0,003	0,014 ± 0,007	0,024 ± 0,006	0,356 ± 0,024	0,018 ± 0,004
Ethyl 9-decenoate	1,149 ± 0,313	0,764 ± 0,3	1,578 ± 0,054	1,468 ± 0,165	1,148 ± 0,079	3,06 ± 0,205
Ethyl decanoate	1,574 ± 0,381	1,156 ± 0,399	2,466 ± 0,005	2,041 ± 0,519	1,577 ± 0,232	9,506 ± 0,839
Ethyl dodecanoate	0,023 ± 0,001	0,14 ± 0,025	0,04 ± 0,002	0,026 ± 0,005	0,039 ± 0,005	0,128 ± 0,018
Isoamyl octanoate	0,058 ± 0,02	0,05 ± 0,018	0,117 ± 0,025	0,055 ± 0,032	0,067 ± 0	0,178 ± 0,014
Diethyl succinate	0,515 ± 0,032	1,882 ± 0,532	2,669 ± 0,061	0,635 ± 0,007	0,533 ± 0,046	1,337 ± 0,239
Ethyl hydrogen succinate	0,038 ± 0,003	0,12 ± 0,019	0,235 ± 0,113	0,032 ± 0,013	0,033 ± 0,001	0,105 ± 0,006
Isoamyl lactate	0,054 ± 0,004	0,032 ± 0,012	0,059 ± 0,006	0,062 ± 0,008	0,043 ± 0,011	-
Ethyl lactate	0,679 ± 0,03	0,369 ± 0,144	0,671 ± 0,192	0,864 ± 0,077	0,46 ± 0,086	-
Terpenoids						
Linalol	0,119 ± 0,027	0,127 ± 0,033	0,145 ± 0,011	0,123 ± 0,006	0,099 ± 0,014	0,115 ± 0,009
Citronellol	0,072 ± 0,011	0,096 ± 0,021	0,108 ± 0,004	0,076 ± 0,001	0,099 ± 0,007	0,07 ± 0,005
Geraniol	0,018 ± 0,003	0,02 ± 0,004	0,02 ± 0	0,017 ± 0,003	0,02 ± 0	0,011 ± 0,001
Nerolidol	0,044 ± 0,007	0,047 ± 0,004	0,093 ± 0,007	0,074 ± 0,008	0,075 ± 0,008	0,102 ± 0,011
Phenols						
4-Ethyl phenol	0,008 ± 0,001	0,009 ± 0,006	0,013 ± 0	0,009 ± 0,002	0,011 ± 0,001	0,01 ± 0,001
Phenol, 2,4-tertbutyl	0,023 ± 0,001	0,054 ± 0,026	0,038 ± 0,01	0,031 ± 0	0,033 ± 0	0,013 ± 0,001
2,3-Dimethyl phenol	0,024 ± 0,001	0,025 ± 0,002	0,029 ± 0	0,03 ± 0,003	0,031 ± 0,001	0,029 ± 0,001
Others						
Acetoin	0,234 ± 0,272	0,045 ± 0,009	0,02 ± 0,005	0,029 ± 0,001	0,492 ± 0,081	0,698 ± 0,234
2,3-Butanediol	0,098 ± 0,001	0,062 ± 0,025	0,075 ± 0,01	0,09 ± 0,015	0,046 ± 0,002	0,059 ± 0,016
γ-Butyrolactone	0,039 ± 0,004	0,04 ± 0,017	0,038 ± 0,009	0,042 ± 0,003	0,04 ± 0,005	0,043 ± 0,007
Methionol	0,08 ± 0,002	0,1 ± 0,042	0,083 ± 0	0,077 ± 0,005	0,065 ± 0,008	0,066 ± 0,009
β-damascenone	0,114 ± 0,037	0,149 ± 0,026	0,13 ± 0,004	0,123 ± 0,036	0,144 ± 0,019	0,125 ± 0,018

Table 29. Significant differences for each compound during all winemaking process and each inoculation strategy. Different letters imply significant differences (p<0,05).

			Co-inoculation MLF					Sequential MLF					
	Must	AF	P2A	P3A	P3G	P7B	OENOS	P2A	P3A	P3G	P7B	OENOS	ESP
Higher alcohols													
Isobutanol	a	ab	bc	bcd	bcd	ab	b	ab	ab	ab	ab	ab	bcd
3-methylbutanol	-	abc	ab	abc	abc	abc	a	c	abc	bc	a	abc	abc
3-methyl-1-pentanol	-	ab	ab	ab	ab	b	a	b	b	b	ab	b	a
1-hexanol	d	abc	ab	abc	abc	abc	a	c	abc	bc	bc	abc	abc
3-hexen-ol	c	ab	a	a	a	a	a	ab	b	ab	ab	ab	b
Heptanol	a	ab	ab	bc	c	abc	abc	ab	ab	ab	ab	ab	ab
2-ethyl-1-hexanol	a	ab	ab	ab	ab	ab	ab	b	ab	ab	b	ab	ab
2-Nonanol	-	a	bc	d	d	d	bc	cd	bc	d	e	bc	ab
1-octanol	ab	abc	a	ab	ab	ab	a	de	cde	de	e	cde	bcd
2-phenylethanol	a	c	bc	bc	c	c	bc	c	bc	c	c	c	c
Acids													
Acetic acid	a	ab	ab	ab	b	b	ab	ab	b	ab	b	ab	ab
Isobutyric acid	a	b	b	b	b	b	b	b	b	b	b	b	b
Butanoic acid	a	b	b	b	b	b	b	b	b	b	b	b	c
3-Methyl butryric acid	a	bc	bc	bc	c	c	bc	bc	b	bc	bc	bc	bc
Hexanoic acid	a	b	ab	b	b	b	ab	b	b	b	b	b	b
heptanoic acid	ab	ab	b	b	b	b	ab	ab	b	ab	ab	ab	b
Octanoic acid	a	de	a	ab	ab	ab	ab	bc	de	cde	cd	cd	e
Nonanoic acid	a	a	a	a	a	a	a	a	a	a	a	a	a
Decanoic acid	a	d	ab	ab	ab	ab	abc	abc	abc	c	bc	abc	e
Dodecanoic acid	a	bc	ab	abc	abc	abc	abc	bc	cd	c	abc	bc	d
Benzoic acid	a	d	bc	bc	bc	c	ab	bc	abc	abc	abc	ab	ab
Acetate esters													
Ethyl acetate	a	d	ab	ab	ab	ab	a	bc	ab	ab	ab	ab	cd
Isoamyl acetate	-	d	ab	abc	abc	abc	a	bc	bc	bc	abc	abc	abc
Phenylethyl acetate	a	e	b	bc	bc	bc	b	de	de	de	de	de	cd
Hexyl acetate	a	e	ab	ab	ab	bc	ab	d	bc	cd	d	cd	cd
3-hexen-1-ol acetate	a	c	bc	abc	abc	bc	ab	bc	bc	bc	c	abc	abc

Table 29 continuation

			Co-inoculation MLF					Sequential MLF					
	Must	AF	P2A	P3A	P3G	P7B	OENOS	P2A	P3A	P3G	P7B	OENOS	ESP
Ethyl esters and others													
Ethyl butyrate	a	bcd	bc	bcd	bcd	bcd	b	d	cd	cd	bcd	bcd	cd
Ethyl hexanoate	a	bc	abc	bc	bc	bc	ab	c	bc	bc	ab	bc	bc
Ethyl heptanoate	a	a	b	bc	c	bc	bc	a	a	a	a	a	bc
Ethyl 2-hexenoate	-	a	a	a	a	a	a	a	a	a	a	a	b
Ethyl 3-hydroxy hexanoate	a	bc	b	bc	bc	bc	bc	bc	d	c	c	d	d
Ethyl octanoate	a	e	b	bc	bcd	bcd	bc	de	f	e	e	cd	f
Ethyl 7-octenoate	a	a	cd	e	e	de	bc	ab	ab	ab	ab	f	ab
ethyl 9-decenoate	a	e	ab	bc	bc	b	bc	cd	bc	de	d	cd	f
Ethyl decanoate	a	e	ab	ab	ab	ab	abc	bcd	abc	d	cd	bcd	f
Ethyl dodecanoate	c	a	a	a	a	a	a	a	b	a	a	a	b
Isoamyl octanoate	-	b	a	ab	ab	ab	ab	b	b	c	b	c	d
Diethyl succinate	a	ab	abc	e	d	bc	abc	bc	d	f	c	bc	d
Ethyl hydrogen succinate	a	a	a	bc	ab	ab	ab	ab	abc	c	ab	ab	abc
Isoamyl lactate	-	-	ab	ab	ab	d	a	bc	a	cd	cd	ab	-
Ethyl lactate	-	-	ab	ab	ab	bc	a	bc	ab	bc	c	ab	-
Terpenoids													
Linalol	a	b	b	bc	c	c	b	c	c	c	c	bc	c
Citronellol	a	b	b	b	b	b	b	b	b	b	b	b	b
Geraniol	a	e	bcd	d	bcd	bcd	bcd	bcd	bcd	cd	bcd	d	bc
Nerolidol	-	a	a	abc	abc	ab	d	bcd	cd	f	e	e	f
Phenols													
4-ethyl phenol	ab	a	ab	ab	ab	ab	ab	ab	abc	c	abc	bc	bc
Phenol, 2,4-tertbutyl	a	ab	ab	ab	ab	ab	ab	ab	b	b	ab	ab	ab
2,3-dimethyl phenol	d	ab	bcd	bcd	bcd	bc	a	ab	ab	bcd	bcd	cd	bcd
Others													
Acetoin	-	-	a	a	a	a	a	a	a	a	a	b	b
2,3-Butanediol	-	c	c	c	c	c	ab	b	ab	ab	ab	a	ab
b-damascenone	a	a	b	bc	bc	bc	bc	bc	bc	bc	bc	c	bc
Hidroxy propil sulfide	a	b	b	c	b	b	b	b	b	b	b	b	b
γ-butyrolactone	a	cd	bcd	cd	cd	cd	ab	cd	cd	cd	d	cd	d

In order to elucidate a comprehensible overview of the potential relationship between the main aromatic compounds and the followed inoculation strategy, a PCA analysis was carried out (**Figure 26**). The first two components collected 76% of the variance. The first component was related to different esters (ethyl acetate, ethyl butyrate, isoamyl acetate, ethyl decanoate, ethyl 9-decenoate), acids (hexanoic, nonanoic and decanoic), nerolidol and 2-phenylethanol. The second component showed a greater correlation with the esters isoamyl lactate and ethyl dodecanoate, the acids isobutyric acid and heptanoic acid, with heptanol and 2,3-butanediol. It was observed how the different inoculation strategies were differentiated in two distinct groups. The fermentations carried out sequentially were grouped more clearly, whereas in co-inoculations there was a greater dispersion, led by the strains that concluded the fermentation earlier, P2A and OENOS. In any case, the differentiation between the two strategies was clear, that is, their aromatic profile was well differentiated. In the same way, previous vinification stages (must and AF) as well as the failed spontaneous fermentation, all were shown clearly differentiated in the plot, showing a particular aromatic profile. It must be stated that although AF profile is shown near sequential fermentations, in 3D plot it appeared in another level. It should be noted that sequential fermentations showed greater correlation for ethyl acetate, ethyl butyrate, isoamyl acetate, nonanoic and hexanoic acids, 2-phenylethanol, nerolidol and β-damascenone. On the contrary, co-inoculations showed better relationship with isoamyl lactate, isobutyric and heptanoic acids, heptanol and 2,3-butanediol. In that way, sequential inoculation may lead to more fruity and floral character than co-inoculation, regarding esters, 2-phenylethanol, nerolidol and β-damascenone. However, as previously explained, the higher concentration of acids and higher

alcohols in comparison with co-inoculated wines could mask the desired fruity and floral character of Rioja Alavesa wines (Ferreira et al., 2007).

In order to compare the different aromatic profiles obtained through the different bacteria inoculation strategies a sensorial analysis was performed, in which five descriptors (ripe fruit, red fruit, vegetable/herbal, floral and dairy) were evaluated. Sensorial analysis showed that co-inoculated wines, generally, stood out for a greater aroma of ripe fruit and less herbal and dairy notes (**Figure 27**). In the case of the P2A, OENOS and P3G strains, when inoculated sequentially they present a flatter profile where no attribute was highlighted. Indeed, P2A and OENOS strains were the lowest scored under this inoculation regime, however, no remarkable differences in the overall aroma liking between both inoculation strategies were noticed in any strain (**Figure 27**). Co-inoculated wines with P2A and OENOS strains were perceived with more ripe fruit notes and less herbal than their respective sequentially performed wines. Co-inoculated wine with P3G strain was also described as less herbal, although it showed a similar sensory profile under both strategies, as the P3A strain did. Lastly, P7B strain, which as the other co-inoculated wines mainly presented the attribute of ripe fruit aroma, when the inoculation was performed sequentially, the dairy aroma was perceived over the rest. In this case, it must be stated that the wine resulted from the sequential inoculation of P7B showed the highest concentration of both ethyl lactate and isoamyl lactate, which are responsible for milky and creamy notes.

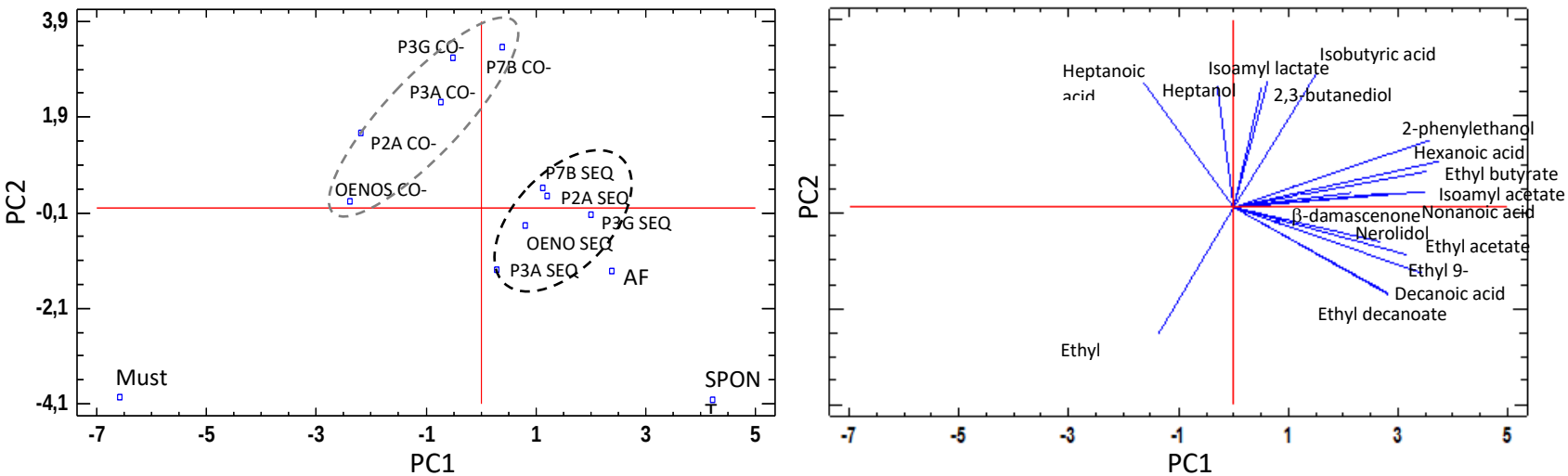


Figure 26. Principal component analysis (PCA) of volatiles profiles showed a differentiation between the inoculation strategies followed. Corresponding loadings showed which aromatic compounds contribute to the differentiation of both strategies.

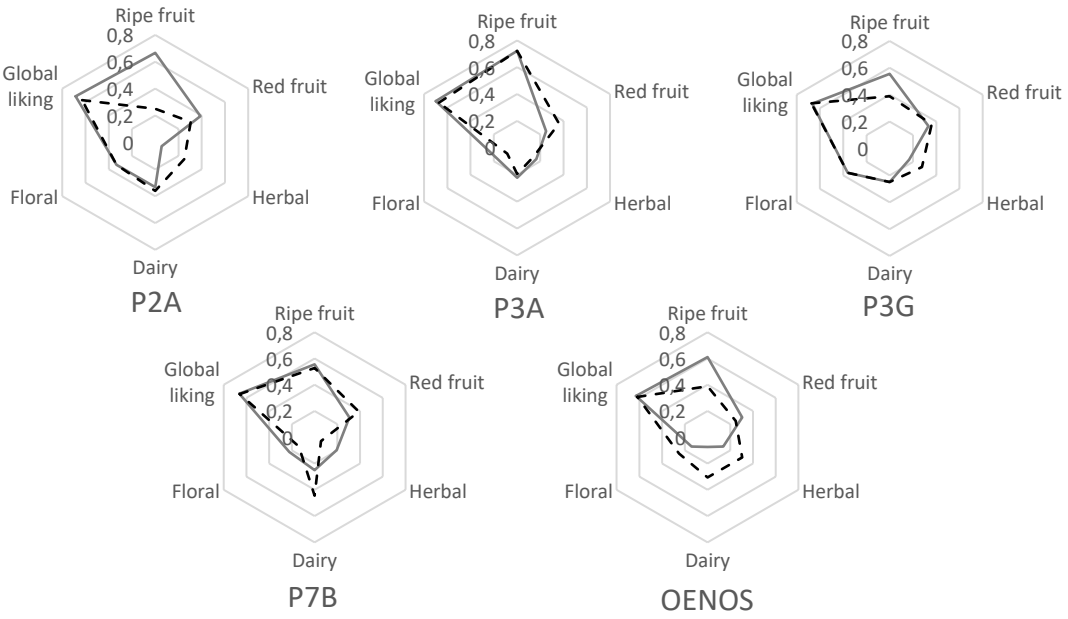


Figure 27. Global aroma liking and aroma attributes detected in sensorial analysis. Results are shown as the mean rate of the percentage of panelists (n=20) that detected each aroma. Straight lines refer to co-inoculation strategy, and dotted lines to wines performed through sequential inoculation.

Massera et al., 2009 demonstrated that co-inoculation tends to retain more fruity descriptors and showed less astringency and bitterness. In the same way, a sensory study of Shiraz wine showed that wines produced through co-inoculation showed more fruity compounds (Abrahamse & Bartowsky, 2012), as the studies performed by Jussier et al. (2006) and Knoll et al. (2011) which showed that more compounds contributing to the fruity character of wine were identified with co-inoculation when analysing Chardonnay and Riesling wines, respectively. Although a trend for more fruity wines has been usually reported, in other studies no significant differences on aromatic profile were detected in this regard (Antalick et al., 2013; Lombardi et al., 2020). In this sense, in the present study, in co-inoculated wines ripe fruit aroma outstood over the rest, however slight differences were detected with other

descriptors. Although all sequentially performed wines presented higher concentration of esters concentration, the content of more fruity descriptors does not always guarantee the enhancement of fruity aroma, since other aromatic compounds can mask the fruity notes. In this sense, the higher concentration of higher alcohols and acids, may have contribute to lower perception of fruity notes in sequentially performed wines, giving rise to more herbal and dairy notes. The effects of inoculation strategy on wine aromatic profile reflected the complex interactive effects of yeast and bacteria strains. Thus, yeast and bacteria co-metabolism, the competitiveness for the nutrients present in the medium and the modification of each others metabolites, resulted in different volatile profiles which led to the perception of differential sensorial properties.

PCA analysis elucidated that co-inoculated wines were better correlated with ripe fruit aroma and global aroma linking, in the right side of the plot (**Figure 28**). Wines performed through sequential inoculation were better correlated with herbal/vegetable and dairy notes, in the left side of the plot. That is, inoculation strategies were not only differentiated by different aromatic profiles, but this differentiation was also observed at sensory level when some attributes stood out above others. In this sense, different correlations were stablished between sensorial attributes and volatile compounds.Overall aroma liking was correlated with 2,3-butanediol, 3-methyl butyric acid and isobutyric acid. In fact, these compounds were detected in higher quantities in co-inoculated wines, which were better qualified. Indeed, OENOS strain which presented the lowest concentration of these compounds in both sequential and co-inoculation was rated with lower punctuation (data not shown). Regarding herbal/vegetable descriptor it was shown to be linked with 3-

methylbutanol (herbaceous/spiritous), which was detected in higher quantities in sequentially performed wines. It was notable the concentration difference of this compound in the sequential fermentations of P2A and P3G in comparison with their co-inoculations. Actually, in these sequentially performed wines the herbal/vegetable attribute was extensively detected. As previously stated, 3-methylbutanol, although in low quantities may enhance wine aroma complexity, when present in high concentration may mask other aromatic attributes and lead to lower wine quality. Regarding red fruit attribute, it was linked with β-damascenone, which is described as key odorant of red wines and which is typically found above its perception threshold in all wines (Ferreira et al., 2007).

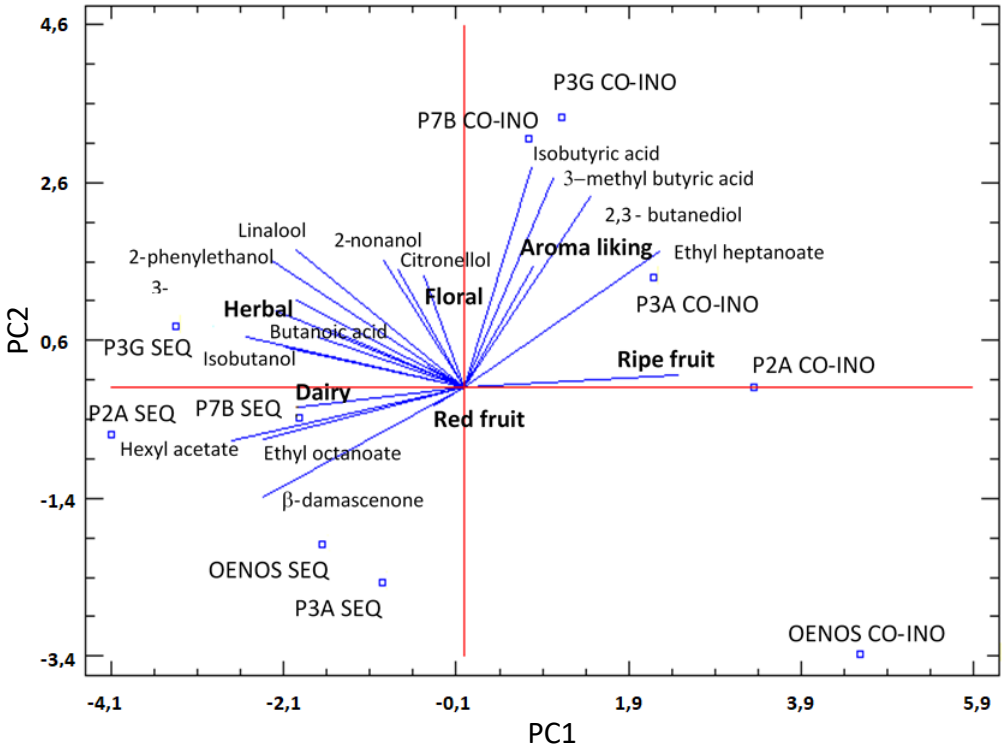


Figure 28. Principal component analysis (PCA) of volatile compounds and sensorial analysis. Corresponding loadings showed which aromatic compounds contribute to the different aroma attributes together with the positioning of each strain in the plot (SEQ: sequential inoculation; CO-INO: co-inoculation).

Floral attribute was correlated mainly with citronellol and 2-nonanol which were detected in similar concentrations in both inoculation strategies. Both compounds were detected around their odour threshold, and they are known to confer fresh/floral/citrus character and waxy/citrus undernotes, respectively (Michlmayr et al., 2012). Regarding dairy aroma attribute it was not correlated with expected compounds, such as acids (conferring cheesy and rancid notes) or with typical esters derived from MLF, as ethyl lactate or isoamyl lactate, responsible for milky notes. This attribute was correlated with hexyl acetate and ethyl octanoate, which are described to contribute to the fruity aroma of wines (Antalick et al., 2012). Finally, ripe fruit descriptor, main aroma detected in co-inoculated wines, was no correlated with any of the volatile compounds analysed. Its description may be the result of complex interactions between different compounds and families of compounds. Indeed, this attribute was mainly detected in co-inoculated wines with P2A and OENOS strains, which showed significantly lower concentration of acids and higher alcohols. This fact could prevent the masking of fruity esters and thus, could enable the perception of desired aromas. In this sense, although sensorial analysis had elucidated different logical interactions between volatile compounds and sensorial perceptions, still some inconclusive results were obtained. This may be due to complex interactions between LAB and yeast, especially when yeast and bacteria co-inoculations were carried out. In the present work it was proved the influence that LAB may have on yeast metabolism and therefore on aromatic profile of wines, which finally is perceived at sensory level. Although further work is needed to deeply elucidate the consequences of LAB and yeast interactions in terms of metabolites production and wine aroma modification, this study reports for the first time the influence of different inoculations

strategies with autochthonous *O. oeni* strains have on Rioja Alavesa Tempranillo wines.

4.5.4. Pilot test in the winery

Finally, it was evaluated the behaviour of P2A strain in a pilot test in the winery. As the rest of previously analysed strains, strain P2A met all the requirements of a new starter culture, such as no production of undesirable compounds (e.g. biogenic amines and volatile phenols), resistance to wine harsh conditions as well as the ability to modulate the sensory profile of wine. Furthermore, the P2A strain showed a great implantation power and a great fermentation vigour as well as the suitability to be used by co-inoculation.

In the winery, 100-liter tanks were inoculated by both inoculation strategies (co-inoculation and sequential inoculation). It was confirmed the suitability of modified OPM medium (Berbegal et al., 2015), for rapid biomass production (a density of 10^9 CFU/ml in merely two weeks) and for fast adaptation to wine conditions. In this sense, in co-inoculated batches, MLF finished together with AF, and when P2A was sequentially inoculated, MLF took similar time to conclude, around 10-12 days. Strain viability was maintained through the process, although higher counts were observed when co-inoculated (**Table 30**). Great availability of nutrients together with progressive adaptation to increase ethanol concentration resulted in better viability when P2A was co-inoculated, in contrary, when sequentially inoculated small viability decrease was detected after 24h of inoculation due to the limiting medium conditions (Brizuela et al., 2017). However, this fact did not alter the consecution of MLF, as with both strategies the time of fermentation process was similar. In this sense, when

sequential inoculation was performed the overall time of winemaking lasted 12 days more in comparison with co-inoculated batches. Regarding spontaneous fermentation, it took one month more to conclude than co-inoculated batches. Thus, it must be highlight that both inoculation strategies significantly reduced the vinification time, and this reduction was more acute when co-inoculation was performed. These results derive mainly from the rapid and total implantation of the P2A strain under both inoculation regimes (**Figure 29**). In addition, when co-inoculated, it is worth noting the compatibility of both yeast-bacteria, carrying out both fermentation processes in parallel. Thus, as previously mentioned, this strategy can turn really advantageous for wineries of the region in terms of cost and time savings as well as for greater control over the process (Cañas et al., 2015; Tristezza et al., 2016).

Table 30. Cell count (logCFU/ml) during the fermentation process.

Strategy	Days after inoculation			Implantation %
	1	6	12	
Co-inoculation	6,02 ± 1,44	8,04 ± 0,01	7,62 ± 0,08	100
Sequential	5,26 ± 0,93	5,26 ± 0,93	6,44 ± 0,21	100

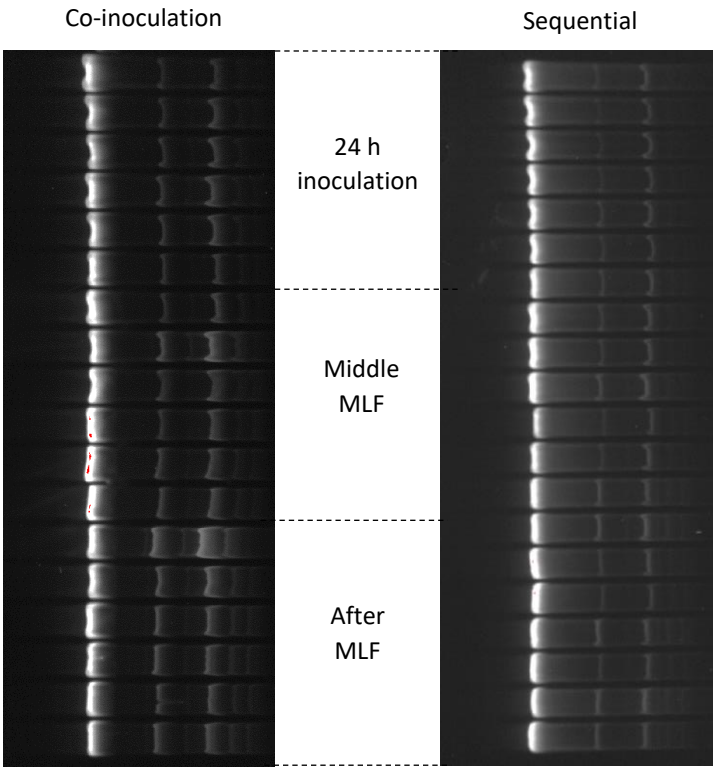


Figure 29 RAPD-PCR profiles obtained at each sampled stage. Ten colonies per stage were submitted to RAPD-PCR analysis, and as all patterns corresponded to P2A strain, only six profiles per stage are shown in the figure.

Regarding physicochemical parameters of wines, after MLF, typical reduction of total acidity was observed (**Table 31**). Furthermore, as the fermentation time increased, the precipitation of tartrate salts or the modification and metabolization of other acids present in the wine, led to a decrease of total acidity. This fact also resulted in the increase of the pH, more acute when MLF was prolonged. Thus, with shorter fermentations, the freshness of the wine could be preserved, as well as safer pH values, preventing the growth of potential spoilage microorganisms. Regarding colour intensity, a reduction associated with MLF was observed. In this sense, the longer the MLF time, the greater stabilization of acetaldehyde and pyruvate associated polymeric

pigments happened (Burns & Osborne, 2015). Co-inoculated wine turned more purple-violet, whereas spontaneously performed wine showed more reddish notes (data not shown).

Regarding biogenic amines concentration, only the presence of putrescine was detected. It was present in must and similar values were maintained during the different fermentation processes (**Table 32**). No production of biogenic amines was detected after MLFs performed through inoculation, nor after spontaneous MLF. However, it is necessary to continue monitoring the concentration of these compounds during the ageing period since a possible accumulation could happen (Berbegal et al., 2017). Anyway, the early wine stabilization associated with the early achievement of MLF (both co-inoculated and sequentially inoculated batches) as well as the rapid and total implantation of P2A strain in both inoculation strategies, make these wines present less risk for BA accumulation during ageing period compared to spontaneous fermentation. All in all, in the winery it was observed the ability of the strain P2A to work on a large scale, the ability to fully implant in a non-sterile environment and rapidly finish the process under both inoculation strategies. Although at winery no remarkable sensorial differences were detected after MLF (data not shown) and it is necessary to wait few months of ageing to evaluate the evolution aromatic compounds and biogenic amines, this pilot test represents a great advance in the pursuit of the first autochthonous malolactic culture from Rioja Alavesa region.

Table 31. Chemical analysis of wines following different inoculation strategies.

			After MLF		
	Must	AF	Co-inoculation	Sequential	Spontaneous
Histamine		-	-	-	-
Agmatine	-	-	-	-	-
Tyramine	-	-	-	-	-
Putrescine	4,02 ± 0,11	4,88 ± 0,1	5 ± 0,18	4,69 ± 0,74	4,65 ± 0,4
Cadaverine	-	-	-	-	-

Table 32. Evolution of biogenic amines through all the winemaking process

		After MLF		
	AF	Co-inoculation	Sequential	Spontaneous
Ethanol (% v/v)	13,17 ± 0,25	-	-	-
pH	3,63 ± 0,02	3,74 ± 0	3,79 ± 0,01	3,85 ± 0,01
Reducing sugars (g/L)	1,975 + 0,474	2,783 + 0,064	2,061 + 0,105	1,987 + 0,176
Malic acid (g/L)	4,14 ± 0,15	< 0,1	< 0,1	< 0,1
Lactic acid (g/L)	< 0,1	1,98 ± 0,13	2,09 ± 0,22	1,93 ± 0,09
Total acidity (g/L)	6,19 ± 0,05	5,7 ± 0,11	5,51 ± 0,05	5,03 ± 0,21
Volatile acidity (g/L)	0,28 ± 0,05	0,31 ± 0,08	0,34 ± 0,07	0,36 ± 0,08
Colour intensity	9,29 ± 0,18	6,72 ± 0,07	5,85 ± 0,39	8,8 ± 0,37
Total phenols (OD280)	5,69 ± 0,24	6 ± 0	5,53 ± 0	5,82 ± 0,25
Days MLF	-	12	12	30

All in all, this work constitutes the first study in which the consequences of different inoculation strategies with autochthonous strains have been analysed over Tempranillo wines from Rioja Alavesa region. Thus, it was observed that co-inoculation strategy may offer to regional winemakers the possibility to ensure a timely completion of the winemaking process prior to stabilisation and ageing period. In co-inoculated batches, the prompt completion of MLF led to a lower production of aroma masking compounds, as acids and higher alcohols, which derived in the perception of greater notes of ripe fruit. Finally, it was confirmed the suitability of strain P2A at winery scale, and although further work may be necessary to firmly confirm its suitability, this study represents another step to clarify the potential use of this strain as an additional strategy for high-quality wines production in Rioja Alavesa region.

5. GENERAL DISCUSSION

The employment of starter cultures for the fermentation industry has become a very common practice to guarantee the production of high-quality food with consistent characteristics. Microorganisms with good technological properties, providing sensory desirable compounds and lacking the ability to promote metabolites of health concern to humans are the main focus of multiple research studies nowadays (Berbegal et al., 2017). In wine industry, the success of winemaking process, in terms of quality and safety, relies mainly on the metabolism of microorganisms present during all vinification process, from vineyard to the fermentation processes and ageing period (Grangeteau et al. 2017, Liu et al. 2017). In this sense, indigenous microbiota can be formed by both beneficial and spoilage microorganisms (Pinto et al., 2015). In wine sector, one of the main factors to take into account regarding the quality and especially safety of the product is the presence of biogenic amines (BAs). These compounds have been described as main responsible for wine intolerance (Konakovsky et al., 2011), especially in individuals lacking or underexpressing the enzymes responsible for their degradation. In this regard, special emphasis has been placed by different international organisms to control the appearance of this compounds (EFSA, 2011; OIV, 2011). Histamine is the most widely studied BA due to its toxicity; however, the accumulation of other BAs, as tyramine, putrescine or cadaverine, also deserve in-depth analysis (Ladero et al., 2010).

It has been observed that the formation of these compounds occurs mainly during spontaneous malolactic fermentation (MLF) by the action of lactic acid bacteria (LAB) strains (Capozzi et al., 2017; Martuscelli et al., 2013). In this sense, although different strategies to prevent the formation of BAs in wine have been described, the

inoculation of safe malolactic starters is considered the main mechanism to avoid their accumulation (Patrignani et al., 2012; Restuccia et al., 2018). Furthermore, the careful selection and processing of healthy grape bunches, correct hygiene care during all the winemaking process or appropriate winemaking conditions, among others, will help to minimize the risk of contamination and therefore the appearance of BAs (OIV, 2011). In that way, selection of LAB strains lacking the ability to produce metabolites of health concern, as BAs, is considered the first step to constitute a collection of strains of potential application in wine industry. However, on many occasions the use of commercial cultures also leads to slow or delayed MLF, where the strain is not fully implanted and the risk of the appearance of potential spoilage microorganisms does not disappear (Ruiz et al., 2010b; Berbegal et al., 2017). The employment of indigenous starter cultures already adapted to specific regional winemaking conditions are a promising strategy that can help to combat this problem, maintaining the sensory characteristics and biodiversity of the region and ensuring an efficient and safe MLF.

In Rioja Alavesa, a worldwide recognized wine region, most of the wineries follow the winemaking process in a traditional way, and whereas yeast starter cultures are widely used, MLF is usually performed spontaneously, arising the risk of the appearance of BA-forming spoilage microorganisms (Berbegal et al., 2017; Izquierdo-Cañas et al., 2008). In this sense, the hypothesis of this thesis was that indigenous LAB strains, owning suitable technical and organoleptic characteristics, have the potential to be used as novel cultures to prevent the production of BAs in wine industry. Therefore, this work was focused on the identification and characterization of potential novel malolactic starters from Rioja Alavesa region. This challenge started with the analysis

of the current situation of BAs in Rioja Alavesa red wines. Higher levels than those found in other studies were detected, especially regarding putrescine levels. Putrescine was detected in highest concentration in all wines, followed by histamine, tyramine and cadaverine, respectively, as it has been usually observed in other studies (EFSA, 2011; Žurga et al., 2019). Although the situation in Rioja Alavesa is not alarming, as stablished toxicological levels for histamine and tyramine are significantly higher (Broquedis et al., 1998; Maintz et al., 2007), it should be taken into account that minimum concentrations can have serious adverse effects on sensitive individuals (Ladero et al., 2010; Smit et al., 2008). In this way, it was detected an opportunity for improvement in order to reduce BAs levels to minimum and increase the competitiveness of these wines. Since BA levels may be a limiting factor for the export of wines (Guo et al., 2015), the reduction of histamine and other BA levels could suppose an opportunity to stand out in the market as a safe and quality product. With the main aim of reducing the content of BAs in Rioja Alavesa red wines, the pursuit of potential indigenous malolactic starters began.

After the processing of several samples of must and wine from all the winemaking process of different wineries, nearly 300 bacterial isolates were obtained. It was elucidated that *Oenococcus oeni* was the predominant species in the different tested wineries, confirming its great adaptability to wine harsh conditions (Berbegal et al., 2017; Franques et al., 2018). Although other typical LAB species were also identified, as *Lactobacillus mali*, *Lactobacillus plantarum*, *Pediococcus parvulus* and *Lactobacillus hilgardii*, *O. oeni* was the leading species responsible for conducting spontaneous MLF. Actually, this species seems to own a plastic genome which enables the rapid adaptation to harsh environments presumably due to the lack of the MisMatch

Repairing system (Marcobal et al., 2008; Borneman et al., 2010). RAPD-PCR analysis elucidated that half of the detected genotypes belonged to *O. oeni,* with each winery sharing few genotypes. That is, winemaking conditions of each winery may create a distinguish ecosystem in which different strains are the best adapted. Indeed, specific genotypes have been identified to specific regions and niches (Bartowsky, 2017). In that way, the selection of most predominant indigenous species may be a criterium to preserve the singularity and biodiversity of those wines.

Climate change entails different problems for oenology, as reduced acidity and increased pH of wines, with the subsequent increased of undesired microbial population. In this regard, the species of *Paenibacillus* and *Staphylococcus* observed in the present study rarely had been found in wine environment (Benavent-Gil et al., 2016; Von Cosmos et al., 2017). Indeed, as far as we know, *P. polymyxa* and *S. warneri* species had not been identified before as BA producers in wine. These species have been described as opportunistic spoilage microorganisms related to food quality losses (Von Cosmos et al., 2017; Fey & Olson, 2010). This feature, together with their aminobiogenic activity, must be taken into consideration due to their spoilage potential. Among LAB strains only those belonging to *L. hilgardii* species showed ability to produce putrescine through agmatine deiminase pathway. None of the other LAB strains showed BA production, in contrast with what some authors have stated (Constantini et al., 2006; Coton et al., 2010; Moreno-Arribas et al., 2003).

Regarding *O. oeni*, which was by far the most abundantly detected species, none of the strains was BA producer. As this strain is the main responsible of spontaneous MLF, many authors have described it as the main producer of BAs in wine (López et

al., 2009; Lucas et al., 2008). However, there is great controversy about it (Garcia-Moruno *et al*., 2012). Either because the methods used to determine *O. oeni* BA production ability have given inconsistent data or have overestimated the concentration of these compounds, in many cases a wrong conclusion has reached regarding *O. oeni* responsibility (García-Moruno et al., 2012). In many other cases, however, its ability to produce BA has been proved (Berbegal et al., 2017; Coton et al., 2010; Landete et al., 2007). In this sense, the controversy is summarized by the fact that the ability to produce BA is not species-dependent characteristic but rather it is a specific attribute of each strain (Ladero et al., 2012). In the present work, both multiplex PCR and phenotypical assays following HPLC analysis were observed to be complementary in other to exhaustively characterize BA-forming bacteria. The low incidence of BA production found among oenological bacteria, makes necessary to look also to the presence of contaminants not related to MLF. In this regard, Costantini et al. (2009) showed that commercial yeast preparations were contaminated with histamine producing *Lactobacillus rossiae* and *L. buchneri*. In the same way, it has been detected the presence of multiple species, as *Gluconobacter oxydans*, *Asaia siamensis*, *Enterobacter sp*., *Serratia sp*. or *Staphylococcus epidermidis,* which have been identified as BA producers in wine (Benavent-Gil et al., 2016; Pinto et al., 2011; Ruiz et al., 2010a). The presence of these species, together with the detection in this work of BA-producing *Paenibacillus* and *Staphylococcus* species, should be considered a detrimental risk factor, and thus, appropriate prevention measures to avoid the presence of spoilage microorganism may be necessary.

Once the identification of non-BA-producing LAB strains had been carried out, these strains were submitted to further characterization. With the premise that strains

must resist low pH values and high ethanol and SO2 concentrations to efficiently implant in wine medium and initiate the MLF (Romero et al., 2018), firstly, technological traits, as growth response under typical winemaking conditions and their fermentation vigour were evaluated. Although non-oenococcal LAB strains showed similar or better growth rates at the different pH and ethanol conditions, when the effect of SO_2 over LAB growth was analysed, the greater stress tolerance of *O. oeni* strains was elucidated. In the same way, when the ability of LAB strains to carry out the MLF was evaluated, non-oenococcal LAB strains rapidly loss their viability, and thus, none was able to start the fermentation process. On the contrary, most *O. oeni* strains retained great viability and were able to successfully finish the process. Indeed, non-oenococcal LAB strains had been all isolated from must samples (Diez-Ozaeta et al., 2019), where nutrient concentration was rich and the presence of moderate concentrations of SO_2 enabled their growth (Volschenk et al., 2006). In this sense, although during winemaking process *O. oeni* growth is usually slower than other species (Lonvaud-Funel, 1999), it finally predominates in wine environment due to its great stress tolerance. Indeed, different stress response mechanisms have been described in *O. oeni* under wine harsh conditions. Changes at transcriptomic and proteomic levels have elucidated, among others, membrane fluidity adjustments, changes in membrane proteins, synthesis of stress proteins, up-regulation of amino acid metabolism and their transport, or the up-regulation of malate transport and citrate utilization as alternative energy sources. (Grandvalet et al., 2008; Maitre et al., 2014; Liu et al., 2017; Margalef-Català et al., 2016). In view of the obtained results, sensorial characterization of LAB strains was only focused on *O. oeni* strains due to their better potentialities as malolactic starter cultures. Thus, MLF does not only lead

to the conversion of malic acid into lactic acid, which results in the reduction of total acidity and the improvement of the microbial stability. This biological process entails many other changes in favour of increasing the sensory complexity of wines (Cappello et al., 2017; Sumby et al., 2019). Citrate metabolism, leading to the production of carbonyl compounds enhancing wine sensorial complexity, the release of trapped odorant compounds by multiple glycosidases or the formation and hydrolysis of esters conferring fruity notes, are different traits that will define the influence of LAB strains on wine organoleptic quality. All the identified *O. oeni* strains harboured the genetic machinery for citrate uptake and utilization, indeed, this trait was phenotypically confirmed in all strains. Production of citrate derived compounds, especially diacetyl but also acetoin, 2,3-butanediol and acetic acid, are all considered important for wine aroma enhancement (Olguín et al., 2009; Bartowsky & Henschke, 2004). The different glycosidase activities revealed the great diversity among strains. All of them showed βGLU activity, main enzyme leading the release of odorants in wine (Liu et al., 2017). Actually, in grapes and wine, glycosides are considered the main source of odorant compounds, such as terpenoids, benzenic compounds or C13-norisoprenoids (D´Incecco et al., 2004; Liu et al., 2017). Particularly noteworthy was the activity of the strain P2A, which showed the highest βGlu and αGlu activities (higher than commercial strains) under the different studied conditions. Less activity was observed when βXyl and αAra enzymes were analysed. Although this enzymes have been considered relevant in the release of impact odorant compounds from oak barrels, as vanillyn or whiskeylactone, little activity among *O. oeni* strains has been described in the bibliography (Bloem et al., 2008; Gagné et al., 2011).

Esters constitute another group of aromatic compounds considered of utmost importance. Both ethyl and acetate esters build an extensive family which contribute to the fruity attribute of wines (Cappelo et al., 2017). In wine, the final content of esters is the result of hydrolysis and synthesis reactions though the activity of several enzymes, as lipases, esterases and alcohol acyl tranferases, as well as, the chemical reactions that occur during wine ageing (Costello et al., 2013). As happened with glycosidase activities, many strains, as P2A, P3F and P7B, retained detectable activities under most restrictive conditions. Nowadays, the addition of commercially available enzymatic mixtures is a common practise among wineries, however, many of them are inhibited under winemaking conditions or may present side enzymatic reactions (Fia et al., 2014). In that way, the characterization of *O. oeni* glycosidase and esterase activities gave an insight on the prospective use of many of the strains in order to enhance the aromatic profile of wine. However, it was still necessary to investigate the performance of the best suited strains in real wine vinifications so as to elucidate their ability to perform a reliable MLF and modulate the sensorial properties of wine.

From a total of seventeen O. oeni strains, the study followed with the in-depth evaluation of seven strains (P2A, P3A, P3G, P3G, P5A, P5B and P7B) and the commercial Viniflora OENOS strain. In the fermentations of Tempranillo must, except for the P5A strain and the spontaneous fermentation that never started, remaining strains were able to exhaust malic acid concentration and maintained great viability after MLF had concluded. This fact is of special relevance since the ability to implant and remain in the wine is fundamental for the success of MLF as well as to prevent the growth of potential spoilage microorganisms (Ong, 2010). It must be highlighted the performance of P2A and P3A strains which completed the process in just 15 days.

In fact, they conclude earlier than Viniflora OENOS strain, which is the main commercial strain used in the region. The differential behaviour of the strains, as previously reported, may be due to different survival strategies and mechanisms that these strains own to face wine harsh conditions (Liu et al., 2017; Margalef-Català et al., 2016). There were no significant differences in the physical-chemical characteristics of wines, highlighting that no strain increased significantly volatile acidity. Since the metabolization of sugars and citric acid by LAB can lead to an excessive production of acetic acid and devalue wine sensory quality, volatile acidity is one of the most important parameters to take into account when performing MLF. As expected, there was no increase in any of the analysed BAs, confirming once again the safety of these strains. Not only health concern metabolite production but also off-flavour development is another threat that commonly arises when controlling the fermentation process. The most common compounds related to wine sensory depreciation are volatile phenols (Chescheir et al., 2015). These compounds impair unpleasant “smoked”, “stable” or “leather” aromas, with very low odour thresholds that mask the fruity and floral attributes of wine (Ferreira et al., 2007). In this sense, it was decided to measure the ability of *O. oeni* strains to hydrolyse esterified hydroxycinnamic acids (HCAs), as they act as precursors of volatile phenols. Only P5C strain showed a significant increase on caffeic, coumaric and ferulic acid concentrations after MLF. Once free HCAs are released, the presence of microorganisms with phenolic acid decarboxylase (pad) and vinyl phenol reductase (vpr) activity will promote the accumulation of volatile phenols (Berbegal et al., 2018). Although the capacity of certain LAB strains to produce these compounds has been observed in the bibliography, they have rarely been described in *O. oeni* (Santamaría

et al., 2018), as it was shown in the present work. However, the main spoilage microorganism and producer of volatile phenols in wine is *Brettanomyces bruxellensis*. This microorganism needs free HCAs to carry out the sequential production of the corresponding vinyl- and ethyl-phenols. Thus, the inoculation of malolactic cultures unable to release free HCAs has been described as a very interesting biocontrol strategy to prevent the proliferation of *Brettanomyces*, and therefore, the accumulation of these compounds (Berbegal et al., 2018; Chescheir et al., 2015; Schopp et al.,2013). This microorganism is typically isolated after AF and before the onset of MLF, when moderate levels of SO_2 and still some nutrients are available. Thus, the total implantation of malolactic starter and the rapid consecution of MLF has been proved as a useful strategy to control *B. bruxellensis* proliferation (Berbegal et al., 2018). In this regard, all strains, except for P5C, were suitable as potential biocontrol agents.

When the different volatile compounds profiles of wines were evaluated, it was elucidated the ability of these strains to build specific aroma profiles. Although yeasts have typically been considered the main responsible for constructing the aroma profile of wines, in the last years a special effort has been made to clarify the role of LAB strains in the evolution of wine aroma profile (Cappello et a., 2017; Brizuela et al., 2018). Whereas different studies have not found major changes in the aromatic profile of wines after performing MLF (Belda et al., 2017; Vilela, 2020), in the present study It was observed how each strain led to the production of characteristic profiles in terms of esters, acids, alcohols or terpenes concentration. The variability found among strains was summarized by the PCA study, which elucidated two distinct group of strains. One of them, constituted by P2A, P3A, P3G and P5C strains, had a greater

relationship with esters; while the other, built by P7B and OENOS, was related with more diverse compounds as acids, terpenes, alcohols and C13-norisoprenoids. That is, while one group of strains may potentially enhance the fruity notes of Rioja Alavesa wines, the other may present more floral and lactic attributes. The sensory complexity of wines does not only depend on the fact that some compounds are in greater or lesser concentration or if they are or not above their perception threshold. The presence of different chemical families that can act synergistically, as well as possible masking effects, must be also considered when defining wine aroma complexity (Ferreira et al., 2007). Thus, although the inoculation of one or another strain may not have a noticeable effect on the sensory perception of wine, their potential to modulate the different aromatic compounds was confirmed.

The selection of novel starters does not only pursue positive impacts in terms of safety and sensorial properties of wine, but it also seeks for improvements leading to the reduction of both processing time and environmental impacts related to winemaking (Berbegal et al., 2017). In this sense, in the last years the strategy of yeast and bacteria co-inoculation is gaining special attention. In the present work, strains P2A, P3A, P3G, P7B and Viniflora OENOS were submitted to both co-inoculation and sequential inoculation strategies. In all co-inoculated batches, a significantly reduction of the winemaking time was observed. In addition, MLF time itself was also reduced with co-inoculation. Yeast and bacteria co-inoculation permitted a significant reduction in overall process time, which turns advantageous to the winery from an economical point of view (Abrahamse and Bartowsky, 2012; Cañas et al., 2015). The wine is also stabilized sooner, reducing potential contaminations with spoilage microorganisms (Cañas et al., 2015; Tristezza et al., 2016). In this sense, it must be highlighted the

behaviour of the strain P2A, which together with the commercial strain, when co-inoculated ended the process together with the AF. This fact underlines the great yeast-bacteria compatibility, since typically, even when co-inoculated, MLF starts once the AF has concluded (Antalick et al., 2013), as it happened with the rest of the strains. Regardless the timing of MLF, yeast and bacteria co-inoculations led to different aroma profiles compared to traditional sequential strategy. In this sense, PCA analysis clearly differentiated both strategies. Generally, in co-inoculations less concentration of higher alcohols, acids and esters was appreciated. This reduction was more acute in those fermentations that were carried out in parallel with the AF (P2A and commercial strains). In that way, the close yeast-bacteria interactions that were favoured with the co-inoculation strategy may have modified the metabolism of these microorganisms. Competition for available nutrients, modifications of each other's metabolites and changes in each metabolic activities, may led to specific aroma profiles in co-inoculated batches (Antalick et al., 2012; Balmaseda et al., 2018; Rossouw et al., 2012). The fact that co-inoculations showed lower concentrations of esters, mainly in the case of P2A and Viniflora OENOS strains, could signify that these wines had less fruity aroma, however, this was not the case. These wines were described as fruitier, with less dairy and herbal notes. In that way, the lower production of higher alcohols and acids, which in high quantities have a negative effect upon wine aroma complexity by masking wine fruity and floral attributes (Ferreira et al., 2007; Antalick et al., 2013), enabled the perception of desired aromas in co-inoculated batches.

In view of the obtained results, it only remained to prove the best suited strain at industrial scale. According to the different analysis performed over the different

research studies, it was decided that the pilot test at winery would be performed with the strain P2A. At winery it was confirmed its great suitability. The implantation with both co-inoculation and sequential inoculation was total and, in this sense, nor spoilage microorganisms neither biogenic amine production was detected throughout the fermentation process. The overall winemaking time was significantly reduced with both inoculation strategies compared to spontaneous fermentation, leading to a prompt wine stabilization. Time reduction not only affects microbial stability, but it also results economically advantageous (e.g. cost and energy reduction at maintaining wine tank temperatures, reduction of overall winemaking process and a prompt market place of the product) (Berbegal et al., 2017; Tristezza et al., 2016). The total implantation and the fast consecution of MLF are of great value in order to use P2A strain as biocontrol agent (e.g. against *Brettanomyces*) to prevent the growth of spoilage microorganisms at critical winemaking stages, as the lapse time between AF and the onset of MLF (Berbegal et al., 2019). In this sense, this strain meets all the characteristics that a novel starter culture should fulfil. It is a safe strain, with a rapid and total implantation capacity, which is also indicated to perform the MLF through co-inoculation. This inoculation strategy is rarely used in Rioja Alavesa and in view of the results, it may present an advantageous strategy for many winemakers in terms of time/cost reduction.

In the last years the selection of indigenous strains, which are already adapted to specific winemaking conditions, is gaining special attention in order to perform a safe and reliable MLF (Franquès et al., 2017; Petruzzi et al., 2017). Each winemaking area has its own terroir, described as the combination of multiple variables as the grape cultivar, climate, geology and winemaking practices. Nowadays, the contribution of

indigenous bacterial ecology to the specific wine´s terroir has also been evidenced (Gilbert et al., 2014; Zarraonaindia et al., 2015). Rioja Alavesa, one of the most valuable wine regions of the world, could have its own malolactic starter in order to gain better control over the fermentation process, keep regional wine character and enhance wine sensorial complexity. Actually, this first strain could entail the beginning of a library of malolactic starters designed for this region so as to stop the dependence on foreign commercial cultures. Indigenous starter selection strategy can be also used to select LAB strains adapted to specific wine conditions, allowing the production of customized MLF starter cultures for specific winery needs (Berbegal et al., 2017). The inoculation of this autochthonous strain is considered an advantageous strategy for wineries that may have problems in terms of biogenic amines concentration as well as wineries that want to add value to their wine as a safe and high-quality product.

One of the main challenges for the wine sector is to face the problems associated with climate change. Main issues identified are (i) the increased of spoilage microorganisms, (II) increased sugar and thus, ethanol concentration, (III) reduced acidity and increased pH, (IV) unbalanced sensorial properties (colour, aroma) and (V) awareness of safety issues (biogenic amines, mycotoxins) (Berbegal et al., 2019). Thus, climate change puts the sustainability and typicity of wines at risk with great consequences on product quality and safety (Whitfield et al., 2018). In this context, taking into account the great socio-economic weight that wine sector represents in the Basque Country, it is highly relevant to develop different strategies to face the challenges that the present and future hold. An enhanced risk of proliferation of spoilage microorganisms due to increased pH values and lower acidities is one of the major issues to consider. Undesired microbial proliferation from fermentation

processes up to ageing process could lead to the accumulation of metabolites of health concern (e.g. biogenic amines) as well as the appearance of sensory defects (Berbegal et al., 2019; Drappier et al., 2019). Main strategy addressed to face these threats is the inoculation of safe starter cultures with great implantation capacity and with the potential to be used as biocontrol agents (Berbegal et al., 2019). Different strains studied in this study accomplish these features, being the strain P2A the main candidate, and although further analysis may be necessary to confirm its potential as malolactic starter, this study represents a great advance in obtaining the first malolactic culture from Rioja Alavesa region.

Future works may confirm the ability of this strain to be fully implanted in the winery after the ageing period, preventing in that way the accumulation of BAs. At sensorial level, it must be confirmed that the typicity of Rioja Alavesa red wines is ensured and that novel sensorial characteristics may even be appreciated, giving an added value to the product. In this sense, the inoculation of novel indigenous malolactic starters should be considered as a strategy for the winemaker to develop novel products with distinct characteristics. With the present work, a new field in the selection of indigenous malolactic cultures from Rioja Alavesa region is opened. All in all, characterization and selection of novel cultures, and combinations of them, with desired features may represent a promising research line to enhance the quality of Rioja Alavesa wines.

6. CONCLUSIONS

1. All types of analysed wines (*year*, *crianza* and *reserva*) showed similar BAs levels, suggesting that main changes occurred after fermentation processes. It was detected the need to reduce the levels of BAs, not only those considered toxicological, as histamine and tyramine, but also special emphasis was on reducing the levels of putrescine, which was detected in excessive levels.

2. It was confirmed the great predominance of *O. oeni* strains during spontaneous MLF in Rioja Alavesa wines

3. Low incidence of BA-producing lactic acid bacteria strains was detected. Only *L. hilgardii* strains were able to produce putrescine via the agmatine deiminase pathway.

4. It has been the first work in which *Staphylococcus warneri* and *Paenibacillus spp.* strains have been described as BA producers in wine.

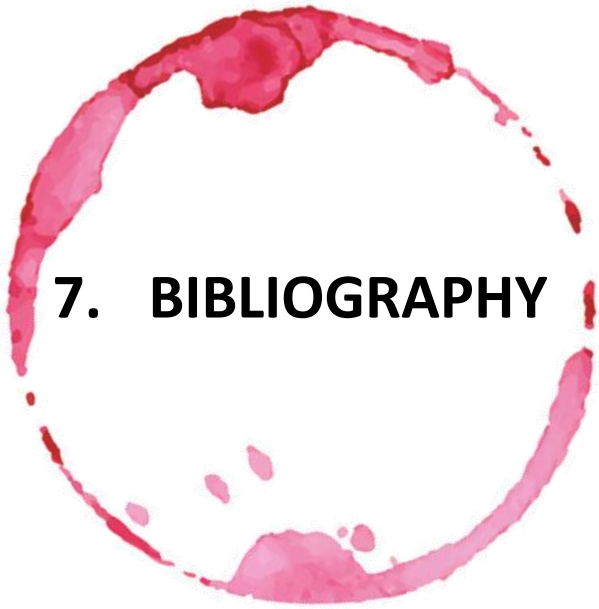
5. The resistance of *O. oeni* strains to wine harsh conditions, over other LAB species during MLF, was confirmed.

6. It was elucidated the ability of *O. oeni* strains to retain key glycosidase and esterase activities under winemaking conditions.

7. No production of BAs was detected in wines produced with selected indigenous *O. oeni* strains. In addition, it was observed the ability of *O. oeni* strains to differentially modulate wine aromatic profile in terms of esters, alcohols, acids or terpenes.

8. Inoculation strategy (co-inoculation vs sequential) influenced the evolution of wine aromatic profile. The observed differences among both strategies were also perceived at sensory level.

9. It was confirmed the suitability of the strain P2A to work in large scale fermentations at winery, resulting an advantageous alternative to significantly reduced the overall winemaking time as well as to better control the fermentative process.



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ANNEXES

ANNEX INDEX

ANNEX 1:

Supplementary Material Study 1

Supplementary Table 1. List of analysed wines (n=70) and their corresponding BA values. Wines are displayed according to their ageing time (*year*, "*crianza"* and "*reserva"*)

		Biogenic amines (mg/L)				
	Year	Histamine	Tyramine	Putrescine	Cadaverine	Total BA
Year						
Amador García	2019	4,09	1,87	6,57	1,40	13,92
Arnalte I	2016	11,94	9,66	32,71	4,13	58,44
Arnalte II (6 months ageing)	2016	5,89	4,34	20,64	1,53	32,39
Artadi	2017	n.d.	n.d.	4,30	1,04	5,34
Baigorri	2016	5,34	4,73	12,22	2,80	25,09
Beltxuri I	2016	6,16	1,38	9,14	1,71	18,40
Beltxuri II (6 months ageing)	2016	5,43	n.d.	10,54	2,12	18,09
Betikoa	2018	2,80	2,08	5,08	n.d.	9,97
Conde de Valdemar	2017	7,00	2,79	9,96	1,50	21,25
Conde de Valdemar	2019	3,36	1,07	8,84	1,54	14,81
Eguren Ugarte	2019	5,02	4,57	18,43	2,20	30,22
El de abajo	2017	3,70	4,02	19,21	n.d.	26,93
Faustino	2019	4,14	5,55	19,53	0,54	29,75
Gomez segura	2017	4,36	6,21	16,37	3,53	30,47
Lar de Paula	2018	2,80	3,43	10,33	3,41	19,97
Luis Cañas I	2016	5,16	3,52	16,93	2,72	28,32
Luis Cañas II	2018	4,48	4,17	17,38	2,23	28,26
Ondalan	2017	1,12	8,69	8,59	1,07	19,47
Ostatu I	2018	4,03	3,57	16,38	2,02	26,00
Ostatu II	2019	2,09	1,82	13,68	1,62	19,22
Patxontxo	2014	3,19	n.d.	9,66	2,36	15,22
Perez Irazu	2017	2,84	2,16	34,01	1,09	40,11
Raices de Oro	2016	11,41	9,64	57,23	6,71	84,98
Crianza						
Alcorta	2006	3,56	2,23	16,98	1,62	24,39
Arabarte	2014	2,65	4,90	26,63	0,94	35,12
Aurum-Murua	2014	7,34	3,56	17,92	5,24	34,06
Baigorri	2016	4,14	4,20	15,58	1,60	25,52
Conde Valdemar	2015	2,00	1,86	18,30	n.d.	22,16
Conde Valdemar	2016	0,86	n.d.	7,51	n.d.	8,37
Dominio Berzal	2015	3,10	6,51	32,36	1,02	42,98
Eguren Ugarte	2015	1,92	3,72	13,65	1,17	20,45
El Buscador	2016	2,20	1,17	13,64	1,58	18,59
El Pacto	2016	5,54	5,10	13,18	1,47	25,28

Supplementary Table 1 continuation

		Biogenic amines (mg/L)				
	Year	Histamine	Tyramine	Putrescine	Cadaverine	Total BA
Crianza						
Fernández de Pierola	2014	2,60	1,10	12,40	4,59	20,69
Izadi	2015	5,44	1,34	10,73	2,41	19,93
Lar de Paula-Terrazas	2014	4,54	5,79	12,92	2,41	25,67
Laukote	2016	3,84	4,37	14,23	2,69	25,13
Lur	2014	n.d.	n.d.	4,11	0,99	5,10
Marques de Caceres	2016	6,90	1,02	8,09	1,69	17,69
Marqués de Vitoria	2014	5,01	4,04	11,34	3,29	23,68
M de Murua	2015	7,66	2,59	13,50	4,70	28,45
Monólogo	2014	4,55	n.d.	14,48	2,58	21,61
Murua VS	2015	11,86	1,79	11,35	4,99	29,99
Ostatu	2015	3,44	0,85	7,91	1,47	13,67
Solaguen	2017	2,24	1,91	7,37	1,52	13,04
Solar de Estraunza	2014	9,97	8,42	22,92	5,03	46,34
Vallobera	2015	1,27	n.d.	15,75	n.d.	17,03
Valserrano	2015	3,75	n.d.	7,55	1,36	12,67
Viña Real	2014	4,61	1,91	10,58	1,81	18,90
Reserva						
Alútiz	2015	4,84	3,51	15,01	2,42	25,78
Amaren	2010	2,92	2,02	4,65	2,35	11,95
Campillo	2016	5,24	3,45	15,25	2,24	26,18
Casa Primicia-Julian Madrid	2008	3,81	n.d.	8,41	3,73	15,95
Conde Valdemar	2004	2,44	7,17	20,44	n.d.	30,05
Conde Valdemar	2012	2,88	1,28	6,69	1,32	12,17
El Coto	1983	1,16	5,63	21,44	n.d.	28,24
Faustino I "Gran Reserva"	2009	4,58	1,78	15,67	2,65	24,68
Faustino V	2015	5,62	2,87	17,05	2,89	28,43
Frías del Val	2011	6,89	2,43	13,87	2,67	25,86
Glorioso	2016	5,38	3,06	16,23	2,39	27,06
Izadi	1999	9,29	7,88	29,51	2,63	49,30
Marqués de Riscal	2013	6,87	4,24	17,58	4,71	33,40
Murua VS	2013	9,63	4,59	17,45	5,06	36,74
Murua VS	2014	2,62	0,80	9,45	2,04	14,92
Pierola	2004	5,33	9,00	24,56	1,07	39,95
Reserva Murua	2013	7,52	3,74	18,94	5,38	35,59
Reserva Murua	2014	3,81	1,51	10,57	2,70	18,59
Torre de Oña	2012	5,88	1,58	12,78	0,60	20,83
Vallobera	2014	1,31	0,47	8,87	1,13	11,78
Viña Arana	2008	6,40	2,84	15,00	1,62	25,86

n.d. (not detected)

ANNEX 2:

Supplementary Material Study 3

Supplementary Table 2. Mean growth rate values (logCFU/ml/day) of each strain under the different conditions of each analysed parameter (pH, ethanol and SO_2). Different letters underline significant differences ($p<0,05$) among strains.

	O. oeni strains											
pH	P1A	P1B	P1C	P1D	P2A	P3A	P3B	P3C	P3F	P3G	P5A	P5B
3,4	0,28ab ± 0,03	0,29ab ± 0,02	0,52bc ± 0,06	0,45b ± 0,08	0,08a ± 0,01	0,2ab ± 0,04	0,11a ± 0,01	0,03a ± 0,01	0,06a ± 0,03	0,24ab ± 0,02	0,25ab ± 0,21	0,06a ± 0,0
3,5	0,31b ± 0,02	0,34b ± 0,02	0,45b ± 0,01	0,43b ± 0,01	0,14a ± 0,01	0,32ab ± 0,06	0,09a ± 0,01	0,48ab ± 0,55	0,09a ± 0,05	0,34ab ± 0,01	0,44b ± 0,38	0,11a ± 0,01
3,6	0,48a ± 0,16	0,41a ± 0,02	0,73a ± 0,24	0,68a ± 0,31	0,20b ± 0,02	0,43a ± 0,04	0,16b ± 0,03	0,18b ± 0,04	0,21ab ± 0,21	0,29ab ± 0,10	0,23ab ± 0,06	0,18ab ± 0,07
3,7	0,37bc ± 0,05	0,44bc ± 0,04	0,76bc ± 0,34	0,53bc ± 0,04	0,24ab ± 0,12	0,59bc ± 0,32	0,26ab ± 0,11	0,27ab ± 0,03	0,13a ± 0,04	0,28ab ± 0,13	0,35bc ± 0,05	0,16ab ± 0,01
3,8	0,30b ± 0,19	0,43b ± 0,03	0,50b ± 0,03	0,75b ± 0,49	0,54b ± 0,62	0,41b ± 0,30	0,18a ± 0,02	0,37b ± 0,09	0,39b ± 0,28	0,41b ± 0,19	0,65b ± 0,63	0,16a ± 0,04

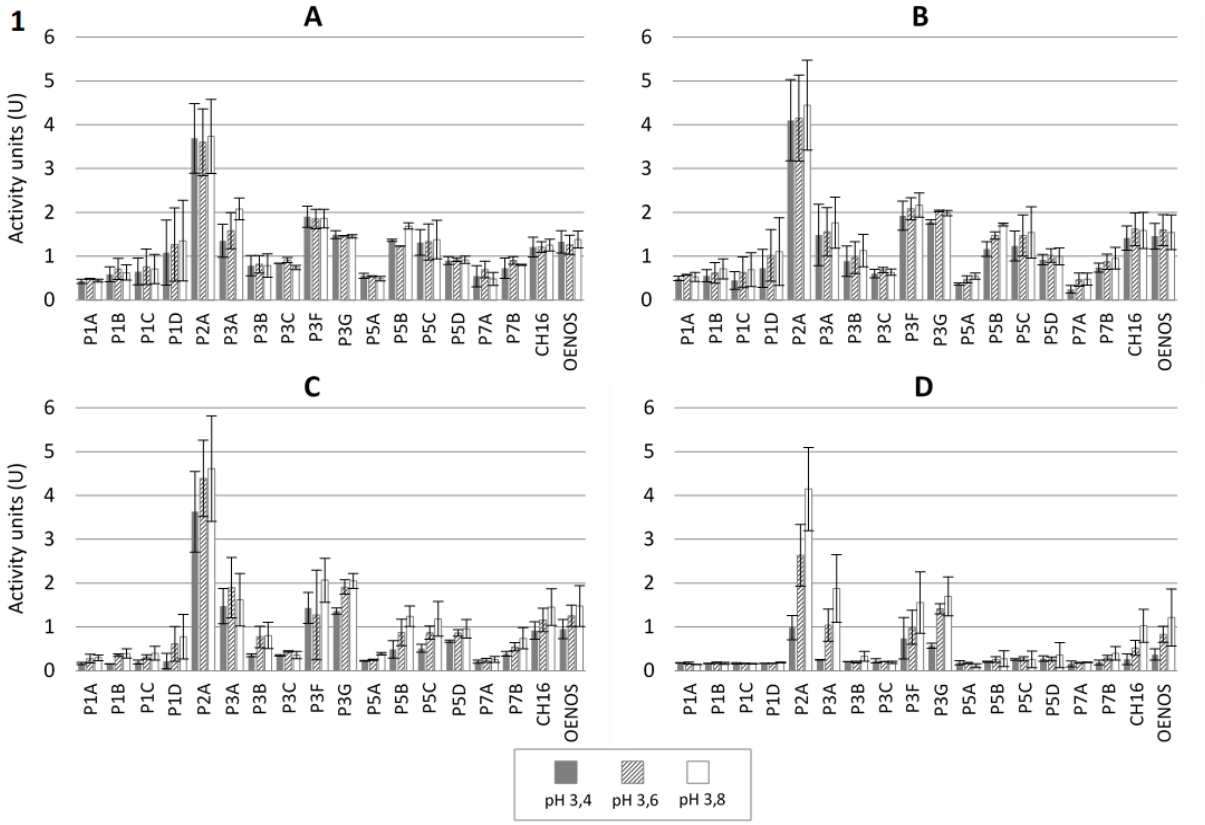
	O. oeni strains						*L. mali* strains			*L. plantarum*
pH	P5C	P5D	P7A	P7B	CH16	OENOS	LM1	LM2	LM3	LP1
3,4	0,07a ± 0,01	0,1a ± 0,04	0,24ab ± 0,11	0,18ab ± 0	0,27ab ± 0,24	0,06a ± 0,01	0,27ab ± 0,04	0,21ab ± 0,02	0,1a ± 0,02	1,24c ± 0,36
3,5	0,13a ± 0,02	0,17a ± 0	0,31ab ± 0,24	0,2a ± 0	0,37ab ± 0,29	0,11a ± 0	0,42b ± 0,12	0,31ab ± 0,11	0,2a ± 0,04	1,49c ± 0,49
3,6	0,2ab ± 0,12	0,23ab ± 0,07	0,41a ± 0,28	0,25ab ± 0	0,31ab ± 0,17	0,21ab ± 0,18	0,81a ± 0,34	0,6a ± 0,27	0,58a ± 0,23	2,12c ± 0,15
3,7	0,17ab ± 0,03	0,18ab ± 0,02	0,44bc ± 0,37	0,2ab ± 0,1	0,19ab ± 0,06	0,17ab ± 0,06	0,91c ± 0,44	0,86bc ± 0,16	0,71bc ± 0,26	2,36d ± 0,06
3,8	0,47b ± 0,36	0,19a ± 0,04	0,49b ± 0,36	0,23a ± 0,08	0,53ab ± 0,62	0,22a ± 0,01	1,02bc ± 0,28	1,11bc ± 0,4	0,86bc ± 0,35	2,74d ± 0,48

Supplementary Table 3. Mean lag period (days) for each strain under the different concentrations of total SO_2.

	O. oeni strains											
ppm total SO_2	P1A	P1B	P1C	P1D	P2A	P3A	P3B	P3C	P3F	P3G	P5A	P5B
5	0 ± 0	0 ± 0	2,19 ± 1,39	0,87 ± 0,95	0,39 ± 0,22	1,45 ± 1,09	1,41 ± 1,99	0 ± 0	0 ± 0	0,48 ± 0,27	0,53 ± 0,28	0 ± 0
15	4,36 ± 1,79	5,07 ± 2,62	4,77 ± 2,53	5,08 ± 3,07	2,67 ± 3,35	7,76 ± 2,75	5,38 ± 7,61	2,7 ± 2,86	0 ± 0	3,51 ± 4,81	4,23 ± 0,9	0,86 ± 1,21
30	5,24 ± 7,41	11,2 ± 1,7	7,15 ± 0,8	8,42 ± 1,12	9,28 ± 2,22	10,13 ± 0,18	-	7,95 ± 1,27	3,75 ± 0,52	5,72 ± 8,09	6,06 ± 0,37	5,88 ± 1,09
40	-	-	-	-	-	-	-	-	-	8,6 ± 0,82	-	7,79 ± 2,28

	O. oeni strains						*L. mali* strains			*L. plantarum*
ppm total SO_2	P5C	P5D	P7A	P7B	CH16	OENOS	LM1	LM2	LM3	LP1
5	0 ± 0	0,23 ± 0,11	0,02 ± 0,03	0,17 ± 0,06	0 ± 0	0,74 ± 1,04	3,41 ± 3,38	2,91 ± 0,6	3,82 ± 1,37	1,98 ± 1,27
15	0 ± 0	0,29 ± 0,41	4,42 ± 1,32	0 ± 0	0 ± 0	0 ± 0	5,94 ± 1,08	6,25 ± 1,31	4,9 ± 1,28	1,92 ± 2,72
30	5,11 ± 2,03	2,59 ± 0,04	7,17 ± 0,49	7,52 ± 2,53	5,37 ± 3,15	0 ± 0	4,16 ± 5,88	9,02 ± 3,81	9,44 ± 2,09	-
40	8,16 ± 0,55	8,79 ± 0,12	6,82 ± 1,88	4,54 ± 0,76	3,92 ± 0,13	-	-	-	-	-

1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B
C
D
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2
3
4
5
6
P1A
P1B
P1C
P1D
P2A
P3A
P3B
P3C
P3F
P3G
P5A
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OENOS
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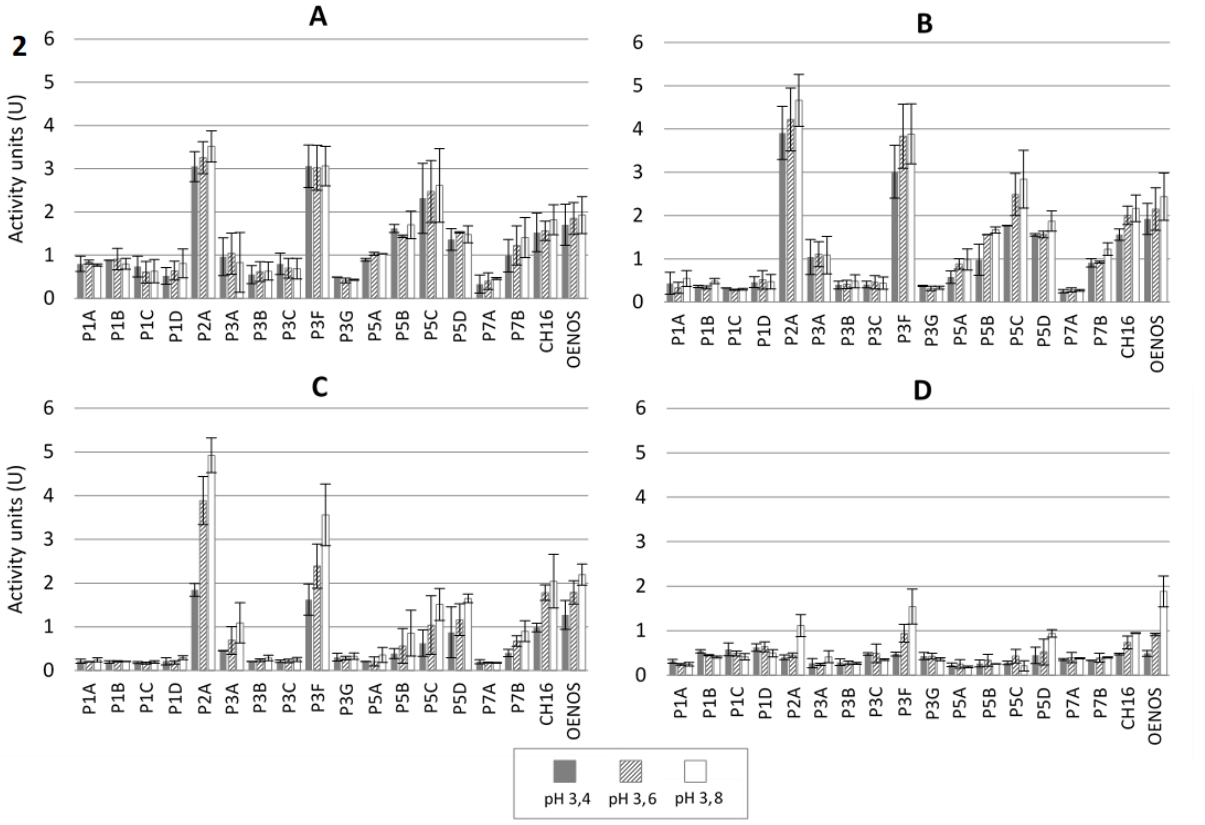
pH 3,8

2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B
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D
Activity units (U)
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P1A
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P3A
P3B
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P3G
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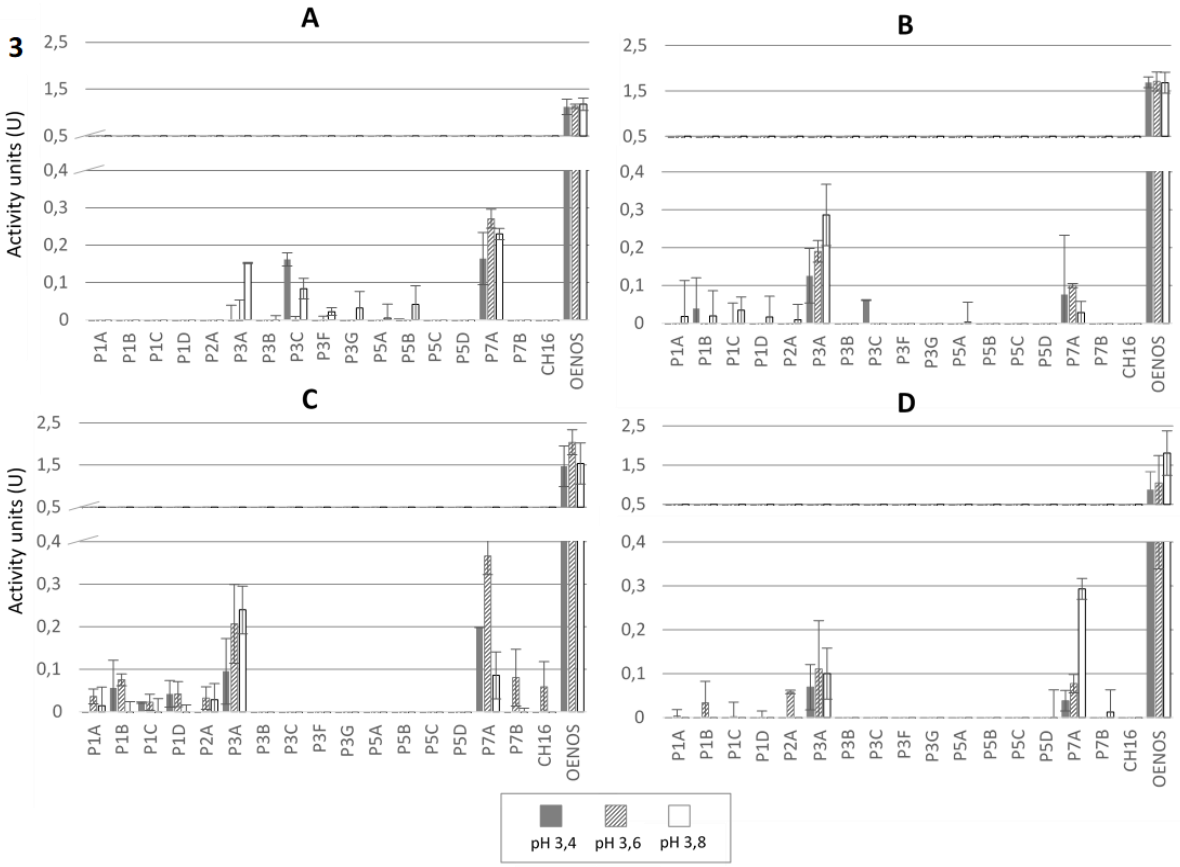
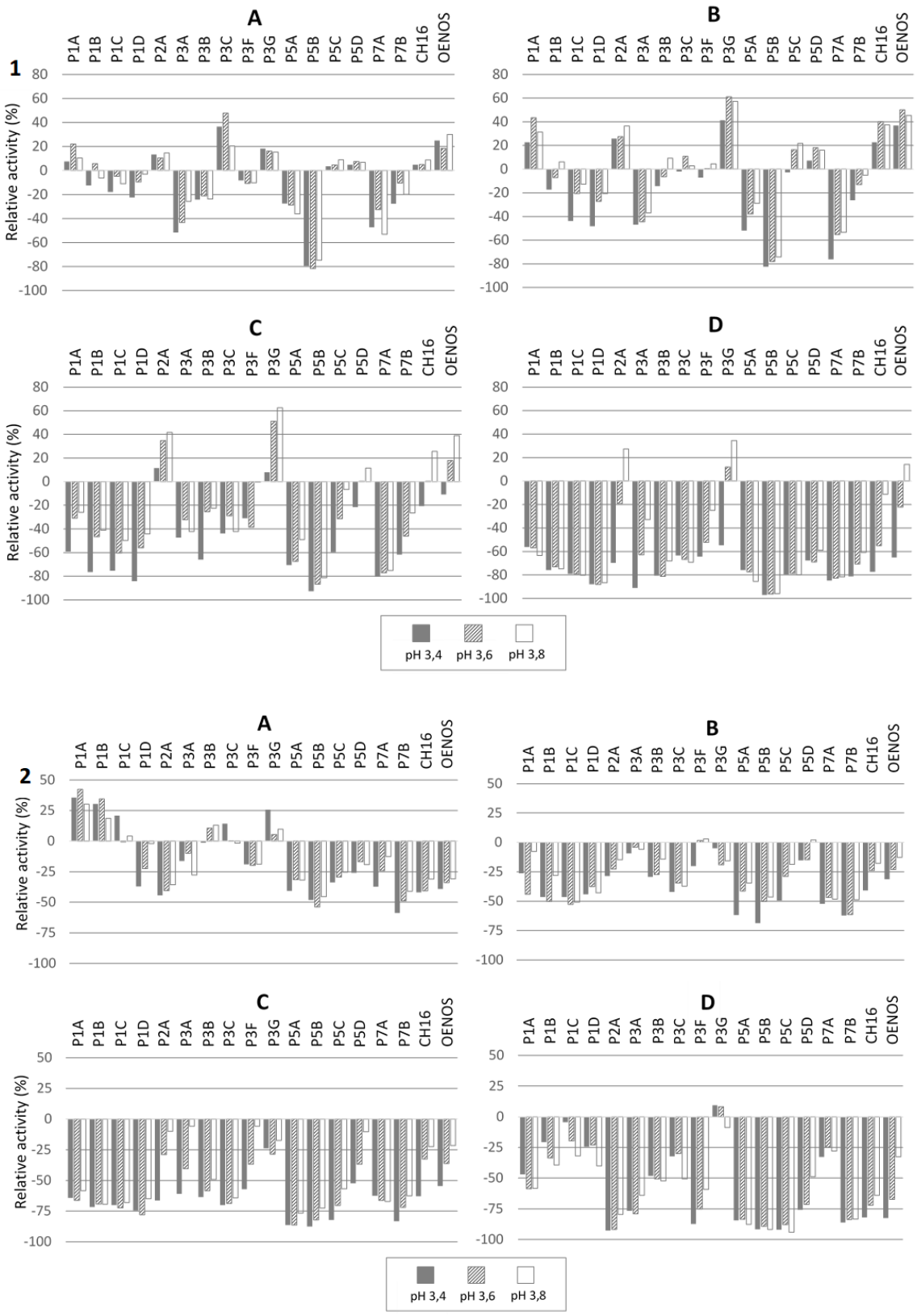
pH 3,6
pH 3,8

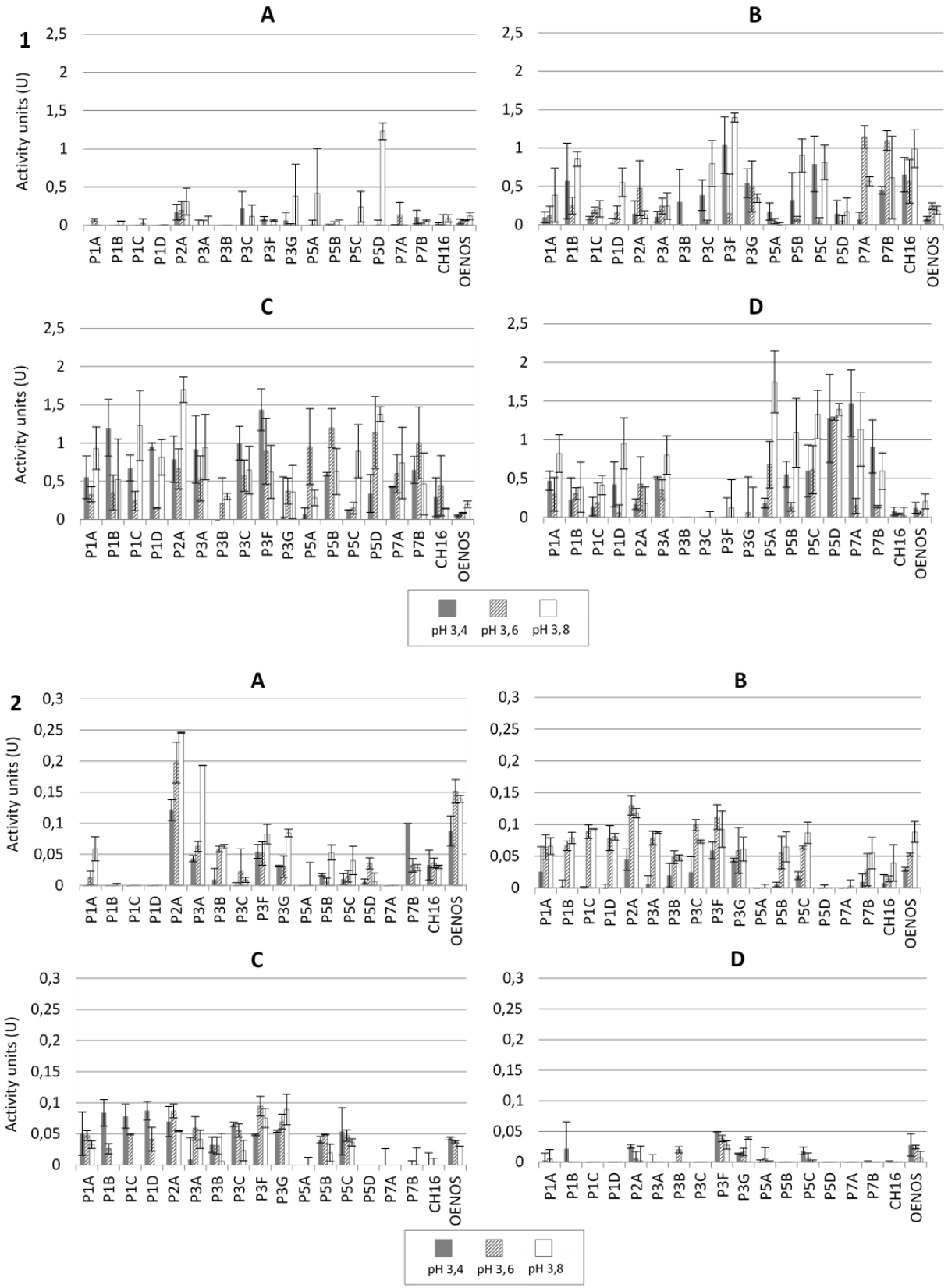
272



Supplementary Figure 1. β-glucosidase (1), α-glucosidase (2) and β-xylosidase (3) activity of all strains under different pH and 0% (A), 10% (B), 12% (C) and 14% v/v ethanol (D) combinations. Activity unit (U) refers to nmol of liberated p-nitrophenol per minute per milligram of cell dry weight



Supplementary Figure 2. Relative β-glucosidase (1) and α-glucosidase (2) activity, compared with the control assay, under different pH and 0% (A), 10% (B), 12% (C) and 14% (D) ethanol combinations



Supplementary Figure 3. Esterase activity of all strains against p-nitrophenyl-acetate (1) and p-nitrophenyl-octanoate (2) under different pH and 0% (A), 10% (B), 12% (C) and 14% v/v ethanol (D) combinations

ANNEX 3:
Publications

Publications derived from the thesis work:

1. Diez-Ozaeta, I., Amárita, F., Lavilla, M., & Rainieri, S. (2019). Ecology of indigenous lactic acid bacteria from Rioja Alavesa red wines, focusing on biogenic amine production ability. *LWT-Food Science and Technology*, 116, 108544. Copyright Elsevier © 2019.

   DOI: 10.1016/j.lwt.2019.108544

2. Diez-Ozaeta, I., Lavilla, M., & Amárita, F. (2020). Technological characterization of potential malolactic starters from Rioja Alavesa winemaking region. LWT-Food Science and Technology, 134, 109916. Copyright Elsevier © 2020.

   DOI: 10.1016/j.lwt.2020.109916

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