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POST-ALCOHOLIC FERMENTATION MICRO-ECOLOGY OF WINES: THE ROLE OF PHENOLIC COMPOSITION AND THE EFFECTS ON QUALITY

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Enology and Viticulture

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Resumo

A investigação apresentada nesta tese explora as interações entre certos compostos fenólicos naturalmente presentes nos vinhos e os microrganismos geralmente presentes neste mesmo meio. As interações estudadas incluem os efeitos no crescimento e metabolismo microbiano, a diversidade intra-específica de *Oenococcus oeni* e a evolução de compostos voláteis e não voláteis durante a fermentação malolática (FML) e subsequente armazenamento dos vinhos. Foi também estudada a capacidade de várias estirpes de bactérias de ácido láctico (BAL) para liberar ácidos hidroxicinâmicos (AHCs) a partir dos correspondens ácidos hidroxicinamoil-tartáricos .

A análise de metabolismo de 16 vinhos diferentes analisados na fase pós-malolática foi realizada usando dados de sequência de amplicons 16S (para bactérias) e ITS2 (para fungos). Foram observados padrões semelhantes em todos os vinhos neste nível de discriminação, tendo sido os géneros *Saccharomyces cerevisiae* e *Acetobacter, Gluconobacter* e *Swaminathania*, os mais abundantes. No que diz respeito aos perfis de compostos fenólicos e voláteis, os vinhos tintos e brancos foram agrupados separadamente e os vinhos franceses e espanhóis tenderam a agrupar-se em conjunto.

Os efeitos do kaempferol e dos ácidos *trans*-caféico e *trans*-caftárico (adicionados a 10 mg/L) no crescimento e metabolismo microbiano foram explorados em vinho puro e em vinho misturado com o meio de cultura MRS (de Man, Rogosa & Sharpe) em experiencias inoculadas e não-inoculadas com *O.oeni*. O impacto mais forte no crescimento microbiano, atividade malolática e atividade metabólica geral no BAL, foi observado para o kaempferol, tendo o ácido *trans*-cafárico mostrando o efeito mais fraco. Os efeitos do cada composto fenólico variaram de acordo com o meio utilizado, o tipo de FML (com ou sem inoculação) e a estirpe de *O. oeni* inoculada (Oenos™ ou CH35™).

Utilizando concentrações encontradas no vinho como referência, foram estudados vários compostos fenólicos, nomeadamente: flavan-3-óis ((+)-catequina e (-)-epicatequina), flavonóis (kaempferol e quercetina), HCA (ácidos *trans*-*p*-cumárico e *trans*-ferúlico) e *trans*-resveratrol em experiências com vinhos não inoculados e inoculados com Oenos™. Dependendo na sua concentração, a presença de (+)-catequina causou um impacto positivo na população de leveduras, ativou ou retardou a degradação do L-ácido málico ou inibiu o crescimento de bactérias. Todos os fenólicos testados, em todas as concentrações, atrasaram o consumo de ácido cítrico por BAL nas amostras inoculadas. O efeito de flavonóis e de *trans*-resveratrol na diversidade de *O. oeni* foi estudado, tendo-se observado ser dependente das estirpes em estudo. O aumento nos níveis de ácido *trans*-*p*-cumárico e *trans*-ferúlico pareceu induzir a liberação de ácido *trans*-caféico a partir de um dos seus precursores, possivelmente através de um aumento na atividade da cinamoil-esterase de algumas estirpes. Os flavan-3-óis e flavonóis testados inibiram o crescimento destas bactérias durante o armazenamento. Os compostos fenólicos testados afetaram sistemas enzimáticos responsáveis pela produção e degradação de importantes metabolitos envolvidos na qualidade organolética dos vinhos.

A presença da enzima cinamoil-esterase (CE) nos microrganismos do vinho é relevante, uma vez que confere a capacidade de modular a composição de ácidos fenólicos de um vinho (que, de outro modo, não biologicamente disponíveis) através da quebra da ligação éster dos ácidos

hidroxicinamoil-tartáricos. Cinco estirpes comerciais de *O. oeni* foram estudadas a este respeito, três exibindo atividade de CE (Oenos™, CiNe™ e CH35™) e duas não (CH16™ e CH11™). A atividade de CE foi detetada em extratos livres de células das três estirpes CE positiva (CE+) e uma das estirpes CE negativa (CE-). A partir da análise comparativa do genoma, não foi detetado nenhum gene exclusivo para as três estirpes CE+ e a inferência de que as diferenças na capacidade de transporte da membrana pudessem estar por trás das diferenças na atividade do CE, também não foi apoiada conclusivamente. Esta faceta merece ser mais explorada, assim como a possibilidade do envolvimento de moléculas de vinho e de mais enzimas na atividade da CE.

Palavras-chave: vinho, compostos phenolicos, bactérias de ácido láctico, compostos voláteis

Abstract

The research presented in this thesis explores the interactions between certain phenolic compounds that are naturally present in wines and the microorganisms that are generally present in this medium. The interactions studied include the effects on microbial growth and metabolism, the intra-specific diversity of *Oenococcus oeni* and the evolution of volatile and non-volatile compounds during malolactic fermentations (MLF) and subsequent storage. The ability of several strains of lactic acid bacteria (LAB) to release hydroxycinnamic acids (HCA) from their tartrate derivative forms ((hydroxy)cinnamoyl-tartaric acids) was also studied.

Metabarcoding analysis of 16 different wines analyzed at the post-malolactic stage was performed using sequence data from 16S (for bacteria) and ITS2 (for fungi) amplicons. Similar patterns were observed in all wines at this level of discrimination, with *Saccharomyces cerevisiae* yeasts and bacteria from the genera *Acetobacter*, *Gluconobacter* and *Swaminathania* being the most abundant taxa. Concerning the profiles of phenolics and volatiles, red and white wines were grouped separately, and French and Spanish wines tended to cluster together.

The effects of kaempferol, *trans*-caffeic and *trans*-caftaric acids (added at 10 mg/L) on the microbial growth and metabolism were explored in non-inoculated and inoculated wine (*O. oeni*) and wine mixed with MRS (de Man, Rogosa & Sharpe). The strongest impact on microbial growth, malolactic and general metabolic activity in LAB, was noted for kaempferol, with *trans*-caftaric acid showing the weakest. The effects of each phenolic compound varied according to the medium used, the type of MLF (inoculated or not) and the *O. oeni* strain inoculated (Oenos™ or CH35™).

Using concentrations normally encountered in wines as a reference, flavan-3-ols ((+)-catechin and (-)-epicatechin), flavonols (kaempferol and quercetin), HCA (*trans*-*p*-coumaric and *trans*-ferulic acids) and *trans*-resveratrol were studied in experiments with non-inoculated wines and wines inoculated with Oenos™. Depending on its concentration, (+)-catechin positively impacted the yeast population, activated or delayed malic acid degradation or inhibited the growth of bacteria. All phenolics tested, at all concentrations tested, delayed citrate consumption in inoculated samples. The effect of flavonols and *trans*-resveratrol on the *O. oeni* diversity was dependent on the strains under study. An increase in *trans*-*p*-coumaric and *trans*-ferulic acid levels appeared to induce the release of *trans*-caffeic acid from one of its precursors, possibly via an increase in cinnamoyl esterase activity of some strains. Flavan-3-ols and flavonols inhibited the growth of these bacteria during storage. The phenolics tested affected specific enzymatic systems responsible for the production and degradation of important metabolites involved in the organoleptic quality of the wines.

Cinnamoyl esterase (CE) activity of wine microbes can be relevant as it confers the capacity to modulate the phenolic acid composition of a wine by liberating these from otherwise seemingly biologically unavailable, tartrate derivatives. Five commercial strains of *O. oeni* were studied in this respect, three exhibiting CE activity (Oenos™, CiNe™ and CH35™) and two not (CH16™ and CH11™). CE activity was detected in cell-free extracts of the three CE positive (CE+) strains and one of the CE negative (CE-) strains. From comparative genome analysis, no gene exclusive to the 3 CE+ was detected and the inference that membrane transport differences might be behind differences in CE activity was also not conclusively supported. This hypothesis needs to be further

explored as does the possibility of the involvement of wine molecules and of more than one enzyme in the CE activity.

Keywords: wine, phenlic compounds, lactic acid bacteria, volatiles

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Publications and Symposia

Publications:

Collombel I., Campos F.M., Hogg T. (2019) Changes in the composition of the lactic acid bacteria behavior and the diversity of *Oenococcus oeni* isolated from red wines supplemented with selected grape phenolic compounds. Fermentation, 5(1), 1; https://doi.org/10.3390/fermentation5010001

Collombel I., Melkonian C., Campos F.M., Molenaar D., Hogg T. Alternative insights into cinnamoyl esterase activity in *Oenococcus oeni*. Under revision for Frontiers in Microbiology.

Collombel I., Campos F.M., Hogg T.A. Influence of phenolic compounds on microbial metabolism during malolactic fermentation in red wines. Submitted to OENO One.

Collombel I., Campos F.M., Hogg T.A. The impact of phenolic compounds on the chemical composition in wines during malolactic fermentation and subsequent storage. In submission form for Beverages.

Symposia:

Collombel I., Campos F.M., Hogg T.A. Post vinification microbial communities' assessment in relation to phenolic composition. **Poster** presented at the *2015 EMBL-EBI workshop (Exploiting Metagenomics approaches in life science research), Cambridge, United Kingdom.*

Collombel I., Campos F.M., Hogg T.A. Impacts of specific phenolic compounds on growth and metabolism of red wine microbiota during the malolactic fermentation. **Poster** presented at the *Viticulture Challenges through Novel Technological Developments Symposium in Copenhagen, Denmark, 2017*.

Collombel I., Campos F.M., Hogg T.A. The effect of added phenolic compounds on the microbiology and chemical composition of red wine during MLF and subsequent storage. **Poster** presented at the *Microbiotec conference in Porto, Portugal, 2017*.

Collombel I., Campos F.M., Hogg T.A. Alternative insights into cinnamoyl esterase activity in *Oenococcus oeni*. **Presentation** given at the *2018 Microwine symposium in ´Cité du vin´, Bordeaux, France* and **poster** presented at *the International Congress on Grapevine and Wine Sciences in Logroño, Spain, 2018.*

Symbols and abbreviations

AAB	Acetic Acid bacteria
Abs	Absorbance
AF	Alcoholic Fermentation
ARISA	Automated Ribosomal Intergenic Spacer Analysis
AU	Absorbance Units
BMLF	Before MLF
bp	base pairs
B-PER	Complete Bacterial Protein Extraction Reagent
CE	Cinnamoyl esterase
CE+ / CE-	Cinnamoyl Esterase positive / negative
CFU	Colony Forming Units
Da	Dalton
DD	Differential Display
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
ESB	Escola Superior de Biotecnologia, Porto, Portugal
EtOH	Ethanol
FAE	Feruloyl Esterase
FID	Flame Ionization Detector
FPLC	Fast Protein Liquid Chromatography
g	Acceleration of Gravity
GC	Gas Chromatography
GRP	Grape Reaction Product
HBA	Hydroxybenzoic Acid
HCA	Hydroxycinnamic Acid
HPLC	High-Performance Liquid Chromatography
HS	Headspace
HTS	High-Throughput Sequencing
ITS	Internal Transcribed Spacer
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Orthology
LAB	Lactic Acid Bacteria

Log	Logarithm to the base 10
M	Molar (moles/liter)
MLF	Malolactic Fermentation
MLST	Multi Locus Sequence Typing
MRS	de Man, Rogosa & Sharpe
MS	Mass Spectrometry
NCFB	National Collection of Food Bacteria, Reading, UK
NIST	National Institute of Standards and Technology
OAV	Odor Activity Value
OPA	o-Phthalaldehyde
ORF	Open Reading Frames
OTU	Operational Taxonomic Unit
p	Probability
PAD	Phenolic Acid Decarboxylase
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pK_A	Acid dissociation constant
ppm	Parts per million
Pt	Portugal
QIIME	Quantitative Insight Into Microbial Ecology
qRT	quantitative Real Time
RAPD	Randomly Amplified Polymorphic DNA
REA-PFGE	Pulsed-Field Gel Electrophoresis of rare Restriction Enzyme Digests
REP	Repetitive Extragenic Palindromic
RFLP	Restriction Fragment Length Polymorphism
RI	Refractive Index
RNA	Ribonucleic Acid
RT	Room Temperature
SADP	Specifically Amplified Polymorphic DNA
SCAR	Sequence Characterized Amplified Regions
SD	Standard Deviation
SDS–PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SNP	Single Nucleotide Polymorphism
SO_2	Sulfur dioxide
SPME	Solid Phase Microextraction

UV	Ultra-Violet
v	Volume
VFA	Volatile Fatty Acid
VNTR	Variable Number of Tandem Repeat analysis
VPR	Vinylphenol Reductase
YMB	Yeast Mold Broth
4-EC	4-Ethylcatechol
4-EP	4-Ethylphenol

Abbreviations of genus names

A.	*Acetobacter*
Brett.	*Brettanomyces*
C.	*Candida*
D.	*Dekkera*
H.	*Hanseniaspora*
L.	*Lactobacillus*
Lach.	*Lachancea*
Lc.	*Leuconostoc*
M.	*Metschnikowia*
O.	*Oenococcus*
P.	*Pediococcus*
Pi.	*Pichia*
S.	*Saccharomyces*
St.	*Starmerella*
T.	*Torulaspora*
Z.	*Zygosaccharomyces*

1. Introduction

Wine is produced and commercialized in many regions of the World and the wine market is constantly evolving with a central role of European producers. Wine conceptualization dates back more than mid-fifth millennium B.C. and comes from an established heritage of traditional practices. Wine is a highly varied product in term of style (red, rosé and white; still, sparkling or fortified) and value and is often paired with food. Many production variables shape the sensory perception of wine including grape variety, environment (soil, climatic conditions, vine exposition, etc.), vinification practices and the microbial activities that they govern.

1.1. General vinification steps

Each step in the winemaking process, from site and vine selection to bottling, can influence greatly the composition of a wine and thus its final appreciation (Figure 1.1.). The process of grape maturation is complex and depends on a number of botanical and agricultural parameters. The sugar concentration, acidity and phenolics´ maturity are all consequences of grape maturation. Phenolics is a term often used in winemaking to describe a wide variety of compounds that can confer flavor, color and texture to a wine. Some of these compounds are the subject of this thesis and these and the concept of wine phenolic compounds are defined in a more detailed way later in this document. An optimal scheduling of grape harvest, considering both operational costs and quality, is indispensable to obtain the right alcohol, phenolics, flavor compounds and polysaccharides content required in the final wine (Ferrer *et al.*, 2008; Tian *et al.*, 2009; Bindon *et al.*, 2013).

Harvest is normally followed by separation of the berries from most of the stems and crushing to liberate the juice and facilitate its maceration with skins and seeds. The incorporation of stems or a delay in destemming process can lead to an increase in wine astringency and bitterness, although these practices are still used in some styles of wine (Pascual *et al.*, 2016).

In the case of red winemaking, the grape solids, made up of skins and seeds (and sometimes stems) will naturally be pushed to the top of a fermentation vessel due to the CO_2 produced during fermentation. This grape solid fraction or *cap*, either has the liquid fraction periodically pumped over it or is pushed down into the liquid fraction to facilitate the extraction of compounds during maceration. The management of the maceration parameters are determining factors in defining the proportion of compounds in wine, certainly this is so for phenolics. The extraction of phenolics from seeds is more affected by the duration of the maceration step and the extraction from skins by the temperature of maceration (Vrhovsek *et al.*, 2002). Must freezing with dry ice is known to strengthen the color intensity and anthocyanins' content of a wine (Busse-Valverde *et al.*, 2011). Enzymes can be added early in the process to promote the diffusion of proanthocyanidins from skins and seeds (Bautista-Ortín *et al.*, 2013).

In the case of white wines, the grape solids fraction is usually removed from the must and this is clarified by filtration, centrifugation or sedimentation before alcoholic fermentation (AF) occurs. Alcoholic fermentation is principally the conversion of grape sugars into ethanol by certain specific species of yeasts, the most important of which is *Saccharomyces cerevisiae.* Through its enzymatic system, *S. cerevisiae* also derivatizes and synthesizes flavor-active compounds from sugar, amino acids and sulfur metabolism (Swiegers *et al.*, 2005a).

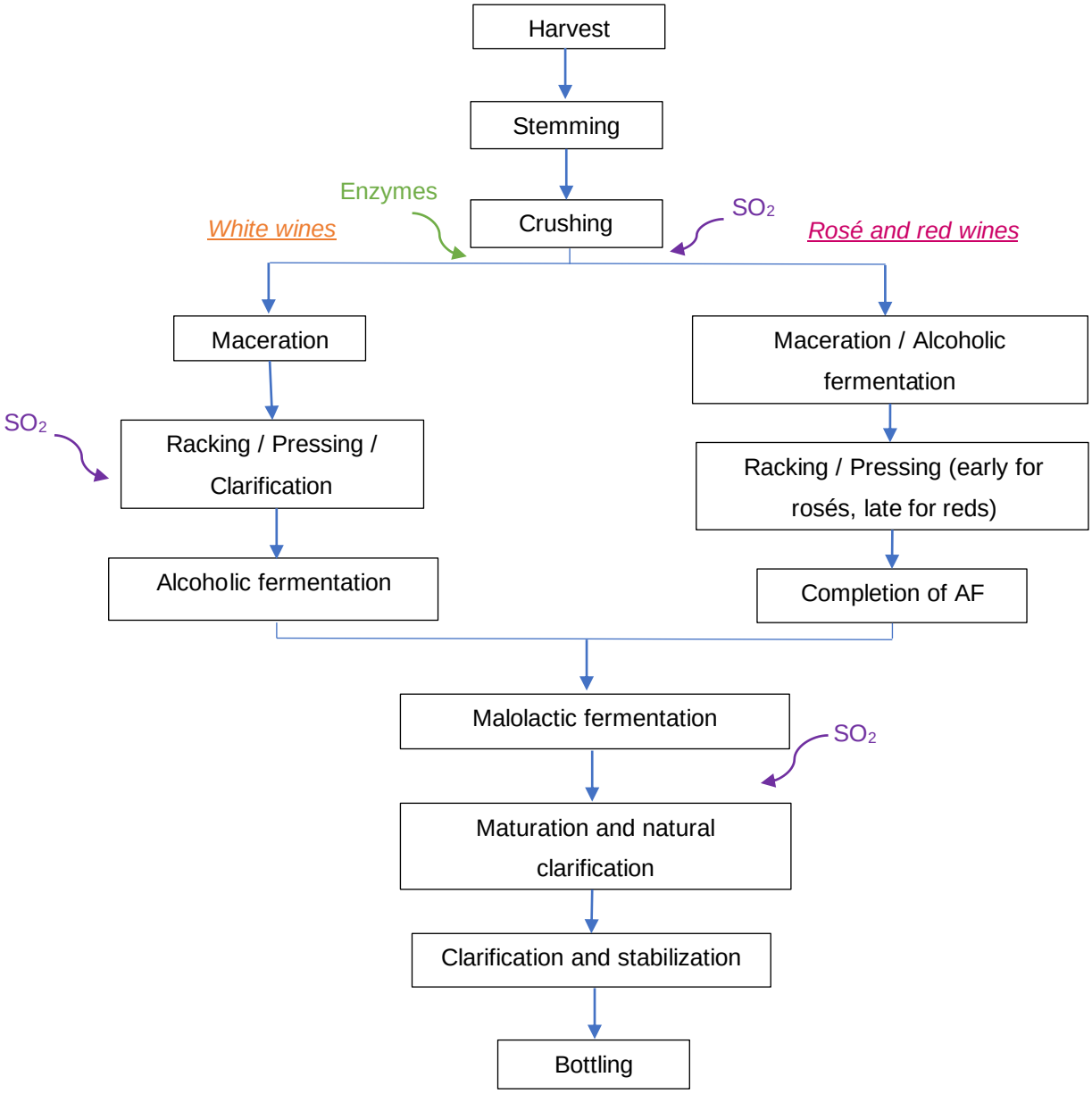


Figure 1.1.- Scheme flow of the winemaking process (Jackson, 2008).

Malolactic fermentation (MLF) is a desirable step in the vinification process of most red wines and some white wines. It is conducted by certain specific species of lactic acid bacteria (LAB), the most important of which is *Oenococcus oeni.* Malolactic fermentation permits a deacidification of the wine (increase of

pH from 0.2 to 0.5 units), decreasing the titratable acidity and wine sourness. Malolactic fermentation also contributes to the microbial stability by the removal of malic acid as a possible carbon substrate, and leads to a modification of the aroma profile of the wine (Rammelsberg *et al.*, 1990; Henick-Kling and Stoewsand, 1993; Laurent *et al.*, 1994; Mcdaniel *et al.*, 2008). Co-inoculated fermentations or simultaneous yeast-bacterial inoculated fermentations may be employed to better control the process and bring specific properties to the final wine (Scudamore-Smith *et al.*, 1990; Jussier *et al.*, 2006; Pan *et al.*, 2011).

Post-MLF fermented wines are considered rough, raw and "green". Therefore, a period of aging, also called maturation, normally ensues MLF and is done in neutral containers, such as stainless steel tanks, or in wood barrels, according to the type of wine targeted and to the desired aroma profile (Escalona *et al.*, 2002; Rivas *et al.*, 2006). In some cases, MLF can also take place during aging in barrels or stainless-steel tanks (Izquierdo-Cañas *et al.*, 2016a; González-Centeno *et al.*, 2017). The addition of toasted wood chips or other formats of wood pieces is also considered in some type of wine (Koussissi *et al.*, 2009).

Absorptive and reactive substances as enzymes, proteins, microbial nutrients and bentonite can be added at a number of stages in the vinification process to reduce or remove the concentration of one or more undesirable components and improve color, flavor and physical stability of the wine (Morris and Main, 1995; Cosme *et al.*, 2009; Lambri *et al.*, 2012). Sulfur dioxide (SO_2) is normally added at different steps of the vinification due to its antimicrobial activity and antioxidant properties. The quantity of SO_2 added during vinification permits the selection of specific strains to conduct fermentations and inhibit potential proliferation of spoilage microorganisms. Supplementary techniques along vinification are used to control the proliferation of spoilage microorganisms producing unwanted compounds (Jackson, 2008). For example, cool temperatures during aging stop or delay the growth of wine microorganisms. Racking, filtration and centrifugation are clarification processes which permit the removal of microbial cells that have flocculated or co-precipitated with tannins and proteins.

1.2. Microbial activity in wine

Microorganisms are naturally present in the wine ecosystem and have many roles along the winemaking process, participating in the overall quality of the final wine (Appendixes 1 and 2).

1.2.1. Pre-fermentation factors shaping the "microbial terroir" of a wine

The variability of microorganisms that can be introduced into the winery and subsequently affect the fermentation processes is linked to the soil microbiota (Burns *et al.*, 2015). The fungal and bacterial consortia on wine-grape surfaces are geographically (region, site and orientation), grape-variety, climate and farming system (sanitary of the harvest, pesticide treatments) specific (Setati *et al.*, 2012; Bokulich *et al.*, 2014; del Carmen Portillo *et al.*, 2016a). Natural ecosystems outside of vineyards also prove a significant source of the microbial diversity of grapes as microorganisms from nearby plants can be transported aerially or via insects (Gilbert *et al.*, 2014; Morrison-Whittle and Goddard, 2018).

Botrytis (*Botrytis cinerea*), *Aspergillus* (*Aspergillus tubingensis*), *Uncinula, Alternaria, Plasmopara, Penicillium* (*Penicillium brevicompactum, Penicillium crustosum, Penicillium glabrum*), *Rhizopus, Oidium* and *Cladosporum* are the principal molds found on grapes (Fleet, 2003; Wang *et al.*, 2015). Yeast diversity is quite high on grape skins, whatever the environmental conditions, the total number of yeasts present increasing from 10^1-10^3 to 10^4-10^6 CFU/g with grape maturation (Fleet, 2003). Depending on the stage of maturity of the grape, *Aureobasidium, Starmerella, Metschnikowia, Hanseniaspora (Kloeckera), Cryptococcus*, *Rhodotorula*, *Candida*, *Debaryomyces* and *Pichia*, among other yeast genera may be detected on the surface and have an impact on the microflora regulation on grapes and fermentations (Fleet, 2003; Renouf *et al.*, 2007). Damaged grapes have higher concentrations in *Candida, Saccharomyces* and *Zygosaccharomyces* than healthy grapes (Fleet, 2003). Grape berry is supposedly the primary source of the important wine spoilage microorganism, *Brettanomyces bruxellensis*, although it is normally barely detected on the surface of grapes (Renouf *et al.*, 2007; Barata *et al.*, 2012). *Gluconobacter oxydans* and *Acetobacter aceti* are the major acetic acid bacteria (AAB) species growing principally on rotten grapes and on grapes affected by *Botrytis cinerea* (Barbe *et al.*, 2001). Acetic acid bacteria are considered spoilage bacteria when detected in wine (González *et al.*, 2005; Fugelsang and Edwards, 2007). Lactic acid bacteria population is also higher on damaged grapes, impacting the fermentative yeasts (Fleet, 2003). Among LAB detected on wine grapes the genera *Lactobacillus, Lactococcus* and *Weissella* can be cited (Bae *et al.*, 2006). Bacteria belonging to the genera *Enterobacter, Enterococcus, Burkholderia, Serratia* and *Staphylococcus* among others, have also been isolated from grapes but apparently do not have the ability to grow in wines (Barata *et al.*, 2012).

Microbial consortia in harvested juice mostly come from grape berry surfaces but microorganisms present on soil, grapevine leaves and bark can also persist (Pinto *et al.*, 2014; Zepeda-Mendoza *et al.*, 2018; Morrison-Whittle and Goddard, 2018).The dominant microorganisms in wine fermentations (*S. cerevisiae* and *O. oeni*) are rarely found on the surface of healthy grapes (Mortimer and Polsinelli, 1999; Bae *et al.*, 2006; Ribéreau-Gayon *et al.*, 2006; Renouf *et al.*, 2007; Barata *et al.*, 2012). Some authors (Stefanini *et al.*, 2016; Ganucci *et al.*, 2018) suggest that the main contributors to fermentation originate from the flora resident in the winery. All along the winemaking process, grapes, must and wine are constantly in contact with specialized equipment surfaces, and under normal operating conditions, winery surfaces, prior to harvest, potentially serve as a source for the ferment *S. cerevisiae* (Bokulich *et al.*, 2013).

1.2.2. Changes in the microbiota during wine fermentations

1.2.2.1. Alcoholic fermentation

Winemaking techniques determine the microbial composition during AF and the fermentation kinetics (Piao *et al.*, 2015). Microbial diversity has been reported to be greater in white wines than red wines (Renouf *et al.*, 2007). Even though most wines are inoculated with starter yeasts to better control the

alcoholic fermentation, this critical vinification step can also be spontaneous, conducted by indigenous wine yeasts.

To grow in must, yeasts need sources of reduced nitrogen (ammonium salts, amino acids, polypeptides), vitamins, organic acids, mineral salts (phosphate, sulfate, chloride, potassium, calcium, magnesium) and carbohydrates (glucose, fructose). Various yeast species intervene in the spontaneous AF. The grape species *C. stellata and H. uvarum* are usually predominant in the early stage of the AF. These species have a weak ethanol tolerance which may increase at lower temperatures of 10 °C to 15 °C (Erten, 2002). *Pichia* and *Metschnikowia* are frequently preponderant in the middle stage of the alcoholic fermentation (del Carmen Portillo and Mas, 2016b; Sternes *et al.*, 2017). Yeasts begin to decline and die off by mid-fermentation, leaving the place to the ethanol-resistant yeast *S. cerevisiae* which usually complete the fermentation (Constantí *et al.*, 1997). In some cases, *Lachancea, Starmerella*, *Torulaspora* and *Schizosaccharomyces* genera persist until the end of fermentation when present at high frequencies in the initial population (Holm Hansen *et al.*, 2001; Pinto *et al.*, 2015; Wang *et al.*, 2015).

Yeasts are known to interact among themselves, competing for nutrients and causing possible effects on the fermentation kinetics (Medina *et al.*, 2012). Moreover, the specific metabolic activity of each yeast species and strain in addition to their interactions, contribute to the final composition in flavor compounds in wine, and thus to its sensory characteristics (Stefanini *et al.*, 2016). Strains of *H. uvarum* can produce killer toxins with activity toward sensitive strains of *S. cerevisiae* (Fugelsang and Edwards, 2007). *Metschnikowia pulcherrima* strains were observed to possess effective antimicrobial action on undesired wild spoilage yeasts, such as *Brettanomyces*, *Hanseniaspora* and *Pichia* genera, but did not show any influence on the growth of *S. cerevisiae* (Oro *et al.*, 2014). *Saccharomyces cerevisiae* can also have a negative impact on the viability of *T. delbrueckii* (Taillandier *et al.*, 2014).

To enhance the quality and improve complexity of wines, co-inoculation of different yeast species and strains is more and more use in wineries. The non-Saccharomyces species *Hanseniaspora vineae, Starmerella bacillaris, Lach. thermotolerans, Pi. kluyveri*, and *T. delbrueckii* have been shown to bring desirable aroma notes when co-inoculated with *S. crevisiae* (Comitini *et al.*, 2017). Toro and Vazquez. (2002) reported that mixing *S. cerevisiae* with *C. cantarellii* or inoculating *S. cerevisiae* three days after *C. cantarellii* provided better organoleptic features to Syrah wines. Gobbi *et al.* (2013) found that pairing *S. cerevisiae* and *Lach. thermotolerans* enhanced the wine acidity and the concentrations of 2-phenylethanol and glycerol. The inoculation of *S. cerevisiae* 48 h after *T. delbrueckii* ensured the growth of *T. delbrueckii* and consequently a decrease of volatile acidity and a higher isoamyl acetate production (Taillandier *et al.*, 2014). Inoculating grape musts with *M. pulcherrima* and *S. uvarum* decreased alcohol concentration and enhanced the production of 2-phenylethanol and 2-phenylethyl acetate (Varela *et al.*, 2016). The co-inoculation of different *S. cerevisiae* strains has been shown to release higher concentrations of alcohols and acetaldehydes than single strains (Barrajón *et al.*, 2011).

Although alcoholic fermentation is mainly carried out by yeasts, *Zymomonas mobilis*, a bacteria detected in must, was found to also possess this ability (Moreno-Arribas and Polo, 2009). Changes in the environment during alcoholic fermentation and the competitive interactions with yeasts, can substantially

reduce the bacterial diversity and survival. The major species of LAB present at the beginning of the AF include *L. plantarum, L. casei, L. hilgardii, Lc. mesenteroides*, and *P. damnosus*. Other species such as *O. oeni* and *L. brevis* can also be present to a lesser extent (Boulton *et al.*, 2013). As for AAB, *A. aceti* remains the main species detected during AF (González *et al.*, 2005).
To reduce the risk of spoilage and unpredictable changes of wine flavor, SO_2 is almost always added to musts in most wine styles. The addition of SO_2 modifies population dynamics and generally favours the dominance of *S. cerevisiae* during fermentation (Grangeteau *et al.*, 2017).

1.2.2.2. Malolactic fermentation

The combination of high acidity and ethanol content makes the post-alcoholic fermentation wine environment highly selective with only a few species of microorganisms able to interact with the medium. The active wine microflora is dominated by LAB during MLF since antimicrobial compounds produced during this stage, such as lactic acid and bacteriocins, inhibit the growth of other bacterial species (Rammelsberg *et al.*, 1990; Henick-Kling and Stoewsand, 1993; Bartowsky and Henschke, 2004).
It may take weeks or months for the indigenous bacterial population to achieve an adequate size to initiate MLF and very often winemakers will raise the temperature of the wine to favor its occurrence. By inoculating with a commercial starter culture, the winemaker can be more confident in ensuring the successful completion of MLF, reduce the risk of potential spoilage bacteria or bacteriophages and bring to the wine specific characteristics.

1.2.2.2.1. Lactic acid bacteria description

Lactic acid bacteria are microaerophilic Gram-positive species and the predominant LAB families able to survive in wine are *Leuconostocaceae* and *Lactobacillaceae*. Residual sugars can be metabolized to two molecules of lactic acid or to one molecule of lactic acid plus carbon dioxide plus ethanol or acetic acid via the fermentative pathways of LAB (Figure 1.2.) (Moreno-Arribas and Polo, 2009). In wine, *Leuconostocaceae* are usually represented by the strictly heterofermentative genera *Leuconostoc* and *Oenococcus*, and *Lactobacillaceae* by the strictly homofermentative genus *Pediococcus* and by the genus *Lactobacillus* which can be strictly homofermentative, facultative heterofermentative or strictly heterofermentative. Lactic acid bacteria essentially degrade malic and citric acids coming originally from grapes which consequently softens the wine (Figure 1.3.).

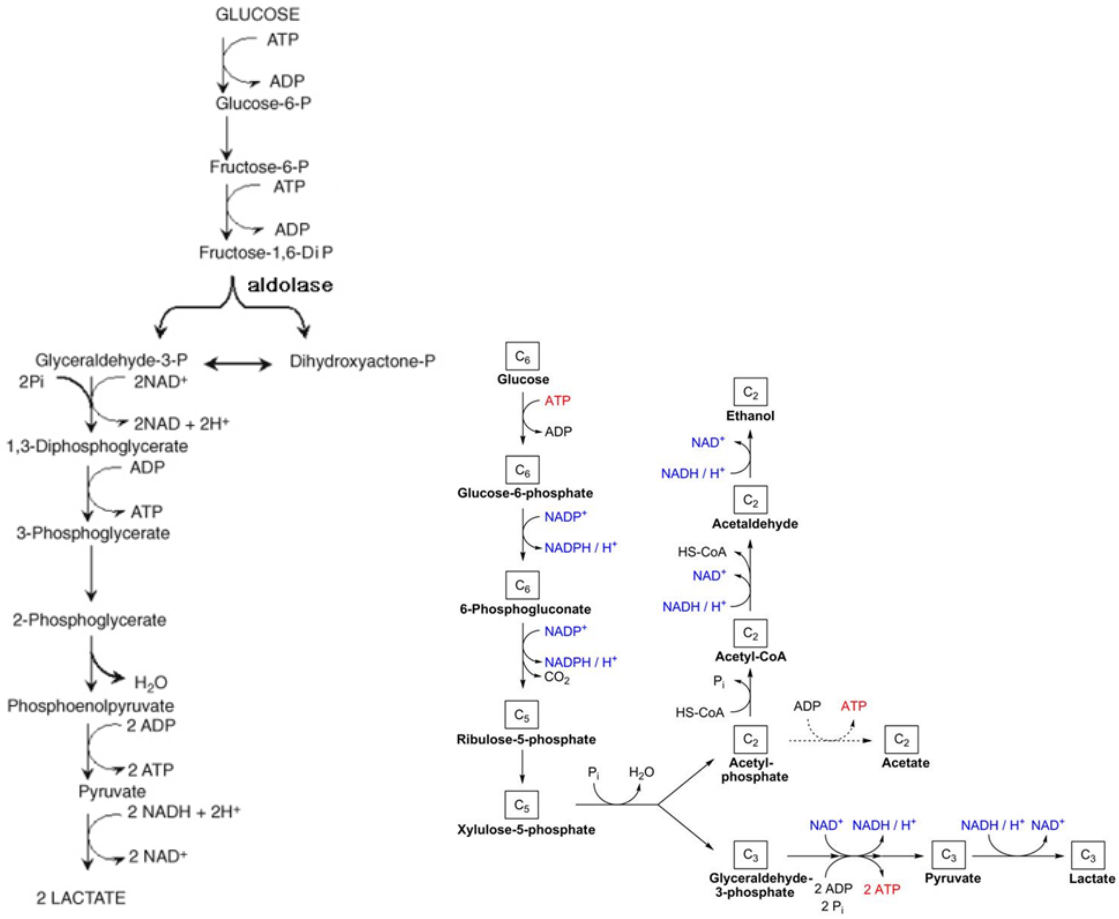


Figure 1.2. – Homofermentative (left) and heterofermentative (right) glucose pathways of LAB (Fugelsang and Edwards, 2007).

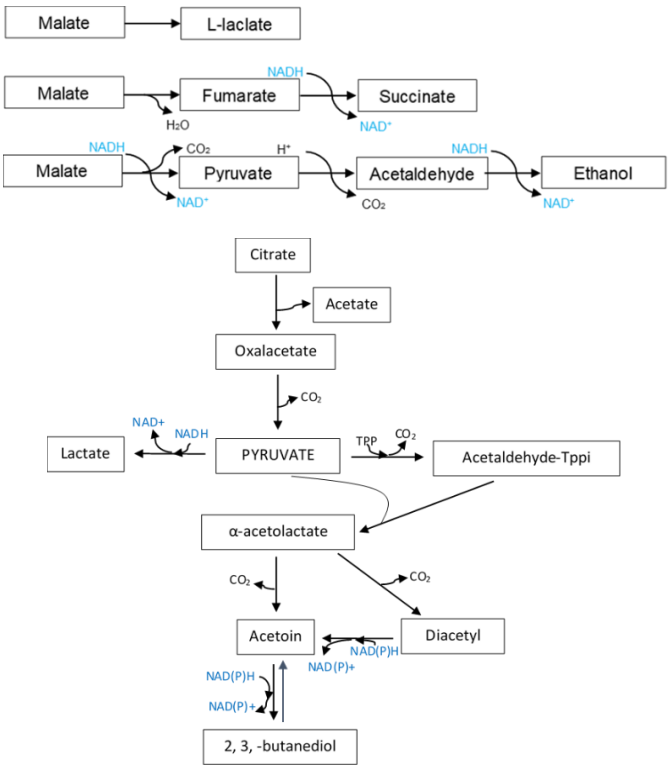


Figure 1.3. - Malic and citric acids degradation in wine by LAB (Mozzi *et al.*, 2016).

The population of LAB can grow to 10^7 CFU/mL in the interval between the end of AF and the start of the MLF. The preferable wine conditions for LAB growth are relatively high pH values (> 3.5), sulfur dioxide (SO_2) concentrations below 50 ppm, temperatures between 25 °C and 30 °C and ethanol levels lower than 13% v/v (Lerm *et al.*, 2010).

With its resistance to harsh wine conditions, *O. oeni* is normally the most important wine LAB conducting MLF. However, some *L. plantarum*, *P. damnosus* and *P. parvulus* strains can perform MLF as favorably as *O. oeni* under wine conditions, with low production of biogenic amines or exopolysaccharides and exerting positive effects on organoleptic properties of wine (du Toit *et al.*, 2011; Juega *et al.*, 2014; Strickland *et al.*, 2016). Lactic acid bacteria can interact among themselves by inhibitory or mutualism effects. For instance, *O. oeni* has been observed to produce essential amino acids for *P. pentosaceus* growth (Fernández and de Nadra, 2006).

If left unchecked, some strains of wine LAB can cause compositional changes which result in a range of spoilage conditions such as, among others, the "tourne" disease (increase in volatile acid content), the production of off-flavor compounds by the metabolization of citric acid and the production of potent N-heterocycles associated with mousy off-flavor (Lonvaud-Funel, 1999; Costello *et al.*, 2001; Liu, 2002; Lonvaud-Funel, 2002).

Strains of *O. oeni* are still the most commercialized starters nowadays, although the commercialization of *L. plantarum* strains is raising. Indeed, *Lactobacillus plantarum* were found to have a tolerance to harsh wine conditions similar to that of *O. oeni*, and to possess a wide range of enzymes capable of

producing a large number of aroma compounds (du Toit *et al.*, 2011; Lerm *et al.*, 2011; Iorizzo *et al.*, 2016).

1.2.2.2.2. Strain-level diversity of Oenococcus oeni in wine

Taxonomically, *O. oeni* species is ordered into three groups, with A and B being the two major phylogenetic groups, and C a putative group composed of a unique strain isolated from cider (Bridier *et al.*, 2010; Campbell-Sills *et al.*, 2015; Campbell-Sills *et al.*, 2017). Group A exclusively contains strains found in wine. All strains from cider, except that attributed to group C, are located in group B, while strains from Champagne and Burgundy wines are only from group A. It appears that most of the strains isolated from malolactic ferments derive from the domestication of ancestral *O. oeni* strains during the process of the industrialization of wine and cider, rather than responding to geographical constraints (Campbell-Sills *et al.*, 2015).

The strain-level diversity of *O. oeni* populations in wine ecosystems is very high, and it can be region- or winery-specific, often contributing to recognized differences in wines (Cafaro *et al.*, 2016; El Khoury *et al.*, 2016). *O. oeni* has been shown to genetically adapt according the type of wine (white or red), driven by the pH and the phenolic compounds present (Breniaux *et al.*, 2018).

1.2.2.2.3. Yeast diversity in non-inoculated wines

Spoilage yeasts, such as *Pichia, Candida*, and *Saccharomycodes* can resist, grow and retard, if not inhibit MLF (Fleet, 2003). Some studies (Serpaggi *et al.*, 2012; Salma *et al.*, 2013; Capozzi *et al.*, 2016) have demonstrated that strains of *S. cerevisiae, Z. bailii*, *C. stellata* and *Brett. bruxellensis* when exposed to SO_2 can enter a Viable-But-Non-Culturable state and survive for more than a month depending on the pH of the environment. At this state cells do not grow on culture media, but they are still viable and maintain a detectable metabolic activity which may affect fermentation performance and wine flavor.

1.2.2.3. Co-inculations with yeasts and lactic acid bacteria

Possible stuck MLF due to bacterial growth inhibition in high ethanol wines can be avoided by co-inoculation of both yeast and bacteria cultures at the beginning of AF (Zapparoli *et al.*, 2009).

When *O. oeni* is inoculated in must, which has a far higher sugar content than wine, acetic acid can be produced from sugars via the heterofermentative pathway. Nevertheless, in a study where *S. cerevisiae* and *O. oeni* were co-cultivated (Nehme *et al.*, 2010), no inhibition and no increase in volatile acidity (essentially a winemaking term for acetic acid) were observed. In other studies (Abrahamse and Bartowsky, 2012; Cañas *et al.*, 2012; Cañas *et al.*, 2015), the inoculation of musts with LAB during AF did not affect the rate and duration of AF, nor induced significant changes in the profiles of volatile compounds. Depending on the grape varieties studied, volatile acidity was not significantly higher when

yeast and bacteria were inoculated at the same time. Some co-inoculated wines were also lower in biogenic amines and higher in hexanoic and octanoic acids contents.

Rossouw *et al.* (2012) studied the impact of the co-inoculation of *O. oeni* on the transcriptome of *S. cerevisiae* in synthetic must and found that several genes were differentially expressed, responding to chemical changes in the fermenting must linked to bacterial metabolic activities or to the presence of a competing organism.

1.2.3. The development of microbial populations in wine after MLF

Despite a lack of nutrients and oxygen, some LAB species as *O. oeni*, *P. damnosus, P. parvulus*, *P. pentosaceus, L. brevis* and *L. casei* can survive in wine after MLF is completed and still grow, contributing to the wine spoilage (Wibowo *et al.*, 1985; Couto and Hogg, 1994; Couto *et al.*, 2006; Lerm *et al.*, 2010). If the wine is not topped up to maintain a low oxygen level, and monitored regularly during barrel maturation, or if there is a poor oxygen management during bottling and storage, AAB as *A. pasteurianus* can grow and spoil the wine (Bartowsky and Pretorius, 2009a).

Several yeast genera as *Brettanomyces, Kluyveromyces, Schizosaccharomyces, Torulaspora, Zygosaccharomyces*, *Saccharomycodes*, *Pichia* and *Candida* among others, can proliferate in post-fermentation wines (during aging in stainless steel tank, oak barrels and bottles for example) generating sensory faults as cloudiness, sediment and off-odors (production of volatile phenols, acetaldehyde, acetoin, acetic acids etc.) (Du Toit and Pretorius, 2000; Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003; Kheir *et al.*, 2013).

1.3. Molecular techniques for the identification of microorganisms and their functionalities during vinification

1.3.1. Microorganisms´ identifications

Culture-dependent methods such as direct plating or following filtration concentration, form the basis of most approaches used to isolate colonies for further analysis. An approximate microbial quantification can be obtained using the plating method, although this method itself is not representative of the total microbial population in suspension as not all microorganisms can grow on agar plate cultures or on the culture media presented to them.

Culture-independent molecular techniques as DNA based methods, do not require a prior isolation stage as DNA is extracted and amplified directly from the environment. These methods permit the identification of most of the microbes present in the analyzed solution including the non-cultivable microorganisms. At present these techniques have a lower discriminatory capacity than methods applied to isolated strains.

Therefore, according to the level of discrimination, studies generally associate conventional direct plating and culture-independent molecular approaches to describe with more accuracy the microbial diversity in a medium.

1.3.1.1. Family to species level

Polymerase chain reaction (PCR)-based techniques including random amplification of polymorphic deoxyribonucleic acid (DNA)-PCR (RAPD-PCR), PCR-denaturing gradient gel electrophoresis (PCR-DGGE), species-specific multiplex PCR, automated ribosomal intergenic spacer analysis (ARISA) and metabarcoding sequencing can be used on DNA samples extracted directly from the suspension studied, with no need of prior isolation, but these are as yet limited to differentiation to the species level at most. Some examples of these techniques which have been used in grapes and wines environments are listed below with some details on their functioning.

Drożdż *et al.* (2015) isolated and identified yeast species from the surface of different varieties of white and red grapes grown in cool climates by RAPD-PCR using the M13 primer.

PCR-DGGE has been broadly used to make the inventory of fungal and bacterial species found in a wine environment (Prakitchaiwattana *et al.*, 2004; Bae *et al.*, 2006; Renouf *et al.*, 2007; García-Ruiz *et al.*, 2013a; Mayrhofer *et al.*, 2014; González-Arenzana *et al.*, 2017). Since the detection limit of a PCR-DGGE is normally around 10^4 CFU/mL, a preliminary enrichment of the cultures and the amplification of diverse genes with the use of several primer sets is normally endorsed. The choice of the gene targeted and the corresponding PCR-DGGE primer set are significant for the discrimination of the species. To identify the corresponding bands obtained by PCR-DGGE, known species can be used as reference markers for comparison (band-matching), and/or the bands are excised and sequenced by metabarcoding. The yeast populations associated with wine grapes were analyzed in Prakitchaiwattana et al (2004) by PCR-DGGE targeting the D1/D2 domain of the 26S rDNA gene. For wine bacteria, the gene coding for the beta subunit RNA polymerase (rpoB gene), which is present as a unique copy in the genome, usually provides more phylogenetic resolution than the 16S ribosomal DNA gene (16S rDNA) which is repeated, with differences between the copies, leading sometimes to ambiguous profiles. Renouf *et al.* (2007) used rpoB PCR-DGGE analysis to study the bacterial evolution along the winemaking process of Bordeaux wines and García-Ruiz *et al.* (2013a) investigated the *O. oeni* population from fermented red wines treated or not with phenolic extracts with the same technique. Bae *et al.* (2006) used enrichment cultures and both plate culture isolation and 16S rRNA PCR-DGEE analysis to determine the diversity of LAB species associated with Australian wine grapes and Mayrhofer *et al.* (2014), by studing the suitability of different PCR-DGGE primer sets, found WLAB1/WLAB2^{GC} targeting the 16S rDNA gene to be most suitable for studying the occurrence of LAB in wine. Both rpoB and 16S rDNA sequences can be targeted for PCR-DGEE as González-Arenzana *et al.* (2017) did to study the diversity of LAB communities in must, alcoholic and malolactic Tempranillo fermented wine. PCR-DGGE provides qualitative data but can´t be applied to quantify populations. For this particular

purpose, a quantitative real time-PCR (qRT-PCR) can be employed to detect and enumerate a particular species or strain in a medium. Cho *et al.* (2011) used this technique to follow the survival of the MLF starter *L. plantarum*, and Tofalo *et al.* (2012) to detect the wine spoiler *Brettanomyces* in wines.

Specifically amplified polymorphic DNA-PCR (SAPD-PCR) is a molecular fingerprinting method based on the amplification of specific gene sequences to rapidly identify isolated LAB species (Sebastian *et al.*, 2011). Petri *et al.* (2013) developed a species-specific multiplex PCR method to directly identify LAB species from must and wine without prior isolation. Species-specific sequence characterized amplified regions (SCAR) primers were generated based on the sequences of specific gel bands resulting from the fingerprinting of diverse known LAB species by (nested) SAPD-PCR (Petri *et al.*, 2013). However, the detection limit of this method is high (10^4-10^5 CFU/mL).

Setati *et al.* (2012) investigated the spatial distribution of yeasts communities on grape berries within and between individual vineyard management units using ARISA fingerprinting. Another study (Campisano *et al.*, 2014) used ARISA fingerprinting to evaluate the consequence of organic production and integrated pest management on bacterial communities in Merlot and Chardonnay grapevines cultivars.

With the fast evolution of genetic analyses, high-throughput sequencing (HTS) approaches are broadly applied in microbiology permitting the detection of the presence of rare taxa. Within the wine field, HTS has already been employed to principally investigate the microbial diversity in vineyard soils, on wine-grape surfaces, grapevines, winery equipment, in grape musts and fermented wines (González *et al.*, 2005; Bokulich *et al.*, 2012; Bokulich *et al.*, 2013; Bokulich *et al.*, 2014; David *et al.*, 2014; Pinto *et al.*, 2014; Piao *et al.*, 2015; Pinto *et al.*, 2015; Setati *et al.*, 2015; Bokulich *et al.*, 2016; Hong *et al.*, 2016; Stefanini *et al.*, 2016; del Carmen Portillo *et al.*, 2016a; del Carmen Portillo and Mas, 2016b; Grangeteau *et al.*, 2017; Gobbi *et al.*, 2019). High-throughput sequencing has made it possible to detect microbial species overlooked in culture-based methods and community fingerprinting approaches. For instance, by the use of metabarcoding, rare bacterial taxa including *Methylobacterium, Sphingomonas, Acinetobacter, Pseudomonas, Wolbachia*, and *Paracoccus*, as well as rare yeast genera counting *Kazachstania, Schizosaccharomyces*, and *Debaryomyces*, were shown to persist from the vineyard environment and throughout wine fermentation (Bokulich *et al.*, 2012; David *et al.*, 2014; Piao *et al.*, 2015; Pinto *et al.*, 2015; Setati *et al.*, 2015; del Carmen Portillo and Mas, 2016b; Grangeteau *et al.*, 2017). Depending on the material analyzed and the microbial communities studied, diverse sampling strategies, DNA extraction protocols, target genes, hypervariable regions of the gene, primers set amplifying the gene sequence, PCR parameters, cleaning method of the PCR fragments and sequencing systems can be used for metabarcoding (Table 1.1.). Different methods can produce different results on the same genetic material and bias can be introduced along the process. For metabarcoding amplicon sequencing, the bacterial 16S rRNA gene is classically targeted while in fungi the 18S rRNA, 26S rRNA and the ITS1-5.8S rRNA-ITS2 are the most common target structures. For bacterial identification, the V4 domain of 16S rRNA gene is often amplified with the primer set 515F / 806R (Bokulich *et al.*, 2013; Bokulich *et al.*, 2014; Bokulich *et al.*, 2016). However, mitochondrial and chloroplast DNA can also be amplified with these V4 region primers and thus overcome the sequencing (Beckers *et al.*, 2016). Grape

materials contain many interfering agents for molecular analysis (impurities, phenols, metal ions, salts, etc.), therefore additional purification steps are necessary, which can introduce bias by altering the original microbial community. Moreover, possible sources of DNA contamination can occur along the DNA sequencing protocol generating misleading results. The large amounts of raw sequencing reads obtained are then processed through computational analyses. In general, with the use of a range of software, sequences are trimmed, filtered, blasted against databases of known microorganisms, clustered in operational taxonomic unit (OTU) and subjected to phylogenetic assignment. Operational taxonomic units are assumed to be originating from a specific organism, nevertheless they can represent multiple species. It is preconized to use different reference databases to avoid incorrect assignments or unclassified species.

Different molecular techniques can be associated to analyze the microbial ecosystem in a suspension. For example, Wang *et al.* (2015) analyzed the diversity of fungi in grape musts and fermented wines combining different molecular techniques. Isolates were identified by 5.8S-ITS-Restriction Fragment Length Polymorphism (RFLP) and 26S-D1/D2 metabarcoding sequencing. In parallel, DNA was extracted directly from samples and analyzed by qPCR, PCR-DGGE and massive sequencing (Wang *et al.*, 2015). González *et al.* (2005) also analyzed the influence of yeast inoculation and SO_2 addition on AAB isolated from wine fermentations by pairing several molecular methods: RFLP of PCR-amplified 16S rDNA to differentiate AAB at the genus and species level and PCR of Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) and Repetitive Extragenic Palindromic (REP-PCR) to differentiate AAB at the strain level (González *et al.*, 2005).

Table 1.1. – Metabarcoding amplicon sequencing parameters applied to wine materials.

Material	DNA extraction	Microbial community	Gene sequence targeted	Primers set	Cleaning PCR products	Sequencer	Reference
Wine equipment + from grapes to wines aging in barrels	ZR-96 Fecal DNA extraction kit (Qiagen)	Bacteria	V4 region of bacterial 16S rRNA gene	515F (5´-GTGCCAGCMGCCGCGGTAA-3´) / 806R (5´-GGACTACHVGGGTWTCTAAT-3´)	Qiaquick spin kit (Qiagen)	Illumina MiSeq	(Bokulich *et al.*, 2013; Bokulich *et al.*, 2014; Bokulich *et al.*, 2016)
		Yeasts	Fungal internal transcribed spacer I (ITS1)	BITS (5´-NNNNNNNNCTACCTGCGGARGGATCA-3´) / B58S3 (5´-GAGATCCRTTGYTRAAAGTT-3´)			
Grapevine leaves, grape musts and fermented wines	QIAamp DNA Stool Mini Kit for grapevine leaves and DNeasy Plant minikit for juices (Qiagen)	Bacteria	V6 region of bacterial 16S rDNA gene	V6_F (5´-ATGCAACGCGAAGAACCT-3´) / V6_R (5´-TAGCGATTCCGACTTCA-3´)	High Pure 96 UF Cleanup Plates (Roche)	454 pyrosequencing (Roche)	(Pinto *et al.*, 2014; Pinto *et al.*, 2015)
		Yeasts	ITS2 and D2 regions of fungal 26S rRNA gene	ITS2_F (5´-GCATCGATGAAGAACGC-3´) / ITS2_R (5´-CCTCCGCTTATTGATATGC-3') and D2_F (5´AAGMACTTTGRAAAGAGAG-3´) / D2_R (5´-GGTCCGTGTTTCAAGACG-3´)			
Grape musts and wine samples	DNeasy Plant Mini kit (Qiagen)	Bacteria	V4 region of bacterial 16S rRNA gene	515F / 799R (5´-CVGGGTATCTAATCCBGTT-3´)	GeneRead Size Selection lit (Qiagen)		(del Carmen Portillo *et al.*, 2016a)
Fermented wines	QIAamp DNA Mini kit (Qiagen)	Bacteria and yeasts	16S and 18S rRNA genes			454 pyrosequencing (Roche)	(del Carmen Portillo and Mas, 2016b)

Grapes	Proteinase K + lysozyme + CTAB + phenol/chloroform/isoamyl alcohol	Yeasts	ITS1-5.8S rDNA-ITS2	ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) / ITS4 (5´-TCCTCCGCTTATTGATATGC-3´)	Zymoclean™ Gel DNA recovery kit (The Epigenetics Company™)	Illumina MiSeq	(Setati *et al.*, 2015)
Fermented wines	Fast DNA SPIN Kit for Soil (MP Biomedical)	Bacteria	V1–V3 region of bacterial 16S rRNA gene	28F (5´-ccatctcatccctgcgtgtctccgactcagxxxxxxxxxGAGTTTGATCNTGGCTCAG-3´) / 519R (5´-cctatcccctgtgtgccttggcagtctcagGTNTTACNGCGGCKGCTG-3´)	None	454 pyrosequencing (Roche)	(Piao *et al.*, 2015)
Chinese Rice Wines	Fast DNA SPIN Kit for Soil (MP Biomedicals)	Bacteria	V3–V4 region of bacterial 16S rRNA gene	forward primer (5′ -ACTCCTACGGGAGGCAGCAG-3′) / reverse primer (5′ -GGACTACHVGGGTWTCTAAT-3′)	Quan-IT™ PicoGreen® kit (Invitrogen)	Illumina MiSeq	(Hong *et al.*, 2016)
		Yeasts	ITS2	forward primer (5′ -GCATCGATGAAGAACGCAGC-3′) / reverse primer (5′ -TCCTCCGCTTATTGATATGC-3′)			
Viticultural habitats (soil, vine bark and ripe fruit), native forests (soil and fruit from different species), grape musts and fermented wines	Zymo Research Soil Microbe DNA MiniPrep kit	Yeasts	D1/D2 regions of fungal 26S rRNA gene	NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3´) / NL4 (5´-GGTCCGTGTTTCAAGACGG-3')	AmpureXP beads	454 pyrosequencing (Roche)	(Morrison-Whittle and Goddard, 2018)
Grape musts and fermented wines	DNeasy Plant Mini kit (Qiagen)	Yeasts	D1/D2 regions of fungal 26S rRNA gene	NL1 / NL4	Quan-IT™ PicoGreen® kit (Invitrogen)	454 pyrosequencing (Roche)	(Wang *et al.*, 2015)

Grape musts and fermented wines	Lysis buffer + bead beater + phenol/chloroform/isoamyl alcohol	Yeasts	18S rRNA gene	FR1 (5'-ANCCATTCAATCGGTANT-3´) / FF390 (5´-CGATAACGAACGAGACCT-3´)	MinElute gel extraction kit (Qiagen)	454 pyrosequencing (Roche)	(Grangeteau *et al.*, 2017)
Grape musts and fermented wines	FastDNA Spin Kit for Soil (MP biomedicals)	Bacteria	V1–V3 region of bacterial 16S rRNA gene	F8 (5'-AGAGTTTGATCMTGGCTCAG-3') / R533 (5'-TTACCGCGGCTGCTGGCAC-3')	AMPure XP beads kit (Beckman Coulter	454 pyrosequencing (Roche)	(Stefanini *et al.*, 2016)
		Yeasts	ITS1-5.8S rDNA-ITS2	ITS1 / ITS4			
Grape musts and fermented wines	FastDNA Spin Kit for Soil (MP Biomedical)	Yeasts	ITS2	ITS7_F ('5-GTGARTCATCGAATCTTTG-3') / ITS4_R	QiaQuick columns (Qiagen)	Illumina MiSeq	(Sirén *et al.*, 2019)
Fermented wines	PowerFood Microbial DNA Isolation Kit (Mobio)	Yeasts	Fungal internal transcribed spacer I (ITS1)	BITS / B58S3	minElute (Qiagen)	Illumina MiSeq	(Sternes *et al.*, 2017)
Vineyard soil	FastDNA Spin Kit for Soil (MP Biomedical)	Bacteria	V3-V4 region of the bacterial 16S rDNA gene	341F (5´-CCTAYGGGRBGCASCAG-3´) / 806R	HighPrep™ PCR reagent (MAGBIO)	Illumina MiSeq	(Gobbi *et al.*, 2019)

1.3.1.2. Strains level

To reach an intra-species discrimination level, a prior isolation stage is required. Several molecular methods are employed to investigate the strain diversity of species of wine LAB. Pulsed-field gel electrophoresis of rare restriction enzyme digests (REA-PFGE) is one of these methods, frequently applied in the wine area and has proved to be a quick tool. PFGE was found to be more discriminative than RAPD-PCR, to study the *O. oeni* and *L. plantarum* communities, and their fluctuation in wine (López *et al.*, 2008). The level of discrimination of PFGE depends on the restriction enzyme chosen. In Simpson *et al*. (2001) study, on 33 assigned strains from 6 species within the genus *Pediococcus*, 30, 32, and 28 PFGE patterns were produced by the genomic digestion with the restriction enzymes *Apa*I, *Not*I, and *Asc*I respectively.

To achieve a finer differentiation of isolated strains, molecular methods are usually combined. Gracia-Ruiz *et al.* (2013a) analysed 43 *O. oeni* strains isolated from inoculated and non-inoculated wines treated or not with phenolics extracts and obtained 27 specific profiles using the restriction enzyme *Not*I. Five of the PFGE profiles were then characterized by the presence of 16 significant genetic markers (García-Ruiz *et al.*, 2013a). Cafaro *et al.* (2016) isolated 32 *O. oeni* strains from two different wineries and obtained 8 different REA-PFGE patterns with the restriction enzyme *Apa*I. The enzyme *Sfi*I was found to be less discriminative than *Apa*I. Differential Display PCR (DD-PCR) analysis of the same 32 strains discriminated 16 different patterns using the primer M13 with the primer OPA9 being found to be less discriminative than M13 (Cafaro *et al.*, 2016). In another study (Bridier *et al.*, 2010), 513 strains of *O. oeni* were isolated from numerous wine types and ciders collected worldwide, and a total of 363 unique REA-PFGE patterns were obtained using the restriction enzyme *Not*I. Based on the REA-PFGE results, 235 strains were selected for multilocus sequence typing (MLST). After DNA extraction, amplifying and sequencing seven housekeeping targeted genes of the DNA of each *O. oeni* strain allowed to determine 127 different sequence types, grouped into 3 principal phylogenic groups (Bridier *et al.*, 2010). Multiple-locus variable number of tandem repeat analysis (VNTR) typing of *O. oeni* was found to be highly discriminating, faster and more reliable than REA-PFGE or MLST methods. Of the 236 strains of *O. oeni* studied in Claisse and Lonvaud-Funel (2012), 201 VNTR types were obtained against 136 PFGE profiles and 110 MLST patterns. In yet another study (El Khoury *et al.*, 2016) using multilocus variable analysis (MLVA), 2,997 *O. oeni* strains were isolated from various French wines and British ciders, revealing 514 different genotypes. A second typing method based on single nucleotide polymorphism (SNP) analysis was applied to the 514 profiles for supplementary discrimination, delineating 11 groups of phylogenetically related strains (El Khoury *et al.*, 2016).

1.3.2. Microbial functionalities

1.3.2.1. Whole-Metagenome-Sequencing (WMS) - metagenomics

Contrary to metabarcoding amplicon sequencing, WMS detects very low abundance members of microbial community and reveals their functional gene composition. The steps involved in the shotgun metagenomic sequencing include DNA extraction, library preparation, sequencing, assembly, annotation and statistical analysis. Recent studies have been focusing their work on the analyze of grapes and wines´ microbial communities using a metagenomic approach. Salvetti *et al.* (2016) used a WMS approach to analyze the microbial consortium of grape berry surfaces at the end of the withering process performed under two different sets of conditions ("traditional withering," or "accelerated withering,"). The main functional differences found between the two processes covered the amino acid and carbohydrate metabolism and transport, transcription, intracellular trafficking, secretion and vesicular transport, and defense mechanisms. Nevertheless, among all the eukaryotic and prokaryotic communities identified, some were common to both conditions with 15 putative genomes dominating the microbial community of the two samples (Salvetti *et al.*, 2016). In their research work, Zepeda-Mendoza *et al.* (2018) used shotgun sequencing-based metagenomics to characterize the impact of various combinations of strains of *Brett. bruxellensis* and *O. oeni* (one with and one without cinnamoyl esterase activity releasing hydroxycinnamic acids) on the taxonomic and potential functional profile of the microbiome of Cabernet Sauvignon wine. One of the main results of this study was that the effect on the microbial profiles depended on the *B. bruxellensis* and *O. oeni* strains being combined (Zepeda-Mendoza *et al.*, 2018). Sirén *et al.* (2019) combined both metabarcoding and shotgun sequencing to characterize the microbial community of Riesling musts collected from 4 different vineyards, during AF. WMS analysis on two of the vineyards revealed a high abundance of *Metschnikowia* during fermentation that might serve as a biocontrol agent against bacteria, via a putative iron depletion pathway. The results of the study also suggest that bacteria might sit-and-wait until *Saccharomyces* activity slows down (Sirén *et al.*, 2019).

1.3.2.2. Transcriptomics

Transcriptomics is the direct study of the genes expressed via the analysis of mRNA in cell populations. This can be employed to determine which genes are up- or down-regulated when a cell population is exposed to different environmental stimuli. The high cost associated with the sequencing of each sample normally limits the number of samples that can be surveyed. Due to the high sensitivity of RNA, its extraction is delicate and preferably requires a kit like the High Pure RNA Isolation Kit (from Roche, Mannheim, Germany).

Some studies about transcriptomics have been recently applied to wine microbiota. For example, Godoy *et al.* (2016) analyzed the resistance of a *Brett. bruxellensis* strain to *p*-coumaric acid using a transcriptome analysis. The results of the study showed the induction of the expression of a proton pump and the efflux of toxic compounds due to a generalized stress caused by the entrance of *p*-coumaric acid

into the yeast cell. According to the authors, these mechanisms could also be involved in the outflux of nitrogen compounds, decreasing the overall concentration and triggering the expression of nitrogen metabolism genes (Godoy *et al.*, 2016). Capozzi *et al.* (2016) also analyzed the transcriptome of a *Brett. bruxellensis* strain to comprehend its transformation and recovery from the Viable-But-Non-Culturable state caused by the addition and removal of SO_2 respectively. The research results suggested the role of genes/proteins involved in redox cell homeostasis and the recovery of cell after SO_2 removal rather than a simple regrowth (Capozzi *et al.*, 2016). Another transcriptomics approach was used in Mendes *et al.* (2017) work to identify new genes in four *S. cerevisiae* strains in association with the formation of flavor active compounds during the fermentation process. Increased expression of genes related with tetracyclic and pentacyclic triterpenes metabolism, involved in sterol synthesis, and genes related with formation of higher alcohols in the Ehrlich pathway, were observed in some of the studied strains (Mendes *et al.*, 2017). Liu *et al.* (2017) investigated the acid stress transcriptomic response in *O. oeni* and found that most functional gene categories affected by acid were membrane transport, amino acid metabolism and carbohydrate metabolism.

1.3.2.3. Combined transcriptomics and proteomics

Proteomics analysis can also be used to corroborate the transcriptomics results. Olguín *et al.* (2015) and Margalef-Català *et al.* (2016) combined transcriptomic and proteomic approaches to study the adaptation of a *O. oeni* strain (PSU-1) to wine stress conditions (low pH and high ethanol content). Significant changes in gene expression and protein synthesis were noticed in cells as a consequence of the stress applied. For example, an apparent use of L-malate and citrate by the strain as an alternative energy source to sugar metabolism was observed as response to wine stress conditions (Margalef-Català *et al.*, 2016; Liu *et al.*, 2017). Moreover, a potential inhibition of transport and cell envelope biosynthesis by ethanol (12% v/v) has also been suggested (Olguín *et al.*, 2015).

1.4. Wine chemical compounds

Microorganisms interact with wine metabolites (volatile and non-volatile components) along the vinification contributing to the sensory perception of the wine (Francis and Newton, 2005; Piao *et al.*, 2015). A number of groups of compounds are modulated along the winemaking process and these are briefly discussed in this section.

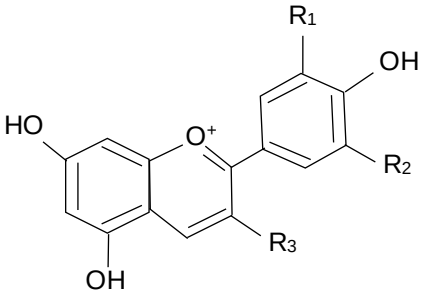
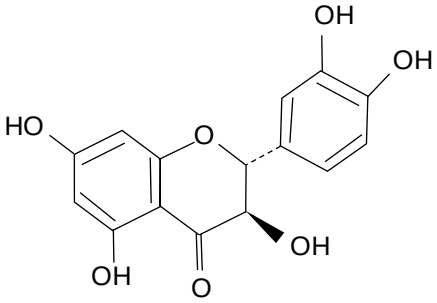
1.4.1. Phenolic compounds in wine

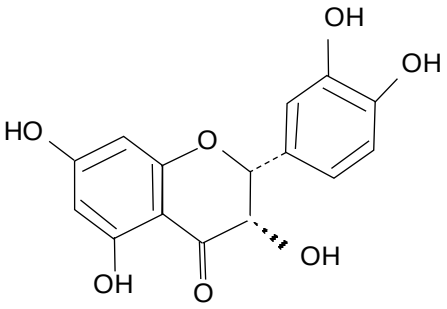
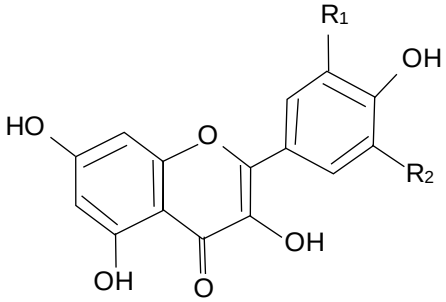
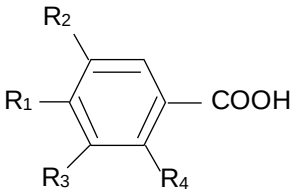
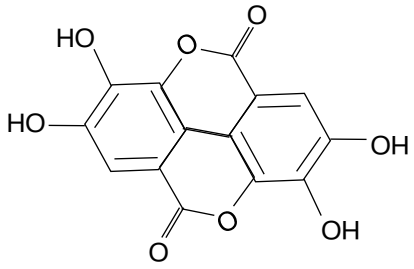
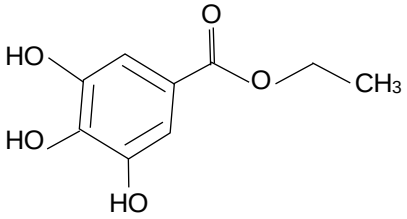
Phenolic compounds are important components participating to the overall organoleptic profile of wines as they are responsible for wine color, are precursors of wine flavors and contribute to the astringency and bitterness of wines (Hernández *et al.*, 2006; Ribéreau-Gayon *et al.*, 2006; Bouzanquet *et al.*, 2012; Zhang *et al.*, 2016).

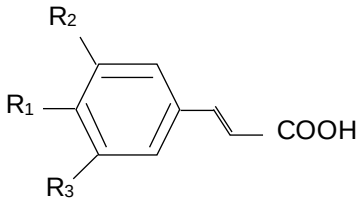
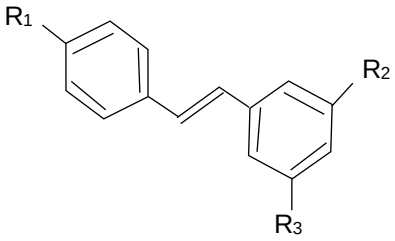
1.4.1.1. Main classes of phenolic compounds

Phenolic compounds are a class of organic molecules which contain at least one hydroxyl group (-OH) attached to an aromatic ring. They are synthesized and located in the skin, seeds and stems of wine grapes and are usually classified in two groups, according to their chemical structure: flavonoid and non-flavonoid compounds (Table 1.2.). Flavonoid compounds comprise anthocyanins, flavan-3-ols, flavonols and other flavone derivatives, while non-flavonoid compounds include mainly phenolic acids - hydroxybenzoic acids (HBA) and hydroxycinnamic acids (HCA) - phenolic alcohols, phenolic aldehydes and stilbenes (Monagas *et al.*, 2005). Hydroxycinnamic acids occur principally as esters of tartaric acid and ethanol (forming ethyl and diethyl esters) but may also be associated with anthocyanins, or other organic acids (Waterhouse, 2002; Pozo-Bayón *et al.*, 2003).
Malvidin-3-O-glucoside, gallic acid, (+)-catechin*, trans*-caftaric acid (tartrate derivative of *trans*-caffeic acid), quercetin and *trans*-resveratrol are the most abundant compounds in their respective groups encountered in red wines (Ginjom *et al.*, 2011).

Table 1.2. – Chemical structures of the main wine associated phenolic compounds (adaptation from (Figueiredo *et al.*, 2008)).

Group	Chemical structure	Name
Flavonoids		
Anthocyanins		R_1=OH, R_2=H : Cyanidin R_1=R_2=OH : Delphinidin R_1=OCH_3, R_2=H : Peonidin R_1=OCH_3, R_2=OH : Petunidin R_1=R_2=OCH_3 : Malvidin R_3=O-Glucose, 3-O-acetylglucose or 3-O-coumaroylglucose
		(+)-Catechin

Flavan-3-ols		(-)-Epicatechin
Flavonols		R_1=R_2=H : Kaempferol R_1=H, R_2=OH : Quercetin R_1=R_2=OH : Myricetin
Non-flavonoids		
Hydroxybenzoic acids		R_1=OH, R_2=R_3=R_4=H : *p*-Hydroxybenzoic acid R_1= R_2=OH, R_3=R_4=H : Protocatechuic acid R_1=OH, R_2=OCH_3, R_3=R_4=H : Vanillic acid R_1=R_2=R_3=OH, R_4=H : Gallic acid R_1=OH, R_2= R_3=OCH_3, R_4=H : Syringic acid R_1= R3=H, R2=R_4=OH : Gentisic acid
		Ellagic acid
Ethyl ester of gallic acid		Ethyl gallate

Hydroxycinnamic acids		R_1=OH, R_2=R_3=H : *p*-coumaric acid R_1=R_2=OH, R_3=H : Caffeic acid R_1=OH, R_2=OCH_3, R_3=H : Ferulic acid R_1=OH, R_2=R_3=OCH_3 :Sinapic acid
Trans-stilbenes		R_1=R_2=R_3=OH : *Trans*-resveratrol

1.4.1.2. Factors influencing the abundance of phenolic compounds during vinification

The phenolics are mainly derived from grapes and vary in terms of specific nature and abundance in the grape according to variety, climate, harvest date etc. Part of the phenolic compounds extracted into the must/wine will depend on the practices of maceration (musts freezing and heating, duration of maceration, enzymatic treatments, stems, tannins and woodchips additions, ethanol content produced during AF etc). The phenolic compounds present in a final wine will also depend on processing by microbes (during fermentations) and procedures of aging and wine treatments (fining, filtration etc).

1.4.1.2.1. Location of phenolics in grape berries

Total phenolics distribution in red grape berries has been estimated as follow: 50% skin, 44% seeds and the remaining 6% in the pulp (Singleton, 1969). More precisely, anthocyanins, flavonols and stilbenes are found in the skin, HCA and their derivatives in the pulp and skin, and flavan-3-ols in the seeds, skin and sometimes in the pulp. Seeds mostly include (+)-catechin, (-)-epicatechin, gallic acid and epicatechin gallate. Skins mostly include *trans*-resveratrol, *trans*-caftaric acid and quercetin derivatives (Anastasiadi *et al.*, 2010).

1.4.1.2.2. Factors influencing berry composition

The phenolic compounds present in wine are grape variety dependent. For instance, Stavridou *et al.* (2016) have shown that (+)-catechin concentration increased with aging time in Cabernet Sauvignon wines but decreased in Merlot wines. Seasonality is also known to influence color and phenolic content of red grapes while *p*-coumaric acid concentrations have been found to increase with stress periods of vine (Kilinc and Kalkan, 2003; Van der Merwe *et al.*, 2011). According to Tian *et al*. (2009), most of the phenolic acids and flavan-3-ol in musts increase with harvest time delay. On the contrary, mechanical harvesting, through reaction with oxidative radicals may decrease the phenolics content of musts (Olejar *et al.*, 2015).

1.4.1.2.3. Maceration effects

Various maceration processes were found to facilitate the extraction of phenolics from grape solids. These include must freezing and heating, saignée and duration of maceration among others (Vrhovsek *et al.*, 2002; Sacchi *et al.*, 2005). Other maceration practices can promote differential release of phenolics from grape solids and induce molecular changes in the phenolics. For example, some enzymatic treatments were found to preferentially release anthocyanins, HBA and HCA from grape skins and transform flavonols (Arnous and Meyer, 2010). The presence of stems during maceration has been shown to enhance the concentration of flavan-3-ols, especially (+)-catechin while gallic and ellagic acids increased with the addition of tannins and woodchips (Gambuti *et al.*, 2007; Pascual *et al.*, 2016). The addition of SO_2 before fermentation was also observed to increase the contents in anthocyanins, flavan-3-ols, quercetin and *trans*-resveratrol (Gambuti *et al.*, 2007).

Maceration and AF usually occur simultaneously in red wines, with the ethanol generated by yeasts increasing grape cell membrane permeability, thus enhancing the extraction of anthocyanins from skins and partially from seeds (Canals *et al.*, 2005; Jackson, 2008). Depending on the desired final concentration of phenolic compounds, the maceration can be shorter, equal or longer than AF. Wine yeasts can influence the extraction of grape anthocyanins during maceration and fermentation and stimulate the formation of more stable anthocyanin forms linked to pyruvic acid and acetaldehyde production (Fulcrand *et al.*, 1998; de Freitas and Mateus, 2011; Morata *et al.*, 2016). Nevertheless, wine yeasts can also promote anthocyanin degradation or interactions with other compounds resulting in color loss (Monagas *et al.*, 2007). In wine, the concentrations of most phenolic compounds are normally at the highest point during AF, and stabilize or slowly decrease thereafter (Ginjom *et al.*, 2011). Sun *et al.* (2011) noticed that the intensities of astringency and bitterness in a red wine was linked to its phenolics´ composition and decreased after alcoholic fermentation.

1.4.1.2.4. Malolactic fermentation

The concentrations of anthocyanins and total polyphenols normally decrease during MLF (Vrhovsek *et al.*, 2002). However few phenolics such as *trans*-ferulic acid can increase in concentration due to the hydrolysis of their tartrate derivatives ((hydroxy)cinnamoyl-tartaric acids) by chemical or microbial activities (Hernández *et al.*, 2006; Hernandez *et al.*, 2007; Cabrita *et al.*, 2008). Most variations of the phenolic compounds‘ composition of a wine during MLF are linked to LAB activity (Hernández *et al.*, 2006; Hernandez *et al.*, 2007). Nevertheless, the metabolic pathways that LAB use to degrade these compounds are relatively poorly studied. Recently, Devi *et al.* (2018b) analyzed the adaptations of *L. plantarum* and *O. oeni* in response to phenolics stresses during wine fermentation and found that these bacteria were able to degrade phenolic compounds into volatile phenols, aromatic alcohols, and phenyl-propionic acids, thus indicating the possible involvement of oxidoreductases, decarboxylases, and demethylases. In this study (+)-catechin was metabolized but no metabolic product was detected (Devi

et al., 2018b). It was shown by Poussier *et al.* (2003) that *O. oeni*, as *S. cerevisiae*, could affect the concentration in stilbenes in red wines.

1.4.1.2.5. Aging

The phenolic profile of a wine continues to evolve during post-fermentation phases of production and depends on the aging techniques employed. For example, in white wines significant decreases of phenolic compounds have been observed during storage, which resulted in a change in the color from pale yellow to yellow-brown (Recamales *et al.*, 2006). Li *et al.* (2009) found the concentration in HBA in red wines to first increase and then decrease during storage. Bimpilas *et al.* (2015) described a decline in some red wines in the monomeric anthocyanins and flavonol glycosides concentrations after one year of storage, involving polymerization reactions, co-pigmentation and enzymatic hydrolysis. Decreases in anthocyanins and flavan-3-ol dimmers in red wines were also highlighted in Castillo-Sánchez *et al.* (2008). Lower storage temperature may improve the color quality of a wine (Gómez-Plaza *et al.*, 2000). Mature wines in oak barrels may bring the wines elevated levels of HBA derivatives, notably ellagic acid, and low levels of anthocyanins, flavan-3-ols, gallic acid, *p*-coumaric acid, quercetin and *trans*-resveratrol (Gambuti *et al.*, 2007; Stavridou *et al.*, 2016). The origin and age of oak barrels doesn´t appear to affect the phenolic composition of aged wines (Ginjom *et al.*, 2011; Stavridou *et al.*, 2016). Micro-oxygenation of the wine, through and between wood staves of oak barrels, was shown to stabilize the wine color and decrease its astringency (Gambuti *et al.*, 2012). Aging in bottles for two years would mostly release *trans*-*p*-coumaric acid from *p*-coumaroyl-acylated anthocyanins and decrease the flavonols content in red wines (Monagas *et al.*, 2005).

1.4.1.2.6. Clarification and stabilization

Techniques before bottling such as filtration and fining by addition of natural or synthetic polymeric agents have been used to modulate the concentration of phenolics in wine (Castro *et al.*, 2016). Filtration is the most common procedure performed to reduce suspended material in post-fermentative wine including microorganisms (Malfeito-Ferreira, 2011). Therefore, anthocyanins adsorbed by yeast cell walls during fermentation may be eliminated with yeasts during filtration (Morata *et al.*, 2003). Various fining agents (bentonite, egg white, polyvinylpolypyrrolidone etc.) were observed to decrease the *trans*-resveratrol content of the wine (Threlfall *et al.*, 1999).

1.4.1.3. Principal effects of phenolics on the wine microbiota

During the fermentation processes and aging of wines, phenolic compounds can behave as activators or inhibitors of growth and metabolism of specific microbial species and strains depending on their chemical structures and concentrations (Rozès *et al.*, 2003; Campos *et al.*, 2009b).

1.4.1.3.1. The consequences of the addition of phenolic extracts on wine microorganisms

By adding phenolic fractions of Malbec wine characterized by high HCA concentrations to a synthetic wine-like medium, Stivala *et al.* (2015) detected a decrease in bacterial viability of *P. pentosaceus*, an exopolysaccharide-producing wine spoilage bacterium, and thus a decrease in exopolysaccharide production. García-Ruiz *et al.* (2012) showed that various phenolic extracts from different origins were found to damage the integrity of the cell membranes of two LAB (*L. plantarum* and *O. oeni*) and two AAB (*A. aceti* and *G. oxydans*) cultivated in medium cultures. In the same study, the addition of eucalyptus extract (particularly rich in *trans*-resveratrol, gallic acid and quercetin) in wine, delayed the progress of non-inoculated MLF and MLF inoculated with three *O. oeni* strains (García-Ruiz *et al.*, 2012). In another study from the same authors (García-Ruiz *et al.*, 2013a), eucalyptus extract and almond extract (particularly rich in (+)-catechin and tyrosol) were shown to have different impacts on the *O. oeni* diversity of the studied red wines. Another study (Chasseriaud *et al.*, 2015) observed the total phenolic extracts from red wines made from different grape varieties, added in parallel to a laboratory medium and a white wine medium, to have a positive effect on the growth and the activities of two *O. oeni* strains, more or less stronger depending on the tannins composition.

1.4.1.3.2. Impact of specific phenolics on wine yeasts and acetic acid bacteria

Of the 15 phenolic compounds analyzed by Pastorkova *et al.* (2013) for their antimicrobial effects in medium cultures, *trans*-resveratrol possessed the strongest inhibitory effect against the spoilage yeasts *D. bruxellensis, H. uvarum, S. cerevisiae, Z. bailii*, and *Z. rouxii*, and the spoilage acetic acid bacteria *A. aceti, A. oeni, A. pasteurianus.* Myricetin, *p*-coumaric and ferulic acids exhibited selective growth inhibitory effects against these microorganisms, and (+)-catechin and caffeic acid apparently none (Pastorkova *et al.*, 2013). The inhibitory effect of *trans*-ferulic acid on *Dekkera spp.* was found to be amplified by the ethanol content, *D. anomala* being less sensitive than *D. bruxellensis* (Harris *et al.*, 2010).

1.4.1.3.3. Impact of specific phenolics on wine lactic acid bacteria

Several studies have been published focusing on the impact of specific phenolic compounds on wine LAB. Free anthocyanins and gallic acid were found to activate the cell growth and the rate of malic acid degradation of some *O. oeni* strains (Vivas *et al.*, 1997). Moreover, in concentrations normally found in wine, gallic acid and (+)-catechin were observed to enhance the growth and metabolism of *L. hilgardii* (Alberto *et al.*, 2001). Nevertheless, in another study, the production of acetic acid from citric acid by *O. oeni* was delayed by the addition of gallic acid (Reguant *et al.*, 2000). In the same study, MLF conducted by this specific *O. oeni* strain was apparently stimulated by the presence of quercetin and (+)-catechin, but impaired by the addition of HCA. In another study (Rozès *et al.*, 2003) it was found that mixing HCA

with gallic acid and (+)-catechin activated the cell growth of this strain and increased the production of acetic acid by reducing the rate of sugar consumption and enhancing citric acid consumption. The cell growth of other LAB (*P. pentosaceus* and *L. plantarum*) was also found to be activated by some levels of (+)-catechin (de Llano *et al.*, 2016). In a more recent study (Devi and Anu-Appaiah, 2018a), (+)-catechin only exercised a slight stress on the *L. plantarum* and *O. oeni* strains tested.

The growth of a commercial *O. oeni* strain has been found to be inhibited by quercetin and kaempferol, but barely affected by myricetin, (+)-catechin and (-)-epicatechin (Figueiredo *et al.*, 2008). Gacía-Ruiz *et al*. (2009) found kaempferol to damage the cell membrane of *L. hilgardii* and *P. pentosaceus* strains. In another study from the same authors (García-Ruiz *et al.,* 2011), kaempferol and *trans*-resveratrol had the strongest negative impact on the growth of a single wine-isolated strain of *L. hilgardii* and *P. pentosaceus* and four wine-isolated strains of *O. oeni* while HCA and HBA had only a limited inhibitory effect on these bacteria and flavanol-3-ols, a negligible one.

Hydroxycinnamic acids were found to inhibit the growth of a commercial *O. oeni* strain and a wine-isolated *L. hilgardii* strain and to delay the metabolism of glucose and citric acid (Campos *et al.*, 2009a). In the same work, HCA also increased the yield of lactic and acetic acids production from glucose by the heterofermentative pathway of the *O. oeni* strain. In addition to their antimicrobial effects, the presence of HCA has been observed to increase the cell membrane permeability and delay the malic acid degradation of a variety of microorganisms including the LAB *L. collinoides*, *L. brevis*, *L. hilgardii* and *O. oeni* (Stead, 1993; Campos *et al.*, 2003; Campos *et al.*, 2009b). According to Devi and Anu-Appaiah (2018a), the microbial response to exposure to HCA would be in part manifested as changes in membrane and enzyme compositions.

The experiments described in this chapter were performed in culture medium with the addition of varying concentrations of phenolic compounds.

1.4.2. Wine volatile compounds related to post-alcoholic fermentation stage and microbial activity

The volatile compounds might or might not be relevant to the aroma or flavor of a wine and can have a positive or negative impact on its sensorial quality. The composition of the volatile fraction of wine, defined as the sum of all the volatiles that are in the headspace of a wine in detectable quantities, derives from grape composition, alcoholic fermentation, malolactic fermentation, subsequent storage or aging regime. All are important steps in shaping the aromatic nature of a wine, generally regarded to be a combination of fruit derived compounds (sometimes modulated by microbes) and aromatic products of microbial metabolism.

1.4.2.1. Main classes of volatiles in wine

The volatile fraction of wine is mostly derived from AF and contains among others ethanol, acetaldehyde, higher alcohols and their acetates, volatile fatty acids (VFA) and their ethyl esters (Appendix 3). During

MLF, LAB can generate additional volatile compounds from non-volatile grape constituents such as residual sugars and amino acids, and can metabolize the pre-MLF volatile compounds or absorb them to their cell walls (Laurent *et al.*, 1994; Bartowsky and Henschke, 1995).

Acetaldehyde (ethanal), which brings a fruity note to wines, is derived from AF but can also be produced by the oxidative metabolism of some spoilage AAB occurring mainly after MLF. This compound can bind with the added SO_2, reducing the effectiveness of its antimicrobial activity and its antioxidative effect. Depending on its concentration, acetaldehyde can also simulate or inhibit microbial growth (Liu and Pilone, 2000).

Esters are important volatiles contributing to the fruity aromas of the wine and are some of the main volatile compounds affected by malolactic bacteria (Liu, 2002; Swiegers *et al.*, 2005b). Acetate esters (ethyl acetate, isoamyl acetate, 2-phenylethyl acetate, etc.) are products of the condensation of higher alcohols with acetyl-CoA and ethyl fatty acid esters (ethyl butanoate, ethyl hexanoate, ethyl octanoate, etc.) are formed by the esterification of VFA.

Higher alcohols and VFA are the principal volatiles formed from the metabolism of amino acids. For example, isobutanol (2-methyl-1-propanol or isobutyl alcohol), isoamyl alcohol (3-methyl-1-butanol), methionol and 2-phenylethanol are formed from the respective amino acids; valine, leucine, methionine and phenylalanine (Styger *et al.*, 2011). In concentrations found in wine, the aromatic impact of higher alcohols depends on their chemical structures and the aromatic context of the wine. For example, De-la-Fuente-Blanco *et al.* (2016) proved the sensory importance of the pair isobutanol-isoamyl alcohol, added in wine models at levels of concentration within the natural range of occurrence, compared to methionol and 2-phenylethanol which brought negligible aroma to the wine analyzed. The volatile fatty acids of wine (acetic acid, hexanoic acid, octanoic acid, etc.) are mostly short-chain (less than six carbons) and medium-chain (six to twelve carbons) fatty acids. Even though VFA are related with undesirable odors at concentrations higher than 20 mg/L, they were revealed to be essential for the perception of fresh fruit and to mask the animal character of ethylphenols (Romano *et al.*, 2009; San-Juan *et al.*, 2011; Sánchez-Palomo *et al.*, 2017).

Volatile phenols (4-ethylphenol [4-EP], 4-ethylcatechol [4-EC], etc.) are synthesized in wines from HCA and their derivatives through microbial processes (Chatonnet *et al.*, 1992; Couto *et al.*, 2006). Above certain concentrations, volatile phenols are considered off-flavors, causing a deterioration of wine quality.

Terpenes (linalool, nerol, α-terpineol, etc.) are hydrocarbon natural products biosynthesized in the grapevine from interconnected isoprene units and are present in wine in free and bound (in a form of glycosides) forms (Marais, 1983). The typical aroma description of terpenes in wine is floral/fruity (Appendix 3).

Sulfur compounds in wine are products of enzymatic reactions resulting mostly from yeast metabolism and can be sensed by unpleasant smells when encountered in concentrations above their thresholds (Moreira *et al.*, 2002).

1.4.2.2. Principal effects of post-alcoholic fermentation microbial activity on wine aroma

The changes in the volatile composition of a wine were found to be linked to its inter- and intra-microbial species diversity during its production (Cappello *et al.*, 2016). Hence, through a number of activities, LAB are mainly responsible for the modifications in wine aroma and flavor during MLF.

Acetaldehyde was found to generate flavor components when catabolized by bacteria (Liu and Pilone, 2000). Davis *et al.* (1988) suggested that the formation of VFA from lipids during MLF was principally due to lipase activities from LAB. Some LAB strains are able to release terpenes by the hydrolyze of aroma precursors (Ugliano *et al.*, 2003; Hernandez-Orte *et al.*, 2009). However, some esters and terpenes produced during AF can be degraded during MLF, leading to a loss in the fruity and floral characters of the wine (Bartowsky, 2005). The inoculation of wines by some commercial starters has been shown to increase the concentrations in higher alcohols and VFA in some situations (Maicas *et al.*, 1999; Ugliano and Moio, 2005; Malherbe *et al.*, 2012). Several studies have been done on the impact of MLF on wine esters. García-Ruiz *et al.* (2013b) described the wines after MLF as containing less isoamyl acetate, isobutyl acetate, ethyl butyrate, 2-phenylethyl acetate, hexyl acetate, ethyl acetate and ethyl hexanoate, but more diethyl succinate and ethyl lactate than before MLF. Riu-Aumatell *et al.* (2006) also observed that long aging times in contact with lees decreased the content in hexyl, 2-phenylethyl and isoamyl acetates in the wine studied and increased the concentrations in diethyl succinate. The increase in diethyl succinate and ethyl lactate after MLF has been found to be related to the bacterial production of succinic and lactic acids (Herjavec *et al.*, 2001; Ugliano and Moio, 2005; Jeromel *et al.*, 2008; Malherbe *et al.*, 2012). The decrease in the esters concentration was associated with the esterase activity of LAB and with the acidic hydrolysis of these compounds (Matthews *et al.*, 2007). However, depending on the strain evaluated, Maicas *et al.* (1999) found an increase in isoamyl acetate, 2-phenylethyl acetate and ethyl hexanoate, while Malherbe *et al.* (2012) found an increase in ethyl octanoate, ethyl 2-methylpropanoate, and ethyl propionate by MLF. Lactic acid bacteria possess a substantial collection of enzymes involved in the synthesis and hydrolysis of esters (Sumby *et al.*, 2010). Intracellular esterases from *O. oeni* and *L. hilgardii* have been characterized by Sumby *et al.* (2009; 2013b) and shown to metabolize volatile aromatic esters. An alcohol acyltransferase activity was also identified in *O. oeni* and *L. plantarum* strains, contributing to the increase of the concentrations of esters in wine (Costello *et al.*, 2013).

By their effects on the microbial population, phenolics can indirectly impact the volatile composition of wine. Garcia-Ruiz *et al.* (2013b) and Rodriguez-Bencomo *et al.* (2014) found that the addition of two antimicrobial extracts prior to MLF, one mainly represented by *trans*-resveratrol, gallic acid and quercetin (eucalyptus extract), and the other one by (+)-catechin and tyrosol (almond extract), led to some compositional changes in the volatile composition according to whether the wine was red or white and MLF was induced or not. The addition of eucalyptus extract decreased the concentrations in most of the esters (except diethyl succinate) in red wines, but the opposite effect was observed in white wines. A decrease of the content in alcohols and an increase of the content in volatile phenols were observed in

both type of treated wines. Only in the inoculated red wines, did the eucalyptus decreased the content of octanoic acid and increased the content of hexanoic acid (García-Ruiz *et al.*, 2013b; Rodríguez-Bencomo *et al.*, 2014).

1.4.2.3. Enzymatic systems involved in the production of volatile phenols in wine

1.4.2.3.1. Production of volatile phenols from hydroxycinnamic acids

Hydroxycinnamic acids can be converted to volatile phenols by specific yeasts and bacteria through two different biochemical pathways. The most commonly reported pathway for the microbial metabolization of HCA involves two enzymes; a phenolic acid decarboxylase (PAD) which decarboxylates HCA into their corresponding vinylphenols, and a vinylphenol reductase (VPR) which reduces the latter to the corresponding ethylphenols (Figure 1.4.a). This biochemical pathway has been found to especially occur in *Brettanomyces/Dekkera* yeasts but is also known to occur in some *S. cerevisiae* and *Pi. guilliermondii* strains as well as in bacteria as *P. pentosaceus, P. damnosus*, *O. oeni*, *Lc. mesenteroides, L. mali*, *L. brevis, L. collinoides* and *L. plantarum* (Chatonnet *et al.*, 1992; Chatonnet *et al.*, 1995; Barata *et al.*, 2006; Couto *et al.*, 2006; Campos *et al.*, 2009a). The activities of PAD and VPR are variable among the strains and principally modulated by the nature and concentration of the substrate (Silva *et al.*, 2011; Filannino *et al.*, 2015; Rosimin and Kim, 2015; Sturm *et al.*, 2015). Some strains of the genus *Brettanomyces/Dekkera* were shown to possess the two enzymes metabolizing “free” HCA into volatile phenols but others only have the cinnamate decarboxylase enzyme (Harris *et al.*, 2008). Bloem *et al.* (2007) only found *Lactobacillus* and *Pediococcus* strains to convert *trans*-ferulic acid into 4-vinylguaiacol. Another PAD, which also displays inducible reductase activity, was detected by Barthelmebs *et al.* (2000) in a *L. plantarum* strain. Depending on the induction conditions, the enzyme was found to be able to reduce caffeic, *p*-coumaric, and ferulic acids into their respective propionic acids: dihydrocaffeic, dihydrop-coumaric (phloretic), and dihydroferulic acids (Figure 1.4.b). The reductase activity was observed to usually take over the decarboxylase activity when glucose is added and to be better induced with ferulic acid than with *p*-coumaric acid (Barthelmebs *et al.*, 2000). Phenyl propionic acids, just like vinylphenols, may then be metabolically reduced to ethylphenols. The acid phenol reductase activity has also been observed in strictly heterofermentative non wine-related LAB species (*Weissella spp., L. curvatus* and *L. rossiae*), using HCA as external acceptors of electrons (Filannino *et al.*, 2014). Besides a strong reductase activity, the addition of HCA may also cause a shift from alcohol dehydrogenase to acetate kinase activities in the heterofermentative pathway of the studied LAB and increases the NAD+/NADH ratio and the quantity of intracellular ATP (Reguant *et al.*, 2000; Campos *et al.*, 2009a).
Vinylphenols and ethylphenols are two families of compounds presenting unpleasant aromas, contrary to phenyl propionic acids which are odorless, stable and non-toxic (Ribéreau-Gayon *et al.*, 2000; Dias *et al.*, 2003a; Schopp *et al.*, 2013). *p-C*oumaric acid may also be adsorbed on *Brettanomyces* cells, decreasing its availability as substrate (Salameh *et al.*, 2008).

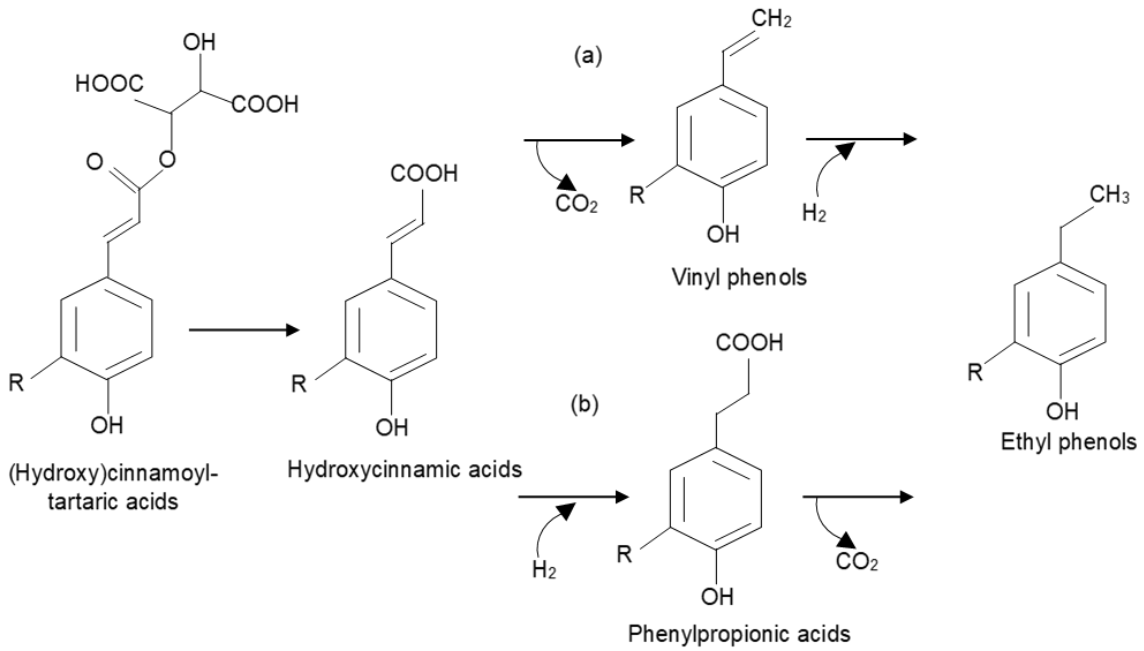


Figure 1.4. – Biochemical pathways of the conversion of (hydroxy)cinnamoyl-tartaric acids into ethylphenols adapted from Dugelay *et al.* (1993) and Barthelmebs *et al.* (2000). (a) decarboxylase activity and (b) reductase activity of PAD enzymes.

A few *Brett. bruxellensis* strains have also been observed to directly produce ethylphenols from *p*-coumaroyl glucose, feruloyl glucose and ethyl coumarate, but not from *p*-coumaroyl and feruloyl L-tartaric acids (coutaric and fertaric acids) (Hixson *et al.*, 2012; Hixson *et al.*, 2016). Hydroxycinnamic acids are known to exist in wine mainly as their tartrate derivatives, with *trans*-caftaric acid being the most abundant (Ribéreau-Gayon *et al.*, 2006). These acids are apparently not themselves substrate for the PAD enzymes and so are not direct precursors of volatile phenols (Schopp *et al.*, 2013).

1.4.2.3.2. Release of hydroxycinnamic acids from their derivatives forms

Hydroxycinnamic acids can be partially hydrolyzed from their tartaric acid derivatives ((hydroxy)cinnamoyl-tartaric acids) during winemaking, via chemical and enzymatical pathways.
Cinnamoyl-tartaric acids are susceptible to slowly hydrolyze through the vinification and the wine storage due to the acidity of the wine. The rate of the reactions depends on the wine pH, which normally varies between 3.0 and 3.9 (Waterhouse, 2002). Enzyme preparations added to the must during maceration to enhance the phenolic compounds extraction from grapes, have been shown to also release HCA from their esterified forms (Dugelay *et al.*, 1993; Arnous and Meyer, 2010). Also, during MLF, the release of "free" HCA has been previously linked to the disappearance of its corresponding tartrate derivative forms (Hernández *et al.*, 2006; Cabrita *et al.*, 2008). Among other microorganisms tested, a few *S. cerevisiae* strains, two *O. oeni* strains (Oenos™ and CiNe™ strains from Chr. Hansen) and a strain of a probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533 have been shown to possess cinnamoyl esterase

activity enable to cleave the ester bond of (hydroxy)cinnamoyl-tartaric acids, releasing tartaric acid and the corresponding HCA (Figure 1.4.) (Monagas *et al.*, 2007; Burns and Osborne, 2013; Chescheir *et al.*, 2015; Madsen *et al.*, 2016). The substrate for this cinnamoyl esterase enzymatic activity is apparently limited to the *trans*-isomers of the (hydroxy)cinnamoyl-tartaric acids (Hernandez *et al.*, 2007).

1.4.3. Amino acids, biogenic amines and wine bacteria

Microbial enzymatic activities in wine can convert amino acids into a range of volatile and non-volatile compounds, playing an important role in determining the organoleptic qualities of wine (Vincenzini *et al.*, 2017). Lactic acid bacteria require amino acids to grow and these requirements are strain dependent (de Nadra *et al.*, 2003). Strains of *O. oeni*, *L. brevis, L. hilgardii, L. plantarum, Lc. mesenteroides*, *P. parvulus* and *P. damnosus* are often found to produce various biogenic amines causing detrimental effects on wine quality and safety (Lonvaud-Funel, 1999; 2001; Guerrini *et al.*, 2002; Walling *et al.*, 2005; Rosi *et al.*, 2009; Coton *et al.*, 2010; Sebastian *et al.*, 2011). *Enterococcus faecium* have been isolated from wines by Capozzi *et al.* (2011) and were also found to produce biogenic amines. Histamine, tyramine and putrescine are the most abundant biogenic amines in wine, respectively produced from histidine, tyrosine and arginine (ornithine being the intermediate component between arginine and putrescine).

1.5. Research aims

The combination of high acidity and ethanol content makes the post-alcoholic fermentation wine environment highly selective with only very few species of yeast and bacteria able to interact with the medium. However, malolactic fermentation and, when it occurs, bacterial spoilage, can profoundly affect the chemical composition of wine and impact the final quality of the product.

Metabarcoding is a high-throughput technique based on the sequencing of amplicons from highly conserved regions in eukaryotic and prokaryotic cells, permitting the detection and quantification of most of the genomes present including those that are not otherwise detectable via cultural methods. In the first chapter, with the use of metabarcoding and chemical techniques, the microbial population and the metabolites content of 16 post-fermented wines with various origins and technical processes was analyzed.

Naturally-occurring phenolic compounds are known to influence the behavior of wine microbes, but this is yet still poorly defined. Most of the studies cited on the effects of phenolic compounds on wine microorganisms have been performed in culture media, with concentrations of phenolic compounds far higher than those found in wines, and certainly not under real wine conditions. The media used to test specific microbial reactions may have a significant impact on the overall metabolism. For example, the addition of wine into MRS broth culture containing *trans-p*-coumaric acid was shown to change the proportions in 4-vinylphenol and 4-ethylphenol produced by *L. plantarum*, implying a wine effect on the two enzymes involved in the volatile phenols production from HCA (Fras *et al.*, 2014).

In the second chapter, the effects of the single addition of different phenolics in pre-malolactic wines and wines mixed with MRS broth, non-inoculated and inoculated with different commercial *O. oeni* strains, was studied during the exponential phase of the LAB growth, on the microbial performance and the chemical composition.

The composition of phenolics in a wine is mostly regulated by pre-fermentation techniques. In the third and fourth chapters, the impact of the addition, in post-alcoholic fermentation wine, of specific groups of phenolic compounds, was evaluated with concentrations within the range that are encountered in real wine situations, on the behavior of wine LAB, and more specifically, on the diversity of MLF starter *O. oeni* strains and on the organoleptic properties of the wines related to their volatiles and phenolics' compositions, during spontaneous and inoculated MLF and subsequent storage.

Although the production of volatile phenols from phenolic acids has been quite studied in wine, little is known about the prior processes that determine the availability of "free" precursor molecules from tartrate derivatives. A cinnamoyl esterase activity, which enables the cleavage of the ester bond of (hydroxy)cinnamoyl-tartaric acids, releasing "free" phenolic acids, has been previously observed in some wines during MLF. This enzymatic activity has been found to be linked to specific strains of microorganisms. The objective of the fifth and last chapter was to better understand the basis of the differential activity between LAB strains using bioinformatic and enzymatic tools.

At the end of this disertation, the main findings are discussed in relation to what is currently known in this area and a number of possible new studies are proposed.

2. General materials and methods

2.1. Oenococcus oeni strains

For most of the experimental work the five commercial *Oenococcus oeni* strains; Viniflora® Oenos™, CiNe™, CH35™, CH16™ and CH11™, from Ch. Hansen (Hørsholm, Denmark) were used. Viniflora® bacterial cultures are natural strains originated from must samples that have been isolated and are sold for their specific particularities. Oenos™ was the first *O. oeni* strain developed for direct inoculation in wine and nowadays is the most commercialized MLF starter. CiNe™ does not metabolize citric acid and is mostly used to produce white and rose wines. The strains CH35™ and CH11™ are often used in wines that have difficulties undergoing MLF due to low temperature or low pH. The strain CH16™ was originally isolated from high-alcohol red wine (up to 16% (v/v) alcohol) and therefore is recommended as MLF starter for wines with high alcohol content.
Strains from Ch. Hansen were lyophilized, packed in pouches and store at -20 °C (Table 2.1.).

Table 2.1. – Strains of commercial *O. oeni* (from Ch. Hansen) used in this work.

Strains	Batch n°	CFU / g
Oenos™	3239704	$6.9\ 10^{11}$
CiNe™	3292333	$5.0\ 10^{11}$
CH35™	3239720	$1.4\ 10^{11}$
CH16™	3239711	$6.4\ 10^{11}$
CH11™	3995452	$7.7\ 10^{11}$

2.2. Growth medium

The liquid growth medium used in most of the experiments to cultivate LAB was a liquid MRS medium from BIOKAR Diagnostics (Allonne, France) supplemented with 10 mg/L cycloheximide from Sigma-Aldrich (Steinheim, Germany) and 5% (v/v) absolute ethanol from Carlo Erba (Val-de-Reuil, France). Ethanol added at this concentration has been found to stimulate growth of wine lactic acid bacteria (Couto and Hogg, 1994). The initial pH of the liquid MRS medium was adjusted to 4.5 using (37% m/v) hydrogen chloride.

2.3. Growth measurement

2.3.1. Lactic acid bacteria in suspensions containing wine

Decimal dilutions were prepared by transferring 100 µL aliquots between sterile 2 mL Eppendorf tubes containing 900 µL Ringer's solution (Sigma-Aldrich, Germany). All Eppendorf tubes were homogenized with a rotative vortex before transferal of the aliquots. The drop-count technique described by Miles and Misra (1938) was used on MRS agar medium, prepared with the same composition than liquid MRS medium with additional 2% (w/w) agar from Liofilchem (Roseto degli Abruzzi, Italy). All plates were incubated aerobically at 25 °C for 8-10 days before bacterial Colony Forming Units (CFU) were counting.

2.3.2. Lactic acid bacteria in liquid MRS medium

Bacterial growth in liquid MRS medium was determined indirectly by absorbance at 600 nm wavelength with a UV/VIS UNICAM 8620 spectrophotometer (UNICAM, Cambridge, UK) and optical cells of 1 cm path length. Distilled water was used to adjust the base (“zero”) absorbance of the spectrophotometer. Dilutions of the samples with distilled water were made when the absorbance value exceeded 1 AU.

2.3.3. Yeast in suspensions containing wine

The dehydrated culture media used to quantify yeasts in wine was Yeast Mold Broth (YMB) from Thermo Fisher Scientific (Waltham, MA, USA) supplemented with 34 mg/L chloramphenicol (Sigma-Aldrich, Germany), 5% (v/v) absolute ethanol and 2% (w/w) agar. The initial pH of the medium was adjusted to 4.5 using (37% m/v) hydrogen chloride. As for the bacterial counting, 100 µL sample was directly diluted into 900 µL Ringer's solution in 2 mL sterile Eppendorf tubes, diluted, plated on YMB agar medium and incubated at 30 °C for 48 h before yeast counting.

2.4. Phenolic compounds solutions

Concentrated solutions of phenolic compounds were prepared by weighing an appropriate amount to a sterile tube in an analytical scale and dissolving the compounds in pure (99.5% v/v) ethanol. All commercial phenolic compounds had a purity of at least 90% and were obtained from Sigma-Aldrich (Steinheim, Germany) except myricetin (purity 99%), ferulic acid methyl ester, ellagic acid and malvidin-3-O-glucoside chloride (purities ≥ 95%) which were obtained from Extrasynthese (Genay, France). All solutions were prepared before use to minimize oxidation.

2.5. Analyze of phenolic compounds

The analyze of phenolic compounds was adapted from the method described by Oliveira *et al.* (2015). Identification and quantification were conducted by an High-Performance Liquid Chromatography

(HPLC) system with a Diode Array Detector (DAD) from Waters Corporation (Milford, MA, USA). The stationary phase used was a Kromasil® C18 HPLC column 5 µm x 250 mm x 4.6 mm from Sigma-Aldrich (Steinheim, Germany). A binary HPLC solvent system was used with the following mobile phases: phase A, composed of acetonitrile from Fisher Chemical (Pittsburgh, PA, USA) with 0.2% of trifluoroacetic acid (TFA) (Sigma-Aldrich, Germany) and phase B, a mixture of acetonitrile and ultra-pure water 5:95 v/v with 0.2% TFA with the following gradient: 0-1 min (100% B); 1-30 min (100% to 79% B); 30-42 min (79% to 73% B); 42-55 min (3% to 42% B), 55-60 min (42-100% B) and 60-61 min (100% B).
The absorption spectra of all peaks were recorded between 212 and 600 nm. Hydroxybenzoic acids and flavan-3-ols were detected at 280 nm, hydroxycinnamic acids, their derivatives, and stilbenes at 320 nm, flavonols at 360nm and anthocyanins at 528 nm. Phenolic compounds were identified according to UV-Vis spectra, and retention times of known standards (*trans*-*p*-coumaric acid, *trans*-caffeic acid, *trans*-caftaric acid, *trans*-ferulic acid, sinapic acid, syringic acids, (+)-catechin, kaempferol, quercetin, gallic acid, *trans*-resveratrol, (-)-epicatechin, myricetin, malvidin-3-glucoside and ellagic acid). The concentrations were calculated according to the standard calibration curves. The compounds with unavailable standards were identified using the relative retention times and the UV-Vis data from (Lamuela-Raventós and Waterhouse, 1994; Hernandez *et al.*, 2007; Ginjom *et al.*, 2011; Oliveira *et al.*, 2015), and quantified as malvidin-3-O-glucoside equivalents for the anthocyanins and *trans*-caftaric acid equivalents for the (hydroxy)cinnamoyl-tartaric acids using the corresponding calibration curves. Samples were syringe-filtered (with a 0.45 µm cellulose acetate filter) and directly injected without dilution.

2.6. Sugars and organic acids analysis

The analytical methods used have been previously employed in our laboratory (Campos *et al.*, 2009a). Organic acids (citric acid, acetic acid, malic acid, lactic acid), and sugars (glucose, fructose) were analyzed by HPLC-UV-RI, with a Ultra-Violet (UV) detector at 210 nm (K-2501) and a refraction index (RI) detector (K-2301), both from KNAUER (Berlin, Germany). The column used was an Aminex HPX-87H 300×7.8 mm from Bio-Rad laboratories (Hercules, CA, USA) at a temperature of 55 °C, and the mobile phase a solution of 2.5 mM sulfuric acid at a flow-rate of 0.5 mL/min. To avoid molecules of interest to get embedded into the tail formed by anthocyanins with this analytical method, samples were diluted five-fold, and syringe-filtered (with a 0.45 µm cellulose acetate filter) before injection (20 µL).

2.7. GC-FID analysis for the identification and the quantification of higher alcohols, acetaldehyde and methanol

A wine sample of 5 mL was mixed with 50 µL of 4-methyl-2-pentanol at 10 g/L (as internal standard) from Merck (Darmstadt, Germany), 0,5 g of Na_2SO_4 (for dehydration) and a magnetic stirring bar in a 15 mL glass vial. The vial was tightly capped with a PTFE-silicone septum (Supelco, Bellefonte, PA, USA).

The solution was incubated in a water bath at 40 °C for 30 min under continuous stirring and extracted with a solid phase microextraction (SPME) fiber assembly Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS) needle size 24 ga (Supelco, USA) by exposition to the headspace (HS). After extraction, the fiber was removed from the vial and the sample manually injected (1 µL) in a Varian C*P*-8410 Auto-Injector from Agilent Technologies (Santa Clara, CA, USA) for 10 min at 220 °C in split mode (30 mL/min). The identification and quantification of higher alcohols, acetaldehyde and methanol was conducted on a Varian 3900 Gas Chromatograph System with flame ionization detector (GC-FID). Chromatographic separations were done by using a C*P*-Wax 57CB column (Agilent Technologies, USA) with the following characteristics: 50 m length, 0.25 mm internal diameter and 0.2 µm stationary phase film thickness. The carrier gas was hydrogen at a flow rate of 1.2 mL/min. The column oven temperature program was: initial temperature 40 °C for 5 min, 40 °C to 80 °C at a rate of 3 °C/min, 80 °C to 200 °C at a rate of 15 °C/min, and then held for 10 min. The total run time was 36 min. Each sample was extracted twice, and each extract injected once. The volatile compounds were identified according to the retention times of known standards (purities ≥ 98%): acetaldehyde (ethanal), 1-propanol (n-propyl alcohol), 1-butanol, isobutanol (2-methyl-1-propanol or isobutyl alcohol), 2-methyl-1-butanol (active amyl alcohol), isoamyl alcohol (3-methyl-1-butanol) and methanol from Sigma-Aldrich (Steinheim, Germany). The concentrations were calculated according to the internal standard method.

2.8. Statistical analysis

In order to show the changes in wine composition during the experiments, all the assays were performed with a minimum of two replicates.

Each sample was analyzed once for its L-malic acid concentration with the L-malic acid essay kit from Megazyme (Bray, Ireland), once for its sugars, organic acids and phenolic compounds´ contents by HPLC and twice for its volatile compounds' composition by GC. The reproducibility of the non-volatiles' analyses was assured by repeating the samples at the beginning, middle and end of each analytical session, and in all cases the obtained values were similar (Measures of dispersion: < 0.8 mg/L for L-malic acid analyzed with Megazyme kit; < 0.05 g/L for sugars and organic acids analyzed by HPLC-UV-RI and between 0.003 and 1 mg/L depending on the type of phenolic analyzed by HPLC-DAD).

Protein concentration was analyzed by the Bradford method (Martina and Vojtech, 2015) and performed in triplicate on each sample. Microbial counts were done in triplicate for each experimental trial.

Data were subjected to statistical analysis using JMP13 for Windows XP (Taikoo Shing, Hong Kong, China), at a confidence level of 95% ($p = 0.05$). One-way analysis of variance (ANOVA) was used to test the effect of the type of MLF (non-inoculated and inoculated) on microbial and chemical parameters. Dunnett´s test was run to compare the means of each sample relatively to the controls while Tukey-Kramer HSD (honestly significant differences) test was run to compare yield values between samples of different sizes.

3. Microbial and chemical diversity of wines from different European countries

3.1. Abstract

Wine is a complex biological and chemical matrix, the product of microbial interactions and mechanisms playing a critical role in the aroma profile of the wine and thus in its quality. Although widely studied, a lot remains to be explored and understood in the wine interactions. In this work, high-throughput sequencing (HTS) tools of metabarcoding and chromatography techniques were used to investigate the microbial diversity and the chemical composition of 16 post-malolactic wines with different origins. Potential recurrent genetic patterns and apparent correlations between microbiota and metabolomes of wines were of particularly interest. *Saccharomyces cerevisiae* was by far the predominant yeast in all wines analyzed. A differential abundance of non-Saccharomyces was noticeable among wines, with *Lachancea thermotolerans* and *Torulaspora delbrueckii* being the major common species. Rhodospirillales, principally characterized by the acetic acid bacteria from the genera *Acetobacter*, *Gluconobacter* and *Swaminathania*, was the main bacterial order detected in most of the samples, highlighting possible contaminations. Lactobacillales, which may strongly correspond to the malolactic fermentation starter *Oenococcus oeni*, was the second most abundant bacterial order in the wines. Rare family taxa as *Enterobacteriaceae*, *Cellulomonadaceae*, *Sphingomonadaceae*, *Pseudomonadaceae* and *Methylobacteriaceae* were also observed in some of the wines. The analyze of metabolites showed that the red and white wines tested in this study were grouped separately. The analysis of non-volatile compounds revealed that Portuguese and French/Spanish wines tended to form two distinct clusters, the first one characterized by higher content in hydroxycinnamic acids (HCA) and derivatives, and the second one by higher concentrations in anthocyanins and flavan-3-ols. The Principal Component Analysis (PCA) of the volatile fraction showed French and Spanish wines grouped again whereas Portuguese wines were separated in three different groups. Lastly, no particular correlations were observed between microbiota and metabolomes of the post-malolactic wines. Although metabarcoding techniques can improve our knowledge of the wine microbiome, other molecular or enzymatic tools are still necessary to explore its potential.

3.2. Introduction

Many factors from the vine to bottling influence the final quality and appreciation of a wine, starting with the involvement of numerous microorganisms metabolizing wine flavor and aroma along the process.

The vinification process is largely governed by the fermentations and the microorganisms responsible for it with *Saccharomyces cerevisiae* and *Oenococcus oeni* being the predominant species leading the

alcoholic (AF) and malolactic (MLF) fermentations, respectively. The fermentations can be conducted by the grape and wine environment microbiota (spontaneous) or controlled by the addition of chosen strains with sought-after characteristics (inoculated with starter cultures). Various yeast species intervene in the spontaneous AF and the specific nature of these will vary along with the increasing ethanol content. The grape genera *Candida and Hanseniaspora* are usually predominant in the early stages of the AF, followed by *Pichia* and *Metschnikowia* in the middle stage (del Carmen Portillo and Mas, 2016b; Sternes *et al.*, 2017). Fungal diversity of wines begins to decline and die off by mid-fermentation, leaving the place to the ethanol-resistant yeast *S. cerevisiae* which completes the fermentation (Constantí *et al.*, 1997; Sirén *et al.*, 2019). *Torulaspora delbrueckii* and *Lachancea thermotolerans*, present on the grape berry surface, are less tolerant to the low available oxygen conditions encountered during maceration than *S. cerevisiae* (Holm Hansen *et al.*, 2001). In some cases in which they are present at high concentrations in the initial population, *Lachancea, Starmerella*, and *Schizosaccharomyces* genera can persist until the end of fermentation (Pinto *et al.*, 2015; Wang *et al.*, 2015). The interactions and metabolic activities of each yeast species and strain contribute to the final wine composition in flavor compounds (Stefanini *et al.*, 2016). Possible sluggish fermentation can occur as the result of competition for nutrients between *Saccharomyces* and non-*Saccharomyces* yeasts (Fugelsang and Edwards, 2007; Medina *et al.*, 2012; Oro *et al.*, 2014; Taillandier *et al.*, 2014). Nevertheless, co-inoculation of specific *Saccharomyces* and non-*Saccharomyces* strains is often use in wineries to enhance the quality and improve the complexity of the wine. When co-inoculated with *S. cerevisiae*, yeasts as *H. vinae, St. bacillaris, Lach. thermotolerans, Pi. kluyveri*, *T. delbrueckii*, *C. cantarellii*, *M. pulcherrima* and *S. uvarum* can provide distinct organoleptic features to the wine (Toro and Vazquez, 2002; Maturano *et al.*, 2012; Gobbi *et al.*, 2013; Taillandier *et al.*, 2014; Varela *et al.*, 2016; Comitini *et al.*, 2017). Lactic acid bacteria (LAB) dominate MLF and essentially degrade L-malic and citric acids into L-lactic and acetic acids respectively. Residual sugars can be metabolized into lactic acid or lactic acid, carbon dioxide and ethanol or acetic acid via the fermentative pathways of LAB (Moreno-Arribas and Polo, 2009). Like the yeasts during AF, LAB can interact among themselves and in some cases cause compositional changes which result in a range of spoilage conditions (Lonvaud-Funel, 1999; Costello *et al.*, 2001; Liu, 2002; Lonvaud-Funel, 2002; Fernández and de Nadra, 2006). *Oenococcus oeni* strains are still the most commercialized starters nowadays. Although, with their tolerance to harsh wine conditions and their broad range of relevant enzymes *L. plantarum* strains are become more important on the market (du Toit *et al.*, 2011; Lerm *et al.*, 2011; Iorizzo *et al.*, 2016). Microbial diversity has been generally considered as relatively constant after the completion of the MLF (Bokulich *et al.*, 2016). Lactic acid bacteria can remain in wine after fermentations are completed despite a shortage of nutrients and oxygen, and may contribute to its spoilage (Wibowo *et al.*, 1985; Lerm *et al.*, 2010). The most frequent contaminants found in wines after fermentations completed with the remaining LAB include strains from the yeast genera *Brettanomyces, Candida, Hanseniaspora, Pichia*, *Kluyveromyces, Schizosaccharomyces, Torulaspora, Zygosaccharomyces and Saccharomycodes* among others and the acetic acid bacteria (AAB) from the genera *Acetobacter* and *Gluconobacter* (Du

Toit and Pretorius, 2000; Loureiro and Malfeito-Ferreira, 2003; Bartowsky and Pretorius, 2009a; Boulton *et al.*, 2013; Kheir *et al.*, 2013). The faults caused by these microorganisms in wines (after fermentation) include bitterness, off-flavors, turbidity, viscosity, sediment and film formation. Sulfur dioxide (SO_2) can be added at different stages of the vinification as an effective antimicrobial agent. The quantity of SO_2 added during vinification permits the selection of specific strains to conduct fermentations and to inhibit potential proliferation of spoilage microorganisms (Grangeteau *et al.*, 2017).

With the fast evolution of genetic analyses, high-throughput sequencing approaches are broadly applied in microbiology permitting the detection of the presence of rare and with low abundance taxa. Within the wine field, HTS have already been employed to principally investigate the microbial diversity in vineyard soils, on wine-grape surfaces, grapevines, winery equipment, in grape musts and fermented wines (González *et al.*, 2005; Bokulich *et al.*, 2012; Bokulich *et al.*, 2013; Bokulich *et al.*, 2014; David *et al.*, 2014; Pinto *et al.*, 2014; Piao *et al.*, 2015; Pinto *et al.*, 2015; Setati *et al.*, 2015; Bokulich *et al.*, 2016; Hong *et al.*, 2016; Stefanini *et al.*, 2016; del Carmen Portillo *et al.*, 2016a; del Carmen Portillo and Mas, 2016b; Grangeteau *et al.*, 2017; Gobbi *et al.*, 2019). Metabarcoding techniques demonstrated the presence of rare and non-cultivable bacterial taxa including *Methylobacterium, Sphingomonas, Acinetobacter, Pseudomonas, Wolbachia*, and *Paracoccus*, as well as rare yeast genera including *Kazachstania, Schizosaccharomyces*, and *Debaryomyces* in the vineyard environment and throughout wine fermentation (Bokulich *et al.*, 2012; David *et al.*, 2014; Piao *et al.*, 2015; Pinto *et al.*, 2015; Setati *et al.*, 2015; del Carmen Portillo and Mas, 2016b; Grangeteau *et al.*, 2017).

Microorganisms possess enzymes capable of hydrolyzing or otherwise catabolizing wine constituents (carbohydrates, proteins, peptides and lipids) into aroma precursors which can further be converted into a variety of aroma compounds (Swiegers *et al.*, 2005a). The volatile fraction of wine that can be attributed to microbial activity is predominantly derived from AF and contains among others ethanol, acetaldehyde, higher alcohols and their acetates, as well as volatile fatty acids (VFA) and their ethyl esters (Liu and Pilone, 2000; Styger *et al.*, 2011; Knight *et al.*, 2015). Lactic acid bacteria can also generate new volatile compounds from non-volatile grape constituents such as residual sugars and amino acids, can transform pre-MLF volatile compounds and absorb others on their cell walls (Davis *et al.*, 1988; Laurent *et al.*, 1994; Bartowsky and Henschke, 1995; Maicas *et al.*, 1999; Liu, 2002; Ugliano *et al.*, 2003; Swiegers *et al.*, 2005b; Hernandez-Orte *et al.*, 2009; Malherbe *et al.*, 2012; Costello *et al.*, 2013). Specific volatile compounds can contribute to the aroma perception of wines when their concentrations are above their threshold values (Appendix 3). However, by additive or synergic effect among compounds with similar aroma nature, a volatile compound with an odor activity value (OAV) below one can still contribute to the final aroma of a wine (Vilanova *et al.*, 2012).

Phenolic compounds in wine come mainly from the grape and their concentrations depend on the pre-fermentations techniques (Anastasiadi *et al.*, 2010; Van der Merwe *et al.*, 2011; Olejar *et al.*, 2015; Setford *et al.*, 2017). These metabolites participate to the overall organoleptic profile of the wine and may be used as precursors for the production of volatiles (Dias *et al.*, 2003b; Hernández *et al.*, 2006; Harris *et al.*, 2010; Bouzanquet *et al.*, 2012; Zhang *et al.*, 2016). The phenolics are also well known to

behave as activators or inhibitors of microbial growth and metabolism depending on their chemical structures and concentrations (Rozès *et al.*, 2003; Campos *et al.*, 2009b).

In this study, we combined metabarcoding sequencing and metabolomic analysis in order to determine if a constant pattern existed in the microbial and metabolites compositions of 16 post-fermented wines originated from different areas and produced with different technical processes. This experiment was also conducted to assess whether the wines microbiota and metabolomes of the 16 wines were correlated or distinct.

3.3. Materials and methods

3.3.1. Sampling

Six Portuguese red wines, three French red wines, three Spanish red wines and four German white wines elaborated in 2015 in wineries with different grape varieties, with or without addition of SO_2 and which went through inoculated or non-inoculated fermentations, were collected after bottling (Table 3.1.). Only the red wines went through the malolactic fermentation.

Table 3.1.- Post-malolactic wines.

Wine	Type of wine	Grape variety	Country	Region	Winery	SO_2 addition	Alcoholic fermentation	Malolactic fermentation
P1	Red	Touriga Nacional	Portugal	Alentejo	Herdade das servas	No	-	-
P2	Red	Touriga Nacional	Portugal	Ribatejo	Falua	No	-	-
P3	Red	Touriga Nacional	Portugal	Douro	L. Feitoria	No	-	Spontaneous
P4	Red	Tinta roriz	Portugal	Douro	L. Feitoria	No	-	Spontaneous
P5	Red	Alicante Boushei	Portugal	Alentejo	-	After MLF	-	-
P6	Red	Touriga Nacional	Portugal	Alentejo	-	After MLF	-	-
F1	Red	Merlot	France	Bordeaux	Boudon	-	Inoculated *S. cerevisiae* / *M. pulcherrima* Primaflora® VR BIO (Oenolia, Paris, France)	Spontaneous
F2	Red	Presse Merlot with dominance Cabernet Sauvignon	France	Bordeaux	Boudon	-	Spontaneous	Spontaneous
F3	Red	Cabernet Sauvignon bio	France	Bordeaux	Château de Lavison	After MLF	Inoculated *S. cerevisiae* ZYMAFLORE® 011 BIO (Laffort, Bordeaux, France)	Spontaneous
S1	Red	Grenache	Spain	-	-	Before AF: 6 ppm free + 45 ppm total	Inoculated *S. cerevisiae* ZYMAFLORE® RX60 (Laffort, France)	Inoculated *O. oeni* Lalvin VP41™ (Lallemand, Montreal, QC, Canada)
S2	Red	Tempranillo	Spain	-	-	Before MLF: 30 ppm free + 60 ppm total	Inoculated *S. cerevisiae* Lalvin 71B™ (Lallemand, Canada)	Spontaneous
S3	Red	Graciano	Spain	-	-	Before MLF: 25 ppm free + 50 ppm total	Spontaneous	Spontaneous

G1	White	Riesling	Germany	Mußbach	Staatsweingut	No	Spontaneous	No MLF
G2	White	Riesling	Germany	Gimmeldingen	A. Christmann	No	Spontaneous	No MLF
G3	White	Riesling	Germany	Deidesheim	Basserman-Jordan	No	Spontaneous	No MLF
G4	White	Riesling	Germany	Kindenheim	Axel Neiss	No	Spontaneous	No MLF

- No data

3.3.2. DNA extraction

Each bottle of wine was gently swirled before being poured into three 50 mL sterile Falcon tubes. Cells were pelleted by centrifugation at 4500 g for 10 min and subsequently washed three times with 10 mL of ice-cold phosphate buffered saline (PBS).

Fifty milligrams of pellet were mixed with 100 µL DNA/RNA free water in a 2 mL tube containing G2 DNA/RNA enhancer® and 0.1 mm beads (Ampliqon, Odense, Denmark), and incubated at room temperature (RT) for 2 min. The G2 DNA/RNA enhancer® is a commercial product capable of improving the amount of DNA recovered after the lysis step without introducing bias that can influence the resulting microbial community composition (Gobbi *et al.*, 2019). One milliliter of the lysis buffer (20 mM Tris-HCl- pH 8.0, 2 mM EDTA and 40 mg/ml lysozyme) was next added to the tube and incubated at 37 °C for one hour. The solution was then transferred in a new sterile 2 mL tube without the beads, and mixed with 1 mL CTAB/PVP lysis buffer (2% CTAB, 2% PVP, 1.4 mole NaCl, 1% 2-mercaptoethanol, 10 mM Tris-HCl- pH 8.0, 20 mM EDTA, 0.05% Teen-20 and 10% Proteinase K). After incubation at 65 °C for another hour, the solution was centrifugated at 4500 g for 3 min. One milliliter of the supernatant was purified with an equal volume of phenol-chloroform in a third 2 mL sterile tube, incubated at RT for 5 min and centrifugated at 1600 g for 10 min. The aqueous upper phase was shifted to a 15 mL sterile tube and the DNA extract was further purified with a MinElute PCR Purification kit and the QIAvac 24 plus (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA was finally eluted in 105 µL buffer EB (10 mM Tris·Cl, pH 8.5), incubated at 37 °C for 15 min and centrifugated at 13000 g for a minute. DNA extracts were quantified using a Qubit 1.0 fluorometer with dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Sample DNA concentrations obtained by this method were in the 1-10 ng/µL range.

3.3.3. Quantitative real-time PCRs

Quantitative real-time PCRs (qPCRs) were used before metabarcoding PCRs to detect any PCR inhibitors, and to determine the number of PCR cycles required. For both qPCR and metabarcoding, we used fusion primers ITS7_F and ITS4_R targeting the fungal internal transcribed spacer 2 region (ITS2), and 341F/806R primers targeting the V3-V4 domain of the bacterial 16S rDNA gene (Table 1.1). An exclusive 8 base pairs (bp) multiplex identifier tag (MIDtag) and MiSeq sequencing adapters were already contained into the fusion primers. The primers 341F/806R were also completed with adapters for Illumina MiSeq sequencing.

Each ITS2 qPCR reaction contained 2 µL of template and 23 µL of mastermix with 14.5 µL AccuGene molecular biology water (Lonza, Basel, Switzerland), 2.5 µL GeneAmp 10X PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$ (Applied Biosystems), 0.25 mM dNTPs, 1 µL SYBR Green (Invitrogen, Carlsbad, CA, USA), 0.25 µL AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1 µL of each forward and reverse ITS fusion primers (10 µM).

Each 16S qPCR reaction contained 1 µL of template, 18 µL AccuGene molecular biology water (Lonza), 2.5 µL GeneAmp 10X PCR Buffer II (Applied Biosystems), 1 µL SYBR Green (Invitrogen), 0.5 µL AccuPrime™ *Pfx* DNA Polymerase (Thermo Fisher Scientific), and 1 µL of each forward and reverse primers (10 µM).

PCRs were performed using a MX3005 qPCR machine (Agilent Technologies, Santa Clara, CA, USA). The qPCR cycling conditions included initial denaturation at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and an extension at 68 °C for 40 s.

3.3.4. Library preparation and sequencings

The ITS2 metabarcoding PCRs were carried out in an AB 2720 Thermal cycler (Applied Biosystems), and were based on the same mastermix as the one used in the qPCR, except for replacing 1 µL SYBR Green with 1 µL AccuGene molecular biology water. Primers with different 8 bp multiplex identifier tags were combined to index the samples. The cycling conditions included initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s, with a final extension performed at 72 °C for 10 min. PCR products were visualized with negative controls by electrophoresis using 2% agarose gels. The quality and quantity of the libraries were measured on the Bioanalyzer 2100 (Agilent technologies). The libraries were pooled at equimolar concentration (10 ng) and purified with QiaQuick columns (Qiagen) following the manufacturer's protocol to remove primer dimers.

The 16S amplicon library preparation was performed by a two-step PCR process. The 16S metabarcoding PCRs were carried out in a Veriti Thermal Cycler (Applied Biosystems). The first PCRs contained 5 µL of template, 1.5 µL of sterile water, 0.5 µL of 10 mg/mL bovine serum albumin (BSA), 12 µL of AccuPrime™ SuperMix II (Thermo Fisher Scientific) and 0.5 µL of each forward and reverse primers (10 µM). The first PCRs cycling conditions were a pre-incubation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 68 °C for 40 s, with a final extension performed at 68 °C for 4 min. The second PCRs permitted to add dual index barcodes to the samples. Amplifications were performed with 12 µL of AccuPrime™ SuperMix II (Thermo Fisher Scientific), 2 µL of primers complete with indexes and P7/P5 ends, 7 µL of sterile water and 5 µL of the first PCR products. The cycling conditions included initial denaturation at 98 °C for 1 min, followed by 13 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 20 s, and extension at 68 °C for 40 s, with a final extension performed at 68 °C for 5 min. The PCR products were then cleaned-up with HighPrep™ PCR reagent (MAGBIO, Lausanne, Switzerland) to remove primer dimers. The PCR products were visualized with negative controls by electrophoresis using 1.5% agarose gels and pooled together in an equimolar amount (10 ng).

The Amplicon pools were sequenced on Illumina MiSeq instrument in 250 bp paired-end mode.

3.3.5. Sequencing Data Analysis

3.3.5.1. Yeasts

Raw reads were first merged and demultiplexed using vsearch v2.1.2 (Rognes *et al.*, 2016), and then cleaned with cutadapt v1.11 (Martin, 2011) to remove adapter sequences and low-quality bases. Sequencing data were next analyzed and visualized with the Quantitative Insight Into Microbial Ecology (QIIME) v1.9.1 open-source software package (Caporaso *et al.*, 2010). Reads smaller than 300 bp were trimmed with vsearch, followed by dereplication. Usearch v9.0.2132 (Edgar, 2010) was used to filter singletons, cluster the reads to operational taxonomic units (OTUs) with the UPARSE-OTU algorithm and map back the reads to OTUs (including singletons) with 97% similarity. The OTU table was created with Python 3.6.0 and converted into BIOM format for assigning taxonomy in the next step. OTUs were aligned with the reference UNITE+INSD database released on 2017.12.01 (UNITE Community 2017) for taxonomic assignment to species level with a dynamic use of clustering thresholds. The OTU table was finally filtered by removing the plant OTUs from the analysis and all OTUS that had no taxonomic assignment (labeled as “No blast hit.”). OTUs with fewer than 10 reads and samples with fewer than 1000 reads were also discarded according to previously used criteria (Werner *et al.*, 2012; Oliver *et al.*, 2015).

3.3.5.2. Bacteria

The 16S amplicon metabarcoding sequence analyses were also processed with QIIME following the bioinformatics protocol described in Gobbi *et al.* (2019). OTUs clustering and taxonomic assignment were performed with a reference database and 99% of similarity. To identify the bacterial diversity ~460 bp fragment of the 16S rRNA variable region V3-V4 was amplified using the specific bacterial primer set 341F/806R. Due to their homology with the region V4 of 16S rDNA gene, wine chloroplast and mitochondrial DNA were amplified with these V3-V4 region primers and thus caused contaminating sequences (Beckers *et al.* 2016). Therefore, OTUs corresponding to chloroplasts and mitochondria were removed from the analysis. OTUs with no taxonomic assignment to family level in QIIME were also removed.

3.3.6. Analysis of non-volatile compounds

The analyses of phenolic compounds, sugars and organic acids in this chapter were done using the same HPLC methods described in sections 2.5. and 2.6. respectively.

3.3.7. Analysis of volatile compounds

Acetaldehyde (ethanal), 1-propanol (n-propyl alcohol), 1-butanol, isobutanol (2-methyl-1-propanol or isobutyl alcohol), 2-methyl-1-butanol (active amyl alcohol), isoamyl alcohol (3-methyl-1-butanol), methanol and ethyl acetate were analyzed by GC-FID as described in section 2.7.

For the analysis of other volatile compounds, 5 mL of sample wine was mixed with 1 g of Na_2SO_4 (for dehydration) and 20 µL of 3-octanol (Sigma-Aldrich, Steinheim, Germany) at 50 mg/L (internal standard) in a 15 mL glass vial sealed with a PTFE-silicone septum (Supelco, Bellefonte, PA, USA). The sample was pre-incubated 5 min at 40°C, and the headspace (HS) extracted by solid phase microextraction (SPME) with a Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS) 50/30 mm fiber from Supelco for five more minutes at the same temperature. After extraction, the wine samples were injected in split/splitless mode (30 s, 30 mL/min) through an automatic injector set at 220 °C and analyzed with a Varian C*P*-450 GC gas chromatograph coupled to a Varian 240 MS mass spectrometer (Agilent Technologies). Chromatographic separations were done by using a FactorFour VF-WAXms column (Agilent Technologies) with the following characteristics: 15 m length, 0.15 mm intern diameter, 0.15 µm film thickness. The carrier gas was helium at a flow rate of 1 mL/min. The column oven temperature program was: initial temperature 40 °C for 1 min, 40 °C to 220 °C at a rate of 4 °C/min, then held for 2.5 min and the total run time was 48.5 min. Mass spectral data were collected over a range of 33 to 150 m/z in full-scan mode. The identification of volatile compounds was achieved on the basis of their mass spectra and linear retention index. Mass spectrometric information of each chromatographic peak was compared to the NIST 14 mass spectral library. The concentrations of the volatile compounds were calculated by the internal standard method and the analyses were performed in duplicate. The following wine volatile compounds were monitored: esters (ethyl butyrate [ethyl butanoate], ethyl hexanoate, ethyl heptanoate, ethyl octanoate, ethyl nonanoate, ethyl decanoate, ethyl-2-furoate, diethyl succinate, isoamyl acetate, isoamyl octanoate, hexyl acetate, 2-phenylethyl-acetate, isobutyl acetate, linalyl acetate and ethyl lactate), alcohols (1-hexanol, *trans*- and *cis*-3-hexenol, *trans*-2-hexenol, benzyl alcohol, 2-phenylethanol, furfuryl alcohol, vanillyl alcohol and methionol), terpenes (linalool, α-terpineol, β-citronellol, nerol and geraniol), volatile fatty acids (isobutyric, isovaleric, hexanoic, octanoic, decanoic, valeric, dodecanoic and butyric acids), volatile phenols (4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol, 4-vinylphenol [4-VP], 4-ethylcatechol, vanillin, eugenol, isoeugenol and guaiacol), carbonyl compounds (benzaldehyde, 2-phenylacetaldehyde, cinnamaldehyde, sinapaldehyde, hydroxybenzaldehyde, coniferaldehyde, syringaldehyde, α-ionone, β-ionone, 2-furfural and 5-methyl-furfural) and sulfur compounds (dimethyl disulfide, methional and benzothiazole).

3.3.8. Statistics

The statistical methods used for the data analysis were: one-way analysis of variance (ANOVA) to determine the minimum significant difference ($p < 0.05$) in the DNA extracted quantity and the microbial biodiversity of the 16 analyzed wines and Principal Component Analysis to explore the relationship between analyzed metabolites and between samples.

The JMP14 program for Windows XP (Taikoo Shing, Hong Kong) was used for data processing.

3.4. Results and Discussion

The main objective of the present study was to analyze the microbial and chemical compositions of 16 bottled wines from different countries to evaluate their potential similarities and differences.

3.4.1. Metabarcoding

To determine the fungal and bacterial communities' diversities of the wines, metabarcoding techniques were performed by amplifying the ITS2 and 16S genes.

The DNA quantity measured *via* Qubit after extraction was lower than 50 ng/mL for the samples S1 and G1. As seen in figure 3.1., the Portuguese wines P1 and P3 had the greatest DNA yields compared to the other samples. In the contrary, P2, G4, the French wines and the Spanish wines seemed to possess the least quantity in DNA after the extraction protocol application.

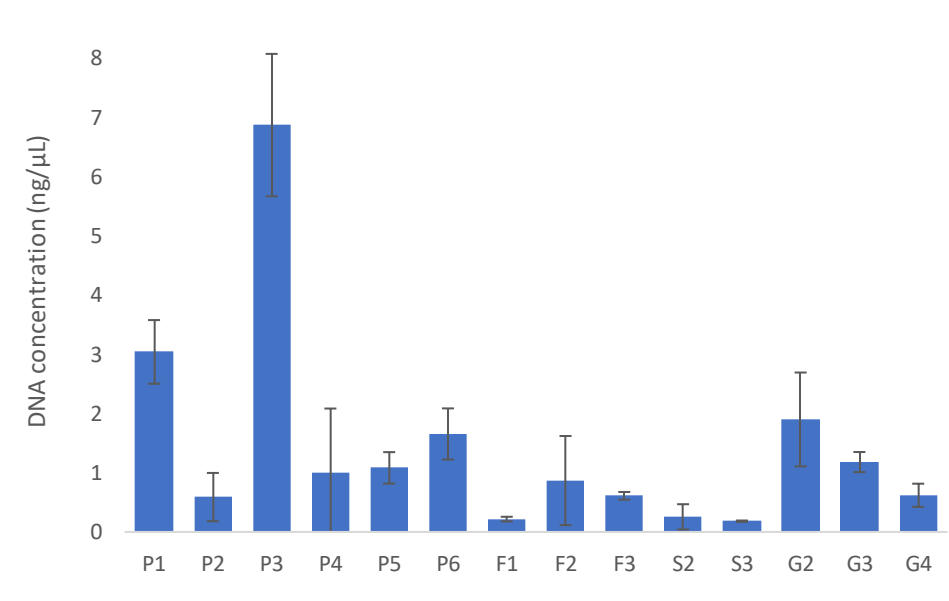


Figure 3.1. - Qubit quantification after DNA extraction (ng/µL). Error bars represent the standard deviation of three determinations. One-way ANOVA with *p*-value < 0.0001.

In total, 7.80 million reads were generated for ITS2 sequencing, yielding 67 OTUs. After all filtering this was reduced to 6.87 million reads representing only 25 OTUs retained for subsequent analyses.

For 16S amplicon sequencing, samples P1, S2, G2, G3 and G4 contained a very low amount of good quality reads due to their poor quantity in amplified 16S rDNA genes. Therefore, these samples were discarded for the bacterial diversity analysis. After chloroplasts, mitochondria and unknown families´ removal, we obtained 16.33 thousands reads clustered in 411 OTUs for the eleven remained samples.

Analysis of OTUs profiles suggests that fungal community richness was higher for the samples P2 and S1, and lower for P1, G2 and G3 (Table 3.2.). The bacterial community richness was greater for F2 and F3, and lower for the samples P3 and P6. Although their poor DNA extract quantities (Figure 3.1.), the

wines P2 and S1 represented the greatest richness in fungal OTUs and the wines F2 and F3 the greatest richness in bacterial OTUs. In a similar way, the Portuguese wines P1 and P3 had the highest quantities in DNA extract, but the lowest fungal and bacterial OTUs richness for P1 and the lowest bacterial richness for P3. This observation could be explained by a potential presence of contaminants or / and non-microbial DNA in the wines P1 and P3.

In all the analyzed samples, more than 98 % of the identified yeasts belonged to the genus *Saccharomyces*.

A compositional bar chart of the non-*Saccharomyces* community at species resolution level (Figure 3.2.a) shows that although the relative abundances varied across wines, *Lachancea thermotolerans* was the predominant non-*Saccharomyces* species in the majority of the samples except for P1, F1, F3, G1 and G4. *Lach. thermotolerans* is an aerobic fungal species usually found on grape surfaces and in some cases able to persist until the end of the alcoholic fermentation (Bokulich *et al.*, 2014; Pinto *et al.*, 2015). In co-inoculation with *S. cerevisiae*, *Lach. thermotolerans* can enhance wine acidity and levels of in 2-phenylethanol and glycerol (Gobbi *et al.*, 2013). The less studied *Lach. lanzarotensis* was also relatively abundant in the Portuguese wine P2. Yeasts from the *Lachancea* genus are also known to ferment sugars to lactic acid during wine fermentation (Varela and Borneman, 2017; Porter *et al.*, 2019).

Torulaspora delbrueckii was the predominant non-*Saccharomyces* species in the Portuguese wine P1 and the second most predominant in the French wines as well as in wines P3, P4, S2, S3 and G3 (Figure 3.2.a). *T. delbrueckii* can bring desirable aroma notes (such as isoamyl acetate) and decrease volatile acidity when co-inoculated with *S. crevisiae* (Taillandier *et al.*, 2014; Comitini *et al.*, 2017). This species is the first non-*Saccharomyces* to be commercially available for use in wine production with this characteristics (Jolly *et al.*, 2014).

Portuguese wines P2 and P6 showed high levels of *Zygosaccharomyces* (Figure 3.2.a). In wine *Zygosaccharomyces* normally originate from damaged grapes and decline early in the fermentation process. Yeasts from this genus are highly resistant to low pH and usually associated with spoilage (Varela and Borneman, 2017).

The grape-associated yeast species *Curvibasidium sp, Sporobolomyces roseus* and *Cryptococcus_sp* together represented more than 40% of the total non-*Saccharomyces* community abundance in the wines F3, G1 and G4 (Figure 3.2.a). *Curvibasidium* has previously been identified in German grapes (Brysch-Herzberg and Seidel, 2015) while *Cryptococcus* is one of the genera most often found on the surface of grape berry, also being present in the winery environment, and known to have an impact on the microflora regulation on grapes and in fermentations (Fleet, 2003; Ocón *et al.*, 2013).

Rhodotorula graminis, essentially present in the German wine G2, is part of the genus *Rhodotorula* which, like *Zygosaccharomyces* and *Cryptococcus,* is supposedly greatly affected by the decrease in oxygen and increase in ethanol content in the wine during fermentation (Romano *et al.*, 2003). *Rhodotorula* and *Cryptococcus* are yeast species usually associated with grapes but also having been isolated from wine bottle corks (Fleet, 2003).

Other grape-associated yeast species such as *Kazachstania servazzii* and *Naganishia adeliensis* were only detected in G1 (Figure 3.2.a). *Kazachstania servazzii* yeast has previously been detected on Danish grapes and is known to be involved in food spoilage (Lederer *et al.*, 2013; Spanoghe *et al.*, 2017).

Schizophyllum commune was exclusively observed in the Portuguese wine P1 (Figure 3.2.a). This fungal species is a wood-decaying basidiomycete with lactate dehydrogenase activity (Okamura-Matsui *et al.*, 2001; Mirfat *et al.*, 2014).

Cystofilobasidium macerans was the predominant non-*Saccharomyces* species found in G4, also present in G1, and *Pseudohyphozyma pustula* the predominant one in F1 (Figure 3.2.a).

It is interesting to notice that the fermentative starter *Metschnikowia pulcherrima* used in the production of the French wine F1 was not detected in the respective sample.

The 16S sequencing analysis used in this study did not permit to identify bacteria further than their genus taxa. The compositional bar chart of the bacterial community made on order resolution (Figure 3.2.b) shows that Rhodospirillales was the most abundant bacterial taxa in all the wine samples except F3. Bokulich *et al.* (2012) also found Rhodospirillales to be the most dominant order of bacteria detected in fermented wines tested in the study. This order was defined by the acetic acid bacteria from the Acetobacteraceae family and more precisely from the genera *Acetobacter*, *Gluconobacter* and *Swaminathania*, highlighting a possible contamination of the samples (Figure 3.3.). Acetic acid bacteria are strictly aerobic bacteria, growing predominantly at grape (particularly overripe/rotten grapes) and wine surfaces in permanent contact with air and oxidizing ethanol through acetaldehyde to acetic acid (Joyeux *et al.*, 1984). These bacteria may also develop in bottled red wines sealed with natural cork closures and stored in a vertical upright position (Bartowsky and Henschke, 2008). The effect of SO_2 seems relevant since the wines P5, P6, F3 and S3, to which SO_2 was added during vinification, had lower *Gluconobacter* abundances than the other analyzed wines (Figure 3.3.). SO_2 was also added to the Spanish wine S3 but in lower quantity, which may explain why its concentration in Gluconobacter was higher than for P5, P6, F3 and S3. More than 20% of the *Acetobacter* analyzed in our samples were identified as *Acetobacter aceti*. According to González *et al.* (2005), *Acetobacter aceti* remains the main species detected in fermented wines. Contrary to *Acetobacter* and *Gluconobacter*, bacteria from the genus *Swaminathania* have not been previously associated with grapes or wines.

The order Lactobacillales was present in all samples studied except G1 (Figure 3.2.b). The family *Leuconostocaceae* predominated in this order, which may strongly correspond to the MLF starter *O. oeni*. No MLF starter species was found in the German white wine G1 as it was predictable. The Portuguese red wine P3 which underwent spontaneous MLF had the greatest abundance of Lactobacillales among all the wines analyzed in this study.

The order Enterobacteriales, characterized by the family *Enterobacteriaceae*, regroups with other wine spoilage-related species, usually found on the surface of grape berry (Pinto *et al.*, 2014). The Portuguese wine P4 from Douro region had by far the greatest abundance of Enterobacteriales among all the wines

analyzed in this study (Figure 3.2.b). *Enterobacteriaceae* was also the most abundant family found during alcoholic fermentation in Douro wines by Pinto *et al.* (2015).

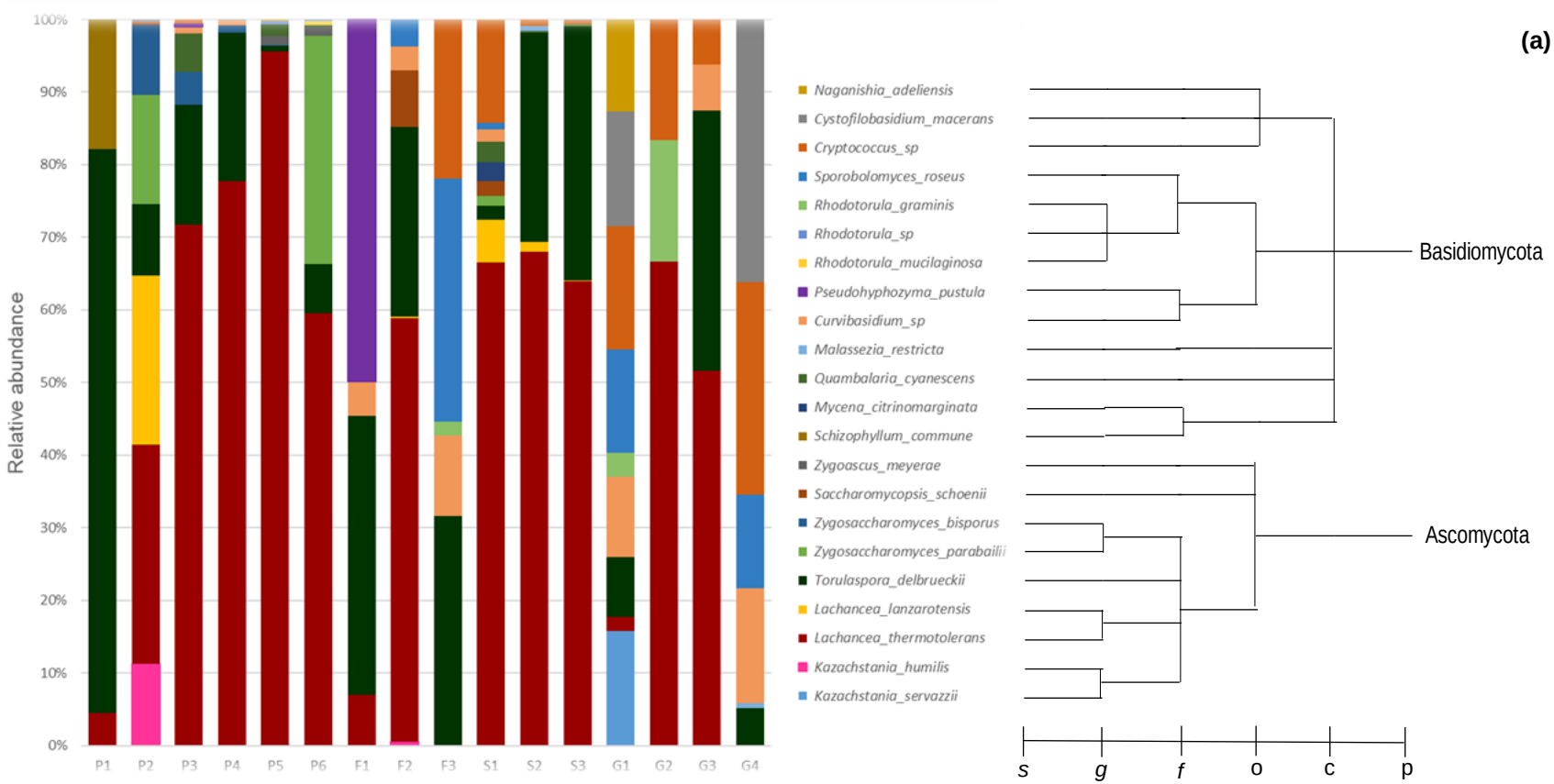
The orders Sphingomonadales, Rhizobiales and Pseudomonadales, represented mainly by the genera *Sphingomonas*, *Methylobacterium* and *Pseudomonas* respectively, were found in some of the analyzed samples (Figure 3.2.b). This bacteria were also previously found on grapes and in wines during fermentation (Piao *et al.*, 2015; del Carmen Portillo and Mas, 2016b). The relative abundance of *Sphingomonas* was higher in the wines F1, F3 and S1, with the genus *Methylobacterium* being especially relevant in samples F1, F3 and G1 and *Pseudomonas* in sample S1. In some studies (Piao *et al.*, 2015; Pinto *et al.*, 2015; Bokulich *et al.*, 2016), *Enterobacteriaceae, Sphingomonas, Methylobacterium* and *Pseudomonas* appear to increase in relative abundance during fermentation and potentially contribute to wine characteristics.

The French wine F3 also showed a noticeable abundance of the Actinomycetales and Campylobacterales orders (Figure 3.2.b). In this present study, Actinomycetales were represented principally by the family *Cellulomonadaceae* usually found in vineyard soils (Burns *et al.*, 2015) while Campylobacterales were characterized by the genus *Arcobacter* which has previously been found in fermented botrytized wine (Bokulich *et al.*, 2012).

Table 3.2. - Summary of generated filtered reads and OTUs observed per sample.

		P1	P2	P3	P4	P5	P6	F1	F2	F3	S1	S2	S3	G1	G2	G3	G4
Yeasts - ITS2	Reads	102.97 ± 60.54	430.04 ± 65.10	386.75 ± 137.16	264.32 ± 68.20	88.94 ± 34.89	143.01 ± 71.96	164.04 ± 6.05	233.45 ± 59.31	111.29 ± 18.07	174.95 ± 8.10	239.83 ± 32.99	238.52 ± 18.9	143.19 ± 44.91	295.57 ± 16.58	202.04 ± 137.26	143.39 ± 96.98
	OTUs	4 ± 1	11 ± 1	8 ± 1	8 ± 2	10 ± 1	8 ± 0	6 ± 0	7 ± 0	7 ± 1	12 ± 2	7 ± 4	8 ± 1	9 ± 1	4 ± 1	5 ± 1	6 ± 2
Bacteria - 16S	Reads	ND	0.63 ± 0.0	0.16 ± 0.0	0.35 ± 0.07	0.18 ± 0.09	0.27 ± 0.0	0.68 ± 0.38	3.64 ± 0.54	1.03 ± 0.61	0.54 ± 0.08	ND	0.47 ± 0.00	0.43 ± 0.40	ND	ND	ND
	OTUs	ND	38 ± 1	19 ± 0	32 ± 4	24 ± 4	20 ± 0	44 ± 6	113 ± 8	79 ± 17	53 ± 0	ND	39 ± 6	29 ± 11	ND	ND	ND

Unity reads: thousands. ND - no data obtained because of low quality reads. Average of triplicates ± Standard deviation (SD)


(a)
Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
P1
P2
P3
P4
P5
P6
F1
F2
F3
S1
S2
S3
G1
G2
G3
G4
Naganishia_adeliensis
Cystofilobasidium_macerans
Cryptococcus_sp
Sporobolomyces_roseus
Rhodotorula_graminis
Rhodotorula_sp
Rhodotorula_mucilaginosa
Pseudohyphozyma_pustula
Curvibasidium_sp
Malassezia_restricta
Quambalaria_cyanescens
Mycena_citrinomarginata
Schizophyllum_commune
Zygoascus_meyerae
Saccharomycopsis_schoenii
Zygosaccharomyces_bisporus
Zygosaccharomyces_parabailii
Torulaspora_delbrueckii
Lachancea_lanzarotensis
Lachancea_thermotolerans
Kazachstania_humilis
Kazachstania_servazzii
Basidiomycota
Ascomycota
s
g
f
o
c
p

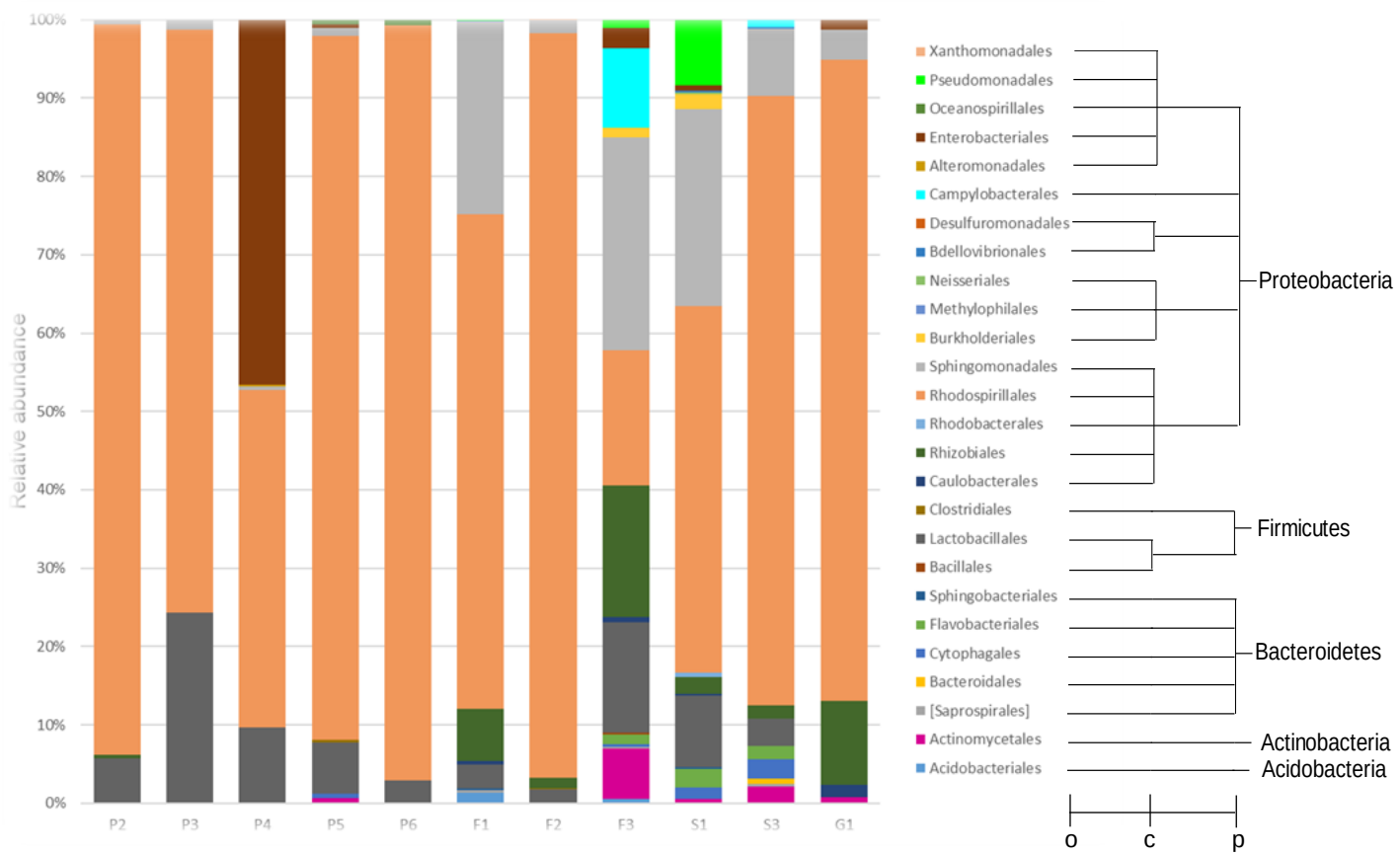


Figure 3.2.- Compositional bar charts of the microbial population associated with the post-malolactic fermentation wines. (a) species-level fungal community abundance without the genus *Saccharomyces* (non- *Saccharomyces* community), and (b) order-level bacterial community abundance. Samples P1, S2, G2, G3 and G4 were discarded for the bacterial diversity analysis as they contained very low amounts of good quality reads. Each column represents average abundance of microbial taxa detected in all 16 studied wines. p – phylum, c – class, o – order, *f* – family, *g* – genus, *s* – species.

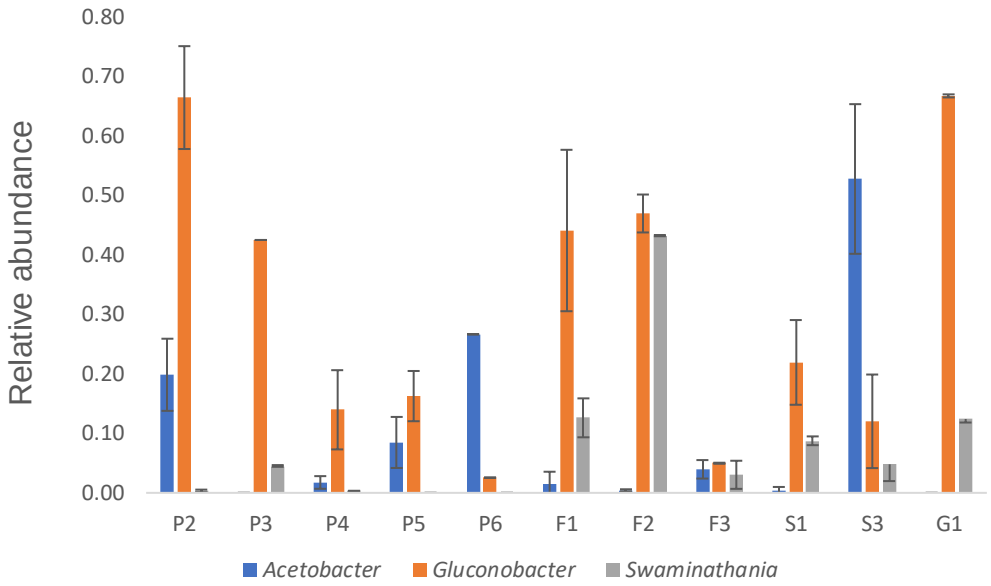


Figure 3.3. - Abundance of genera detected belonging to the bacterial order Rhodospirillales. Each column represents average relative abundance (maximum 1.0) of select bacterial taxa detected in all samples. Error bars represent the standard deviation of three determinations. One-way ANOVA with *p*-value < 0.0001. Samples P1, S2, G2, G3 and G4 were discarded for the bacterial diversity analysis as they contained very low amounts of good quality reads.

3.4.2. Chemical analysis

The metabolites compositions of the 16 studied post-malolactic wines were analyzed with HPLC-DAD and HPLC-UV-RI for the non-volatile compounds and by GC-FID and GC-MS-SPME for the volatiles.

3.4.2.1. Analysis of non-volatile compounds

The phenolic compounds with commercially unavailable standards were quantified by HPLC-DAD as malvidin-3-O-glucoside equivalent concentration for anthocyanins, *trans*-caftaric acid equivalents for (hydroxy)cinnamoyl-tartaric acids and gallic acid equivalents for ethyl gallate, using the corresponding calibration curves (Table 3.3.). Organic (citric, acetic, malic and lactic) acids, sugars (glucose and fructose), glycerol (g/L) and ethanol (%) were analyzed by HPLC-UV-RI.

The Principal Component Analysis of the main non-volatile compounds identified and quantified in the studied wines showed a clear distinction between white and red wines (Figure 3.4.). Grape pomace, is rapidly removed from the must for white wines production, explaining the absence or low content in phenolic compounds, and particularly anthocyanins and flavonols, in white wines (Singleton, 1969).

In the study presented, the Portuguese wines and the French/Spanish wines tested appeared to form two distinct clusters, the first one characterized by higher content in HCA and their derivatives, and the second one by higher concentrations in anthocyanins and flavan-3-ols (Figure 3.4.).

Comparing the red wines, several differences between samples could be noticed; flavonols occurred in significantly higher concentration in the Portuguese wine P2 (36.56 mg/L) and in lower concentrations in the Spanish wines S2 and S3 (0.82 and 0.37 mg/L respectively); the highest level of *trans*-resveratrol was observed in the French wine F1 (19.61 mg/L) and the lowest levels in the wines P1 and S2 (1.52 and 1.17 mg/L respectively); P2, P3, S1 and S3 shown the greatest concentrations in hydroxybenzoic acids (~41.31 mg/L) and the Portuguese wine P1 the lowest (13.64 mg/L).
All red wines analyzed contained acetic acid levels of 0.4-0.9 g/L except for the Portuguese wine P6 (14.1 % EtOH v/v) which had 1.26 g/L. This level indicates that acetic acid bacteria degraded the wine P6.

Table 3.3. – Phenolics compounds identification based on HPLC-DAD of 16 post-malolactic wines.

Phenolic compounds	Retention time (min)	λ_{max1}	λ_{max2}	λ_{max3}	Identification
Anthocyanins					
Delphinidin-3-glucoside	21.3	288	328	527	Oliveira *et al.* (2015); Ginjom *et al.* (2011)
Petunidin-3-glucoside	24.5	279	328	528	Oliveira *et al.* (2015); Ginjom *et al.* (2011)
Peonidin-3-glucoside	27.0	277	346	517	Oliveira *et al.* (2015); Ginjom *et al.* (2011)
Malvidin-3-glucoside	27.4	275	350	528	Available standard
Delphinidin-3-O-acetylglucoside	29.1	279	532		De Villiers *et al.* (2004)
Cyanidin-3-O-acetylglucoside	32.0	279	532		Lamuela-Raventós and Waterhouse (1994)
Petunidin-3-O-acetylglucoside	32.1	281	536		Lamuela-Raventós and Waterhouse (1994)
Peonidin-3-O-acetylglucoside	33.4	280	536		Lamuela-Raventós and Waterhouse (1994)
Malvidin-3-O-acetylglucoside	34.9	278	322	530	Oliveira *et al.* (2015); Ginjom *et al.* (2011)
trans-Peonidin-3-O-coumaroylglucoside	38.9	280	319	534	Lamuela-Raventós and Waterhouse (1994)
trans-Malvidin-3-O-coumaroylglucoside	40.6	281	317	533	Oliveira *et al.* (2015); Ginjom *et al.* (2011)
Flavonols					
Quercetin	46.8	255	370		Available standard

Kaempferol	52.6	265	366	Available standard
Hydroxycinnamic acids				
cis-Caftaric acid	17.6	276	322	Singleton *et al.* (1985)
trans-Caftaric acid	18.5	298	329	Available standard
cis-Coutaric acid	20.5	286	312	Hernandez *et al.* (2007)
trans-Caffeic acid	22.0	295	325	Available standard
trans-Coutaric acid	22.9	286	314	Oliveira *et al.* (2015)
trans-Fertaric acid	24.5	298	333	Oliveira *et al.* (2015)
trans-*p*-Coumaric acid	28.1	298	310	Available standard
trans-Ferulic acid	29.8	294	322	Available standard
Stilbenes				
trans-Resveratrol	41.4	305	320	Available standard
Hydroxybenzoic acids				
Gallic acid	7.4	272		Available standard
Protocatechuic acid	13.4	259	294	Available standard
Ethyl gallate	27.8	214	273	Ginjom *et al.* (2011), Hernandez *et al.* (2007)
Flavan-3-ols				
(+)-Catechin	18.9	279		Available standard
(-)-Epicatechin	25.7	278		Available standard

(Singleton *et al.*, 1985; Lamuela-Raventós and Waterhouse, 1994; De Villiers *et al.*, 2004; Hernandez *et al.*, 2007; Ginjom *et al.*, 2011; Oliveira *et al.*, 2015).

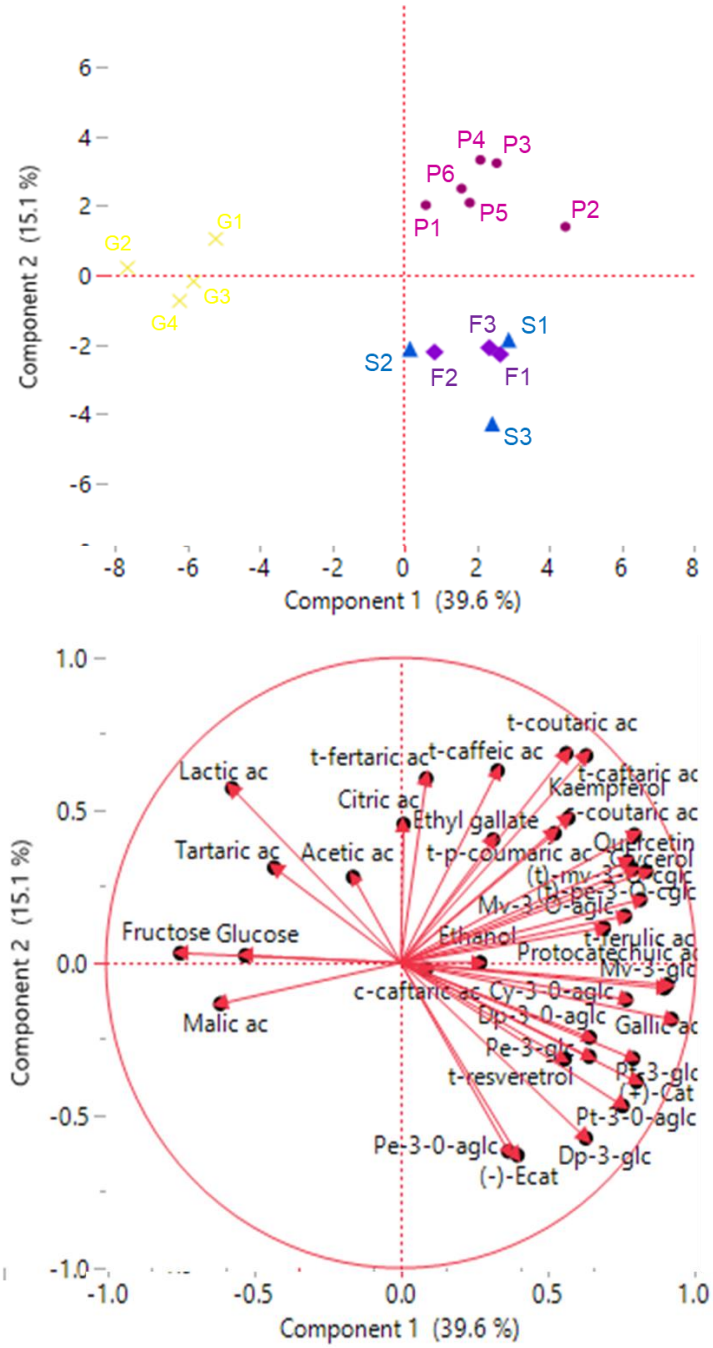


Figure 3.4.- Principal Component Analysis results of the main non-volatile compounds observed in 16 post-MLF wines. Key: dp: delphinidin; cy: cyanidin; pt: petunidin; pe: peonidin; mv: malvidin; glc: glucoside; aglc: acetyl-glucoside; cglc: coumaroyl-glucoside; *t*: *trans*; *c*: *cis*; ac: acid; cat: catechin; ecat: epicatechin.

3.4.2.2. Analysis of volatile compounds

Isobutyl acetate, ethyl lactate, linalyl acetate, butyric acid, vanillyl alcohol, cinnamaldehyde, sinapaldehyde, hydroxybenzaldehyde, coniferaldehyde, syringaldehyde, vanillin and 4-ethylcatechol were not detectable with the HS-SPME-GC-MS system used. Furfuryl alcohol, geraniol, valeric acid, dodecanoic acid, guaiacol, dimethyl-disulfide, methional, benzothiazole, 5-methyl-furfural and 2-methyl-1-propanol (isobutanol) were not detectable in the wine samples.

Principal component analysis was performed to determine the correlation between the volatile compounds analyzed and the wines studied. Components PC1 and PC2 described 54.8% of the total variance in the data and provide discriminatory information (Figure 3.5.). Four clusters were formed: (a) white German wines; (b) Portuguese wines P2, P5 and P6; (c) French wines, Spanish wines and Portuguese wines P3 and P4; (d) Portuguese wine P1.

Cluster (a) was only represented by the German white wines, distinctly grouped from the red wines. This observation agree with previously published results analyzing the volatile composition of red and white wines from different Spanish regions (Aznar and Arroyo, 2007). Cluster (a) was essentially defined by high levels of the esters ethyl hexanoate and ethyl octanoate (with fruity aromas), their corresponding volatile fatty acids hexanoic and octanoic acids (fatty and cheesy aromas), 2-phenylethylacetate (floral/fruity aroma), linalool (floral/citrus aroma), *cis*-3-hexenol (herbaceous aroma) and the volatile phenols 4-vinylphenol and 4-vinylgaiacol (clove-like and pharmaceutical aromas, respectively). The high concentrations in esters in the German wines could be explained by a lower and better controlled temperature during AF than for the other analyzed wines and by the nature of the grape used (Table 3.4.). *Cis*-3-hexenol could describe relatively unripe grapes at the moment of harvest. The high concentrations in hexanoic acid and 4-VP could be related to *Brettanomyces spp.* activity. Although no *Brettanomyces spp.* were identified by metabarcoding analysis in any of the wines (Figure 3.2.a). All these compounds may impact the German wines aroma since their concentrations were above their sensory thresholds (Table 3.4.). Although, OAV were obtained comparing with odor thresholds calculated in hydro-alcoholic solutions or other matrices (Appendix 3), which does not necessarily mean that these compounds can be perceived in these particular wines. Even though VFA are related with undesirable odors at concentrations higher than 20 mg/L, they seem to be important for the perception of fresh fruit and to mask the animal character of ethylphenols (Romano *et al.*, 2009; San-Juan *et al.*, 2011; Sánchez-Palomo *et al.*, 2017).

Cluster (b) was mainly characterized by isoamyl acetate (banana aroma), ethyl heptanoate (fruity aroma), benzyl alcohol (solvent-like aroma), nerol (rose aroma), decanoic acid (rancid aroma), eugenol (smoky aroma), isoeugenol (floral/rose aroma) and β-ionone (violet aroma), all with concentrations above their thresholds (Table 3.4.).

2-Phenylethanol and 2-phenylacetaldehyde (with floral/honey aromas) were the two main volatile compounds, with concentrations above their thresholds, distinguishing the wines belonging to the cluster

(c) from the other wines. Isovaleric acid (cheese aroma) was also found in great concentrations in the French and Spanish wines (Table 3.4.).

Most of the higher alcohols (methionol, 2-phenylethanol, active amyl alcohol and isoamyl alcohol) and eugenol/isoeugenol were odor-active compounds present in the Portuguese wine P1 (Table 3.4.). A potential correlation with the non-*Saccharomyces* community of P1 mainly composed of *T. delbrueckii and Schizophyllum commune* could explain these observation (Figure 3.2.a). Although, lower amounts of higher alcohols would be expected with the implication of *T. delbrueckii*, it is not necessarily the case with mixed populations (Belda *et al.*, 2015).

2-Phenylethanol was lower in the white wines (cluster (a)), as expected since they have fewer amino acids, but also missing in the red wines of cluster (b) (Table 3.4).

The high levels of linalool found in the Portuguese wines P1, P2, P3 and P6 corroborate previous researches about the terpenic richness of Touriga Nacional grape variety (Table 3.4) (Falqué *et al.*, 2004; De Pinho *et al.*, 2007; Symington and Rogerson, 2007).

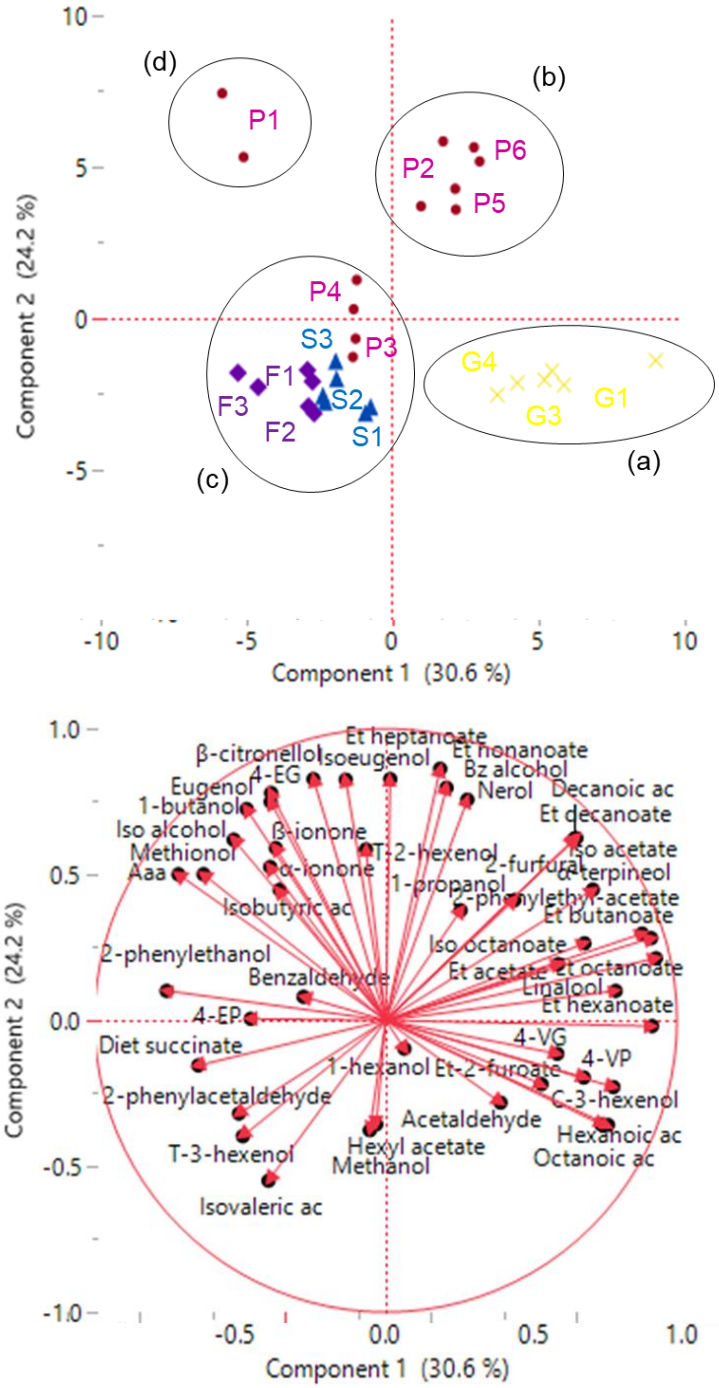


Figure 3.5. - Principal component analysis of the main volatile compounds observed in 16 post-malolactic wines. Concentrations in mg/L. Key: et: ethyl; ac: acid; 4-EP: 4-ethylphenol; 4-EG: 4-ethylguaicol; 4-VP: 4-vinylphenol; 4-VG: 4-vinylguaiacol; iso: isoamyl; diet: diethyl; bz: benzyl; Aaa: Active amyl alcohol.

Table 3.4. – Concentrations of the volatile compounds analyzed in the 16 post-malolactic wines.

Volatile compounds	Thresholds	m/z	P1	P2	P3	P4	P5	P6	F1	F2	F3	S1	S2	S3	G1	G3	G4
Esters																	
Ethyl acetate	12.27[4]		**59.59 ± 8.43**	**136.81 ± 23.22**	**130.98 ± 17.60**	**73.96 ± 10.25**	**118.43 ± 15.58**	**143.37 ± 15.21**	**103.14 ± 11.96**	**117.15 ± 16.40**	**51.79 ± 8.06**	**101.52 ± 12.92**	**50.96 ± 5.04**	**90.45 ± 15.35**	**99.55 ± 23.93**	**138.84 ± 17.48**	**115.77 ± 21.28**
Ethyl hexanoate	0.014[9]	88	**0.76 ± 0.11**	**1.42 ± 0.24**	**1.23 ± 0.17**	**1.29 ± 0.18**	**1.87 ± 0.25**	**1.51 ± 0.16**	**0.57 ± 0.07**	**0.28 ± 0.04**	**0.65 ± 0.05**	**0.73 ± 0.09**	**0.47 ± 0.05**	**1.03 ± 0.17**	**4.58 ± 1.10**	**3.58 ± 0.45**	**2.86 ± 0.53**
Ethyl octanoate	0.58[3]	88	0.32 ± 0.05	**1.16 ± 0.20**	**0.66 ± 0.09**	**0.90 ± 0.12**	**1.37 ± 0.18**	**1.55 ± 0.16**	0.05 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.20 ± 0.03	0.18 ± 0.02	0.19 ± 0.03	**2.88 ± 0.69**	**1.58 ± 0.20**	**1.59 ± 0.29**
Ethyl decanoate µg/L	200[9]	88	58.14 ± 8.22	**296.87 ± 50.38**	165.73 ± 22.27	**252.68 ± 35.02**	**309.03 ± 40.64**	**370.97 ± 39.35**	7.27 ± 0.84	3.75 ± 0.52	4.67 ± 0.73	28.38 ± 3.61	24.05 ± 2.38	24.78 ± 4.21	**245.38 ± 58.99**	168.07 ± 21.15	130.50 ± 23.99
Isoamyl acetate	0.03[9]	43	**12.89 ± 1.82**	**22.95 ± 3.90**	**6.72 ± 0.90**	**16.91 ± 2.34**	**34.18 ± 4.50**	**25.43 ± 2.70**	**4.78 ± 0.55**	**6.45 ± 0.90**	**5.57 ± 0.87**	**7.00 ± 0.89**	**6.27 ± 0.62**	**11.38 ± 1.93**	**16.64 ± 4.00**	**19.02 ± 2.39**	**23.05 ± 4.24**
Isoamyl octanoate µg/L		127	1.35 ± 0.19	4.46 ± 0.76	2.16 ± 0.29	7.35 ± 1.02	3.45 ± 0.45	2.74 ± 0.29	0.18 ± 0.02	0.12 ± 0.02	0.34 ± 0.05	0.33 ± 0.04	0.45 ± 0.04	0.45 ± 0.08	7.58 ± 1.82	3.62 ± 0.46	3.52 ± 0.65
2-phenylethyl-acetate µg/L	250[4]	43	90.09 ± 12.74	159.94 ± 27.14	31.90 ± 4.29	113.67 ± 15.75	180.91 ± 23.79	201.02 ± 21.32	16.97 ± 1.97	26.22 ± 3.67	30.82 ± 4.79	16.19 ± 2.06	42.48 ± 4.21	38.87 ± 6.60	**335.13 ± 80.57**	**251.20 ± 31.62**	165.96 ± 30.51
Diethyl succinate	200[3]	101	6.79 ±0.96	0.12 ± 0.02	10.42 ± 1.40	3.48 ±0.48	0.04 ±0.01	0.24 ±0.03	3.49 ±0.40	3.21 ± 0.45	4.67 ± 0.73	1.43 ± 0.18	5.75 ± 0.57	6.53 ± 1.11	0.96 ± 0.23	0.51 ± 0.06	1.40 ± 0.26
Ethyl butanoate	0.02[9]	88	**0.53 ± 0.08**	**1.34 ± 0.23**	**0.79 ± 0.11**	**0.55 ± 0.08**	**1.43 ± 0.19**	**1.61 ± 0.17**	**0.44 ± 0.05**	**0.32 ± 0.05**	**0.35 ± 0.05**	**0.97 ± 0.12**	**0.49 ± 0.05**	**0.84 ± 0.14**	**1.75 ± 0.42**	**1.65 ± 0.21**	**1.26 ± 0.23**
Hexyl acetate	0.7[7]	43	0.10 ± 0.01	0.03 ± 0.00	0.23 ± 0.03	0.13 ± 0.02	0.44 ± 0.06	0.02 ± 0.00	0.02 ± 0.00	0.06 ± 0.01	nd	**3.84 ± 0.49**	**6.88 ± 0.68**	**13.30 ± 2.26**	**2.23 ± 0.54**	**2.26 ± 0.28**	**2.00 ± 0.37**
Ethyl heptanoate µg/L	2.2	88	**4.09 ± 0.58**	**4.63 ± 0.79**	2.05 ± 0.28	1.10 ± 0.15	**6.31 ± 0.83**	**5.09 ± 0.54**	0.50 ± 0.06	0.60 ± 0.08	0.40 ± 0.06	1.17 ± 0.15	1.43 ± 0.14	**4.07 ± 0.69**	0.65 ± 0.16	0.25 ± 0.03	0.51 ± 0.09
Ethyl nonanoate µg/L	850	88	1.52 ± 0.21	2.04 ± 0.35	1.24 ± 0.17	1.19 ± 0.16	1.93 ± 0.25	3.25 ± 0.34	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.40 ± 0.05	0.72 ± 0.07	0.58 ± 0.10	0.59 ± 0.14	0.40 ± 0.05	0.32 ± 0.06
Ethyl-2-furoate	16[17]	112	0.07 ± 0.01	0.13 ± 0.02	0.03 ± 0.00	0.02 ± 0.00	0.07 ± 0.01	0.05 ± 0.00	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.14 ± 0.03	0.27 ± 0.03	0.58 ± 0.11

Alcohols																	
Trans-3-hexenol µg/L	400[9]	67	3.30 ± 0.47	0.41 ± 0.07	0.21 ± 0.03	1.15 ± 0.16	0.13 ± 0.02	0.31 ± 0.03	6.34 ± 0.74	51.40 ± 7.20	33.32 ± 5.18	5.58 ± 0.71	17.33 ± 1.72	6.02 ± 1.02	0.54 ± 0.13	0.35 ± 0.04	0.44 ± 0.08
Trans-2-hexenol µg/L	400[9]	67	39.85 ± 5.64	nd	15.23 ± 2.05	18.17 ± 2.52	nd	75.41 ± 8.00	nd	nd	6.68 ± 1.04	nd	nd	15.96 ± 2.71	3.64 ± 0.87	2.62 ± 0.33	3.40 ± 0.62
Benzyl alcohol	200[6]	108	9.21 ± 1.30	48.53 ± 8.24	1.18 ± 0.16	2.24 ± 0.31	31.81 ± 4.18	35.17 ± 3.73	1.99 ± 0.23	0.46 ± 0.06	0.64 ± 0.10	1.18 ± 0.15	0.26 ± 0.03	1.38 ± 0.23	0.04 ± 0.01	0.06 ± 0.01	0.13 ± 0.02
Methanol			nd	nd	nd	nd	nd	nd	nd	nd	0.98 ± 0.15	5.08 ± 0.65	nd	2.79 ± 0.47	nd	nd	2.15 ± 0.40
Cis-3-hexenol	0.4[9]	67	0.06 ± 0.01	nd	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.06 ± 0.01	nd	nd	nd	nd	0.02 ± 0.00	nd	**1.53 ± 0.37**	**0.67 ± 0.08**	**0.79 ± 0.14**
Higher alcohols																	
Methionol	1[4]	106	**27.31 ± 3.86**	**3.16 ± 0.54**	**5.86 ± 0.79**	**13.63± 1.89**	**3.19 ± 0.42**	**4.92 ± 0.52**	**6.42 ± 0.74**	**5.11 ± 0.72**	**7.52 ± 1.17**	**2.70 ± 0.34**	**3.69 ± 0.37**	**6.41 ± 1.09**	0.92 ± 0.22	0.83 ± 0.10	**1.06 ± 0.20**
1-hexanol	4[7]	56	2.75 ± 0.39	3.47 ± 0.59	2.24 ± 0.30	2.31 ± 0.32	1.11 ± 0.15	2.86 ± 0.30	2.27 ± 0.26	2.88 ± 0.40	1.69 ± 0.26	**4.24 ± 0.54**	3.91 ± 0.39	2.67 ± 0.45	**4.17 ± 1.00**	1.27 ± 0.16	2.28 ± 0.42
2-phenylethanol	14[9]	122	**384.03 ± 54.31**	1.60 ± 0.27	**67.81 ± 9.11**	**330.37 ± 31.93**	nd	nd	**169.31 ± 19.63**	**114.36 ± 16.01**	**300.67 ± 46.77**	**52.50 ± 6.68**	**78.74 ± 7.80**	**145.37 ± 24.67**	**27.82 ± 6.69**	**31.65 ± 3.98**	**34.02 ± 6.25**
1-propanol	500[7]		59.07 ± 8.35	64.29 ± 10.91	78.77 ± 10.58	37.30 ± 5.17	**694.08 ± 91.29**	114.60 ± 12.15	47.17 ± 5.47	50.01 ± 7.00	45.02 ± 7.00	114.81 ± 14.61	53.39 ± 5.29	80.41 ± 13.65	87.50 ± 21.04	73.07 ± 9.20	64.14 ± 11.79
1-butanol	150[3]		104.70 ± 14.81	103.49 ± 17.56	75.01 ± 10.08	89.29 ± 12.37	48.86 ± 6.43	86.38 ± 9.16	66.64 ± 7.73	51.72 ± 7.24	65.97 ± 10.26	34.47 ± 4.39	40.33 ± 3.99	48.91 ± 8.30	27.19 ± 6.54	19.32 ± 2.43	22.52 ± 4.14
2-methyl-1-butanol	7[10]		**151.02 ± 21.36**	**85.88 ± 14.57**	**62.65 ± 8.42**	**108.48 ± 15.03**	**51.31 ± 6.75**	**73.09 ± 7.75**	**88.40 ± 10.25**	**69.14 ± 9.68**	**124.31 ± 19.34**	**32.60 ± 4.15**	**44.33 ± 4.39**	**71.26 ± 12.09**	**27.37 ± 6.58**	**23.55 ± 2.96**	**26.85 ± 4.94**
Isoamyl alcohol	30[4]		**450.31 ± 63.68**	**293.04 ± 49.73**	**197.76 ± 26.57**	**336.30 ± 46.61**	**206.08 ± 27.10**	**274.48 ± 29.11**	**272.61 ± 31.61**	**46.42 ± 6.50**	**338.38 ± 52.64**	**163.00 ± 20.75**	**220.31 ± 21.81**	**260.16 ± 44.15**	**128.63 ± 30.93**	**133.28 ± 16.78**	**108.89 ± 20.02**
Terpenes																	
Linalool µg/L	25[9]	93	**140.33 ± 19.85**	**122.66 ± 20.82**	**63.74 ± 8.56**	15.94 ± 2.21	14.41 ± 1.90	**335.96 ± 35.63**	15.84 ± 1.84	11.56 ± 1.62	20.16 ± 3.14	20.92 ± 2.66	11.87 ± 1.18	23.41 ± 3.97	**415.13 ± 99.80**	**356.22 ± 44.84**	**367.07 ± 67.49**
β-citronellol µg/L	100[3]	67	32.41 ± 4.58	10.76 ± 1.83	5.38 ± 0.72	9.37 ± 1.30	10.96 ± 1.44	16.746 ± 1.78	3.88 ± 0.45	4.54 ± 0.63	5.95 ± 0.93	6.85 ± 0.87	4.76 ± 0.47	4.88 ± 0.83	5.83 ± 1.40	4.33 ± 0.55	4.63 ± 0.85

Nerol µg/L	300[12]	93	25.52 ± 3.61	239.51 ± 40.65	nd	nd	275.54 ± 36.24	**308.27 ± 32.70**	nd	3.12 ± 0.44	3.07 ± 0.48	nd	nd	nd	nd	nd	nd
α-terpineol µg/L	250[9]	93	52.94 ± 7.49	34.02 ± 5.77	18.20 ± 2.45	13.66 ± 1.89	52.57 ± 6.91	50.78 ± 5.39	3.31 ± 0.33	2.43 ± 0.34	3.65 ± 0.57	2.53 ± 0.32	2.55 ± 0.25	3.11 ± 0.53	54.22 ± 13.03	68.16 ± 8.58	66.60 ± 12.80
Volatile Fatty Acids																	
Isobutyric acid	0.23[9]	73	**1.94 ± 0.27**	**1.68 ± 0.29**	**0.33 ± 0.04**	**0.76 ± 0.11**	**1.78 ± 0.23**	**0.73 ± 0.08**	**1.11 ± 0.13**	**0.65 ± 0.09**	**1.21 ± 0.19**	**1.29 ± 0.16**	**1.25 ± 0.12**	**1.96 ± 0.33**	**0.52 ± 0.13**	**0.43 ± 0.05**	**0.97 ± 0.18**
Isovaleric acid	0.033[9]	60	nd	nd	nd	nd	nd	nd	**1.44 ± 0.17**	**0.52 ± 0.07**	**4.36 ± 0.68**	**2.70 ± 0.34**	**3.28 ± 0.33**	**1.93 ± 0.33**	nd	**0.29 ± 0.04**	**1.78 ± 0.33**
Hexanoic acid	0.42[9]	60	**0.71 ± 0.10**	**0.61 ± 0.10**	**0.79 ± 0.11**	**1.31 ± 0.18**	0.17 ± 0.02	**1.62 ± 0.17**	**1.37 ± 0.16**	**1.10 ± 0.16**	**0.80 ± 0.12**	**1.93 ± 0.25**	**1.22 ± 0.12**	**1.16 ± 0.20**	**6.90 ± 1.66**	**5.68 ± 0.72**	**6.87 ± 1.26**
Octanoic acid µg/L	500[9]	60	214 ± 30	60 ± 1	249 ± 33	**727 ± 101**	20 ± 0	60 ± 10	346 ± 40	306 ± 43	260 ± 40	376 ± 48	278 ± 28	254 ± 43	**3546 ± 853**	**2916 ± 367**	**3347 ± 615**
Decanoic acid	1[9]	60	0.30 ± 0.04	**1.77 ± 0.30**	0.01 ± 0.00	0.74 ± 0.10	**1.47 ± 0.19**	**1.49 ± 0.16**	0.42 ± 0.05	0.23 ± 0.03	0.18 ± 0.03	0.27 ± 0.03	0.19 ± 0.02	0.21 ± 0.04	1.00 ± 0.24	0.81 ± 0.10	0.82 ± 0.15
Volatile phenols																	
4-ethylphenol µg/L	600[7]	122	21.35 ± 3.02	22.31 ± 3.79	10.29 ± 1.38	12.36 ± 1.71	nd	4.32 ± 0.46	2.41 ± 0.28	10.05 ± 1.41	107.29 ± 16.69	3.36 ± 0.43	1.31 ± 0.13	nd	nd	nd	nd
4-vinylguaiacol µg/L	1100[5]	135	75.85 ± 10.73	51.65 ± 8.77	nd	nd	nd	nd	11.71 ± 1.36	3.98 ± 0.56	4.47 ± 0.70	nd	nd	nd	**1169.08 ± 281.06**	106.88 ± 13.45	56.34 ± 10.36
4-vinylphenol	0.18[5]	120	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**4.58 ± 1.10**	**1.41 ± 0.18**	**0.36 ± 0.07**
4-ethylguaicol µg/L	1100[7]	152	23.99 ± 3.39	14.39 ± 2.44	1.89 ± 0.25	6.94 ± 0.96	11.26 ± 1.48	4.53 ± 0.48	1.32 ± 0.15	2.45 ± 0.34	12.35 ± 1.92	nd	nd	nd	nd	nd	nd
Eugenol µg/L	6[6]	164	**50.69 ± 7.17**	**11.80 ± 2.00**	3.12 ± 0.42	4.24 ± 0.59	**13.82 ± 1.82**	**12.67 ± 1.34**	4.83 ± 0.56	2.55 ± 0.36	2.90 ± 0.45	3.99 ± 0.51	4.84 ± 0.48	**8.36 ± 1.42**	nd	nd	nd
Isoeugenol	0.006[8]	164	**0.33 ± 0.05**	**0.33 ± 0.06**	nd	nd	**0.10 ± 0.01**	**0.07 ± 0.01**	nd	nd	nd	nd	nd	nd	nd	nd	nd
Carbonyl compounds																	

Benzaldehyde	200[2]	105	0.98 ± 0.14	0.36 ± 0.06	0.15 ± 0.02	0.13 ± 0.02	0.05 ± 0.01	0.90 ± 0.10	1.12 ± 0.13	1.38 ± 0.19	0.70 ± 0.11	0.19 ± 0.02	0.06 ± 0.01	0.20 ± 0.03	0.54 ± 0.13	0.13 ± 0.02	0.55 ± 0.10
2-phenylacetaldehyde µg/L	1[2]	91	**49.66 ± 7.02**	**11.73 ± 1.99**	**24.54 ± 3.30**	**26.16 ± 3.63**	nd	**4.39 ± 0.47**	**242.66 ± 28.14**	**357.65 ± 50.07**	**270.40 ± 42.06**	**12.41 ± 1.58**	**2.48 ± 0.25**	**1.24 ± 0.21**	nd	nd	**25.46 ± 6.52**
Acetaldehyde	100[7]		nd	25.35 ± 4.30	nd	nd	21.68 ± 2.85	14.08 ± 1.49	6.22 ± 0.72	13.66 ± 1.91	15.83 ± 2.46	66.49 ± 8.46	nd	58.05 ± 9.85	42.53 ± 10.23	33.06 ± 4.16	25.83 ± 4.75
2-furfural	0.5[4]	95	0.04 ± 0.01	0.29 ± 0.05	nd	nd	0.44 ± 0.06	0.24 ± 0.03	0.13 ± 0.02	0.12 ± 0.02	0.07 ± 0.01	0.14 ± 0.02	0.08 ± 0.01	0.13 ± 0.02	0.14 ± 0.03	0.05 ± 0.01	0.23 ± 0.04
α-ionone µg/L	0.09[6]	121	**6.43 ± 0.91**	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ß-ionone µg/L	0.09[6]	177	**13.95 ± 1.97**	**1.47 ± 0.25**	nd	nd	**0.42 ± 0.05**	**0.33 ± 0.03**	nd	nd	nd	nd	nd	nd	nd	nd	nd

Concentration values in mg/L except indicated. Average of two measurements ± standard deviation. In bold, compounds with concentrations above thresholds. nd: not detected. Aroma notes are given in Appendix 3.

2 (Escudero *et al.*, 2007): Thresholds calculated in beer; 3 (Etiévant, 1991) and 7 (Swiegers *et al.*, 2005a): Thresholds calculated in wine; 4 (Guth, 1997): Thresholds calculated in 10% ethanol; 5 (Boidron *et al.*, 1988): Thresholds calculated in synthetic wine containing 12% ethanol, 8 g/L glycerol and different salts; 6 (Gomez-Miguez *et al.*, 2007) and 8 (Culleré *et al.*, 2004): Thresholds calculated in 10% water / ethanol mixture containing 5 g/L of tartaric acid at pH 3.2; 9 (Ferreira *et al.*, 2000): Thresholds determined in 11% v/v aqueous ethanol with 7 g/L glycerol and 5 g/L tartaric acid, at pH 3.4; 10 (Salo, 1970): Thresholds calculated in hydro-alcoholic solution; 12 (García-Ruiz *et al.*, 2013b); 17: (Lopez *et al.*, 2002): Thresholds calculated in 10% water / ethanol solution at pH 3.2.

3.5. Conclusion

This study demonstrates a relatively constant pattern in the microbial population of the 16 different post-malolactic wines.

For both volatiles and non-volatiles, the red and white wines studied gathered in distinct groups. This phenomenon was probably linked to the origin of the grapes and the vinification processes used (maceration time, etc.) which differ according to the type of wine produced. A possible regional classification clustering French and Spanish wines together separately from the Portuguese wines was noticeable for the analyzed chemicals although, many other factors (other than place of origin) like grape variety, grape maturity, vinification protocol, etc. could have affected the phenolics and volatiles composition of grapes and wines (Aleixandre-Tudo *et al.*, 2015; Olejar *et al.*, 2015; Stavridou *et al.*, 2016; González-Centeno *et al.*, 2017).

The aroma profile of the Portuguese wine P1 was rather different from the others as this wine contained higher concentrations in higher alcohols. A potential correlation with its particular yeast population (*T. delbrueckii and Schiz. commune*) could explain this observation. No other interesting correlations were observed between the microbiota and the metabolomes of the wines. The phenolics content in a wine is mostly influenced by pre-malolactic practices (Sacchi *et al.*, 2005; Del Llaudy *et al.*, 2008; Setford *et al.*, 2017) and the volatile fraction of wine is predominantly derived from the fermentations (Bartowsky and Henschke, 1995; Styger *et al.*, 2011). The microbial richness decreases naturally after fermentations (Bokulich, 2016). Moreover, SO_2 can be applied as additional antimicrobial agent during vinification emphasizing the decrease in the microbial diversity. The wines analyzed in this study were collected post fermentations, therefore the microorganisms responsible for the metabolites production may have already be inactive or died off.

4. Influence of the addition of phenolic compounds on microbial behavior and metabolism of lactic acid bacteria in red wines

(Publications based on this chapter: Collombel I., Campos F.M., Hogg T.A. Influence of phenolic compounds on microbial metabolism during malolactic fermentation in red wines. Submitted to OENO One.)

4.1. Abstract

Phenolic compounds are important components of wine and are known to have an impact on the physiology of wine microbes in growth medium. However, the activity of these compounds on wine microorganisms, and more specifically on wine lactic acid bacteria (LAB), has not been extensively studied in real wine conditions.

In this chapter, the influence of the addition of kaempferol, *trans*-caffeic and *trans*-caftaric acids on the microbial behavior was investigated in inoculated (*Oenococcus oeni* Oenos™ or CH35™) and non-inoculated pre-malolactic fermentation (MLF) wines and wines mixed with MRS (de Man, Rogosa & Sharpe) broth (mixed media).

All tested phenolics decreased the growth rate of LAB in non-inoculated wines. Kaempferol had a perceptible LAB inhibitory impact in non-inoculated mixed media and *trans*-caffeic acid in inoculated mixed media. The addition of kaempferol declined the yeasts´ concentration in wines inoculated with Oenos™. Kaempferol and *trans*-caffeic acid delayed the malic acid degradation and lactic acid production in most of the samples. In mixed media inoculated with Oenos™, more acetic acid was produced with the addition of these two compounds comparatively to the control. Lactic acid bacteria volatile products as diethyl succinate, ethyl octanoate and terpenes were also diminished by the addition of kaempferol and *trans*-caffeic acid. In addition, in wine, the cinnamoyl esterase activity of Oenos™ was apparently inhibited by kaempferol and *trans*-caffeic acid.

Trans-caftaric acid had a weak effect on MLF. The impact of kaempferol and *trans*-caffeic acid on microbial population and metabolism depended whether the MLF was spontaneous or induced, on the *O. oeni* strain used as starter and whether the wine was mixed with MRS medium or not.

4.2. Introduction

Malolactic fermentation is a desirable step in the vinification process of most red wines, mainly leading to microbial stability, deacidification and modification of the aroma profile of the wine (Rammelsberg *et al.*, 1990; Henick-Kling and Stoewsand, 1993; Laurent *et al.*, 1994; Mcdaniel *et al.*, 2008). This crucial

step in the winemaking process is conducted by some species of LAB, the most important of which is *Oenococcus oeni*. Lactic acid bacteria can metabolize residual grape sugars into lactic acid or lactic acid, carbon dioxide and ethanol or acetic acid via the two main fermentative pathways of LAB (Moreno-Arribas and Polo, 2009). Lactic acid bacteria also degrade malic and citric acids which consequently softens the wine (Rozès *et al*., 2003). Malolactic fermentation can be spontaneous, due to microflora present in the winery or from grape materials or be induced by inoculation with selected starters, chosen according to their technological or quality attributes. *Oenococcus oeni* strain Viniflora® Oenos™ from Christian Hansen (Hørsholm, Denmark) is probably the most commercialized and studied MLF starter nowadays. CH35™ is another *O. oeni* strain commercialized by Ch. Hansen and used in wines that have difficulties undergoing MLF due to low temperature or low pH.

Phenolic compounds are initially synthesized and located in grapes, with concentrations in wine depending on many practices employed in the winemaking process (Poussier *et al.*, 2003; Sacchi *et al.*, 2005; Castillo-Sánchez *et al.*, 2008; Del Llaudy *et al.*, 2008; Olejar *et al.*, 2015; Setford *et al.*, 2017). Kaempferol and *trans*-caffeic acid are phenolic compounds belonging respectively to the flavonols and hydroxycinnamic acids (HCA) families, mostly studied in the wine context for their antibacterial activity, damaging the bacteria membranes (Campos *et al.*, 2003; Figueiredo *et al.*, 2008; García-Ruiz *et al.*, 2009; Campos *et al.*, 2009b).

The presence of *trans*-caffeic acid has been observed to delay the metabolism of glucose, malic and citric acids in wine LAB strains, and to increase the yield of lactic and acetic acids production from glucose (Campos *et al.*, 2009a). The microbial response to exposure to HCA may be manifested as changes in membrane and enzyme compositions (Devi and Anu-Appaiah, 2018a). The previous described effects on wine microorganisms were made in modified MRS media with concentrations in kaempferol around 10 mg/L and in *trans*-caffeic acid above 100 mg/L. Hydroxycinnamic acids exist in wine mainly as their tartrate derivatives ((hydroxy)cinnamoyl-tartaric acids), with *trans*-caftaric acid being the most abundant (Ribéreau-Gayon *et al.*, 2006). During MLF, the release of *trans*-caffeic acid has been previously linked to the disappearance of *trans*-caftaric acid (Hernández *et al.*, 2006; Cabrita *et al.*, 2008). Among other LAB tested, *O. oeni* Oenos™ has been found to possess cinnamoyl esterase activity enable to cleave the ester bond of tartaric salts (hydroxy)cinnamoyl-tartaric acids releasing phenolic acids (Burns and Osborne, 2013; Chescheir *et al.*, 2015). The reported concentrations in post-MLF wines of kaempferol, *trans*-caffeic acid and *trans*-caftaric acid are respectively 2.58 – 5.40 mg/L, 1.68 – 37.96 mg/L and 0.16 – 110 mg/L (Rossouw and Marais, 2004; Hernández *et al.*, 2006; Hernandez *et al.*, 2007; Komes *et al.*, 2007; Zoechling *et al.*, 2009; Lima *et al.*, 2018).

The volatile fraction of wine is predominantly derived from alcoholic fermentation and contains ethanol, esters, alcohols, volatile fatty acids and acetaldehyde among others (Appendix 3). Through a number of activities, LAB are mainly responsible for the modifications in wine aroma and flavor during MLF, generating volatile compounds from non-volatile grape constituents such as residual sugars and amino acids, transforming pre-MLF volatile compounds and absorbing others on their cell walls (Laurent *et al.*,

1994; Bartowsky and Henschke, 1995). Lactic acid bacteria possess a substantial collection of enzymes involved in the synthesis and hydrolysis of esters (Sumby *et al.*, 2010a; Antalick *et al.*, 2012). Some LAB strains are able to release terpenes by hydrolyzing their aroma precursors (Ugliano *et al.*, 2003; Hernandez-Orte *et al.*, 2009). The changes in the volatiles composition of a wine was found to be linked to its inter- and intra-microbial species diversity (Maicas *et al.*, 1999; Malherbe *et al.*, 2012; Cappello *et al.*, 2016; Benítez-Cabello *et al.*, 2019). Through their effects on the microbial population, phenolics are known to indirectly impact the volatiles composition of wines (García-Ruiz *et al.*, 2013b; Rodríguez-Bencomo *et al.*, 2014).

The impact of phenolic extracts on the progress of MLF, on the diversity, growth, cell membrane integrity and activities of isolated LAB have recently been investigated in wine (García-Ruiz *et al.*, 2012; García-Ruiz *et al.*, 2013a; García-Ruiz *et al.*, 2013b; Chasseriaud *et al.*, 2015). However, the influences of single phenolic compounds such as kaempferol, *trans*-caffeic and *trans*-caftaric acids on the microbial population and metabolism have not been extensively studied in wine conditions. Mixing wine with modified MRS media is known to change the microbial activities (Fras *et al.*, 2014). Therefore, this chapter aims to characterize the impact of these three phenolics, added at the same concentrations in wine and in wine mixed with MRS broth, on the microorganisms and their activities during non-inoculated MLF and MLF inoculated with *O. oeni* Oenos™ and CH35™.

4.3. Materials and Methods

4.3.1. Wine

The red wine used for the experiment was obtained from the Dão region in Northern Portugal from the 2015 harvest. The main analytical parameters were as follows: 11.6 ± 0.4 % (v / v) alcohol, pH 3.70 ± 0.01, 0.906 ± 0.100 g/L malic acid and 0.694 ± 0.057 g/L lactic acid. The wine was collected before completion of malolactic fermentation and stored at 4 °C before the initiation of the experiment.

The initial concentrations in kaempferol, *trans*-caffeic and *trans*-caftaric acids in the pre-malolactic wine were 0.95 ± 0.12 mg/L, 6.31 ± 0.30 mg/L and 119.43 ± 2.62 mg/L respectively. Kaempferol and *trans*-caffeic acid were chosen in this research for their antimicrobial activity proven before in modified MRS media. *Trans*-caftaric acid was also tested in this work because it is the esterified form of *trans*-caffeic acid with tartaric acid and the most abundant HCA found in wine.

4.3.2. *Oenococcus oeni* culture suspensions preparation

The commercial *O. oeni* strains used (Oenos™ and CH35™) were Viniflora® (described in section 2.1.). In the following experiment, 0.05 g of selected *O. oeni* strain was straightly inoculated into 50 mL of wine in a pasteurized falcon tube to get a bacterial suspension of around 10^8 CFU/mL.

4.3.3. Malolactic fermentation

Inoculated and non-inoculated MLF experiments were carried out in parallel. Lactic acid bacteria population at the beginning of these fermentations was around 10^4 CFU/mL. The commercial *O. oeni* strains Viniflora® Oenos™ and CH35™ were prepared as described above and added to 550 mL wine or 165 mL wine mixed with 385 mL MRS broth (30/70 v/v) at approximatively 1 % v/v into 1 L autoclaved flasks to obtain initial concentrations of around 10^6 CFU/mL. The ratio wine/MRS was used based on previous results (Fras *et al*., 2014). MRS broth is composed of glucose 20 g/L, triammonium citrate 2 g/L and sodium acetate trihydrate 5 g/L among other components.

Solutions of kaempferol, *trans*-caffeic acid and *trans*-caftaric acid (purities 98%) from Sigma-Aldrich (Steinheim, Germany) were previously prepared in pure (99.5% v/v) ethanol as described in section 2.4 before being added with a concentration of 10 mg/L into the samples, later distributed into 50 mL sterile falcon tubes - one tube corresponding to a specific time-point.

Control wines and wines containing phenolic compounds, all in duplicate, were incubated at 25 °C in the dark with no agitation.

Wine samples were monitored for 21 days of incubation and mixed samples for 10 days, since the time needed for microbial growth was longer in wine than in MRS broth.

At each time-point, 100 µL sample of each assay was collected and directly diluted for plating on MRS and YMB (Yeast Mold Broth) agar media (sections 2.3.2 and 2.3.3.). Yeast and bacterial growth were monitored by the drop-count technique (as described in sections 2.3.1 and 2.3.3.).

4.3.4. Analysis of non-volatile compounds

The concentrations in organic acids and sugars as well as the concentrations in phenolic compounds were verified before and during incubation by HPLC-UV-IR (as described in section 2.6) and HPLC-DAD (as described in section 2.5) respectively.

4.3.5. Analysis of volatile compounds

Higher alcohols, acetaldehyde and methanol were analyzed only in wines before and after 21 days incubation by GC-FID as described in section 2.7.

The HS-SPME-GC-MS system described in section 3.3.7. was used to analyze the evolution of the predominant volatiles detected in the wine studied in this chapter: esters (ethyl octanoate, ethyl decanoate, diethyl succinate, isoamyl acetate and 2-phenylethyl acetate), alcohols (*trans*-3-hexenol, benzyl alcohol, 2-phenylethanol and methionol), terpenes (α-terpineol and β-citronellol), volatile fatty acid (isobutyric acid) and the carbonyl compounds (benzaldehyde and 2-phenylacetaldehyde).

4.3.6. Statistical analysis

As explained in section 2.8., data were subjected to statistical analysis using JMP13 for Windows XP (Taikoo Shing, Hong Kong, China), at a confidence level of 95% ($p = 0.05$). One-way analysis of variance (ANOVA) was used to test the effect of the type of MLF (non-inoculated, inoculated with Oenos™ and inoculated with CH35™) on microbial and chemical parameters. Dunnett´s test was run to compare the means of each sample relatively to the controls.

4.4. Results

4.4.1. Microbial behavior

The bacterial stationary phase was reached after 21 days of incubation in wines and 10 days in mixed media (Figure 4.1.). The initial concentrations in LAB were around 10^4 CFU/mL (4 log(CFU/mL)) for the non-inoculated samples and the samples inoculated with CH35™ (Figure 4.1.a, c, d and f), and around 10^6 CFU/mL (6 log(CFU/mL)) for the samples inoculated with Oenos™ (Figure 4.1.b and e). The starter Oenos™ did not appear to have any *lag* phase when inoculated in wine or mixed media contrary to CH35™.

All phenolics tested in the study delayed the exponential phase of the LAB in non-inoculated wines with no other effects being observed on LAB growth in the other wines (Figure 4.1.a).

By comparing the controls to the treated samples (Dunnett´s test), the addition of 10 mg/L kaempferol significantly affected the bacterial growth in non-inoculated mixed media (Figure 4.1.d). The bacterial growth in inoculated mixed media was also impacted by the addition of 10 mg/L *trans*-caffeic acid although this effect was only noticeable after 8 days incubation for samples inoculated with Oenos™ and 4 days incubation for the ones inoculated with CH35™ (Figure 4.1.e and f).

Yeasts´ concentrations decreased over time in wines but were relatively constant in mixed media (Figure 4.2.). The nutrients contained in MRS broth could help yeasts maintain their concentration in the mixed media.

After 21 days of incubation, no culturable yeasts were detected in wines inoculated with Oenos™ (Figure 4.2.b). By statistically comparing the controls to the treated wines (Dunnett´s test), the addition of 10 mg/L kaempferol was observed to impact significantly the yeasts 'population during MLF conducted by Oenos™ (Figure 4.2.b). After 21 days of incubation, no more culturable yeasts were detected in wines inoculated with CH35™ except for the ones with addition of 10 mg/L of *trans*-caffeic and *trans*-caftaric acids (Figure 4.2.c).

No significative effects were observed on the yeasts´ populations by the addition of 10 mg/L of the tested phenolic compounds in mixed media (Figure 4.2.d, e and f).

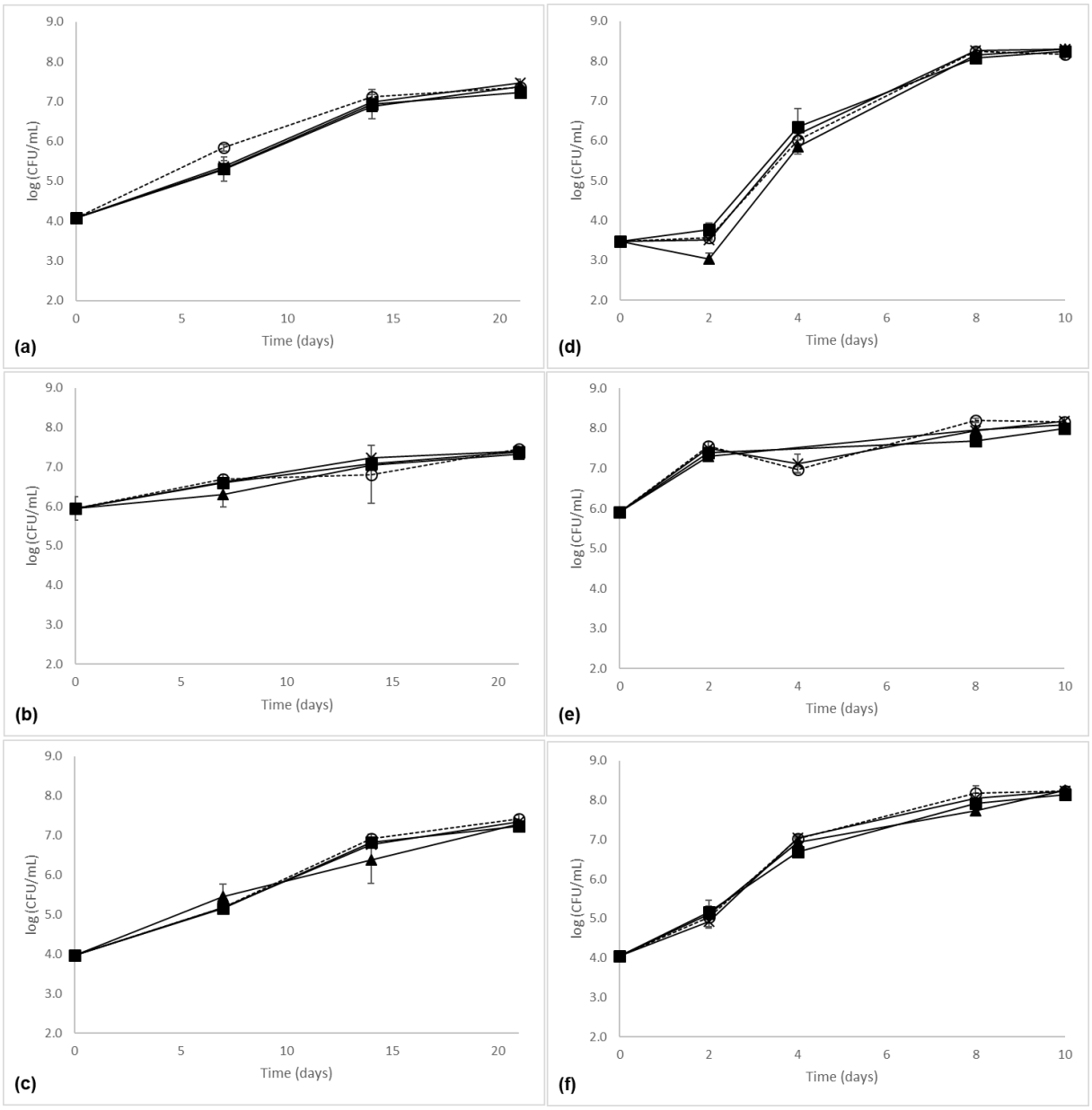


Figure 4.1. - Lactic acid bacteria concentrations (Log_{10} CFU/mL) at 25 °C in wines (a) non-inoculated, (b) inoculated with Oenos™, (c) inoculated with CH35™ and in mixes of wine/MRS broth (30/70 v/v, pH 4.5, 5% v/v ethanol) (d) non-inoculated, (e) inoculated with Oenos™, (f) inoculated with CH35™, supplemented with 10 mg/L of (▲) kaempferol, (■) *trans*-caffeic acid and (x) *trans*-caftaric acid and compared to controls (○). Error bars represent standard deviations of three replicates.

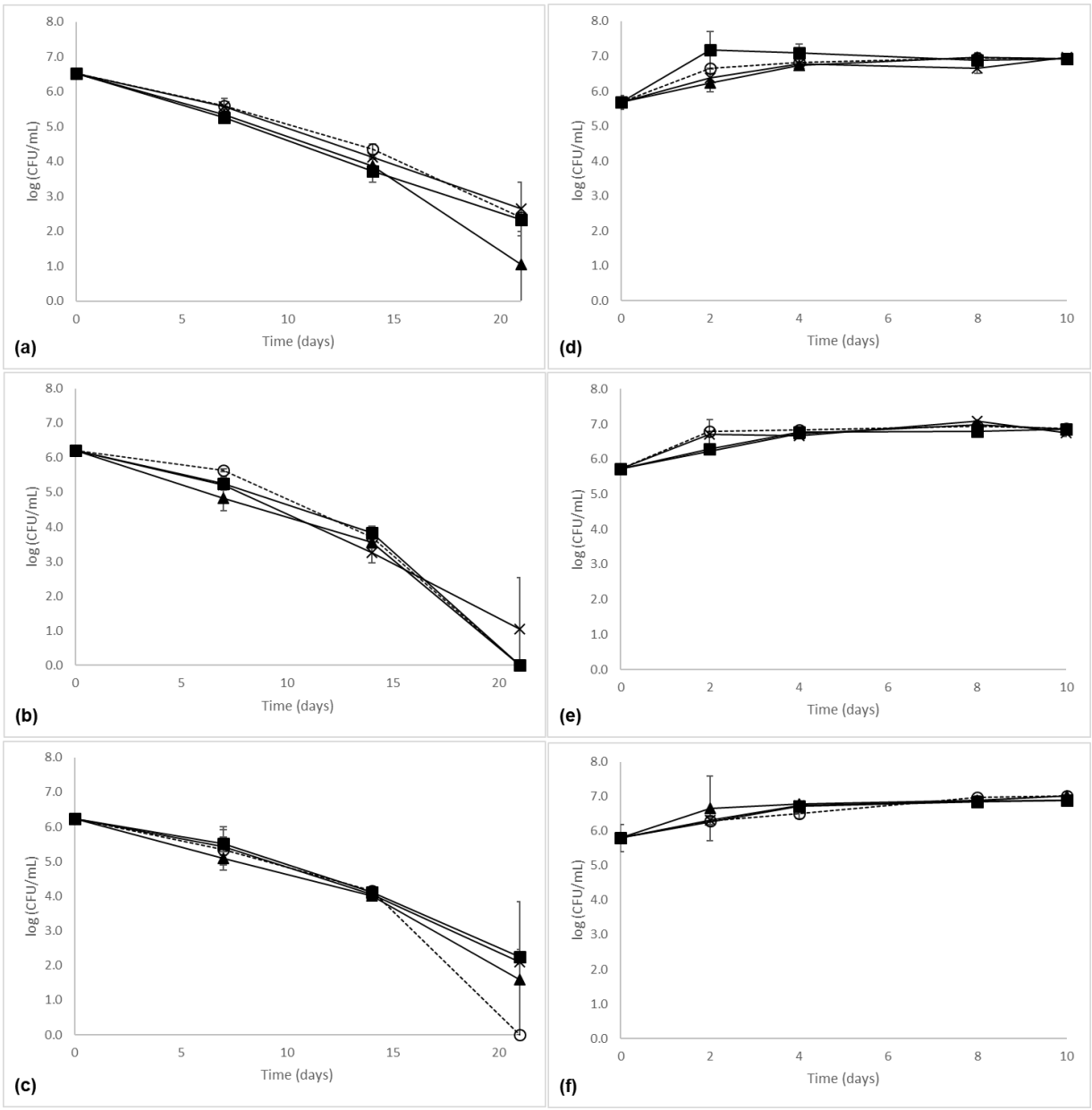


Figure 4.2. - Yeasts´concentrations (Log_{10} CFU/mL) at 25 °C in wines (a) non-inoculated, (b) inoculated with Oenos™, (c) inoculated with CH35™ and in mixes of wine/MRS broth (30/70 v/v, pH 4.5, 5% v/v ethanol) (d) non-inoculated, (e) inoculated with Oenos™, (f) inoculated with CH35™, supplemented with 10 mg/L of (▲) kaempferol, (■) *trans*-caffeic acid and (x) *trans*-caftaric acid and compared to controls (○). Error bars represent standard deviations of three replicates.

4.4.2. The metabolism of sugars and organic acids

Glucose, citric and acetic acids were in highest concentrations in mixed media than in wines, which can be explained by MRS broth being composed of glucose 20 g/L, triammonium citrate 2 g/L and sodium acetate trihydrate 5 g/L among other components. No glucose was detected in wines by the method

used in this experiment (detection limit: 0.30 g/L) and no glucose was left in mixed samples after 2 days incubation. Also, no citric acid was detected in wines after 7 days incubation and in mixed samples after 4 days incubation. No significative differences between treated and non-treated samples were found for glucose and citric acid. Acetic acid increased during the 21-day incubation period in all wines (from 0.257 ± 0.015 g/L to 0.552 ± 0.031 g/L) without significative differences between treated and non-treated wines. More acetic acid was found after 8 days incubation in mixed samples inoculated with Oenos™ and supplemented with 10 mg/L of kaempferol and *trans*-caffeic acid (Control: 3.585 ± 0.008 g/L, kaempferol: 3.833 ± 0.012 g/L, *trans*-caffeic acid: 3.742 ± 0.003 g/L).

Malic acid degradation was faster when the samples were inoculated (Figure 4.3.). Kaempferol and *trans*-caffeic acid added at 10 mg/L delayed malic acid degradation and lactic acid production in wines. The negative impacts of these two compounds on LAB metabolism were greater in inoculated wines (Figure 4.3. b and c). In mixed samples, the inhibitory effect of kaempferol was noticeable on malic acid degradation in non-inoculated samples after 2 days incubation (Figure 4.3. d) while the inhibitory effect of *trans*-caffeic acid was observable as a decrease in lactic acid production in mixed samples inoculated with Oenos™ after 8 days incubation (Figure 4.3. e). At the concentration tested, *trans*-caftaric acid did not appear to have any impact on malic acid consumption (and lactic acid production) (Figure 4.3.).

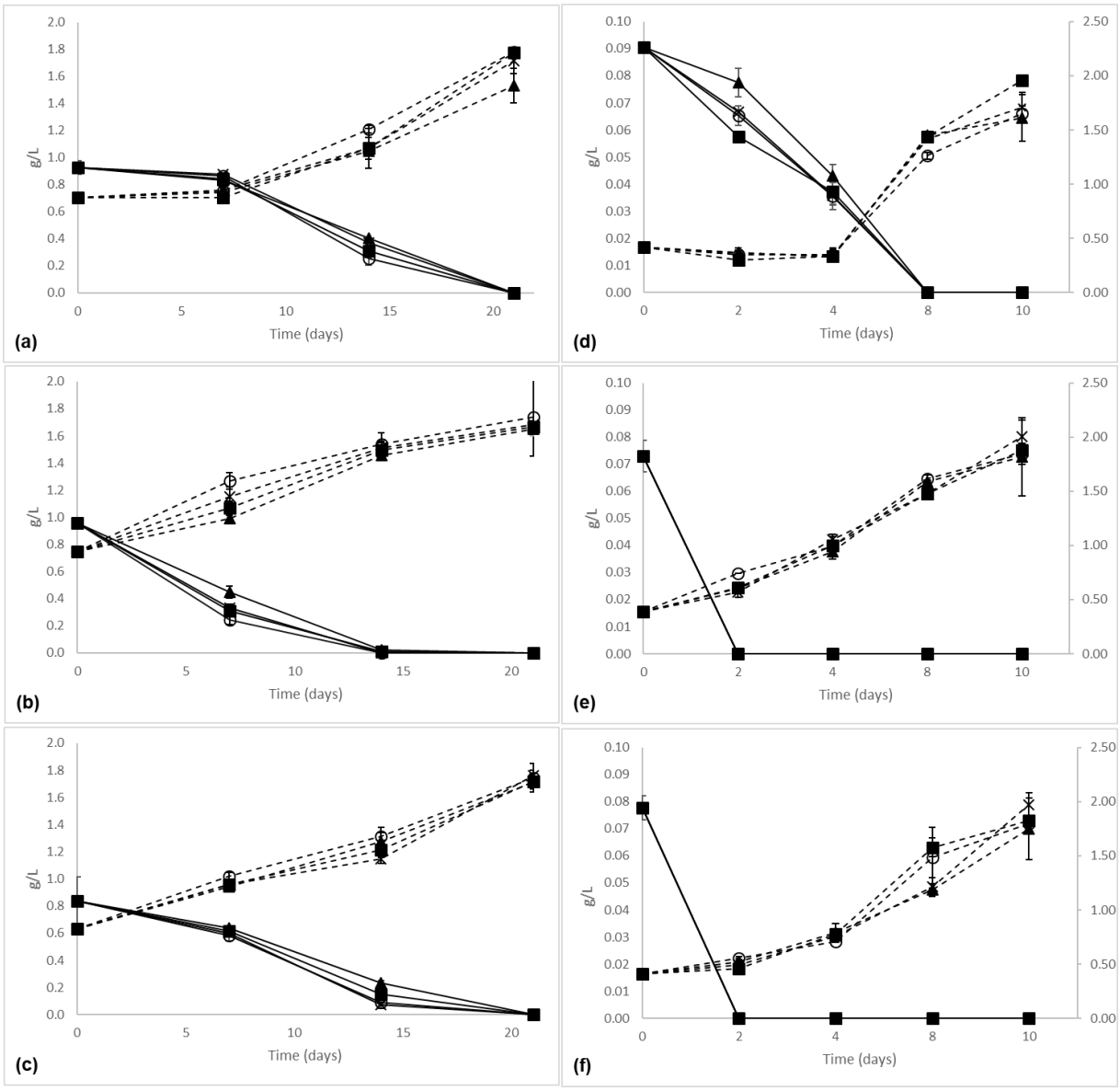


Figure 4.3. – Malic acid degradation (plain lines) and lactic acid production (dashed lines) in wines (a) non-inoculated, (b) inoculated with Oenos™, (c) inoculated with CH35™ and in mixes of wine/MRS broth (30/70 v/v, pH 4.5, 5% v/v ethanol) (d) non-inoculated, (e) inoculated with Oenos™, (f) inoculated with CH35™, supplemented with 10 mg/L of (▲) kaempferol, (■) *trans*-caffeic acid and (x) *trans*-caftaric acid and compared to controls (○). Error bars represent standard deviations of two replicates. A secondary axis for lactic acid concentration was used in in mixed samples (g/L).

4.4.3. Phenolic compounds evolution during malolactic fermentations

In all samples supplemented with 10 mg/L of kaempferol, the concentration of this compound decreased with time from 10.95 ± 0.10 mg/L before incubation to 1.36 ± 0.23 mg/L after incubation.

Over the incubation period, the concentrations in *trans*-caffeic and *trans*-caftaric acids were relatively constant in the samples except the ones inoculated with Oenos™ (Figure 4.4.). In wines inoculated with Oenos™, *trans*-caftaric, *trans*-coutaric and *trans*-fertaric acids decreased and their corresponding HCA *trans*-caffeic, *trans*-*p*-coumaric and *trans*-ferulic acids increased (Figure 4.4.a, b and c). This activity was inhibited in wines by the addition of 10 mg/L kaempferol and *trans*-caffeic acid. In mixed samples inoculated with Oenos™ the decrease in (hydroxy)cinnamoyl-tartaric acids and the increase in HCA "free" forms were also observed except that "free" HCA were decreasing after 4 days incubation (Figure 4.4.d, e and f). Only in the mixed samples inoculated with Oenos™ and supplemented with *trans*-caftraric acid, the *trans*-caffeic acid concentration was higher comparatively to the other samples (Figure 4.3.d).

The anthocyanin concentration decreased over time in all samples. The addition of 10 mg/L kaempferol and *trans*-caftaric acid in wines and mixed samples inoculated with Oenos™ appeared to slightly but significantly increase this degradation (Figure 4.5.). The same observation was made in non-inoculated mixed samples.

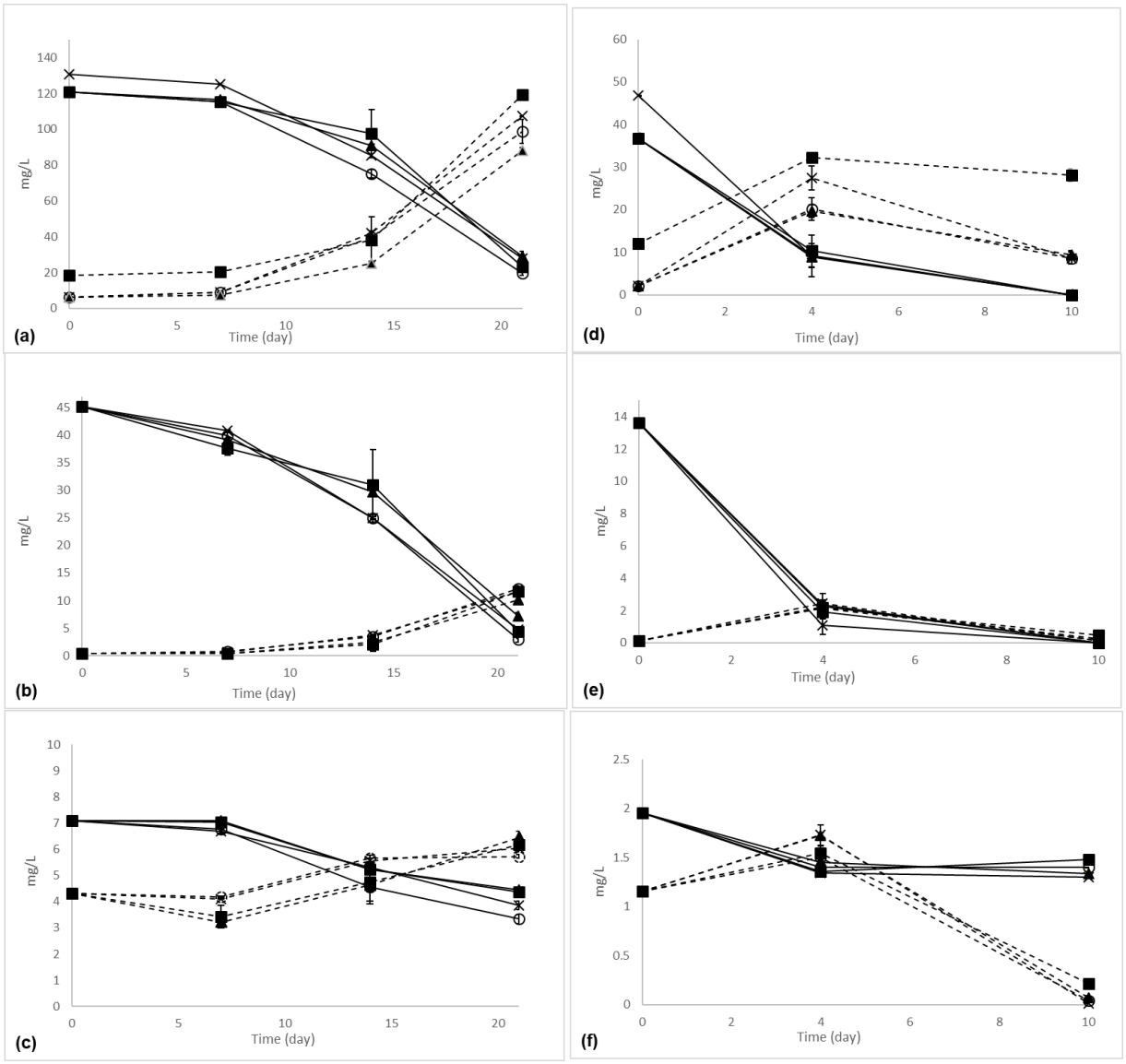


Figure 4.4. – (Hydroxy)cinnamoyl-tartaric acids degradation (plain lines) and "free" HCA production (dashed lines) in wines inoculated with Oenos™ (a, b and c) and in mixes of wine/MRS broth (30/70 v/v, pH 4.5, 5% v/v ethanol) inoculated with Oenos™ (d, e and f), supplemented with 10 mg/L of (▲) kaempferol, (■) *trans*-caffeic acid and (x) *trans*-caftaric acid and compared to controls (○). (a and d) *trans*-caftaric / *trans*-caffeic acids, (b and e) *trans*-coutaric / *trans*-*p*-coumaric acids, (c and f) *trans*-fertaric / *trans*-ferulic acids. Error bars represent standard deviations of two replicates.

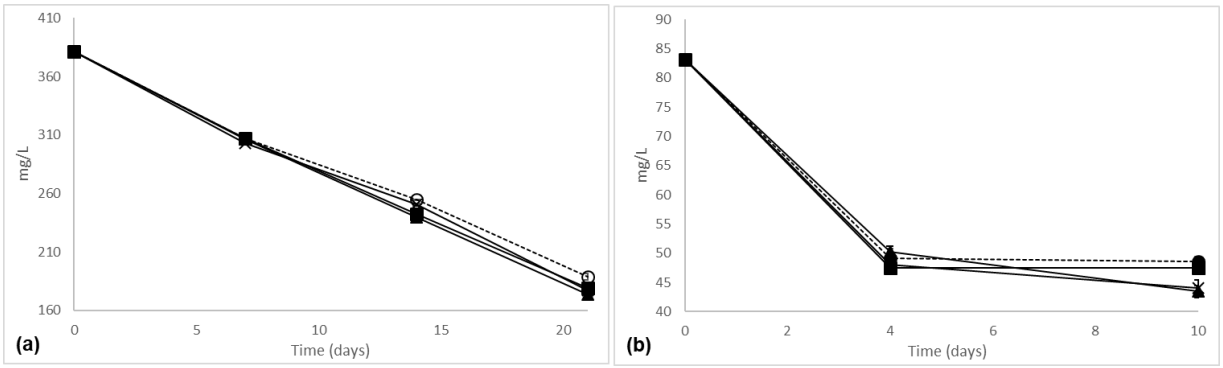


Figure 4.5. – Anthocyanins degradation in (a) wines and (b) in mixes of wine/MRS broth (30/70 v/v, pH 4.5, 5% v/v ethanol) inoculated with Oenos™, supplemented with 10 mg/L of (▲) kaempferol, (■) *trans*-caffeic acid and (x) *trans*-caftaric acid and compared to controls (○). Error bars represent standard deviations of two replicates.

4.4.4. The evolution of volatile compounds during malolactic fermentations

The composition of wine with respect to the volatile compounds present was more influenced by the type of MLF (inoculated, inoculated with Oenos™ or inoculated with CH35™) than by the addition of 10 mg/L kaempferol, *trans*-caffeic and *trans*-caftaric acids (Table 4.1.).

The non-inoculated wines contained higher concentrations in *trans*-3-hexenol (grass) and the inoculated wines higher concentrations of α-terpineol (floral, solvent), isobutyric acid (fatty, cheese), benzaldehyde (almond), and 2-phenylacetaldehyde (floral, honey). Ethyl octanoate (fruity) and ethyl decanoate (floral) were found in higher concentrations in wines inoculated with Oenos™ and diethyl succinate (fruity), 2-phenylethyl-acetate (rose, floral), benzyl alcohol (solvent), methionol (cabbage, potato) and 2-phenylethanol (rose, floral) in higher concentrations in wines inoculated with CH35™ (Table 4.1.).

Compared to the controls, more *trans*-3-hexenol and isobutyric acid were found in non-inoculated wines supplemented with 10 mg/L kaempferol after seven days incubation. More 2-phenylethyl-acetate and benzyl alcohol were found in the same samples after 21 days incubation (Table 4.1.).

The addition of the phenolics tested in wine inoculated with Oenos™ decreased the content in diethyl succinate 21 days after incubation. The addition of 10 mg/L kaempferol increased the concentration in methionol in wines inoculated with Oenos™ 21 days after incubation (Table 4.1.).

As for the wines inoculated with CH35™, the content in ethyl octanoate was lower after 21 days incubation when 10 mg/L kaempferol was initially added to the wines (Table 4.1.).

Some of these compounds may impact the wines aroma since their concentrations were above their thresholds, although it does not necessarily mean that these compounds could be perceived in these particular wines as the odor thresholds were calculated in hydro-alcoholic solutions or other matrices (Appendix 3).

Ethyl octanoate and ethyl decanoate were also found in higher concentrations in mixed media inoculated with Oenos™ while 2-phenylethyl-acetate, benzyl alcohol, isobutyric acid and 2-phenylacetaldehyde were also found in higher concentrations in mixed media inoculated with CH35™ (Table 4.2.). Contrary to what was observed in wines, α-terpineol was in greater content in non-inoculated mixed media and *trans*-3-hexenol in higher concentration in mixed media inoculated with CH35™. More isoamyl acetate (banana) was also detected in mixed media inoculated with CH35™.

Compared to the controls, 2-phenylethanol, ethyl octanoate and β-citronellol (lemon) were found in lower concentrations in non-inoculated mixed media supplemented with 10 mg/L kaempferol and *trans*-caffeic acid after 4 days incubation for the first compound and after 10 days incubation for the last two compounds (Table 4.2.). Less diethyl succinate was found during the incubation in non-inoculated mixed media supplemented with *trans*-caffeic acid, and less α-terpineol was observed after 10 days incubation in non-inoculated mixed media supplemented with all phenolics tested.

The content in isoamyl acetate was lower in mixed media inoculated with CH35™ and supplemented with kaempferol after 4 days incubation comparatively to the controls. More isobutyric acid was found in mixed media inoculated with CH35™ and supplemented with all phenolics tested comparatively to the controls. After 10 days incubation, more benzaldehyde was measured in mixed media inoculated with CH35™ and supplemented with 10 mg/L kaempferol than in the controls (Table 4.2.).

Table 4.1. – Volatile compounds composition (mg/L) of non-inoculated and inoculated wines before, 7 and 21 days after incubation at 25 °C, with or without (controls) addition of phenolics.

Volatile compounds	Odor thresholds	Before incubation		
		Non-inoculated	Oenos™	CH35™
		Control		
Esters				
Ethyl octanoate	0.58[3]	1.53 ± 0.36	1.76 ± 0.44	1.32 ± 0.50
Ethyl decanoate	0.20[9]	0.49 ± 0.12	0.52 ± 0.12	0.32 ± 0.19
Isoamyl acetate	0.03[9]	6.60 ± 0.54	8.38 ± 1.21	5.39 ± 1.77
2-Phenylethyl-acetate	0.25[4]	0.21 ± 0.06	0.26 ± 0.07	0.26 ± 0.07
Diethyl succinate	200[3]	44.88 ± 2.54	42.72 ± 6.22	48.94 ± 1.10
Alcohols				
Trans-3-hexenol	0.4[9]	0.01 ± 0.00	0.10 ± 0.00	0.04 ± 0.00
Benzyl alcohol	200[6]	0.99 ± 0.14	0.79 ± 0.14	1.21 ± 0.08
Methanol	-	30.58 ± 16.95	30.58 ± 16.95	30.58 ± 16.95
Higher alcohols				
Methionol	1[4]	14.92 ± 4.31	13.81 ± 4.89	23.48 ± 2.05
2-Phenylethanol	14[9]	348.56 ± 56.95	338.54 ± 41.94	354.48 ± 15.27
1-Propanol	500[7]	39.23 ± 3.20	39.23 ± 3.20	39.23 ± 3.20
1-Butanol	150[3]	175.46 ± 4.13	175.46 ± 4.13	175.46 ± 4.13
2-Methyl-1-propanol (Isobutanol)	40[9]	nd	nd	nd
2-Methyl-1-butanol (Active amyl alcohol)	7[10]	89.05 ± 3.09	89.05 ± 3.09	89.05 ± 3.09
3-Methyl-1-butanol (Isoamyl alcohol)	30[4]	296.08 ± 6.70	296.08 ± 6.70	296.08 ± 6.70
Terpenes				
β-Citronellol	0.1[3]	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01
α-Terpineol	0.25[9]	0.09 ± 0.02	0.08 ± 0.07	0.08 ± 0.04

Volatile Fatty Acids				
Isobutyric acid	0.23[9]	6.11 ± 1.45	6.90 ± 2.29	10.15 ± 1.72
Carbonyl compounds				
Benzaldehyde	200[2]	0.07 ± 0.01	0.05 ± 0.04	0.15 ± 0.10
2-Phenylacetaldehyde	0.001[2]	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01
Acetaldehyde	100[7]	35.11 ± 9.92	35.11 ± 9.92	35.11 ± 9.92

Volatile compounds	**After 7 days incubation**											
	Non-inoculated				**Oenos™**				**CH35™**			
	Control	Kaempf.	Caffeic	Caftaric	Control	Kaempf.	Caffeic	Caftaric	Control	Kaempf.	Caffeic	Caftaric
Esters												
Ethyl octanoate	0.71 ± 0.14	0.65 ± 0.06**	0.63 ± 0.01	0.89 ± 0.04	0.85 ± 0.35	0.92 ± 0.08*	0.72 ± 0.07	0.76 ± 0.08	0.88 ± 0.12	0.72 ± 0.06	0.55 ± 0.00	0.76 ± 0.03
Ethyl decanoate	0.12 ± 0.04	0.09 ± 0.01**	0.09 ± 0.00	0.11 ± 0.02	0.11 ± 0.05	0.17 ± 0.03*	0.10 ± 0.01*	0.09 ± 0.01	0.13 ± 0.07	0.08 ± 0.01**	0.06 ± 0.00**	0.11 ± 0.01
Isoamyl acetate	4.29 ± 0.05	4.66 ± 0.40	3.94 ± 0.10	4.51 ± 0.40	3.67 ± 1.15	4.70 ± 0.30	3.48 ± 1.45	3.48 ± 1.71	3.79 ± 0.50	4.50 ± 0.01	3.44 ± 0.00	4.18 ± 1.17
2-Phenylethyl-acetate	0.12 ± 0.02**	0.23 ± 0.11	0.19 ± 0.06	0.10 ± 0.00**	0.16 ± 0.05	0.18 ± 0.06	0.18 ± 0.13	0.15 ± 0.02**	0.29 ± 0.05*	0.21 ± 0.07	0.22 ± 0.00	0.26 ± 0.03*
Diethyl succinate	39.98 ± 3.02	37.48 ± 2.88	36.63 ± 1.22	35.72 ± 3.61**	35.70 ± 6.54	40.74 ± 3.99	37.08 ± 6.39	36.85 ± 2.09**	46.78 ± 1.95	42.56 ± 4.79	50.44 ± 0.00	48.34 ± 1.73*
Alcohols												
Trans-3-hexenol	0.10 ± 0.01 **b**	0.63 ± 0.05* **a**	0.11 ± 0.00*	0.10 ± 0.00	0.05 ± 0.03	0.05 ± 0.02**	0.06 ± 0.01**	0.10 ± 0.00*	0.06 ± 0.03	0.04 ± 0.00**	0.06 ± 0.00**	0.06 ± 0.02**
Benzyl alcohol	0.64 ± 0.06**	0.60 ± 0.06**	0.68 ± 0.06	0.55 ± 0.02**	0.56 ± 0.14**	0.83 ± 0.10	0.76 ± 0.22	0.71 ± 0.06	1.17 ± 0.03*	1.05 ± 0.19*	1.05 ± 0.19	1.28 ± 0.19*
Higher alcohols												

Methionol	7.55 ± 0.96**	8.61 ± 1.35	9.34 ± 2.17	6.01 ± 0.24**	8.92 ± 0.19**	13.15 ± 2.51	12.33 ± 5.77	11.46 ± 1.53**	24.80 ± 3.92*	19.40 ± 6.53	29.30 ± 0.00	25.99 ± 6.31*
2-Phenylethanol	254.51 ± 25.76**	264.60 ± 23.28	237.77 ± 18.70	207.27 ± 14.98**	264.83 ± 48.17**	254.91 ± 36.59	263.29 ± 61.93	252.99 ± 36.57**	366.45 ± 1.66*	292.86 ± 60.82	406.40 ± 0.00	379.54 ± 21.94*
Terpenes												
β-Citronellol	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
α-Terpineol	0.03 ± 0.01	0.02 ± 0.00**	0.02 ± 0.00*	0.03 ± 0.00	0.02 ± 0.02	0.04 ± 0.01*	0.03 ± 0.00*	0.02 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.00
Volatile Fatty Acids												
Isobutyric acid	5.10 ± 0.85** **b**	9.08 ± 0.06 **a**	6.69 ± 0.06***	6.54 ± 0.41	8.81 ± 0.00	10.55 ± 2.75	9.17 ± 0.53**	9.96 ± 2.62	9.37 ± 061*	8.87 ± 1.12	11.77 ± 0.00*	10.46 ± 0.17
Carbonyl compounds												
Benzaldehyde	0.13 ± 0.01	0.15 ± 0.03**	0.16 ± 0.02**	0.16 ± 0.00**	0.14 ± 0.09	0.25 ± 0.03*	0.20 ± 0.02	0.22 ± 0.02*	0.23 ± 0.02	0.21 ± 0.00	0.26 ± 0.00*	0.26 ± 0.03*
2-Phenylacetaldehyde	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00**	0.05 ± 0.01**	0.05 ± 0.02	0.06 ± 0.01	0.07 ± 0.00*	0.05 ± 0.01**	0.08 ± 0.00	0.07 ± 0.01	0.08 ± 0.00*	0.09 ± 0.01*

Volatile compounds	**After 21 days incubation**											
	Non-inoculated				**Oenos^{TM}**				**CH35^{TM}**			
	Control	Kaemp.	Caffeic	Caftaric	Control	Kaempf.	Caffeic	Caftaric	Control	Kaempf.	Caffeic	Caftaric
Esters												
Ethyl octanoate	0.44 ± 0.00	0.66 ± 0.21	0.47 ± 0.06**	0.45 ± 0.04**	0.69 ± 0.10	0.48 ± 0.11	0.69 ± 0.08*	0.62 ± 0.03*	0.63 ± 0.01 **a**	0.49 ± 0.01 **b**	0.54 ± 0.04	0.62 ± 0.00
Ethyl decanoate	0.05 ± 0.00	0.09 ± 0.03	0.06 ± 0.01	0.04 ± 0.00***	0.08 ± 0.01	0.09 ± 0.02	0.12 ± 0.04	0.08 ± 0.00**	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.10 ± 0.00*

Isoamyl acetate	3.25 ± 0.00	3.64 ± 0.64	2.74 ± 0.47	3.29 ± 0.13	3.72 ± 0.38	3.62 ± 0.00	3.76 ± 0.85	2.73 ± 0.31	3.51 ± 0.24	3.28 ± 0.14	2.82 ± 0.58	3.26 ± 0.00
2-Phenylethyl-acetate	0.12 ± 0.00 **b**	0.35 ± 0.03 **a**	0.15 ± 0.08	0.10 ± 0.01***	0.24 ± 0.08	0.24 ± 0.01	0.17 ± 0.03	0.14 ± 0.01**	0.29 ± 0.00	0.21 ± 0.10	0.26 ± 0.03	0.27 ± 0.00*
Diethyl succinate	56.40 ± 0.00	68.90 ± 4.10*	61.19 ± 3.42**	53.24 ± 3.92**	74.29 ± 1.75 **a**	52.73 ± 0.55** **b**	63.02 ± 1.22** **b**	54.05 ± 0.37** **b**	75.57 ± 0.00	66.08 ± 0.78*	71.99 ± 1.90*	75.15 ± 0.00*
Alcohols												
Trans-3-hexenol	0.02 ± 0.00	0.38 ± 0.32	0.12 ± 0.00*	0.09 ± 0.00*	0.08 ± 0.04	0.04 ± 0.01	0.03 ± 0.01**	0.05 ± 0.01**	0.11 ± 0.02	0.03 ± 0.01	0.03 ± 0.01**	0.03 ± 0.00**
Benzyl alcohol	1.33 ± 0.00 **b**	1.58 ± 0.00 **a**	1.42 ± 0.17	1.10 ± 0.05**	1.37 ± 0.10	1.39 ± 0.44	1.17 ± 0.08**	1.05 ± 0.06**	2.27 ± 0.00	1.81 ± 0.27	2.05 ± 0.29*	2.13 ± 0.00*
Methanol	10.59 ± 0.00	20.35 ± 0.00	17.37 ± 11.20	28.54 ± 4.77	40.98 ± 10.74	25.14 ± 10.32	35.32 ± 2.29	39.37 ± 11.10	21.30 ± 5.56	24.61 ± 6.68	29.84 ± 28.50	30.58 ± 0.00
Higher alcohols												
Methionol	3.57 ± 0.00**	8.01 ± 1.29**	5.64 ± 1.99**	3.62 ± 0.29**	8.41 ± 0.37 **b**	16.39 ± 3.12* **a**	7.98 ± 0.23	6.36 ± 1.57	12.55 ± 0.00*	9.26 ± 0.74**	13.05 ± 2.01*	9.80 ± 0.00*
2-Phenylethanol	251.09 ± 0.00	331.25 ± 45.43	258.73 ± 27.46**	203.40 ± 11.49**	281.91 ± 78.17	332.08 ± 19.44	256.45 ± 7.59**	260.52 ± 24.27	359.12 ± 0.00	289.44 ± 67.61	336.84 ± 8.65*	349.56 ± 0.00*
1-Propanol	46.15 ± 0.00	16.13 ± 0.00	21.92 ± 11.36	31.47 ± 8.10	7.88 ± 4.26	10.61 ± 5.07	28.13 ± 9.04	30.88 ± 26.55	39.63 ± 14.87	36.01 ± 19.40	39.17 ± 7.78	12.61 ± 0.00
1-Butanol	147.92 ± 0.00	86.69 ± 0.00	84.62 ± 7.30	134.36 ± 51.91	83.39 ± 22.13	83.31 ± 5.15	135.94 ± 30.71	136.02 ± 47.42	125.33 ± 57.29	133.04 ± 38.56	140.76 ± 28.56	97.77 ± 0.00
2-Methyl-1-propanol	nd	0.41 ± 0.00	nd	0.49 ± 0.09	nd	0.22 ± 0.32	0.49 ± 0.17	0.50 ± 0.71	0.80 ± 0.37	0.40 ± 0.56	nd	nd
2-Methyl-1-butanol	77.85 ± 0.00	47.45 ± 0.00	64.00 ± 0.09**	77.54 ± 17.72	72.03 ± 5.10	60.75 ± 11.58	83.59 ± 2.25	92.67 ± 15.22	85.41 ± 26.25	74.07 ± 11.70	95.49 ± 12.11*	82.53 ± 0.00

3-Methyl-1-butanol	262.86 ± 0.00	167.34 ± 0.00	223.12 ± 2.22**	265.76 ± 57.57	245.32 ± 20.15	209.98 ± 46.19	284.75 ± 9.42	323.91 ± 60.74	303.52 ± 94.21	255.86 ± 33.56	334.15 ± 49.91*	274.07 ± 0.00
Terpenes												
β-Citronellol	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
α-Terpineol	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00**	0.02 ± 0.00***	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00*	0.02 ± 0.00**	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.00*	0.03 ± 0.00*
Volatile Fatty Acids												
Isobutyric acid	5.89 ± 0.00	13.15 ± 3.21	9.45 ± 1.28	6.42 ± 0.88	8.81 ± 0.42	11.22 ± 1.01	10.80 ± 2.65	8.98 ± 1.80	12.63 ± 0.00	9.98 ± 0.22	9.62 ± 0.90	1.01 ± 0.00
Carbonyl compounds												
Benzaldehyde	0.38 ± 0.00	0.40 ± 0.01**	0.34 ± 0.01	0.29 ± 0.04	0.40 ± 0.02	0.36 ± 0.03**	0.38 ± 0.11	0.37 ± 0.05	0.43 ± 0.10	0.52 ± 0.03*	0.51 ± 0.03	0.51 ± 0.03
2-Phenyl acetaldehyde	0.09 ± 0.00	0.11 ± 0.01	0.08 ± 0.00	0.07 ± 0.01**	0.10 ± 0.01	0.11 ± 0.02	0.11 ± 0.00	0.10 ± 0.00*	0.11 ± 0.04	0.13 ± 0.02	0.11 ± 0.02	0.11 ± 0.02*
Acetaldehyde	15.12 ± 0.00	22.75 ± 0.00	19.14 ± 0.01	20.47 ± 6.43	21.23 ± 1.70	22.47 ± 2.47	23.89 ± 9.53	33.64 ± 2.10	21.09 ± 8.31	19.16 ± 7.11	30.57 ± 1.27	30.57 ± 1.27

n = 4 ± standard deviations. nd = not detectable (Limits of detection: 2-methyl-1-propanol = 20 µg/L). Kaempf. = Kaempferol.

Bold letters on the right indicate statistically significant differences of the sample with the control at that specific time point (Dunnett´s $p < 0.05$).

Stars on the up right indicate statistically significant differences between non-inoculated, inoculated with Oenos™ and inoculated with CH35™ samples with the same treatment at the same time point (ANOVA $p < 0.05$). * > ** > *** mean values from the highest to the lowest.

2 (Escudero *et al.*, 2007): Thresholds calculated in beer; 3 (Etiévant, 1991) and 7 (Swiegers *et al.*, 2005a): Thresholds calculated in wine; 4 (Guth, 1997): Thresholds calculated in 10% ethanol; 6 (Gomez-Miguez *et al.*, 2007): Thresholds calculated in 10% water / ethanol mixture containing 5 g/L of tartaric acid at pH 3.2; 9 (Ferreira *et al.*, 2000): Thresholds determined in 11% v/v aqueous ethanol with 7 g/L glycerol and 5 g/L tartaric acid, at pH 3.4; 10 (Salo, 1970): Thresholds calculated in hydro-alcoholic solution.

Table 4.2. – Volatile compounds composition (mg/L) in non-inoculated and inoculated mixed samples before, 4 and 10 days after incubation at 25 °C, with or without (controls) addition of phenolics.

Volatile compounds	Odor thresholds	Before incubation		
		Non-inoculated	Oenos™	CH35™
		Control		
Esters				
Ethyl octanoate	0.58[3]	0.15 ± 0.01	0.17 ± 0.07	0.22 ± 0.02
Ethyl decanoate	0.20[9]	0.02 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Isoamyl acetate	0.03[9]	1.28 ± 0.02	1.38 ± 0.06	1.51 ± 0.15
2-Phenylethyl-acetate	0.25[4]	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
Diethyl succinate	200[3]	5.60 ± 0.35	6.13 ± 0.21	6.14 ± 0.14
Alcohols				
Trans-3-hexenol	0.4[9]	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.01
Benzyl alcohol	200[6]	0.13 ± 0.00	0.15 ± 0.00	0.13 ± 0.00
Higher alcohols				
Methionol	1[4]	1.40 ± 0.10	2.12 ± 0.34	2.39 ± 0.26
2-Phenylethanol	14[9]	33.84 ± 0.53	36.91 ± 0.64	38.73 ± 0.11
Terpenes				
β-Citronellol	0.1[3]	0.004 ± 0.000	0.004 ± 0.000	0.006 ± 0.001
α-Terpineol	0.25[9]	0.03 ± 0.00	0.04 ± 0.00	0.05 ± 0.01
Volatile Fatty Acids				
Isobutyric acid	0.23[9]	1.83 ± 1.71	1.95 ± 1.31	2.44 ± 2.70
Carbonyl compounds				
Benzaldehyde	200[2]	0.32 ± 0.02	0.39 ± 0.01	0.25 ± 0.02
2-Phenylacetaldehyde	0.001[2]	0.21 ± 0.02	0.29 ± 0.02	0.20 ± 0.01

Volatile compounds	After 4 days incubation											
	Non-inoculated				Oenos™				CH35™			
	Control	Kaempf.	Caffeic	Caftaric	Control	Kaempf.	Caffeic	Caftaric	Control	Kaempf.	Caffeic	Caftaric
Esters												
Ethyl octanoate	0.17 ± 0.03	0.10 ± 0.02**	0.11 ± 0.01	0.19 ± 0.02	0.17 ± 0.01	0.17 ± 0.01*	0.11 ± 0.00	0.17 ± 0.06	0.12 ± 0.01	0.10 ± 0.03**	0.12 ± 0.02	0.15 ± 0.04
Ethyl decanoate	0.02 ± 0.01	0.02 ± 0.00**	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00*	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00**	0.02 ± 0.00	0.03 ± 0.01
Isoamyl acetate	1.04 ± 0.06**	1.05 ± 0.01	1.09 ± 0.14	1.22 ± 0.01*	0.95 ± 0.07**	1.00 ± 0.10	0.92 ± 0.00	0.95 ± 0.06**	1.29 ± 0.00* **a**	1.10 ± 0.05 **b**	1.30 ± 0.00	1.23 ± 0.05*
2-phenylethyl acetate	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00**	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00*	0.04 ± 0.01	0.04 ± 0.00	0.06 ± 0.04
Diethyl succinate	6.49 ± 0.10* **a**	5.87 ± 0.15**	5.06 ± 0.35 **b**	5.91 ± 0.58	5.64 ± 0.14**	6.39 ± 0.13*	5.74 ± 0.00	6.66 ± 0.25	6.50 ± 0.41*	5.73 ± 0.03**	6.30 ± 0.53	6.99 ± 0.89
Alcohols												
Trans-3-hexenol	0.01 ± 0.00	0.01 ± 0.00***	0.01 ± 0.00**	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00**	0.01 ± 0.00**	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.00*	0.03 ± 0.00*	0.02 ± 0.01
Benzyl alcohol	0.20 ± 0.01**	0.19 ± 0.01	0.18 ± 0.02	0.21 ± 0.01**	0.23 ± 0.00*	0.20 ± 0.01	0.22 ± 0.00	0.24 ± 0.01	0.25 ± 0.01*	0.22 ± 0.01	0.43 ± 0.24	0.26 ± 0.02*
Higher alcohols												
Methionol	4.59 ± 0.25	3.67 ± 0.41	3.72 ± 0.62	3.92 ± 1.03	3.98 ± 0.14	4.44 ± 1.11	4.90 ± 0.00	5.25 ± 0.17	4.42 ± 0.25	3.49 ± 0.44	3.67 ± 0.02	3.67 ± 0.94
2-phenylethanol	43.52 ± 0.23* **a**	38.89 ± 1.75	36.33 ± 0.40** **b**	36.33 ± 0.40 **b**	39.74 ± 0.46**	42.13 ± 0.37	42.08 ± 0.00*	44.96 ± 2.79	44.83 ± 1.23*	42.16 ± 1.49	41.12 ± 1.17*	40.32 ± 6.81
Terpenes												
β-citronellol	0.003 ± 0.001	0.003 ± 0.001	0.004 ± 0.000	0.004 ± 0.000	0.004 ± 0.000	0.005 ± 0.001	0.005 ± 0.000	0.005 ± 0.001	0.004 ± 0.000	0.004 ± 0.000	0.004 ± 0.001	0.005 ± 0.001
α-terpineol	0.05 ± 0.01*	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01*	0.03 ± 0.01**	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01**	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00**

Volatile Fatty Acids												
Isobutyric acid	2.33 ± 0.25	2.12 ± 0.04**	2.24 ± 0.22**	2.60 ± 0.02	2.45 ± 0.01	2.88 ± 0.39*	2.11 ± 0.00**	1.86 ± 0.04	2.99 ± 0.49	3.16 ± 0.10*	3.41 ± 0.14*	2.78 ± 0.72
Carbonyl compounds												
Benzaldehyde	0.06 ± 0.01	0.06 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.09 ± 0.01	0.06 ± 0.02	0.07 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.02
2-phenylacetaldehyde	0.04 ± 0.01	0.03 ± 0.00**	0.03 ± 0.00	0.02 ± 0.00**	0.06 ± 0.01	0.03 ± 0.01**	0.03 ± 0.00	0.02 ± 0.00**	0.04 ± 0.00	0.05 ± 0.00*	0.04 ± 0.01	0.03 ± 0.00*

Volatile compounds	**After 10 days incubation**											
	Non-inoculated				**Oenos™**				**CH35™**			
	Control	Kaempf.	Caffeic	Caftaric	Control	Kaempf.	Caffeic	Caftaric	Control	Kaempf.	Caffeic	Caftaric
Esters												
Ethyl octanoate	0.11 ± 0.01 **a**	0.07 ± 0.00 **b**	0.09 ± 0.01 **b**	0.09 ± 0.00	0.09 ± 0.00	0.12 ± 0.02	0.10 ± 0.03	0.11 ± 0.03	0.11 ± 0.04	0.10 ± 0.02	0.11 ± 0.00	0.11 ± 0.02
Ethyl decanoate	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Isoamyl acetate	1.25 ± 0.06	1.15 ± 0.00	1.17 ± 0.05	1.25 ± 0.07	1.09 ± 0.25	1.13 ± 0.24	1.05 ± 0.14	1.05 ± 0.17	1.34 ± 0.11	1.19 ± 0.19	1.28 ± 0.12	1.37 ± 0.00
2-Phenylethyl acetate	0.03 ± 0.00**	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00**	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.05 ± 0.01*	0.04 ± 0.00	0.03 ± 0.01	0.04 ± 0.00
Diethyl succinate	7.46 ± 0.13* **a**	6.70 ± 0.00	5.84 ± 0.44 **b**	6.17 ± 0.28	5.77 ± 0.29***	6.73 ± 0.62	8.83 ± 2.68	6.50 ± 0.62	6.37 ± 0.06**	6.00 ± 0.58	5.90 ± 0.29	6.37 ± 0.18
Alcohols												
Trans-3-hexenol	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00**	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01**	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01*	0.03 ± 0.00
Benzyl alcohol	0.68 ± 0.09	0.65 ± 0.00	0.61 ± 0.11	0.62 ± 0.02	0.60 ± 0.07	0.60 ± 0.10	0.63 ± 0.08	0.64 ± 0.06	0.59 ± 0.07	0.60 ± 0.06	0.49 ± 0.39	0.55 ± 0.00

Higher alcohols												
Methionol	3.82 ± 0.24	3.43 ± 0.00	4.19 ± 0.13**	4.20 ± 0.08	4.01 ± 0.15	4.37 ± 0.01	5.25 ± 0.31*	4.97 ± 0.88	4.92 ± 0.70	3.73 ± 0.34	3.32 ± 0.32***	3.54 ± 0.01
2-Phenylethanol	45.24 ± 2.69	42.73 ± 0.00	39.84 ± 1.77	42.14 ± 1.04	38.20 ± 4.60	43.08 ± 8.75	40.95 ± 7.70	43.44 ± 3.63	41.86 ± 5.18	41.97 ± 2.93	38.85 ± 3.23	38.40 ± 0.18
Terpenes												
β-Citronellol	0.004 ± 0.000 **a**	0.003 ± 0.000 **b**	0.004 ± 0.000 **b**	0.004 ± 0.000	0.004 ± 0.000	0.004 ± 0.001	0.004 ± 0.001	0.004 ± 0.000	0.004 ± 0.001	0.004 ± 0.000	0.004 ± 0.000	0.004 ± 0.000
α-Terpineol	0.03 ± 0.00* **a**	0.02 ± 0.00 **b**	0.02 ± 0.00 **b**	0.02 ± 0.00 **b**	0.02 ± 0.00**	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00**	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Volatile Fatty Acids												
Isobutyric acid	2.49 ± 0.24	2.20 ± 0.00	2.35 ± 0.16**	2.43 ± 0.16**	2.33 ± 0.29	2.80 ± 0.66	2.32 ± 0.10**	2.12 ± 0.05***	2.59 ± 0.04 **b**	3.55 ± 0.09 **a**	3.49 ± 0.19* **a**	3.51 ± 0.01* **a**
Carbonyl compounds												
Benzaldehyde	0.07 ± 0.01	0.07 ± 0.00	0.07 ± 0.02	0.06 ± 0.01	0.06 ± 0.04	0.07 ± 0.03	0.06 ± 0.04	0.04 ± 0.01	0.05 ± 0.00 **b**	0.09 ± 0.00 **a**	0.07 ± 0.01	0.06 ± 0.01
2-Phenylacetaldehyde	0.05 ± 0.01	0.07 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.03	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.00	0.06 ± 0.00	0.05 ± 0.02	0.05 ± 0.01

n = 4 ± standard deviations. Kaempf. = Kaempferol.

Bold letters on the right indicate statistically significant differences of the sample with the control at that specific time point (Dunnett´s $p < 0.05$).

Stars on the up right indicate statistically significant differences between non-inoculated, inoculated with Oenos™ and inoculated with CH35™ samples with the same treatment at the same time point (ANOVA $p < 0.05$). * > ** > *** mean values from the highest to the lowest.

2 (Escudero *et al.*, 2007): Thresholds calculated in beer; 3 (Etiévant, 1991): Thresholds calculated in wine; 4 (Guth, 1997): Thresholds calculated in 10% ethanol; 6 (Gomez-Miguez *et al.*, 2007): Thresholds calculated in 10% water / ethanol mixture containing 5 g/L of tartaric acid at pH 3.2; 9 (Ferreira *et al.*, 2000): Thresholds determined in 11% v/v aqueous ethanol with 7 g/L glycerol and 5 g/L tartaric acid, at pH 3.4.

4.5. Discussion

The results indicate that the phenolics tested influence the microbial development and metabolism in wines and mixed media, impacting their chemical composition. The effects depend on the type of MLF (non-inoculated and inoculated), on the type of starters used (Oenococcus oeni Oenos™ or CH35™) and on the media used, whether pure wine or wine mixed with MRS broth.

All phenolics tested were inhibitory for the growth of LAB in non-inoculated wines. In mixed media, kaempferol was more potent in this respect in non-inoculated samples and *trans*-caffeic acid in inoculated ones. The inhibitory effect of kaempferol and *trans*-caffeic acid agree with those previously reported in experiments performed in culture media with other isolated strains of LAB (Campos *et al.*, 2003; Figueiredo *et al.*, 2008; García-Ruiz *et al.*, 2011). However, in the cited studies *trans*-caffeic acid was tested at concentrations above 100 mg/L in growth medium. The inhibitory effect of these compounds is thought to be linked to the damage of the LAB cell membrane (García-Ruiz *et al.*, 2009; Campos *et al.*, 2009b; Devi and Anu-Appaiah, 2018a).

Malolactic fermentations were considered to be over when malic acid was no longer detectable in the samples, corresponding to 21 days after incubation in non-inoculated wines and wines inoculated with CH35™, 14 days after incubation in wines inoculated with Oenos™, 8 days after incubation in non-inoculated mixed experiments and 2 days after incubation in inoculated mixed experiments. As expected, MLF was faster in inoculated experiments, particularly in those that were wine-medium mixtures. In addition to their antimicrobial effects, kaempferol and *trans*-caffeic acid were observed to delay the malic acid degradation and the lactic acid production. This result is in accordance with Campos *et al.* (2009a) who observed the malolactic activity of some LAB to be clearly diminished with the addition of 500 mg/L *trans*-caffeic acid in modified MRS medium.

More acetic acid was found in mixed media inoculated with Oenos™ and supplemented with 10 mg/L of kaempferol and *trans*-caffeic acid. As suggested by Campos *et al.* (2009a), the addition of *trans*-caffeic acid could have induced a shift in the glucose metabolism pathway of Oenos™ towards acetate production in a medium containing MRS broth.

The starter Oenos™ is known to liberate HCA from their tartrate derivatives through its cinnamoyl esterase activity (Burns and Osborne, 2013; Chescheir *et al.*, 2015). The addition of 10 mg/L kaempferol and *trans*-caffeic acid inhibited this activity in wines.

In both wine and mixed media, the esters ethyl octanoate and ethyl decanoate were found in significantly higher concentrations in samples inoculated with Oenos™ and supplemented with kaempferol comparatively to the non-inoculated and inoculated with CH35™ samples with the same treatment. 2-Phenylethyl-acetate, benzyl alcohol, isobutyric acid and 2-phenylacetaldehyde were found in higher concentrations in samples inoculated with CH35™ than in non-inoculated samples and samples inoculated with Oenos™. This last observation was particularly statistically significant for samples supplemented with *trans*-caftaric acid. As described in previous works, inoculation with commercial LAB starters enhances the ethyl esters, higher alcohols and VFA contents in the media, causing changes on the odor profile of the samples (Maicas *et al.*, 1999; Ugliano and Moio, 2005;

Hernandez-Orte *et al.*, 2009; Antalick *et al.*, 2012; Malherbe *et al.*, 2012). Diethyl succinate was present in lower concentrations with the addition of the phenolics tested in wines inoculated with Oenos™ and of *trans*-caffeic acid in non-inoculated mixed media. This observation could be explained by the inhibitory effect of the phenolics on the bacteria synthesizing diethyl succinate (Maicas *et al.*, 1999; Malherbe *et al.*, 2012). Addition of the phenolics tested led to other modifications of the volatiles´ profile of the samples, probably impacting their overall organoleptic perception. Devi and Anu-Appaiah (2018a) suggested possible changes in the enzymatic activity of the microorganisms and in other chemical reactions during wine fermentation by the exposure to phenolic compounds, affecting the levels of volatile compounds.

Of the three phenolics studied and added at the same concentration, *trans*-caftaric acid exerted the least impact on the microbial growth and metabolism. Initially present at 120 mg/L in the analyzed wine and at 35 mg/L in the mixed medium, the additional concentration (10 mg/L) probably did not make a considerable difference. Kaempferol had the strongest influence on malolactic fermentations of all tested compounds. The reported concentrations in kaempferol in post-malolactic wines are between 2.58 and 5.40 mg/L (Rossouw and Marais, 2004; Zoechling *et al.*, 2009). Therefore, the addition of 10 mg/L was quite considerable in relative terms, which might explain the greater effects observed with this compound.

Most of the previous researches on the impact of phenolic compounds on wine microorganisms were made in modified MRS media and have not been extensively studied in wine conditions. Fras *et al.* (2014) investigated the influence of wine on some LAB metabolism and found that the addition of wine to MRS changed somehow the LAB behavior. Hence, in this work we chose to compare experiments performed with wines and wines mixed with MRS broth and as expected, different effects were observed. The yeasts population decreased in wines but was relatively constant in mixed media, HCA increased over time in wines inoculated with Oenos™ but decreased in mixed media inoculated with Oenos™ after 4 days incubation. Methionol decreased in wines but increased in mixed media. A possible explanation could be that (sulfur-containing) amino acid content increased in mixed media too. The addition of wine to MRS broth changed the pattern of the production of volatiles by isolated LAB as has previously been demonstrated with volatile phenols (Fras *et al.*, 2014).

5. Impact of phenolic compounds on growth, metabolism and diversity of *Oenococcus oeni* during MLF and wine storage

(Publications based on this chapter: Collombel I., Campos F.M., Hogg T., 2019, Changes in the composition of the Lactic acid bacteria behavior and the diversity of Oenococcus oeni isolated from red wines supplemented with selected grape phenolic compounds. Fermentation, 5(1), 1; https://doi.org/10.3390/fermentation5010001)

5.1. Summary

Phenolic compounds are important components of wine and are known to have an impact on the physiology of wine microbes.

The influence of groups of phenolic compounds on the microbial growth and on the metabolism of lactic acid bacteria (LAB) in inoculated and non-inoculated red wines was investigated during malolactic fermentation (MLF) and subsequent storage. Moreover, representative *Oenococcus oeni* strains from non-treated wines and wines treated with flavonols and *trans*-resveratrol were isolated and analyzed by pulsed-field gel electrophoresis of rare restriction enzyme digests (REA-PFGE).

Yeast counts decreased faster in the inoculated samples with the addition of the phenolics tested. Twenty-eight days after MLF initiation, strains from all samples had reached the death phase, except those supplemented with *trans*-resveratrol. In the non-inoculated samples, the onset of lactic acid production was apparently delayed by all compounds tested. Depending on the concentration, (+)-catechin affected positively the yeast population and activated the malic acid degradation.

Increased levels of phenolics also delayed citrate consumption in inoculated samples. At the end of MLF the concentration of acetic acid was lower for the non-inoculated wines treated with the highest concentrations added in flavonols and hydroxycinnamic acids (HCA).

PFGE analysis revealed 22 genetic profiles, some of which were characteristic of specific samples. The results suggest that the commercial starter culture used in the inoculated wines did not dominate during MLF.

The effect of the phenolics studied was dependent on the origin and concentration of each as well as the incubation stage and whether the wines were inoculated. The effect of flavonols and *trans*-resveratrol also seemed to be strain-dependent.

5.2. Introduction

Malolactic fermentation is a desirable step in the vinification process of most red wines, which involves the transformation of L-malic to L-lactic acid. It is normally carried out by specific species of LAB. The result of a successful MLF is an increased microbial stability of wine through the

consumption of key carbon sources and other nutrients, which might otherwise be used by spoilage microorganisms, and through the production of antimicrobial compounds by LAB (Rammelsberg *et al.*, 1990; Henick-Kling and Stoewsand, 1993). The organoleptic properties of wine can be altered during MLF by the consumption and liberation of various metabolites (Laurent *et al.*, 1994; Mcdaniel *et al.*, 2008). Residual sugars can be metabolized into lactic acid or lactic acid, carbon dioxide and ethanol or acetic acid via the fermentative pathways of LAB. Acetic acid and hence the volatile acidity, can also increase in wine from the degradation of citric acid during MLF (Davis *et al.*, 1986). Malolactic fermentation can be spontaneous, due to the microflora present in the winery or in grapes, or it can be induced by inoculation with one of a number of selected starters, chosen according to their technological or quality attributes. Due to their adaptation to the wine environment, and in particular their tolerance to wine's acidity and alcohol concentration, strains of *O. oeni* are normally the predominant LAB responsible for the MLF. Thus, starter cultures for MLF are also predominantly selected from this species. The strain-level diversity of *O. oeni* populations in wine ecosystems is high, and it can be region- and winery-specific, often contributing to recognized differences in wines (Cafaro *et al.*, 2016; El Khoury *et al.*, 2016). For the same ethanol level *O. oeni* has been shown to genetically adapt according the type of wine (white or red), driven by pH and the phenolic compounds present (Breniaux *et al.*, 2018).

According to Bridier *et al.* (2010), taxonomically, the *O. oeni* species is ordered into three groups, with A and B being the two major phylogenetic groups, and C a putative group composed of a unique strain isolated from cider. Group A exclusively contains strains found in wine. All strains from cider, except that attributed to group C, are located in group B, while strains from Champagne and Burgundy are only found in group A (Bridier *et al.*, 2010). Campbell-Sills *et al.* (2015, 2017) suggested that most of the strains isolated from malolactic ferments derive from the domestication of ancestral *O. oeni* strains during the process of the industrialization of wine and cider, rather than responding to geographical constraints.

Several molecular techniques have been applied to determine the diversity of LAB in red wines without a prior culture step. Techniques based on polymerase chain reaction (PCR), including 16S metabarcoding sequencing (del Carmen Portillo and Mas, 2016b), PCR-DGGE (denaturing gradient gel electrophoresis) (García-Ruiz *et al.*, 2013a) and species-specific multiplex PCR (Petri *et al.*, 2013) have been used on samples taken directly from wines, but these are as yet limited to differentiation to the species level at most. To reach an intra-species discrimination level, a prior isolation stage is still required. Randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis of rare restriction enzyme digests (REA-PFGE) have been employed to investigate the strain diversity of several species of wine LAB (López *et al.*, 2008). PFGE proved to be a quick tool to study the *O. oeni* community, and their genetic variation in wine and the level of discrimination of PFGE depends on the restriction enzyme used (Bridier *et al.*, 2010; García-Ruiz *et al.*, 2013a; Cafaro *et al.*, 2016). To achieve a finer differentiation of isolated strains, other methods such as multilocus sequence typing (MLST) (Bridier *et al.*, 2010; García-Ruiz *et al.*, 2013a; El Khoury *et al.*, 2016), multiple locus variable number of tandem repeat analysis (VNTR) (Claisse and Lonvaud-

Funel, 2012), differential display PCR (Cafaro *et al.*, 2016), and single nucleotide polymorphism (SNPs) (El Khoury *et al.*, 2016) have been applied in this field.

In the wine context, phenolic compounds are naturally occurring molecules that derive from grape material or wood used for aging. They are important components of wine, contributing many sensorially and technologically-relevant traits to the finished product. Phenolics constitute a highly diverse group of compounds, having in common the presence of at least one phenolic ring in their structure, but varying greatly in structure and size. Many factors in the winemaking process influence the phenolics composition and concentration of the final wines. These begin with viticulture practices (including grape variety and clone selection) and continue in the winery procedures and parameters, amongst others. Must freezing, cryogenic maceration, extended maceration, enzyme regime and alcoholic fermentation temperature have been reported to increase phenolics concentrations in wines (Poussier *et al.*, 2003; Sacchi *et al.*, 2005; Castillo-Sánchez *et al.*, 2008; Del Llaudy *et al.*, 2008; Olejar *et al.*, 2015; Setford *et al.*, 2017). On the other hand, according to Olejar *et al.* (2015), mechanical harvesting contributes to decreases in phenolics through reactions with oxidative radicals. In a recent study (Caridi *et al.*, 2017), one *S. cerevisiae* strain used as starter for alcoholic fermentation was shown to enhance red wine content in phenolic compounds, especially in *trans*-resveratrol, *trans*-caffeic acid, quercetin and (-)-epicatechin.

Phenolic compounds found in wines are normally classified in two groups: flavonoids, which include anthocyanins, flavan-3-ols, condensed tannins and flavonols (and other flavone and flavanone derivatives), and the non-flavonoids, which comprise hydroxycinnamic acids and stilbenes among others (Monagas *et al.*, 2005). Wine flavan-3-ols, mainly (+)-catechin and (-)-epicatechin, are primarily synthesized in seeds and stems, and are the precursors of procyanidins and condensed tannins, which contribute to the astringency and bitterness of wines. The main flavonols found in grapes and wines are quercetin, myricetin, and kaempferol. Hydroxycinnamic acids are mostly found in grapes in their bound forms, located in the vacuoles of the skin and pulp cells. In their "free" forms (*trans*-*p*-coumaric, *trans*-ferulic and *trans*-caffeic acids), HCA are important compounds in the oxidation processes of wine, and act as color stabilizers and flavor precursors (Hernández *et al.*, 2006; Bouzanquet *et al.*, 2012; Lima *et al.*, 2018). *Trans*-resveratrol is the most important stilbene in wine, known for its putative health effects and antioxidant activity (Burns *et al.*, 2000), mostly coming from red grape skins, being biosynthesized via the phenylalanine pathway as a defense response to biotic and abiotic stresses (Hasan and Bae, 2017).

Some phenolic compounds have been shown to have a species and strain-dependent impact on the activity of bacteria, which can be relevant to wine quality. Phenolics can activate or inhibit growth and metabolism of wine microorganisms, depending on their structures and concentrations (Rozès *et al.*, 2003; Campos *et al.*, 2009b). Hydroxycinnamic acids, and especially *trans*-*p*-coumaric acid, have been shown to exhibit a strong inhibitory effect on the growth and survival of malolactic starters and wine-spoilage strains (Stead, 1993; Campos *et al.*, 2003; Bloem *et al.*, 2007; Harris *et al.*, 2010). In addition to their antimicrobial effects, the presence of HCA has been observed to increase the cell membrane permeability of some wine LAB, to delay their metabolism of glucose and citric acid, and to increase the yield of lactic and acetic acid production from glucose (Campos *et al.*, 2009a; Campos

et al., 2009b). According to Devi and Anu-Appaiah (2018a) the microbial response to exposure to HCA is, in part, manifested as changes in membrane and enzyme compositions. Quercetin and (+)-catechin can either stimulate cell growth and metabolism or have an antimicrobial effect, depending on their concentrations and the microorganisms targeted. Treutter *et al.* (2006) mention that flavonols possess antimicrobial activities linked to their antioxidant properties and Vaquero *et al.* (2007) investigated the negative impacts of both quercetin and (+)-catechin against pathogenic bacteria. (+)-Catechin has been observed to increase the cell density of *Lactobacillus hilgardii* (Alberto *et al.*, 2001), and both quercetin and (+)-catechin can stimulate MLF by *O. oeni* under certain conditions (Reguant *et al.*, 2000). Moreover, previously published researches (de Llano *et al.*, 2016) suggest that some levels of (+)-catechin can activate the cell growth of some *Pediococus pentosaceus* and *L. plantarum* strains. Devi and Anu-Appaiah (2018a) noted that among all the phenolic compounds tested, (+)-catechin exercised the least stress on the LAB tested. As for *trans*-resveratrol, this stilbene was described as a strong inhibitor against some contaminant yeasts and acetic acid bacteria (Pastorkova *et al.*, 2013). Furthermore, as reported by García-Ruiz *et al.* (2011), both stilbenes and flavonols may have a negative impact on the growth of the *O. oeni, L. hilgardii* and *P. pentosaceus* strains isolated from wine. In addition, flavonols, especially kaempferol, have been found to inactivate LAB by damaging their membranes (García-Ruiz *et al.*, 2009). The concentration of the compounds tested seems to be critical to any effect, and most of the studies on this subject have been performed in culture media, with concentrations of phenolic compounds far higher than those found in wines, and certainly not under real wine conditions.

In the present study, the development of the malolactic microbiota was studied throughout and following MLF conducted with and without *O. oeni* starter inoculation. This study evaluated the impact of the addition of specific groups of phenolic compounds on the behavior of wine microbiome, and more specifically, on the diversity of MLF starter *O. oeni* strains. For this purpose, a post-alcoholic fermentation red wine, supplemented with varying concentrations of flavan-3-ols, HCA, flavonols and *trans*-resveratrol was used. The development of specific *O. oeni* strains was followed in the samples treated with flavonols and *trans*-resveratrol. The novelty of the work lies in the fact that it was performed directly in post-alcoholic fermentation wine supplemented with concentrations of phenolics that are within the range encountered in real wine situations.

5.3. Material and Methods

5.3.1. Impact of phenolic compounds on growth and metabolism of wine microorganisms during spontaneous malolactic fermentation

5.3.1.1. Wine parameters

The red wine used for the experiment (13.18 ± 0.36 % (v / v) alcohol, pH 3.54 ± 0.02, 0.789 ± 0.03 g/L malic acid and 1.128 ± 0.022 g/L lactic acid) was a varietal wine from the Touriga Franca variety collected before malolactic fermentation in the Douro region in Northern Portugal from the 2016 harvest (stored at 4 °C before the initiation of the experiment). Touriga Franca is one of the most

widely grown variety in the Douro Demarcated Region, normally being blended with other varieties for the still and fortified wines of the region. The wine used had been through a spontaneous (non-inoculated) alcoholic fermentation with no addition of SO_2 at the end of fermentation.
The initial concentrations of phenolic compounds in the wine were: (+)-catechin 18.69 ± 1.47 mg/L; (-)-epicatechin 20.88 ± 0.38 mg/L; kaempferol 0.07 ± 0.01 mg/L; quercetin 2.44 ± 0.36 mg/L; *trans*-*p*-coumaric acid 0.30 ± 0.08 mg/L; *trans*-ferulic acid 0.08 ± 0.04 mg/L; *trans*-resveratrol 0.78 ± 0.14 mg/L.

5.3.1.2. Spontaneous malolactic fermentation

The flavonols quercetin and kaempferol, the HCA *trans*-*p*-coumaric and *trans*-ferulic acids, the stilbene *trans*-resveratrol and the flavan-3-ols (+) catechin and (-)-epicatechin (purities > 90%) were obtained from Sigma-Aldrich (Steinheim, Germany) and prepared in ethanol as described in section 2.4. The initial concentrations of these compounds in wine, except (+)-catechin, were tripled at the beginning of MLF, to mimic pre-fermentation variations, with values within the range encountered in real wine situations (Table 5.1). The concentration of the flavan-3-ol (+)-catechin was also tripled but with plus or minus 5 mg/L (addition of 35 and 45 mg/L of (+)-catechin instead of 40 mg/L).
Solutions of phenolic acids were added into 1 L autoclaved flasks containing the wine, later distributed into 50 mL sterile falcon tubes, each tube corresponding to a specific time-point (0, 3, 7, 9, 12 and 15 days incubation). The initial concentrations in phenolic compounds were verified before incubation by HPLC-DAD (as described in section 2.5). All treated wines (with addition of phenolics) were compared to the non-treated wine (control). All assays were prepared in duplicate and incubated at 25 °C in the dark without agitation. At each time-point, 100 µL sample of each wine was collected and directly diluted by plating on MRS (de Man, Rogosa & Sharpe) and YMB (Yeast Mold Broth) agar media for cells counting (prepared as described in sections 2.3.2 and 2.3.3.). Another 1 mL volume of sample was taken for immediate analysis of the wine content in L-malic by enzymatic reactions with the L-malic acid essay kit (Megazyme, Bray, Ireland) (detection limit = 0.25 mg/L) and the UV/Vis spectrophotometer set at a wavelength of 340 nm. Another sample of 1 mL was kept in a commercial freezer (−20 °C) for metabolite (sugars and organic acid) analysis by HPLC-UV-IR (as described in section 2.6).

Table 5.1. - Minimal and maximal concentrations (mg/L) in phenolic compounds in wines (during alcoholic fermentation, MLF, aging and in bottles) as reported in the literature.

Phenolic compounds	Bottled red wines (Zoechling *et al.*, 2009)		White wine during fermentation and short storage (Komes *et al.*, 2007)		Red wines during MLF (Hernández *et al.*, 2006)		Red wines during aging (Hernández *et al.*, 2006)		Red wine inoculated with different *O. oeni* strains (Hernandez *et al.*, 2007)		Red wine (Rossouw and Marais, 2004)	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Flavan-3-ols												
(+)-catechin	22.00	141.70	1.84	3.03	16.54	19.82	7.11	22.44	11.50	23.50	41.82	50.40
(-)-epicatechin	7.50	94.50	0.67	4.68	9.89	11.35	3.18	15.84	3.69	7.53	29.94	40.58
Flavonols												
Quercetin	nd	7.40	-	-	14.09	14.69	3.56	8.73	0.98	4.72	11.19	18.27
Kaempferol	nd	5.40	-	-	-	-	-	-	-	-	2.58	3.60
HCA												
Trans-p-coumaric acid	1.30	6.60	0.34	2.63	0.70	21.34	10.01	26.30	2.06	16.01	7.36	11.47
Trans-ferulic acid	0.30	1.00	2.02	3.20	-	-	0.73	1.41	nd	0.84	-	-
Stilbene												
Trans-resveratrol	0.07	3.95	-	-	0.59	1.01	nd	0.79	0.63	5.25	-	-

Min: minimum; Max: maximum; nd: not detected; - not quantified in the referred literature

5.3.2. Impact of phenolic compounds on growth and metabolism of wine microorganisms at the end of spontaneous and induced malolactic fermentations and during subsequent storage

5.3.2.1. Wine parameters

The same wine as section 5.3.1.1 was used but with a different metabolites composition due to a shorter time storage at 4 °C (3 months storage time for this section against 9 months for the previous section): 14.6 ± 0.3 % (v / v) alcohol; pH 3.48 ± 0.05; 1.510 ± 0.145 g/L malic acid; 0.757 ± 0.046 g/L lactic acid; (+)-catechin 28.34 ± 1.05 mg/L; (-)-epicatechin 12.60 ± 0.51 mg/L; kaempferol 1.84 ± 0.08 mg/L; quercetin 3.19 ± 0.25 mg/L; *trans*-*p*-coumaric acid 0.53 ± 0.07 mg/L; *trans*-ferulic acid 0.16 ± 0.11 mg/L; *trans*-resveratrol 1.30 ± 0.11 mg/L.

5.3.2.2. Malolactic fermentations and subsequent storage

The same protocol described in section 5.3.1.2 was applied for this experiment with some modifications.

The indegeneous LAB population of the wine at the beginning of these fermentations was around 10^4 CFU/mL. Non-inoculated (spontaneous) and inoculated MLF experiments were carried out in parallel. For the inoculated experiments, the commercial *O. oeni* strain Viniflora® Oenos™ was prepared as described in section 4.3.1 and directly inoculated into the wine at an initial concentration of 10^6 CFU/mL.

The initial concentrations of flavan-3-ols, flavonols, hydroxycinnamic acids and *trans*-resveratrol in the wine were doubled (2x) and tripled (3x), at levels consistent with those encountered in the real wine situations (Table 5.1.).

Control wines and wines containing phenolic compounds were prepared in duplicate and incubated 28 days at 25 °C and 142 days at 12°C in the dark with no agitation (Figure 5.1).

During the first incubation at 25 °C, the wine contents in L-malic acid and L-lactic acid were monitored by HPLC-UV-RI (as described in section 2.6). The end of MLF was considered to be reached when L-malic acid was no longer detected (detection limit determined by the statistics of the calibration curve: 0.09 g/L).

Samples (100 µL) were collected at the beginning of MLF - prior to the addition of phenolic compounds, and after 14, 28, 90 and 170 days incubation and directly diluted for plating and counting. Another 1 mL sample was kept in a commercial freezer (−20 °C) for later metabolite analysis.

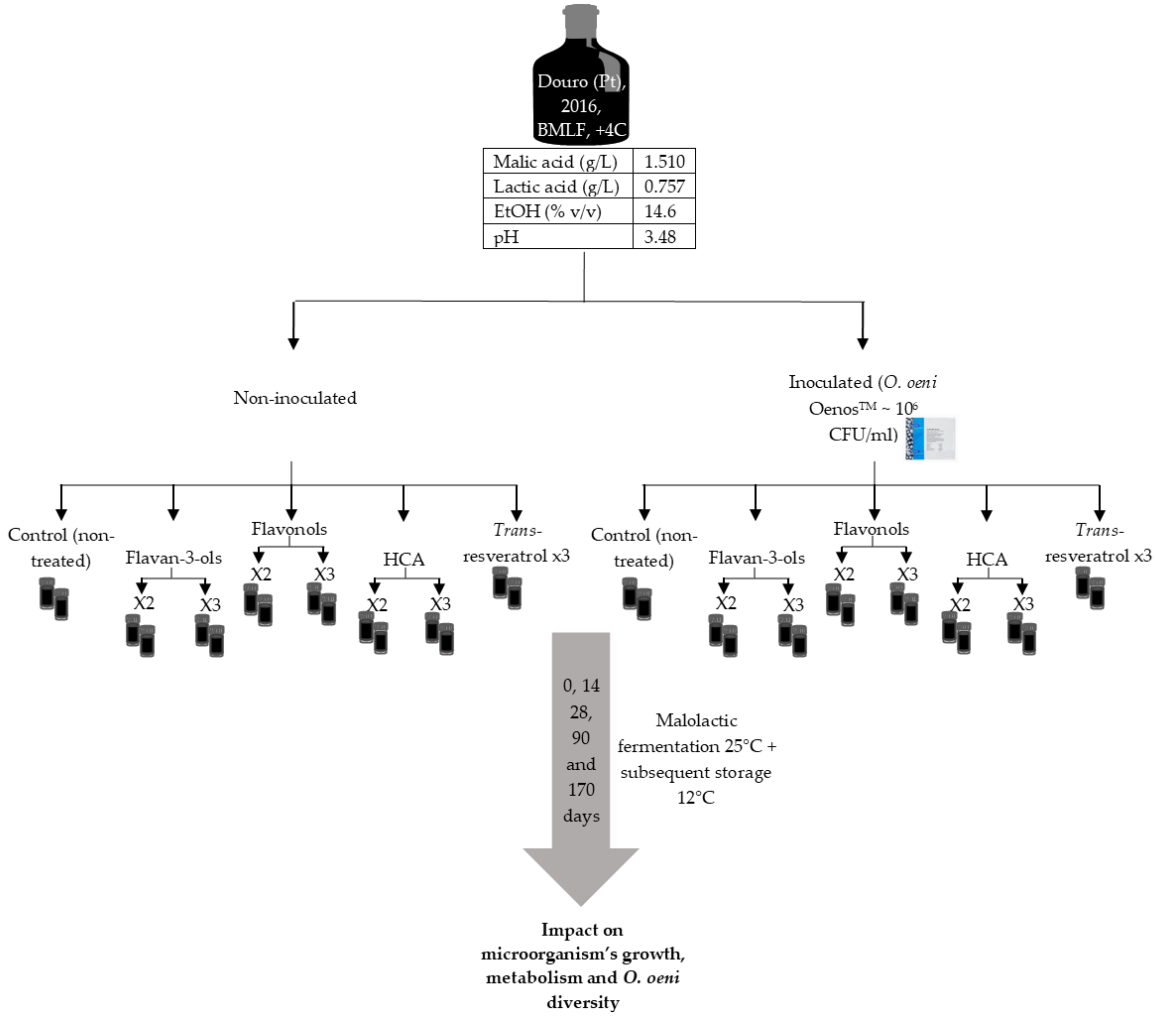


Figure 5.1. - Overall experimental process. Legend: Pt: Portugal; BMLF: Before malolactic fermentation.

5.3.3. Diversity of *O. oeni* isolated from red wines treated with flavonols and *trans*-resveratrol at the beginning of the MLF and 28 days after its initiation

5.3.3.1. Lactic acid bacteria isolation and identification

Each wine sample was plated on 3 Petri dishes split in 4 different dilutions from which 3 colonies (from 2 of the 4 dilutions containing more than 10 colonies and less than 100 colonies) were randomly picked from the first plate, 4 from the second one and 3 from the last one. Therefore, 10 isolated colonies out of around 330 were randomly chosen from samples at the beginning of the MLF and 28 days after its initiation both for the non-treated wines and the wines with double concentration of flavonols and *trans*-resveratrol. The isolates were sub-cultured onto the same agar medium for purification and each pure colony was then grown in liquid MRS medium and stored at −80 °C with 30% (*v* / *v*) glycerol (Sigma-Aldrich, Germany) before molecular analysis. The 80 isolated LAB strains were initially identified as *O. oeni* from their appearance under microscope (observed at ×1000 magnification).

5.3.3.2. Pulsed-field gel electrophoresis of rare restriction enzyme digests technique

5.3.3.2.1. Commercial strains as standards

The five commercial *O. oeni* strains from Ch. Hansen described in section 2.1 plus the strains Alpha™ and VP41™ from Lallemand (Montreal, QC, Canada) were used in this study to optimize the REA-PFGE protocol, and for comparison with the indigenous strains. After re-suspension into peptone water (10 g peptone, 5 g NaCl in 1 L H_2O, pH 7.2), 10^6 CFU/mL pre-cultures were prepared in MRS broth.

5.3.3.2.2. Method

The REA-PFGE protocol described by García-Ruiz *et al.* (2013) was followed with some modifications. Except where otherwise stated, all suspensions were grown without agitation at 25 °C. Pre-cultures of 2 mL in MRS broth were made for each isolated strain from stock cultures and grown for 7 days. The cultures used for digestion and electrophoresis were prepared by inoculating 4 mL of MRS medium with 40 µL (1 % *v*/*v*) of the pre-cultures and growing these for 6 days to reach an absorbance of approximately 0.5 at a 600 nm wavelength (as described in section 2.3.2.). Cells were harvested by centrifugation (5 min, 10000 *g*) at room temperature and the supernatant was discarded. Afterwards, the pellets were washed twice with 1 mL of 1 × TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) before being resuspended in 200 µL of $T_{100}E$ (10 mM Tris-HCl, 100 mM EDTA, pH 8). The obtained cell suspensions were mixed with an equal volume of 1 % (w/v) SeaKem™ Gold Agarose (Lonza, Basel, Switzerland), which was pre-melted and kept at 60 °C. The mixtures were placed in PFGE molds to obtain three plugs per strain which were let to solidify for 20 min at room temperature. Cells embedded in the agarose plugs were lysed for 3 h at 37 °C in 1 mL lysozyme (10 mg/mL buffer) from Thermo Fisher Scientific (Waltham, MA, USA). The lysis buffer was replaced with 1 mL of 2 mg/mL Pronase E from *Streptomyces griseus* (Sigma-Aldrich) / 1.5 % N-lauryl sarcosyl buffer in $T_{100}E$, and incubated for 16 h at 37 °C. The plugs were rinsed four times for 30 min with 4 mL 1 × TE in a shaking water bath set at 55 °C. The 5 mL tubes containing the plugs were filled with 5 mL 1 × TE and stored at 4 °C before restriction enzyme digestion. A half-plug of each strain was digested with 20 U *Not*I restriction endonuclease (10 U/mL, Thermo Fisher Scientific) in a volume of 200 µL for 16 h at 37 °C. As a size marker and normalization reference, plugs of *Salmonella* serotype Braenderup H98122 restricted with *Xba*I (10 U/mL, Thermo Fisher Scientific) 50 U 2 h / 37 °C were prepared and kept at 4 °C before electrophoresis. Standards were placed in the gel every six lanes. The digested DNA fragments were separated by electrophoresis in a 1 % SeaKem™ Gold Agarose in 3 L 0.5× TBE buffer (0.1 M Tris, 0.09 M boric acid, 0.01 M EDTA, pH 8) with a CHEF-DRIII apparatus from Bio-Rad laboratories (Hercules, California, CA, USA). Electrophoresis was performed for 20 h at 15 °C, 6 V/cm, with interpolation pulse time of 2.5 s to 25 s, and an included angle of 120°. Gels were stained with ethidium bromide (0.7 mg/mL) and washed twice in deionized water, and images were taken using a Bio-Rad ChemiDoc™ MP System (Bio-Rad laboratories). The fingerprint data were analyzed using BioNumerics 6 software (Applied Maths NV, Sint-Martens-

Latem, Belgium). The Dice coefficient with 1 % optimization and 2 % band position tolerance settings and the clustering algorithm UPGMA were chosen to compare the PFGE profiles.

5.3.4. Statistical Analysis

As explained in section 2.8., data were subjected to statistical analysis using JMP13 for Windows XP (Taikoo Shing, Hong Kong, China), at a confidence level of 95% (p = 0.05). Dunnett´s test was run to compare the means of each sample relatively to the controls.

5.4. Results

5.4.1. Impact of phenolic compounds on growth and metabolism of wine microorganisms during spontaneous malolactic fermentation

5.4.1.1. Impact of phenolics on microbial populations

During spontaneous malolactic fermentation, the LAB populations (Figure 5.2.a) increased while the yeast populations (Figure 5.2.b) decreased.

The addition of flavonols was inhibiting the bacterial development. Flavan-3-ols ((+)-catechin 45 mg/L) and HCA also had a negative impact on the bacterial growth but slighter and not significative. Fifteen days after the initiation of MLF, less bacteria were found in samples supplemented with *trans*-resveratrol (Figure 5.2.a).

The yeast concentration was slightly higher along MLF in wines initially supplemented with flavan-3-ols ((+)-catechin 35 mg/L) (Figure 5.2.b).

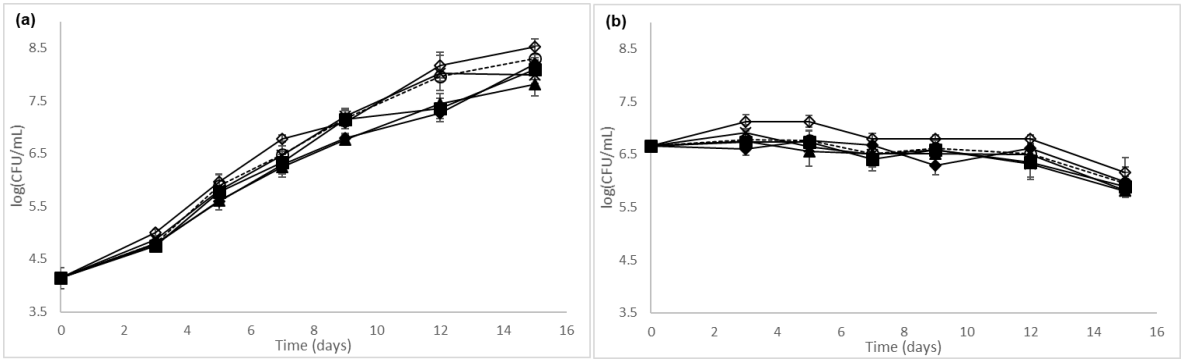


Figure 5.2. - (a) Lactic acid bacteria and (b) yeast populations during incubation at 25 °C in uninoculated wines supplemented with (◊) flavan-3-ols ((+)-catechin 35 mg/L), (♦) flavan-3-ols ((+)-catechin 45 mg/L), (▲) flavonols, (■) HCA and (x) *trans*-resveratrol and compared to controls (○). Error bars represent standard deviations of three replicates.

5.4.1.2. Impact of phenolics on bacterial metabolism

No citric acid was detected in the wine samples (detection limit: 0.26 g/L).

Wines supplemented with flavan-3-ols ((+)-catechin 35 mg/L) consumed L-malic acid faster (Figure 5.3). No L-malic acid was detectable in the samples supplemented with flavan-3-ols ((+)-catechin 35

mg/L) after 12 days instead of 15 days for the controls. The biggest differences regarding sugar and organic acids metabolism were observed 15 days after the initiation of MLF with a delay in the malic acid degradation, and in lactic acid production in the samples supplemented in flavan-3-ols ((+)-catechin 45 mg/L), flavonols and HCA (Figure 5.3.a and b).

Less acetic acid was produced in the wines supplemented in flavonols and HCA, highlighting a possible negative impact from those compounds on the citric acid degradation or the heterofermentative pathway from glucose by the indigenous LAB (Figure 5.3.c).

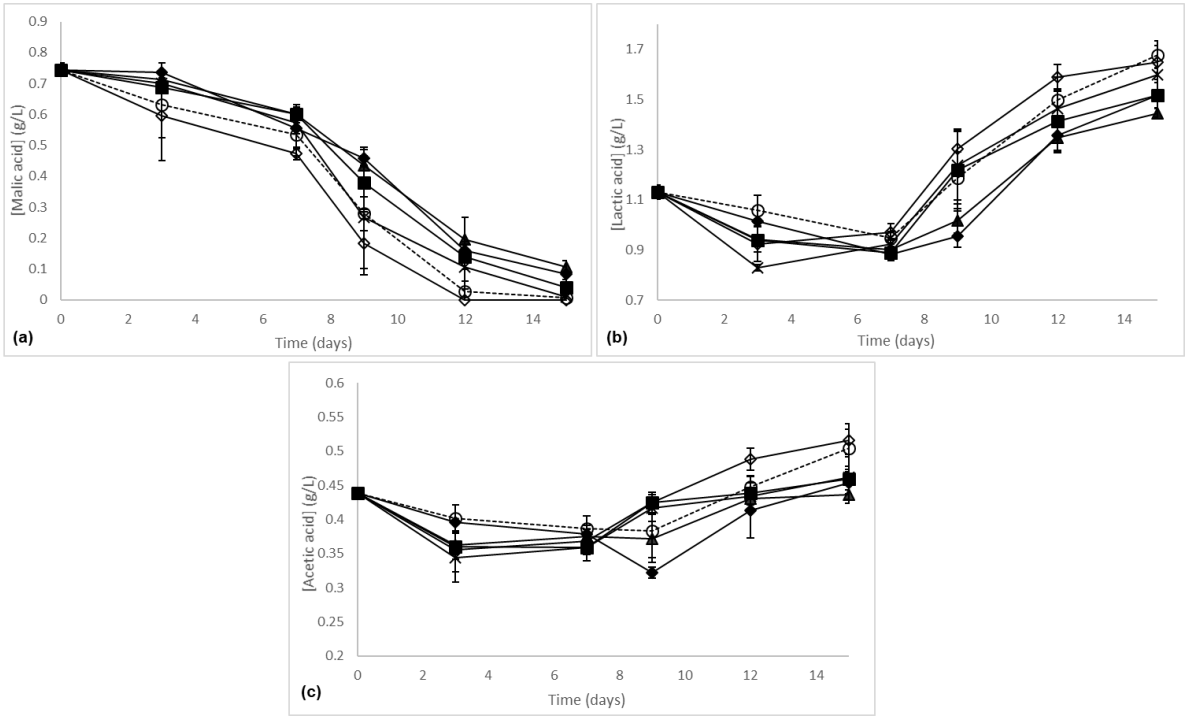


Figure 5.3. - (a) Malic, (b) lactic and (c) acetic acids´ concentrations during incubation at 25 °C in wines supplemented with (◊) flavan-3-ols ((+)-catechin 35 mg/L), (♦) flavan-3-ols ((+)-catechin 45 mg/L), (▲) flavonols, (■) HCA and (x) *trans*-resveratrol and compared to controls (○). Error bars represent standard deviations of four replicates.

5.4.2. Impact of phenolic compounds on growth and metabolism of wine microorganisms at the end of spontaneous and induced malolactic fermentations and during subsequent storage

5.4.2.1. Impact of phenolics on microbial populations

A significant reduction in the total bacterial growth was observed after 14 days of incubation for both spontaneous and inoculated samples containing three times their initial concentrations in flavonols and HCA (Figure 5.4.a). At the lower concentrations tested, no impact was detected by flavonols, and only the bacterial growth rate of the wines undergoing spontaneous MLF was affected by HCA. At the single concentration under which it was tested (three times the original concentration), *trans*-resveratrol showed an inhibitory effect similar to the double concentration of HCA on the non-inoculated samples.

The lactic acid bacteria counts were lower after 28 days of incubation than after 14 days, assuming that LAB were in the death phase (Figure 5.4.b). Inferior LAB populations were noticed after 28 days of incubation for wines containing flavan-3-ols (two times the original concentrations in non-inoculated wines and three times the original concentrations in inoculated wines). In the contrary, in the case of both the inoculated and non-inoculated samples supplemented in *trans*-resveratrol, the bacterial population was higher than for the other samples. No significant effect from the flavonols and HCA addition was observed at this stage.

While all the colonies observed on MRS agar from wines incubated at 25 °C looked the same to the naked eye, strains of different shape, that looked like *Lactobacillus* under microscope, were detected when plating the wines stored at 12 °C. Except for the wines treated with 3x HCA which had lower concentration in *Lactobacillus* (5.53 ± 0.15 log (CFU/mL)) than the controls (6.42 ± 0.13 log (CFU/mL)) after 3 months storage (90 days incubation), no differences between wines were observed at this stage.

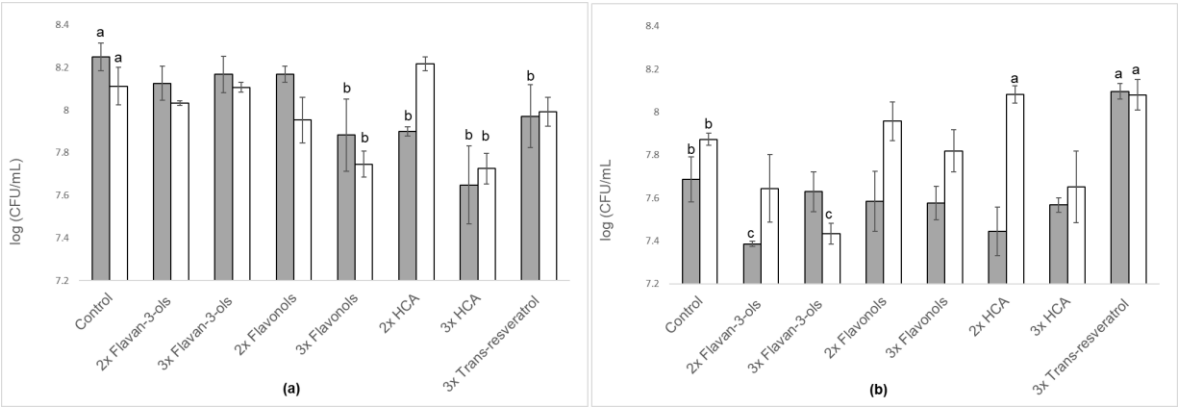


Figure 5.4. – Lactic acid bacteria counts at 14 (a) and 28 (b) days after the beginning of MLF in wine samples supplemented with phenolic compounds. Grey bars - spontaneous MLF. White bars - MLF inoculated with *O. oeni* Oenos™. a, b and c indicate values statistically significantly different at $p < 0.05$, n = 3, Dunnett´s test.

Yeast concentrations were decreasing in wine with time, from ~$9.5*10^6$ CFU/mL at the beginning of MLF to ≤ $3.5*10^3$ CFU/mL 170 days after. Differences between samples appeared after the end of MLF (day 14), the strongest effects being observed for the wines inoculated with Oenos™ (Figure 5.5). Yeast concentrations decreased faster in the inoculated samples with the addition of all the phenolics tested. Higher the concentrations in phenolics were, stronger the inhibitory effect was.

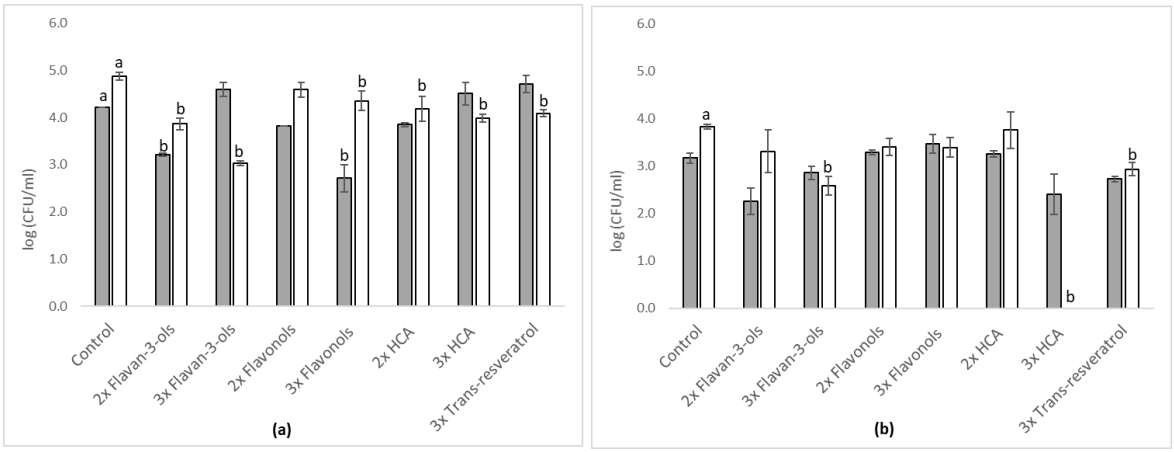


Figure 5.5. - Yeast counts at 28 (a) and 90 (b) days after the beginning of MLF in wine samples supplemented with phenolic compounds. Grey bars - spontaneous MLF. White bars - MLF inoculated with *O. oeni* Oenos™. a and b indicate values statistically significantly different at $p < 0.05$, $n = 3$, Dunnett´s test.

5.4.2.2. Sugar and organic acids metabolism

Malolactic fermentation was considered to be finished in all the wines 14 days after its initiation, as L-malic acid was no longer detectable at this time point.

Bacterial metabolism was affected differently, depending on the type of MLF (spontaneous or induced), and on the phenolic compounds added. Table 5.2 shows that the addition of all phenolic compounds, except for flavan-3-ols, delayed the production of lactic acid in the samples undergoing spontaneous MLF. In the inoculated wines, only flavan-3-ols, at the triple concentration, suppressed lactic acid production (Table 5.3). At the end of MLF, the concentration of acetic acid appeared to be lower for the non-inoculated wines treated with triple concentrations of flavonols, HCA and *trans*-resveratrol (respectively, 0.34 ± 0.03 g/L, 0.32 ± 0.03 g/L and 0.33 ± 0.05 g/L, against 0.43 ± 0.00 g/L for the controls). However, the contrary effect was observed after 28 days of incubation for the samples with triple concentrations of HCA and *trans*-resveratrol where higher concentrations of acetic acid were observed. Citric acid was already completely consumed at the end of MLF in the wines that went through the spontaneous MLF. In the samples inoculated with Oenos™, the addition of the phenolics decreased citrate consumption during MLF when compared to the control (Table 5.3).

Table 5.2. - Effect of phenolic compounds addition on sugars and organic acids metabolism of wine LAB after 28 days of spontaneous MLF.

Treatments	**Fructose (g/L)**		**Lactic Acid (g/L)**		**Acetic Acid (g/L)**	
	Before	After	Before	After	Before	After
Control	0.84 ± 0.04[y]	0.62 ± 0.06	0.78 ± 0.05	2.77 ± 0.03	0.26 ± 0.00	0.59 ± 0.02
2x Flavan-3-ols		0.51 ± 0.06		2.71 ± 0.00		0.69 ± 0.02
3x Flavan-3-ols		0.47 ± 0.02		2.76 ± 0.00		0.59 ± 0.02

2x Flavonols	0.49 ± 0.09	2.38 ± 0.12	0.59 ± 0.00
3x Flavonols	0.51 ± 0.01	2.39 ± 0.05	0.59 ± 0.00
2x HCA	0.46 ± 0.02	2.41 ± 0.17	0.59 ± 0.00
3x HCA	0.49 ± 0.02	2.29 ± 0.10	0.65 ± 0.02
3x *Trans*-resveratrol	0.41 ± 0.01	2.38 ± 0.07	0.69 ± 0.02

y values represent the mean ± standard deviation of two replicates.

Table 5.3 - Effect of phenolic compounds addition on sugars and organic acids metabolism of wine LAB after 28 days of MLF in wines inoculated with *O. oeni* Oenos™.

Treatments	**Citric acid (g/L)**		**Fructose (g/L)**		**Lactic acid (g/L)**		**Acetic acid (g/L)**	
	Before	After [h]	Before	After	Before	After	Before	After
Control	1.46 ± 0.07[y]	nd	0.87 ± 0.02	0.41 ± 0.01	0.78 ± 0.03	2.66 ± 0.02	0.26 ± 0.01	0.72 ± 0.02
2x Flavan-3-ols		0.63 ± 0.08		0.47 ± 0.03		2.65 ± 0.00		0.71 ± 0.00
3x Flavan-3-ols		0.80 ± 0.21		0.50 ± 0.01		2.46 ± 0.10		0.71 ± 0.00
2x Flavonols		0.80 ± 0.37		0.52 ± 0.02		2.65 ± 0.00		0.71 ± 0.00
3x Flavonols		0.61 ± 0.10		0.49 ± 0.04		2.65 ± 0.00		0.71 ± 0.00
2x HCA		0.69 ± 0.05		0.42 ± 0.01		2.65 ± 0.00		0.71 ± 0.00
3x HCA		0.93 ± 0.29		0.51 ± 0.02		2.65 ± 0.00		0.71 ± 0.00
3x *Trans*-resveratrol		0.76 ± 0.26		0.42 ± 0.05		2.65 ± 0.00		0.71 ± 0.00

y values represent the mean ± standard deviations of two replicates; h citric acid consumed at end-MLF (day 14). nd = not detected (detection limit of citric acid = 0.26 g/L).

5.4.3. Diversity of *O. oeni* isolated from red wines treated with flavonols and *trans*-resveratrol at the beginning of the MLF and 28 days after its initiation

Pulsed-field gel electrophoresis of rare restriction enzyme digests analysis of genomic DNA from 7 commercial *O. oeni* strains showed that the *Not*I enzyme, used at 20 U, yielded more discriminating restriction fragments than *Apa*I (20 U and 40 U), producing different patterns consisting of 8–12 bands in the range of 33.3 kb–1135 kb (Figure 5.6).

A total of 70 *O. oeni* colonies isolated from spontaneous and inoculated fermentations, as well as 7 commercial starters were characterized genotypically by REA-PFGE. It was not possible to grow 7 of the isolates, and for 3 further isolates, it was not possible to obtain a restriction enzyme digest (Table 5.4). Cluster analysis of the PFGE profiles of the 70 *O. oeni* isolates revealed 22 genotypes

with specific profiles, 3 of these (P, T, V) contained commercial strains (Figure 5.7). Most of the isolated strains digested with *Not*I shown predominantly 8, 9, or 10 bands.

On inspecting the dendrogram (Figure 5.7), the percentage of similarity between the unrelated profile varied from 62.5% to 96%. The 7 commercial strain profiles were clustered together at 85% similarity. CiNe™ and Oenos™ profiles were ≥95% similar to CH16™ and CH35™.

The results also showed a clear distinction between most of the *O. oeni* strains isolated at the beginning of incubation, and those isolated 28 days later. The strains of profiles A, B, C (5 strains), D and E were isolated specifically from the wines at the beginning of the MLF. Some strains from the profiles L and N were detected at both time-points (Table 5.4).

Differences in the *O. oeni* strain diversity were observed between inoculated and non-inoculated samples supplemented with the same family of phenolic compounds. Considering the 10 colonies isolated out of around 330 per sample, the commercial strain Oenos™, used as a starter for the inoculated wine samples, did not appear to predominate during the malolactic fermentation. In fact, only one of the colonies isolated from the inoculated non-treated wines (WP265O) after 28 days of incubation, had the same PFGE profile as this starter strain (V). Two other strains considered by this analysis to be part of the same PFGE profiles of, respectively, Alpha™ (P) and CH11™ (T), were found. The first one was in the non-inoculated wine treated with flavonols, and the second one was in the inoculated, non-treated wine. Among the 22 PFGE profiles, 12 were characterized by a unique strain, with the inoculated, non-treated wine containing the most of these (Figure 5.7).

Profiles L, F, and N showed the highest number of strains with 15, 13, and 8 isolates, respectively. The profile L was mostly present after 28 days of incubation in the non-supplemented samples, and F in the samples supplemented with flavonols, in the case of inoculated and non-inoculated wines (Table 5.4).

Concerning the *O. oeni* diversity in the wines supplemented with phenolic compounds compared to the non-supplemented ones, the profiles X, Z and M were found only in non-supplemented wines (Table 5.4). X and Z were unique strain profiles from inoculated samples, and profile M was composed of three strains from non-inoculated samples. I (three strains) and Q (unique strain) were two PFGE profiles only found in inoculated wines with added *trans*-resveratrol, contrary to J (two strains) and O (three strains) which were present in all the samples after 28 days of incubation, but the ones that were treated with the stilbene were studied (Table 5.4). G and H (one strain each) were exclusively observed in wines treated with flavonols, contrary to Y (three strains). The strains from profile K were isolated in wines treated with both the phenolics studied (Table 5.4).

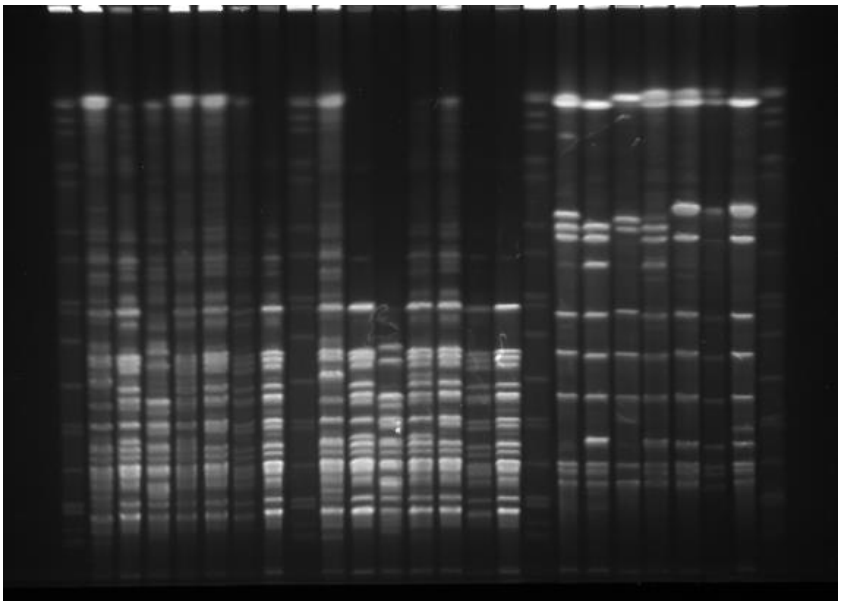


Figure 5.6. - REA-PFGE gel of 7 commercial *O. oeni* strains (CH11™, CH16™, CH35™, Oenos™ and CiNe™ from Ch. Hansen and Alpha and VP41 from Lallemand) using 2 different restriction enzymes (*Apa*I and *Not*I) at different concentrations (20 and 40 U). From left to right: 20 U *Apa*I CH11™, 20 U *Apa*I CH16™, 20 U *Apa*I Alpha, 20 U *Apa*I CH35™, 20 U *Apa*I Oenos™, 20 U *Apa*I CiNe™, 20 U *Apa*I VP41, Control, 40 U *Apa*I CH11™, 40 U *Apa*I CH16™, 40 U *Apa*I Alpha, 40 U *Apa*I CH35™, 40 U *Apa*I Oenos™, 40 U *Apa*I CiNe™, 40 U *Apa*I VP41, Control, 20 U *Not*I CH11™, 20 U *Not*I CH16™, 20 U *Not*I Alpha, 20 U *Not*I CH35™, 20 U *Not*I Oenos™, 20 U *Not*I CiNe™, 20 U *Not*I VP41.

Table 5.4.- *Oenococcus oeni* strains isolated from spontaneous and inoculated red wines fermented in the absence/presence of phenolic compounds: flavonols and *trans*-resveratrol.

Red wine	Treatment	Sampling Time (days)	*O. oeni* Isolated	PFGE Profile	Red Wine	Treatment	Sampling Time (days)	*O. oeni* Isolated	PFGE Profile	Commercialized Strains	PFGE Profile
Non-inoculated	Control	0	WP201C	nd	Inoculated	Control	0	WP201O	nd	Oenos™	V
			WP202C	nd				WP202O	N	CH11™	T
			WP203C	L				WP203O	L	CH16™	R
			WP204C	N				WP204O	D	CH35™	S
			WP205C	A				WP205O	E	CiNe™	W
			WP206C	C				WP206O	C	Alpha™	P
			WP207C	B				WP207O	F	VP41™	U
			WP208C	L				WP208O	F		
			WP209C	N				WP209O	C		
			WP210C	C				WP210O	C		
		28	WP266C	M			28	WP263O	Z		
			WP267C	L				WP264O	X		
			WP268C	nd				WP265O	V		
			WP269C	M				WP266O	F		
			WP270C	Y				WP267O	L		
			WP271C	N				WP268O	T		
			WP272C	L				WP269O	N		
			WP273C	J				WP270O	L		
			WP274C	L				WP271O	O		
			WP275C	L				WP272O	nd		
	Flavonols	28	WP294C	L		Flavonols	28	WP291O	nd		

			WP295C	L				WP292O	J
			WP296C	nd				WP293O	L
			WP297C	P				WP294O	H
			WP298C	F				WP295O	F
			WP299C	M				WP296O	F
			WP2100C	K				WP297O	F
			WP2101C	F				WP298O	F
			WP2102C	G				WP299O	O
			WP2103C	O				WP2100O	N
	Trans-resveratrol	28	WP2114C	L		*Trans*-resveratrol	28	WP2111O	nd
			WP2115C	M				WP2112O	nd
			WP2116C	Y				WP2113O	I
			WP2117C	F				WP2114O	I
			WP2118C	nd				WP2115O	F
			WP2119C	N				WP2116O	F
			WP2120C	N				WP2117O	L
			WP2121C	F				WP2118O	Y
			WP2122C	K				WP2119O	Q
			WP2123C	L				WP2120O	I

nd: strains that did not grow when cultivated for REA-PFGE.

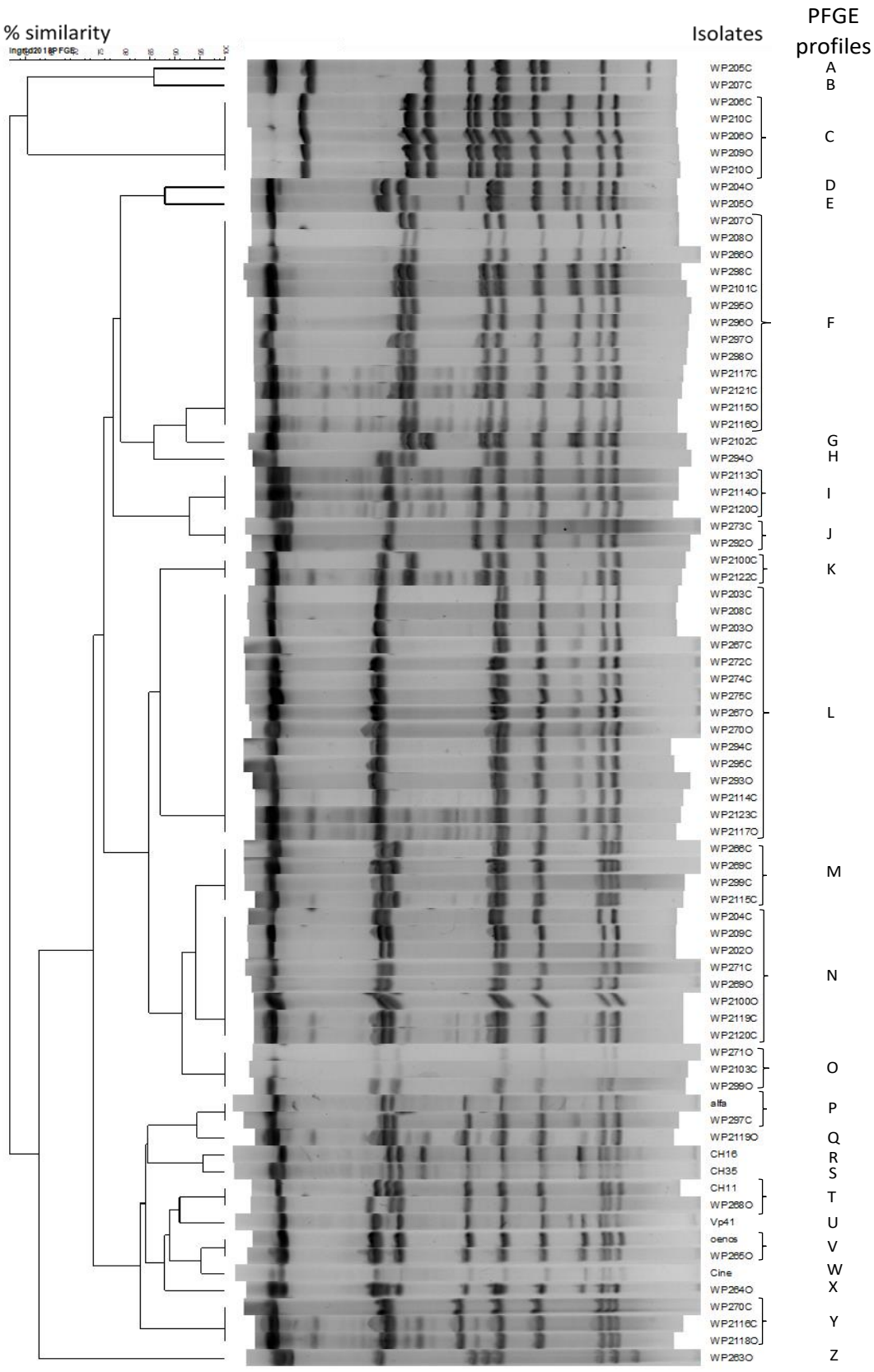

% similarity
Isolates
PFGE profiles
WP205C A
WP207C B
WP206C
WP210C
WP206O C
WP209O
WP210O
WP204O D
WP205O E
WP207O
WP208O
WP266O
WP298C
WP2101C
WP295O
WP296O F
WP297O
WP298O
WP2117C
WP2121C
WP2115O
WP2116O
WP2102C G
WP294O H
WP2113O
WP2114O I
WP2120O
WP273C J
WP292O
WP2100C K
WP2122C
WP203C
WP208C
WP203O
WP267C
WP272C
WP274C
WP275C
WP267O L
WP270O
WP294C
WP295C
WP293O
WP2114C
WP2123C
WP2117O
WP266C
WP269C M
WP299C
WP2115C
WP204C
WP209C
WP202O
WP271C N
WP269O
WP2100O
WP2119C
WP2120C
WP271O
WP2103C O
WP299O
alfa P
WP297C
WP2119O Q
CH16 R
CH35 S
CH11 T
WP268O
Vp41 U
oenos V
WP265O
Cine W
WP264O X
WP270C
WP2116C Y
WP2118O
WP263O Z

Figure 5.7.- Dendrogram based on the *Not*I Pulsed-field gel electrophoresis of rare restriction enzyme digests (REA-PFGE) profiles of the 19 unrelated patterns of the 80 *O. oeni* strains isolated in this study, and 7 commercial *O. oeni* strains.

5.5. Discussion

In order to analyze the effect of an increase of particular classes of phenolics on MLF and subsequent storage, the concentrations of flavan-3-ols, hydroxycinnamic acids, flavonols, and *trans*-resveratrol were doubled and tripled in inoculated and non-inoculated, post-alcoholic fermentation wines. The results obtained indicate that the effect of the phenolics on growth and metabolism of wine microorganisms, as well as on the intraspecific diversity of *O. oeni*, was influenced by the type of phenolic compounds and their concentrations, the stage of the incubation and whether the wines were inoculated or not.

The addition of all the compounds tested caused a delay in the lactic acid production for the non-inoculated wines. The reduction of the lactic acid production rate by the microbiota of non-inoculated samples treated with the phenolics studied is concomitant with the inhibitory nature of the compounds at the concentrations tested.

The flavan-3-ol (+)-catechin had a mixed effect during the spontaneous MLF depending on its concentration and the microbial population. At lower concentration added in the wine used in the first part of this study, (+)-catechin had a positive impact on the yeast population and activated malic acid degradation. At slightly higher concentration added in the same wine (+ 10 mg/L), this compound inhibited the bacterial growth and delayed the lactic acid production. These observations are in agreement with previous studies showing the variable effect of (+)-catechin on different LAB (Reguant *et al*., 2000; Alberto *et al.*, 2001; Vaquero *et al.*, 2007; de Llano *et al*., 2016).

In wines inoculated with Oenos™, the decrease in yeast population was accelerated by the addition of phenolic compounds.

Except for the wines treated with triple HCA concentrations which had lower concentration of *Lactobacillus*, no microbial differences were noticeable between the samples during storage at 12 °C.

Less acetic was found at the end of MLF (14 days) in non-inoculated wines which had an initial concentration in *trans*-resveratrol (around 1.30 mg/L) tripled. At a lower initial concentration (around 0.78 mg/L), the increase to three times its concentration didn't seem to impact the LAB metabolism of the wine. Acetic acid production was also apparently repressed in the non-inoculated samples by triple HCA concentration at the end of MLF (14 days), although it increased after 28 days in both samples supplemented with triple HCA and *trans*-resveratrol concentrations. A possible shift in the metabolic pathway of glucose consumption in Oenos™ towards acetic acid production, caused by hydroxycinnamic acids was previously observed by Campos *et al.* (2009a) in experiments performed in growth medium.

All tested compounds caused an apparent delay in citric acid degradation by the LAB in inoculated wines. A similar observation on citrate metabolism was shown by Campos *et al.* (2009a), using the same *O oeni* stain that was inoculated in the growth medium and supplemented with HCA and other benzoic acids.

Similarly to the results reported by García-Ruiz *et al.* (2011), *trans*-resveratrol had a negative impact on growth and metabolism of LAB in non-inoculated samples as flavonols and HCA. Nevertheless, in this study, 28 days after initiation of incubation, the bacterial concentrations were slightly higher in the wines treated with *trans*-resveratrol.

Natural phenolic extracts have been tested on the *O. oeni* intra-diversity in wine but the effect of the addition of the individual families of phenolic compounds so far has not been explored (García-Ruiz *et al.*, 2013a; Breniaux *et al.*, 2018). The peculiar activity of flavonols and *trans*-resveratrol against the *O. oeni* distribution in the wines was investigated in a preliminary way in this current study. REA-PFGE has been used successfully for strain typing *O. oeni* in red wines (García-Ruiz *et al.*, 2013a; Cafaro *et al.*, 2016; El Khoury *et al.*, 2016). In this work, a REA-PFGE technique was employed on the 80 representative isolates from the non-treated wines, and the wines treated with flavonols and *trans*-resveratrol and identified as *O. oeni* by microscopic observation. *Not*I was used as a restriction enzyme to study the diversity and evolution of the wine *O. oeni* population. The isolates showed 22 different genetic profiles, indicating a considerable intra-specific diversity in the wines. One of the 80 colonies that were randomly selected out of around 330 for subculture from the wines with no prior addition of phenolic compounds presented the same pattern as Oenos™, the starter that was employed in the experiment. The post-alcoholic fermentation wines used in this study had indigenous LAB populations of around 10^4 CFU/mL. Together with a high diversity of *O. oeni* strains, these factors could explain why Oenos™, in inoculated experiments appeared not to dominate the indigenous microbiota in this particular wine. The diversity of *O. oeni* was more influenced by fermentation time than the type of MLF (inoculated or not) and the type of phenolic compounds tested under study. Indeed, as observed in the dendogram of the PFGE profiles, two clear clusters, corresponding to the two strain isolation time points, grouped all the patterns. The effect of phenolics on *O. oeni* seems also to be strain-dependent. Some authors (García-Ruiz *et al.*, 2011) have observed a strong inhibitory effect for quercetin against four different *O. oeni* strains, with an IC_{50} value of 0.148 to 0.454 g/L, while others (Reguant *et al.*, 2000) have shown that the addition of 5–25 mg/L of quercetin activated malic acid degradation by other *O. oeni* strains. In our study, the profile L, grouping the highest number of strains, was less represented in the phenolics-treated samples. Moreover, the profiles X, Z, and M were found only in the wines with no prior addition of phenolic compounds. A possible interpretation is that these strains are less tolerant to flavonols and stilbenes. The same goes for the strains from profiles I and Q, which are possibly enhanced by *trans*-resveratrol, whilst strains from profiles G and H might be activated by flavonols. On the other hand, strains from profiles J and O could be negatively impacted by *trans*-resveratrol and those from profile Y, by flavonols. These results seem to indicate that flavonols and *trans*-resveratrol could affect *O. oeni* positively or negatively, depending on the specific strain.

In summary, the effects of the addition of specific phenolic compounds on microbial behavior during MLF and subsequent storage in wine have been described in this chapter, highlighting the importance, in this respect, of the pre-malolactic winemaking techniques that influence the phenolics composition of the wine. More particularly, the increase of flavonols and *trans*-resveratrol concentrations at this stage could

influence the malolactic bacterial population at the strain level and therefore possibly also the metabolic activities occurring during this period. More work should be focused on the specific effect of these compounds on each PFGE profile. Moreover, a broader approach to this study could be apply to different wines with other starters.

6. The impact of phenolic compounds on the chemical composition in wines during malolactic fermentation and subsequent storage

(Publications based on this chapter: Collombel I., Campos F.M., Hogg T.A. The impact of phenolic compounds on the chemical composition in wines during malolactic fermentation and subsequent storage. In submission form for Beverages.)

6.1. Summary

The concentrations of phenolic compounds in wine are regulated by many pre-malolactic fermentation factors and are well known to impact the microbial population of the wine, thus being susceptible to alter its organoleptic properties. Specific groups of phenolics were added, post-alcoholic fermentation in red wines and their volatile and phenolic compositions were analyzed by HPLC-DAD and GC-FID during malolactic fermentation (MLF) and subsequent storage. All the phenolics added diminished over time and some were no longer detectable in wines after 170 days incubation. The addition of *trans*-*p*-coumaric and *trans*-ferulic acids in wines induced an increase in *trans*-caffeic acid and a degradation of *trans*-caftaric acid, probably via the cinnamoyl esterase activity of some lactic acid bacteria (LAB). The addition of flavonols possibly caused an inhibition of these bacteria as the contents in *trans*-caftaric and *trans*-coutaric acids were higher, 170 days after the beginning of MLF, in inculated wines with flavonols added compared to the controls. Depending on their concentrations and the time of incubation, flavan-3-ols apparently inhibit the ability of some LAB to hydrolyze and/or synthesize volatile esters. Wines inoculated with Oenos™ were more sensitive to the addition of hydroxycinnamic acids (HCA) and *trans*-resveratrol during MLF (14 days) than non-inoculated wines as less 1-hexanol, 2-phenylethanol, isobutyric, isovaleric, octanoic and decanoic acids were detected in these wines compared to the controls. Moreover, 170 days after the beginning of MLF, HCA and *trans*-resveratrol had an opposite impact on the diethyl succinate content whether the wines were inoculated or not. The addition of phenolic compounds in wines affected specific microbial enzymatic systems leading to the production and degradation of important metabolites involved in the organoleptic aspect of wines.

6.2. Introduction

Malolactic fermentation is one of the main steps in the winemaking process of red wine and is conducted by specific species of LAB. With the naturally low pH of wine and the increase of the alcohol content during alcoholic fermentation, only a few microorganisms can grow in a post-alcoholic fermented wine. *Oenococcus oeni* is the most important wine LAB that conducts MLF. Although MLF can be spontaneous,

starter cultures are also widely employed to better control MLF. In these cases, starters are predominantly selected from *O. oeni*. Besides the deacidification (due to the conversion of L-malic acid to L-lactic acid) and microbial stabilization effects, MLF can also alter the organoleptic properties of wines (Laurent *et al.*, 1994). Some yeasts and acetic acid bacteria (AAB) can resist the high acidity and alcohol content of wines and some of these are the cause of wine spoilage. During MLF and subsequent storage, various metabolites are enzymatically consumed and liberated, impacting on the overall composition of a wine. Both volatile and non-volatile components contribute to the aroma and flavor perception of wines (Francis and Newton, 2005).

The volatile fraction of wine is predominantly derived from alcoholic fermentation (AF) and contains among others ethanol, acetaldehyde, higher alcohols and their acetates, volatile fatty acids and their ethyl esters. Volatile compounds can potentially contribute to the aroma perception when their concentrations are above their threshold values. However, by additive or synergic effect among compounds with similar aroma nature, a volatile compound with an odor activity value (OAV = compound concentration/compound odour threshold) below one can still contribute to the final aroma of a wine (Vilanova *et al.*, 2012). During MLF, LAB can generate additional volatile compounds from non-volatile grape constituents such as residual sugars and amino acids, and can metabolize the pre-MLF volatile compounds or adsorb them to their cell walls (Laurent *et al.*, 1994; Bartowsky and Henschke, 1995).

The changes in the volatiles composition of a wine has been found to be linked to the inter- and intra-species diversity of its microbiome (Cappello *et al.*, 2016). Hence, through a number of metabolic activities, LAB are mainly responsible for the modifications in wine aroma and flavor during MLF.

Esters are important volatiles contributing to the fruity aromas of wines and are some of the main volatile compounds affected by malolactic bacteria. Acetate esters are products of the condensation of higher alcohols with acetyl-CoA, and ethyl fatty acid esters are formed by the esterification of volatile fatty acids (VFA) with ethanol (Liu, 2002; Swiegers *et al.*, 2005). García-Ruiz *et al.* (2013b) described the wines after MLF as containing less isoamyl acetate, isobutyl acetate, ethyl butyrate, 2-phenylethyl acetate, hexyl acetate, ethyl acetate and ethyl hexanoate, but more diethyl succinate and ethyl lactate than before MLF. The increase in diethyl succinate and ethyl lactate observed in some wines during MLF may be explained by a bacterial production of succinic and lactic acids by LAB (Herjavec *et al.*, 2001; Ugliano and Moio, 2005; Jeromel *et al.*, 2008; Malherbe *et al.*, 2012). On the other hand, the decrease in esters' concentrations observed during MLF has been associated with the esterase activity of LAB and with the acidic hydrolysis of the esters (Matthews *et al.*, 2007). However, by comparing different strains of LAB regarding the production of esters, Maicas *et al.* (1999) found some of the tested stains to increase isoamyl acetate, 2-phenylethyl acetate and ethyl hexanoate contents during MLF, and Malherbe *et al.* (2012) found other strains to increase ethyl octanoate, ethyl 2-methylpropanoate, and ethyl propionate concentrations. Sumby *et al.* (2010; 2013a) observed a relationship between ester hydrolysis and synthesis activities of *O. oeni* strains, and variations in concentrations of ethyl butanoate, ethyl hexanoate and ethyl octanoate, which lead to variations in the sensory profile of the wines. Moreover,

other activities such as the acyl coenzyme A: alcohol acyltransferase, were identified in *O. oeni* and *L. plantarum* by Costello *et al.* (2013) which may contribute to the increase of ester concentrations during MLF.

Isobutanol, isoamyl alcohol (3-methyl-butan-1-ol), active amyl alcohol (2-methyl-butan-1-ol), methionol and 2-phenylethanol are the most relevant higher (or fusel) alcohols in wine and are formed from the respective amino acids; valine, leucine, isoleucine, methionine and phenylalanine (Styger *et al.*, 2011). In concentrations found in wine, the aromatic impact of higher alcohols depends on their chemical structures and the aromatic context of the wine. For example, De-la-Fuente-Blanco *et al.* (2016) proved the sensory importance of the pair isobutanol-isoamyl alcohol, added in wine models at levels of concentration within the natural range of occurrence, compared to methionol and 2-phenylethanol which brought negligible aroma to the wine analyzed.

The formation of VFA from lipids during MLF due to lipase activity from LAB was suggested by Davis *et al.* (1988). Even though VFA are related with undesirable odors at concentrations higher than 20 mg/L, at lower concentrations they were revealed to be essential for the perception of fresh fruit and to mask the animal character of ethylphenols (Romano *et al.*, 2009; San-Juan *et al.*, 2011; Sánchez-Palomo *et al.*, 2017). The inoculation of wines by commercial starters may increase the concentrations of higher alcohols and VFA (Maicas *et al.*, 1999; Ugliano and Moio, 2005; Malherbe *et al.*, 2012).

Acetaldehyde (ethanal), which can bring a fruity note to wines but is also considered a defect above certain levels, is derived from AF and can be produced by the oxidative metabolism of some spoilage AAB occurring mainly after MLF. This compound can bind with the added SO_2, reducing the effectiveness of its antimicrobial activity and its antioxidative effects. Depending on its concentration, acetaldehyde can also simulate or inhibit microbial growth (Liu and Pilone, 2000).

Terpenes (linalool, nerol, α-terpineol, etc.) are hydrocarbon natural products biosynthesized in the grapevine from connected isoprene units, which normally are associated with floral aromas, and are present in wine in free and bound (glycoside) forms (Marais, 1983). Grape maturity, skin-contact and heat treatment increase the concentrations of most of terpenes (Maraisa and Rapp, 1988). During MLF process, some LAB strains were able to release terpenes by hydrolyzing its aroma precursors (Ugliano *et al.*, 2003; Hernandez-Orte *et al.*, 2009).

Volatile phenols (4-ethylphenol, 4-ethylcatechol, etc.) are synthesized in wines from HCA and their derivatives through microbial processes (Chatonnet *et al.*, 1992; Couto *et al.*, 2006). Above certain concentrations, volatile phenols are considered off-flavors, causing a deterioration of wine quality.

Lactic acid bacteria require amino acids to grow and their enzymatic activities convert amino acids into a range of non-volatile and volatile compounds such as biogenic amines and ethyl carbamate precursors that are relevant to the wine safety (Lonvaud-Funel, 2001; de Nadra *et al.*, 2003; Vincenzini *et al.*, 2017).

Non-volatile phenolic compounds are important components of wine, normally classified in two groups (flavonoids and non-flavonoids) and derived from grape material with composition and concentration in post-malolactic fermented wines influenced by numerous practices employed in the winemaking process (Olejar *et al.*, 2015; Setford *et al.*, 2017). Anthocyanins are responsible for the initial purple-red color of

young red wines, while flavan-3-ols are the precursors of procyanidins and condensed tannins contributing to the astringency and bitterness of wines (Moreno-Arribas and Polo, 2009). The non-volatile phenolic compounds have generally been reported to decrease after AF completion, resulting in a change of color and a decline of astringency and bitterness in wines (Recamales *et al.*, 2006; Castillo-Sánchez *et al.*, 2008; Li *et al.*, 2009; Sun *et al.*, 2011; Bimpilas *et al.*, 2015).

Variations in the phenolic composition of a wine during MLF are usually linked to LAB activities although, the mechanisms of phenolics degradation/reduction are still unclear (Poussier *et al.*, 2003; Hernández *et al.*, 2006; Hernandez *et al.*, 2007). Some LAB were found to use phenolic compounds as electron acceptors or precursors of aromatic compounds such as volatile phenols, aromatic alcohols and phenyl-propionic acids indicating the possible involvement of diverse enzymatic systems (Filannino *et al.*, 2014; Devi *et al.*, 2018b). For example, HCA can be released in wines during MLF due to the cinnamoyl esterase activity of some LAB strains, or degraded due to the decarboxylase and reductase activities of some LAB and yeasts (Couto *et al.*, 2006; Kheir *et al.*, 2013; Chescheir *et al.*, 2015).

Some phenolic compounds are also known to have a strain-specific effect on wine microorganisms depending on the nature of the compounds and of their concentrations, potentially impacting the MLF and the final wine quality (Collombel *et al.*, 2019). Garcia-Ruiz *et al.* (2013) and Rodriguez-Bencomo *et al.* (2014) found that the addition of two antimicrobial extracts, an eucalyptus extract containing *trans*-resveratrol, gallic acid and quercetin, and an almond extract rich in (+)-catechin and tyrosol, led to compositional changes in wine volatiles depending on whether the wine was red or white and MLF was induced or not. For example, the addition of eucalyptus extract decreased the concentrations of most esters except diethyl succinate in red wines, but the opposite effect was observed in white wines (García-Ruiz *et al.*, 2013b; Rodríguez-Bencomo *et al.*, 2014).

The aim of this present study was to investigate whether the addition of specific groups of phenolic compounds to red wines may affect their compositions in volatile and phenolic compounds, during inoculated and non-inoculated MLF and subsequent storage.

6.3. Material and methods

6.3.1. Microvinification

The pre-malolactic fermented red wine, the inoculated (with Oenos™) and non-inoculated (spontaneous) MLF experiments, the phenolic compounds added before incubation and their concentrations (their initial concentrations in wine doubled [2x] and tripled [3x]), the growth conditions (28 days at 25 °C and 142 days at 12 °C in the dark with no agitation) and the samples collection times (beginning of MLF prior to addition of phenolics, and 14, 28 and 170 days after the beginning of MLF) used in this work were exactly the same as described in section 5.3.2. (experimental process explained in figure 5.1). All wines treated with phenolics were compared to non-treated wines (controls). This present work was performed in

parallel to the one described in chapter 5. The collected samples were kept in a commercial freezer (−20 °C) for later analyses.

Malolactic fermentation was considered to be finished in all wines 14 days after its initiation, since L-malic acid was no longer detectable by HPLC-UVIR (description of the HPLC method in section 2.6) at this time point (detection limit: 0.09 g/L).

6.3.2. Analysis of phenolic compounds

The identification and quantification of phenolic compounds were performed by HPLC-DAD as described in section 2.5. All samples were analyzed at the beginning of MLF prior to addition of phenolics, and 14 and 28 days after the beginning of MLF, while only controls and samples with their initial concentrations in phenolics tripled were analyzed 170 days after the beginning of MLF.

6.3.3. Analysis of volatile compounds

Higher alcohols, acetaldehyde and methanol were identified and quantified by SPME-GC-FID as described in section 2.7. All wine samples were analyzed by SPME-GC-FID at the beginning of MLF prior to addition of phenolics, and 28 days after the beginning of MLF.

The analysis of other volatile compounds was carried out by a modified version of the method described by Bertrand (1981). A wine sample volume of 40 mL was added to 10 mL ultrapure water, both volumes measured with volumetric pipettes, and 50 µL 3-octanol at 500 mg/L (internal standard) in a 100 mL volumetric flask. The sample was successively extracted with 4+2+2 mL of a 50:50 (v/v) mix of diethyl ether/n-hexane (Honeyweel, Charlotte, NC, USA) – (Carlo Erba, Val-de-Reuil, France) by stirring for 5 min. The organic phases were collected, mixed, dehydrated using Na_2SO_4 (Merck, Darmstadt, Germany), transferred into a 1.5 mL GC vial and concentrated approximately to one-third of the original volume under a stream of nitrogen before analysis. The injector was heated to 220 °C in split/spitless mode (30 s, 30 mL/min) and 1 µL of the extract was injected into a Varian 3900 GC-FID (Walnut Creek, CA, USA) with a FFA*P*-type column (50 m × 0.25 mm × 0.2 µm) from SGE (Austin, TX, USA) with the carrier gas flow adjusted to 1 mL/min. The oven temperature program was: initial temperature 40 °C for 1 min, 40 °C to 220 °C at a rate of 2 °C/min, with a final plateau of 30 min at this temperature. The total run time was 121 min. Each sample was extracted twice, and each extract injected once. The volatile compounds were identified using the retention times of known standards and quantified according to the internal standard method. The following wine volatile compounds were monitored: esters (ethyl butyrate [ethyl butanoate], ethyl hexanoate, ethyl heptanoate, ethyl octanoate, ethyl nonanoate, ethyl decanoate, ethyl-2-furoate, diethyl succinate, isoamyl acetate, isoamyl octanoate, hexyl acetate, 2-phenylethyl-acetate, isobutyl acetate, linalyl acetate, ethyl lactate, ethyl-2-methylbutanoate, methyl octanoate and isoamyl hexanoate), alcohols (1-hexanol, *trans*- and *cis*-3-hexenol, *trans*-2-hexenol, benzyl alcohol, 2-phenylethanol, furfuryl alcohol, vanillyl alcohol and methionol), terpenes (linalool, α-terpineol, β-citronellol, nerol and geraniol), volatile fatty acids (isobutyric, isovaleric, hexanoic, octanoic, decanoic,

valeric, dodecanoic and butyric acids), volatile phenols (4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol, 4-vinylphenol, 4-ethylcatechol and vanillin), carbonyl compounds (benzaldehyde, 2-phenylacetaldehyde, hydroxybenzaldehyde, α-ionone and β-ionone) and the sulfur compound dimethyl disulfide. All samples were analyzed by solvents extraction and GC-FID at the beginning of MLF prior to addition of phenolics, and 14 and 28 days after the beginning of MLF, while only controls and samples with their initial concentrations in phenolics tripled were analyzed 170 days after the beginning of MLF.

6.3.4. Analysis of amino acids by solvent extraction and HPLC-fluorescence detection

Amino acid identification and quantification was conducted by HPLC-fluorescence using an autosampler for prior sample derivatization. Three reagents were necessary for this analytical method: reagent A was prepared with 3 mL of internal standard solution (20 mg/L of homoserine + norvaline in HCL 0.1 M), 120 µL of 2-mercaptoethanol and 500 mg of sodium tetraphenylborate completed to 25 mL with borate buffer (6.2 g H_3BO_3 in 1 L in deionized water at pH = 9.5); reagent B was obtained by dilution of 3.5 g iodoacetic acid in 100 mL borate buffer and reagent C was prepared by dilution of 225 mg OPA (*o*-phthaldialdehyde) and 5 mL methanol in 50 mL borate buffer. A volume of 0.5 mL of 2-mercaptoethanol was added to reagent C and the flask was filled with nitrogen, before covering it with aluminum fold and storing it in the fridge at 4 °C. For each sample, a vial of reagent B was filled and put first on the autosampler. A sample of 100 µL of wine was manually syringe-filtered (with a 0.45 µm cellulose acetate filter) before being derivatized for 3 min in the autosampler with 250 µL of reagent A and 250 µL of reagent B. Reagent C (250 µL) was then added and mixed for 3.5 min with the previous mixture. A volume of 10 µL of the derivatized mixture was injected in a Chromolith® Performance RP18 column (4.6 x 100 mm) from Merck (Darmstadt, Germany). The mobile phase (flow rate of 0.8 mL/min) was composed of 2 solvents: solvent A was prepared with 20 g $Na_2HPO_4*2H_2O$, 7.4 g propionic acid, 40 mL dimethyl sulfoxide and 130 mL acetonitrile completed with deionized water to 2 L, with final pH = 6.65 and solvent B, made of 330 mL methanol, 70 mL dimethyl sulfoxide, 400 mL acetonitrile and 200 mL deionized water. The following gradient was employed: 0-30 min (100% A); 30-40 min (90% A); 40-45 min (80% A); 45-80 min (75% A), 80-110 min (60% A), 110-120 min (15% A) and 120-123 min (95% A). Amino acids were identified according to retention times of the known standards; aspartic acid, cysteine, serine, threonine, arginine, valine, isoleucine, glutamine, tryptophan, glutamic acid, asparagine, histidine, alanine, tyrosine, methionine, phenylalanine and leucine (Purities ≥ 98% - Sigma-Aldrich, Steinheim, Germany). The concentrations were calculated according to the internal standard method. Only controls and samples with their initial concentrations in phenolics doubled were analyzed by HPLC-fluorescence at the beginning of MLF prior to addition of phenolics, and 14 days after the beginning of MLF.

6.3.5. Statistical Analysis

As described in section 2.8, chemical data were subjected to statistical analysis using JMP13 for Windows XP (Taikoo Shing, Hong Kong, China), at a confidence level of 95% ($p = 0.05$). One-way

analysis of variance (ANOVA) was used to test the effect of the type of MLF (non-inoculated and inoculated), Student´s *t*-test was performed to compare the samples collection times, while Dunnett´s test was run to compare the means of each sample relatively to the controls.

6.4. Results

6.4.1. Chemical changes in wines during MLF and 28 days after the beginning of MLF by the addition of phenolics

6.4.1.1. Amino acids composition changes during MLF

In all samples analyzed for their contents in amino acids, aspartic acid, cysteine, threonine, arginine, valine and isoleucine increased during malolactic fermentation (14 days), histidine and glutamine decreased and glutamic acid, asparagine, serine alanine, tyrosine, methionine, tryptophan, phenylalanine and leucine remained stable. Table 6.1. shows a representation of these variations in non-inoculated and inoculated samples with no addition of phenolics (controls). Only the concentration in threonine was slightly affected by the addition of HCA and *trans*-resveratrol in wines during MLF (Figure 6.1.). No other significant effects by the addition of phenolics on the amino acids contents in wines were found.

Table 6.1. - Contents in amino acids (mg/L) in non-inoculated and inoculated wines with no addition of phenolics (controls) at the beginning of MLF prior to addition of phenolics, and 14 days after the beginning of MLF (end MLF).

	Non-inoculated wines		Inoculated wines	
	Beginning of MLF	**End MLF**	**Beginning of MLF**	**End MLF**
Aspartic acid	4.73 ± 0.01	11.51 ± 0.79	4.92 ± 0.01	12.07 ± 0.58
Glutamic acid	24.00 ± 0.16	20.30 ± 1.25	22.48 ± 0.01	20.14 ± 1.01
Cysteine	0.72 ± 0.00	1.47 ± 0.10	0.86 ± 0.04	1.57 ± 0.22
Asparagine	7.05 ± 0.03	6.96 ± 0.45	6.72 ± 0.05	7.42 ± 0.53
Serine	3.47 ± 0.04	3.98 ± 0.33	3.54 ± 0.07	3.67 ± 0.39
Histidine	6.00 ± 0.09	4.49 ± 0.42	5.95 ± 0.03	4.79 ± 0.66
Glutamine	6.83 ± 0.05	3.90 ± 0.08	6.44 ± 0.07	4.35 ± 0.39
Threonine	3.57 ± 0.05	4.21 ± 0.09	3.71 ± 0.01	4.28 ± 0.16
Arginine	4.84 ± 0.08	7.75 ± 0.42	4.66 ± 0.05	7.48 ± 0.14
Alanine	17.79 ± 0.20	17.51 ± 0.97	17.24 ± 0.02	17.37 ± 0.60
Tyrosine	3.24 ± 0.07	2.89 ± 0.01	3.31 ± 0.05	3.24 ± 0.22
Valine	2.24 ± 0.00	3.24 ± 0.20	2.28 ± 0.05	3.53 ± 0.10
Methionine	0.57 ± 0.14	0.70 ± 0.03	0.61 ± 0.04	0.49 ± 0.05
Tryptophan	1.17 ± 0.09	0.92 ± 0.12	1.10 ± 0.11	0.91 ± 0.07
Phenylalanine	2.19 ± 0.08	2.26 ± 0.12	2.25 ± 0.09	2.22 ± 0.29
Isoleucine	1.08 ± 0.04	1.49 ± 0.08	1.16 ± 0.06	1.50 ± 0.12
Leucine	1.52 ± 0.02	1.49 ± 0.06	1.61 ± 0.00	1.64 ± 0.22

n = 2 ± standard deviations

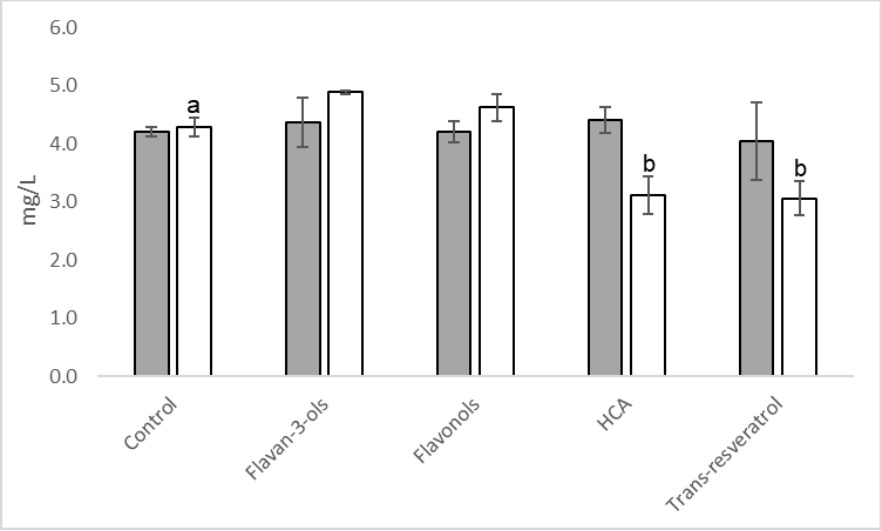


Figure 6.1. – Concentrations in threonine 14 days after the beginning of MLF in wine samples supplemented with phenolic compounds. Grey bars - spontaneous MLF. White bars - MLF inoculated with *O. oeni* Oenos™. a and b indicate values statistically significantly different at $p < 0.05$, n = 2, Dunnett´s test.

6.4.1.2. Phenolics composition

The most statistically significant changes of the composition of phenolics in wines described below occurred during MLF (14 days) ($p < 0.05$, Student´s *t*-test).

In this experiment, the anthocyanins delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, delphinidin-3-O-acetylglucoside, malvidin-3-O-glucoside-ethyl-catechin, malvidin-3-O-acetylglucoside, (*trans*)-peonidin-3-O-coumaroylglucoside and (*trans*)-malvidin-3-O-coumaroylglucoside were identified by HPLC-DAD and quantified as malvidin-3-glucoside equivalents. As predicted, the concentrations in anthocyanins decreased over time, mainly during MLF (Figure 6.2.). At the end of MLF, the concentrations in malvidin-3-O-glucoside-ethyl-catechin were slightly higher in wines with the addition of flavan-3-ols compared to the controls (Figure 6.3). Even though the differences between concentrations in malvidin-3-O-glucoside-ethyl-catechin were small, they were significant in statistical terms.

Hydroxybenzoic acids (HBA represented by ellagic, gallic and syringic acids in this study) slightly but significantly increased since the initiation of MLF in the non-inoculated wines (Figure 6.3. a) but only after the end of MLF in the inoculated wines (Figure 6.3. d). The concentrations in flavan-3-ols ((+)-catechin and (-)-epicatechin) and flavonols (represented by mercytin, kaempferol and quercetin in this study) significantly decreased in wines during MLF and stabilized during early storage (28 days incubation) at 25 °C (Figure 6.3. b, e, d and f). The addition of other groups of phenolics did not affect the concentrations in HBA, flavan-3-ols and flavonols.

In all samples the (hydroxy)cinnamoyl-tartaric acids *trans*-caftaric, *trans*-coutaric and *trans*-fertaric acids decreased over time (Figure 6.5.). While *trans*-caffeic and *trans*-*p*-coumaric acids slightly increased in wines over time (mainly during MLF), *trans*-ferulic acid tended to disappear (Figure 6.5.). Twenty-eight days after the beginning of MLF, *trans*-caffeic acid was found in statistically higher concentrations in wines with initial concentrations in *trans*-*p*-coumaric and *trans*-ferulic tripled compared to the controls (Figure 6.5. a and d). By comparison with the controls, *trans*-caftaric and *trans*-coutaric acids were in lower quantity in non-inoculated wines with initial concentrations in *trans*-*p*-coumaric and *trans*-ferulic doubled (Figure 6.5. a and b).

Trans-resveratrol added in wines at the beginning of MLF was apparently consumed during the incubation at 25 °C (from 3.92 ± 0.62 to 0.50 ± 0.09 mg/L and from 3.68 ± 0.20 to 0.39 ± 0.02 mg/L in non-inoculated wines and wines inoculated with Oenos™ respectively).

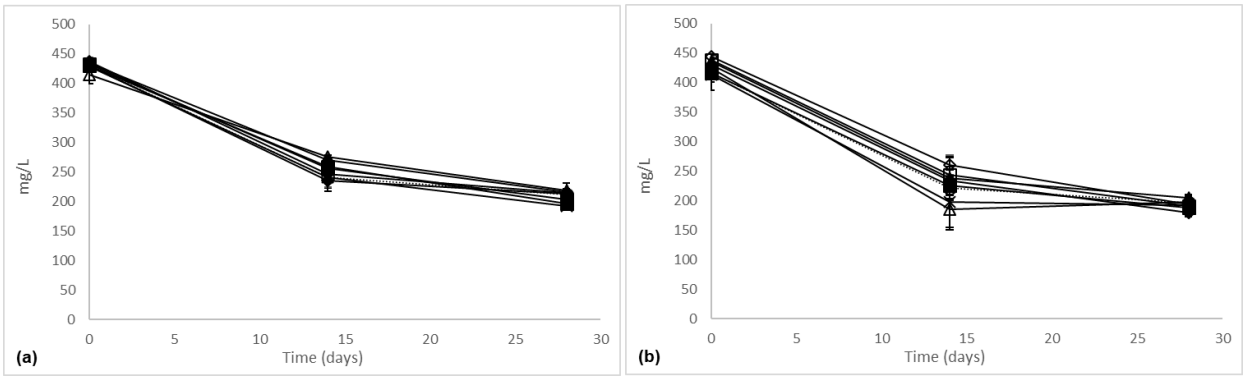


Figure 6.2. - Concentrations of anthocyanins in wines (a) non-inoculated and (b) inoculated with Oenos™ supplemented with (◊) 2x flavan-3-ols, (♦) 3x flavan-3-ols, ( ) 2x flavonols, (▲) 3x flavonols, (□) 2x HCA, (■) 3x HCA and (x) 3x *trans*-resveratrol and compared to controls (○) during incubation at 25 °C. Error bars represent standard deviations of two replicates.

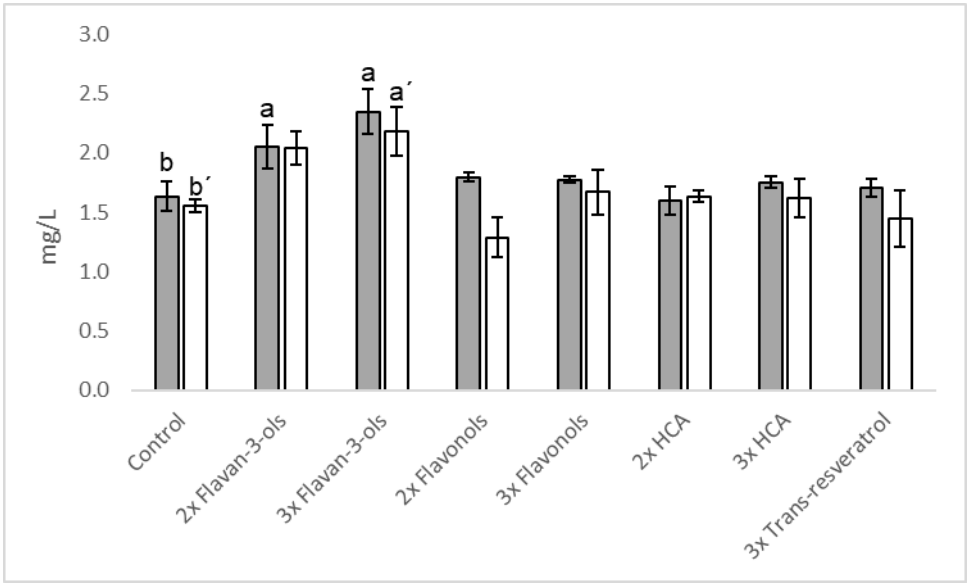


Figure 6.3. - Concentrations in malvidin-3-O-glucoside-ethyl-catechin 14 days after the beginning of MLF in wine samples supplemented with phenolic compounds. Grey bars - spontaneous MLF. White bars - MLF inoculated with *O. oeni* Oenos™. a and b indicate values statistically significantly different at $p < 0.05$, $n = 2$, Dunnett´s test.

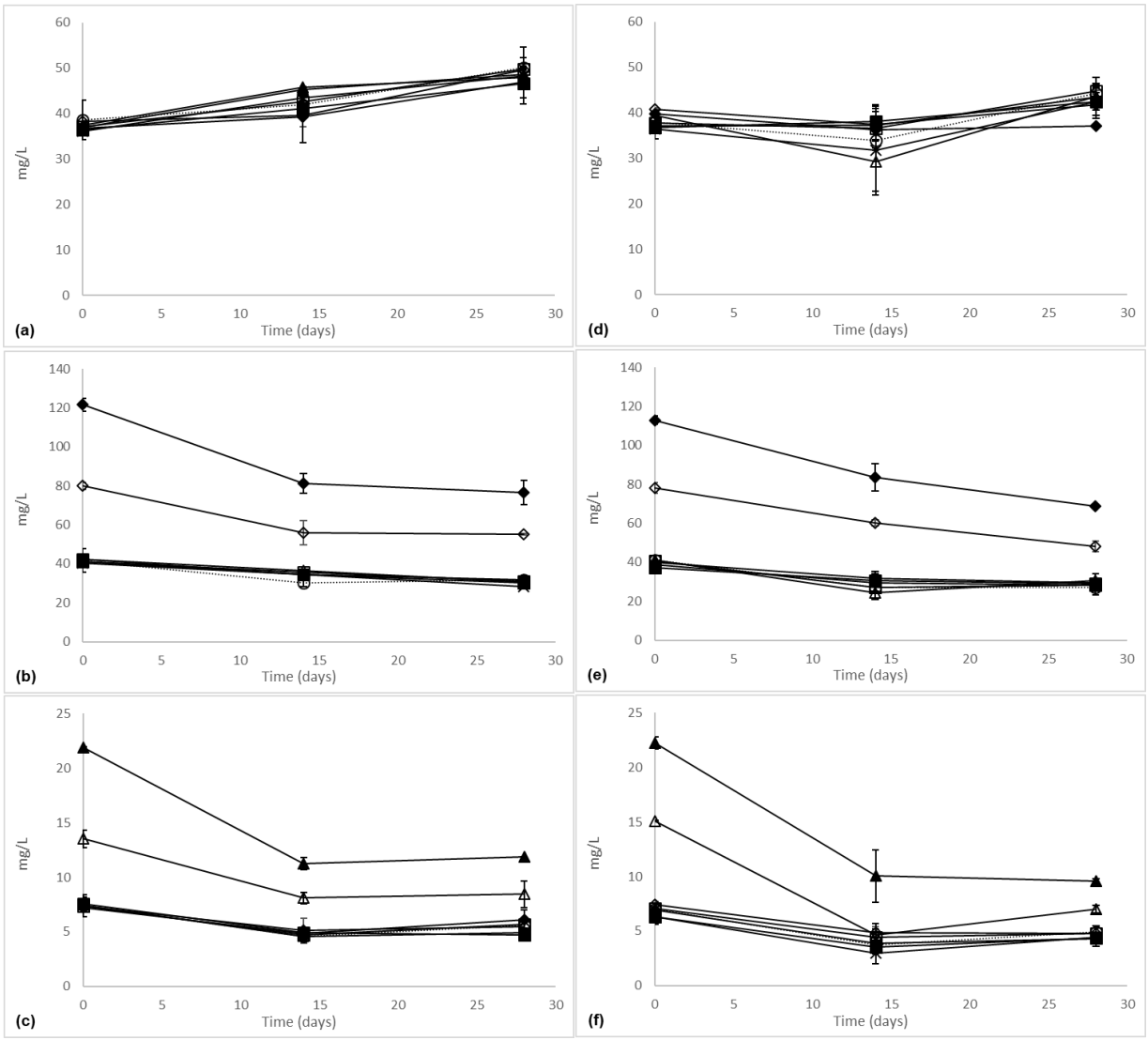


Figure 6.4. - Concentrations of (a and d) HBA, (b and e) flavan-3-ols and flavonols (c and f) in wines (a, b and c) non-inoculated and (d, e and f) inoculated with Oenos™ supplemented with (◊) 2x flavan-3-ols, (♦) 3x flavan-3-ols, (△) 2x flavonols, (▲) 3x flavonols, (□) 2x HCA, (■) 3x HCA and (x) 3x *trans*-resveratrol and compared to controls (○) during incubation at 25 °C. Error bars represent standard deviations of two replicates.

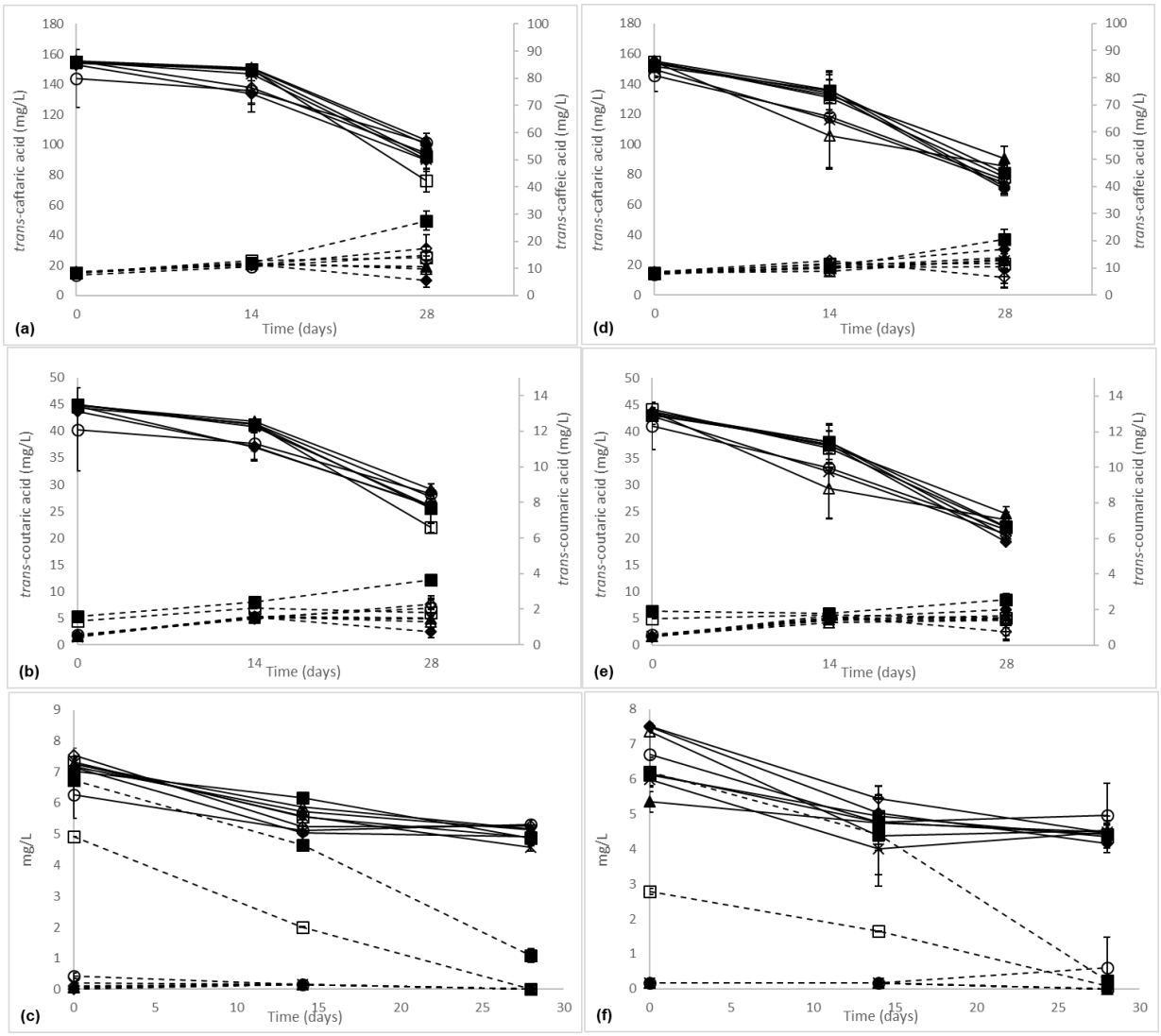


Figure 6.5. – Concentrations of (hydroxy)cinnamoyl-tartaric acids (first axis-plain lines) and "free" HCA (second axis-dashed lines) in non-inoculated wines (a, b and c) and in wines inoculated with Oenos™ (d, e and f), supplemented with (◊) 2x flavan-3-ols, (♦) 3x flavan-3-ols, ( ) 2x flavonols, (▲) 3x flavonols, (□) 2x HCA, (■) 3x HCA and (x) 3x *trans*-resveratrol and compared to controls (○) during incubation at 25 °C. (a and d) *trans*-caftaric / *trans*-caffeic acids, (b and e) *trans*-coutaric / *trans-p*-coumaric acids, (c and f) *trans*-fertaric / *trans*-ferulic acids. Error bars represent standard deviations of two replicates.

6.4.1.3. Volatile compounds composition

Tables 6.2. and 6.3. report the concentrations of the different volatile compounds (n = 34) detected in the wines used in this study during incubation at 25 °C for 28 days.

The non-inoculated wines contained generally higher concentrations in volatile compounds compared to the wines inoculated with Oenos™ (Table 6.3.), and more specifically higher concentrations in esters (fruity, floral).

Most of the esters decreased in concentration over time except for isobutyl acetate, linalyl acetate, isoamyl octanoate and diethyl succinate. Compared to the controls, 28 days after the beginning of MLF, some esters showed greater concentrations in wines with initial addition of double flavan-3-ols concentration. More isoamyl octanoate and ethyl decanoate were found in non-inoculated wines supplemented with double flavan-3-ols concentration (Table 6.2.), and more ethyl hexanoate, ethyl octanoate, linalyl acetate and 2-phenyl-ethylacetate were found in the inoculated ones (Table 6.3.). With concentrations above the threshold (determined in a water/ethanol/glycerol/tartaric acid solution by Ferreira *et al.* (2000)), only ethyl hexanoate characterized by a fruity/green apple note could perceptibly differentiate the inoculated wines treated with double flavan-3-ols from the controls, 28 days after the beginning of MLF.

The concentrations in alcohols (grass, harsh, bitter) and VFA (fatty, cheese) analyzed in this study varied differently over time according to the type of compound (Tables 6.2. and 6.3.). *Trans*-2-hexenol, *trans*-3-hexenol, benzyl alcohol, valeric acid and hexanoic acid slightly increased during incubation at 25 °C, while 1-propanol, 1-butanol, 2-mehyl-1-butanol (active amyl alcohol), octanoic acid, decanoic acid and dodecanoic acid decreased, and methanol, hexanol, 2-phenylethanol, 2-methyl-1-propanol (isobutanol), 3-methyl-1-butanol (isoamyl alcohol), isobutyric acid and isovaleric acid tended to be stable. Wines inoculated with Oenos™ were more sensitive to the addition of HCA and *trans*-resveratrol during MLF (14 days) than non-inoculated wines. Less 1-hexanol, 2-phenylethanol, isobutyric, isovaleric, octanoic and decanoic acids were detected in inoculated wines with addition of HCA and *trans*-resveratrol compared to the controls (Table 6.3.). Octanoic and decanoic acids were also found in lower concentrations at the end of spontaneous MLF in wines with double HCA concentration (Table 6.2.). With concentrations above the thresholds (determined in a water/ethanol/glycerol/tartaric acid solution by Ferreira *et al.* (2000)), 2-phenylethanol, isobutyric, isovaleric and octanoic acids could theoretically lead to perceptible differences between the wines treated with HCA and *trans*-resveratrol from the controls, at the end of MLF.

No general trend was observed in the variation of concentration in the terpenes analyzed (linalool and β-citronellol) over 28 days incubation at 25 °C. Nevertheless, linalool, defined by a lemon/floral aroma, was present at concentrations above threshold (determined in a water/ethanol/glycerol/tartaric acid solution by Ferreira *et al.* (2000)) only in some of the wines that undergone spontaneous MLF (Table 6.2.).

Table 6.2. - Volatile compounds composition of non-inoculated wines before, 14 and 28 days after incubation at 25°C, with or without (controls) addition of phenolics.

Volatile compounds	Odor thresholds	Before MLF	14 days after MLF initiation							
			Control	2x Flavan-3-ols	3x Flavan-3-ols	2x Flavonols	3x Flavonols	2x HCA	3x HCA	3x *Trans*-resveratrol
Esters										
Ethyl hexanoate	14 [9]	157.02 ± 2.78	71.71 ± 1.97	79.15 ± 0.15	80.47 ± 3.92	75.36 ± 2.04	72.09 ± 0.42	76.04 ± 1.52	76.95 ± 0.84	71.31 ± 12.07
Ethyl octanoate	580 [3]	226.90 ± 4.93	158.25 ± 8.09 **b**	165.73 ± 10.11	175.93 ± 3.31	185.89 ± 8.41 **a**	159.68 ± 8.80	156.27 ± 2.57	165.09 ± 1.02	153.58 ± 5.10
Ethyl decanoate	200 [9]	23.73 ± 0.65	20.74 ± 0.14	19.26 ± 2.96	22.56 ± 0.22	21.15 ± 0.69	19.62 ± 1.21	17.62 ± 0.13	20.02 ± 0.86	22.87 ± 4.70
Isobutyl acetate	1 600 [2]	477.83 ± 6.30	414.41 ± 39.31	413.73 ± 11.58	432.70 ± 17.78	368.49 ± 61.10	434.72 ± 41.60	379.44 ± 93.20	534.07 ± 88.45	375.09 ± 137.63
Isoamyl acetate (mg/L)	0.03 [9]	1.25 ± 0.00	0.96 ± 0.02	0.97 ± 0.01	0.97 ± 0.02	0.95 ± 0.00	0.93 ± 0.01	0.94 ± 0.01	0.99 ± 0.00	0.82 ± 0.21
Linalyl acetate	–	22.57 ± 1.24	21.14 ± 0.06	21.31 ± 0.17	20.50 ± 6.68	21.71 ± 1.20	21.62 ± 0.75	21.59 ± 0.06	21.91 ± 2.22	20.14 ± 2.54
Isoamyl octanoate	–	15.26 ± 0.05	11.70 ± 0.96	12.46 ± 1.24	12.80 ± 0.07	11.01 ± 0.32	9.98 ± 1.27	9.63 ± 1.03	12.39 ± 1.01	12.51 ± 1.31
2-Phenylethyl-acetate	250 [4]	103.69 ± 1.19	90.03 ± 1.66	91.31 ± 5.21	93.01 ± 7.95	86.36 ± 2.58	88.46 ± 4.89	93.89 ± 1.85	90.88 ± 1.68	89.63 ± 0.12
Diethyl succinate (mg/L)	200 [3]	0.60 ± 0.03	1.72 ± 0.08	1.70 ± 0.01	1.80 ± 0.02	1748.91 ± 53.84	1.69 ± 0.04	1.68 ± 0.07	1.73 ± 0.01	1.73 ± 0.10

Alcohols										
Trans-3-hexenol	400 [9]	37.74 ± 13.39	29.79 ± 0.92	31.38 ± 6.88	26.43 ± 9.32	35.60 ± 12.61	33.43 ± 1.09	33.85 ± 7.70	41.23 ± 0.93	28.49 ± 5.12
Trans-2-hexenol	400 [9]	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzyl alcohol (mg/L)	200 [6]	0.86 ± 0.04	1.01 ± 0.11	0.95 ± 0.15	1.11 ± 0.01	1.10 ± 0.26	1.01 ± 0.10	0.89 ± 0.14	1.02 ± 0.07	0.96 ± 0.09
Methanol mg/L	–	23.69 ± 1.53	–	–	–	–	–	–	–	–
Higher alcohols										
1-Hexanol (mg/L)	4 [7]	1.60 ± 0.08	1.81 ± 0.08	1.73 ± 0.10	1.79 ± 10.44	1.74 ± 0.05	1.75 ± 0.05	1.68 ± 0.07	1.80 ± 0.06	1.73 ± 0.16
2-Phenylethanol (mg/L)	14 [9]	135.80 ± 13.24	130.68 ± 12.31	123.57 ± 30.97	129.94 ± 10.44	135.89 ± 21.82	135.20 ± 10.75	117.37 ± 17.30	144.30 ± 10.19	131.74 ± 20.85
1-Propanol (mg/L)	500 [7]	6.84 ± 0.54	–	–	–	–	–	–	–	–
1-Butanol (mg/L)	150 [3]	60.23 ± 6.10	–	–	–	–	–	–	–	–
Isobutanol	40 000[9]	361.62 ± 51.45	–	–	–	–	–	–	–	–
2-methyl-1-butanol (mg/L)	7 [10]	62.81 ± 5.27	–	–	–	–	–	–	–	–
Isoamyl alcohol (mg/L)	30 [4]	211.12 ± 17.31	–	–	–	–	–	–	–	–
Terpenes										

Linalool	25 [9]	28.51 ± 2.08	19.07 ± 2.86	23.71 ± 8.04	24.24 ± 3.35	20.21 ± 7.64	14.95 ± 1.46	nd	17.14 ± 1.37	15.02 ± 9.56
β-Citronellol	100 [3]	16.97 ± 1.54	15.97 ± 0.59 **a**	16.06 ± 2.32	15.53 ± 0.79	13.81 ± 0.16	14.69 ± 0.74	14.42 ± 0.46	11.93 ± 1.37 **b**	13.54 ± 0.29
Volatile Fatty Acids										
Isobutyric acid (mg/L)	0.23 [9]	8.13 ± 0.96	6.85 ± 0.71	6.91 ± 1.71	7.51 ± 0.01	7.56 ± 1.85	7.45 ± 0.73	6.05 ± 1.69	8.14 ± 0.88	7.07 ± 1.60
Isovaleric acid (mg/L)	0.033 [9]	3.87 ± 0.45	3.95 ± 0.34	3.86 ± 0.61	4.09 ± 0.10	4.01 ± 0.51	4.03 ± 0.26	3.54 ± 0.54	4.22 ± 0.23	3.96 ± 0.57
Valeric acid	8 100[3]	34.55 ± 48.87	nd	nd	62.65 ± 3.18	64.26 ± 11.18	55.64 ± 7.01	56.87 ± 3.79	73.88 ± 5.29	nd
Hexanoic acid (mg/L)	0.42 [9]	12.63 ± 0.66	14.22 ± 0.67	14.15 ± 0.47	14.75 ± 0.47	14.31 ± 0.52	13.94 ± 0.19	13.16 ± 0.89	13.79 ± 0.07	13.72 ± 0.67
Octanoic acid (mg/L)	0.50 [9]	1.53 ± 0.01	1.02 ± 0.01 **a**	1.01 ± 0.07	1.00 ± 0.05	1.02 ± 0.02	0.93 ± 0.03	0.85 ± 0.04 **b**	0.94 ± 0.00	0.98 ± 0.03
Decanoic acid	1 000 [9]	274.69 ± 4.51	83.67 ± 3.71 **a**	86.31 ± 8.24	78.98 ± 5.41	80.31 ± 2.28	74.14 ± 1.91	64.27 ± 1.88 **b**	73.92 ± 0.57	86.11 ± 1.01
Dodecanoic acid	6 100 [11]	31.53 ± 0.22	14.35 ± 1.33	15.44 ± 4.00	19.16 ± 1.67	14.16 ± 4.29	14.32 ± 5.31	13.18 ± 2.15	15.10 ± 0.31	16.66 ± 6.54
Volatile phenols										
4-Vinylguaiacol	40 [4] or 1100 [5]	nd	nd	nd	nd	nd	nd	14.14 ± 5.27	42.71 ± 10.31	nd
Carbonyl compounds										
Benzaldehyde	200 000 [2]	16.10 ± 4.05	11.67 ± 1.84	10.09 ± 2.98	nd	nd	10.76 ± 1.24	nd	nd	nd

2-Phenylacetaldehyde	1 [2]	69.83 ± 6.99	76.91 ± 6.77	68.55 ± 19.74	77.47 ± 11.49	72.70 ± 17.52	83.42 ± 9.50	56.56 ± 36.91	86.94 ± 17.00	84.61 ± 14.28
Acetaldehyde	100 000 [7]	61.96 ± 3.13	–	–	–	–	–	–	–	–

Volatile compounds	**28 days after MLF initiation**							
	Control	**2x Flavan-3-ols**	**3x Flavan-3-ols**	**2x Flavonols**	**3x Flavonols**	**2x HCA**	**3x HCA**	**3x *Trans*-resveratrol**
Esters								
Ethyl hexanoate	59.14 ± 3.05	66.89 ± 0.37	61.83 ± 0.96	60.49 ± 0.88	60.47 ± 2.72	60.82 ± 2.51	63.79 ± 1.40	57.19 ± 10.12
Ethyl octanoate	83.43 ± 2.84 **b**	94.08 ± 7.42	85.76 ± 1.61	79.61 ± 1.23	85.42 ± 1.90	95.04 ± 0.21	97.51 ± 5.33 **a**	92.56 ± 2.40
Ethyl decanoate	7.78 ± 1.38 **b**	11.82 ± 1.78 **a**	8.49 ± 0.71	6.30 ± 0.47	6.90 ± 0.13	12.80 ± 0.60 **a**	10.76 ± 1.38	8.31 ± 1.27
Isobutyl acetate	479.14 ± 136.58	507.63 ± 48.87	458.95 ± 25.84	401.63 ± 102.11	396.68 ± 5.75	467.77 ± 51.37	387.59 ± 57.02	349.62 ± 148.10
Isoamyl acetate (mg/L)	0.80 ± 0.02	0.87 ± 0.01	0.80 ± 0.01	0.80 ± 0.02	0.81 ± 0.03	0.80 ± 0.03	0.83 ± 0.02	0.58 ± 0.33
Linalyl acetate	26.59 ± 1.77	30.05 ± 2.34	32.66 ± 1.31	29.55 ± 1.30	23.32 ± 3.04	29.93 ± 3.61	28.00 ± 1.58	30.39 ± 2.95
Isoamyl octanoate	11.84 ± 0.70 **b**	15.15 ± 1.40 **a**	14.01 ± 0.04	12.87 ± 0.29	14.59 ± 0.95	12.80 ± 0.22	10.96 ± 0.24	11.12 ± 1.54
2-Phenylethyl-acetate	82.22 ± 0.23	82.58 ± 0.87	76.36 ± 0.29	78.11 ± 2.57	78.50 ± 2.65	80.27 ± 2.28	80.06 ± 1.73	80.32 ± 3.62

Diethyl succinate (mg/L)	3.23 ± 0.13	3.32 ± 0.17	3.17 ± 0.08	3.07 ± 0.15	3.18 ± 0.06	3.34 ± 0.29	3.10 ± 0.08	3.14 ± 0.20
Alcohols								
Trans-3-hexenol	42.22 ± 1.01	54.79 ± 11.76	44.75 ± 5.48	44.12 ± 11.51	37.63 ± 18.33	38.83 ± 7.85	32.77 ± 2.02	39.21 ± 1.52
Trans-2-hexenol	nd	nd	nd	nd	nd	39.35 ± 1.50	nd	71.29 ± 16.91
Benzyl alcohol (mg/L)	2.82 ± 0.78	2.81 ± 0.30	3.12 ± 0.31	2.42 ± 0.40	2.62 ± 0.05	3.05 ± 1.04	2.06 ± 0.00	2.70 ± 0.52
Methanol mg/L	25.59 ± 4.78	31.03 ± 4.97	22.60 ± 2.43	31.03 ± 0.45	30.67 ± 2.67	23.81 ± 8.58	26.60 ± 4.63	24.28 ± 9.21
Higher alcohols								
1-Hexanol (mg/L)	1.94 ± 0.10	2.00 ± 0.12	1.92 ± 0.03	1.85 ± 0.11	1.91 ± 0.02	1.93 ± 0.18	1.81 ± 0.04	1.82 ± 0.16
2-Phenylethanol (mg/L)	139.78 ± 22.63	142.25 ± 13.53	137.21 ± 7.27	120.12 ± 23.90	127.43 ± 0.28	144.20 ± 28.74	121.77 ± 10.25	127.88 ± 20.70
1-Propanol (mg/L)	3.58 ± 0.05	7.99 ± 6.47	18.39 ± 8.80	7.74 ± 0.59	3.81 ± 0.07	11.75 ± 0.88	9.99 ± 6.78	3.64 ± 0.11
1-Butanol (mg/L)	46.34 ± 13.44	63.92 ± 20.20	46.70 ± 12.68	63.71 ± 1.68	48.94 ± 2.20	54.86 ± 11.59	54.62 ± 3.42	47.05 ± 0.24
Isobutanol	421.42 ± 263.03	503.63 ± 337.62	468.94 ± 114.58	592.40 ± 8.00	432.12 ± 45.29	504.53 ± 48.65	461.97 ± 147.45	587.00 ± 322.17
2-methyl-1-butanol (mg/L)	53.90 ± 7.80	63.13 ± 6.58	54.53 ± 10.00	62.57 ± 1.71	56.41 ± 1.34	59.27 ± 5.04	54.98 ± 2.86	55.92 ± 3.71
Isoamyl alcohol (mg/L)	208.49 ± 9.30	221.16 ± 30.07	210.51 ± 2.71	215.96 ± 2.23	194.24 ± 3.80	204.64 ± 22.44	199.74 ± 2.56	192.33 ± 8.26

Terpenes								
Linalool	24.30 ± 3.72	29.86 ± 3.44	24.76 ± 4.03	18.16 ± 4.88	17.20 ± 7.06	25.59 ± 16.90	18.69 ± 0.72	31.75 ± 17.10
β-Citronellol	13.78 ± 1.13	14.31 ± 1.25	14.97 ± 1.11	13.52 ± 0.72	14.16 ± 0.98	15.66 ± 2.09	13.71 ± 1.58	13.79 ± 0.54
Volatile Fatty Acids								
Isobutyric acid (mg/L)	7.69 ± 1.91	7.91 ± 0.75	7.72 ± 0.60	6.46 ± 1.75	6.91 ± 0.19	8.45 ± 2.01	6.60 ± 0.76	6.82 ± 1.13
Isovaleric acid (mg/L)	4.27 ± 0.59	4.34 ± 0.41	4.23 ± 0.17	3.78 ± 0.63	4.01 ± 0.02	4.32 ± 0.70	3.79 ± 0.30	3.75 ± 0.45
Valeric acid	93.17 ± 10.75	76.96 ± 8.53	108.47 ± 2.47	53.83 ± 7.67	nd	63.91 ± 0.26	nd	31.74 ± 44.89
Hexanoic acid (mg/L)	16.91 ± 0.81	17.01 ± 0.76	17.03 ± 0.26	16.10 ± 0.35	16.53 ± 0.07	16.90 ± 1.73	15.71 ± 0.32	16.07 ± 0.93
Octanoic acid (mg/L)	0.93 ± 0.03	1.05 ± 0.00	0.85 ± 0.06	0.92 ± 0.07	0.88 ± 0.04	0.92 ± 0.08	1.00 ± 0.06	0.87 ± 0.04
Decanoic acid	55.98 ± 5.85	60.44 ± 0.96	nd	50.59 ± 11.77	41.89 ± 0.11	59.28 ± 6.30	61.57 ± 8.70	49.79 ± 1.87
Dodecanoic acid	11.01 ± 3.69	11.38 ± 2.58	13.68 ± 1.40	12.69 ± 0.25	36.17 ± 34.58	13.55 ± 2.23	20.01 ± 2.65	10.57 ± 5.00
Carbonyl compounds								
Benzaldehyde	nd	nd	nd	nd	nd	20.26 ± 0.35	nd	nd
2-Phenylacetaldehyde	120.55 ± 42.33	128.62 ± 21.43	106.65 ± 10.24	96.79 ± 38.87	88.66 ± 1.37	129.78 ± 53.01	93.99 ± 1.78	94.73 ± 14.22

Acetaldehyde	13.34 ± 0.82	14.35 ± 8.26	10.48 ± 6.91	15.47 ± 10.17	13.04 ± 4.63	10.72 ± 0.40	11.65 ± 1.64	9.51 ± 0.44

Concentration values in µg/L except where indicated. n = 4 ± standard deviations.

- not analyzed at this samples collection time; nd = not detectable in the wines.

Bold letters on the right indicate statistically significant differences of the sample with the control at that specific time point (Dunnett´s $p < 0.05$).

2 (Escudero *et al.*, 2007) and 11 (Meilgaard, 1975): Thresholds calculated in beer; 3 (Etiévant, 1991) and 7 (Swiegers *et al.*, 2005a): Thresholds calculated in wine; 4 (Guth, 1997): Thresholds calculated in 10% ethanol; 5 (Boidron *et al.*, 1988): Thresholds calculated in synthetic wine containing 12% ethanol, 8g/L glycerol and different salts; 6 (Gomez-Miguez *et al.*, 2007): Thresholds calculated in 10% water / ethanol mixture containing 5 g/L of tartaric acid at pH 3.2; 9 (Ferreira *et al.*, 2000): Thresholds determined in 11% v/v aqueous ethanol with 7 g/L glycerol and 5 g/L tartaric acid, at pH 3.4; 10 (Salo, 1970): Thresholds calculated in hydro-alcoholic solution.

Table 6.3. - Volatile compounds composition of inoculated wines (Oenos™) before, 14 and 28 days after incubation at 25°C, with or without (controls) addition of phenolics.

Volatile compounds	Odor thresholds	Before MLF	14 days after MLF initiation							
			Control	2x Flavan-3-ols	3x Flavan-3-ols	2x Flavonols	3x Flavonols	2x HCA	3x HCA	3x *Trans*-resveratrol
Esters										
Ethyl hexanoate	14 [9]	175.20 ± 2.68 **	63.71 ± 28.14	77.90 ± 0.38 *	69.21 ± 11.58	60.96 ± 1.82 *	71.33 ± 0.44	66.46 ± 4.13	60.74 ± 5.44	55.74 ± 13.92
Ethyl octanoate	580 [3]	250.66 ± 8.62	100.20 ± 120.75	176.96 ± 18.14	128.23 ± 13.32 *	129.89 ± 0.48 *	171.58 ± 4.15	142.44 ± 18.95	121.08 ± 1.58 *	124.86 ± 0.07 *
Ethyl decanoate	200 [9]	21.72 ± 2.37	13.03 ± 11.03	15.97 ± 5.50	11.39 ± 2.42 *	13.63 ± 0.46 *	17.90 ± 2.92	18.34 ± 0.70	13.71 ± 0.99 *	14.40 ± 1.32
Isobutyl acetate	1 600 [2]	529.90 ± 117.04	535.17 ± 147.80	595.86 ± 30.83	492.54 ± 16.17	538.30 ± 12.85	472.42 ± 95.20	305.97 ± 10.40	316.37 ± 51.23	300.44 ± 103.11
Isoamyl acetate (mg/L)	0.03 [9]	1.37 ± 0.04 **	0.93 ± 0.13	0.99 ± 0.00	0.93 ± 0.06	0.92 ± 0.02	0.95 ± 0.00	0.90 ± 0.02	0.80 ± 0.16	0.68 ± 0.36
Linalyl acetate	–	25.79 ± 0.14	21.82 ± 1.82	17.83 ± 3.74	18.52 ± 1.28	17.90 ± 0.43	17.30 ± 1.74	18.73 ± 0.37 *	16.61 ± 2.23	19.22 ± 1.57
Isoamyl octanoate	–	15.34 ± 0.57	10.29 ± 2.40	10.26 ± 1.98	8.92 ± 1.33	9.07 ± 1.15	10.04 ± 0.77	8.32 ± 0.03	10.69 ± 0.07	10.21 ± 0.74

2-Phenylethyl-acetate	250 [4]	115.06 ± 1.84 ****	91.25 ± 4.91	90.38 ± 5.81	83.74 ± 0.37	82.59 ± 5.04	87.14 ± 0.11	86.47 ± 3.32	85.57 ± 0.03	88.60 ± 1.00
Diethyl succinate (mg/L)	200 [3]	0.63 ± 0.03	1.27 ± 0.83	1.69 ± 0.07	1.19 ± 0.02 *	1.20 ± 0.01 *	1.63 ± 0.05	1.45 ± 0.00 *	1.11 ± 0.14 *	1.15 ± 0.03 *
Alcohols										
Trans-3-hexenol	400 [9]	30.44 ± 4.59	36.2 ± 10.95	35.69 ± 3.22	31.28 ± 2.86	32.33 ± 10.30	33.28 ± 9.88	27.29 ± 0.94	23.94 ± 0.63 *	28.04 ± 1.41
Trans-2-hexenol	400 [9]	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzyl alcohol (mg/L)	200 [6]	0.80 ± 0.11	1.13 ± 0.46	1.22 ± 0.19	0.87 ± 0.01 *	1.04 ± 0.12	0.93 ± 0.07	0.57 ± 0.01	0.60 ± 0.07 *	0.66 ± 0.02 *
Methanol mg/L	–	24.68 ± 0.59	–	–	–	–	–	–	–	–
Higher alcohols										
1-Hexanol (mg/L)	4 [7]	1.70 ± 0.10	1.79 ± 0.14 **a**	1.78 ± 0.05	1.61 ± 0.00 *	1.74 ± 0.03	1.75 ± 0.05	1.52 ± 0.02 **b**	1.52 ± 0.12 **b**	1.46 ± 0.04 **b**
2-Phenylethanol (mg/L)	14 [9]	129.96 ± 16.65	146.74 ± 24.33 **a**	145.71 ± 14.24	118.28 ± 5.03	140.24 ± 5.25	125.49 ± 10.63	82.78 ± 0.54 **b**	93.62 ± 8.84 **b** *	91.29 ± 3.97 **b**
1-Propanol (mg/L)	500 [7]	15.80 ± 7.80	–	–	–	–	–	–	–	–
1-Butanol (mg/L)	150 [3]	80.94 ± 17.98	–	–	–	–	–	–	–	–

Isobutanol	40 000[9]	548.53 ± 178.83	–	–	–	–	–	–	–	–
2-methyl-1-butanol (mg/L)	7 [10]	73.88 ± 6.97	–	–	–	–	–	–	–	–
Isoamyl alcohol (mg/L)	30 [4]	247.59 ± 21.25	–	–	–	–	–	–	–	–
Terpenes										
Linalool	25 [9]	nd	10.72 ± 0.43	nd	nd	nd	nd	8.74 ± 0.21	7.73 ± 1.27 *****	8.29 ± 1.48
β-Citronellol	100 [3]	15.43 ± 1.94	14.46 ± 0.60	15.98 ± 0.23	10.40 ± 3.08	14.19 ± 0.58	13.42 ± 2.96	14.14 ± 1.10	14.31 ± 0.10	14.93 ± 1.84
Volatile Fatty Acids										
Isobutyric acid (mg/L)	0.23 [9]	5.75 ± 1.40	8.83 ± 2.82 **a**	8.89 ± 1.31	6.86 ± 0.43	8.39 ± 0.42	6.80 ± 0.98	4.90 ± 0.15 **b**	4.86 ± 0.53 **b ***	4.68 ± 0.38 **b**
Isovaleric acid (mg/L)	0.033 [9]	3.83 ± 0.43	4.35 ± 0.66 **a**	4.33 ± 0.35	3.57 ± 0.09 *****	4.13 ± 0.12	3.86 ± 0.29	2.66 ± 0.02 **b**	2.93 ± 0.34 **b ***	2.83 ± 0.10 **b**
Valeric acid	8 100[3]	102.50 ± 9.54	65.54 ± 2.23	88.23 ± 11.77	93.91 ± 15.62	81.47 ± 13.04	73.31 ± 10.79	49.97 ± 5.58	73.34 ± 15.40	50.25 ± 7.56
Hexanoic acid (mg/L)	0.42 [9]	13.22 ± 0.30	14.35 ± 1.42	14.46 ± 0.31	12.52 ± 0.13 *****	13.22 ± 0.31	13.83 ± 0.54	12.41 ± 0.20	12.18 ± 1.42	12.12 ± 0.19
Octanoic acid (mg/L)	0.50 [9]	1.66 ± 0.05	1.35 ± 0.35 **a**	0.98 ± 0.01	0.85 ± 0.01 **b**	0.95 ± 0.01 *****	0.92 ± 0.01 **b**	0.94 ± 0.03 **b**	0.94 ± 0.01 **b**	0.97 ± 0.04

Decanoic acid	1 000 [9]	281.83 ± 7.26	135.58 ± 43.34 **a**	80.63 ± 0.95 **b**	58.98 ± 2.45 **b ***	76.65 ± 1.85 **b**	72.03 ± 0.55 **b**	69.86 ± 0.17 **b**	72.76 ± 2.18 **b**	76.89 ± 1.34 **b ***
Dodecanoic acid	6 100 [11]	37.00 ± 11.22	21.93 ± 13.62	15.13 ± 0.49	3.91 ± 5.53	10.87 ± 1.70	17.29 ± 6.60	11.95 ± 0.37	10.22 ± 2.05	15.04 ± 1.93
Volatile phenols										
4-Ethylphenol	600 [7]	nd	nd	nd	nd	nd	nd	nd	nd	nd
4-Vinylguaiacol	40 [4] or 1100 [5]	nd	nd	nd	nd	nd	nd	5.26 ± 0.28	22.90 ± 1.17	nd
Carbonyl compounds										
Benzaldehyde	200 000 [2]	12.13 ± 0.91	nd	11.25 ± 1.10	9.22 ± 0.54	nd	10.60 ± 3.19	nd	7.13 ± 0.67	7.21 ± 0.44
2-Phenylacetaldehyde	1 [2]	64.29 ± 23.35	95.11 ± 36.65	111.53 ± 26.29	84.52 ± 10.82	108.12 ± 14.99	76.02 ± 7.56	46.36 ± 1.91	54.60 ± 7.61	50.57 ± 1.01
Acetaldehyde	100 000 [7]	51.57 ± 18.60	–	–	–	–	–	–	–	–

Volatile compounds	**28 days after MLF initiation**							
	Control	**2x Flavan-3-ols**	**3x Flavan-3-ols**	**2x Flavonols**	**3x Flavonols**	**2x HCA**	**3x HCA**	**3x *Trans*-resveratrol**
Esters								
Ethyl hexanoate	54.73 ± 4.15 **b**	81.42 ± 2.54 **a ****	54.06 ± 3.61	50.07 ± 1.28 *****	54.58 ± 1.35	52.89 ± 9.79	53.61 ± 0.08	51.23 ± 0.97
Ethyl octanoate	72.31 ± 13.26 **b**	99.17 ± 8.74 **a**	64.77 ± 2.65 *****	59.29 ± 0.49 *****	71.65 ± 2.39 *****	77.98 ± 2.32 *****	64.64 ± 3.11 *****	65.59 ± 2.37 *****

Ethyl decanoate	8.86 ± 1.30	12.89 ± 0.41	9.31 ± 2.40	6.61 ± 0.25	12.11 ± 2.25	8.68 ± 1.70	6.65 ± 0.28	7.54 ± 0.97
Isobutyl acetate	392.12 ± 74.74	402.34 ± 125.98	459.97 ± 66.52	466.31 ± 30.78	500.34 ± 169.09	436.58 ± 184.94	417.24 ± 72.81	329.83 ± 84.68
Isoamyl acetate (mg/L)	0.78 ± 0.02	1.00 ± 0.04 ****	0.81 ± 0.02	0.76 ± 0.00	0.76 ± 0.01	0.57 ± 0.30	0.74 ± 0.00 *	0.76 ± 0.03
Linalyl acetate	28.59 ± 2.72 **b**	37.29 ± 3.76 **a**	28.96 ± 0.07	27.46 ± 0.12	26.22 ± 0.57	29.47 ± 1.64	27.70 ± 1.04	28.00± 1.22
Isoamyl octanoate	12.15 ± 1.65	16.25 ± 0.96	13.28 ± 2.31	10.59 ± 0.24 *	9.85 ± 0.49 *	7.77 ± 2.61	12.04 ± 1.55	10.71 ± 0.44
2-Phenylethyl-acetate	80.24 ± 0.84 **b**	104.60 ± 5.52 **a** **	85.98 ± 1.54 **	77.92 ± 3.96	77.38 ± 2.29	76.65 ± 3.58	78.36 ± 1.23	76.86 ± 2.64
Diethyl succinate (mg/L)	2.73 ± 0.19	3.42 ± 0.54	2.40 ± 0.15 *	2.08 ± 0.08 *	2.93 ± 0.21	3.09 ± 0.13	2.39 ± 0.08 *	2.31 ± 0.05 *
Alcohols								
Trans-3-hexenol	45.47 ± 6.35	49.82 ± 18.46	44.19 ± 5.63	35.96 ± 1.87	47.00 ± 0.96	40.56 ± 10.27	52.25 ± 6.76	44.94 ± 4.80
Trans-2-hexenol	65.85 ± 0.30	72.10 ± 17.23	51.76 ± 6.01	60.01 ± 0.53	58.79 ± 11.09	73.00 ± 6.81	56.12 ± 5.40	59.08 ± 2.44
Benzyl alcohol (mg/L)	2.24 ± 0.14	2.69 ± 1.30	2.51 ± 0.55	2.58 ± 0.00	2.77 ± 0.62	3.09 ± 0.66	2.50 ± 0.58	2.02 ± 0.12
Methanol mg/L	30.27 ± 1.72	30.15 ± 1.09	30.13 ± 12.79	31.8 ± 2.06	32.02 ± 8.66	21.91 ± 8.63	29.18 ± 5.33	28.44 ± 1.02
Higher alcohols								
1-Hexanol (mg/L)	1.82 ± 0.07	2.17 ± 0.36	1.88 ± 0.09	1.78 ± 0.05	1.89 ± 0.12	1.91 ± 0.16	1.88 ± 0.07	1.79 ± 0.06

2-Phenylethanol (mg/L)	122.99 ± 20.85	133.80 ± 54.65	136.13 ± 22.62	122.69 ± 7.98	139.45 ± 31.55	145.01 ± 25.52	130.29 ± 22.97	106.60 ± 6.18
1-Propanol (mg/L)	6.92 ± 1.88	8.96 ± 1.75	2.00 ± 2.82	4.42 ± 1.17	3.95 ± 0.12	4.32 ± 0.07 *	4.23 ± 1.26	7.29 ± 3.48
1-Butanol (mg/L)	60.10 ± 6.29	68.55 ± 5.69	83.50 ± 40.04	57.49 ± 0.38 *	52.80 ± 3.42	50.39 ± 3.23	51.06 ± 7.32	60.64 ± 12.34
Isobutanol	505.98 ± 9.79	670.04 ± 84.58	1184.15 ± 999.50	484.46 ± 164.19	497.47 ± 18.13	453.03 ± 64.12	559.32 ± 67.51	520.79 ± 114.06
2-methyl-1-butanol (mg/L)	63.93 ± 2.20	61.52 ± 0.95	71.20 ± 15.72	62.72 ± 1.91	58.67 ± 2.25	57.54 ± 5.12	58.08 ± 5.28	62.62 ± 3.35
Isoamyl alcohol (mg/L)	213.21 ± 8.32	217.15 ± 9.21	243.28 ± 65.86	206.93 ± 4.47	195.96 ± 12.98	196.39 ± 8.67	195.79 ± 12.17	214.48 ± 13.02
Terpenes								
Linalool	11.74 ± 1.08 *	14.32 ± 0.27 *	11.81 ± 1.12 *	nd	12.46 ± 0.23	nd	23.50 ± 2.19	12.07 ± 6.08
β-Citronellol	14.24 ± 2.26	16.28 ± 3.98	16.87 ± 1.34	17.37 ± 0.66 **	15.91 ± 0.73	13.78 ± 2.08	14.37 ± 2.94	13.38 ± 2.28
Volatile Fatty Acids								
Isobutyric acid (mg/L)	6.33 ± 1.59	6.76 ± 3.26	7.83 ± 1.34	7.60 ± 0.30	8.09 ± 2.27	8.21 ± 1.97	7.25 ± 1.61	5.21 ± 0.87
Isovaleric acid (mg/L)	3.75 ± 0.59	4.13 ± 1.59	4.21 ± 0.54	7.40 ± 0.30	4.22 ± 0.80	4.38 ± 0.60	4.06 ± 0.52	3.39 ± 0.24
Valeric acid	nd	nd	nd	nd	93.32 ± 28.83	nd	nd	nd

Hexanoic acid (mg/L)	16.15 ± 0.51	19.71 ± 3.01	16.93 ± 0.80	15.87 ± 0.33	16.29 ± 0.92	17.00 ± 0.61	16.61 ± 0.86	15.87 ± 0.43
Octanoic acid (mg/L)	1.01 ± 0.07 **b**	1.29 ± 0.03 **a** **	1.08 ± 0.08	0.93 ± 0.05	0.89 ± 0.01	0.81 ± 0.06 **c**	0.97 ± 0.06	0.95 ± 0.06
Decanoic acid	65.23 ± 12.71	77.44 ± 7.08	58.89 ± 11.25	43.83 ± 7.70	49.55 ± 2.93	nd	50.07 ± 1.10	47.78 ± 5.86
Dodecanoic acid	14.16 ± 2.25	13.72 ± 5.38	9.60 ± 2.30	9.19 ± 1.60	17.06 ± 6.09	13.41 ± 1.58	7.28 ± 0.09 *	11.19 ± 2.76
Volatile phenols								
4-Ethylphenol	nd	16.73 ± 2.82	nd	nd	nd	nd	nd	nd
4-Vinylguaiacol	nd	nd	nd	nd	nd	nd	nd	nd
Carbonyl compounds								
Benzaldehyde	12.67 ± 1.71	9.27 ± 1.41	nd	9.86 ± 1.18	10.35 ± 0.15	nd	nd	3.57 ± 5.05
2-Phenylacetaldehyde	88.82 ± 21.46	94.51 ± 33.93	111.25 ± 19.16	112.59 ± 9.80	114.59 ± 31.36	131.95 ± 45.95	103.13 ± 26.04	71.96 ± 48.01
Acetaldehyde	11.40 ± 1.90	11.09 ± 0.52	12.08 ± 0.33	11.62 ± 0.51	12.92 ± 2.33	11.75 ± 1.65	15.77 ± 1.27	9.10 ± 0.78

Concentration values in µg/L except where indicated. n = 4 ± standard deviations.

- not analyzed at this samples collection time; nd = not detectable in the wines.

Bold letters on the right indicate statistically significant differences of the sample with the control at that specific time point (Dunnett´s $p < 0.05$). Stars on the up right indicate statistically significant differences between inoculated and non-inoculated samples with the same treatment at the same time point (ANOVA $p<0.05$). * Mean value significantly higher for the non-inoculated sample compared to the inoculated one, ** Mean value significantly higher for the inoculated sample compared to the non-inoculated one.

2 (Escudero *et al.*, 2007) and 11 (Meilgaard, 1975): Thresholds calculated in beer; 3 (Etiévant, 1991) and 7 (Swiegers *et al.*, 2005a): Thresholds calculated in wine; 4 (Guth, 1997): Thresholds calculated in 10% ethanol; 5 (Boidron *et al.*, 1988): Thresholds calculated in synthetic wine

containing 12% ethanol, 8g/L glycerol and different salts; 6 (Gomez-Miguez *et al.*, 2007): Thresholds calculated in 10% water / ethanol mixture containing 5 g/L of tartaric acid at pH 3.2; 9 (Ferreira *et al.*, 2000): Thresholds determined in 11% v/v aqueous ethanol with 7 g/L glycerol and 5 g/L tartaric acid, at pH 3.4; 10 (Salo, 1970): Thresholds calculated in hydro-alcoholic solution.

6.4.2. Chemical changes in wines with added phenolics 170 days after the beginning of MLF

Only controls and samples from experiments in which the initial concentrations in phenolics was tripled were analyzed for their compositions in phenolic and volatile compounds 170 days after the beginning of MLF.

6.4.2.1. Phenolic compounds composition

The concentrations in anthocyanins decreased from 28 to 170 days after the beginning of MLF and the wines supplemented with flavan-3-ols still had higher contents in malvidin-3-O-glucoside-ethyl-catechin than the controls (Figure 6.6).

No changes in the HBA concentrations were noticed over time (47.52 ± 2.58 mg/L in average in all the wines 170 days after the beginning of MLF).

All the analyzed HCA and their bound forms with tartaric acid decreased from 28 days to 170 days after the beginning of MLF (Table 6.4). *Trans*-caftaric and *trans*-coutaric acids were found in lower concentrations in the inoculated wines compared to the non-inoculated ones. This observation was statistically significant for the wines supplemented with flavan-3-ols and HCA (Figure 6.7. a and b). The contents in *trans*-caffeic and *trans*-*p*-coumaric acids were also statistically lower in wines inoculated with Oenos™ and supplemented with HCA than in non-inoculated wines (Figure 6.7. c and d). Both *trans*-caftaric and *trans*-coutaric acids were detected in lower quantities in wines with their initial concentrations in HCA tripled, although this result was only statistically significant for the non-inoculated wines (Figure 6.7. a and b). Even though the results were not statistically significant, it is interesting to notice that *trans*-caffeic acid was found in lower concentration in inoculated wines supplemented with HCA compare to the controls (Figure 6.7. c). Finally, the contents in *trans*-caftaric and *trans*-coutaric acids were higher in inoculated wines with addition of three times their initial concentration in flavonols (Figures 6.4. a and b).

No more *trans*-ferulic acid, kaempferol and *trans*-resveratrol were detected in any of the wines 170 days after the beginning of MLF. Although added three times their initial concentrations in some wines, the quantities in quercetin, (+)-catechin and (-) epicatechin did not show any statistic differences between wines 170 days after the beginning of MLF (quercetin: 1.48 ± 0.68 mg/L, (+)-catechin: 18.14 ± 2.33 mg/L and (-) epicatechin: 17.98 ± 1.32 mg/L in average in all wines).

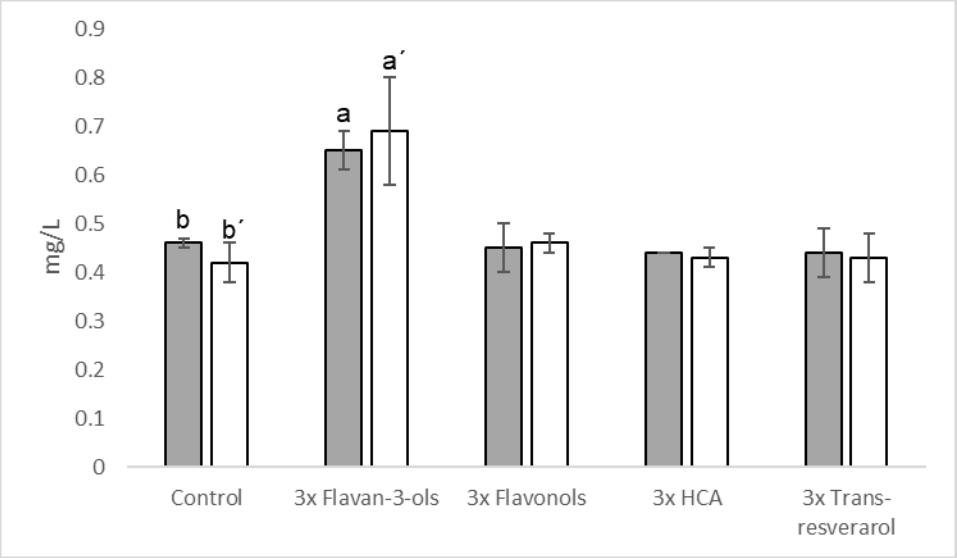


Figure 6.6. - Concentrations in malvidin-3-O-glucoside-ethyl-catechin 170 days after the beginning of MLF in controls and wines with their initial concentrations in phenolics tripled. Grey bars - spontaneous MLF. White bars - MLF inoculated with *O. oeni* Oenos™. a and b indicate values statistically significantly different at $p < 0.05$, $n = 2$, Dunnett´s test.

Table 6.4. - Minimum and maximum concentrations (mg/L) in the principal hydroxycinnamic acids and their bound forms with tartaric acid in all wines analyzed 28 days and 170 days after the beginning of MLF.

		28 days	**170 days**
***trans*-caftaric acid**	Minimum	61.57	2.1
	Maximum	106.09	32.43
***trans*-coutaric acid**	Minimum	19.38	0.58
	Maximum	29.86	11.22
***trans*-fertaric acid**	Minimum	4.04	3.8
	Maximum	5.62	4.53
***trans*-caffeic acid**	Minimum	4.99	0.82
	Maximum	30.01	6.88
***trans-p*-coumaric acid**	Minimum	0.59	0.04
	Maximum	3.77	1.56
***trans*-ferulic acid**	Minimum	nd	nd
	Maximum	1.24	nd

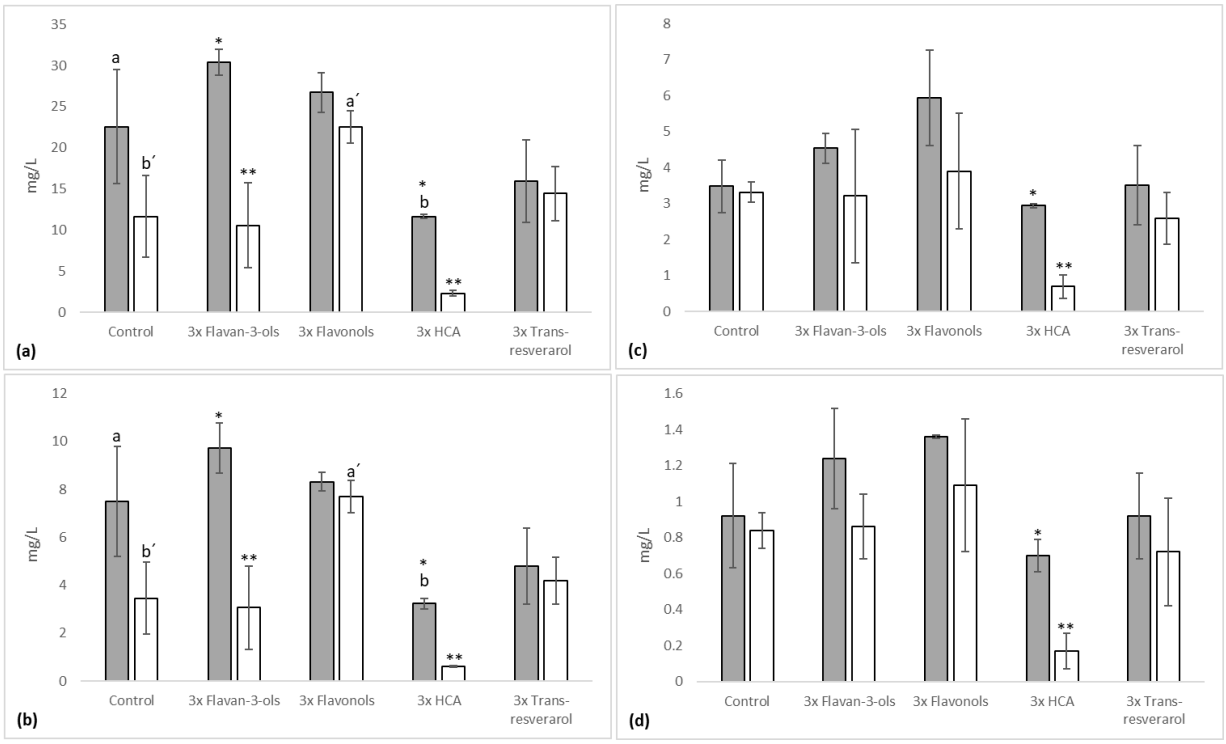


Figure 6.7. - Concentrations in (a) *trans*-caftaric acid, (b) *trans*-coutaric acid, (c) *trans*-caffeic acid and (d) *trans-p*-coumaric acid 170 days after the beginning of MLF in controls and wines with their initial concentrations in phenolics tripled. Grey bars - spontaneous MLF. White bars - MLF inoculated with *O. oeni* Oenos™. n = 2. a, a´, b and b´ indicate values statistically significantly different between non-treated wines and wines treated with phenolics (Dunnett´s test, $p < 0.05$). * and ** indicate values statistically significantly different between inoculated and non-inoculated samples with the same treatment (ANOVA, $p<0.05$).

6.4.2.2. Volatile compounds composition

Tables 6.5. and 6.6. report the concentrations of the different volatile compounds that were detected in the wines used in this study after 170 days incubation (28 days at 25 °C and 142 days at 12 °C).

Esters (fruity, floral) represented the largest group of volatile compounds identified. From 28 days after the beginning of MLF to 170 days after, ethyl hexanoate (from 58.95 ± 8.07 µg/L to 116.18 ± 14.95 µg/L in all samples), ethyl octanoate (from 80.55 ± 13.34 µg/L to 113.52 ± 14.93 µg/L in all samples), isoamyl octanoate (from 12.25 ± 2.29 µg/L to 21.24 ± 4.25 µg/L in all samples) and diethyl succinate (from 2.93 ± 0.43 mg/L to 10.07 ± 1.40 mg/L in all samples) increased over time in all wines, while ethyl decanoate (from 9.11 ± 2.42 µg/L to 6.21 ± 1.81 µg/L in all samples), isobutyl acetate (from 428.55 ± 88.46 µg/L to 67.19 ± 17.33 µg/L in all samples), isoamyl acetate (from 779.64 ± 127.00 µg/L to 42.89 ± 21.21 µg/L in all samples) and 2-phenyl-acetate (from 81.03 ± 6.94 µg/L to 63.14 ± 2.77 µg/L in all samples) decreased. For both inoculated and non-inoculated samples, ethyl hexanoate showed lower concentrations in the

wines with initial addition of flavan-3-ols than in the control wines (Figure 6.8. a). Inoculation with Oenos™ combined with the addition of *trans*-resveratrol and HCA also affected the production of ethyl hexanoate after 170 days of incubation (Figure 6.8. a). The concentration in diethyl succinate in the non-inoculated wines treated with HCA and *trans*-resveratrol was higher than in the controls (Figure 6.8. b). Inoculation with Oenos™ combined with the addition of *trans*-resveratrol and HCA had the opposite effect. Although, more diethyl succinate was produced in control wines inoculated with Oenos™ and less in wines inoculated with Oenos™ and combined with the addition of *trans*-resveratrol and HCA compared to the non-inoculated ones (Figure 6.8. b). More methyl octanoate was detected in the non-inoculated wines supplemented with *trans*-resveratrol compared to the controls, and more ethyl-2-methyl-butanoate was detected in the inoculated ones (Tables 6.5. and 6.6.). Ethyl hexanoate (fruity/green apple) and ethyl-2-methyl-butanoate (berry) having concentrations above their thresholds (determined in a water/ethanol/glycerol/tartaric acid solution by Ferreira *et al.* (2000)), could perceptibly differentiate these wines 170 days after the beginning of MLF.

2-Phenylethanol, isobutyric acid and isovaleric acid were the most relevant alcohol and VFA analyzed in this study 170 days after the beginning of MLF in term of variations between samples and their concentrations were above the thresholds determined in a water/ethanol/glycerol/tartaric acid solution by Ferreira *et al.* (2000) (Tables 6.5. and 6.6.). The concentrations in these compounds in wines were relatively constant from 28 days after the beginning of MLF to 170 (Tables 6.2., 6.3., 6.4. and 6.5.). The quantities of 2-phenylethanol and isovaleric acid were higher in non-inoculated wines supplemented with HCA compared to the controls, while the quantity of isobutyric acid was lower in wines inoculated with Oenos™ combined with the addition of HCA than in the controls (Tables 6.5. and 6.6.).

No general trend was observed in the variation of concentration in the terpenes 170 days after the beginning of MLF (Tables 6.5. and 6.6.). Nevertheless, linalool, defined by a lemon/floral aroma, was present in all wines at concentrations above threshold (determined in a water/ethanol/glycerol/tartaric acid solution by Ferreira *et al.* (2000)).

At this time point, volatile phenols (4-ethylphenol and 4-vinylguaiacol) were detected in the samples but in low quantities and with no differences between wines (Tables 6.5. and 6.6.).

Table 6.5. - Volatile compounds composition of non-inoculated wines 170 days after the beginning of MLF, with or without (controls) addition of phenolics.

Volatile compounds	Odor thresholds	Non-inoculated samples				
		Control	Flavan-3-ols	Flavonols	HCA	*Trans*-resveratrol
Esters						
Ethyl hexanoate	14 [9]	130.60 ± 6.83 **a**	110.29 ± 3.72 **b**	117.86 ± 5.61	132.15 ± 1.64	135.38 ± 7.40
Ethyl octanoate	580 [3]	122.01 ± 26.33	122.58 ± 0.53	95.73 ± 0.55	123.82 ± 0.39	139.27 ± 9.93
Ethyl decanoate	200 [9]	5.09 ± 0.88	3.23 ± 0.16	7.28 ± 0.36	7.14 ± 1.00	6.62 ± 1.50
Isobutyl acetate	1 600 [2]	75.75 ± 11.68	44.32 ± 6.47	75.00 ± 4.85	71.57 ± 28.04	75.65 ± 2.30
Isoamyl acetate	30 [9]	34.32 ± 12.90	33.39 ± 0.43	43.12 ± 6.53	83.43 ± 54.68	32.21 ± 1.18
Isoamyl octanoate	_	25.65 ± 2.03	25.06 ± 1.61	19.54 ± 2.12	23.60 ± 2.70	25.06 ± 3.42
2-Phenylethyl-acetate	250 [4]	61.44 ± 3.64	62.54 ± 2.04	62.03 ± 2.10	66.26 ± 2.04	62.56 ± 0.34
Diethyl succinate mg/L	200 [3]	9.66 ± 1.47 **b**	8.99 ± 0.15	10.80 ± 0.75 **a**	12.03 ± 0.03 **a**	11.61 ± 0.47 **a**
Ethyl butanoate	20 [9]	164.76 ± 27.52	175.40 ± 50.31	171.99 ± 35.92	235.32 ± 81.85	172.86 ± 28.47
Hexyl acetate	700 [7]	1.84 ± 0.06	4.50 ± 0.25	2.97 ± 2.45	1.75 ± 0.32	2.22 ± 0.45
Ethyl-2-methyl-butanoate	18 [9]	21.76 ± 0.24	7.78 ± 2.16	15.36 ± 10.94	13.79 ± 6.65	14.27 ± 7.49
Methyl octanoate	_	3.28 ± 0.70 **b**	3.95 ± 0.67	4.77 ± 1.11	5.09 ± 0.78	6.66 ± 0.94 **a**
Isoamyl hexanoate mg/L	_	2.98 ± 0.44	3.48 ± 0.43	1.75 ± 0.47	3.77 ± 1.38	3.09 ± 0.55
Alcohols						
Trans-3-hexenol	400 [9]	61.97 ± 8.41	48.15 ± 2.90	59.51 ± 5.06	48.88 ± 26.13	72.53 ± 10.85
Trans-2-hexenol	400 [9]	92.62 ± 5.49 **b**	90.98 ± 4.39	110.24 ± 6.56 **a**	118.82 ± 0.56 **a**	109.87 ± 2.05 **a**
1-Hexanol mg/L	4 [7]	2.18 ± 0.05 **b**	2.06 ± 0.06	2.33 ± 0.02	2.54 ± 0.02 **a**	2.54 ± 0.09 **a**
2-Phenylethanol mg/L	14 [9]	65.56 ± 2.54 **b**	67.58 ± 4.27	84.58 ± 5.81	101.02 ± 1.25 **a**	92.74 ± 16.06
Terpenes						
Linalool	25 [9]	59.39 ± 2.91	85.42 ± 13.86	47.68 ± 3.24	67.24 ± 16.89	54.17 ± 4.08
β-Citronellol	100 [3]	10.77 ± 0.23	9.47 ± 0.92	12.00 ± 1.55	10.98 ± 1.15	11.93 ± 1.46
Nerol	300 [12]	7.98 ± 1.13	7.01 ± 1.08	6.79 ± 0.25	7.78 ± 1.83	8.50 ± 0.11
Volatile Fatty Acids						
Isobutyric acid mg/L	0.23 [9]	6.89 ± 0.65	6.63 ± 0.32	7.45 ± 0.99	7.80 ± 1.37	7.67 ± 0.08
Isovaleric acid mg/L	0.033 [9]	2.46 ± 0.04 **b**	2.45 ± 0.16	3.09 ± 0.26	3.58 ± 0.12 **a**	3.39 ± 0.53

Valeric acid	8 100 [3]	316.56 ± 23.07	305.23 ± 8.44	358.72 ± 2.23	463.54 ± 142.95	423.40 ± 37.19
Hexanoic acid mg/L	0.42 [9]	17.24 ± 0.08 **b**	16.76 ± 0.53	18.11 ± 0.75	19.04 ± 0.24 **a**	19.70 ± 0.28 **a**
Volatile phenols						
4-Ethylphenol	600 [7]	5.69 ± 0.44	7.91 ± 2.13	3.26 ± 0.01	5.16 ± 1.06	4.62 ± 2.02
Carbonyl compounds						
Benzaldehyde	200 000 [2]	34.09 ± 3.41	39.12 ± 0.60	32.76 ± 3.26	44.50 ± 3.77	45.37 ± 10.38

Concentration values in µg/L except where indicated. n = 4 ± standard deviations. nd = not detectable in the wines.

Bold letters on the right indicate statistically significant differences of the sample with the control at that specific time point (Dunnett´s $p < 0.05$).

2 (Escudero *et al.*, 2007): Thresholds calculated in beer; 3 (Etiévant, 1991) and 7 (Swiegers *et al.*, 2005a): Thresholds calculated in wine; 4 (Guth, 1997): Thresholds calculated in 10% ethanol; 9 (Ferreira *et al.*, 2000): Thresholds determined in 11% v/v aqueous ethanol with 7 g/L glycerol and 5 g/L tartaric acid, at pH 3.4; 12 (Almudena García-Ruiz *et al.*, 2013b): Thresholds calculated in 10% water/ethanol solution containing 5 g/l tartaric acid.

Table 6.6. - Volatile compounds composition of inoculated (Oenos™) wines 170 days after the beginning of MLF, with or without (controls) addition of phenolics.

Volatile compounds	Odor thresholds	Inoculated samples				
		Control	Flavan-3-ols	Flavonols	HCA	*Trans*-resveratrol
Esters						
Ethyl hexanoate	14 [9]	123.50 ± 2.45 **a**	92.86 ± 12.23 **b**	117.80 ± 0.58	98.24 ± 2.55 **b ***	103.09 ± 1.32 **b ***
Ethyl octanoate	580 [3]	107.63 ± 9.47	101.99 ± 1.45 *	104.42 ± 8.55	102.75 ± 7.19	114.99 ± 3.42
Ethyl decanoate	200 [9]	8.80 ± 0.78 **	4.54 ± 2.04	7.63 ± 1.84	6.56 ± 0.09	5.22 ± 0.45
Isobutyl acetate	1 600 [2]	66.31 ± 7.26	51.56 ± 19.25	72.55 ± 5.82	47.46 ± 6.04	91.71 ± 13.81
Isoamyl acetate	30 [9]	46.05 ± 10.95	31.57 ± 10.71	44.90 ± 16.39	30.86 ± 10.51	50.04 ± 12.98
Isoamyl octanoate	_	16.99 ± 1.59 *	18.88 ± 2.18 *	18.44 ± 4.96	16.71 ± 1.47	20.49 ± 4.00
2-Phenylethyl-acetate	250 [4]	65.40 ± 2.25	65.04 ± 5.58	65.13 ± 5.03	63.66 ± 1.11	65.21 ± 2.50
Diethyl succinate mg/L	200 [3]	11.55 ± 0.42 **a** **	7.95 ± 0.55 **b**	10.33 ± 0.25	8.57 ± 0.17 **b ***	9.23 ± 0.67 **b**
Ethyl butanoate	20 [9]	152.32 ± 4.25	114.63 ± 61.31	151.63 ± 7.54	138.91 ± 0.50	161.82 ± 10.36
Hexyl acetate	700 [7]	1.82 ± 0.04	1.37 ± 0.66 *	2.00 ± 0.01	1.33 ± 0.29	1.40 ± 0.41
Ethyl-2-methyl-butanoate	18 [9]	9.97 ± 7.03 **b**	16.80 ± 2.32	18.63 ± 0.43	18.90 ± 0.85	25.07 ± 0.98 **a**
Methyl octanoate	_	4.27 ± 0.66	3.46 ± 0.19	4.14 ± 0.06	6.20 ± 1.36	7.31 ± 1.59
Isoamyl hexanoate mg/L	_	2.72 ± 0.33	2.69 ± 0.34	2.51 ± 0.22	2.40 ± 0.13	4.18 ± 1.67
Alcohols						
Trans-3-hexenol	400 [9]	66.28 ± 12.64	59.27 ± 6.63	60.02 ± 5.58	57.22 ± 1.42	75.04 ± 12.90
Trans-2-hexenol	400 [9]	107.79 ± 0.43	117.70 ± 23.24	103.87 ± 2.17	114.17 ± 0.46 *	119.83 ± 17.26
1-Hexanol mg/L	4 [7]	2.50 ± 0.13	2.28 ± 0.20	2.34 ± 0.07	2.46 ± 0.04	2.68 ± 0.18
2-Phenylethanol mg/L	14 [9]	91.33 ± 7.16 **	85.54 ± 2.73 **	80.35 ± 5.40	90.89 ± 2.33 *	112.24 ± 20.92
Terpenes						
Linalool	25 [9]	54.63 ± 1.11	50.24 ± 3.81	46.76 ± 3.09	45.12 ± 3.98	50.23 ± 6.33
β-citronellol	100 [3]	9.82 ± 0.91	11.71 ± 2.27	8.90 ± 0.24	13.75 ± 1.36	10.87 ± 0.35
Nerol	300 [12]	7.66 ± 1.26	7.15 ± 1.26	6.98 ± 0.03	7.04 ± 0.07	7.98 ± 0.31
Volatile Fatty Acids						
Isobutyric acid mg/L	0.23 [9]	7.67 ± 1.44 **a**	6.74 ± 0.49	6.81 ± 0.12	4.03 ± 0.85 **b**	8.68 ± 0.99
Isovaleric acid mg/L	0.033 [9]	3.32 ± 0.23 **	3.13 ± 0.09 **	2.92 ± 0.20	3.33 ± 0.09	4.04 ± 0.72

Valeric acid	8 100 [3]	450.68 ± 47.46	387.56 ± 164.64	249.74 ± 101.34	364.90 ± 37.53	567.68 ± 378.24
Hexanoic acid mg/L	0.42 [9]	19.25 ± 1.15	18.75 ± 1.09	18.34 ± 0.39	20.36 ± 0.38	21.70 ± 1.32
Volatile phenols						
4-Ethylphenol	600 [7]	2.90 ± 1.50	4.24 ± 2.31	4.75 ± 1.06	3.67 ± 0.24	3.77 ± 0.62
4-Vinylguaiacol	40 [4] / 1 100 [5]	14.37 ± 0.09	nd	nd	nd	nd
Carbonyl compounds						
Benzaldehyde	200 000 [2]	38.31 ± 5.94	38.11 ± 2.39	36.65 ± 0.43	35.60 ± 1.79	51.16 ± 13.25

Concentration values in µg/L except where indicated. n = 4 ± standard deviations. nd = not detectable in the wines.

Bold letters on the right indicate statistically significant differences of the sample with the control at that specific time point (Dunnett´s $p < 0.05$). Stars on the up right indicate statistically significant differences between inoculated and non-inoculated samples with the same treatment at the same time point (ANOVA $p<0.05$). * Mean value significantly higher for the non-inoculated sample compared to the inoculated one, ** Mean value significantly higher for the inoculated sample compared to the non-inoculated one.

2 (Escudero *et al.*, 2007): Thresholds calculated in beer; 3 (Etiévant, 1991) and 7 (Swiegers *et al.*, 2005a): Thresholds calculated in wine; 4 (Guth, 1997): Thresholds calculated in 10% ethanol; 9 (Ferreira *et al.*, 2000): Thresholds determined in 11% v/v aqueous ethanol with 7 g/L glycerol and 5 g/L tartaric acid, at pH 3.4; 12 (Almudena García-Ruiz *et al.*, 2013b): Thresholds calculated in 10% water/ethanol solution containing 5 g/l tartaric acid.

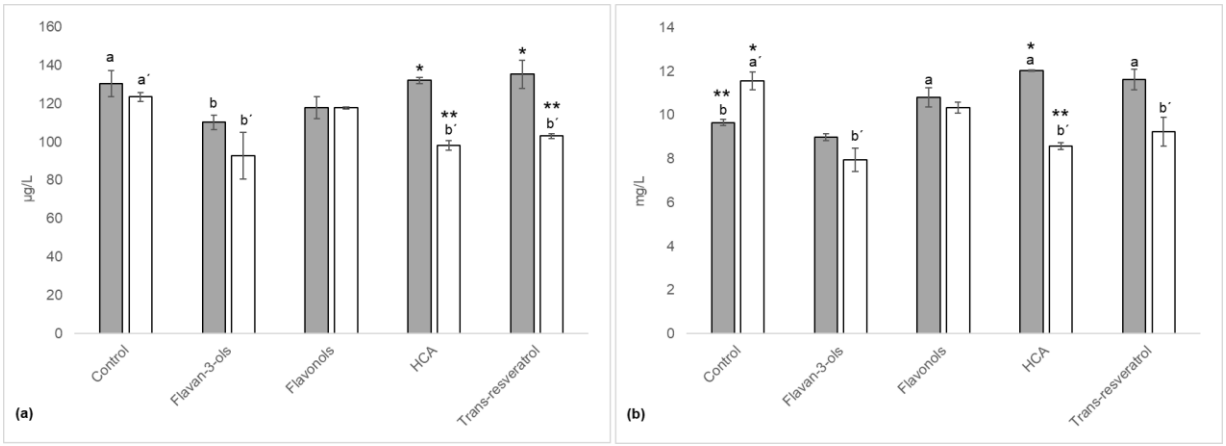


Figure 6.8. - Concentrations in (a) ethyl hexanoate and (b) diethyl succinate 170 days after the beginning of MLF in controls and wines with their initial concentrations in phenolics tripled. Grey bars - spontaneous MLF. White bars - MLF inoculated with *O. oeni* Oenos™. n = 4. a, a´, b and b´ indicate values statistically significantly different between non-treated wines and wines treated with phenolics (Dunnett´s test, $p < 0.05$). * and ** indicate values statistically significantly different between inoculated and non-inoculated samples with the same treatment (ANOVA, $p<0.05$).

6.5. Discussion

The pre-malolactic winemaking techniques influence the composition of wines in phenolic compounds, impacting on the microbial behavior and therefore having consequences on the wines´ final quality. In this study, the changes of the composition of volatiles and phenolics in wines produced by the increase of particular classes of phenolics before the initiation of the malolactic fermentation have been specifically explored during non-inoculated and inoculated MLF and subsequent storage. The increases in phenolics modified the metabolite profiles of the wines as did the MLF process itself and the subsequent storage period.

The levels of all phenolics added diminished over time and some (kaempferol, *trans*-ferulic acid and *trans*-resveratrol) were no longer detectable in wines after 170 days incubation. As it has been described by previous authors (Vrhovsek *et al.*, 2002; Castillo-Sánchez *et al.*, 2008; Bimpilas *et al.*, 2015), anthocyanins declined over time. In the wines supplemented with flavan-3-ols, (+)-catechin was possibly used as precursor of the condensed pigment malvidin-3-O-glucoside-ethyl-catechin (Trikas *et al.*, 2016). Hydroxybenzoic acids generally increased in the first 28 days of incubation, similarly to the results obtained previously by Li *et al.* (2009), but remained relatively stable during the storage at 12 °C. In all the samples the concentration of (hydroxy)cinnamoyl-tartaric acids decreased after 28 days incubation at 25 °C and their corresponding HCA “free” forms increased, suggesting a cinnamoyl esterase activity by the microbiota of the wines as has been observed in other works (Hernández *et al.*, 2006; Cabrita *et al.*, 2008). *Oenococcus oeni* Oenos™, used as starter in this work, is known to possess the activity which could help to explain the observed effect in the inoculated wines (Burns and Osborne, 2013; Chescheir

et al., 2015). Nevertheless, in the spontaneous-MLF wines used in this work the same effect was observed, implying the presence of indigenous microorganisms with this enzymatic activity. After a period of 170 days post-MLF initiation, *trans*-caftaric and *trans*-coutaric acids were found in lower concentrations in the inoculated samples compared to the non-inoculated ones, which could support a greater cinnamoyl esterase activity of *O. oeni* Oenos™ comparatively with the native strains in uninoculated wines. The concentration in *trans*-caffeic acid in the wines supplemented with *trans*-*p*-coumaric and *trans*-ferulic acids was higher than in the other samples 28 days after the beginning of MLF, and their concentration in *trans*-caftaric acid was lower 170 days after. Silva *et al.* (2011) and Rosimin *et al.* (2015) indicated that "free" HCA were inducing the decarboxylase and reductase activities of some LAB, metabolizing HCA into volatile phenols. An induction of the release of *trans*-caffeic acid from *trans*-caftaric acid by the addition of other compounds from the same family could be a possible explanation to this phenomenon. *Trans*-fertaric acid decreased during MLF but remained relatively constant during storage contrary to *trans*-caftaric and *trans*-coutaric acids. This observation indicates that *trans*-fertaric acid was probably not used as substrate by the cinnamoyl esterase during storage. Finally, 170 after the beginning of MLF, the contents in *trans*-caftaric and *trans*-coutaric acids were higher in inoculated wines with addition of two times their initial concentration in flavonols. This result could be explained by an inhibition of specific LAB possessing the cinnamoyl esterase activity by flavonols.

A different susceptibility of LAB to the antimicrobial properties of phenolic compounds leaded to peculiar impacts on the composition of esters in the wines. Most of the esters decreased in the wines after 28 days of incubation and some (isoamyl octanoate, ethyl decanoate, ethyl hexanoate, ethyl octanoate, linalyl acetate and 2-phenyl-ethylacetate) were in higher concentrations in wines with initial addition of double flavan-3-ols concentration. The same observation was described by Rodriguez-Bencomo *et al.* (2014) in wines with addition of phenolic extracts from eucalyptus leaves and almond skins reported to be rich in (+)-catechin (Rodríguez-Bencomo *et al.*, 2014). Some studies (Roguant *et al.*, 2000; Alberto *et al.*, 2001; de Llano *et al.*, 2016) have reported (+)-catechin to have a stimulatory effect on the growth and metabolism of some LAB, while Vaquero *et al.* (2007) found an inhibitory effect of this compound at high concentration towards other bacteria. A potential inhibitory effect of flavan-3-ols on some LAB strains and their metabolism hydrolyzing the esters could explain the higher concentrations in esters in wines with initial concentration of flavan-3-ols doubled compared to the controls. Ethyl hexanoate decreased over 28 days incubation and increased after, to 170 days incubation. Tripling the concentration in flavan-3-ols before the initiation of MLF delayed the production of ethyl hexanoate after 170 days of incubation. According to Sumby *et al.* (2010; 2013a), some *O. oeni* strains could synthesize ethyl hexanoate which could indicate an inhibition of some LAB and their metabolism to synthesize esters by flavan-3-ols, therefore reducing the concentration of this compound. From all the esters analyzed, diethyl succinate was the only one that strictly increased over time. After 170 days incubation, HCA and *trans*-resveratrol had an opposite impact on the diethyl succinate content whether the wines were inoculated or not. Garcia-Ruiz *et al.* (2013b) suggested the capacity of phenolic extracts to regulate the

bacterial production of succinic acid by influencing the growth and/or metabolism of specific LAB and thus the concentration in diethyl succinate, which could explain in this present study, the lower concentrations in diethyl succinate found in inoculated wines supplemented with HCA and *trans*-resveratrol 170 days after the beginning of MFL. This phenomenon could be linked to the decrease in threonine, essential amino acid for the growth of some strains, at the end of MLF in these samples (de Nadra *et al.*, 2003).

Wines inoculated with Oenos™ were more sensitive to the addition of HCA and *trans*-resveratrol during MLF (14 days) than non-inoculated wines as less 1-hexanol, 2-phenylethanol, isobutyric, isovaleric, octanoic and decanoic acids were detected in these wines compared to the controls. Rodriguez-Bencomo *et al.* (2014) also observed a decrease in 2-phenylethanol over MLF by the addition of phenolic extracts. Nevertheless, while 2-phenylethanol and isovaleric acid decreased from 28 days after the beginning of MLF to 170 days after, more of these compounds were found in non-inoculated wines supplemented with HCA.

The results obtained indicate that the effects of the phenolics on the volatile composition of the wines are linked to the wine microbiota. A pre-malolactic increase in flavonols appeared to affect the least the volatiles composition of the wines. Although some of the volatiles analyzed were detected in concentrations in wines above their thresholds (determined in a water/ethanol/glycerol/tartaric acid solution by Ferreira *et al.* (2000)), possibly impacting their organoleptic profile, no sensory analysis was performed so no definitive conclusions about the sensory impact could be drawn.

This work provided a better knowledge of the impact of specific groups of phenolic compounds on the compositions of volatiles and phenolics in wines during MLF and subsequent storage, which could help winemakers to optimize operational pre-malolactic winemaking conditions (harvest parameters, fermentation techniques, use of specific bacteria and enzymes, etc.) in order to emphasize targeted aromas in the final wine.

7. New insights into cinnamoyl esterase activity in *Oenococcus oeni*

(Publications based on this chapter: Collombel I., Melkonian C., Campos F.M., Molenaar D., Hogg T. (submitted and underreview) Alternative insights into cinnamoyl esterase activity in Oenococcus oeni. Frontiers in Microbiology.)

7.1. Summary

Some strains of *Oenococcus oeni* possess cinnamoyl (CE) esterase activity that can be relevant in the malolactic fermentation stage of wine production by liberating hydroxycinnamic acids from their tartrate derivative forms ((hydroxy)cinnamoyl-tartaric acids) and thus can serve as precursors of volatile phenol compounds responsible for sensory faults in wine. The objective of this study was to better understand the basis of this differential activity between strains. After initial screening, five commercial strains of *O. oeni* were selected. Three of the strains were found to exhibit cinnamoyl esterase activity (CE+) and two not (CE-). Unlike its "free" form (*trans*-caffeic acid), *trans*-caftaric acid was not toxic toward *O. oeni*. Although the use of functional annotation of genes revealed genotypic variations between the strains, no specific genes common only to the three CE+ strains could explain the different activity. Pasteurized wine was used as a natural source of (hydroxy)cinnamoyl-tartaric acids in growth and metabolism experiments conducted in MRS medium, whilst commercial *trans*-caftaric acid was used as available substrate for enzyme assays. In the case of the two CE+ strains, Oenos™ and CiNe™, wine-exposed samples showed a more rapid degradation of *trans*-caftaric acid than unexposed ones. Of thirteen lysis protocols tested, only one enzymatic and one physical protocol were able to adequately disrupt the cell wall and membrane of the five commercial *O. oeni* strains studied. The CE activity was present in all cell-free extracts of both wine-exposed and unexposed strains, except in the cell-free extracts of the CE- strain CH11™. This activity may be constitutive rather than induced by exposure to the (hydroxy)cinnamoyl-tartaric acids. *Trans*-caftaric acid was totally degraded to *trans*-caffeic acid by cell-free extracts of the three CE+ strains, whilst cell-free extracts of the CE- strain CH16™ showed significantly lower activity, although higher for the strains in experiments with no prior wine exposure. Only in the case of the CE+ strains exposed to wine did the disrupted cell fraction (cell debris) contain higher protein concentrations that the unexposed ones. The EstB28 esterase gene did not reveal any difference on the upstream regulation and transport functionality between the strains. This study highlights the complexity of the basis of this activity in wine related *O. oeni*. Variable cinnamoyl esterases or / and membrane transport activities in the *O. oeni* strains analyzed and a possible implication of wine molecules could explain this phenomenon.

7.2. Introduction

Hydroxycinnamic acids (HCA) are a group of phenolic acids and are abundantly present across the plant kingdom. These compounds have many roles in many aspects of biology – both in the plants in which they are produced and in bacteria, fungi, plants and animals that interact with them. Examples of these include post-ingestion effects, through food and beverage consumption, on animals and humans (Boudet, 2007; Prasad *et al.*, 2011; Shahidi and Ambigaipalan, 2015; Calabriso *et al.*, 2016) and plant-fungi signaling through soil diffusion (Ragonezi *et al.*, 2014).

In grapes, HCA are mostly encountered in forms that are covalently bound to tartaric acid, glucose and the ethyl group, in the vacuoles of the skin and pulp cells. In wines, “free” HCA may act as color stabilizers, antimicrobial agents and flavor precursors (Campos *et al.*, 2003; Hernández *et al.*, 2006; Bouzanquet *et al.*, 2012; Lima *et al.*, 2018).

Caffeic, *p*-coumaric and ferulic acids are substrates for enzymatic systems of the wine spoilage yeast *Brettanomyces/Dekkera bruxellensis* and a number of wine lactic acid bacteria (LAB), through which volatile phenols are produced and can be responsible for sensory faults in wines (Couto *et al.*, 2006; Kheir *et al.*, 2013). The most commonly reported pathway for the microbial metabolization of HCA involves two enzyme systems: a phenolic acid decarboxylase (PAD) and a vinylphenol reductase (VPR) (Figure 7.1). PAD and VPR activities are variable among the strains and principally modulated by the nature and concentration of the substrate (Barthelmebs *et al.*, 2000; Silva *et al.*, 2011; Filannino *et al.*, 2015; Rosimin and Kim, 2015; Sturm *et al.*, 2015). Some *B. bruxellensis* strains have been observed to directly produce ethylphenols from *p*-coumaroyl glucose, feruloyl glucose and ethyl coumarate, but not from *p*-coumaroyl and feruloyl L-tartaric acids (coutaric and fertaric acids) (Hixson *et al.*, 2012; Hixson *et al.*, 2016).

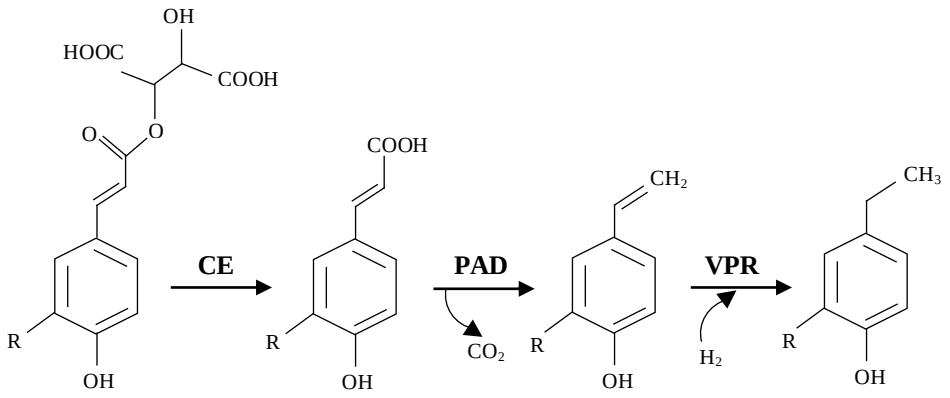
Malolactic fermentation (MLF) is a normally desirable step in the vinification process of most red wines and can be spontaneous, due to grape/winery LAB or induced by selected starters chosen according to the type and quality of wine desired. Due to its great tolerance to alcohol content and acidity, *Oenococcus oeni* strains are usually the most predominant LAB that perform MLF. According to Ribereau-Gayon *et al.* (2006), the HCA that are precursors of volatile phenols exist in wine mainly as their tartrate derivatives, with *trans*-caftaric acid being the most abundant. These molecules have not yet been described as substrates for the PAD enzymes and so are apparently not direct precursors of volatile phenols. During MLF, the release of "free" HCA has been previously linked to the disappearance of its corresponding tartrate derivative form (Hernández *et al.*, 2006; Cabrita *et al.*, 2008)

Cinnamoyl esterases, also called feruloyl esterases, ferulic acid esterases or hydroxycinnamoyl esterases, have been studied in many microorganisms (*Lactobacillus*, *Bacillus*, *Bifidobacteria*) and commercial enzyme preparations used in winemaking, and are described as enzymes involved in the release of HCA from their esterified forms (Donaghy *et al.*, 1998; Comino *et al.*, 2014; Fia *et al.*, 2014; Fritsch *et al.*, 2017). Cinnamoyl esterase activity is apparently inducible and depends both on the

substrates present and on the specific strain (Brezillon *et al.*, 1996). Crepin *et al.* (2004) classified the cinnamoyl esterases into 4 groups based on substrate preferences and supported by primary sequence identity. Ethyl ferulate, methyl ferulate, methyl caffeate, methyl *p*-coumarate, methyl sinapinate, and chlorogenic acid are the most common esters cleaved by the cinnamoyl esterases studied (Crepin *et al.*, 2004). Some cinnamoyl esterases were reported to be extracellular (Brezillon *et al.*, 1996), whilst others were found to be located within the cell (Gobbetti *et al.*, 1996; Castillo *et al.*, 1999). A few of these enzymes produced during fermentation have yet been purified from various bacteria (*Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus plantarum*, *Lactobacillus helveticus*) and fungi (*Penicillium pinophilum*, *Aspergillus awamori*) and characterized to some extent (Castanares *et al.*, 1992; Wang *et al.*, 2004; Kanauchi *et al.*, 2008; Lai *et al.*, 2009; Esteban-Torres *et al.*, 2013; Esteban-Torres *et al.*, 2015; Song and Baik, 2017). However, none of the microorganisms producing these activities studied are wine-related nor are the (hydroxy)cinnamoyl-tartaric acids previously tested as substrates.

Lactic acid bacteria possess a substantial collection of enzymes involved in the synthesis and hydrolysis of esters including the wine-associated LAB *O. oeni* and *L. hilgardii*, catabolizing volatiles aromatic esters and which the intracellular esterases have been characterized by Sumby *et al.* (2009, 2013b). Among LAB, only *O. oeni* Oenos™ and CiNe™ strains in wine and the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533 have been found to possess the cinnamoyl esterase activity enable to cleave the ester bond of (hydroxy)cinnamoyl-tartaric acids, releasing tartaric acid and the corresponding HCA (Figure 7.1.) (Bel-Rhlid *et al.*, 2012; Burns and Osborne, 2013; Chescheir *et al.,* 2015; Madsen *et al.*, 2016). Substrate for this enzymatic activity is limited to *trans*-isomers of the (hydroxy)cinnamoyl-tartaric acids (Hernandez *et al.*, 2007a).

Although the production of volatile phenols from phenolic acids has been widely studied in wine, much less is known about prior processes that determine the availability of free precursor molecules from (hydroxy)cinnamoyl-tartaric acids. The objective of the current study is to better understand the differences between LAB strains regarding their cinnamoyl esterase activity.



(Hydroxy)cinnamoyl-tartaric acids	HCA	Vinylphenols	Ethylphenols
R = H: coutaric acid	*p*-coumaric acid	4-vinylphenol	4-ethylphenol
R = OH: caftaric acid	caffeic acid	4-vinylcatechol	4-ethylcatechol
R = OCH_3: fertaric acid	ferulic acid	4-vinylguaiacol	4-ethylguaiacol

Figure 7.1. - Enzymatical activities linking the production of volatile phenols from its tartrate derivatives forms.

7.3. Material and Methods

7.3.1. Microbial aspect

7.3.1.1. Source and preparation of cultures

Seven commercial *O. oeni* strains were used in this study; Viniflora® Oenos™, CH11™, CH16™, CH35™, CiNe™ from Ch. Hansen (Hørsholm, Denmark) as well as Enoferm Alpha™ and Lalvin VP41™ from Lallemand (Montreal, Canada). The wine-related strain *L. plantarum* NOVA™ from Ch Hansen and the non-wine related strains *L. plantarum* NCFB 1752, *Pediococcus damnosus* NCFB 1832T, *P. pentosaceus* NCFB 990T and *L. brevis subsp. gravesensis* NCFB 1749T from the National Collection of Food Bacteria (Reading, UK) were also tested for their cinnamoyl esterase activity. Three Port-wine isolates were also screened: *L. hilgardii* ESB 19, *L. fructivorans* ESB 92 and *L. collinoides* ESB 99, isolated by Couto and Hogg (1994), from the Escola Superior de Biotecnologia, Portuguese Catholic University (Porto, Portugal). Pre-cultures were grown aerobically at 25 °C with no agitation to late exponential phase (absorbance of about 1.6 AU at 600 nm for *Lactobacillus* and *Pediococcu*s strains and about 0.9 AU for *Oenococcus* strains except for CiNe™ about 0.6 AU and CH35™ about 0.4 AU) in liquid MRS medium (prepared as described in section 2.2).

7.3.1.2. Toxicity evaluation of *trans*-caftaric acid, *trans*-caffeic acid and 4-ehylcatechol against wine *O. oeni*

In order to understand whether the cinnamoyl esterase activity in *O. oeni* is somehow related to a detoxification effect, the toxic levels of *trans*-caftaric acid and its immediate metabolites (*trans*-caffeic acid and 4-ethylcatechol) were evaluated in MRS broth (prepared as described in section 2.2).
Solutions of 4-ethylcatechol (4-EC), *trans*-caftaric and *trans*-caffeic acids were prepared as described in section 2.4. Fresh cultures of the five *O. oeni* strains from Ch. Hansen were cultivated in 96-well x 300 µL microplates containing MRS broth supplemented with 300 mg/L and 150 mg/L of *trans*-caftaric acid, 170 mg/L and 85 mg/L of *trans*-caffeic acid and 130 mg/L and 65 mg/L of 4-EC, representing equivalent molar concentrations (1 and 0.5 mmol/L respectively) for all compounds. Cultures were grown at 25 °C with no agitation for 9 days with 1 day as interval of absorbance measurement. Absorbance was measured (at 600 nm wavelength) with a Synergy™ HTX Multi-Mode Microplate Reader from BioTek Instruments (Winooski, VT, USA).

7.3.2. Enzymatic activity

7.3.2.1. Wine as a natural source of (hydroxy)cinnamoyl-tartaric acids

A red wine (13.28% (v/v) alcohol, pH 3.79, 1.00 g/L malic acid and 1.35 g/L lactic acid) from the Touriga Franca variety collected half-way through malolactic fermentation in the Douro region (in Northern Portugal) from the 2017 harvest (stored at 4 °C before use) was pasteurized 3 min at 50 °C. Prior wine contamination was checked by the drop-count technique as described in section 2.3. (detection limit 500 CFU/mL).

In most of the following experiments, bacteria were cultivated in liquid MRS medium with 30% pasteurized wine (wine-exposed). The HCA / tartrate derivatives composition of the preparations is shown in table 7.1.

Table 7.1. - Concentrations in mg/L of HCA and their tartrate derivatives in a mix of 30% pasteurized red wine and 70 % MRS broth.

trans-caftaric acid	52.76 ± 0.30[y]
trans-caffeic acid	0.56 ± 0.04
trans-coutaric acid	23.79 ± 0.29
trans-*p*-coumaric acid	1.58 ± 0.15
trans-fertaric acid	2.34 ± 0.05
trans-ferulic acid	0.02 ± 0.01

y values represent the mean of three values ± standard deviation

7.3.2.2. Cinnamoyl esterase activity screening

Pre-cultures of LAB were transferred to sterile 100 mL flasks containing 30 mL pasteurized wine and 70 mL MRS broth (30% pasteurized wine).
After growing at 25 °C with no agitation and reaching a concentration of 9.26 (± 0.21) log CFU//mL in 2-4 days for *Lactobacillus* and *Pediococcu*s strains and 5-8 days for *Oenococcus* strains, 1 mL samples were collected and stored at -20 °C before High-Performance Liquid Chromatography (HPLC) analysis. Lactic acid bacteria counts were done in triplicate using the drop-count technique. Each individual assay was performed in duplicate.

7.3.2.3. Localization of the cinnamoyl esterase activity

Cultures of the five *O. oeni* strains from Ch. Hansen were prepared in sterile flasks containing 250 mL liquid MRS medium (unexposed: pH 4.5, 5 % (v/v) alcohol) and 175 mL liquid MRS medium mixed with 75 mL (30%) of pasteurized wine (wine-exposed: pH 4.29, 7.48 % (v/v) alcohol) and incubated at 25 °C with no agitation until the stationary growth phase (5-7 days depending on the strain). After reaching an absorbance (at 600 nm) of 0.6 AU (10^8 to 10^9 CFU/mL), the cells were harvested by centrifugation (7500 g for 10 min at 4 °C). The absorbance was measured for unexposed samples by spectrophotometry (UNICAM, Cambridge, UK). Bacterial counts were done in triplicate for both unexposed and wine-exposed samples using the drop-count technique.

Among the 13 lysis protocols tested onto the CE+ *O. oeni* strain Oenos™ (Table 7.2), only two (one enzymatic and one mechanical) were able to satisfactorily disrupt the membrane and therefor used for the following experiment. The supernatants of the three CE+ strains (extracellular parts) were collected and the pellets were washed twice with sodium phosphate buffer (50 mM, pH 7) for the enzymatical protocol and NaCl 0.15 M for the mechanical protocol. For the enzymatical protocol, the cells were resuspended in 6 mL sodium phosphate buffer with 10 mg/mL of lysozyme from Thermo Fisher Scientific (Waltham, MA, USA), split in four 2 mL sterile tubes containing 200 µL autoclaved glass beads 1 mm diameter (Sigma-Aldrich, Steinheim, Germany) and incubated for one hour at 37 °C. Bacterial cells were then disintegrated three times 20-second cycles with FastPrep®-24 Classic Instrument (MP Biomedicals, Santa Ana, CA, USA) set with a speed of 4 m/s, and cooled five minutes in ice in between each beads-beating. For the mechanical protocol, the cells were resuspended in 6 mL PBS (pH 7.4) and the protein extract was obtained by sonication using a Bandelin Sonopuls HD 2200 homogenizer fitted with an UW 200 probe (Bandelin Electronics, Berlin, Germany) for a total of 20 min. Each disruption cycle lasted 3 min (2 min for the last one) with a power set at 20%. During sonication and 3 min cooling in between each disruption cycle, the cell suspension was immersed in ice. The probe was washed with commercial bleach and ethanol 96,0% v/v before changing of sample. A control without bacteria was also performed for each cell-lysis protocol.

The suspension of disintegrated cells was centrifuged (15000 g for 20 min at 4 °C) to sediment the cell debris. The cell debris were washed twice with 5 mL of NaCl 0.15 M and resuspended in 5 mL of sterile, cold distilled water. The efficiency of the lysis protocols was evaluated using the drop-count technique on the cell suspension before lysis and on the cell debris after washing and resuspension.

The extracellular part and the cell extract of each culture were filtered using sterile filters of 0.22 µm pore size from Elkay Laboratory Products (Basingstoke, UK). *Trans*-caftaric acid was added at a concentration of 10 mg/L and the mixture was incubated at 30 °C for 16 h. Samples were collected before and immediately after substrate addition and after incubation. Samples were analyzed to test their cinnamoyl esterase activity by HPLC analysis.

The cell extracts and cell debris were then stored at -20 °C for further analyses.

Table 7.2. - Cell-lysis protocols adapted from literature tested on *O. oeni* Oenos™.

Washing buffers	Lysis buffers	Lysis protocols
Sodium phosphate buffer (50 mM, pH 7) [2,6]	Sodium phosphate buffer (50 mM, pH 7) [2,6]	Vortex 1 min with autoclaved glass beads 1 mm diameter (Sigma-Aldrich, Germany) [7].
		Disintegrate 3 times 20 s with autoclaved glass beads 1 mm diameter and FastPrep®-24 Classic Instrument (MP Biomedicals, Santa Ana, California, USA) set with a speed of 4 m/s and cool 5 min in ice in between each beads-beating [6].
		Sonicate with a Bandelin Sonopuls HD 2200 homogenizer fitted with an UW 200 probe (Bandelin Electronics, Berlin, Germany) for a total of 2.5 min. Each disruption cycle lasted 30 s with a power set at 100 %. During sonication and 30 s cooling in between each disruption cycle, the cell suspension was immersed in ice [3,4,5].
		Freeze sample at -20 °C for 30 min and vortex 1 min with autoclaved glass beads 1 mm diameter. Repeat the cycle.
	Sodium phosphate buffer (50 mM, pH 7) with 10 mg/mL of lysozyme	Incubate 1 h at 37 °C, disintegrate 3 times 20 s with autoclaved glass beads 1 mm diameter and FastPrep®-24 Classic Instrument set with a speed of 4 m/s and cool 5 min in ice in between each beads-beating [3,4].
	Lysis buffer (Tris-HCl, NaCl, EDTA)	Incubate 1 h 30 at 37 °C.

	with 10 mg/mL of lysozyme	
	Lysis buffer (Tris-HCl, NaCl, EDTA)	Freeze with liquid nitrogen and thaw at room temperature by putting the tube in water while stirring. Repeat the cycle 3 times.
		Sonicate sample 3 times 30 s with a power set at 70 %. Each disruption cycle was spaced with 2 min cooling on ice. Sonicate a last time 30 s at 90 % [3,4,5].
		Sonicate sample for a total of 6 min. Each disruption cycle lasted 20 s with a power set at 25 %. During sonication and 25 s cooling in between each disruption cycle, the cell suspension was immersed in ice [4].
PBS (pH 7.4) [3]	Lysis buffer 50 mM Tris-HCl with 0.1 mg/mL of lysozyme [3]	Incubate 1 h at 37 °C and sonicate 10 min in ice with a power set at 20% and pulses of 10 s [3].
TAE 1X [7]	TAE 1X with 50 mg/mL of lysozyme [7]	Incubate 2 h 30 at 37 °C, disintegrat 3 times 20 s with autoclaved glass beads 1 mm diameter and FastPrep®-24 Classic Instrument set with a speed of 4 m/s and cool 5 min in ice in between each beads-beating [6,7].
	B-PER (Complete Bacterial Protein Extraction Reagent) from Thermo Fisher Scientific	Freeze pellet 30 min. Add 5 mL B-Per reagent, 10 µL lysozyme 50 mg/mL and 10 µL DNaseI 2500 U/mL for 1 g pellet. Homogenized and incubate 1 h at room temperature.
0.15 M NaCl [5]	PBS (pH 7.4) [5]	Sonicate sample for a total of 20 min. Each disruption cycle lasted 3 min (2 min for the last one) with a power set at 20%. During sonication and 3 min cooling in between each disruption cycle, the cell suspension was immersed in ice [5.]

1 (Lai *et al.*, 2009); 2 (Esteban-Torres *et al.*, 2013); 3 (Cafaro *et al.*, 2014); 4 (Couto and Hogg, 1994); 5 (Ya-hui *et al.*, 2012); 6 (Silveira *et al.*, 2004); 7 (Silva *et al.*, 2004).

7.3.2.4. Suitability of ferulic acid methyl ester as substrate for the cinnamoyl esterase activity

A parallel experiment to the former section was conducted in liquid MRS with and without 100 mg/L of ferulic acid methyl ester - that has been used as substrate for the cinnamoyl esterase activity in other

studies of this type (Crepin *et al.*, 2003; Esteban-Torres *et al.*, 2013; Esteban-Torres *et al.*, 2015). The esterase activity was evaluated in the extracellular parts and the cell extracts of the five *O. oeni* strains by adding 100 mg/L of ferulic acid methyl ester before incubation for 16 h at 30 °C.

7.3.2.5. Measurement of the cinnamoyl esterase activity

The three CE+ *O. oeni* strains Oenos™, CiNe™ and CH35™ were grown in pure MRS liquid medium (unexposed) or in 70 % MRS liquid medium with 30% pasteurized wine (wine-exposed) as described in the previous sections. The cells were harvested by centrifugation (7500 g for 10 min at 4 °C), and the pellets were washed twice with KH_2PO_4 buffer (0.15 M, pH 4.5, 9% EtOH), as described by Campos *et al.* (2003) with the cells being resuspended in the same buffer with 10 mg/L of *trans*-caftaric acid. The cinnamoyl esterase activity was measured by following the increase in concentration of "free" *trans*-caffeic acid over 5 h in 25 °C by HPLC.

In order to evaluate the stimulation of the cinnamoyl esterase activity of the CE+ strain Oenos™, cultures were grown without agitation for 5 days and 10 h at 25 °C in: 1 - pure liquid MRS medium (unexposed); 2 – 10% pasteurized wine; 3 – 20% pasteurized wine; 4 – 30% pasteurized wine (around 50 mg/L of natural *trans*-caftaric acid as shown in table 7.1); 5 - 50 mg/L of *trans*-caftaric acid; 6 - liquid MRS medium incubated first for 5 days with substitution by 20% pasteurized wine and incubated a second time for 10 h and 7 - liquid MRS medium incubated first for 5 days followed by an addition of 50 mg/L *trans*-caftaric acid and incubated a second time for 10 h. The cells were harvested by centrifugation and the pellets were washed with KH_2PO_4 buffer and resuspended in the same buffer with 10 mg/L of *trans*-caftaric acid. The cinnamoyl esterase activity was measured over 13 h in 25 °C by HPLC.

7.3.2.6. Bioinformatics analyses

The entire genomes of the five commercial *O. oeni* strains were provided by Ch Hansen and some were available in the GenBank sequence database (GenBank accession numbers: CiNe™ AZJV00000000 (Dimopoulou *et al.*, 2014); CH35™ ALAG00000000 (Borneman *et al.*, 2012)).
Firstly, to predict the open reading frames (ORFs) the Prodigal (PROkaryotic Dynamic Programming Gene-finding ALgorith) software (Hyatt *et al.*, 2012) was used with parameterization for single genomes. The produced ORFs were then used as an input for BlastKoala (Kanehisa *et al.*, 2016), which provides the KEGG Orthology (KOs) assignments. The gene annotation was carried out using the databases, "species_prokaryotes" or "genus_prokaryotes" for the five *Oenococcus* strains. The KO and KEGG pathway enrichment analysis were done by custom python and R scripts (Melkonian *et al.*, 2019); KEGGREST (Tenenbaum, 2016), lattice (Sarkar, 2008), apcluster (Frey and Dueck, 2007; Bodenhofer *et al.*, 2011), Python BioServices (Cokelaer *et al.*, 2013) and pandas (McKinney, 2011). In total, for all five *O. oeni* strains we obtain 857 unique KOs, which mapped to 151 unique KEGG metabolic pathways.

To identify potential genes coding for esterase activity, a local BLAST database was created with nine esterase genes reported in literature (Table 7.3). Therefore, each *O. oeni* strains ORFs were compared to the database and filtered with e-value < 0.05 and identity greater than 50%. For all significant hits of each strain, a multiple sequence alignment was performed together with the nine esterase genes using ClustalW algorithm with default parametrization. The resulting alignments were used to produce pairwise distance matrices and unrooted phylogenetic trees employing the msa (Bodenhofer *et al.*, 2015), seqinr (Charif and Lobry, 2007), phytools (Revell, 2012) and ape (Paradis and Schliep, 2018) R-packages.
A similar process was followed to identify transporter genes - each *O. oeni* strain ORFs was blasted against the local version of TCDB 2.0 database (Saier Jr *et al.*, 2006), filtered with e-value < 0.05 and identity greatter than 70%.

All *O. oeni* strains had a highly significant hit with EstB28 gene (WP_011677767.1, alpha/beta hydrolase) from *Oenococcus oeni* PSU-1 strain. Therefore, the corresponding upstream sequences transporter gene (WP_002823494.1, MFS transporter) and regulator gene (WP_002821683.1, AraC family transcriptional regulator) were also similarly searched in all five *O. oeni* strains.

Table 7.3. - Putative esterases from the literature.

Microbial species studied	Esterase type	Enzyme name	Molecular weight (kDa)	Substrates	Gene involved	Gene sequence link	Article
Talaromyces stipitatus	Feruloyl esterase	FAEC	55.3	Methyl ferulate, methyl caffeate, methyl sinapate, methyl *p*-coumarate	*faeC*	https://www.ncbi.nlm.nih.gov/nuccore/AJ505939	(Crepin *et al.*, 2003)
Lactobacillus johnsonii NCC 533	Ferulic acid esterases	Lj1228 and Lj0536	31	Ethyl ferulate, chlorogenic acid	ND	https://www.ncbi.nlm.nih.gov/nuccore/GU454587.1 https://www.ncbi.nlm.nih.gov/nuccore/GU454586.1	(Lai *et al.*, 2009)
Lactobacillus jonhsonii DPC6026	Cinnamoyl esterase	LJ*P*-0936	ND	ND	ND	https://www.ncbi.nlm.nih.gov/protein/329667314	(Guinane *et al.*, 2011)
Lactobacillus plantarum WCFS1	Feruloyl esterase / carboxyl esterase	Lp_0796	28	Methyl ferulate, methyl caffeate, methyl *p*-coumarate, and methyl sinapinate	*lp_0796*	https://www.ncbi.nlm.nih.gov/protein/YP_004888771.1	(Esteban-Torres *et al.*, 2013)
7 *Lactobacillus plantarum*	Feruloyl esterase	Est-1092	33.5	Methyl ferulate, methyl caffeate	*est-1092*	GenBank accession number CP001617.1.	(Esteban-Torres *et al.*, 2015)
Oenococcus oeni PSU-1	Esterase	EstB28	34.5	Volatile aromatic esters	*estB28*	https://www.ncbi.nlm.nih.gov/nuccore/410695969	(Sumby *et al.*, 2009)

Oenococcus oeni and *Lactobacillus hilgardii*	Esterases	EstCOo8 and EstC34	29	Volatile aromatic esters	*estCOo8* and *estC34*	https://www.ncbi.nlm.nih.gov/nuccore/JX215240.1 https://www.ncbi.nlm.nih.gov/nuccore/418206119	(Sumby *et al.*, 2013b)

ND: no data

7.3.2.7. Transcriptomics approach

To go further in the understanding of the cinnamoyl esterase activity, a transcriptomics approach could be used to analyze gene expression. Therefore, several ribonucleic acid (RNA) extraction protocols were tested with two CE+ and two CE- *O. oeni* strains, as described below.

A preliminary experiment on unexposed Viniflora® Oenos™ (CE+) and CH11™ (CE-) strains was performed to determine the optimal growth parameters and cell-lysis protocol.
Bacterial cells were cultivated from pre-cultures in duplicates in 15 mL and 50 mL liquid MRS broth, at 25 °C, until reaching an absorbance (at 600 nm) of 0.7 AU (mid exponential phase) and 1.1 AU (late exponential phase), corresponding to 4- and 6-days incubation for Oenos™ and 5- and 7-days incubation for CH11™. The cells were harvested by centrifugation (7500 g for 10 min at 4 °C), washed twice with 10 mM Tris-HCl (pH 8) and frozen at -80 ºC before RNA extraction.
The RNA extraction procedure took place under a laminar flow cabinet where the bench and equipment were cleaned with bleach, ethanol and RNA away spray. The cells were disrupted using the High Pure RNA Isolation kit (Roche, Mannheim, Germany) with slight modifications. The cells were incubated 30 min at 37 °C with 1 mg/mL lysozyme, or 1 h at 37 °C with 1 mg/mL lysozyme and disintegrated three times 20-second cycles with FastPrep®-24 Classic Instrument (MP Biomedicals, USA) set with a speed of 4 m/s as described in previous section. A negative control, with no cells, was used to certify the well-functioning of the extraction. RNA extracts were cleaned with a Turbo DNA-free kit (Life Technologies, Carlsbad, CA, USA), verified and quantified by nanophotometer and electrophoresis gel 1% agarose (Gelred stain, 120 V, 45 min, 400 mA).
A second experiment was performed on the four *O. oeni* strains targeted. To obtain a clear and pure RNA extract, the cells were cultivated in 15 mL MRS broth, incubated until a final absorbance of 0.7 AU, lysed with 1 mg/mL of lysozyme and incubated again for 30 min at 37 °C. The extraction was made in duplicate for each strain.

7.3.3. Chemical analyses

7.3.3.1. Analysis of HCA and their derivatives

The identification and quantification of HCA and their derivatives were performed by HPLC- Diode Array Detection (-DAD) as described in section 2.5.

7.3.3.2. Chemical hydrolysis of (hydroxy)cinnamoyl-tartaric acids in wine

A red wine (pH 3.52; 1.510 ± 0.145 g/L malic acid; 0.757 ± 0.046 g/L lactic acid) from the Touriga Franca variety collected before malolactic fermentation in the Douro region in Northern Portugal from the 2016 harvest (stored at 4 °C before the initiation of the experiment) was pasteurized 3 min at 50 °C, split in sterile 50 mL Falcon tubes and stored at 25 °C for 28 days (*O. oeni* growth conditions) and 12 °C for 142

days (wine storage conditions). The concentrations of HCA and their derivatives were followed by HPLC-DAD along the incubations to analyze the chemical hydrolysis of the (hydroxy)cinnamoyl-tartaric acids.

7.3.3.3. Protein quantification

The contents in proteins of the cell extracts and cell debris of the five *O. oeni* strains from CH. Hansen cultivated in unexposed and wine-exposed media were evaluated by the Bradford method (Martina and Vojtech, 2015). To avoid interference with lysozyme, only the cell extracts and cell debris of strains lysed with the mechanical protocol were used for the analysis of protein ´concentration. Protein quantification was performed in triplicate for each sample at once using the same calibration, and the results were given in mg/L for 1 mL suspension and 1 AU of culture.

7.3.3.4. Fast protein liquid chromatography (FPLC)

As a first tentative to extract the cinnamoyl esterases of the three CE+ *O. oeni* strains, a FPLC analysis was performed. The cell extracts of the five *O. oeni* strains from Ch. Hansen obtained after mechanical lysis were defrosted at room temperature and analyzed by gel filtration chromatography using a Superdex™ 200 10/300 GL column coupled to a Superdex Peptide 10/300 GL 8 in a FPLC AKTA-purifier system. The eluent used was 0.05 M phosphate buffer pH 7.0, containing 0.15 M Sodium chloride (ionic strength) and 0.2 g/L of sodium azide (as preservative) and the elution was monitored at 280 nm at a flow rate of 0.5 mL/min.
The cell extracts were passed first to detect any differences between the FPLC profiles of the different strains and then passed twice in FPLC, fractionated, freeze dried and resuspended into 1 mL sterile cold distilled water. The fractions were tested for their cinnamoyl esterase activity by addition of 10 mg/L *trans*-caftaric acid and incubation for 16 h at 30 °C.

7.3.4. Statistical analysis

All experiments were performed with a minimum of two replicates. Data were subjected to statistical analysis using JMP 13 for Windows XP (JMP, Marlow, UK), at a confidence level of 95% (p=0.05). Dunnett´s test was run to compare the means of each sample relatively to the controls while Tukey-Kramer HSD (honestly significant differences) test was run to compare yield values between the samples of different sizes. A one-way analysis of variance (ANOVA) was used to determine the minimum significant difference ($p < 0.05$) between media for the protein results.

7.4. Results

7.4.1. Chemical hydrolysis of (hydroxy)cinnamoyl-tartaric acids in wine

The concentration of the anthocyanin cinnamoyl derivatives analyzed (*trans*-peonidin-3-*O*-coumaroylglucoside and *trans*-malvidin-3-*O*-coumaroylglucoside) decreased over time in the pasteurized wine, possibly attributed to their involvement in coupled oxidation-reduction reactions (Table 7.4). On the other hand, *trans*-caftaric, *trans*-coutaric, *trans*-*p*-coumaric and *trans*-ferulic acids acid concentrations remained relatively constant over time while *cis*-caftaric and *trans*-caffeic acids were stable during the first 28 days of fermentation and decreased during the storage period and *cis*-coutaric acid slightly increased over time.

No increase of "free" HCA was observed at the expense of (hydroxy)cinnamoyl-tartaric acids in pasteurized wine over time (Table 7.4).

Table 7.4. – Concentrations in HCA, tartrate and anthocyanin cinnamoyl derivatives (mg/L) in pasteurized wine incubated at 25 °C for 28 days (*O. oeni* growth conditions) and 12 °C for 142 days (wine storage conditions).

	MLF start	**14 days**	**28 days**	**170 days**
cis-caftaric acid	9.57 ± 0.55(1)	8.20 ± 0.36	7.74 ± 0.82	0.50 ± 0.11
trans-caftaric acid	140.95 ± 10.92	140.54 ± 5.00	130.58 ± 14.93	141.78 ± 0.97
trans-caffeic acid	7.87 ± 0.81	7.94 ± 0.30	7.67 ± 0.81	2.64 ± 0.49
cis-coutaric acid	9.18 ± 0.22	13.22 ± 0.50	15.31 ± 1.66	22.01 ± 0.25
trans-coutaric acid	39.55 ± 4.52	38.68 ± 1.54	35.33 ± 3.82	44.46 ± 0.76
trans-*p*-coumaric acid	1.04 ± 0.02	1.21 ± 0.06	1.36 ± 0.15	1.32 ± 0.11
trans-fertaric acid	6.33 ± 0.97	5.20 ± 0.31	3.49 ± 0.43	3.56 ± 0.45
trans-ferulic acid	0.26 ± 0.01	0.17 ± 0.02	0.17 ± 0.03	0.21 ± 0.00
trans-peonidin-3-O-coumaroylglucoside	3.40 ± 0.14	1.51 ± 0.22	1.00 ± 0.07	0.32 ± 0.02
trans-mv-3-O-coumaroylglucoside	50.25 ± 3.99	24.28 ± 0.86	13.32 ± 1.28	2.24 ± 0.13

(1) values represent the mean ± standard deviation. n = 2. mv – malvidin.

7.4.2. Screening for cinnamoyl esterase activity

Of the 15 strains tested, only *O. oeni* Oenos™, CH35™ and CiNe™ showed cinnamoyl esterase activity (Figure 7.2), and these were expressed at different levels, depending on the strain. The degradation of *trans*-caftaric, *trans*-coutaric and *trans*-fertaric acids by Oenos™ and CiNe™ was almost complete (≥ 88%) whilst only 30% was recorded for CH35™.

The molar conversion of *trans*-coutaric and *trans*-fertaric acids into their HCA "free" forms was less complete than the molar conversion recorded for *trans*-caftaric acid (Table 7.5).

The concentrations in *cis*-caftaric and *cis*-coutaric acids remained unchanged contrary to their *trans*-isomer forms (Figure 7.2).

Table 7.5. - Molar percentages (%n) of "free" HCA released from their tartrate derivatives precursors by the three CE+ *O. oeni* strains.

	Oenos™	CiNe™	CH35™
trans-caffeic / *trans*-caftaric acids	87	89	77
trans-*p*-coumaric / *trans*-coutaric acids	60	65	65
trans-ferulic / *trans*-fertaric acids	40	40	34

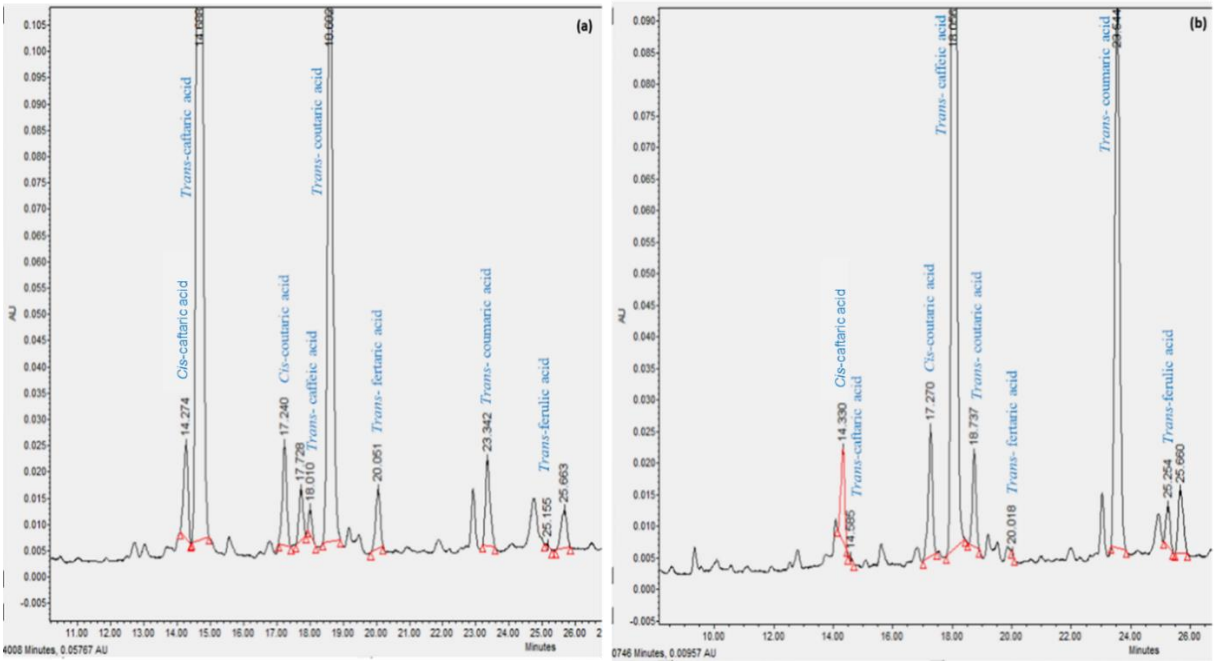


Figure 7.2. – HPLC-DAD chromatograms (at 320 nm wavelength) of the HCA (*trans*-caffeic, *trans*-*p*-coumaric and *trans*-ferulic acids) and corresponding tartrate derivatives (*trans*-caftaric, *cis*-coutaric, *trans*-coutaric and *trans*-fertaric acids) in a *O. oeni* Oenos™ culture grown in 30% pasteurized wine and 70% MRS broth; (a) before and (b) after incubation at 25 °C with no agitation until log (CFU/mL) ~ 9.26 (± 0.21).

7.4.3. Evaluation of *O. oeni* growth inhibition by *trans*-caftaric acid, *trans*-caffeic acid and 4-ethylcatechol

Within the range of concentrations tested, *trans*-caftaric acid apparently did not inhibit the growth of the *O. oeni* strains studied, whilst *trans*-caffeic itself and its corresponding ethylphenol (4-EC), added at the same molar concentrations (0.5 and 1 mmol/L) in culture medium did inhibit, with a stronger effect at the higher concentrations tested (Figure 7.3).

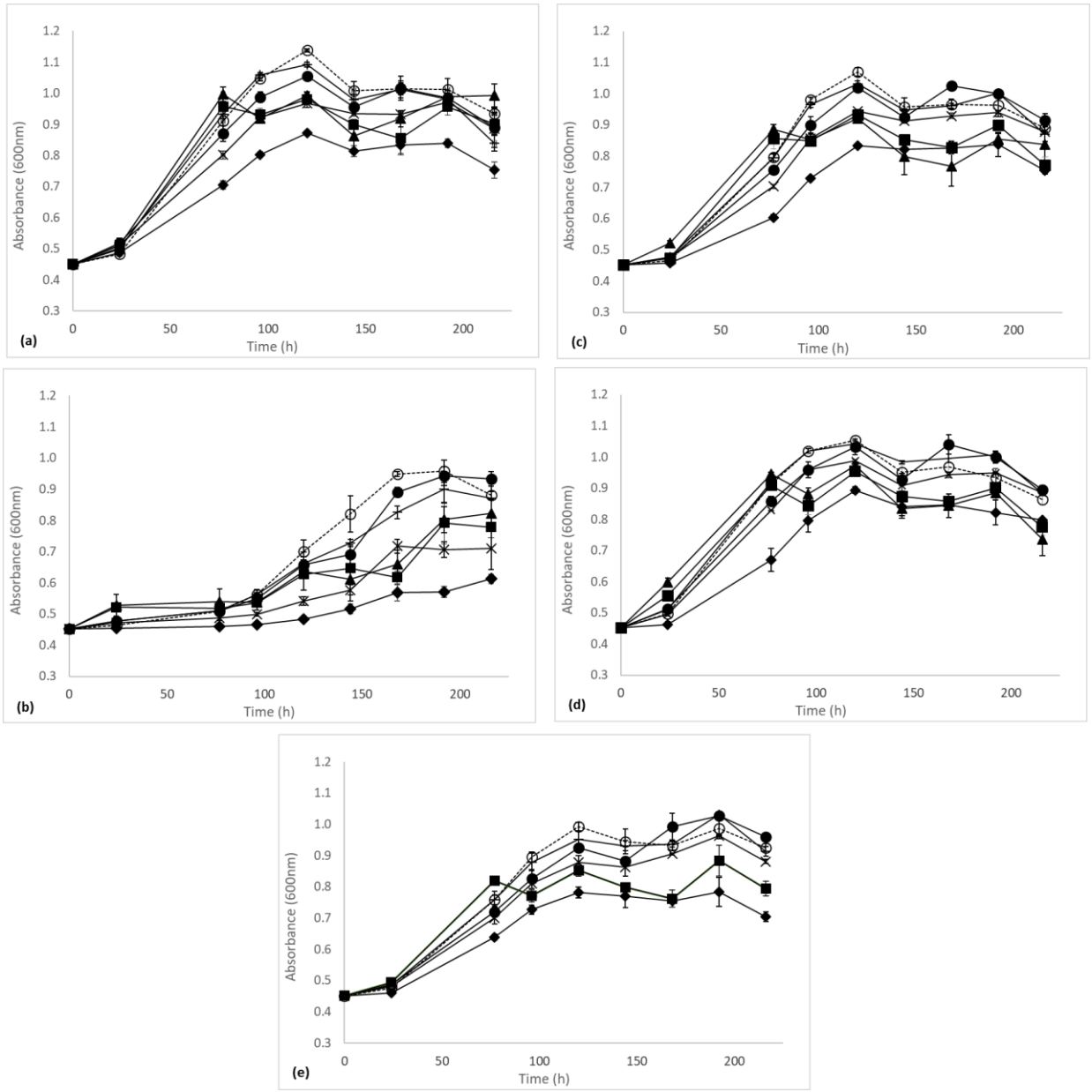


Figure 7.3. - Growth curves of *O. oeni* (a) Oenos™, (b) CiNe™, (c) CH35™, (d) CH16™ and (e) CH11™ in MRS broth medium (pH 4.5, 5% v/v ethanol at 25 °C with no agitation in aerobic conditions) supplemented with (●) 150 mg/L and (+) 300 mg/L of *trans*-caftaric acid, (x) 85 mg/L and (♦) 170 mg/L of *trans*-caffeic acid, (■) 65 mg/L and (▲) 130 mg/L of 4-ethylcatechol, (○) no phenolics added; error bars represent the standard deviation of three determinations.

7.4.4. Genes responsible for cinnamoyl esterase activity in *O. oeni* genomes

The KO annotations of the five *O. oeni* strains were compared with each other and from the 857 KOs 34 were found to be different between the strains. As observed in figure 7.4.a, no known esterase was exclusive to the three CE+ strains either. Nevertheless, one common ortholog to Oenos™ and CiNe™

corresponding to a galactonate dehydratase could be identified (Figure 7.4.a). Consequently, regarding the KEGG pathways enrichment analysis (Figure 7.4.b), Oenos™ and CiNe™ showed a higher expression of enzymes connected to galactose metabolism compared to CH16™ and CH11™. This enrichment is corresponding to the conversion of D-galactonate into 2-dehydro-3-deoxy-D-galactonate (Figure 7.5). A more extensive expression of the enzymes of the galactose pathway was also observed for CH35™ through a unique KO (K02744, PTS-Aga-EIIA, agaF, PTS system, N-acetylgalactosamine-specific IIA component) matching the reactions converting D-galactosamine to D-galactosamine-6P and N-acetyl-D-galactosamine to N-acetyl-D-galactosamine-6P (Figure 7.5).

As has been previously applied, seven published ORFs encoding cinnamoyl esterases in the *Lactobacillus* genus and two others coding esterases in wine *Oenococcus* genus were selected (Table 7.3) and computationally analyzed against the ORFs of the five targeted *O. oeni* strains (Figure 7.6 - example for Oenos™) (Lai *et al.*, 2009). A good hit (99.01% - 99.34 % identity with 0 e-value) for all the strains toward the EstB28 esterase of *O. oeni* PSU-1 was observed (Sumby *et al.*, 2009). On four of the strains (all except for CH35™) two fragmented ORFs were blasted both with good hits as well (95.91% and 96.34 % identities with 4e-123 and 1e-54 e-values) toward the EstCOo8 esterase from Sumby *et al.* (2013b) study. Other ORFs could also be pointed out with relatively good hits (above 70% identity and e-value < 0.05) against FAEC feruloyl esterase from *Talaromyces stipitatus* (Crepin *et al.*, 2003) and Lj0536 ferulic acid esterase from *Lactobacillus johnsonii* NCC 53 (Lai *et al.*, 2009).

A synteny analysis on EstB28 gene and its corresponding regulator and transporter genes was performed to determine if differences between the CE+ and CE- strains could be detected upstream. Once more, significant hits (above 99% identity and e-value 0) towards all *O. oeni* strains were found for transporter gene (WP_002823494.1, MFS transporter) and regulator gene (WP_002821683.1, AraC family transcriptional regulator).

A search of all transporters genes with the usage of TCDB 2.0 database was performed for the five *O. oeni* strains. In total twenty-two ORFs had a significant hit with fifteen commonly shared between the strains (Figure 7.7).

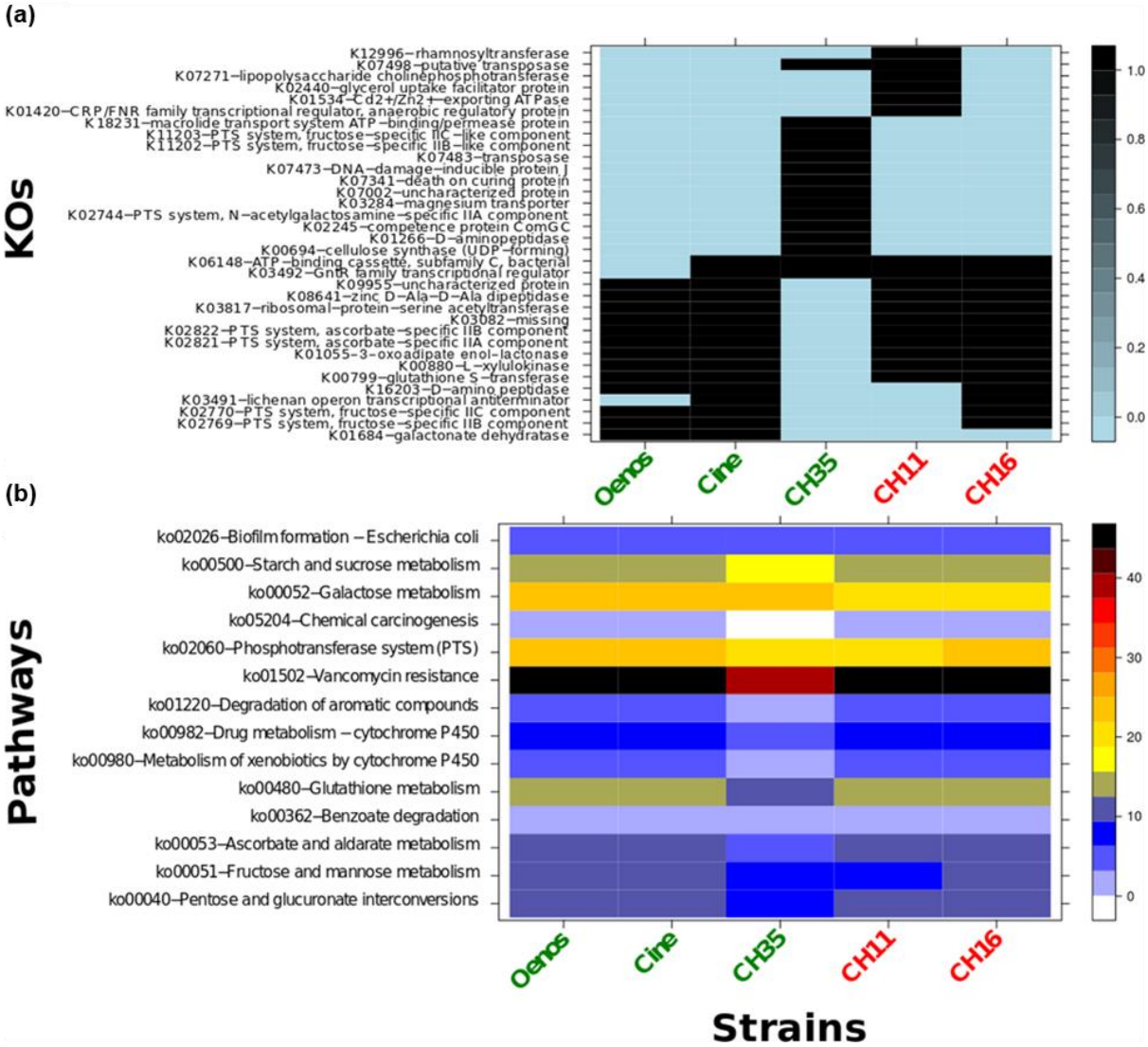


Figure 7.4. – (a) Heatmap representing the distribution of the 34 different KOs within the five *O. oeni* strains. Black - presence of the KO; light blue - absence of the KO; green - CE+ strains and red - CE- strains. Row-names correspond to the KO identifiers and the corresponding KO definition. (b) Heatmap of the KEGG pathways enrichment within the five *O. oeni* strains. In right side the color scheme represents the pathway coverage which is the percentage of the KOs mapping the pathway divided by the total KOs number for the corresponding pathway.

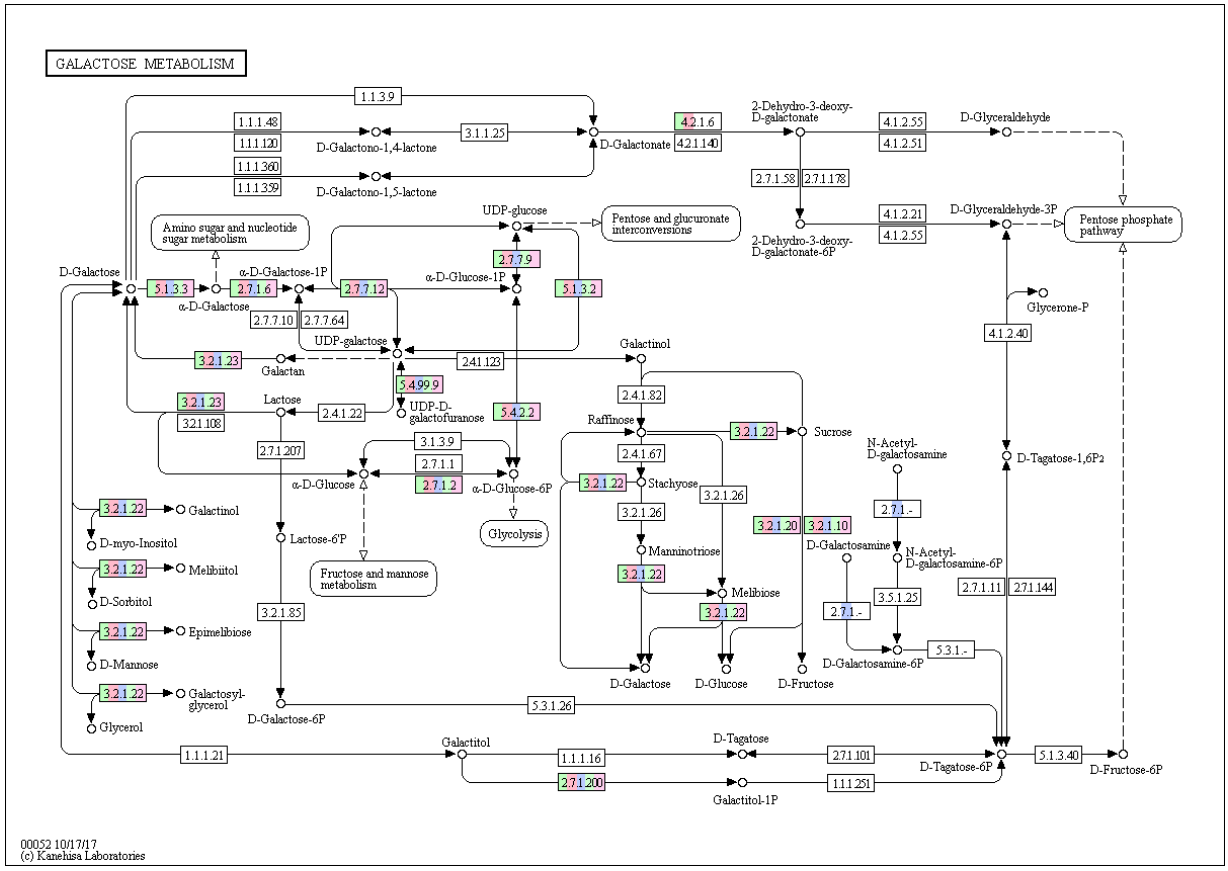


Figure 7.5. - Galactose metabolism. Colors indicate the presence of the KO from left to right in: Oenos™ (green), CiNe™ (red), CH35™ (blue), CH16™ (yellow) and CH11™ (purple).

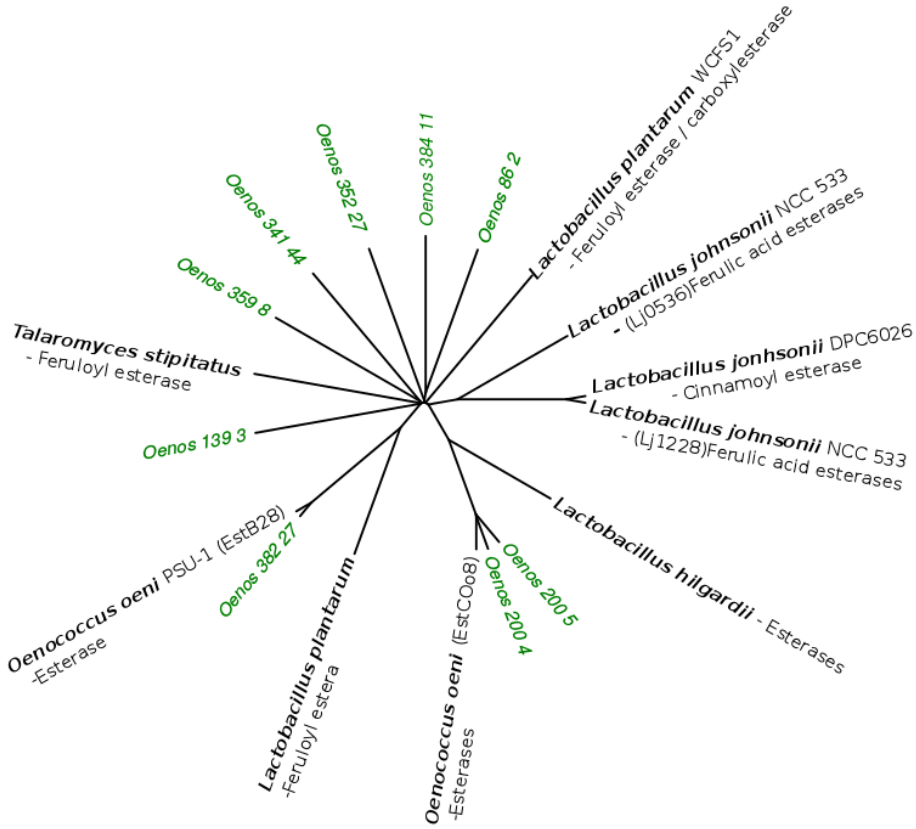


Figure 7.6. - Unrooted phylogenetic tree of all significant hits of Oenos™ ORFs together with the nine esterase genes from the literature (Crepin *et al.*, 2003; Lai *et al.*, 2009; Sumby *et al.*, 2009; Guinane *et al.*, 2011; Esteban-Torres *et al.*, 2013; Sumby *et al.*, 2013b; Esteban-Torres *et al.*, 2015).

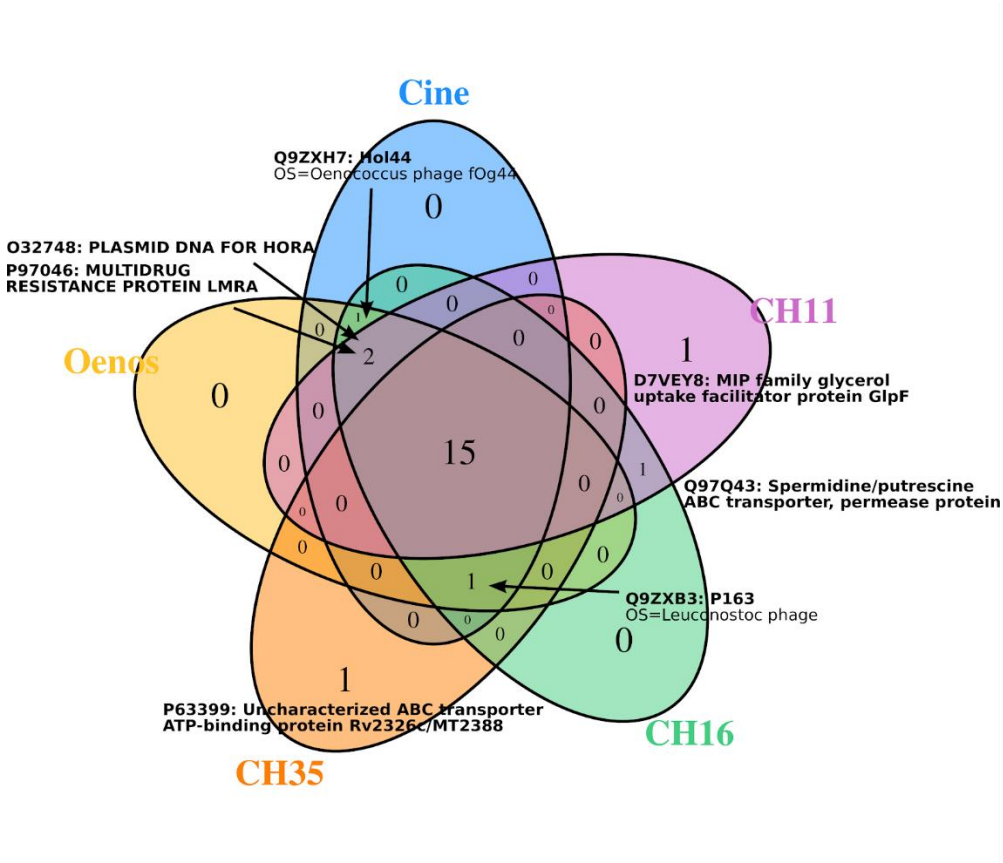


Figure 7.7. - Venn diagram of transporter genes from the five *O. oeni* strains based on custom orthology detection using TCDB 2.0 database.

7.4.5. Cellular effect of prior exposure to wine and localization of enzymatic activity

Prior exposure to wine (30% pasteurized wine) slightly activated the bacterial growth although the level of enhancement was strain dependent (Figure 7.8.).

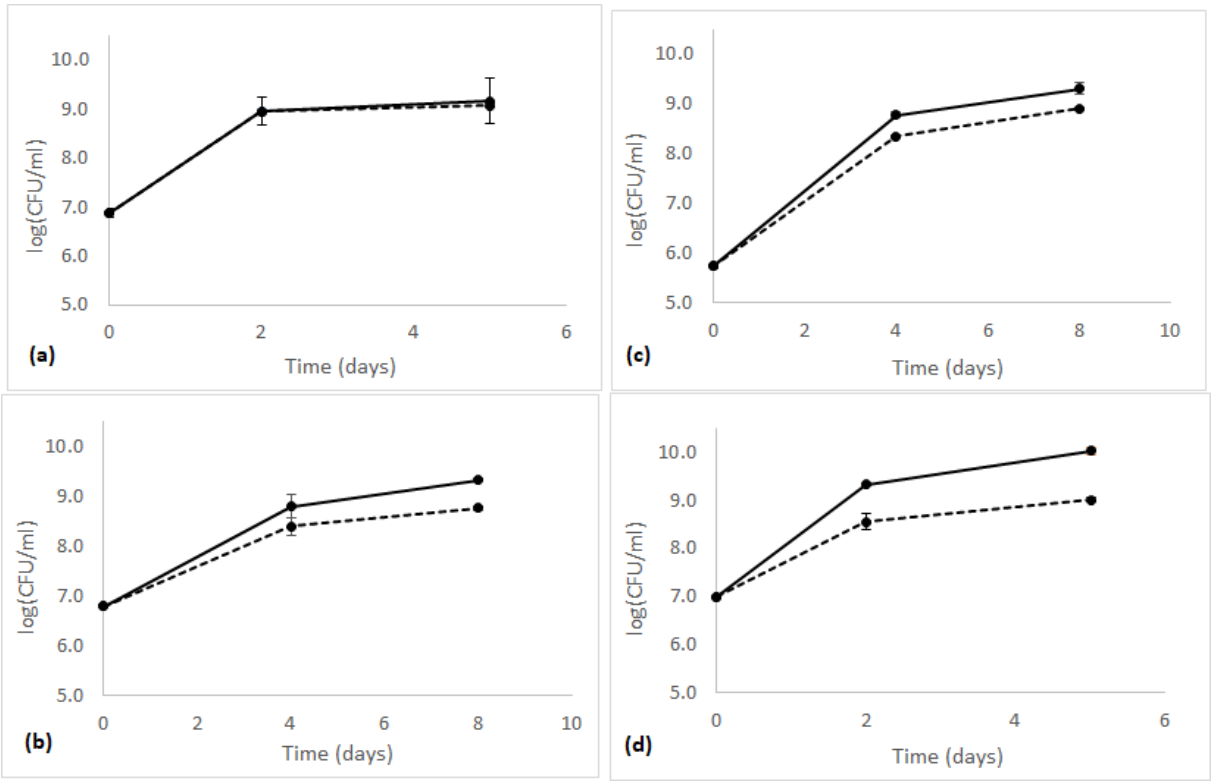


Figure 7.8. - Growth curves of *O. oeni* (a) Oenos™, (b) CiNe™, (c) CH35™ and (d) CH16™ in MRS agar medium (pH 4.5, 5% v/v ethanol at 25 °C). Unexposed (dotted line) and wine-exposed (solid line) strains. Error bars represent the average value of three determinations.

Comparing the efficiency of the two lysis protocols used, 40% of the *O. oeni* cells were disrupted by the mechanical method against 60% by the enzymatic one. No activity was detected in the supernatants of the non-lysed CE+ strains.

All cell-free extracts of the *O. oeni* strains tested in wine-exposed (30% pasteurized wine) and unexposed media, except CH11™, appeared to show a cinnamoyl esterase activity. However, the activity was stronger in the cell-free extracts of the CE+ strains. An almost immediate reaction to exposure to the cell extracts, leading to the total degradation of *trans*-caftaric acid, was observed for extracts from the three CE+. More than 80% of the *trans*-caftaric acid consumed during this period apparently liberated *trans*-caffeic acid. As for CH16™, a partial activity in the cell-free extracts was found, and this was higher in the experiments where there was no prior exposition to the wine (Table 7.6.). Although the cell lysis efficiency was greater with the enzymatical protocol compared to the mechanical one, for the wine-exposed CH16™ strains, the cinnamoyl esterase activity was lower.

Table 7.6. - Cinnamoyl esterase activity of cell-free extracts of *O. oeni* strain CH16™, grown in MRS medium mixed (wine-exposed) or not (unexposed) with 30% pasteurized wine, obtained after applying the mechanical and enzymatic lysis protocols.

		log (CFU/mL) before cell lysis	*Trans*-caftaric acid (mg/L)		*Trans*-caffeic acid (mg/L)		% *trans*-caftaric degraded
			t0	tfinal	t0	tfinal	
Mechanical lysis	Unexposed	8.81 ± 0.12[y]	9.91 ± 0.82	2.44 ± 2.21	0.51 ± 0.35	4.04 ± 0.96	76 ± 22
	Wine-exposed	9.50 ± 0.11	11.21 ± 0.40	8.80 ± 0.60	0.19 ± 0.06	1.14 ± 0.15	22 ± 4
Enzymatic lysis	Unexposed	8.71 ± 0.05	10.77 ± 0.46	3.42 ± 2.49	0.14 ± 0.03	3.21 ± 1.31	68 ± 25
	Wine-exposed	9.44 ± 0.10	11.55 ± 0.25	10.88 ± 0.01	0.11 ± 0.00	0.44 ± 0.13	6 ± 2

y values represent the mean ± standard deviation; n = 4. t0 - immediately after substrate addition and before incubation. tfinal – after incubation at 30°C for 16 h.

The protein concentration in the cell-free extracts of Oenos™, CH16™ and CH11™ strains grown in unexposed medium (pure MRS broth) was two to three times higher than after exposition to 30% pasteurized wine (Table 7.7). Only in the case of the CE+ strains exposed to wine did the disrupted cell fraction (cell debris) contain higher protein concentrations that the unexposed ones.

Table 7.7. - Total protein content (mg/L) in 1 mL of cell-free extracts and cell debris of unexposed and wine-exposed *O. oeni* cultures.

	Oenos™		**CiNe™**		**CH35™**		**CH16™**		**CH11™**	
	Unexposed	**Wine-exposed**	**Unexposed**	**Wine-exposed**	**Unexposed**	**Wine-exposed**	**Unexposed**	**Wine-exposed**	**Unexposed**	**Wine-exposed**
Cell extracts	0.21 ± 0.01[y]	0.08 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.18 ± 0.01	0.06 ± 0.01	0.15 ± 0.01	0.09 ± 0.01
Cell debris	0.08 ± 0.02 **b**	0.12 ± 0.01 **a**	0.11 ± 0.02 **b**	0.14 ± 0.00 **a**	0.05 ± 0.00 **b**	0.08 ± 0.01 **a**	0.11 ± 0.01	0.10 ± 0.00	0.09 ± 0.01	0.09 ± 0.01

y values represent the mean ± standard deviation; n = 6. Values followed by different bold letters are statistically significantly different at p-value < 0.001 using a One-way ANOVA.

7.4.6. Cinnamoyl esterase activity in live cultures; strain differences and prior exposure effects

In the case of the two CE+ strains Oenos™ and CiNe™, *trans*-caftaric acid was degraded faster by the cells previously grown in 30% pasteurized wine (wine-exposed) comparatively to the cells grown without prior exposition to the wine (unexposed) (Figure 7.9. a and b). The reaction was slightly faster for the wine-exposed cells of CiNe™ (Figure 7.9. b). Almost no degradation was registered for CH35™ over 5 h of incubation (Figure 7.9. c).

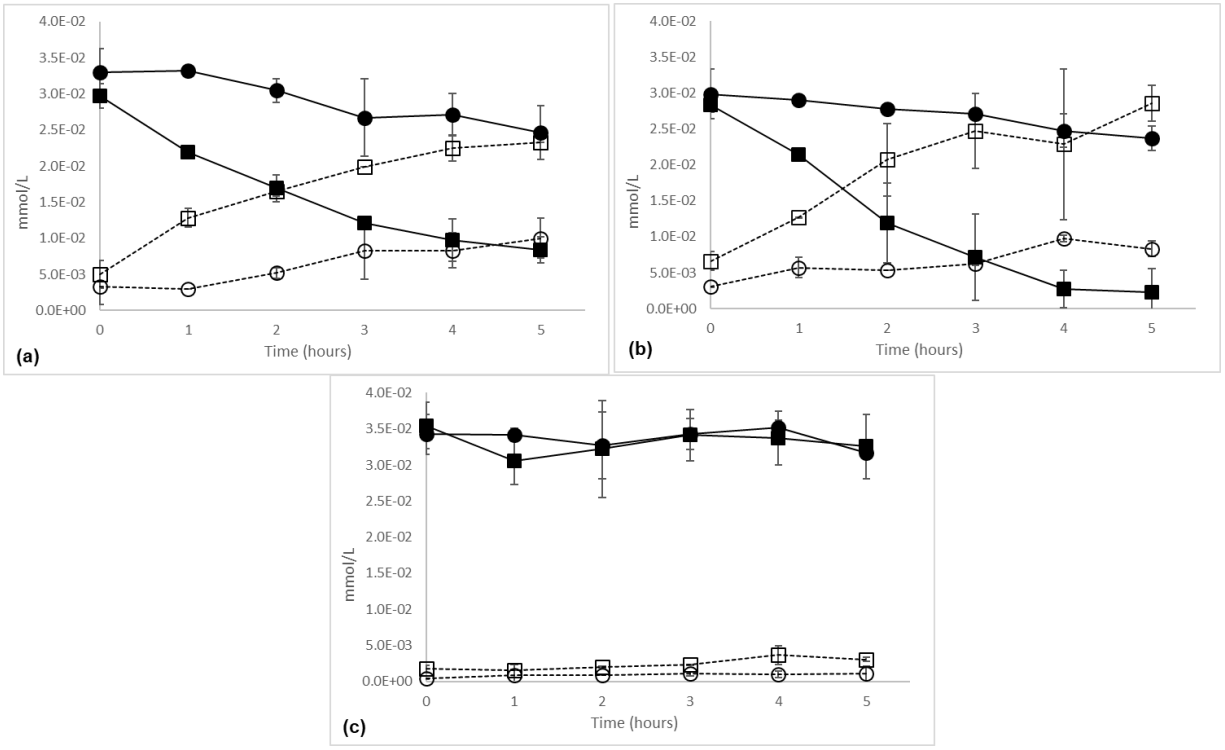


Figure 7.9. - Cinnamoyl esterase activity in live cultures of *O. oeni* CE+ strains (a) Oenos™, (b) CiNe™ and (c) CH35™ previously grown in (■, □) wine-exposed or (●, ○) unexposed media. Solid lines - *trans*-caftaric acid degradation; dotted lines - *trans*-caffeic acid production. Error bars represent the standard deviation of three determinations.

In order to evaluate the stimulation of the cinnamoyl esterase activity, cultures of the CE+ strain Oenos™ were grown without agitation for 5 days and 10 h at 25 °C in different media with a certain time of substrate-exposure (5 days and 10 h or only 10 h). All *trans*-caftaric acid was apparently degraded by the live cultures of *O. oeni* Oenos™ after 5 days and 10 h growth and exposure at 25 °C (Table 7.8).

Table 7.8. - *Trans*-caftaric and *trans*-caffeic acids concentrations in different cultures of *O. oeni* Oenos™ before (t0) and after (tfinal) 5 days and 10 h growth and a certain time of substrate-exposure (5 days and 10 h or only 10 h) at 25 °C.

Medium culture	log (CFU/mL) tfinal	***Trans*-caftaric acid (mg/L)**		***Trans*-caffeic acid (mg/L)**	
		t0	**tfinal**	**t0**	**tfinal**
1 - Unexposed	8.87 ± 0.07[y]	nd	nd	nd	nd
2 – 10% wine-exposed for 5 days and 10 h	9.30 ± 0.07	16.59 ± 0.11	nd	0.19 ± 0.00	8.80 ± 0.14
3 – 20% wine-exposed for 5 days and 10 h	9.73 ± 0.36	33.97 ± 0.13	nd	0.28 ± 0.01	18.13 ± 0.02
4 – 30% wine-exposed for 5 days and 10 h	9.68 ± 0.13	50.74 ± 0.58	nd	0.39 ± 0.02	26.85 ± 0.08
5 - 50 mg/L *trans*-caftaric acid for 5 days and 10 h	8.87 ± 0.10	49.45 ± 1.56	nd	nd	19.97 ± 0.86
6 – 20% wine-exposition for the last 10 h incubation	9.45 ± 0.08	33.97 ± 0.13	20.42 ± 0.00	0.28 ± 0.01	5.68 ± 0.00
7 - 50 mg/L *trans*-caftaric acid for the last 10 h incubation	8.90 ± 0.09	49.45 ± 1.56	29.73 ± 0.05	nd	8.27 ± 0.02

y values represent the mean ± standard deviations; 2 < n < 3; nd-not detected (detection limit of *trans*-caftaric and *trans*-caffeic acids = 0.10 mg/L).

Regardless of the proportion of pasteurized wine added to the culture medium and the time of exposure (5 days and 10h or only 10h), the rate of apparent cinnamoyl esterase activity of *O. oeni* Oenos™ was relatively similar and, in all cases, higher than with added pure *trans*-caftaric acid as a potential stimulator and without any prior addition of wine nor pure *trans*-caftaric acid (Table 7.9).

Table 7.9. – Cinnamoyl esterase activity in cultures of *O. oeni* Oenos™ previously grown in different culture media, in KH_2PO_4 buffer at 25 °C supplemented with *trans*-caftaric acid.

Medium culture	***Trans*-caftaric acid (mg/L)**		***Trans*-caffeic acid (mg/L)**		**% *trans*-caftaric acid degraded**
	t0	**t13**	**t0**	**t13**	
1 - Unexposed	13.72 ± 0.65[y]	9.49 ± 0.62	0.25 ± 0.08	2.13 ± 0.25	31 ± 1 **b**
2 – 10% wine-exposed for 5 days and 10 h	9.33 ± 0.18	2.58 ± 0.15	2.45 ± 0.12	4.81 ± 0.15	72 ± 1 **a**
3 – 20% wine-exposed for 5 days and 10 h	12.65 ± 0.60	3.92 ± 1.38	1.50 ± 0.25	4.84 ± 0.54	69 ± 12 **a**

4 – 30% wine-exposed for 5 days and 10 h	11.82 ± 0.30	3.54 ± 0.58	1.67 ± 0.10	5.08 ± 0.13	70 ± 4 **a**
5 - 50 mg/L *trans*-caftaric acid for 5 days and 10 h	11.20 ± 1.36	7.92 ± 0.12	1.18 ± 0.08	3.24 ± 0.48	29 ± 8 **b**
6 – 20% wine-exposition for the last 10 h incubation	12.97 ± 0.00	2.30 ± 0.00	1.84 ± 0.00	6.28 ± 0.00	82 ± 0 **a**
7 - 50 mg/L *trans*-caftaric acid for the last 10 h incubation	14.89 ± 0.35	9.94 ± 0.94	0.33 ± 0.00	2.65 ± 0.01	33 ± 5 **b**

y values represent the mean ± standard deviation; values followed by different bold letters within a column are statistically significantly different at $p < 0.05$ using a Tukey's-Kramer HSD test, $2 < n < 3$. t0 - immediately after *trans*-caftaric acid addition and before incubation. t13 – after incubation at 25 °C for 13 h.

7.4.7. Tentative of extraction of the cinnamoyl esterases by fast protein liquid chromatography

As a first tentative to extract the potential cinnamoyl esterases of the *O. oeni* strains studied, a FPLC analysis was performed on the cell-free extracts of wine-exposed (30% pasteurized wine) and unexposed stains. The FPLC peaks were greater for the strains with no prior exposition to wine (Figure 7.10.b).

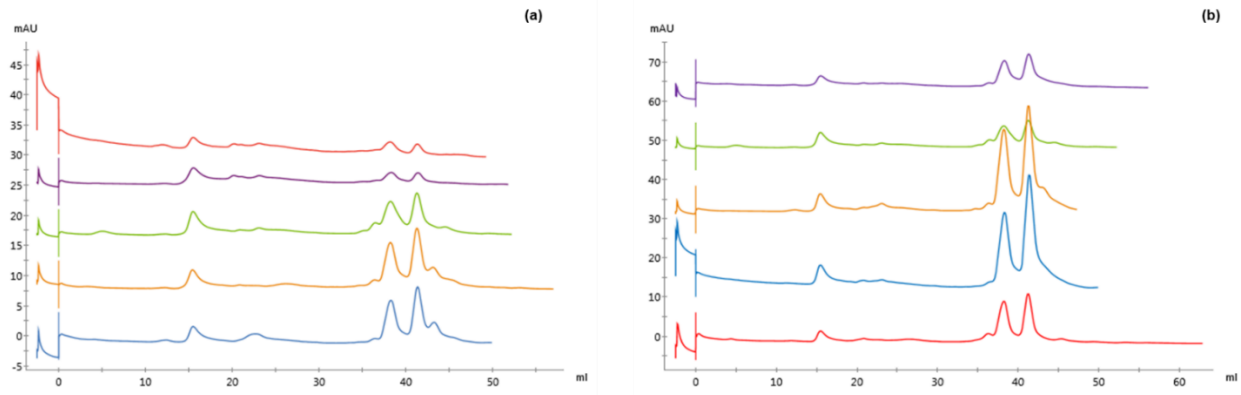


Figure 7.10. – FPLC profiles of the cell-free extracts obtained after mechanical lysis of *O. oeni* strains that grown with (a) or without (b) prior exposure to 30% pasteurized wine: blue – CH16™, orange – CH11™, green – CH35™, purple – CiNe™ and red - Oenos™.

Regardless a prior exposition to wine, the peaks P2 (~ 9 kDa) and P3 (~ 4 kDa) were higher for the two CE- strains CH16™ and CH11™ (Figures 7.10. and 7.11). By fractioning the cell-free extracts of the CE- strain CH11™ and the CE+ strain CiNe™ and comparing the CE activity of the fractions obtained, a slight

activity (8% of *trans*-caffeic acid produced from *trans*-caftaric acid,) was found exclusively for the peak P4 of CiNe™, corresponding to low molecular weight amino acids (< 1 kDa). The strain CH35™ being CE+ too did not show any activity in P4.

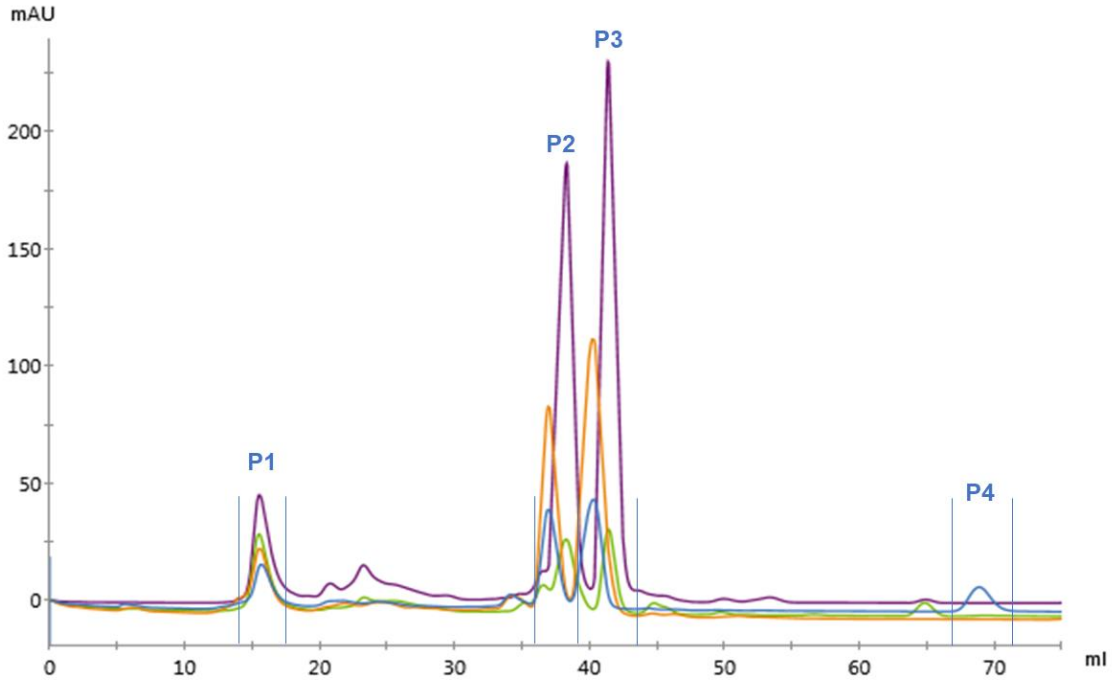


Figure 7.11. – Fractions from the FPLC profiles of the cell-free extracts obtained after mechanical lysis of *O. oeni* strains that grown without prior exposure to 30% of pasteurized wine: orange – CiNe™; green – CH35™; purple – CH16™; red – CH11™. P – peak: P1 ~ 260 kDa, P2 ~ 9 kDa, P3 ~ 4 kDa and P4 < 1 kDa.

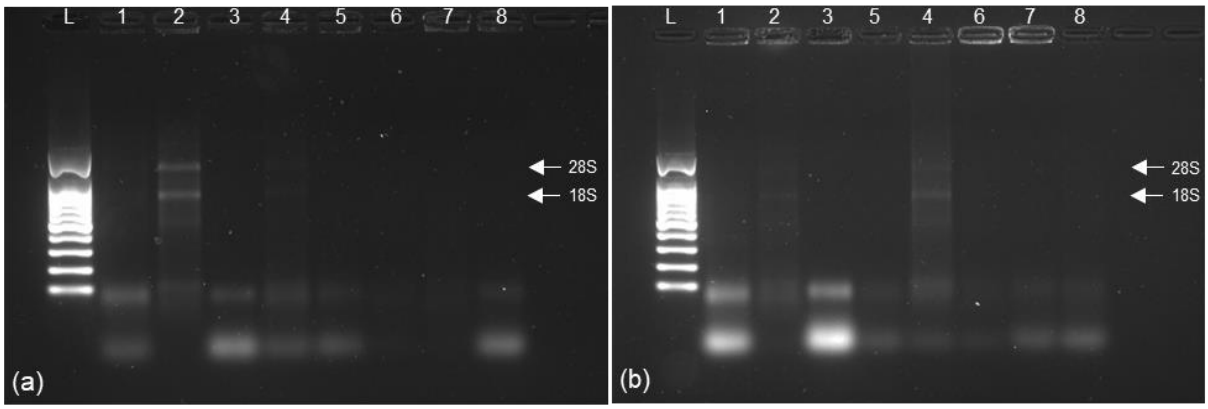
7.4.8. Transcriptomics approach

7.4.8.1. Optimal growth parameters and cell-lysis protocol for RNA extraction

Eight RNA extraction protocols were tested on the CE- CH11™ and the CE+ Oenos™ *O. oeni* strains. Although the RNA purity measured by NanoDrop™ spectrophotometer was rather low for all the extracts (absorbance ratio A260/A230 < 2), suggesting potential contaminations by wash solutions, protein, phenols and DNA (Table 7.11), clear RNA bands (18S and 28S) were obtained on a 1 % agarose gel electrophoresis for the cell extracts of CH11™ culture grown in 15 mL medium broth until 0.7 AU (at 600 nm wavelength) and disrupted with 1 mg/mL of lysozyme incubated for 30 min at 37 °C (Picture 7.1). Therefor, this protocol was selected to extract the RNA from the other *O. oeni* strains studied.

Table 7.10. – RNA purity and concentration using a NanoDrop™ spectrophotometer on the RNA extracts of the CE- CH11™ and the CE+ Oenos™ *O. oeni* strains acquired using different cell-growth parameters and cell-lysis protocols.

***O. oeni* strains**	**Culture size (mL)**	**Growth time at 25 °C (days)**	**Lysis protocol**	**A260 / A230**	**ng/µL**
CH11™	50	5	37°C / 30 min	1.606	184.0
	15			1.650	66.0
	50	7		1.480	223.0
	15			1.653	132.0
Oenos™	50	4		1.433	80.8
	15			1.500	28.8
	50	6		1.628	119.0
	15			1.448	33.6
CH11™	50	5	37°C / 1h + FastPrep®-24	1.678	242.0
	15			1.622	58.4
	50	7		1.124	298.0
	15			1.577	86.4
Oenos™	50	4		1.613	108.0
	15			1.592	48.4
	50	6		1.617	82.8
	15			1.630	82.8



Picture 7.1. – 1% agarose gels of the RNA extracts of the CE- CH11™ and the CE+ Oenos™ *O. oeni* strains acquired using different cell-growth parameters and cell-lysis protocols. (a) – cells incubated 30 min at 37°C with 1 mg/mL of lysozyme; (b) - cells incubated 1 h at 37°C with 1 mg/mL of lysozyme followed by beads beating with FastPrep®-24. L - ladder (GRS ladder 100 bp 50 µg), 1 - CH11™ cultivated in 50 mL liquid MRS medium for 5 days, 2 - CH11™ cultivated in 15 mL liquid MRS medium for 5 days, 3 - CH11™ cultivated in 50 mL liquid MRS medium for 7 days, 4 - CH11™ cultivated in 15 mL liquid MRS medium for 7 days, 5 - Oenos™ cultivated in 50 mL liquid MRS medium for 4 days, 6 - Oenos™ cultivated in 15 mL liquid MRS medium for 4 days, 7 - Oenos™ cultivated in 50 mL liquid MRS medium for 6 days and 8 - Oenos™ cultivated in 15 mL liquid MRS medium for 6 days.

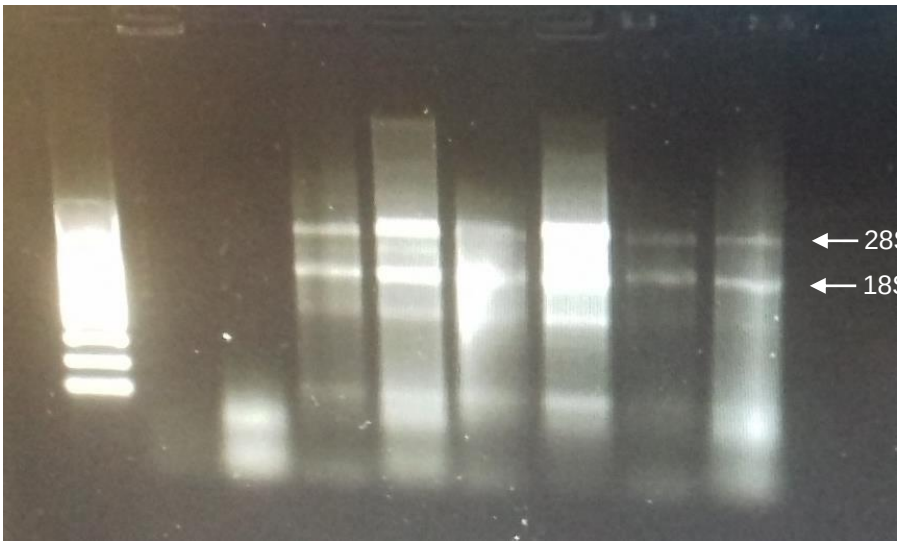
7.4.8.2. RNA extraction of four *O. oeni* strains

The RNA extraction protocol selected in the previous section was applied to the two CE+ *O. oeni* strains Oenos™ and CH16™, and the two CE- *O. oeni* strains CH11™ and CH35™ with or without a RNA cleaning step, and was successful for all the strains tested except for Oenos™ (Table 7.11. and picture 7.2.).

Despite greater absorbace ratios (A260/A230 and A260/A280) and RNA bands (18S and 28S) obtained for the non-cleaned RNA extracts of the strains CH16™, CH11™ and CH35™, cleaning the RNA permitted to remove impurities that could potentially block the sequencing (Table 7.11. and picture 7.2.). Therefore, a preliminary RNA cleaning step is advised before RNA sequencing.

Table 7.11. – Quantity and purity of the RNA extracts of the two CE+ *O. oeni* strains Oenos™ and CH16™, and the two CE- *O. oeni* strains CH11™ and CH35™ cleaned with or without Turbo DNA-free kit.

***O. oeni* strains**	**Turbo DNA-free kit**	**AU (600nm)**	**ng/µL**	**A260/A230**	**A260/A280**
Oenos™	Yes	0.685	41.2	0.343	1.132
	No	0.700	71.6	2.081	1.827
CH35™	Yes	0.816	59.2	0.949	1.682
	No	0.828	114	1.399	1.868
CH16™	Yes	0.656	136	1.504	1.838
	No	0.715	205	2.51	2.032
CH11™	Yes	0.634	193	1.744	1.932
	No	0.653	256	2.504	2.081
Negative control			17.6	0.265	1.222



Picture 7.2. – 1% agarose gel of the RNA extracts of the two CE+ *O. oeni* strains Oenos™ and CH16™, and the two CE- *O. oeni* strains CH11™ and CH35™ cleaned with or without Turbo DNA-free kit. From left to right: Ladder (GRS ladder 100bp 50µg), Oenos™ cleaned, Oenos™ not cleaned, CH16™ cleaned, CH16™ not cleaned, CH11™ cleaned, CH11™ not cleaned, CH35™ cleaned, CH35™ not cleaned, negative control.

7.5. Discussion

Wine spoilage by the production of volatile phenols is thought to be influenced by the liberation of precursor hydroxycinnamic acids from their tartrate derivative forms ((hydroxy)cinnamoyl-tartaric acids) by the cinnamoyl esterase activity of a few specific LAB strains (Chescheir *et al.*, 2015; Madsen *et al.*, 2016). However, the characteristics of the enzymatic activity have not been explored. Various microbial cinnamoyl esterases have been studied, purified and characterized previously but none of these had (hydroxy)cinnamoyl-tartaric acids as recognized substrates (Sumby *et al.*, 2013b; Esteban-Torres *et al.*, 2015; Song and Baik, 2017). Thus, this chapter brings new insights into the enzymatic activity in *O. oeni*. Viniflora® Oenos™, CH35™, Enoferm Alpha™ and Lalvin VP41™ formerly tested by Chescheir *et al.* (2015) for their cinnamoyl esterase activity in wine, Viniflora® CiNe™ and CH11™ described respectively as CE+ and CE- in Madsen *et al.* (2016) study, the LAB reported in Couto *et al.* (2006) work associated in some way with the production of volatile phenols, together with the commercial *O. oeni* strain Viniflora® CH16™ and *L. plantarum* strain NOVA™ were screened in the current study for their cinnamoyl esterase activity in a medium culture using 30% of pasteurized wine as a source of (hydroxy)cinnamoyl-tartaric acids. The three *O. oeni* strains Oenos™, CH35™ and CiNe™ were able to release "free" HCA from their tartrate derivatives at different yields, with *O. oeni* CH35™ having the lowest conversion yield, suggesting that the esterase system might somehow differ between strains. Despite the degradation of *trans*-coutaric and *trans*-fertaric acids by cinnamoyl esterases, as registered by their disappearance from the samples analyzed, their "free" HCA forms (*trans*-*p*-coumaric and *trans*-ferulic acids) did not yield their molar equivalent levels. It is possible that, as these compounds are relatively reactive, the HCA liberated might be converted to other reaction products (Singleton *et al.*, 1985). Another hypothesis would be that the cinnamoyl esterase studied has more affinity towards *trans*-caftaric acid. In accordance with Hernandez *et al.* (2007), the *trans*-forms such as *trans*-caftaric and *trans*-coutaric acid were the only ones cleaved in this process.

The five commercial *O. oeni* from Ch. Hansen were further tested in this study; Oenos™, CH35™ and CiNe™ exhibiting cinnamoyl esterase activity (CE+) and CH11™ and CH16™ not exhibiting it (CE-).

Computation analysis on the genomic information of the five *O. oeni* strains was carried out in an attempt to identify the genetic basis of the observed differences between CE+ and CE- phenotypes. No specific genes common only to the three CE+ strains could explain the different activity. This observation suggests the potential involvement of more than a single enzyme in the cinnamoyl esterase activity of the *O. oeni* strains tested. A good hit was observed in all strains toward a single, known esterase gene related to aroma esters (EstB28). The presence of the corresponding regulator and transporter genes were explored and identified, but the results were not conclusive. Another interpretation could be that genes that may be relevant for the metabolism (or transport) of the targeted substrates are non-expressed in the case of the CE- strains.

The effects of HCA on the growth and metabolism of LAB have been widely reported, both in scenarios related to wine production and others. The decrease in cell culture viability of Oenos™ by "free" HCA has been linked to the increase of the cell membrane permeability by Campos *et al.* (2003, 2009b). At the maximum concentration used (1 mmol/L), unlike its metabolic products *trans*-caffeic acid (from the cinnamoyl esterase metabolism) and 4-EC (from the volatile phenols metabolic pathway), *trans*-caftaric acid had no effect on the growth of the five *O. oeni* strains in MRS medium broth. Since Oenos™, CiNe™ and CH35™ are cinnamoyl esterase positive strains, a stronger inhibitory effect of *trans*-caftaric acid than of *trans*-caffeic acid would be expected assuming that detoxification was the main biological mechanism involved. Therefore, the presence of cinnamoyl esterase activity doesn't seem to be justifiable by a stronger inhibitory effect from *trans*-caftaric acid compared to *trans*-caffeic acid and 4-EC.

In the current study, the addition of 30% of pasteurized wine as source of (hydroxy)cinnamoyl-tartaric acids slightly increased the bacterial growth of CiNe™, CH35™ and CH16™. This observation may be partially explained by the presence of wine components which may have stimulated bacterial growth.

Among thirteen different lysis protocols tested, only two were able to adequately disrupt the cell wall and membrane of the commercial *O. oeni* strains studied; one enzymatic and one mechanical protocol. The esterase enzyme targeted in this study was found in the cell-free extracts of the strains, as those previously studied by Sumby *et al.* (2009 and 2013b).

Sommer *et al.* (2018) suggest that lysozyme addition to wine prior to fermentation may affect the release of HCA from their tartrate derivative forms. In this present study, in the control without bacteria, no *trans*-caftaric acid cleavage was observed even where laboratory grade lysozyme was employed in the lysis protocol.

The cinnamoyl esterase activity was found in both wine-exposed and unexposed cell extracts of the CE+ strains. The significance of this is still unclear but one possibility might be that the enzyme(s) responsible have another, perhaps more generic, role in the biology of the bacteria that show this activity.

Interestingly, the ferulic acid methyl ester used as a substrate in other related studies (Crepin *et al.*, 2003; Esteban-Torres *et al.*, 2015) was observed not to be substrate for the activities shown by the strains tested in this study, neither in the extracellular medium, nor in the cell extracts. This phenomenon could possibly be due to the short side chain of this compound comparatively to the (hydroxy)cinnamoyl-tartaric acids or to tartaric acid (or any acid) moiety which might be necessary for the enzyme activity.

Among the CE+ strains tested, CH35™ was found to have the lowest cinnamoyl esterase activity. However, after lysis and cell contents release, the substrate was almost instantly cleaved. Moreover, CH16™, considered to be CE-, partially degraded *trans*-caftaric acid once the cell contents were liberated. These results suggest a possible role for the wall and/or membrane in the activity studied. The pK_A values of "free" forms of phenolic acids ranges from 4.2 to 4.5 (Ramos-Nino *et al.*, 1996). At the pH of the media used in these experiments (4.3-4.5) about half of the total phenolic acids' concentration would be in the un-dissociated forms and thus is expected to cross the cell membrane by passive diffusion (Campos *et al.*, 2009b). There is a possibility that (hydroxy)cinnamoyl-tartaric acids bind or are

blocked by cell wall components and therefore could not cross the membrane, or that these compounds require some form of active or facilitated membrane transport in order to enter the cells. However, analysis of the Venn diagram of transporter genes from the five *O. oeni* strains did not suggest a common gene present only in the 3 CE+ strains.

A higher activity was noted for the CH16™ cell extracts with no prior exposition to the wine which suggests that some wine components might interfere with the ability of *O. oeni* CH16™ to degrade *trans*-caftaric acid.

The cinnamoyl esterase activity was faster for the CE+ live cultures that have been previously exposed to wine which could possibly be due to membrane protein transporters expressed on prior wine exposure. The protein content was higher in the cell debris of the CE+ strains exposed to wine, which could be explained by the stress application caused by wine addition, as it was observed in a previous study (Garbay and Lonvaud-Funel, 1996). When added to give the same content in *trans*-caftaric acid (50 mg/L), pasteurized wine (30%) was apparently better at stimulating the esterase activity than the pure molecule alone. This observation suggests that other wine compounds may be involved in the stimulation of this enzymatic activity. Previously, other authors have suggested that “free” HCA may induce PAD and VPR activities in wine LAB (Silva *et al.* (2011)) and that the concentration of *p*-coumaric acid is the most significant factor correlated with the expression of the gene coding for PAD in *L. plantarum* isolates (Rosimin *et al.* (2015)). Free HCA could also induce the synthesis of enzymes involved in the metabolism of *trans*-caftaric acid.

Altogether, the results presented suggest the possibility of the involvement of more than merely a single catalytic enzyme in the production of “free” HCA from their tartrate derivative forms in wine by *O. oeni*, a potential stimulation of the activity by wine related molecules and a cell-free extracts location of the esterase molecule itself. Further studies will be needed to characterize the enzymatic activity.

8. General discussion

A relative constant pattern in the microbial population of 16 post-malolactic wines coming from different origins were obtained by high-throughput sequencing (HTS) tools of metabarcoding techniques. Regardless of the type of alcoholic fermentation (inoculated or not), *Saccharomyces cerevisiae* was by far the predominant yeast in all wines analyzed (> 98 % of the identified yeasts). A differential abundance of non-*Saccharomyces* was noticeable among the wines, with *Lachancea thermotolerans* and *Torulaspora delbrueckii*, usually found on the grape berries surface, being the major common species found in the tested wines. Other non-*Saccharomyces* species as *Zygosaccharomyces sp.*, *Curvibasidium sp.*, *Sporobolomyces roseus*, *Cryptococcus sp.*, *Rhodotorula graminis*, *Kazachstania servazzii*, *Naganishia adeliensis*, *Schizophyllum commune*, *Cystofilobasidium macerans* and *Pseudohyphozyma pustula*, which might bring specific aroma notes, were also observed in some of the wines.

Of the bacteria identified, acetic acid bacteria (genera *Acetobacter* and *Gluconobacter*) and *Swaminathania* were dominant in the samples analyzed (except for one). These bacteria are part of generally recognized wine microbiota but could also come from contamination along the vinification (Joyeux *et al.*, 1984; Bartowsky and Henschke, 2008). Members of the Lactobacillales order, which may correspond to the malolactic fermentation starter *O. oeni*, were detected in all red wines independently of the type of malolactic fermentation (inoculated or not), while family taxa such as *Enterobacteriaceae*, *Cellulomonadaceae*, *Sphingomonadaceae*, *Pseudomonadaceae* and *Methylobacteriaceae*, usually detected on the surface of grape berries and/or during wine fermentation, were only found in some of the post-malolactic wines.

Regarding the analysis of the metabolites in wines by chromatography techniques (HPLC, GC-MS), a possible regional classification clustering French and Spanish wines together separately from the Portuguese wines was noticeable, although many other factors (other than place of origin) could affect the phenolics and volatiles composition of the wines studied (Aleixandre-Tudo *et al.*, 2015; Olejar *et al.*, 2015; Stavridou *et al.*, 2016; González-Centeno *et al.*, 2017).

The volatile composition of the Portuguese wine P1 was rather different from the others as this wine contained higher concentrations in higher alcohols and it could be explained by a potential correlation with its particular yeast population (*T. delbrueckii and Schiz. commune*). Most of the microorganisms responsible for the metabolites production may have already died off before the samples collection and therefore not be detectable anymore. Further experiments would, of course, be necessary to elucidade any genome-metabolome relationships.

The phenolic compounds kaempferol, *trans*-caffeic acid and its tartrate derivative form *trans*-caftaric acid, were found to impact differently the microbial population and the chemical composition during malolactic fermentation (MLF) depending on the media (wine or wine mixed with MRS broth), the type

of MLF (inoculated and non-inoculated) and the *O. oeni* strain used as a starter (Oenos™ or CH35™ from Ch. Hansen).

All phenolics tested were inhibitory for the growth of indigenous lactic acid bacteria (LAB) in non-inoculated wines. In mixed media (30% wine mixed with 70% MRS broth), kaempferol was more potent in this respect in non-inoculated samples and *trans*-caffeic acid in inoculated ones. The inhibitory effect of *trans*-caffeic acid was found to be specific towards the commercial starter strains Oenos™ and CH35™. In addition to their antimicrobial effects, kaempferol and *trans*-caffeic acid delayed malic acid degradation and lactic acid production in all samples. Similar observations were reported in experiments performed in culture media with other isolated strains of LAB (Campos *et al.*, 2003; Figueiredo *et al.*, 2008; Campos *et al.*, 2009a; García-Ruiz *et al.*, 2011). However, in the cited studies *trans*-caffeic acid was tested at concentrations above 100 mg/L in growth medium.

More acetic acid was found in mixed media inoculated with Oenos™ and supplemented with 10 mg/L of kaempferol and *trans*-caffeic acid. As suggested by Campos *et al.* (2009a), the addition of *trans*-caffeic acid could have induced a shift in the glucose metabolism pathway of Oenos™ towards acetate production in a medium containing MRS broth. The starter Oenos™ is known to liberate hydroxycinnamic acids (HCA) from their tartrate derivative forms through its cinnamoyl esterase activity (Burns and Osborne, 2013; Chescheir *et al.*, 2015). The addition of 10 mg/L kaempferol and *trans*-caffeic acid inhibited this activity in wines inoculated with Oenos™.

Addition of the phenolics tested led to modifications of the volatiles profile of the samples, which might interfere with their overall organoleptic perception. For example, diethyl succinate (fruity) was present in lower concentrations with the addition of the phenolics tested in wines inoculated with Oenos™ and of *trans*-caffeic acid in non-inoculated mixed media. Devi and Anu-Appaiah (2018a) suggested possible changes in the enzymatic activity of the microorganisms and in other chemical reactions during wine fermentation by the exposure to phenolic compounds, affecting the levels of volatile compounds.

The addition of wine to MRS broth changed the pattern of the production of volatiles by LAB as has previously been demonstrated with volatile phenols in Fras *et al.* (2014) work.

In a second experiment, the initial concentrations of flavan-3-ols ((+)-catechin and (-)-epicatechin), HCA (*trans*-*p*-coumaric and *trans*-ferulic acids), flavonols (kaempferol and myricetin), and *trans*-resveratrol were doubled and tripled in a post-alcoholic fermentation wines to simulate pre-fermentation variations, with values within the range encountered in real wine situations. Two types of MLF were compared in parallel (non-inoculated and inoculated with Oenos™) and the experiment was extended to a contemplate post vinification storage period.

The addition of all the compounds tested caused a delay in lactic acid production during MLF (14 days) for the non-inoculated wines and this phenomenon was concomitant with the inhibitory nature of the compounds at the concentrations tested. Of all phenolics tested, flavonols and HCA, added at triple initial concentrations, had the greatest inhibitory impact on the growth of LAB during MLF in all wines.

The flavan-3-ol (+)-catechin had a mixed effect during the spontaneous MLF depending on its concentration and the microbial population. At lower concentrations, (+)-catechin had a positive impact on the yeast population and activated malic acid degradation, while at higher concentrations this compound inhibited bacterial growth and delayed lactic acid production. These observations are in agreement with previous studies showing the variable effect of (+)-catechin on different LAB (Roguant *et al*., 2000; Alberto *et al*., 2001; Vaquero *et al*., 2007; de Llano *et al*., 2016).

All phenolics tested caused an apparent delay in the citric acid degradation by LAB in wines inoculated with Oenos™ as has been observed in previous works (Campos *et al.*, 2009a). A reduction of acetic acid production by indigenous LAB during spontaneous MLF by the increase to three times the concentrations in flavonols, HCA and *trans*-resveratrol, was observed, probably linked to the antimicrobial attribute of these compounds at these concentrations.

Twenty-two different genetic profiles were detected by Pulsed-Field Gel Electrophoresis of rare Restriction Enzyme Digests (REA-PFGE) techniques applied on 80 *O. oeni* strains isolated right before MLF and after 28 days incubation from non-treated wines and wines treated with triple flavonols and triple *trans*-resveratrol concentrations, indicating a considerable intra-specific diversity in the wines. The strain Oenos™, in inoculated experiments, appeared not to dominate the indigenous microbiota in this particular wine. The diversity of *O. oeni* was more influenced by the time of the samplings (before MLF and after 28 days incubation) than the type of MLF (inoculated or not) and the type of phenolic compounds tested under study. As also observed in previous studies (Reguant *et al.*, 2000; García-Ruiz *et al.*, 2011), the inhibitory or stimulatory effect of phenolics on *O. oeni*, seemed to be strain-dependent. In wines inoculated with Oenos™, the decrease in yeast population was accelerated by the addition of phenolic compounds. The higher the concentrations in phenolic compounds, the stronger the inhibitory effect observed.

The initial increases in phenolics also modified the metabolite profiles of the wines. The levels of all phenolics added diminished over time and some (kaempferol, *trans*-ferulic acid and *trans*-resveratrol) were no longer detectable in wines after 170 days incubation. The decreases of phenolic compounds in wines during MLF and storage have been previously reported by several authors (Vrhovsek *et al.*, 2002; Recamales *et al.*, 2006; Castillo-Sánchez *et al.*, 2008; Li *et al.*, 2009; Bimpilas *et al.*, 2015). In the wines supplemented with flavan-3-ols, (+)-catechin was possibly partially used as precursor of the condensed pigment malvidin-3-O-glucoside-ethyl-catechin (Trikas *et al.*, 2016). In all the samples the concentration of (hydroxy)cinnamoyl-tartaric acids decreased after 28 days incubation at 25 °C and their corresponding HCA “free” forms increased, suggesting a cinnamoyl esterase activity by the microbiota of the wines as has been observed in other works (Hernández *et al.*, 2006; Cabrita *et al.*, 2008). The concentration in *trans*-caffeic acid in the wines supplemented with *trans*-*p*-coumaric and *trans*-ferulic acids was higher than in the other samples 28 days after the beginning of MLF, and their concentration in *trans*-caftaric acid was lower 170 days after. Silva *et al.* (2011) and Rosimin *et al.* (2015) indicated that “free” HCA were inducing the decarboxylase and reductase activities of some LAB, metabolizing HCA into volatile

phenols. An induction of the release of *trans*-caffeic acid from *trans*-caftaric acid by the addition of other compounds from the same family could be a possible explanation to this phenomenon. The cinnamoyl esterase activity decreased with the pre-malolactic addition of flavonols, probably as a result of the inhibitory effect of these compounds on specific LAB possessing the cinnamoyl esterase activity.

The effects of the phenolics on the volatile composition of the wines were linked to the specific wine microbiota. Most of the esters decreased in the wines after 28 days of incubation and some (isoamyl octanoate, ethyl decanoate, ethyl hexanoate, ethyl octanoate, linalyl acetate and 2-phenyl-ethylacetate) were in higher concentrations in wines by doubling the initial concentration of flavan-3-ols. The same observation was described by Rodriguez-Bencomo *et al.* (2014) in wines with addition of phenolic extracts from eucalyptus leaves and almond skins reported to be rich in (+)-catechin. In the contrary, tripling the concentration in flavan-3-ols before the initiation of MLF delayed the production of ethyl hexanoate after 170 days of incubation. According to Sumby *et al. (*2010; 2013a), some *O. oeni* strains could synthesize esters such as ethyl hexanoate, which could indicate an inhibition of some LAB and their metabolism to synthesize esters by flavan-3-ols, therefore reducing the concentration of this compound. Wines inoculated with Oenos™ were more sensitive to the addition of HCA and *trans*-resveratrol during MLF than non-inoculated wines as less 1-hexanol, 2-phenylethanol, isobutyric, isovaleric, octanoic and decanoic acids were detected in these wines compared to the controls. Rodriguez-Bencomo *et al.* (2014) also observed a decrease in 2-phenylethanol over MLF by the addition of phenolic extracts. Some of the volatiles analyzed were detected in concentrations in wines above their thresholds (determined in a water/ethanol/glycerol/tartaric acid solution by Ferreira *et al.* (2000)), possibly impacting their organoleptic profile.

A survey of representative species of LAB for their ability to produce phenolic acids from their tartrate derivative forms was performed, using 15 strains, most of them associated with wine and linked in some way with the production of volatile phenols. Only the three commercial *O. oeni* strains Oenos™, CH35™ and CiNe™ were able to metabolize *trans*-caftaric, *trans*-coutaric and *trans*-fertaric acids to their corresponding “free” HCA *trans*-caffeic, *trans*-*p*-coumaric and *trans*-ferulic acids via a probable cinnamoyl esterase activity. *Trans*-forms such as *trans*-caftaric and *trans*-coutaric acids were the only ones cleaved in this process. The enzymatic activity was expressed at different yields, with *O. oeni* CH35™ having the lowest conversion yield, suggesting that the esterase system might somehow differ between strains. Of *trans*-caftaric, *trans*-coutaric and *trans*-fertaric acids, only *trans*-caftaric acid yielded the molar equivalent level of its released HCA *trans*-caffeic acid. It is possible that cinnamoyl esterases express different affinities towards the different (hydroxy)cinnamoyl-tartaric acids.

The five commercial *O. oeni* from Ch. Hansen were further tested in this study; Oenos™, CH35™ and CiNe™ exhibiting cinnamoyl esterase activity (CE+) and CH11™ and CH16™ not exhibiting it (CE-). No specific genes common only to the three CE+ strains could explain the different activity by computation analysis. An interpretation could be that genes that may be relevant for the metabolism (or transport) of the targeted substrates are present in all the strains but non-expressed in the case of the CE- strains.

The presence of cinnamoyl esterase activity doesn't seem to be justifiable by a stronger inhibitory effect from *trans*-caftaric acid compared to its metabolic products *trans*-caffeic acid and 4-ethylcatechol.
The esterase enzyme targeted in this study was found in the cell-free extracts of the strains, as those previously studied by Sumby *et al.* (2009 and 2013b). Moreover, the cinnamoyl esterase activity was detected in both wine-exposed and unexposed cell extracts of the CE+ strains, which suggest that the enzyme(s) responsible have another, perhaps more generic, role in the biology of the bacteria that show this activity.
Among the CE+ strains tested, CH35™ was found to have the lowest cinnamoyl esterase activity. However, after lysis and cell contents release, the substrate was almost instantly cleaved. Moreover, CH16™, considered to be CE-, partially degraded *trans*-caftaric acid once the cell contents were liberated. These results suggest a possible role for the wall and/or membrane in the activity studied. The cinnamoyl esterase activity was faster for the CE+ cells that have been previously exposed to wine which could possibly be due to membrane protein transporters expressed on prior wine exposure.
When added to give the same content in *trans*-caftaric acid (50 mg/L), pasteurized wine (30%) was apparently better at stimulating the esterase activity than the pure molecule alone. This observation suggests that other wine compounds may be involved in the stimulation of this enzymatic activity. Free HCA could also induce the synthesis of enzymes involved in the metabolism of *trans*-caftaric acid as it was previously suggested for the PAD and VPR activities (Silva *et al.*, 2011; Rosimin and Kim, 2015).
The involvement of more than merely a single catalytic enzyme in the production of free HCA from tartrate derivatives in wine by *O. oeni* should be considered.

Altought metabarcoding sequencing has a lower discriminatory capacity than methods applied to isolated strains, this method is broadly used nowadays in various fields as it is a relatvelly fast technique to identify bacteria and fungy populations directly from the environment and this metagenomic tool is becoming less expensive and more exhaustive as the technolgy develops.

Malolactic fermentation is a more complex step in the vinification process than a simple stabilization of the wine as relevant chemical changes occur associated to the metabolism of specific microbial strains and interactions between strains, modulating the overall acceptance of the wines.

The naturally-occurring phenolic compounds in wines are affected by many pre-malolactic fermentation parameters and can have multiple impacts on wine quality, being responsible for the color, astringency and bitterness of wines and also as precursors of wine flavors. Phenolic compounds can also be used as modulators of microbial ecology and behavior indirectly impacting the wine volatile profile.

As more becomes known it is possible to imagine producers "managing" their fermentations according to some specific elements of the wine composition such as the naturally-occuring phenolics and microflora rather than simply adding enzymatic preparations and fermentation starters.

Even when considering the simple “additif model”, it is interesting that starter companies need to constantly revise their selection criteria for malolactic bacteria. Depending on the intended wine style it is important to consider the starters metabolism and particularities.

The selection of commercial starter culture with no cinnamoyl esterase activity may be helpful in modulating the release of precurosors for the potential production of volatile phenolic components altering the wine aroma when found in high concentrations.

9. Suggestions for future works

Further work on the interactions between wine phenolics and microorganisms and the repercussions on the wine composition and quality would be worthy to consider including the analyze of the taxonomical and functional attributes of malolactic bacteria strains, a deeper understanding of the effects of phenolics on isolated strains and active consortia as well as on the metabolites and aroma profiles of wines by a combined metabolomics and sensory approach. The following experiments and approaches are suggested directly from the work presented in the dissertation.

9.1. Extended molecular analysis of the diversity of Oenococcus oeni in wines treated with phenolics

As a matter of practicality, only representative *Oenococcus oeni* strains from non-treated wines and wines treated with flavonols and *trans*-resveratrol, isolated at the beginning and after 28 days of malolactic fermentation (MLF), were analyzed by pulsed-field gel electrophoresis of rare restriction enzyme digests (REA-PFGE). Other colonies, also identified as belonging to the *O. oeni* species, were isolated from each assay, in inoculated and non-inoculated wines, treated and non-treated wines, along the incubations at 25 °C and 12 °C (0, 14, 28, 90 and 170 days after the initiation of the MLF). It would be interesting to test these colonies too.

Several published studies used a second typing method, as multilocus sequence typing, on the *O. oeni* strains representing different specific REA-PFGE profiles, to genetically better characterize them (Bridier *et al.*, 2010; García-Ruiz *et al.*, 2013a; El Khoury *et al.*, 2016). The markers used in these studies may be important for understanding the selection mechanism of the *O. oeni* strains. Thus, a similar analysis might also be used on strains from PFGE profiles less represented in the phenolics-treated samples (profile L) or found only in particular samples (profiles X, I, G etc.).

9.2. The impact of phenolics on the growth and metabolism of isolated Oenococcus oeni strains

In chapter 5, isolated *O. oeni* strains belonging to some PFGE profiles seemed to be less tolerant to flavonols and *trans*-resveratrol than others (profiles X, Z, M, L). The effects observed were apparently strain-dependent. Further research is needed to determine the impact of each groups of phenolics tested on the growth and metabolism of the isolated strains of each of the 22 profiles obtained by REA-PFGE. To mimic real wine situations, the experiment could be made in pasteurized wine with addition of phenolic compounds with final values within the ranges encountered in wine.

9.3. The impact of hydroxybenzoic acids on the microbial growth and metabolism and on the diversity of Oenococcus oeni in wines

The influence of hydroxybenzoic acids (HBA) in wine and more particularly on lactic acid bacteria (LAB) activities was variable among the studies. Campos *et al.* (2003, 2009a, 2009b) found a slight to non-existent effect of gallic acid on *O. oen*i and *Lactobacillus hilgardii* growth, cell membrane permeability and metabolism. Lorrain *et al.* (2013) added that the sensory perception of most esters studied were not impacted by gallic acid. In Vivas *et al.* (1997) study, gallic acid activated cell growth of *O. oeni* and the rate of malic acid degradation but in Vaquero *et al.* (2007) study gallic acid had a strong antimicrobial activity on some non-LAB. Consequently, the same experiment could be done using some HBA.

9.4. The influence of phenolics on the aroma profile of wines

Relevant changes in the chemical composition of the wines during MLF and subsequent storage were observed further in this work. Wine contains hundreds of volatile compounds, but only a small proportion of the volatiles actively contribute to aroma (Francis and Newton, 2005). Depending of the wine composition, a volatile compound can participate to its final aroma even with an odor active value (OAV) below one (Vilanova *et al.*, 2012). Many studies have associated instrumental and sensory analysis to characterize their wines. Lorrain *et al.* (2013) described the influence of (+)-catechin on the volatility and sensorial perception of isoamyl acetate, ethyl isobutyrate, ethyl butyrate and ethyl octanoate using a triangle test. Devi *et al.* (2018b) analyzed the impact of the co-inoculation on wine phenolic compounds composition and perception using a quantitative descriptive analysis. Therefore, it would be also interesting to evaluate the impact of the pre-malolactic increases in phenolic compounds on the organoleptic profiles of the wines used in this work by sensory analysis.

9.5. Characterization of the induction of the cinnamoyl esterase activity by wine components

The results presented in chapter 7 are consistent with the stimulation of the cinnamoyl esterase activity in *O. oeni* by wine related molecules. At present there is a general lack of knowledge about which wine factors may influence the activity releasing potential substrates for the synthesis of volatile phenols. Previous studies have shown that phenolic acid decarboxylase and vinylphenol reductase synthetization and activity in some LAB were induced by compounds belonging to the same family as their substrates (Silva *et al.*, 2011; Rosimin and Kim, 2015). Moreover, an increase in *trans*-caffeic acid concentration during MLF in wine supplemented with *trans*-ferulic and *trans*-*p*-coumaric acids was observed in chapter 6 of our research. Other studies have shown that tannic acid affected differently the expression of

metabolic enzymes depending on the LAB species (Bossi *et al.*, 2007; Cecconi *et al.*, 2009). Furthermore, the cinnamoyl esterase activity was found in both wine-exposed and unexposed cell extracts of the CE+ strains tested in this study, suggesting that the esterase is rather constitutive than inductive. Thus, it is suggested that the characterization of the esterase activity by wine components in the cinnamoyl esterase positive (CE+) strains studied in this work should be further researched.

9.6. Transcriptomic analysis of the cinnamoyl esterase activity in Oenococcus oeni

Computation analysis on genomic information of the commercial *O. oeni* strains studied in this work could not explain the absence of cinnamoyl esterase activity in two of the five strains. Margalef *et al.* (2016) and Liu *et al.* (2017) have studied the adaptation of *O. oeni* to wine stressful conditions thought transcriptomics approaches. A similar analysis might also be used to study the genes expressed in the wine-exposed commercial *O. oeni* strains of this work comparatively to the unexposed ones.
Optimal growth parameters and cell-lysis protocol are given in chapter 7 to get the greatest purified RNA extracts of the *O. oeni* strains tested for sequencing.

9.7. Purification of the cinnamoyl esterase of wine LAB

There are several published studies on the purification of cinnamoyl esterases in LAB by cloning technique (Lai *et al.*, 2012; Sumby *et al.*, 2013b; Esteban-Torres *et al.*, 2015; Song and Baik, 2017). By genomic analysis, good hits for some specific open reading frames (ORFs) of most of the commercial *O. oeni* strains studied in this work were found toward four microbial esterase genes reported in literature. Lai *et al.* (2009) designed primers based on five ORFs of the CE+ *L. johnsonii* NCC 533 encoding esterases to extract potential genes coding for the cinnamoyl esterase of another targeted CE+ *L. johnsonii* strain. The genes of interest were then amplified and cloned to purify the esterases. Comparable analysis could be made on Oenos ™, CiNe ™ and CH35™. Other studies (Esteruelas *et al.*, 2009; Jaeckels *et al.*, 2013) fractioned concentrated protein extracts of wines by fast protein liquid chromatography (FPLC) to purify proteins of concern. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the fractions obtained was usually performed in order to verify the separation efficiency. Therefore, it could be an alternative to the purification of the cinnamoyl esterases of the 3 CE+ strains studied in this work.

Appendix 1: Main yeasts in the wine ecosystem and their roles in the winemaking process

Phylum	Family	Genus	Relevant species	Main source	Technological significance	References
Basidiomycota	*Auriculibuller*	*Auriculibuller*	*Auri.fuscus*	Grape berries surface		Renouf *et al.* (2007)
	Filobasidiaceae	*Filobasidium*	*Fil. floriform*	Grape berries surface		Kecskeméti *et al.* (2016)
	Tremellaceae	*Cryptococcus*	*Cr. albidus, Cr. foliicola, Cr. laurentii, Cr. nemorosus, Cr. tephrensis, Cr. chernovii, Cr. stepposus*	Grape berries surface, must, winery equipment	Microflora regulation	Renouf *et al.* (2007), Fleet *et al.* (2003), Fugelsang *et al.* (2007)
		Bulleromyces	*Bull. albus*	Grape berries surface		Renouf *et al.* (2007)
	Ustilaginaceae	*Rhodosporidium*	*Rsp. babjevae, Rsp. krachilovae*	Grape berries surface		Renouf *et al.* (2007)
	Sporidiobolaceae	*Rhodotorula*	*R. bacarum, R. glutinis, R. fujisanensis*	Grape berries surface, must, beginning AF	Microflora regulation	Renouf *et al.* (2007), Fleet *et al.* (2003), Bisson *et al.* (1991), Setati *et al.* (2015)
			R. mucilaginosa,	Grape berries surface, must, bottled wine		Renouf *et al. (*2007)
		Sporidiobolus	*Sporo. salmonicolor*	Grape berries surface		Renouf *et al. (*2007)
		Sporobolomyces	*Spor. carnicolor. Spor. longuisculus, Spor. oryzicola, Spor. coprosmae*	Grape berries surface, must, fermented wine		Renouf *et al.* (2007), David *et al.* (2014), Setati *et al.* (2015), De Filippis *et al.* (2017)
Ascomycota	*Aureobasidiaceae*	*Aureobasidium*	*Aure. pullulans*	Grapevine leaves, grape berries surface, must, wine equipment	Microflora regulation	Renouf *et al.* (2007), Fleet *et al.* (2003), Fugelsang *et al.* (2007), Pinto *et al.* (2014)
		Kabatiella	*Kab. microsticta*	Grape berries surface		Setati *et al.* (2015)
	Dipodascaceae	*Yarrowia*	*Y. lipolytica*	Grape berries surface		Renouf *et al.* (2007)
		Starmerella	*Starmerella bacillaris*	Grape berries surface, winery environement, fermented wine	Desirable aroma notes	Setati *et al.* (2015), Wang *et al.* (2015), Comitini *et al.* (2017)
	Metschnikowiaceae	*Metschnikowia*	*M. audauensis, M. frusticola*	Grape berries surface, damaged grapes, must, beginning AF	Microflora regulation	Renouf *et al.* (2007), Fleet *et al.* (2003)

			M. pulcherrima / C. pulcherrima	Grape berries surface, damaged grapes, must, beginning AF	Microflora regulation and wine spoilage: film production and production of acetaldehyde, volatile acids and esters	Renouf *et al.* (2007), Fleet *et al.* (2003), du Toit *et al.* (2000)
	Lipomycetaceae	*Lipomyces*	*Lipo. lipofer, Lipo. tetrasporus*	Grape berries surface		Renouf *et al.* (2007)
	Phaeosphaeriaceae	*Sclerostagonospora*	*Scl. Opuntiae*	Must, beginning AF		Bokulich *et al.* (2014)
	Pichiaceae	*Dekkera / Brettanomyces*	*Brett. (D.) bruxellensis*	Grape berries surface, must, fermented wine, bottled wine, winery equipment	Wine spoilage by production of volatile phenols	Renouf *et al.* (2007), Couto *et al.* (2006)
			D. anomala / Brett. anomulus, Brett. schanderlii, Brett. lambicus, D. intermedia / Brett. intermedius	Wine	Wine spoilage by production of volatile phenols	du Toit *et al.* (2000)
	Saccharomycetaceae	*Hanseniaspora (Kloeckera)*	*K. apiculata / H. uvarum*	Grape berries surface, damaged grapes, must, beginning AF	Microflora regulation and wine spoilage by production of acetic, octanoic and decanoic acids	Renouf *et al.* (2007), Fleet *et al.* (2003), Fugelsang *et al.* (2007), Daek *et al.* (2008), Velázquez *et al.* (1991), Comitini *et al.* (2017)
			H. clermontiae, H. meyeri, H. opuntiae, H. osmophila H. vinae, H. valbyensis, H. thailandica	Grape berries surface, must, damaged grapes,	Microflora regulation	Renouf *et al.* (2007), Fleet *et al.* (2003), Fugelsang *et al.* (2007), Daek *et al.* (2008), Velázquez *et al.* (1991), Maturano *et al.* (2012), Wang *et al.* (2015), Stefanini *et al.* (2016)
		Candida	*C. boidinii, C. bombi, C. cidri, C. fermentati*	Grape berries surface, damaged grapes, must		Renouf *et al.* (2007), Fleet *et al.* (2003), Fugelsang *et al.* (2007)
			C. cantarelli	Damaged grapes, must	Better organoleptic features	Fugelsang *et al.* (2007), Toro *et al.* (2002)
			C. krusei / Issatchenkia orientalis, C. vini	Damaged grapes, must, wine	Wine spoilage by film production and production of acetaldehyde, volatile acids and esters	Fugelsang *et al.* (2007)
			C. steatolytica / Zygoascus hellenicus	Damaged grapes, must, fermented wine	Wine spoilage	Fugelsang *et al.* (2007)

			C. stellata / C. zemplinina	Grape berries surface, damaged grapes, must, beginning AF, winery equipment	Wine spoilage by film production and production of acetaldehyde, volatile acids and esters	Renouf *et al.* (2007), Fleet *et al.* (2003), Fugelsang *et al.* (2007)
		Kazachstania		Grape, fermented wine		Grangeteau *et al.* (2017)
		Pichia	*P. anomala / C. pelliculosa*	Grape berries surface, must, beginning AF, bottled wine	Wine spoilage by film production and production of ethyl acetate, isoamyl acetate and methyl butyl acetate	Renouf *et al.* (2007)
			P. guilliermondii / C. guilliermondii	Must, beginning AF, winery equipment	Wine spoilage by production of volatile phenols	Fugelsang *et al.* (2007), Barata *et al.* (2012)
			P. kluyveri	Must, beginning AF	Desirable aroma note	Comitini *et al.* (2017)
			P. membranaefaciens / C. valida	Grape berries surface, must, beginning AF	Wine spoilage by film production and release of acetaldehyde, esters and acetic acid	Renouf *et al.* (2007), Fleet *et al.* (2003)
			P. terricola / I. terricola	Grape berries surface, must, beginning AF		Renouf *et al.* (2007)
		Debaryomyces	*Db. hansenii*	Grape berries surface, must, fermented wine	Wine spoilage	Renouf *et al.* (2007)
		Lachancea	*Lach. fermentati (ex Z. fermentati)*	Grape, must, fermented wine		Fugelsang *et al.* (2007), Wang *et al.* (2015)
			Lach. thermotolerans	Grape, must, fermented wine	Desirable aroma note	Fugelsang *et al.* (2007), Comitini *et al.* (2017)
			K. hubeiensis, K. lactis, K. marxianus	Grape berries surface, must		Renouf *et al.* (2007), Fugelsang *et al.* (2007)
		Torulaspora	*T. delbrueckii / C. colliculosa*	Grape berries surface, must, wine	Desirable aroma note	Renouf *et al.* (2007), Comitini *et al.* (2017)
		Zygosaccharomyces	*Z. baillii*	Must, wine, bottled wine, winery equipment	Wine spoilage by production of acetic acid and esters + turbidity and sediment	Renouf *et al.* (2007), Fleet *et al.* (2000), Daek *et al.* (2008)
			Z. bisporus, Z. rouxii	Must, wine	Wine spoilage	Daek *et al.* (2008)
			Z. fermentati	Must, wine, winery equipment	Wine spoilage	Fleet *et al.* (2000)

			Z. florentinus	Grape berries surface, must, wine		Renouf *et al.* (2007)
		Kluyveromyces		Fermented and aged wines		Loureiro and Malfeito-Ferreira (2003)
		Saccharomyces	*S. bayanus*	Fermented wine	AF and wine spoilage by production of acetaldehyde	Ribéreau-Gayon *et al.* (2006)
			S. cerevisiae	Grape berries surface, fermented wine, winery equipement	AF and wine spoilage by production of volatil phenols, acetaldehyde and biogenic amines	Renouf *et al.* (2007), Daek *et al.* (2008), Monagas *et al.* (2007), Caruso *et al.* (2002), Bokulich *et al.* (2013)
	Saccharomycodaceae	*Saccharomycodes*	*Sch. Ludwigii*	fermented wine, bottled wine	Wine spoilage by production of acetaldehyde	du Toit *et al.* (2000), Fleet *et al.* (2003)
	Schizosaccharomycetaceae	*Schizosaccharomyces*	*Sc. pombe*	Must, bottled wine, winery equipment	Wine spoilage	Fleet *et al.* (2000), Fugelsang *et al.* (2007)
			Sc. japonicus	Fermented wine		Pinto *et al.* (2015)

AF – alcoholic fermentation

Appendix 2: Main bacteria in the wine ecosystem and their roles in the winemaking process

Phylum	Family	Genus	Relevant species	Main source	Technological significance	References
Acetic acid bacteria (AAB)						
Proteobacteria	*Acetobacteraceae*	*Acetobacter*	*A. aceti, A. cerevisiae*	Grape, fermented wine, wine equipment	Wine spoilage by production of acetic acid, acetaldehyde and ethyl acetate	Daek *et al.* (2008), Bartowsky *et al.* (2009b)
			A. malorum, A. tropicalis, A. oeni	Wine		Daek *et al.* (2008)
			A. pasteurianus	Grape, wine (barrel and bottle)		Daek *et al.* (2008), Bartowsky *et al.* (2009a)
		Gluconobacter	*G. cerinus, G. oxydans*	Grape berries surface, *Botrytis CiNerea* grapes, wine		Renouf *et al.* (2007), Barbe *et al.* (2000), González *et al.* (2005)
		Asaia	*As. siamensis*	Grape	Production of lactic acid	Bae *et al.* (2006)
Lactic acid bacteria (LAB)						
Firmicutes	*Leuconostocaceae*	*Oenoccocus*	*O. oeni*	Grape berries surface (rare), must, AF, MLF, bottled wine, winery equipment	MLF + wine spoilage by production of off-flavor compounds + production of esters	Renouf *et al.* (2007), Boulton *et al.* (2013), Costello *et al.* (2013)
			O. kitaharae	Fermented wine		González-Arenzana *et al.* (2017)
		Leuconostoc	*Lc. dextranicum, Lc. fallax, Lc. citreum*	Grape, must, fermented wine		Bae *et al.* (2006), González-Arenzana *et al.* (2017)
			Lc. mesenteroides, Lc. pseudomesenteroides	Grape berries surface, must, AF	Wine spoilage by production of off-flavor compounds	Renouf *et al.* (2007), Bae *et al.* (2006), Boulton *et al.* (2013), González-Arenzana *et al.* (2017)
		Weissella	*W. paramesenteroides*	Grape berries surface, wine	MLF + wine spoilage	Renouf *et al.* (2007), Bae *et al.* (2006)
			W. uvarum, W. cibaria, W. soli	Grape, wine		Nisiotou *et al.* (2014)
		Fructobacillus	*F. tropaeoli, F. ficulneus*	Must, fermented wine		González-Arenzana *et al.* (2017)

	Lactobacillaceae	*Lactobacillus*	*L. brevis*	Grape, must, AF	Wine spoilage by production of off-flavor compounds	Bae *et al.* (2006), Boulton *et al.* (2013), Costello *et al.* (2001)
			L. buchneri	Grape, must, wine	Wine spoilage by production of biogenic amines	Bae *et al.* (2006)
			L. casei	Grape berries surface, must, AF	Wine spoilage	Renouf *et al.* (2007), Bae *et al.* (2006), Boulton *et al.* (2013)
			L. cellobiosus / L. fermentum, L. delbrueckii / L. leichmannii, L. diolivorans, L. jensenii, L. paracasei	Grape, must, wine		Fugelsang *et al.* (2007)
			L. collinoides	Fortified wine	Wine spoilage by production of off-flavor compounds	Couto and Hogg (1994)
			L. curvatus, L. pentosus, L. rhamnosus	Grape, must, wine		Bae *et al.* (2006), González-Arenzana *et al.* (2017)
			L. fructivorans, L. heterohiochii, L. trichodes	Grape, wine, fortified wine	Wine spoilage	Couto and Hogg (1994)
			L. hilgardii / L. vermiforme	Grape, must, AF, fortified wine	Wine spoilage by production of off-flavor compounds	Bae *et al.* (2006), Boulton *et al.* (2013)
			L. kefiri / L. yamanashiensis, L. mali	Damaged grapes, wine, fortified wine	Wine spoilage by production of volatile phenols	Bae *et al.* (2006)
			L. kunkeei	Grape, must, wine	Wine spoilage by production of acetic acid --> retard or inhibit fermentation	Bae *et al.* (2006), Boulton *et al.* (2013)
			L. lindneri	Grape		Bae *et al.* (2006)
			L. plantarum	Grape berries surface, must, AF, MLF	MLF + wine spoilage by production of volatile phenols and biogenic amines + production of esters	Renouf *et al.* (2007), Bae *et al.* (2006), Boulton *et al.* (2013), Costello *et al.* (2013)
			L. sanfranciscansis	Grape berries surface, grape, must, wine		Renouf *et al.* (2007), Bae *et al.* (2006)
			L. zeae	Ageing wine	Spoilage by production of biogemic amines	Moreno-Arribas *et al.* (2008)

		Pediococcus	*P. acidilactici*	Grape berries surface	Wine spoilage	Renouf *et al.* (2007)
			P. damnosus	Grape berries surface, must, AF	MLF + wine spoilage by production of volatile phenols and biogenic amines	Renouf *et al.* (2007), Boulton *et al.* (2013), Lonvaud-Funel *et al.* (1999), Walling *et al.* (2005), Juega *et al.* (2014)
			P. inopinatus	Grape, wine	MLF + wine spoilage by production of biogenic amines	Strickland *et al.* (2016)
			P. parvulus	Grape berries surface, wine, bottled wine	MLF + wine spoilage by production of biogenic amines	Renouf *et al.* (2007), Lonvaud-Funel *et al.* (1999), Walling *et al.* (2005), Strickland *et al.* (2016)
			P. pentosaceus	Grape, wine	Wine spoilage by production of volatile phenols	Fernández and de Nadra (2006), Lerm *et al.* (2010)
	Enterococcaceae	*Enterococcus*	*Ec. avium*	Grape		Bae *et al.* (2006)
			Ec. durans	Grape	Wine spoilage	Bae *et al.* (2006)
			Ec. faecium	Grape berries surface, wine	Wine spoilage by production of biogemic amines	Renouf *et al.* (2007), Bae *et al.* (2006), Boulton *et al.* (2013), Capozzi *et al.* (2011)
			Ec. hermaniensis	Damaged grapes		Bae *et al.* (2006)
Other bacteria						
Proteobacteria	*Moraxellaceae*	*Acinetobacter*	*Ac. baumannii, Ac. calcoaceticus, Ac. Guillouiae, Ac. Johnsonii, Ac. Junii, Aci. Lwoffii, Ac. rhizosphaerae*	Grape, fermented wine	Wine spoilage	Piao *et al.* (2015), Del Carmen Portillo and Mas (2016b)
	Aeromonadaceae	*Aeromonas*	*Aer. hydrophila*	Soil, fermented wine	Wine spoilage	Zepeda-Mendoza *et al.* (2018)
	Burkholderiaceae	*Burkholderia*	*Burk. vietnamiensis*	Grape berries surface		Renouf *et al.* (2007)
	Pseudomonadaceae	*Pseudomonas*	*Ps. jessenii*	Grape berries surface	Wine spoilage	Renouf *et al.* (2007), Piao *et al.* (2015), Del Carmen Portillo and Mas (2016b)
	Enterobacteriaceae	*Enterobacter spp.*	*Eb. gergoviae*	Grapevine leaves, grape berries surface	Wine spoilage	Renouf *et al.* (2007), Pinto *et al.* (2014)
		Serratia	*Ser. rubidaea*	Grapevine leaves, grape berries surface	Wine spoilage	Renouf *et al.* (2007), Pinto *et al.* (2014)
	Sphingomonadaceae	*Zymomonas*	*Zym. mobilis*	Wine	AF	Moreno-Arribas *et al.* (2009)

		Sphingomonas	*Sph. pseudosanguinis,*	Grape, fermented wine		Bokulich *et al*. 2012, Piao *et al.* (2015), Del Carmen Portillo and Mas (2016b)
	Rhodobacteraceae	*Paracoccus*		Grape, fermented wine		Piao *et al.* (2015), Del Carmen Portillo and Mas (2016b)
	Anaplasmataceae	*Wolbachia*	*W. endosymbiont*	Grape, fermented wine		Piao *et al.* (2015), Del Carmen Portillo and Mas (2016b)
	Methylobacteriaceae	*Methylobacterium*	*Met. populi*	Grape, fermented wine		Bokulich *et al*., 2012, Piao *et al. (*2015); Del Carmen Portillo and Mas (2016b)
	Rhodanobacteraceae	*Dyella*	*Dy. japonica*	Soil, fermented wine		Zepeda-Mendoza *et al.* (2018)
	Xanthomonadaceae	*Xanthomonas*	*X. alfalfae*	Soil, fermented wine		Zepeda-Mendoza *et al.* (2018)
Firmicutes	*Bacillaceae*	*Bacillus*	*B. thuringiensis*	Grape	Wine spoilage	Fleet *et al*. (2003)
		Staphylococcus		Grape	Wine spoilage	Barata *et al.* (2012)
	Sporolactobacillaceae	*Sporolactobacillus*	*Spor. inulinus*	Grape	Production of lactic acid	Bae *et al.* (2006)
	Clostridiaceae	*Clostridium*		Stored and bottled wines		du Toit *et al.* (2000)
Actinobacteria	*Microbacteriaceae*	*Leifsonia*	*Leif. xyli*	Grape berries surface, Grape		Renouf *et al.* (2007), du Toit *et al.* (2000)
		Micrococcus	*Mic. luteus*	Soil, fermented wine		Zepeda-Mendoza *et al.* (2018)

AF – alcoholic fermentation; MLF – malolactic fermentation

Appendix 3: Main volatile compounds detected in wines

			Reported concentrations in wine (µg/L)													
			(1): 17 white and 23 red Spanish wines		(2): 3 Spanish and 2 Uruguayan premium red wines		(6): 9 Zalema white wines	(7): in wines		(12): Spanish red wine during MLF		(13): Spanish white wine during MLF		(14): wines		(15): Changli Cabernet Sauvignon wines	
Volatile compounds	**Odor thresholds (µg/L)**	**Odor**	Min.	Max.	Min.	Max.	Mean	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
Esters																	
Ethyl acetate	12270 [4]	Nail polish[7], fruity[7]	–	–	10440	66600	5000	22500	63500	13700	84200	14400	110000	46400	120000	11400	90000
Ethyl hexanoate	14 [9]	Fruity[2], green apple[6], anise[2], strawberry[1]	206	1 640	29	227	783	30	3 400	67	641	401	2 332	160	770	400	1 300
Ethyl octanoate	580[3]	Fruity[9], fat[9] sweet[1]	137	2 610	24	102	773	50	3 800	316	917	1 630	5 750	150	660	130	740
Ethyl decanoate	200[9]	Floral[9], soap[9], grape[1]	47	696	4.1	15	544	nd	2 100	256	785	1 480	14 800	180	300	4	100
Isobutyl acetate	1 600[2]	Fruity[1], apple[1], solvent[2]	–	–	10	57	71	10	1 600	9.4	120	3.7	122	20	60	70	180
Isoamyl acetate	30[9]	Banana[1], fruity[1]	31	5 520	120	221	1 091	100	3 400	105	2 380	50	7 230	100	770	200	2 800
Linalyl acetate	–	Herbal[9]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isoamyl octanoate	–	Fruity[9]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2-phenylethyl-acetate	250[4]	Rose[1], floral[1]	31	394	16	41	214	nd	18 500	97	152	131	322	20	90	80	500
Diethyl succinate	200 000[3]	Fruit[1], wine[1]	1 210	61 110	6 240	31 500	2 586	–	–	189	3 430	102	147	2 620	7 110	4 800	52 800
Ethyl butanoate	20[9]	Floral[1], strawberry [1]	111	532	69	170	375	10	1 800	39	401	34	770	180	400	500	1 900
Hexyl acetate	700[7]	Green[1], herbaceous[1]	1.0	390	–	–	–	nd	4 800	12	21	nd	633	50	280	10	20
Ethyl-2-methyl-butanoate	18[9]	Strawberry[1], berry[1], cider[1]	–	–	9.2	32	11	–	–	1.4	17	19	29	–	–	–	–
Methyl octanoate	–	Orange, wax	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isoamyl hexanoate	–	Fruity, pineapple	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ethyl heptanoate	2.2	Fruity	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ethyl nonanoate	850	Floral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ethyl-2-furoate	16 000[17]	Balsamic	–	–	6.4	17	21	–	–	–	–	–	–	–	–	–	–
Alcohols																	
Trans-3-hexenol	400[9]	Grass[2]	–	–	–	–	–	–	–	78	94	48	65	–	–	600	2 100
Trans-2-hexenol	400[9]	Grass[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	150	800

Benzyl alcohol	200 000[6]	Solvent[1]	86	2 420	70	1 859	37	_	_	228	585	53	155	80	850	500	2 000
Methanol	_	Alcohol[9]	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Cis-3-hexenol	400[9]	Fresh[1], cut grass[1]	_	_	66	234	353	_	_	43	69	101	148	80	150	700	1 500
Higher alcohols																	
Methionol	1 000[4]	Cabbage, potato	_	_	1 624	3 750	252	_	_	_	_	_	_	300	2 900	_	_
1-hexanol	4 000[7]	Green[1], dry[1]	740	4 380	780	1 478	824	300	12 000	728	981	710	1 180	880	1 310	11 400	28 400
2-phenylethanol	14 000[9]	Rose[1], floral[1]	15 110	112 300	46 207	96 292	22 548	4 000	197 000	43	52	17 300	23 100	7 200	43 200	30 800	140 100
1-propanol (n-propyl alcohol)	500 000[7]	ripe fruit[1], Pungent[7], harsh[7]	7 510	44 800	_	_	_	9 000	68 000	_	_	_	_	_	_	5 800	20 400
1-butanol	150 000[3]	Bitter[1], solven[1]t, chemical[1]	294	3 090	1 900	2 502	2 066	50	8 500	_	_	_	_	_	_	1 600	4 700
Isobutanol (2-methyl-1-propanol or isobutyl alcohol)	40 000[9]	Higher[2], green[6], fresh[6]	2 750	69 800	28 700	89 670	20 639	9 000	174 000	_	_	_	_	21 900	55 800	31 000	105 200
2-methyl-1-butanol (active amyl alcohol)	7 000[10]	Bitter[1], harsh[1]	10 280	124 900	_	_	_	_	_	_	_	_	_	_	_	_	_
Isoamyl alcohol (3-methyl-1-butanol)	30 000[4]	Bitter[1], harsh[1], Sweet[6], higher[6]	49 200	457 900	112 800	277 139	149 528	6 000	490 000	_	_	_	_	72 300	178 000	164 400	567 500
Terpenes																	
Linalool	25[9]	Lemon[1], fresh[1], floral[2], Muscat[2]	1.0	40	nd	10	11	1.7	10	5.8	7.4	4.7	7.1	_	_	10	130
β-citronellol	100[3]	Lemon[1]	_	_	1.2	6.6	2.2	15	42	8.1	10	2.9	6.1	_	_	_	_
Nerol	300[12]	Rose	_	_	_	_	_	_	_	2.7	6.5	nd	4.0	_	_	_	_
α-terpineol	250[9]	wood[9], soil[9]	4.0	34	13	24	26	_	_	nd	22	nd	6.0	_	_	10	100
Volatile Fatty Acids																	
Isobutyric acid (2-methylpropanoic acid)	230[9]	Phenolic[1], fatty[1]	_	_	670	4 260	411	_	_	_	_	_	_	1 060	2 490	40	200
Isovaleric acid (3-methylbutyric acid)	33[9]	Cheese[1], spicy[6]	_	_	1 651	2 180	349	_	_	_	_	_	_	510	1 490	_	_
Valeric acid	8 100[3]	Fatty[1], pungent[1]	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Hexanoic acid	420[9]	Fatty[1]	1 520	14 740	1 031	3 328	9 499	_	_	2 690	4 260	8 140	30 600	1 210	5 450	100	1 700
Octanoic acid	500[9]	Sweat[9], cheese[9]	_	_	546	2 135	9 767	_	_	2 850	3 700	8 070	9 070	1 090	6 580	700	1000
Decanoic acid	1 000[9]	Rancid[9], fat[9]	_	_	92.8	3 010	16 861	_	_	_	_	810	2 710	190	1 430	_	_
Dodecanoic acid	6 100[11]	Dry[8], metallic[8]	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Butyric acid	173[9]	cheese[8]	_	_	nd	1 360	996	_	_	_	_	_	_	777	2 130	_	_
Volatile phenols																	
4-ethylphenol	600[7]	Medicinal[7], barnyard[7]	_	_	nd	174	_	12	6 500	8.3	30	8.3	30	_	_	_	_

4-vinylguaiacol	40[4] or 1 100[5]	Clove-like[7], phenolic[7]	–	–	19	334	463	1.4	710	–	–	–	–	–	–	–	–
4-vinylphenol	180[5]	Pharmaceutical[7]	–	–	nd	27	105	40	450	7.2	15	532	907	–	–	–	–
4-ethylguaicol	110[7]	Phenolic[7], sweet[7]	–	–	1.2	39	0.1	1.0	444	1.2	1.5	1.3	1.6	–	–		
Eugenol	6[6]	smoky, clove	–	–	17	60	–	–	–	19	29	19	31	–	–	1.0	6.0
Isoeugenol	6[8]	floral[8]	–	–	1.6	5.5	1.0	–	–	–	–	–	–	–	–	–	–
Carbonyl compounds																	
Benzaldehyde	200 000[2]	Bitter[1], almond[1]	1.0	47	–	–	–	–	–	–	–	–	–	–	–	–	–
2-phenylacetaldehyde	1[2]	Floral[2], honey[2]	–	–	1.5	9.9	1.5	–	–	–	–	–	–	–	–	–	–
Acetaldehyde (ethanal)	100 000[7]	Sherry[7], nutty[7], bruised apple[7]	–	–	–	–	2 963	10 000	75 000	–	–	–	–	2 730	26 000	–	–
2-furfural	500[4]	sweet[8]	41	308	8.8	31	60	–	–					30	1 360		
α-ionone	0.09[6]	raspberry, violet, sweet fruity	–	–	nd	0.7	0.2	–	–	nd	5.6	–	–	–	–	2.0	6.0
ß-ionone	0.09[6]	raspberry, violet[8], sweet fruity	–	–	0.1	0.2	–	–	–	–	–	–	–	–	–	1.0	9.0

Min: minimum; Max: maximum; nd: not detectable; - not quantified in the referred literature

1 (Aznar and Arroyo, 2007); 2 (Escudero *et al.*, 2007) and 11 (Meilgaard, 1975): Thresholds calculated in beer; 3 (Etiévant, 1991) and 7 (Swiegers *et al.*, 2005a): Thresholds calculated in wine; 4 (Guth, 1997): Thresholds calculated in 10% ethanol; 5 (Boidron *et al.*, 1988): Thresholds calculated in synthetic wine containing 12% ethanol, 8g/L glycerol and different salts; 6 (Gomez-Miguez *et al.*, 2007) and 8 (Culleré *et al.*, 2004): Thresholds calculated in 10% water / ethanol mixture containing 5 g/L of tartaric acid at pH 3.2; 9 (Ferreira *et al.*, 2000): Thresholds determined in 11% v/v aqueous ethanol with 7 g/L glycerol and 5 g/L tartaric acid, at pH 3.4; 10 (Salo, 1970): Thresholds calculated in hydro-alcoholic solution; 12 (García-Ruiz *et al.*, 2013b); 13: (Rodríguez-Bencomo *et al.*, 2014); 14: (Ortega *et al.*, 2001); 15: (Tao and Li, 2009); 16: (Oliveira *et al.*, 2004): Thresholds calculated in 10% water/ethanol solution containing 5 g/L tartaric acid; 17: (Lopez *et al.*, 2002): Thresholds calculated in 10% water / ethanol solution at pH 3.2.

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