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Malolactic bacterial starters in winemaking: study of implantation and biogenic amines during malolactic fermentation and storage, and their role in ochratoxin A reduction

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Presented by

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Certificamos,

Que la memoria intitulada "Malolactic bacterial starters in winemaking: study of implantation and biogenic amines during malolactic fermentation and storage, and their role in ochratoxin A reduction", presentada por Paola Daniela Olmos Rizzo para optar al grado de Doctor en Ciencia de los Alimentos, ha estado realizada bajo nuestra dirección y considerada acabada, autorizamos su presentación para ser evaluada por la comisión correspondiente.

Vilafranca del Penedès (Barcelona), 15 de Mayo de 2013.

Dr. Santiago Mínguez

Dra. Anna Puig Pujol

Quiero dedicar esta tesis doctoral a

Jean-Baptiste, Marco y Julia

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Todos somos ignorantes…

…solo que no todos ignoramos las mismas cosas

Albert Einstein

Summary

Malolactic bacterial starters are used in the production of high quality and safe wines because of their capabilities to produce low levels of biogenic amines and perform the malolactic fermentation under controlled conditions. However, in certain conditions, inoculated wines can have high biogenic amines content which is in contradiction with the expected performance of the bacterial starter. In the present work, it was demonstrated, by typification of bacterial strains using RAPD-PCR, that malolactic starters can present different levels of implantations and this is correlated with the biogenic amines produced during the malolactic fermentation, which can explain the unexpected results when malolactic starters are used. In order to understand how the implantation is affected by oenological practices, lysozyme, nutrients, co-inoculation with yeast, or application of different seeding methods (direct inoculation or *pied de cuve)* were studied regarding their impact on the level of implantation and biogenic amines production.

On the other hand, biogenic amines in bottled wine evolve all along the storage period. Their content increase, decrease or stay constant, which creates incertitude regarding the levels of biogenic amines that the wine will have in the moment of its commercialization. In the second part of this thesis, we demonstrated the presence of microorganisms with decarboxylase capabilities and exocellular amine-decarboxylase enzymes in the bottled wine. The presence of amine-degrading enzymes is also suspected. The link between the latter and the biogenic amines found at the end of a year of storage were related and different histamine profiles were determined for the wines: histaminolitic, histaminogenic and histamine-stable. Some indicators to measure the risk of histamine development are proposed.

In the search for technological means to help wine-makers to elaborate safe wines, the use of biological tools to reduce the levels of ochratoxin A (OTA) in wines was investigated. In the third part of this thesis, malolactic starters were screened by their property to reduce OTA during the malolactic fermentation. Some malolactic starters were able to obtain high OTA reductions in wines containing 13% ethanol. OTA reduction by starters seems to be related to the pH. The interaction between protein-polyphenols haze formation as function of ethanol and pH and the interaction that this might have on absorption phenomenon is discussed. Modification of cell wall by lyophylization process and its consequences on adsorption properties are also analyzed. The results are encouraging but more investigation is needed to understand OTA reduction mechanism carried-out by malolactic bacterial strains in wines.

Resumen

La utilización de bacterias iniciadoras de fermentación maloláctica es de fundamental importancia para los elaboradores de vino comprometidos con la producción de vinos de alta calidad y sin riesgos para la salud, ya que estas bacterias han sido seleccionadas como poco productoras de aminas biógenas. Sin embargo, en ciertas condiciones se obtienen vinos con altos niveles de aminas biógenas, aun cuando estos vinos han sido inoculados con bacterias seleccionadas. En el presente trabajo, con la tipificación de bacterias aisladas de la fermentación maloláctica usando un método basado en RAPD-PCR, se demostró que el estárter maloláctico puede tener diferentes niveles de implantación y que a su vez, esto se correlaciona con la producción de aminas biógenas durante la fermentación maloláctica, lo cual explicaría los altos niveles de aminas obtenidos en vinos inoculados con estárteres malolácticos. Para entender como las diferentes practicas enológicas afectan la implantación del estárter maloláctico, lisozima, nutrientes, co-inoculación con levaduras, o siembra del estárter por diferentes métodos (inoculación directa o pie de cuba) fueron estudiados respecto al nivel de implantación y producción de aminas biógenas.

Luego de la fermentación maloláctica, las aminas biógenas pueden aumentar, degradarse o estar constantes durante el almacenamiento del vino una vez que este ha sido embotellado. Esto puede crear incertidumbre respecto al nivel que las aminas biógenas tendrán en el momento de su comercialización. En la segunda parte de esta tesis, se puso en evidencia la presencia de microorganismos y enzimas libres en el vino embotellado capaces de producir histamina durante su almacenamiento; también se sospecha la presencia de enzimas capaces de degradar histamina. Se estudió la relación entre esto, y el contenido de aminas al final de un año de almacenamiento; diferentes perfiles de vinos se identificaron: histaminogénicos, histaminoliticos e histamin-estables. Indicadores para estimar el riesgo de producción de histamina fueron propuestos.

Siguiendo con el interés de investigar tecnologías que ayuden a producir vinos sin peligro para la salud del consumidor, el uso de técnicas biológicas para reducir los niveles de ocratoxina A (OTA) en vinos fue investigado. En la tercera parte de esta tesis, estárteres malolácticos fueron evaluados respecto a su capacidad para reducir OTA durante la fermentación maloláctica. Algunos estárteres, fueron capaces de reducir OTA en vinos conteniendo 13% de etanol. La reducción de OTA parece relacionarse con el pH del vino. La interacción entre la formación de aglomerados de protein-polifenoles en función del etanol y pH, y la interacción que esto podría tener en la capacidad de adopción fue discutido. La modificación de la pared celular debido al proceso de liofilización de los estárteres como causa posible de la capacidad reductora de los estárteres fue también abordado. Los resultados son prometedores, sin embargo, sería necesario profundizar en la investigación para entender los mecanismos ligados a la reducción de OTA producido por los estárteres malolácticos en vinos.

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Abbreviations

1/2	middle
%	percentage
°C	Celsius degrees
AAB	Acetic acid bacteria
AFLP	amplified fragment length polymorphism
agdi	gene coding agmatine deiminase
ALF	alcoholic fermentation
ANOVA	analysis of variance
ARDRA	amplified ribosomal DNA restriction analysis
b.w.	body weight
BA	biogenic amines
CFU	colony forming unit
CO_2	carbon dioxide
DAO	diamino oxidase enzyme
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DOC	denominación de origen calificada
EFSA	European Food Safety Authority
ES	spontaneous
et al.	*et alii* (and others)
FAO	Food and Agriculture Organization of the United Nations
Fig.	figure
FISH	fluorescent in situ hibrydation
g	grams
HACCP	hazard analysis of critical control points
HDC	histidine decarboxylase
hdc	histidine decarboxylase gene
HL	hectoliter
HMD	histidin medium decarboxylase
HPLC	High-Performance Liquid Chromatography
IgE	inmunoglobulin E
IN	inoculated
INCAVI	Institut Calatà de la Vinya i el Vi
ITA-PCR	PCR analysis of the 16S-23S rRNA gene internal transcribed spacer
ITV France	Institute Technique du Vin
L	liter
L.	Lactobacillus
LAB	lactic acid bacteria
Lc.	Leuconostoc
LOD	limit of detection
LOQ	limit of quantification
lys.	lysozyme
M	100 base pair ladder molecular weight marker
MAO-A	monoamine oxidase A
MAOI	monoamine oxidase inhibitors
MCO	multicopper oxidase
mg	milligram
mL	milliliter
MLF	malolactic fermentation
MLO	Medium for *Leuconostoc oenos*
Mn	manganese
MT	mother tank
n.a.	non applicable
n.e.d.	non enought data
na	non applicable
NAD	nicotinamide adenine dinucleotide
nm	nanometer
O.	Oenococcus
OCD	ornithin decarboxylase
odc	gene coding ornithin decarboxylase
OIV	Organization International de la Vigne et du Vin
OTA	ochratoxin A
P.	Pediococcus
ppm	parts per million
pvpp	polyvinylpolypyrrolidone
R%	OTA reduction
RAPD	random amplified polymorphic DNA
RASFF	Rapid Alert System for Food and Feed
rep-PCR	repetitive sequence-based PCR
RFLP	restriction fragments length polymorphic analysis
RFLP-PFGE	restriction fragments length polymorphism by pulsed-field gel electrophoresis
RNA	ribonucleic acid
SD	standard deviation
SO_2	Sulphur dioxide
TBE	tris-borate EDTA
TDC	tyrosine decarboxylase
tRNA-PCR	coding intergenic space of transfer RNA
*tyr*dc	gene coding histidin decarboxylase
UAB	Universitat Autonoma de Barcelona
WHO	World Health Organization
µm	micrometer
Y-M	Yeast and moulds

PROLOGUE

WINE & FOOD SAFETY

Food safety and consumers

With the improvement of living standards, consumers have become increasingly concerned about health and general physical well-being. The rapid pace of change in science and technology, changes in legislation and the current socio-economic and socio demographic realities have all had a marked impact on the food we buy today.

« *About 37 per cent of European consumers believe that their health could be at risk from the food they eat»*

reported the Eurobarometer in the last « Food related Risks » survey published in 2010. The intensification of farming, such as the use of pesticides, and the industrialization of food production, using additives and preservatives to improve taste, appearance and shelf-life, for example, can be causes for concern among many consumers.

The absence of danger or their reduction should be the main objective of any food safety system. During production or commercialization of food, many factors might contribute to sanitary dangers in the food chain, as per example, the indiscriminate use or abuse of pesticides in agriculture, environmental contamination or bad sanitary conditions in production sites, uncontrolled food production or processes, etc.

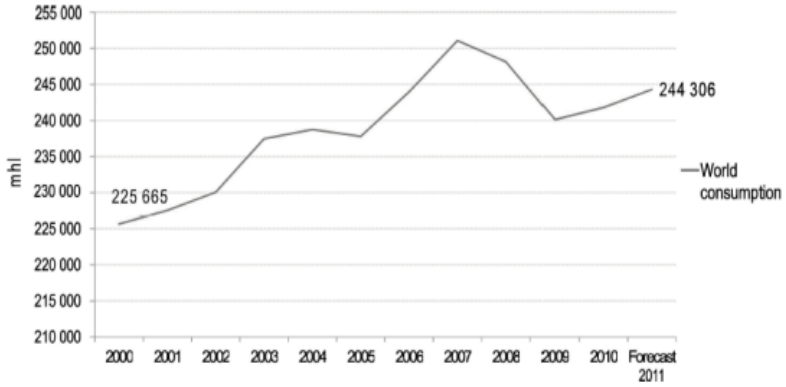
All these factors of change have given rise to the increasing need for risk managers and risk assessors to be vigilant so that they can respond to both known and emerging risks in order to protect consumers.

Wine

“Wine” is defined, according to the International Oenological Codex (OIV, 2013), as the beverage resulting exclusively from the partial or complete alcoholic fermentation (ALF) of fresh grapes, whether crushed or not, or of grape must. Its actual alcohol content shall not be less than 8,5%vol.

With a global wine consumption trend constantly increasing (Fig. 1), Spain is in the 3rd position of main wine producer countries with 38 000 million HL per year, and in 8th position of the top ten of wine consumers (OIV, 2012).

Figure 1. Global wine consumption ***(source OIV 2012)***



The increasing consumption trend together with the food safety concern of consumers, put the safety of wine in the focus of attention of public organisms, as FAO (Food and Agriculture Organization of the United Nations), WHO (World Health Organization), EFSA (European Food Safety Authority) and OIV (Organization International de La Vigne et du Vin).

A lot research has been done to determine the potential risk associated with wine consumption. Regarding the origin of the substances found in wine that could have a risk for the human health, they can be classified as follows (Suárez Lepe and Íñigo, 2003):

- Contaminants: for example, pesticides and heavy metals
- Additives: sulphur dioxide
- Molecules related to the metabolism of microorganisms: biogenic amines (histamine, tyramine, etc), ethyl carbamate or ochratoxin A.

Safety, in terms of the absence of chemical contaminants, physical and toxic agents of biological origin, must be the most important and indispensable requirement of quality. This PhD study focuses on the toxins present in wines related to the metabolism of microorganisms, as biogenic amines and ochratoxin A.

GENERAL AIM OF THE THESIS

The use of malolactic bacterial starters is of uncontestable importance to produce high quality and safe wines due to their capabilities to produce low levels of biogenic amines and to perform the malolactic process under controlled conditions.
However, in certain conditions, the winemakers obtain high levels of biogenic amines which are in contradiction with the expected performance of the bacterial starter. The monitoring of the process, in terms of the identification of the inoculated bacterial starter among the autochthonous bacterial population is of paramount importance to understand the level of implantation of the bacterial starters and thus, understand the deviations. In the first part of this thesis, the main objectives were 1) to apply molecular techniques for the typification of *O. oeni* during the malolactic fermentation in order to determine the level of implantation of the inoculated starter and 2) to evaluate how the level of implantation affects the biogenic amines production during the malolactic fermentation.

Even if the biogenic amines can be managed until a certain level via inoculation of malolactic starters, degradation or synthesis of biogenic amines can occur in bottled wine all long the storage. This creates uncertainty regarding the levels of biogenic amines that the wine might have in the moment of its commercialization. In the second part of this thesis, the main objectives were 3) to accelerate the amine decarboxylation reaction in wines in order to estimate the potential production of biogenic amines in those wines and 4) to evaluate decarboxylase activity from microorganisms and cell-free wines as indicators of histaminogenesis in wines.

Following with the interest to investigate technological strategies to help wine-makers to elaborate safe wines, the use of biological tools to reduce the levels of ochratoxin A (OTA) in wines was investigated. In the third part if this thesis, the main objective was 5) to determine the OTA-reduction capability of lyophilized malolactic starters for direct inoculation in microvinifications.

Specific objectives are presented in the corresponding chapters.

STRUCTURE OF THE THESIS

Three research axes were followed in this thesis:

Chapter 1: Malolactic Fermentation in Wines: influence of the level of implantation of lactic acid bacteria starter on the production of biogenic amines during malolactic fermentation. This investigation was carried-out in Institut Català de la Vinya i el Vi (INCAVI), Vilafranca del Penedès, Catalunya, Spain.

Chapter 2: Evolution of biogenic amines content in bottled wines during storage. This research was also performed in INCAVI.

Chapter 3: Role of malolactic starters in ochratoxin A reduction during malolactic fermentation. This last research was performed in Institut Technique de la Vigne et du vin (ITV- France, currently named ICV), Beaune, Burgundy, France.

GENERAL INTRODUCTION

Biogenic amines in Wines

BIOGENIC AMINES IN WINES

OCCURRENCE AND REGULATORY FRAME

A.1 Biogenic amines levels in wines

The biogenic amines content in food has been of paramount interest between the European authorities during the last years. In a report published by EFSA (2011), the issues related to ingestion of biogenic amines contained in the food was analyzed. The results concerning alcoholic beverages obtained from public call for data (June 2010) are shown in Table 1.

Table 1. Biogenic amines content in wines *(source EFSA, 2011).*

Histamine

Food category	Sub-category	n	ND	Mean	P5	Median	P95	Max
Alcoholic beverages	Beer	188	9.6%	1.4	<0.5	0.7	4.8	21.6
	Fortified and liqueur wines	28	32%	1.1	<0.1	0.7	2.8	2.9
	Wine, red	300	10%	3.6 – 3.7	<0.1	1.4 – 1.5	12.3 – 12.4	34.3
	Wine, white	225	22%	0.8 – 0.9	<0.1	0.3	2.6	55
	Wine, white, sparkling	45	73%	1	<0.1	<0.1	5.2	9.8
Total for alcoholic beverages		786	18%	2 – 2.1	<0.1	0.6 – 0.7	8.8 – 9.2	55

Tyramine

Food category	Sub-category	n	ND	Mean	P5	Median	P95	Max
Alcoholic beverages	Beer	188	0%	6.1	1.4	3.2	24.7	46.8
	Fortified and liqueur wines	28	3.6%	6	0.1	1.5	21.3	22.5
	Wine, red	296	12%	2.7 – 2.9	<0.2	1.6 – 1.8	7.8 – 8.5	18.5
	Wine, white	224	17%	1.1 – 1.2	<0.1	0.8	4.3 – 4.5	10
	Wine, white, sparkling	45	56%	4.9	<0.1	<0.1	26.4	47.3
Total for alcoholic beverages		*781*	*13%*	*3.3 – 3.4*	*<0.1*	*1.7*	*11.5 – 11.6*	*47.3*

Putrescine

Food category	Sub-category	n	ND	Mean	P5	Median	P95	Max
Alcoholic beverages	Beer	188	32%	3.3 – 3.5	<0.4	3.3	8.3	17.8
	Fortified and liqueur wines	28	0%	1.4	0.3	0.9	3.6	4.3
	Wine, red	120	5.0%	4.2 – 4.8	0.3 – 1	3.4 – 3.7	9.5 – 11.5	21.6
	Wine, white	100	3.0%	1.4 – 1.5	0.2	1	3.9 – 4.3	5.7 – 10
	Wine, white, sparkling	45	11%	5.2	<0.1	2.4	15	46.4
Alcoholic beverages		*481*	*16%*	*3.2 – 3.4*	*<0.4*	*2.3 – 2.4*	*8.6 – 9*	*46.4*

The statistics are presented using a bounded approach for the handling of non-detected/non-quantified data; therefore they are displayed as ranges. The upper bound of the range estimates the non-detected/non-quantified values using the reported limit of detection (LOD) or limit of quantification (LOQ) respectively. The lower bound of the range instead assumes the no detected/non-quantified values as zero. When the lower bound and the upper bound of the range are coincident, only one number is presented. When the lower bound is zero, the range is represented by the upper bound prefixed by '<'. The table contains the number of samples (n), the percentage of non detected (ND), the mean, several percentiles to describe the occurrence distribution (P5, P50 or median, P95 and max).

The average values of histamine, tyramine and putrescine in wines were not high (Table 1) due to the high dispersion of data. Regarding the maximum levels, the maximum for histamine was 55 mg/L, for tyramine, it was 47,3 mg/L and for putrescine it was 46,4 mg/L which are values to take into consideration from a safety point of view even if the frequency of events is marginal.

The biogenic amines values considered potentially dangerous for health when consuming wine as defined by Vidal-Carou (1987) are: histamine (2-10 mg/L), tyramine (10-80 mg/L) and phenyl ethylamine (3 mg/L). For the Biological Hazard group of experts (EFSA) the risky values for health are difficult to define as the variations in individuals' sensitivity may also be the result of interaction with other biogenic amines, other diet constituents such as alcohol or medication with DAO (diamino oxidase enzyme) inhibitors. These uncertainties may lead either to an underestimation of the adverse effect to occur in sensitive people or to an overestimation of the risk for healthy people (EFSA, 2011).

As per the results of the Rapid Alert System for Food and Feed (RASFF), several consumers were affected by histamine intoxication (reported as 'consumer complaint', 'food poisoning' and "food borne outbreaks') between 2005 to 2010. All cases were caused by fish and products thereof.

Taking into account the notifications made on the RASFF system (webgate.ec.europa.er/rasff-window/portal/, product category : wine), no cases related to biogenic amines levels in wines were reported since RASFF creation in 1979, this is due to the lack of legislation on biogenic amines limits for wines. Additionally, due to the fact that the clinical frame for establishing symptoms associated with biogenic amines in wine is nowadays source of many controversies, the corresponding impact on public health is difficult to establish.

A.2 Biogenic amines in wines: Worldwide references

The presence of biogenic amines in wines has been studied since 1950's. From these studies it is generally concluded that histamine, tyramine and putrescine are the most frequent BA in wines (Vidal and Bover, 2001).

Table 2 is shows the levels of BA found in wines from non-European countries and in Table 3 the biogenic amines found in wines from European countries.

Table 2. Biogenic amines content in wines from non European countries
(updated from Vidal and Bover, 2001)

Reference	Type of Wine	Histamine	Tyramine	Phenyl ethylamine	Putrescine	Cadaverine
Ough 1971 [a] US - California	White Rosé Red	0,3 - 11,4 0,6 - 2,8 0,2 – 15,5				
Zee *et al.* 1983 Canada and US	White - Canada White - US Red – Canada Red – US	1,86 3,56 3,66 7,33	n.d. 3,18 4,27 8,64		1,25 1,69 2,19 5,49	n.d. n.d. 0,32 0,81
Ibe *et al.* 1991 [c] Japan	White Red	Max. 9,9 Max. 10,0	Max. 7,8 0,1 – 9,5	Max. 10,4 0,2 – 5,2	0,1 – 10,4 0,7 – 29	Max. 0,4 0,03 – 0,5
Daeschel *et al.* 1996 US-Oregon	Red – Pinot noir Red – C.sauvignon	n.d. – 23,98 0,16 – 2,85	n.d. – 8,31 n.d. – 0,93	n.d. – 0,89 n.d. – 0,14	2,43 – 203,1 4,54 – 19,81	n.d. – 2,1 0,06 – 1,5
Souza *et al.* 2005 Brazil	Red – C.sauvignon Red – Cab. Franc Red - Merlot	0,23 – 1,73 n.d. – 1,37 0,67 – 1,67	0,4 – 1,07 0,3 – 0,83 0,33 – 0,5	0,2 - 1,37 0,17 - 0,37 0,2 – 1,13	1,27 – 4,33 0,77 – 1,43 0,97 – 1,10	
Massera *et al.* 2009 Argentina	Red - Malvec	<0,05 - 1,97			5,31 – 9,25	
De Scenzo, 2009.US-California	Global figures on 284 wines (red + white+ rosé)	<1 – 72	<1 – 20		<1 – 296	<1 – 4

n.d : non determined

Some considerations regarding this compilation of data (Table 2 and 3):

- Since 1964, the analytical methods to measure the biogenic amines have certainly evolved a lot therefore the results might not be totally comparable.
- In some studies, the wines were taken from the shops, in other cases after bottling, etc. Therefore they were not measured having the same aging time and again, the results might not be comparable.

- In some studies, the maximum and minimum amounts of BA in wines are not available, the results are expressed in average and the range of occurrence of BA in wines is not given.
- When maximum and minimum values are presented, the frequency of occurrence is not presented, which makes the impact of the value in the studied wine samples population difficult to measure.

Regarding the biogenic amines in wines from non-European countries (Table 2), globally the values are similar to those presented by EFSA (Table 1), exceptions are the maximum levels for putrescine (203 and 298 mg/L) and maximum level for histamine (72 mg/L) in Californian wines. As for wines from European countries, the biogenic amines values are also close to those in Table 1, with exception of results recently published about wines produced in Czech Republic (Bunka *et al.,* 2012) where extremely high levels of BA were found in white wines: histamine (78 mg/L), tyramine (410 mg/L) and putrescine (400 mg/L).

Surveys about occurrence of biogenic amines in wines do not consider the market volumes associated to the analyzed samples. Therefore, it is not possible to establish the importance of the impact on public health of the extreme samples. To establish this, data on market share, production volumes and consumption need to be considered. For example, an extreme value of histamine, represented by one sample, can actually represent 0,1% of wine sold or 30%, nobody knows. This dimension would certainly totally change the perception of the public health problem associated to biogenic amines in wines.

A.3 Regulatory restrictions for biogenic amines in wines

Although currently legal limits are not established for any biogenic amines in wines, some countries have established recommended limits of histamine in wines; these are much lower than for other food categories because of the suspected synergic effect with ethanol.

Switzerland fixed for a decade a limit of 10 mg/L of histamine for the importation of wine (OSEC, 2002) until the recent publication of Ordinance on Foreign substances in Foodstuffs (OSEC, 2012) where this requirement for the importation of wine to the Swiss territory was eliminated.

Table 3. Biogenic amines content in wines from European countries.
(*updated from Vidal and Bover, 2001).*

Reference and origin of samples	Type of Wine	Histamine	Tyramine	Phenyl ethylamine	Putrescine	Cadaverine
Horwitz et al. 1964[a] Europe	Chianti		25,4			
	Jerez		3,6			
	Oporto		n.d.			
	Riesling		0,6			
	Sauterne		0,4			
Zappavigna and Cerutti 1973[a] Italy	Chianti	n.d. – 3	n.d. – 3			
	Prossecco	1 – 2	n.d. – 1			
	Barbera	n.d. – 3	n.d. – 4			
	Sparkling	n.d. – 1	n.d. – 1			
	Others	n.d. – 3	n.d. – 2			
Lafon-Lafourcade, 1975 [a] France	White	0,1 – 6,3				
	Red	n.d. – 21				
Tejedor and Marine (1979) [a] Spain	White	0,6 – 5				
	Red	2,8 – 8,8				
Vidal-Carou, 1983[a] Spain	White	0,5 – 2,2				
	Rosé	0,4 – 2,7				
	Red	4,0 – 10,1				
Zee et al. 1983 Europe	White – FR	4,35	6,54		2,26	1,43
	White - GR	3,71	5,87		1,29	1,30
	White– Italy	0,87	1,35		1	0,12
	White -PR	1,12	4,43		2,38	1,45
	White – SP	5,77	1,26		1,87	n.d.
	Red – FR	8,14	6,54		7,63	0,99
	Red – Italy	4,07	4,05		3,10	0,49
	Red – PR	1,24	n.d.		0,91	n.d.
	Red – SP	5,77	3,51		4,79	1,09
Lehtonen, 1986[b] Spain	Red	0,8		0,8	13,0	0,2
Mayer and Pause, 1987 [c] Switzerland	White	1,5	8,6	1,7	11,1	0,1
	Red	2,0	4,8	1,7	21,1	0,3
Vidal-Carou et al. 1989 Spain, Catalonia	White sparkling	Traces– 3,31	0,36– 2,4			
Vidal-Carou et al. 1990 Spain, Catalonia	White	0,25 – 0,4	0,3– 1,05			
	Rosé	0,2 – 0,3	0,3 – 0,7			
	Red	0,25 – 8,5	n.d. – 8,3			
Maxa et al. 1992 [c] Germany	White	0,2 – 4,2	0,9 – 8,8	0,2 – 14,7	0,6 – 4,7	<0,01
	Red	0,2 – 6,3	1,2– 11,8	0,4 – 11,7	1,0 – 24,0	<0,01
Bauza et al. 1995 France, Rhône	Red	0,4 – 7	0,2 – 7,4	0,5 – 4	2,6 – 57,7	
Busto-Busto 1996 [b] Spain, Catalonia	White	n.d. – 3,46	n.d.– ,33		1,93 – 3,88	n.d. – 1,43
	Rosé	0,46 – 5,18	n.d.		2,64 – 4,01	n.d. – 0,34
	Red	0,66 – 13,50	n.d.		n.d. – 5,04	n.d. – 0,71
Vazquez-Lasa et al. 1998 [d] Spain	White	0,84	0,89		3,01	0,28
	Rosé	1,21	0,95		3,84	0,40
	Red - J	8,72	4,98		32,97	0,61
	Red - C	6,67	5,78		31,35	1,74
	Red - R	6,92	4,0		33,79	1,25
	Red - G-R	5,12	5,98		36,10	1,32
Gerbaux and Monamy 2000, FR- Burgundy	White-Ch	n.d. – 5	n.d. – 7		n.d. – 15	
	Red –PN	4 – 15	1 – 7		10 – 45	
Kalkan Yildirim et al. 2007, Turkey	Red -Org	0,628			5,5	
	Red–NO	1,14			3,68	
Kovacevic Ganic et al.2009,Croatia	Red	0,07 – 1,65	n.d.– ,52		0,48 – 3,1	0,08 – 0,87
Konakovsky et al. 2011 Austria	Red "high quality"	0,5 – 26,9	1,1– 10,7	0,16	2,9 - 122	0,58
Patrignani et al. 2012 Italy, Apulia	Red "primitivo"	1,49 – 16,34		Max. 2,12	5,41 – 9,51	
Bunka et al. 2012 Czech Republic	White	1 - 78	1- 410		15 - 400	
	Red	n.d. – 23	n.d.- 81		2 - 27	

FR: France; GR: Germany; PR: Portugal; J: "joven"; C: "crianza"; R: "reserva"; G-R: "gran reserva"; n.d: non detected

Austria rejects wines which contain more than 10 mg/L of histamine and lower top limits have been recommended in other European countries (Vidal and Bover, 2001) as Germany (2 mg/L), Belgium (6 mg/L), France (8 mg/L), Finland (5 mg/L) and Holland (3,5 mg/L) (Lethonen, 1996). Although until now these limits are only recommendations, the sector anticipates the possibility that these values become legal maximum limits that block the commercialization of wines in these countries.

Regarding the biogenic amines content in wines (Tables 1, 2 and 3), it is frequent to find wines exceeding these recommended limits. Therefore, producers attempt to control the presence of BAs and take actions to minimize the risks for their products during the wine-making process and in this way guarantee the wine is safe for consumption and does not present problems for its commercialization.

A.4 Awareness of Wine sector in biogenic amines issue

Through the OIV, the wine business has been implicated in the issue of contaminants in wines. Resolution OENO 4/97 asked for the study of the means required reducing the biogenic amine content in wines; also this was included in the strategic plan of the OIV (2009-2012), which intends in particular to propose the means to detect and limit the presence of contaminants in wine-based products. Based on these efforts, the « OIV Code of good vitivinicultural practices in order to minimize the presence of biogenic amines in wine-based products » was published in 2011. This guideline consists in practical actions that winemakers can apply at vineyard, grape harvest, and production levels to reduce biogenic amines in wines.

BIOGENIC AMINES
SCIENCE AND TECHNOLOGY

B.1 Biogenic amines: definition and role in animal cells

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (Maijala. *et al*., 1993; Silla Santos, 1996, Landete *et al.*, 2005; Suárez Lepe and Íñigo, 2003).

They are organic bases with low molecular weight and are synthesized by microbial, vegetable and animal metabolisms (ten Brink *et al.,* 1990). Biogenic amines in food and beverages are formed by enzymes of raw material or are generated by microbial decarboxylation of amino acids (ten Brink *et al.,* 1990; Halasz *et al*., 1994) but it has been found that some of the aliphatic amines can be formed "in vivo" by amination from corresponding aldehydes (Maijala *et al*., 1993).

The chemical structure of biogenic amines can either be (Silla Santos, 1996):

- aliphatic (putrescine, cadaverine, spermine, spermidine);
- aromatic (tyramine, phenyl ethylamine);
- Heterocyclic (histamine, tryptamine).

In humans, histamine and tyramine are involved in functions of nervous system and blood pressure (Lonvaud-Funel, 2001). Amines such as polyamines, putrescine, spermidine, spermine and also cadaverine are indispensable components of living cells and they are important in the regulation of nucleic acid fraction and protein synthesis and probably also in the stabilization of membranes (Bardocz *et al.*, 1993; Maijala *et al*., 1993; Halasz *et al*., 1994; Silla Santos, 1996, Lonvaud-Funel, 2001).

B.2 Toxicology of biogenic amines

Biogenic amines are undesirable compounds in food and beverages as its consumption at high concentrations generate negative effect on human health. Sneezing, flushing, headache, asthma attacks, nausea, hypo or hypertension and different anaphylactic reactions are frequently the symptoms associated to intoxication with food rich in biogenic amines (Vidal-Carou, 2007).

Overall, healthy individuals can detoxify dietary biogenic amines by acetylation and oxidation reactions mediated by the enzymes, monoamine oxydase, diamine oxydase and N-methyltransferase.

However, very high amounts of biogenic amines in food may present a risk to all individuals (Taylor, 1986; Bardocz, 1995). Toxic effects of biogenic amines can develop through both increased availability (due to high amounts ingested with food) and/or impaired biogenic amine degradation (due to increased sensitivity of individuals).

Basically, the adverse effects of histamine are resulting from the unbalance between the histamine accumulation in blood and the capability of the individual to metabolize the histamine. The accumulation of histamine can be a consequence of (Vidal-Carou, 2007):

- Allergic reaction : IgE mediated
- Pseudo allergic reaction : no IgE mediated
- Intoxication : ingestion of high amounts of histamine
- Intolerance: reduced capability of the individual to metabolize the histamine.

For patients with histamine intolerance and chronic headache, a histamine-free diet is the treatment of choice, because even small amounts of histamine may cause them adverse health effects (Jarisch, 2004).

A review regarding health effects of tyramine in food was carried out by McCabe-Sellers *et al.* (2006) which concluded that the presence of 6 mg in one or two usual servings is thought to be sufficient to cause a mild adverse affect while 10–25 mg will produce severe adverse effects in patients treated with classical MAOI (monoamine oxidase inhibitors) treatment (McCabe, 1986). Other reports conclude that 50 to 150 mg of tyramine would be well tolerated by patients under new generation MAOI treatment, so called RIMA (reversible inhibitors of MAO-A) (Korn *et al.,* 1988; Dingemanse *et al.,*1998; Patat *et al.,* 1995).

In summary, the sensibility to biogenic amines varies from one individual to the other depending on the detoxifying capacity of amino oxidase enzymes of the body. If these enzymes are inactivated by drugs or alcohol, their detoxifying capability decrease (Landete *et al.*, 2003; Lonvaud-Funel, 2001).

B.3 Wine intolerance

The relation between the ingestion of wine, an increase in plasma histamine, and the occurrence of sneezing, flushing, headache, asthma attacks, and other anaphylactic reactions and a reduction of symptoms by antihistamines has been shown in various studies (Wantke *et al.,* 1993 and 1994; Jarisch *et al.,* 1992; Jarisch and Wantke,1996; Maintz and Novak, 2007).

However, among the multitude of substances contained in wine, some biogenic amines such as tyramine (Littlewood *et al.,* 1988) and sulfites (Dahl *et al.,* 1986) have been supposed to contribute to symptoms summarized as "wine intolerance" or "red wine asthma" (Jansen *et al.,* 2003 ; Dahl *et al*., 1986, Gershwin *et al*., 1985).

A recent study shows that wine intolerance was found to be more common than expected (7% of the studied population). The interpretation of data suggests intolerance to alcohol, biogenic amines, or other ingredients of wine rather than to an immunologically mediated allergy (Wigand *et al*., 2012).

Alcohol contained in wines, is a potent inhibitor of diamine oxidase (Zimatkin *et al.,* 1999; Izquierdo-Pulido *et al*., 1996) enzyme responsible of histamine degradation in the body. Putrefactive amines (putrescine and cadaverine) which are commonly found in wines, an others as tyramine, tryptamine and phenyl ethylamine, inhibit the metabolism of histamine by competing with it in the gastrointestinal tract, which results in an increased intestinal uptake and urinary excretion of unmetabolized histamine (Maintz and Novak, 2007).

Few clinique research studies have been performed regarding wine intolerance and the role of biogenic amines in wines is currently controversial:

- No evidence of the relationship between histamine content in wine and wine intolerance was found by Luthy and Schatter (1983).
- In contrary, Wantke et al. (1994) observed that symptomatic individuals had higher histamine content in blood than no-symptomatic individuals after ingestion of wine containing 0,05 mg histamine.

- No relationship was found between histamine or other biogenic amines content and wine intolerance in healthy or sensible individuals by Kanny and Gerbaux (2000) and Kanny *et al.* (2001).In these studies, it was postulated that the acetaldehyde (ethanal) which is an ethanol metabolite in the body might acts as histamine-releasing substance. The involvement of the histamine-releasing acetaldehyde in wine intolerance has previously been suggested (Lowenberg *et al.,* 1981; Shimoda *et al.,* 1996; Zimatkin and Anichtchik, 1999), in particular for consumers who have significant reduced acetaldehyde dehydrogenase activity such as the Japanese population (Harada and Agarwal, 1981).

Given the fact that the mechanism involved in wine intolerance and the role that biogenic amines and wine components play in this syndrome are currently controversial, the governments have determined recommended maximum histamine levels in wines as precautionary measure as previously exposed (*section A.3*).

As appointed by Bodmer *et al*. (2000), it is the population intolerant to the wine that justifies the efforts from scientists, and especially wine industry to minimize as much as possible the biogenic amines levels in wine. It is worth reminding that in the current knowledge on the topic, the biogenic amines do not have a positive effect in the wine and all indicate that their presence might have potentially negative repercussions for some individuals (Vidal and Bover, 2001).

B.4 Factors influencing variability of biogenic amines content in wines

The variability in biogenic amines content in wines has been studied all along the wine-making process and several factors were identified as affecting the BA content in the final product. Some of these factors increase the concentration of the amino acids in the medium, while other favor the development of microorganisms with the ability to produce amines (Torrea Goñi and Ancín Azpilicueta, 2001).

Regarding the factors that influence the amino acid content (Table 4), some of them are hard to manage as type of soil, *Vitis vinifera* variety or degree of maturation as they are

intrinsic to the zone of production. Other factors are more controllable such as oenological practices which help to control the amino acids content in the wines.

The activity of microorganisms during the fermentative process has high impact on the content of biogenic amines in wines (Table 5).

Table 4. Factors affecting biogenic amines content in wines by increasing amino acids concentration during wine-making process.

Factors affecting biogenic amines content in wines	References
Type of soil	Baucom *et al.,* 1986; Marques *et al.,* 2008
Vitis vinifera variety	Zee *et al*., 1983; Beatriz *et al*., 1998; Nicolini *et al.,* 2003; Bertoldi *et al*., 2004; Landete *et al.,* 2005a; Marques *et al.,* 2008; Hernandez-Orte *et al*., 2008; Del Prete *et al*., 2009
Degree of maturation of the grape	Ough, 1971; Herbert *et al*., 2005; Del Prete *et al*., 2009
Natural Amino acids content of grapes	Soufleros *et al*., 1998; Marques *et al.,* 2008
Contact of must and grape skin	Ough, 1971; Íñigo and Bravo 1980; Guitart *et al*. 1997; Martin-Alvarez *et al*. 2006; Garcia-Marino *et al*. 2010
Addition of nutrients	Gloria *et al*., 1998; Gonzalez Marco *et al.,* 2005; Corzani, 2008; Garcia-Marino *et al*., 2010, Smit *et al*., 2012; Batch *et al.,* 2010
Duration of wine contact with yeast lees and marcs	Bauza *et al*., 1995a; Coton *et al*., 1999; Lonvaud-Funel, 2001; Marques *et al.,* 2008
Strain of *S. cerevisiae* used	Rosi *et al,.* 2009; Torrea and Ancín, 2001

During fermentation, besides the contribution of contaminating bacteria, the microbiota responsible for fermentation can also show aminogenic activity. Moreover, the proteolysis, yeast lysis and acidification usually accompanying fermentation processes increase the availability of precursor free amino acids and favors decarboxylation reactions (ten Brink *et al*., 1990)

Table 5. Factors affecting biogenic amines content in wines by influencing the development of microorganisms with the ability to produce biogenic amines.

Factors affecting biogenic amines content in wines	References
Degree of maturation of the grape	Ough 1971; Herbert *et al*., 2005
Metabolism of yeast during alcoholic fermentation	Vidal-Carou *et al.* 1990b; Torrea Goni and Ancin Azpilicueta 2001 and 2002; Caruso et al. 2002; Valero *et al.* 2003; Manfroi *et al.* 2009
Alcohol content	Landete *et al*. 2004; Vidal-Carou *et al*. 1990b
Sulfur dioxide concentration	Rivas-Gonzalo *et al.* 1983; Vidal-Carou *et al.* 1990b; Garcia-Marino *et al*. 2010
pH	Aerny, 1990; Vidal-Carou *et al.* 1990b; Lonvaud-Funel 2001; Landete *et al.* 2004 and 2005; Corzani 2008; López *et al.* 2012
Metabolism of LAB during MLF	Vidal-Carou *et al.* 1990b; Lonvaud-Funel and Joyeux 1994; Coton *et al.,*1998; Lonvaud-Funel 2001; Guerrini *et al.,* 2002; Landete *et al.,* 2005a; Palacios *et al.,* 2005 ; López *et al.* 2012; Izquierdo-Cañas *et al*. 2008; Garcia-Marino *et al*. 2010
Use (or not) of LAB starters	Gindreau *et al*. 1997 and 2003 ; Davis *et al*. 1985 ; Gerbaux and Monamy 2000; Lonvaud-Funel 2001; Marques *et al.* 2008; Lopez *et al*. 2012; Hernandez-Orte *et al*. 2008; Garcia-Marino *et al*. 2010
Time and storage conditions	Ough *et al.,* 1981; Gonzalez Marco and Ancin Azpilicueta 2006; Landete *et al.,* 2005a; Hernandez-Orte *et al*. 2008
Possible microbial contamination during winemaking	Zee *et al*. 1983; Vidal-Carou *et al.* 1991; Buteau *et al.* 1984; Vidal-Carou *et al.* 1991; Lehtonen 1996; Bravo-Abad 1996; Izquierdo-Cañas *et al*. 2008
Wood aging	Bauza *et al.* 1995a; Bauza *et al*. 1995b; Gerbaux and Monamy 2000

B.5 Synthesis of biogenic amines in wines

Apart of the BA present in the must coming from the grapes, the biogenic amines produced during the winemaking process are the result of the metabolism of yeast (Ough and Daudt, 1981; Torrea and Ancín, 2001 and 2002; Manfroi *et al.,* 2009; Del Prete *et al*., 2009) and the lactic acid bacteria (LAB) via the decarboxylation of amino acids (Landete *et al.,* 2005; Lonvaud-Funel, 2001). The conditions that need to be gathered for synthesis of biogenic amines in food and wines are:

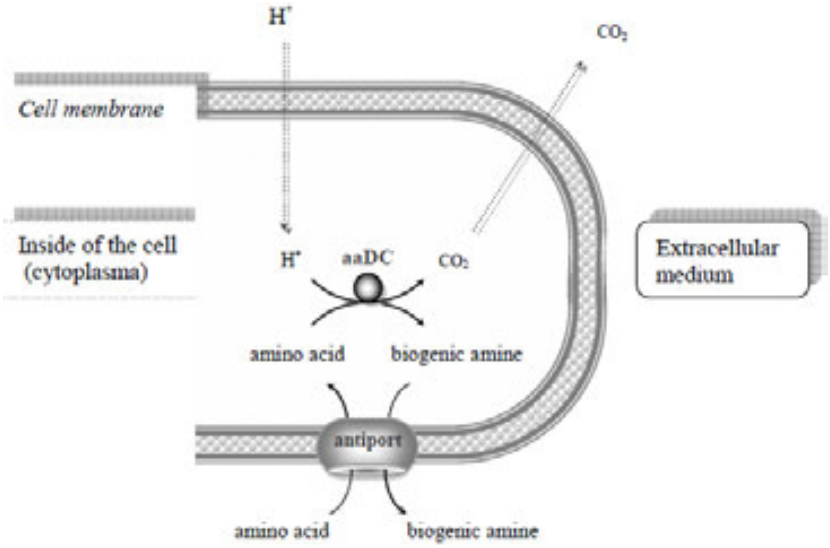
- Availability of free amino acids (Soufleros *et al*., 1998).
- Presence of decarboxylase-positive microorganisms (Landete *et al.*, 2005; ten Brink et al., 1990).
- Conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity as pH, temperature, etc. (ten Brink *et al.*, 1990; Silla Santos, 1996; Coton *et al.,* 1998; Gardini *et al.,* 2005).

- Absence of other fermentable substrates to obtain energy (Lonvaud-Funel, 2001).
- Presence of pyridoxal 5'phosphate, co-enzymatic factor of decarboxylase enzymes (Vidal-Carou and Bover Cid, 2001)

B.6 Amino acids decarboxylation as adaptive response to stress

In prokaryotic cells, the physiological role of BA synthesis by biodegradative decarboxylase mainly appears to be related to defense mechanisms used by bacteria to withstand acidic environments (Rhee *et al.,* 2002). The low pH favors the decarboxylase activity (Landete *et al.*, 2005; Lonvaud-Funel, 200) supporting the amines generation.

Figure 2. Biogenic amines biosynthesis pathway in bacteria. Amino acid decarboxylase (aaDC). ***(source EFSA 2011, adapted from Bover-Cid, 2000)***



Decarboxylation increases survival under acidic stress conditions via the consumption of protons and the excretion of amines and CO_2, helping to restore the internal pH (Figure 2).

Biogenic amines production may also offer a way of obtaining energy, since the electrogenic amino acid/amine antiport can lead to generation of proton motive force (Molenaar *et al*., 1993). This function is particularly important to microorganisms lacking a respiratory chain for generating high yields of ATP (Vido *et al.,* 2004).

The LAB strains with amines decarboxylase ability can survive longer in the wine as this property allows obtaining additional energy when all the fermentable substrates were consumed, as it is the case of bottled wine (Lonvaud-Funel, 2001). The decarboxylase reaction, which induces an increase of pH, can stimulate bacterial survival in difficult conditions.

B.7 Impact of biogenic amines on sensory properties of wines

Despite of the negative effect that biogenic amines might have on human health, some amines are also significant to wines in terms of flavour (González-Marco and Ancín-Azpilicueta, 2006). In general, a weakening of the flavour impression is attributed to amines, whereby an unpleasant bitter aftertaste has been described in wines with high amine levels (Manfroi *et al.,* 2009). Furthermore, putrescine and cadaverine can negatively affect the sensory quality of wines for some authors (García-Villar *et al.,* 2007). On the other hand, Gerbaux and Monamy (2000) studied the sensorial perception of high levels of biogenic amines in hydroalcoholic solutions and Chardonnay and Pinot Noir wines. They concluded that histamine, tyramine and putrescine at the maximum concentrations detected in those wines (20mg/L, 16 mg/L and 60 mg/L respectively) do not have negative impact on flavor of the wine as they were not detected by the panelists. The putrescine at 60 mg/L in hydro-alcoholic solutions (not in wine) was described by tasters as chlorine, rotten, and mousey in this study. In other research, Palacios *et al.* (2005b) studied defective molecules resulting from uncontrolled malolactic fermentations, including the putrescine and cadaverine, in red wines at different concentrations. Globally, the putrescine at 1 and 10 ppm in wines, was perceived as dirty, rotten, fish, burnt and the cadaverine, at the same concentrations, was described as human perspiration, humidity, rotten, chemical among others descriptors. Moving to 50 and 100 ppm, the descriptors were even more unpleasant.

B.8 Control methods: curatives treatments in wine

At the end of the vinification process and before bottling, curative methods as clarification can be applied to decrease the biogenic amines content but a modification on sensory quality of the wine can be expected. Clarification can be carried out by physical methods (sedimentation, floatation, centrifugation and filtration) or by fining

agents addition (gelatin, albumin, casein, bentonite) or by pectolytic enzymes addition (Ribéreau-Gayon *et al*., 2006).

The bentonite induces a precipitation of proteins and amino acids, even of biogenic amines in wines if they are present at the moment of the application of bentonite (Schneyder, 1973).

- Kally and Body-Szalkai (1996) observed that in red wines, 80 g/HL of bentonite reduced histamine content by 60 % and more with higher amount of bentonite. The wine color must be considered, because bentonite reduces it.
- Mannino *et al*. (2006) carried out trials on different fining agents (bentonite, tannin and gelatin) to verify the possibility to reduce the dosage of bentonite necessary to remove amines. They found that the addition of tannin before bentonite addition is useful to reduce amines content 2-6 times. Therefore, it is possible to decrease the amount of bentonite added to wine and to preserve wine sensory characteristics.

On the other hand, the use of silica induces a good decrease of nitrogen compounds, while pectolytic enzymes increase the amino acids content in relation to their activity on proteins and peptides (Guitart *et al.,* 1998).
The clarification carried out with physical treatments not always induces a decrease in biogenic amines content (Corzani, 2008).

Apart from clarification, other methodologies have been postulated as the use of amine-oxidase positive bacteria (Leuschner *et al.,* 1998) or fungi (Cueva-Sanchez *et al.*, 2012) or treatment with gamma-irradiation in model-system (Kim *et al.*, 2004). Despite of their effectiveness, their use may be controversial because elimination of biogenic amines could mask improper hygienic and manufacturing practices (Vidal-Carou *et al*., 2007).

According to the EFSA (2011), methods that attempt to destroy biogenic amines are not according to general principles of food hygiene which rely on prevention rather than eliminating problems after they appear. The preventive methods to minimize biogenic amine occurrence in food should be mainly focused on the food processing level, including raw materials handling and the fermentation process, as they constitute the most important factors for the biogenic amine accumulation in fermented products (OIV, 2011).

B.9 Control methods: preventive strategies to manage biogenic amines during wine-making process

As the curative methods might imply the decrease of the sensory attributes of wines, compromising its final quality, the preventive methods to reduce the risk of biogenic amines generation during the process are preferred. The main aspects to consider are hygiene during all the process, microbial activity during MLF and stabilization of wine after MLF:

- Ensuring hygiene all along the wine-making process by the application of GMP (good manufacturing practices) and HACCP (hazard analysis of critical control points) methodologies might help to manage biogenic amines production by contaminants microorganisms (Cerutti and Remondi, 1972; Vidal and Bover, 2001; Bodmer *et al*., 2000; Domingo *et al.,* 2012).
- The microbiological stabilization of wines after the MLF is also important to avoid the production of BA during aging or storage of bottled wine. Some works pointed-out the use of SO_2 to be key for the management of BA in this period of wine-making process (Vidal-Carou *et al.,* 1990; Gerbaux *et al*., 1997). Other authors have found the SO_2 to be less effective due to the high pH of wine at this stage (Bauza *et al.* 1985; Vidal and Bover, 2001). SO_2 also was found not to be efficient enough to stop decarboxylating enzymes in wines (Lonvaud-Funel, 2001). Moreover, the use of SO_2, which is generalized in oenology, cannot be used in excess due to its negative impact on quality of wines (Gerbaux and Monamy, 2000). In the contrary, the lysozyme, an enzyme extracted from hen egg white, is efficient in controlling biogenic amines generation when it is used at 125 to 250 mg/L after the MLF (Gerbaux *et al*., 1997; Gerbaux and Monamy, 2000). The lysozyme activity is not reduced at high pH but it does not have the antioxidant properties that SO_2 has, therefore, the use of both simultaneously was recommended (Gerbaux *et al.,* 1997).
- As the lactic acid bacteria during the malolactic fermentation play a fundamental role on the biogenic amines content of wines, the control of the MLF is the option that the oenologist has to reduce these metabolites. Controlled MLF is understood as the technique to inoculate the wine using preparations made of selected *O. oeni* strains with the property to be poor biogenic amines producers, among of other important properties (Krieger, 2005; Vidal and Bover, 2001; Lonvaud-Funel, 2001). Several works pointed-out a reduction of biogenic amines generation during the MLF when

LAB starters were used (Gerbaux and Monamy, 2000; Gindreau *et al*., 2003; Izquierdo-Pulido *et al*., 1999; Vidal and Bover, 2001; Landete *et al*., 2005). The importance of MLF and LAB starters will be further developed in Chapter 1.

It is generally accepted today that the reduction of growth of strong amine-producing bacteria through the optimization of handling, processing and storage conditions are the most effective methods to manage biogenic amines production in food (Vidal-Carou *et al*., 2007; EFSA, 2011).

GENERAL INTRODUCTION

Ochratoxin A in wines

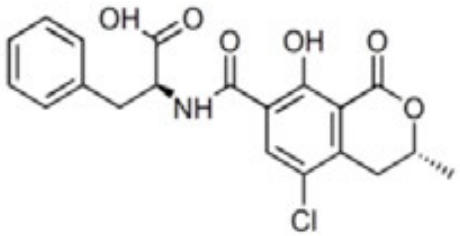
OCHRATOXIN A IN WINE

C.1 Ochratoxin A: Definition

The ochratoxins have been the first group of characterized mycotoxins after the aphatoxins and they currently draw special attention and interest of the scientific community and public health organisms because of their nephrotoxic, carcinogenic, teratogenic, immunotoxic and possibly neurotoxic and genotoxic properties. After consumption of contaminated food, its presence can be detected in blood, whey and tissues of patients (EFSA, 2006).

The chemical structure of ochratoxin A (OTA) consist in a chlorine-containing dihydroisocumarin linked through the 7-carbonyl group to 1-β-phenylalanine

Figure 3. Molecular structure of Ochratoxin A



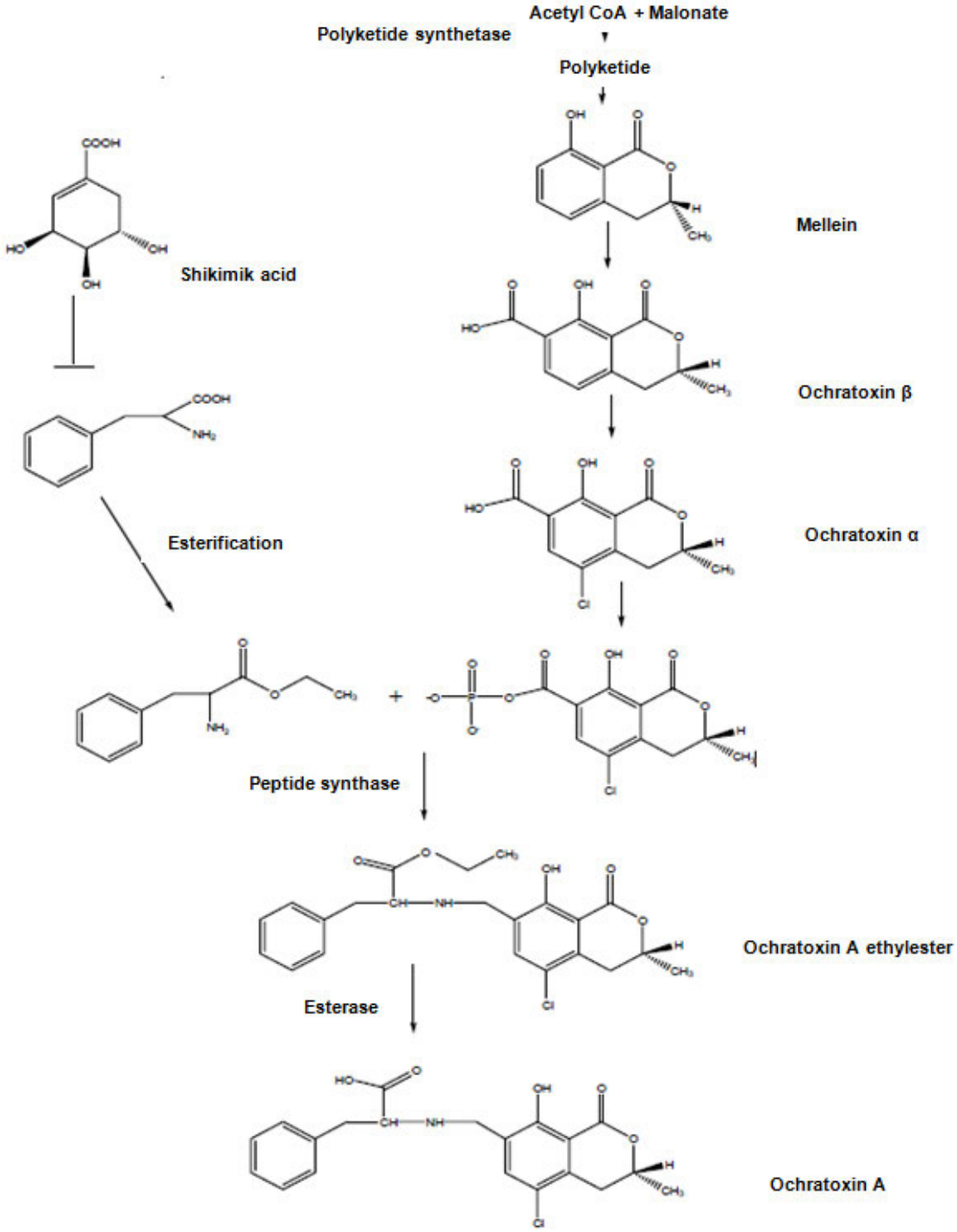
The particularity of OTA is its high stability. It has been shown that it possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule (Khoury and Atoui, 2010).

C.2 Biosynthesis of Ochratoxin A

Although much information exists concerning the various toxigenic properties of OTA, unlike other important mycotoxins, not very much is known about the OTA biosynthetic pathway in any fungal species (Khoury and Atoui, 2010). It is widely believed that the isocoumarin group is a pentaketide formed by acetate and malonate via a polyketide synthesis pathway (Niessen *et al,.* 2005). Thus, a polyketide synthase (PKS), which is considered as the key enzyme, is involved in the OTA biosynthesis in a similar way than other polyketide mycotoxins such as fumonisins and aflatoxins (Khoury and Atoui, 2010).

Huff and Hamilton (1979) proposed a biosynthetic pathway based on a mechanistical model according to the structure of OTA (Figure 4).

Figure 4. Schematic representation of the hypothetical OTA biosynthetic pathway as proposed by Huff and Hamilton, 1979 ***(source Khoury and Atoui, 2010).***



C.3 Toxicology of OTA

Due to the nephrotoxic, carcinogenic, teratogenic, immunotoxic and possibly neurotoxic and genotoxic properties of OTA, it is receiving increasing attention worldwide because of the hazard it poses to human and animal health. In humans, OTA has been determined to be the causal agent of Balkanic Endemic Nephropathy, a chronic nephropathy described in several rural regions of Bulgaria, Romania, Serbia, Croatia and Bosnia and associated with an increased incidence of tumors of the upper urinary tract (EFSA, 2006).

In 1993, the International Agency for Research on Cancer (IARC) classified OTA into group 2B (IARC, 1993). This category includes molecules with limited proves of carcinogenicity in humans and animals for experimentation.

C.4 OTA in food

OTA has been widely detected in food of vegetal origin mainly in the following food categories, Table 6.

Table 6. Occurrence of OTA in food

Food category	References
Cereals (barley, wheat, maize, oat, etc.) and their derivate products	Speijers and Van Egmond, 1993; Trucksess *et al*., 1999,
Green coffee	Trucksess *et al*., 1999
Spices	Hubner *et al*., 1998
Coffee drink	Bucheli *et al*., 1998; Burdaspal and Legarda, 1998
Beer	Jorgensen, 1998
Grapes juices and wines	Zimmerli and Dick, 1996; Ospital, 1998; Visconti *et al*., 1999; Markaki *et al*., 2001; Pietri *et al*., 2001; Belli *et al*., 2002 and 2004; Romero *et al.,* 2007

Regarding the occurrence of OTA levels in food, the EFSA compiled the information coming from difference sources, the average OTA values per food category are shown in Table 7. The average values from both sources for wines are from 0,32 to 0,36 µg/L of OTA; maximum values and its frequency are not indicated.

Table 7. Average OTA contamination levels of relevant food category and number of samples included calculating the average *(source EFSA, 2006)*

	JECFA (FAO/WHO, 2001)		SCOOP (EC, 2002)	
	n	mean (µg/kg)	n	mean (µg/kg)
Cereals and products	1538	0.20 (a)	5180	0.29
Beer	975	0.02	496	0.03
Wine	1828	0.32	1470	0.36
Grape juice	87	0.39	146	0.55
Cocoa	171	0.55	547	0.24
Pork (edible offal)	3603	0.17	1860	0.20
Roasted coffee (b)	2085	0.62	1184	0.72
Instant coffee (c)	767	0.76	-	-

a) average contamination level of 1.02 µg/kg was reported by JECFA for raw cereals ;
b) ground coffee expressed as dry matter ; c) expressed as dry matter

C.5 OTA in diet and intake

In order to determine the daily intake of OTA of European consumers, EFSA (2006) evaluated the food consumption habits based on three country-specific scenarios, considering alternatively cereals and wine (as main contributors in Italy), wine and fruit juice (the main contributors in France) and cereals and fruit juice (the main contributors in Sweden). These scenarios are presented in Table 8.

These data indicate that the dietary exposure for high consumers ranges from 6 to 8 ng/kg b.w. (body weight) per day corresponding to about 40 to 60 ng/kg b.w. per week (EFSA, 2006).

Regarding the different scenarios, cereals, wine and fruit juice (including grape juice) appear to be the main contributors of OTA in the European diet.

Table 8. Model diets for high consumers based on three scenarios assuming that the total dietary exposure is represented by the sum of the exposure at the 97.5th percentile associated with the two food categories that had been identified as main contributors to exposure and the average exposure from other food categories *(source EFSA, 2006)*

	EFSA Code (a)	Number of samples	Concentration (b)	Scenario 1 France	Scenario 2 Italy	Scenario 3 Sweden
			OTA µg/kg	OTA ng/kg b.w. per day		
Cereals and cereal products	1	5180	0.29	1.05	2.33	2.75
Sugar and confectionery (c)	2	547	0.24	0.15	0.08	0.11
Hot beverages (c, d)	8	1184	0.72 (a)	0.18	0.08	0.39
Beer	9A	496	0.03	0.01	0.02	0.07
Wine	9B	1470	0.36	3.92	3.18	0.23
Edible offal	10A	1860	0.20	0.01	0.01	0.02
Fruit juices (c)	7A	146	0.55	2.88	0.16	4.45
TOTAL				**8.2**	**5.9**	**8.0**

a) EFSA opinion on exposure assessment (EFSA, 2005);
b) Mean concentrations from SCOOP 3.2.7 (EC, 2002);
c) Consumption of fruit juices, hot beverages and confectionery was matched in the applied model calculations with the OTA concentrations in grape juice, coffee and chocolate products. This may lead to an overestimate of the number of consumers per category and to an overestimate of average levels of exposure in the total population. However, it probably does not lead to an overestimate of the rate of exposure of high consumers, since in most cases high consumers of a single food commodity are not high consumers of other food commodities within the same category. For example, the high consumers of grape juice do not consume large quantities of other fruit juices at the same time ;
***d)** Assuming a coefficient of 18 to convert "solid coffee" in liquid coffee*

C.6 OTA in wines

The presence of OTA in wine varies from undetectable levels (with LOD 0,003 µg/L) to levels higher than 10 µg/L. Several studies pointed out that wines produced in the Mediterranean area usually have higher OTA content than the wines produced in septentrionals areas (Majerus and Otteneder, 1996; Ospital, 1998).

Regarding the levels of contamination, it is generally accepted that the level of contamination from low to high levels follows this trend: white wines, rosé and the highest are the red wines, exceptions are the liquor wines which can also have high levels of OTA (Zimmerli and Dick, 1996; Majerus and Otteneder, 1996; Ospital *et al*., 1998; Burdaspal and Legarda, 1999).

C.7 Regulatory frame

In the European Union, maximum permitted levels of OTA in wines and grape must based drinks currently is 2 µg/L (Reglament CE 123/2005).

In order to reduce risks associated with OTA content in wines, preventive and corrective measures were taken into consideration through the application of HACCP (Hazard Analysis Critical Control Point) system in an FP5 EU project WINE-OCHRA RISK (Risk Assessment and Integrated Ochratoxin A) Management in Grapes and Wine. The control plan based on the HACCP approach involves strategies for prevention, control, good manufacturing practices and quality control at all stages of production, from the field to the final consumer (Varga and Kozakiewicz, 2006; OIV, 2005; Mínguez *et al.,* 2006).

C.8 Fungi responsible for contamination in grapes

C.8.1 *Black aspergilli*

Fungi responsible for the presence of OTA in grapes have been identified as belonging to the *black aspergilli*, *Aspergillus* section *Nigri*. These species are considered as opportunistic pathogens of grape and may cause bunch rot (sour rot) or berry rots and raisin mould (Varga and Kozakiewicz, 2006). *Black Aspergilli* usually attack damaged berries and are also responsible for vine canker of grapes. *A. carbonarious, A. niger* aggregate and *A.aculeatus* belong to the *black aspergilli group and* have been found to produce OTA in grapes (Cabañes *et al.,* 2002; Battilani *et al.,* 2003).

C.8.2 Main OTA producers

Aspergillus carbonarius and *A. niger* are the main producers (Battilani *et al*., 2003; Varga and Kozakiewicz, 2006). These species are very invasive because they colonize and penetrate berries, even without skin damage.

C.8.3 Effect of temperature and season

Black aspergilli can produce OTA at a wide range of temperatures, this permits continuous production in the field. This has to be taken into account in commodities such as grapes, raisins and wine where *black aspergilli* can grow. The moulds will develop most rapidly between veraison and maturation. The growth of these moulds is possible at air humidity levels of 70 % to 90 % and temperatures in the range of 12-39°C, optimum 28 °C.

OTA can be found in grapes one month before harvest and OTA contamination in wine was observed to increase with grape maturity (Mínguez, 2003; Rousseau, 2004).

C.8.4 Geographical distribution

In countries with colder temperate climates such as Germany, Northern Hungary, Czech Republic or northern parts of Portugal, France and Italy, *black Aspergilli* have not been isolated from grape berries in spite of the presence of OTA in wine (Abrunhosa *et al.,* 2001).

Battilani *et al.* (2001) identified OTA producing *Penicillium* species from grapes collected in Northern Italy and France suggesting that *Penicillium* species could be responsible for OTA contamination of grapes in these regions (Varga and Kozakiewicz, 2006).

C.9 Factors affecting contamination of grapes with OTA producer fungi

Several factors could influence fungal colonization of grapes. Climatic factor, water activity, temperature, grape varieties, grape bunch shape, susceptibility of vine varieties, aeration level of grape bunch and health status of grapes are the main factors influencing germination, growth and sporulation of these fungi (Table 9).

Table 9. Factors affecting contamination of grapes by OTA producing black *Aspergillus* fungi

Factors affecting fungi contamination	References
Climatic conditions	Rousseau, 2004; Belli *et al.,* 2005
Location of vineyard :	
1-Mediterranean basin : southern regions of France and Italy, Greece and certain regions of Portugal and Spain 2-southern parts of Hungary 3-southern parts of Portugal	1-Rousseau 2004; Belli *et al.,* 2005; Tjamos *et al.,* 2006; Battilani *et al,* 2006a; Bau *et al.,* 2005; Gomez *et al.,* 2006 2-Varga *et al.* 2005 3-Abrunhosa *et al*. 2001
Health of the grapes : rotten or damaged berries were found to contain more OTA than healthy berries	Rousseau, 2004
Grape varieties	Battilani *et al.* 2004
Larvae of grape moth and other insects (*Eudemis, Cochylis sp.*), act as vectors for conidial dispersal of OTA-producing fungi	Rousseau, 2004
Skin thickness : more fragile skin susceptible to be contaminated by moulds	Rousseau, 2004
Use of fungicides	Mínguez *et al*., 2005; Varga and Kozakiewicz ,2006

OTA IN WINES

PREVENTIVE AND CORRECTIVE METHODS TO REDUCE OTA

Preventive methods are the most efficient to reduce risk of occurrence of OTA in wines. Currently, complete OTA removal from foodstuff is not feasible, but many efforts from vineyard to end wine product need to be made to achieve the minimization of OTA in wines (Amézqueta *et al.,* 2009).

Different codes were developed to support wine makers to reduce the risk of OTA in their products: OIV released in 2005 a guideline for the prevention of OTA; this one was adapted in 2006 for the situation of Catalan wines (Mínguez *et al.,* 2006) and in 2007, Codex Alimentarious adopted the "Code of practice for the prevention and reduction of ochratoxin A contamination in wine". The application of those guidelines is recommended in viticulture regions in which the climatic conditions are favorable to the formation of OTA in vine products in order to reduce endemic risk which favors the onset of the most damaging vine diseases. These guidelines focus on implantation of vineyards, prevention at vineyard level, during harvest and during winemaking.

D.1 Cultivation practices

The preventive strategies to minimize OTA in grapes at cultivation level, are first of all, optimizing the vineyard establishment, allowing as much as possible the aeration of the vine and avoiding the huimid areas (adecuated plant disposition, to avoid contact of grapes with the soil, and to promote the uniform ripening of the grapes). The selection of plant material is key as well. Vigorous rootstock and varieties which, which are often characterized by having less compact grape bunches are preferred as they are less prompt to developing moulds (Mínguez *et al.,* 2006; Codex Alimentarious, 2007).

D.2 Maintenance of vine

By controlled growing techniques and pest control, the risk of OTA in grapes can be reduced (Mínguez *et al.* 2006; Codex Alimentarious, 2007):
Growing techniques should reduce excess of vigor as limiting inappropriate nitrogenous fertilizer applications. Transfer of soil particles to the grapes, grapes bunches overcrowding, cracks on the skin should also be avoided as they are sources of mould penetration. Using marc containing toxigenic fungi as a fertilizer in the vineyards is also not recommended.

Good practices for the maintenance of vine can be listed as follows:

- Carry out leaf removal where the risk of sun burn is slow, in order to enable the aeration of clusters.
- Avoid lesions on the berries and skin damage caused by diseases, insects, phytotoxicity and sun burn. Careful vine protection plans in order to control dangerous fungal diseases affecting grape quality (mainly oïdium disease, acidic rot).
- Preventing the attacks of grape berry moths, grape mealybugs and grape leafhoppers, which favor mould development on damaged berries.
- Remove shriveled/desiccated berries.

D.3 Practices at harvest

Only healthy grape harvest can ensure optimal quality and safety of vitivinicultural products (Mínguez *et al.*, 2006; Codex Alimentarious, 2007). Consequently, only a healthy grape harvest guarantees the avoidance of the risk of quality loss and food safety issues for consumers.

In high risk OTA areas, it is not recommended to retard the harvest date.

When grapes are extensively contaminated by mould, the grapes cannot be used for making concentrated musts or wine, they can only be used for distillation.

For moderately contaminated grapes with toxigenic moulds the following actions are recommended if the grapes are to be used in wine production:

- Grapes need to be sorted, in order to discard grapes with black mould or grapes damaged by insects. Mould, or contaminated must be eliminated before or during harvest
- It is important to clean containers after each load, especially in the case of harvests where the containers may have been used to harvest grapes that may be rotten.

D.4 Prevention during wine-making

Under conditions with a risk of OTA contamination, it is recommended to measure the level of OTA in the musts to be used in winemaking (Mínguez *et al.* 2006; Codex Alimentarious, 2007).

D.4.1 Pre-fermentation operations and treatments

- Avoid skin maceration in the case of OTA high-risk harvests or carry out short maceration. In the case of a significant contamination of red grapes, evaluate possibility of carrying out rosé winemaking.
- Adapt pressing rate to the health status of the grape; in case of contamination, carry out small volume, low pressure quick pressings.
- In the case of contaminated grapes, avoid using pectolytic enzymes for racking must or maceration. Quick clarifications with must filtration, centrifugation and floatation are preferable.
- Avoid post-harvest heating treatments and aggressive and prolonged macerations.
- In the case of contamination by OTA, it is preferable to treat the grapes and the musts with the lowest possible and most effective doses of oenological charcoal in order to avoid possible loss of aromatic and polyphenolic compounds.

D.4.2 Fermentation treatments

- Carry out, as far as possible, fermentation and maceration in smooth walled containers to avoid sources of contamination linked to previous fermentations.
- Dry active yeasts or inactive yeasts can help reduce the OTA level.
- For alcoholic or malolactic fermentations, use yeasts or bacteria which have adsorbent properties for OTA; ensure that these characteristics are guaranteed by the supplier. Note that these products only enable a partial reduction of OTA.

D.4.3 Maturing and clarification treatments

- Maturing on lees can help in reducing the OTA level. The impact of this technique on the organoleptic quality of wine must be evaluated.

D.5 Corrective methods: Chemical

Although Rousseau and Blateyron (2002) emphasized that the occurrence of OTA in wine may be decreased by about 80% using appropriate vineyard management, decontamination procedures can be useful to reduce the OTA level in wine when it exceeds the legal limits after having made all the efforts with the application of preventive programs. Table 10 shows the wine fining agents used for clarification of wine that have been evaluated for OTA removal.

Table 10. OTA removal in wines with chemical methods ***(updated from Quintela et al. 2013)***

Wine fining agent	References	OTA removal (%)
Activated carbon	Castellari *et al*. 2001	~25 ~72
	Gambuti *et al.* 2005	~96
	Var *et al.* 2008	87
	Olivares-Marín *et al.* 2009	<5 to 54 with different agents
	Mínguez, 2003	
Bentonite	Castellari *et al.* 2001	8
	Gambuti et al. 2005	~19
	Kurtbay *et al.* 2008	0-44
	Var *et al.* 2008	23
Chitin	Bornet and Teissedre 2008	35-67
	Quintela *et al.* 2012b	15-29
Chitosan	Bornet and Teissedre 2008	24-83
	Kurtbay *et al.* 2008	59-100
	Quintela *et al.* 2012b	3-67
Egg albumin	Castellari *et al*. 2001	~8 ~48
	Quintela *et al.* 2012b	14-16
Gelatin	Castellari *et al*. 2001	~2 ~20
	Quintela *et al.* 2012b	16-39
Oak wood pieces	Savino *et al.* 2007	20-65 (chip) 23-75 (powder)
Potassium caseinate	Castellari *et al.* 2001	~4 ~24
PVPP	Castellari *et al.* 2001	<1-6
	Gambuti et al. 2005	~15
	Quintela *et al.* 2012b	30-40

Pvpp: polyvinylpolypyrrolidone

Activated carbon has been reported to be able to remove large quantities of OTA in wines (Mínguez, 2003), but according to the Council Regulation EC n° 1493/1999, their use is only authorized for treatment of white wines. Moreover, decrease of colored polyphenols and wine odorants was observed in wines treated with activated carbon (Quintela *et al.* 2013).

Bentonite has as well the property to adsorb OTA in wines. However, total polyphenols and total anthocyans are strongly affected (Var *et al.,* 2008) by bentonite.

Some of the fining agents commonly used may cause adverse reactions in sensitive wine consumers. The European Commission Directive (EC, 2007) established that wines treated with coadjuncts derivated from eggs, fish and milk must be indicated in the labeling as they are major allergens (Quintela *et al.,* 2013). This is the case of casein, potassium caseinate and egg albumin.

Non allergic biodegradable polymers such as chitin, chitosan and derivates were introduced in the International Code of Oenological practices in 2009 (OIV, 2009) as fining agents for wines. Their degree of acetylation leads to different physicochemical properties and therefore, different levels of OTA reduction can be obtained. This might explain the variable results obtained by Bornet and Teissedre (2008) and Quintela *et al.* (2012b), Table 10.

Oak wood fragments can also be an interesting technology for OTA reduction, but this treatment is forbidden in some protected wine areas, as for example Rioja Qualified Designation of Origin (DOC Rioja).

In conclusion, the choice of the fining agent for reduction of OTA in wines needs to consider the following aspects:

- Regulatory frame in producing country and in target country for commercialization.
- Regulation standards in protected wine production areas.
- Maximum dosage allowed.
- Impact of treatment on sensory properties, principally polyphenols and aromatic compounds.
- Labeling and the concomitant consumer perception.
- Added cost to the product.

D.6 Corrective methods: Physical methods

There are few studies of physical methods to reduce OTA level in wine. This could be due to the fact that the practices proposed may not be economically feasible for the wine industry (Quintela *et al*., 2013).

Solfrizzo *et al.* (2010) found that OTA can be effectively removed up to 50-65% by repassage of contaminated musts or wines over no or little contaminated pomaces from the same or different grape varieties. The wine quality parameters were only affected when the pomace came from a different variety, thus the effect was related to the intrinsic characteristics of the pomace variety.
Regarding filtration treatments, wine filtration through a 0,45 mm membrane reduced the OTA level about 80%, however it does not show a significant decrease in the toxin through a 10 mm membrane (Gambuti *et al*., 2005).

Finally, thermal treatments do not affect the OTA concentration (Gambuti *et al*., 2005; Rousseau, 2004) as the ochratoxin A is thermostable.

As reviewed in this section, ochratoxin A represents a real hazard to consumers' health which justifies the efforts for the development of technologies minimizing its presence in wines.

The following chapters present the different axes of research pursued in the framework of this PhD thesis.

CHAPTER 1

CHAPTER 1

Malolactic Fermentation in Wines: influence of the level of implantation of lactic acid bacteria starter on the production of biogenic amines

1. 1 INTRODUCTION: MALOLACTIC FERMENTATION

1.1.1 Lactic acid bacteria in wines

Musts and wines are very selective media, which can support growth of only few species of lactic acid bacteria (LAB). Four genus are represented in wines: *Lactobacillus, Pediococcus, Leuconostoc* and *Oenococcus.* During alcoholic fermentation, the LAB population is mainly composed of *Pediococcus, Lactobacillus* and *Oenococcus oeni* in less proportion. The homofermentative lactobacilli, the major type present in grapes, disappear quickly after the start of alcoholic fermentation in favor of *Leuconostoc mesenteroides* which, at the end of the fermentation, is replaced by *O. oeni* (Moreno-Arribas *et al*., 2003).

Among lactic acid bacteria, *O. oeni* is the main species present in wine after the ALF and the best adapted to carry out the malolactic fermentation at the low pH of wine (Wibowo *et al*., 1985).

Table 11. List of the most widespread lactic acid bacteria species in grape must and wine *(adapted from Ribéreau-Gayon et al., 2000)*

Morphology	Metabolism	Species
Lactobacilli	Facultative heterofermenters (Group II)	*Lactobacillus casei* *Lactobacilus plantarum*
	Strict heterofermenters (Group III)	*Lactobacilus brevis* *Lactobacilus hilgardii*
Cocci	Homofermenters	*Pediococcus damnosus* *Prediococcus pentosaceus*
	Heterofermenters	*Oenococcus oeni (Leauconostoc oenos)* *Leuconostoc mesenteroides subsp. mesenteroides*

1.1.2 Malolactic Fermentation

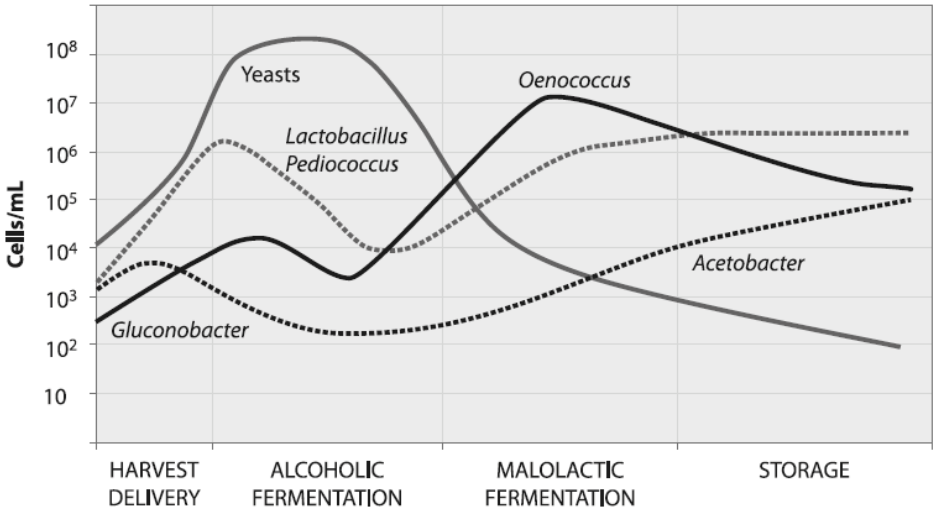
The term malolactic fermentation (MLF) describes the enzymatic conversion of L-malic acid to L-lactic acid and CO_2 by cells of lactic acid bacteria in wines (Lonvaud-Funel, 2001; Costello, 2005, Muñoz and Moreno-Arribas, 2011)

The malolactic fermentation is a facultative step in the wine-making process, which is more often performed in red wines. The oenologist decides whether to stop MLF by application of SO_2 after the alcoholic fermentation (ALF) or the MLF can be encouraged by maintaining conditions favorable to bacterial growth and survival, such as warmer temperatures, minimal or no SO2 addition, and delayed racking.

In the wine-making process, the MLF is the second fermentation of the wine that usually occurs after the ALF when the reducing sugars have been metabolized by the yeast. In terms of processing, MLF is considered finished when the malic acid is completely degraded in the wine. At this stage the oenologists consider that the wine is ready to be moved to clarification and fining steps before the bottling operations.

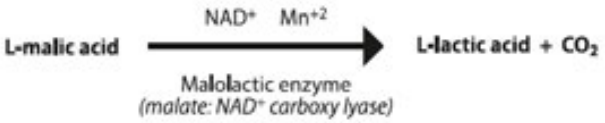
All these changes are accompanied with changes in the micro biota of the wines (Figure 5).

Figure 5. Growth cycle of lactic acid bacteria in wine during winemaking and storage (*source Krieger, 2005*).



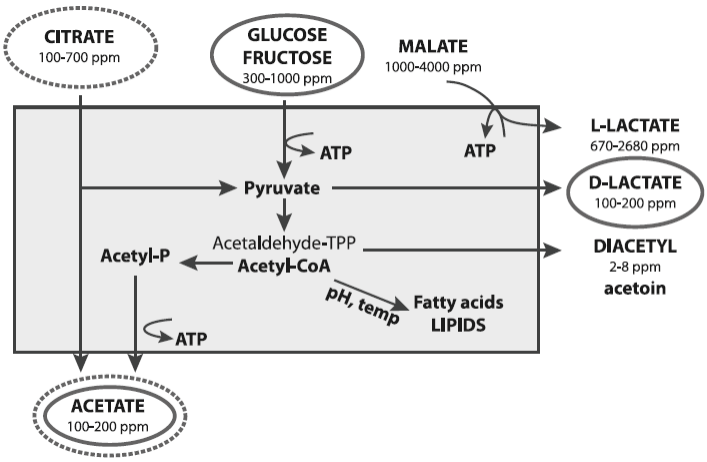
1.1.3 Biochemistry of malolactic fermentation

Kunkee and Morenzoni in 1970 revealed in *Oenococcus oeni* a single enzyme, commonly known as the "malolactic enzyme," that exhibits two separate enzyme activities which act simultaneously on L-malic acid. The predominant "malolactic activity" of the enzyme malate NAD+ carboxyl lyase, catalyzes the direct conversion (decarboxylation) of the dicarboxylic acid L-malic acid to the monocarboxylic acid L-lactic acid, and requires NAD^+ and Mn^{+2} as co-factors (Costello, 2005) :



In addition to the desacidification reaction that characterizes the MLF, it is becoming increasingly recognized that a diverse range of other metabolic activities are associated with the growth and development of LAB in wine, which can have a significant influence on wine quality (Costello, 2005) Fig. 6.

Figure 6. Metabolism of heterofermentative lactic acid bacteria *(source Costello 2005).*



1.1.4 Types of MLF

The MLF can be conducted in different ways (Davis *et al.,* 1985):

- Stimulation of natural flora, called spontaneous MLF.
- Induction of MLF by the inoculation of malolactic starters at the end of ALF or during the ALF.
- Induction of MLF by inoculation of a volume of wines where MLF is undergoing.
- Passage of the wine over supports of immobilized LAB or enzymes.

1.1.5 Impact of MLF in wines

In case MLF is performed in the wine, some global consequences for the quality of the wine can be highlighted:

- Desacidification: specially interesting to moderate the perceived acidity of wines in regions where cold climates deliver grapes naturally rich in organic acids (Krieger, 2005)
- Modification in aroma: LAB are known to produce some flavour-active compounds, including acetaldehyde, acetic acid, diacetyl, acetoin, and 2,3-butanediol. The negative or positive effect on wine of these compounds is dependent on their concentration (Krieger, 2005; Palacios *et al.,* 2005ab; Davis *et al.,* 1985; Lonvaud-Funel, 1999)
- Microbiological stability by reducing fermentable substrates as L-malic acid (Masqué and Bordons, 1996; Davis *et al*., 1985).

1.1.6 LAB producing biogenic amines in wines

After several years of controversies between the scientists and although yeast can contribute to the biogenic amines generation during wine-making, it is currently accepted that an important part of biogenic amines are produced during the MLF and the lactic acid bacterial metabolically active at this stage, are the main responsible for their production.

Several works have been done to determine the LAB species that are involved in the generation of biogenic amines and globally all of them are able to produce at least one type of biogenic amines, Table 12.

As there is no general rule indicating the species that are BA producers or not and this property seems more or less well widespread in the wines LAB species, it was postulated that the capability to produce biogenic amines by LAB is function of genetics and environment:

Different strains of the same species have different capabilities to decarboxylate amino acids. This capability is linked to the presence of the gene responsible for codification of the descarboxylase enzymes, for example the *hdc* gene which is responsible of codification of enzyme histidine decarboxylase (HDC) (Lonvaud-Funel, 2001; Landete *et al.,* 2007).

Table 12. Positive results on ability of lactic acid bacteria isolated from wine to produce biogenic amines.

LAB genus or species	Biogenic amine	References
Pediococcus spp.	Histamine	Delfini, 1989
Pediococcus parvulus	Histamine	Landete *et al.,* 2005; Landete *et al.,* 2007
Oenococcus oeni	Histamine	Lonvaud-Funel and Joyeux, 1994 Le Jeune *et al*. 1995; Coton *et al.* 1998 and 1999; Guerrini *et al*. 2002; Landete et al. 2005; Landete *et al.* 2007; Rosi *et al*. 2009; Coton *et al.* 2010
Lactobacillus brevis, *Lactobacillus hilgardii* *Lc. mesenteroides*	Tyramine	Moreno-Arribas *et al.,* 2000 Moreno-Arribas *et al.,* 2003 Liu, 2002
Lc. mesenteroides *Lactobacillus brevis* *Lactobacillus hilgardii*	Phenyl ethylamine	Moreno-Arribas *et al.,* 2000 Bover-Cid *et al.,* 2001
Oenococcus oeni	Putrescine Cadaverine	Guerrini *et al*., 2002
L*actobacillus buchneri*	Putrescine	Moreno-Arribas *et al*., 2003
Lactobacillus hilgardii	Putrescine	Arena *et al*., 2001 Landete *et al.,* 2007
Oenococcus oeni	Cadaverine	Guerrini *et al*. 2002
Lactobacillus brevis	Tyramine Phenyl ethylamine	Landete *et al.,* 2005 and 2007a-b
Lactobacillus hilgardii	Histamine Tyramine	Landete *et al.,* 2005 and 2007a-b;
Lactobacillus hilgardii	Histamine	Farias *et al*., 1993; Coton *et al.,* 2010
Oenococcus oeni	Putrescine	Coton *et al.,* 1999, Landete *et al.,* 2007
L. hilgardii *L. mali* *Lc. mesenteroides*	Phenyl ethylamine Histamine Histamine	Landete *et al.,* 2007
Oenococcus oeni	Tyramine	Rosi *et al*., 2009
Lactobacillus brevis	Tyramine	Coton *et al.,* 2010

A LAB strain having, for example, the *hdc* gene will not always express it. The expression of the *hdc* gene*,* will depends of the environment conditions as for example availability of other nutrients, as glucose or L-malic acid (Lonvaud-Funel and Joyeux, 1994; Landete *et al.,* 2005, Landete *et al.,* 2008).

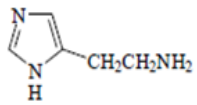
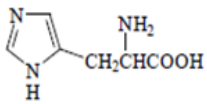
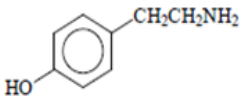
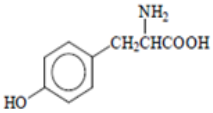
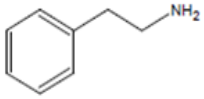
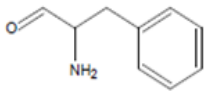
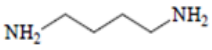
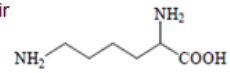
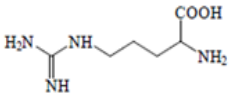
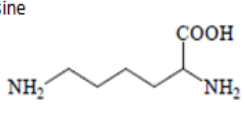
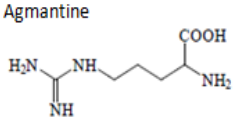
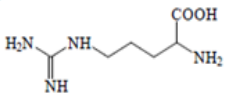
On the other hand, the activity of the decarboxylating enzymes will also be affected by the nutrients and physic-chemical conditions. This was principally studied for HDC and TDC enzymes.

1.1.7 Biogenic amines, precursors and enzymes

Many types of biogenic amines have been detected in both white and red wine: histamine, tyramine, putrescine, cadaverine tryptamine, monomethylamine, 2-phenethylamine and spermidine (Zee *et al.,* 1983; Lehtonen *et al*., 1996; Bauza *et al*., 1995a; Silla Santos, 1996). The first being the most frequently found in wines (Tables 1, 2 and 3, in General Introduction section).

Table 13 shows the molecular structure of the BA most frequently found in wines, together with their amino acid precursor and the enzyme responsible of the reaction.

Table 13. Biogenic amines most frequently found in wines, their precursors and enzymes implicated in their synthesis.

Biogenic Amine	Type of Amine	Precursor	Enzyme
Histamina 	Heterocyclic	Histidine 	Histidine decarboxylase (HDC)
Tyramine 	Aromatic	Tyrosine 	Tyrosine decarboxylase (TDC)
Phenil ethyl amine 	Aromatic	Phenilalanina 	Phenilalanina decarboxylase
Putrescine 	Aliphatic	Ornithir 	Ornithin decarboxylase
		Agmantine 	Agmatine deiminase
Cadaverine 	Aliphatic	Lysine 	Lysine Decarboxylase (LDC)
Agmantine 	Aliphatic	Arginine 	Arginine decarboxylase

1.1.8 Factors affecting HDC and TDC

As mentioned before, generally the factors that affect development of LAB, will affect the biogenic amines content (Table 5). Additionally to the impact that these factors have on bacterial growth, they might also influence directly the enzymes responsible of the decarboxylation of amino acids as observed principally for histidine decarboxylase (HDC) and tyramine decarboxylase (TDC) enzymes:

- Before the isolation and characterization of HDC enzyme done by Coton *et al*. in 1998, a hint about the possible impact of nutrients on HDC activity was observed. The concentration of histidine (as function of the maceration with lees in this study) was correlated with histamine content in the studied wines (Lonvaud-Funel and Joyeux 1994). They also observed that a strain of *Oenococcus oeni* (named *Leuconostoc oenos* 9204) produced more histamine in synthetic media in cultures without malic acid or glucose. To less ethanol, less histamine was produced, and in presence of L-malic and L-lactic acids, the production of histamine by LAB was reduced (Rollan *et al*., 1995). On the contrary, Farias *et al*. (1993) found that HDC activity was stimulated by presence of L-malic and citric acids and inhibition by SO_2 and ethanol at the usual concentrations found in wines.
- When the HDC enzyme was isolated and characterized (Coton *et al*., 1998) it was found that its optimal pH was 4.8 and that its affinity varies according to the pH, the lower HDC activity being at pH 7.6. Histamine, citric acid and lactic acid were found to be a competitive inhibitor of the HDC enzyme. Additionally, it was found that the HDC enzyme was very stable and that its activity remains present in wines for a long time, up to a point where the bacterial population dies and the HDC enzyme is liberated in the wine (Coton *et al*., 1998).

Regarding the TDC enzyme, before to its isolation and characterization, Moreno-Arribas and Lonvaud-Funel (1999) found that tyramine, lactic acid, citric acid, ethanol (12% or more) and glycerol inhibited the production of tyramine by wine LAB. The maximal tyramine production was found at pH 5.0. TDC activity was found to be dependent on the presence of piridoxal 5-phosphate. The TDC enzyme from *Lactobacillus brevis* IOEB 9809 was purified and characterized in 2001 by the same team, and the previous hint in *in-vitro* cells were confirmed (Moreno-Arribas and Lonvaud-Funel, 2001).

1.1.9 Detection of genes for codification of HDS, TDC and ODC enzymes

As the decarboxylating activity of wine LABs is first of all dependent on having the gens that code for the decarboxylase enzymes, it was found very important to detect this property in wine LABs to better understand the BA content in wines. These techniques are also used for the early diagnosis of the risk to produce amines by LAB during the wine-making process. The DNA-DNA hybridation technique directly in colonies was the first technique used (Coton *et al.,* 1999). Then, other PCR-based methodologies were developed based on the design of primers specific for the gene sequences that codify amino acid decarboxylase enzymes:

- Primers for the detection of *hdc* gene which codify the HDC enzyme were developed by Le Jeune *et al.* (1995). Landete *et al.* (2005) improved that methodology. Later on, Constantini *et al.* (2006), De las Rivas *et al.* (2006) and Fernández *et al.* (2006) developed other primers and methodologies. The last one, applied to real time qPCR. Other primers are also available for the detection of the *hdc g*ene in Gram- negative bacteria (Landete *et al*., 2007) but these ones are not relevant for wine LABs.
- Several primers for the detection of gene *tyr*dc which codify the TDC enzyme were developed by Lucas and Lonvaud-Funel (2002). Landete *et al.* (2007b-c) found high correlation between the presence of the *tyr*dc gene and production of tyramine in synthetic media.
- Marcobal *et al.* (2004) and Constantini *et al.* (2006) developed primers for the detection of *odc* gene which codify for the ODC enzyme (ornithin decarboxylase responsible for the production of putrescine).
- It is worth mention that although the primers that amplify a specific fragment of lysine decarboxylase enzyme (responsible of decarboxylation of lysine to convert into cadaverine) are available, they do not concern wine LABs (De las Rivas *et al.,* 2006)
- A multiplex PCR method for the simultaneous detection of the three genes was developed by Marcobal *et al*. (2005). Only one strain of *L. plantarum* and *O. oeni* were identified as putrescine producers.

- Other multiplex method was developed by Coton *et al.* (2010) for the simultaneous detection of the four genes: *hdc*, t*yrdc*, *odc* (ornithine decarboxylase generating putrescine) and *agdi* (agmatine deiminase generating putrescine).

All these techniques are useful tools for the selection and characterization of strains for their industrialization as malolactic starters (Lonvaud-Funel, 1998; Bou and Powell, 2005; Le Jeune *et al.,* 1995).

1.1.10 *Oenococcus oeni* and *hdc, tyrdc* and *odc* genes

High correlation between the detection of the gen *hdc* by PCR and histamine production in synthetic media was found by Landete *et al.* (2005c). This study (136 wine LAB strains analyzed) found that the species exhibiting the highest frequency of histamine production was *Oenococcus oeni*, results also observed by Guerrini *et al.* (2002). However, the concentration of histamine produced by *Oenococcus oeni* was lower than the one produced by strains belonging to species of *Lactobacillus* and *Pediococcus* which have been detected as spoilage and high histamine-producing bacteria in wines.

In another study (Landete *et al.,* 2007c), 41 strains of *Oenococcus oeni* from wine were analyzed for their capability to produce tyramine and phenyl ethylamine in synthetic media and *tyr*dc gene detection. No *Oenococcus oeni* strains were tyramine or phenyl ethylamine producers.

Pramateftaki *et al.* (2012) in an extensive research in wines LAB from Greek wines did not found *O. oeni* having genes *hdc* or *tyr*dc but they did find strains having the *odc* gene. Their capability was also observed in decarboxylating media containing arginine. This discovery was also observed in wines rich in putrescine. In other study, *O. oeni* strains isolated from wine were able to produce putrescine or cadaverine (Guerrini *et al.,* 2002).

On the other hand, Garcia-Moreno and Muñoz (2012) analyzed the published data about the capability *O. oeni* to produce histamine, and they found that existing data support the idea that *O. oeni* is not an histamine producing species in wines. One of the explanations to the lack of alignment between data is that in most of the cases, the tested strains come from culture collections and they may lose the plasmid containing the *hdc* gene (Garcia-Moreno and Muñoz, 2012).

1.1.11 Controlling the MLF process is required

Izquierdo-Cañas *et al.* (2008) reported that indigenous bacteria can drive the MLF in industrial-scale at technologically well equipped wineries producing acceptable levels of histamine (<10 mg/L). However, up to now, everything pointed out that the best strategy to avoid biogenic amines generation during the MLF, is conducting the process with a known *O. oeni* strain selected for its property to be poor biogenic amines producer. The objective being to avoid uncontrolled MLF, as can be the case of spontaneous MLF.

Spontaneous MLF implies several risks, such as a considerable increase in volatile acidity, consumption of residual sugars and formation of undesirable metabolites, such as biogenic amines (Palacios *et al.,* 2005a; Marcobal *et al.,* 2006; López *et al.,* 2008 and 2011).

1.1.12 Commercial *O. oeni* starters

The inoculation of commercial malolactic starters is a strategy used in wine production to minimize problems relating to uncontrolled malolactic fermentations (Lonvaud-Funel, 1995). It also promotes a reliable and rapid malic acid bioconversion, thus ensuring better control and predictability of the reaction (López *et al.,* 2008; Krieger, 2005; Gerbaux and Monamy, 2000).

Many studies between 1957 and 1968 have described the stimulation of MLF by inoculation of LAB strains. These studies have lead to the development of several commercial strains of malolactic bacteria and have revealed some important factors, such as pre-culture conditions and time of inoculation that affect the ability of the bacteria to grow and conduct the MLF (Davis *et al*., 1985).

The malolactic bacteria strains are selected on certain characteristics, including a lower production of biogenic amines. Initially, they are also able to impose themselves on autochthonous micro flora and to develop the MLF which gives the wine its desired characteristics.

In the criteria for the selection of malolactic LAB starters, the non production of biogenic amines is considered third-order criteria (Table 14).

Nowadays, many commercial starter cultures are available to induce malolactic fermentation. Most consist in strains of lactic acid bacteria, mainly *O. oeni*, which have a

high malolactic activity and tolerance to wines with low pH and high ethanol content (González *et al.,* 2011).

These starter cultures have been commercialized in various forms:

- Fresh cultures: they must be produced and sold directly in the producing regions, or used immediately in the producing wineries.
- Frozen cultures: the transport over long distances is complicated by the difficulty to guarantee that the required temperature is maintained.
- Lyophilized cultures: no cold chain needed but problems are also associated with lyophilized cultures, as they might lose viability when they are inoculated directly into the wine (Krieger *et al.,* 1993).

Consequently, most efforts are now focused on the development of lyophilized malolactic starter cultures that can be directly inoculated into wine without prior treatments (González *et al.,* 2011).

Additionally, frozen and fresh starters detriment the carbon foot print and energy profile of the wine due to the need of cold chain for their conservation. Therefore, regarding sustainability and environmental responsibility, the lyophilized cultures are preferred.

Table 14. Criteria for the selection of Lactic Acid Bacteria to induce Malolactic Fermentation in Wine ***(adapted from González et al., 2011)***

First-order criteria
Resistance to low pH
Resistance to ethanol
Tolerance to low temperatures
Reduced metabolism of hexose and pentose sugars
Second-order criteria
High viability following propagation in a standardized medium
Short propagation time in a standardized medium
High production of biomass in a standardized medium
Rapid survival kinetics in a standardized medium
Rapid degradation of malic acid in a tartaric acid buffer (pH 4.5) and in standardized wine
Third-order criteria
Production of appropriate organoleptic characteristics in the wine
Resistance to phages
Sulfite resistance
No formation of biogenic amines
Potential to form diacetyl and acetoin
Limited formation of volatile acids
No degradation of glycerin
No production of extracellular polysaccharides
Little formation of D-lactic acid

1.1.13 Benefits of using malolactic LAB starters

The benefits of using malolactic starters are (González *et al.*, 2011):

- Rapid onset of malolactic fermentation at the most appropriate moment: if the bacterial population has been adequately controlled, at the end of alcoholic fermentation the wine will contain very few bacteria and, therefore, may require weeks or even months before an adequate spontaneous bacterial population is developed. The use of an inoculum containing 10^6 cells/mL can help to avoid significant delays.

- Maintaining wine quality: the bacterial population never comprises a single microorganism. Spontaneous malolactic fermentation is carried out by different strains of *O. oeni* and, often, other bacterial species. Greater variability in this population increases the risk of negative effects on fermentation or of undesirable metabolites being produced. Improvements in the quality of wine were attributed to the utilized starter cultures during the MLF (López *et al.*, 2008; Palacios, 2005a). Moreover, the typology of the wine can be designed by the use of malolactic starters as they confer specific notes or flavour profiles to the wines (Rosi *et al.,*1999)

- Control over the type of wine produced: the use of selected bacterial cultures ensures that the desired quality of wine by the producers can be obtained. This last point is very important since malolactic fermentation is not only a process of de-acidification of the wine but also, depending on the strain used, an opportunity to obtain additional advantages by preventing the production of secondary metabolites that can have a negative effect on the wine. In wines inoculated after alcoholic fermentation, the biogenic amines production during MLF was significantly lower than in spontaneous MLF (Gerbaux and Monamy 2000; López *et al*., 2008). The same was observed for the ethyl carbamate synthesis during MLF, where the use of malolactic starters reduced its production (Romero, 2010).

1.1.14 Monitoring of the MLF process in wine cellar

During the winemaking process, it is fundamental to monitor the progress of the MLF by its primary chemical action, the conversion of L-malic acid into L-lactic acid, and also the microbial flora present in the wine during the conversion. The monitoring of the process is key to keep the process parameters under control.

Regarding the chemical composition, the two most important chemical parameters to monitor during the course of MLF are the depletion of L-malic acid and the rise in volatile acidity. Some methodologies have been used for this purpose in wine cellars (Kollar and Brown, 2005):

- Paper chromatography
- Thin layer chromatography
- Reflectance
- Enzymatic analysis
- Capillary electrophoresis
- High performance liquid chromatography (HPLC)

To follow up the microorganism development during the MLF, some microbiological techniques are used in the wine cellar (Kollar and Brown, 2005):

- Direct microscope observation. Fortunately, few bacteria can grow in wine, and because of their relatively distinct cellular appearance under the microscope, *Oenococcus*, *Pediococcus*, *Lactobacillus* and *Acetobacter* species are identifiable and their relative abundance can be estimated. Using epifluorescence light coupled to phase contrast microscope, an idea of the viable cells population can be estimated (Romero, 2010).
- Viable culturing techniques.

1.1.15 Performance of malolactic LAB starters

The use of malolactic LAB starters is uncontestable regarding the benefits it brings to the wine. However, in many occasions, and depending on different factors (initial microbial load, pH, temperature, technical production technique, etc.), the inoculated starter strain does not implant itself entirely (Gindreau *et al*., 1997 and 2003; Tenorio and Santamaria, 2005). As a result, autochthonous lactic acid bacteria carry out the malolactic fermentation jointly with the starter, giving rise to unpredictable results (López *et al.,* 2008, Ruiz *et al.,* 2010; Maicas, 2001; Claisse and Lonvaud-Funel, 2012)

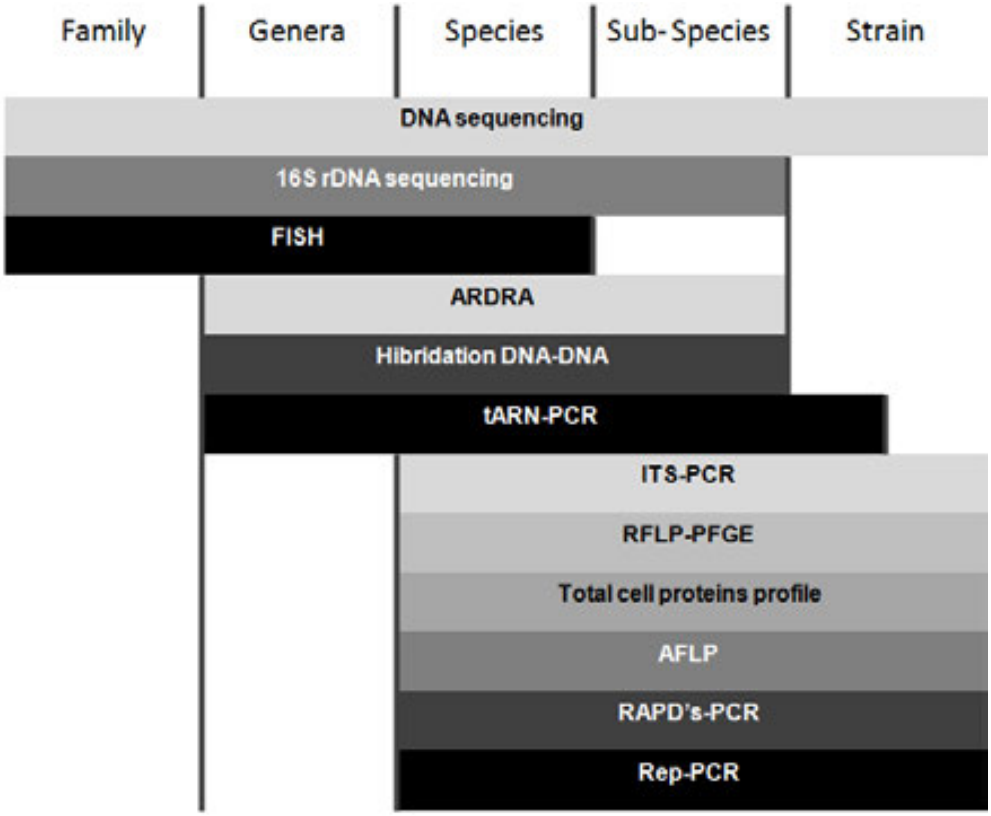
Therefore, it is important to determine the level of implantation of the inoculated malolactic starter in order to determine the participation of autochthonous bacteria during the malolactic fermentation. This parameter enables to evaluate the efficiency of the inoculums from a quantitative standpoint, determine the extent to which they are related to deviations of the product, as well as the extent to which the presence of the selected strain has minimized the risk of formation of biogenic amines.
None of the methods, chemical, microbiologic, or microscopic for the monitoring of the MLF in wine cellar, allow determining the specific implantation of the malolactic starter over the wild bacterial population. As discussed previously, some strains of wild *O. oeni* or strains of other wine LAB species, are able to produce biogenic amines while the selected LAB starters in principle are poor producers. To know and monitor the level of implantation of the starter is key to control the process and master the deliverables. With this objective, only the molecular tools that analyze the DNA (or RNA) of the strains driving the MLF are able to provide such information.

1.2. INTRODUCTION: Molecular tools for identification and typing of wine bacteria

1.2.1 Molecular Tools

Several molecular tools for the identification of microorganism are currently available; some of them allow identification at species level and this is useful to detect contaminant bacteria in wines for example. Some of these techniques allow indentifying until strain level as indicated in Figure 7, this is particularly interesting when the characterization of the population that drive the MLF need to be determined by analyzing the genetic polymorphism of *O. oeni* during the MLF.

Figure 7. Discriminating level of molecular techniques ***(source Blasco, 2009)***



FISH: fluorescent in situ hybridation; ARDRA: amplified ribosomal DNA restriction analysis.; tRNA-PCR: coding intergenic space of transfer RNA analysis; ITA-PCR: PCR analysis of the 16S-23S rRNA gene internal transcribed spacer; RFLP: restriction fragments length polymorphic analysis; RFLP-PFGE: restriction fragments length polymorphism by pulsed-field gel electrophoresis; AFLP: amplified fragment length polymorphism; RAPD: random amplified polymorphic DNA; rep-PCR: repetitive sequence-based PCR

1.2.2 Identification of wine LAB

Moving towards the identification of the bacteria present in must and wines for research and technical purposes, several works described methodologies for their identification through molecular tools as summarized in Table 15.

Table 15. Molecular Tools to identify lactic acid bacteria in wines.

Molecular Tool	Microorganism identified	Reference
Hybridization DNA-DNA (in colony)	Several species of *Lactobacillus.* (except L. *hilgardii* and *L. brevis*), *Pediococcus spp.*	Lonvaud-Funel *et al*. 1989 Lonvaud-Funel *et al.* 1991
	Leuconostoc mesenteroides and *Oenococcus oeni*	
Hybridization DNA-DNA (in cells "in-situ")	*Leuconostoc spp. Lactobacillus Spp.* *Pediococcus spp.*	Sohier and Lonvaud-Funel *et al.* 1998
Hybridization DNA-DNA (in purified DNA)	Leuconostoc and Lactobacilli Leuconostoc *spp.*	Dicks *et al.* 1990
FISH (Hybridization DNA-rRNA)	Several species of *Lactobacillus , Pediococcus spp. Leuconostoc mesenteroides and Oenococcus oeni*	Blasco *et al*. 2003 Blasco 2009
Specific PCR	Several species of *Lactobacillus, Pediococcus spp., Leuconostoc mesenteroides* *Oenococcus oeni* *Weisella paramesenteroides*	Zapparoli *et al.* 1998 Dellaglio *et al.* 1998 Bartowsky and Henschke, 1999 Sohier *et al.* 1999 Blasco 2009 Bravo-Ferrada *et al*., 2011 Petri *et al.* 2013
Real-time quantitative PCR (specific PCR)	*Oenococcus oeni*	Pinzani *et al*. 2004
Multiplex PCR	*Pediococcus acidilactici and P. pentosaceous* *Lactobacillus platarum, L. pentosus, L. paraplantarum*	Mora *et al.* 1997 Torriani *et al.* 2001
RAPD-PCR	*Oenococcus oeni* Several species of *Lactobacillus spp.*	Guerrini *et al*. 2003 Rodas *et al.* 2005
ITS –PCR (region V3 rRNA)	*Oenococcus oeni*	Guerrini *et al.* 2003
16S-ARDRA and RFLP-ITS	Several species of *Lactobacillus, Pediococcus, Leuconostoc mesenteroides and Oenococcus oeni*	Chenoll *et al.* 2003 Rodas *et al.* 2003 and 2005 Ruiz *et al*. 2008 Blasco 2009 Izquierdo-Cañas *et al*. 2009 Bravo-Ferrada *et al.* 2011 Ruiz *et al*. 2010 Pramateftaki *et al.* 2012
Ribotyping	*Oenococcus oeni* Several species of *Lactobacillus spp.*	Viti *et al.* 1996 Rodas *et al.* 2005
Amplification and restriction analysis of rpoB gene region	*Oenococcus oeni*	Renouf *et al.* 2006 Claisse *et al.* 2007 Bravo-Ferrada *et al.* 2011

Currently, the choice of the technique will mainly depend on the laboratory facilities and team expertise. Both aspects explain why this type of analysis is rarely practiced in routine laboratories of winemaking sites.

Nevertheless, the FISH method (Blasco *et al*., 2003 and 2009) which is based on the *in situ* hybridization with specific probes targeting 16rRNA gene, marked with fluorocroms can be highlighted due its simplicity and versatility. Using this method it is possible to identify the most common species of LAB and acetic acid bacteria in must and wines in one analysis and directly in wine sample, but still a microscope with fluorescence champs is needed.

Regarding research studies, two methods have been the preferred in recent years: the 16S ARDRA (amplified ribosomal DNA restriction analysis) method developed by Rodas *et al.* (2003) which is useful for the identification of the common LAB species found in must and wines, and specific PCR that uses primers of the malolactic gene (Zaparolli *et al.*, 1998) for the identification of *O. oeni*. By using other primers, it is also currently possible to identify other wine LAB species than *O. oeni* by specific PCR (Table 15).

1.2.3 Molecular methods for typing of *O. oeni* strains

Several studies have demonstrated that *Oenococcus oeni* is a quite homogeneous species and strains are difficult to differentiate especially when isolates from the same region are analyzed (Lechiancole *et al*., 2006). Additionally, the discrimination of different strains or genotypes of *Oenococcus oeni* is necessary for monitoring the MLF measuring the success of the malolactic LAB starters over the autochthonous microflora and during strains selection process. Therefore, molecular techniques that allow an interspecific identification of *O. oeni* strains have been developed to make it possible. Table 16 shows the molecular methods that have been used for the typification of *O. oeni*. Two techniques are most commonly used:

- RAPD-PCR (random amplified polymorphic DNA): this method uses one short primer with random sequence that is able to hybridize with several DNA sites generating polymorphism of the DNA fragments. Several primers have been used for typification of *O. oeni* as M13 (Stendid *et al*., 1994), COC (Cocconcelli *et al*., 1995), 16R and 17R (Tompkins *et al.,* 1996). After the amplification, the fragments are usually separated by agarose gel electrophoresis or alternatively by capillary electrophoresis (Marquez *et al,* 2011)

• RLFP-PFGE (restriction fragments length polymorphism by pulsed-field gel electrophoresis). This technique employs restriction enzymes that digest microbial DNA, which is then subjected to electrophoretic separation. Enzymes ApaI, SfiI, NotI and SmaI are frequently used for typification of strains of *O. oeni* (Pozo-Bayon *et al.,* 2009).

Table 16. Molecular methods for strain typing of LAB isolated from wine.

Molecular Tool	Strain Typing	Reference
PFGE-RFLP of plasmids DNA	*Oenococcus oeni*	Prevost *et al*. 1995
REA analysis PFGE-RFLP	*Oenococcus oeni* *Oenococcus oeni* Several species of *Lactobacillus*	Viti *et al*. 1996 Lonvaud-Funel 2008 Gindreau *et al*. 1997 and 2003 Daniel *et al*. 1992 Kelly *et al*. 1993 Pardo *et al*. 1998 Zapparoli *et al*. 2000 Guerrini *et al*. 2003 Rodas *et al*. 2005 Tenorio *et al*. 2005 Izquierdo-Cañas *et al*. 2007 López *et al*. 2007 and 2008; Pramateftaki *et al*. 2012
RAPD-PCR	*Oenococcus oeni*	Zapparoli *et al*. 2000 Bartowsky *et al*. 2003
RAPD-PCR (primer M13)	*Oenococcus oeni* Several species of *Lactobacillus*	Izquierdo-Cañas *et al.* 2009 Bravo-Ferrada *et al.* 2011 Marques *et al*. 2011
RAPD-PCR (primers COC and others)	Several species of *Lactobacillus*	Rodas *et al*. 2005
RAPD-PCR (primer M13) + PFGE	*Oenococcus oeni* *Lc. mesenteroides*	Ruiz *et al*. 2008 and 2010.
Ribotyping	*Oenococcus oeni* Several species of *Lactobacillus*	Viti *et al.* 1996 Satokari *et al*. 2000 Rodas *et al*. 2005
Multiplex RAPD-PCR	*Oenococcus oeni*	Reguant and Bordons 2003 Reguant *et al*. 2003
MLST (housekeeping gene sequencing)	*Oenococcus oeni*	De Las Rivas *et al*. 2004
VNTR (Multiple-locus variable number of tandem repeat analysis)-PCR	*Oenococcus oeni*	Claisse and Lonvaud-Funel 2012

1.2.4 Implantation of malolactic LAB starters

Studies concerning strain involvement in wine modifications during MLF are of major interest. Numerous works evaluating the starter performance comparing their kinetics and sensorial aspects are available, but data concerning implantation are often lacking, even if they are a prerequisite for all subsequent analysis.

In recent years, and thanks to the development of efficient strain typing molecular methods, some results on implantation of starters during the MLF were achieved:

- The implantation of the starter was followed in twenty-one MLF in 5 different cellars (Gindreau *et al*., 1997). PFGE patterns from bacterial population harvested by centrifugation of the wine at the end of MLF were performed. One pattern per MLF was obtained. Nine of the 21 MLF were fully implanted (+), in five batches, the profile of the starter was not found (-) and in 6 other batches the starter profile was found mixed with other bacteria (+/-). In some batches the inoculation was done when MLF was already ongoing (as per L-Lactic acid results) but the kinetics was not affected. As the sampling was done at the end of MLF it was not possible to determine the level of participation of the autochthonous bacterial population during the MLF.

- Gindreau *et al.* (2003) analyzed the implantation of malolactic starters by PFGE patterns. This time around, the sampling was done at the beginning of L-malic acid consumption and the bacterial population was harvested by centrifugation. Four different starters (starters name encrypted) were evaluated: 2 of them did not survive after inoculation stress, 2 of them (BL3 and BL4) survived and implanted well as their PFGE pattern is the only one recognized in the analysis of bacterial DNA from wine. From a sensorial standpoint, the observed differences between the inoculated and spontaneous MLF wines were weak. Only starter BL3 was perceived as different from spontaneous MLF. Regarding biogenic amines generation during MLF, histamine, tyramine and putrescine content were similar in inoculated or spontaneous MLF.

- Tenorio *et al.* (2005a) studied the impact of the use of lysozyme in Tempranillo must and wine on the implantation level of the malolactic starter. PFGE patterns of 10 isolated LAB from wine at 60% of consumption of malic acid were analyzed. The lysozyme reduced the bacterial population and therefore the MLF of wines treated with lysozyme were longer than the wines without lysozyme. Nevertheless, the implantation of the starter seems not to be affected by the use of lysozyme as it was about 100%.

- Masqué *et al.* (2007) studied the level of implantation as function of inoculation time of three starters, *O. oeni* starters Elios1 and EQ54 and *L. plantarum* starter C11, in Tempranillo and Merlot wines. The level of implantation was analyzed using RAPD-PCR technique, sampling at 2/3 of malic acid consumption and analyzing profiles of isolated colonies. The level of implantation was from 0%, 93% or 100% for strain EQ54; 0%, 21%, 58%, 73%, 90% or 100% for strain Elios1 and for C11 the level of implantations were 0%, 7%, 50% and 100%. Better implantations were found when the starters were inoculated at the beginning of ALF. In Tempranillo the biogenic amines were very low <1ppm in all conditions. In Merlot wines, slightly higher biogenic amines were found in wines co-inoculated with yeast and that had no implantation of LAB starter.

- López *et al.* (2008) studied the implantation of three non commercial starters isolated from La Rioja wines (two *O. oeni* and one strain of *Lactobacillus plantarum*) and the commercial malolactic starter Uvaferm Alpha during the MLF. The MLF was monitored by implantation analysis in five points. PFGE patterns of 18 LAB isolated in each sampling point were compared with the pattern of the starter. Full implantation was observed for the commercial starter (100% all samples) while *L. plantarum* couldn't implant (100% at inoculation, then 0% until the end of MLF). For one non-commercial starter *O. oeni* the implantation was high (100% all samples) and for the other one, the implantation decreased during the process (100%, 100%, 67%, 50%, 50%). A good correlation between MLF duration and the percentage of implantation of the inoculated strain was observed in all cases. The concentration of histamine seemed to correlate with the level of implantation of the inoculated strain, and the lowest value was

obtained for the wines inoculated with the commercial strain at 100 % implantation. The initial wine showed a low concentration of histamine (0,13 mg/L), and after spontaneous MLF wines displayed the highest concentration of this biogenic amine (3,85 mg/L), whereas histamine concentrations in the inoculated wines were intermediate.

- Romero (2010), carried out an investigation on the production of ethyl carbamate during MLF and inoculation with commercial bacterial starters (Lallemand) at different stages. The level of implantation was analyzed using RAPD-PCR technique, sampling at 2/3 of malic acid consumption and analyzing profiles of isolated colonies. Depending on the inoculation time, Lalvin 31 reaches implantation levels of 0%, 93% and 100% while Elios 1 obtained 77%, 90% and 100%.
- López *et al.* (2011) compared inoculated malolactic fermentations (MLF) of Tempranillo Rioja wines (Spain) with two lactic acid bacteria (LAB) strains and spontaneous MLF on characteristics of bacterial population and biogenic amines. Inoculation with selected *Oenococcus oeni* preparation (Uvaferm Alpha and Beta) shortened MLF duration up to 19 days and lead to wines presenting the characteristics of being more fresh and fruity, especially when implantation was 100%. A modification in the concentration of volatile and nitrogenous compounds and a good correlation between analytical and sensory attributes were also noted. In addition, the low initial amino acid concentration and the consumption of these compounds by the inoculated yeast strain during alcoholic fermentation resulted in wines with very low total biogenic amines levels (under 3,75 mg/L) after MLF and 3 month storage period in all cases.
- López *et al.* (2012) studied the implantation of malolactic starter at different pH and its influence on BA production. Uvaferm alpha presented 89% implantation at pH 3.4 and 62% implantation at pH 3.7. A good correlation between the implantation and duration of MLF was observed.

These studies highlight that the implantation of the malolactic starter is highly variable and depends on wine characteristics and winemaking conditions. There is no consensus with regards to the optimal conditions favoring implantation of the malolactic starter. Thus, more investigation on the topic is needed.

1.3. INTRODUCTION: INOCULATION TECHNIQUES

The performance of the malolactic starter during the MLF when studied by molecular tools using typification of the bacterial population present during the process has shown to result in different levels of implantations (Gindreau *et al.,* 1997 and 2003; Tenorio *et al,.* 2005; López *et al*. 2008, 2011 and 2012; De Revel *et al*., 2003, Masqué *et al.,* 2007, Romero, 2010).

Several techniques to manage the MLF have been developed to enhance the beneficial effect of the MLF, by optimizing the adaptation of the LAB starter, by eliminating wild bacterial population or just to improve the process timing. Within the mentioned technologies, the use of different inoculation method, as direct inoculation or mother tank technique, the practice of co-inoculation with yeast before the finalization of alcoholic fermentation or the addition of nutrients or processing aids as the lysozyme are at disposal of the oenologist for the management of the MLF. It is logical to think that as these techniques influence the LAB population, they should also influence the level of implantation of the starter.

1.3.1 Direct inoculation

Malolactic bacterial starters for direct inoculation (DI) that are currently available are the result of more than 30 years of research on the topic. The first malolactic starter culture for direct inoculation into wines, without previous adaptation step, was introduced in the market in 1993 (González *et al.,* 2001). Table 17 shows the different types of adaptation steps used in the different types of starters (liquid, frozen, standard freeze dried and direct inoculation type).

Direct inoculation starters consist in a lyophilized malolactic bacteria isolated from wine. The biomass is produced in bioreactors via a controlled process and then this biomass is lyophilized for its conservation.

The benefit on the use of LAB starters have been previously presented in this thesis (*"Introduction: Malolactic Fermentation" section*).

The capacity to survive following direct inoculation in wine and the maintenance of this capacity when strains are prepared as lyophilized cultures are of major practical importance in winemaking. These commercial lyophilized preparations thus demonstrate that it is possible to produce malolactic starter cultures that do not require the usual reactivation or pre-adaptation steps prior to their use.

Table 17. Preparation of commercial malolactic bacterial cultures for inoculation in wines (*adapted from Specht 2012)*

Property	Type of malolactic bacteria starter			
	Frozen	Liquid Suspension	Traditional Freeze-Dried (Standard)	Direct Inoculation
Time for Starter Preparation	48 hours before inoculation	10-fold expansion in 3-7 days	3-14 days	0-15 minutes
Starter Preparation	Thaw in room temperature water, not in refrigerator. Mix 3 L water, 3 L grape juice and 30 g yeast extract. Adjust pH to 4.0 with calcium carbonate or other permitted buffer and mix thoroughly. Add 170 g of thawed culture, seal carboy and mix thoroughly. Hold at 18°-24°C for 48 hours before inoculation.	Clean settled juice with no added SO2. If possible, heat juice to 60°C. Adjust sugar level to 18° Brix with water. If juice is not available, substitute with a mix of 50% finished wine (<10 ppm free SO2 and low total SO2), 25% water and 25% apple juice). Adjust pH to 3.5-3.6 with calcium carbonate. If inoculating wine at <pH 3.2, adjust pH again to 3.4 as an intermediate step. Add culture and maintain temperature at 22°-26°C. Monitor to 100% malic acid degradation then expand again as a 10% inoculum at each build-up stage or inoculate. If finished wine was used to prepare the starter, expand culture by doubling the starter volume with wine until the starter volume is 5%-10% of the amount to be inoculated	Rehydrate in a 50:50 water: wine mix. Wine should be pH >3.3 and total SO2 < 30 mg/L. Monitor malic acid drop and when ~2/3 is converted to lactic acid, expand as a 5% inoculum into wine. Make sure pH >3.3 and alcohol <12.5%. Monitor malic acid drop and when ~2/3 is converted to lactic acid, expand as a 4% inoculum into wine.	Not required but may be suspended in clean chlorine-free warm water to help in handling.

This eliminates the risk of contamination and reduces the time required during wine-making process. These cultures are easy to use. The lyophilized product is simply added directly to the wine following alcoholic fermentation. This can be done during racking or, alternatively, with a pump or by recirculation. Certain optimal conditions in the wine to be inoculated have been established for the use of these cultures: it should contain no free SO_2, it should not contain added sulfites (with a maximum total SO_2 of 40 mg/L in red wine and 30 mg/L in white wine), and it should be maintained at an optimal temperature of 23 °C (Gonzáles *et al.,* 2011).

Malolactic strains directly inoculated into wine improved significantly the control of the malolactic fermentation (Nielsen *et al,.* 1996; Lonvaud-Funel, 2001; López *et al*., 2008).

1.3.2 Inoculation by mother tank (*pied de cuve*) technique

In some wines, an unsuccessful MLF may be stimulated by inoculation with wine already undergoing MLF (Davis *et al*., 1985). This already started MLF might be spontaneous or inoculated with malolactic starters. This technique is known by the name of *pied de cuve* and the deposit from where the inoculums-wine is taken is usually called “mother tank”.
The main disadvantages with this approach are that a suitable wine must be available, and that the bacteria in the inoculums-wine may not be suited to grow in the wine to be inoculated. Substantial death of cells has been noted using this approach (Vetsch, 1973). It might be necessary to inoculate large volumes of the wine undergoing MLF, and this may not be practical. Inoculums-wine from 5% to 50% has been recommended (Castino et al., 1975; Davis *et al.,* 1985). Practical difficulties associated with the manipulation of large volumes can be solved by utilizing the cells from an active wine after centrifugation or filtration (Davis *et al*., 1985).
On the other hand, unexpected as this can sound, currently this *pied de cuve* technique might be used in cellars to save money on the quantity of malolactic starter used. This means that a first batch is usually inoculated with commercial LAB starter following supplier recommendation, and once this MLF is ongoing, other wines are inoculated following *pied de cuve* or mother tank method.
This inoculum-wine which level of implantation is unknown, confronts the wild bacterial population and the results in terms of level of implantation in the resulting wine are unpredictable. Even if malic acid consumption shows satisfactory results, it is well known that the type of LAB that drives the MLF will have high impact on sensory quality of the wine and as well on biogenic amines content.

One can wonder if this technique, after the complication it represents because of manipulation of large volumes of wines is really efficient and if the process is really under control.

1.3.3. Time of inoculation

The point of winemaking process where the malolactic starter should be inoculated into the wine has been a source of controversy. The general view in France is to recommend MLB inoculation after completion of the alcoholic fermentation, to avoid the risk of producing acetic acid and D-lactic acid, which is referred as lactique disease or "*piqûre lactique*" (Krieger, 2005; Ribéreau-Gayon *et al.,* 1975) while inoculation of bacteria during the alcoholic fermentation is practiced by several Californian winemakers (Davis *et al.,* 1985; Blackburn, 1984). The latter practice is based on the fact that during ALF the bacteria do not stress for the presence of high ethanol content and the free SO_2 has been reduced by the formation of SO_2-binding compounds by yeast leading in principle to a better adaptation of the bacteria to the wine (Davis *et al.,* 1985).

After several researches, it is generally acepted that acetic acid will not be produced during growth of bacteria and active MLF. The general view is that acetic acid will be mainly produced when half of L-malic acid is degraded and the bacteria began to utilize the citric acid during the MLF (Krieger, 2005).

In practice, the inoculation of bacteria can be done (Davis *et al.* 1985; Krieger ,2005):

- Simultaneously with yeast inoculation (known as co-inoculation).
- At any stage of alcoholic fermentation.
- After completion of alcoholic fermentation (known as sequential inoculation).

Regarding the implantation of malolactic starters in relation with inoculation time, or production of biogenic amines, some research has been done:

- Biogenic amines were studied in wines where LAB starters were inoculated at 12h from inoculation of yeast starter, at middle of ALF, with remaining sugars (10g/L) and sequential inoculation by Masqué *et al.,* 2007. It was observed that the level of implantation (measured using RAPD-PCR) of the three LAB starters (two *O. oeni* and one *L. plantarum*) was higher and the MLF faster when the inoculation was performed at the beginning of ALF in Tempranillo wines while for Merlot wines the better implantations according to inoculation time was strain dependant. In

Tempranillo wines, the biogenic amines were lower than 1ppm and a difference on inoculation time was not observed. In Merlot wines, the biogenic amines were slightly higher in the wines where the LAB implantation was poor.

- Co-inoculation reduced the biogenic amines in Shiraz wine while the Pinotage wine obtained lower biogenic amines with sequential inoculation (Smit *et al.,* 2012). In this study one can suspect a different level of implantation of the starter but this was not demostrated in the research.
- Simultaneous yeast- bacteria and sequential inoculation in Malvec did not released different levels of biogenic amines, also the sensory properties of the wines were not affected by the time of inoculation (Massera *et al.,* 2009).
- Romero (2010) carried out an investigation on the production of ethyl carbamate during MLF and on inoculation with commercial bacterial starters (Lalvin 31 and Elios 1, Lallemand) at different stages. The level of implantation was determined by using RAPD-PCR technique, sampling at 2/3 of malic acid consumption and analyzing profiles of isolated colonies. Depending on the inoculation time, the levels of implantation were:
    - High (93 and 90%), for inoculation 12h after yeast inoculation.
    - Maximum (100% and 100%), for inoculation at the middle of ALF.
    - Variable (0% Lalvin 31 and 77% Elios 1) for inoculation with remaining 10g/L of glucose and fructose.
    - Contradictory (0% Lalvin 31 and 100% Elios 1) for standard inoculation of bacteria at the end of ALF.

The results from these studies suggest that the survival of the LAB starters can be compromised in certain co-inoculation conditions .

1.4. INTRODUCTION: LYSOZYME

1.4.1 Lysozyme to replace SO_2 in wines

Sulphur dioxide (SO_2) is commonly used as a preservative. It is an antioxidant, protecting wine phenols from oxidation and an inhibitor for must endogenous oxidases. It has bacteriostatic properties, which prevent the onset of undesirable fermentations and is useful for the extraction of skin pigments (Ribéreau-Gayon *et al.,* 2006b).

However, there is a general trend towards the reduced use of SO_2 in wine processing because, over certain ingestion doses, this compound could have toxic effects on human health. Moreover, SO_2 is commonly known to trigger adverse reactions in certain people who can be sensitive to its presence (Taylor *et al.*, 1986; Sonni *et al*., 2010 and 2011). It is also important to reduce the amount of SO_2 in wine, since this compound is also found in many food products as an additive, and the amount consumed is accumulative in the organism.

Additionally, the antimicrobial activity of SO_2 decreases as wine pH increases, making it more difficult to microbiologically stabilize wines with low acidity (López *et al*., 2009).

Since the early 1990's, the use of lysozyme has been proposed to control malolactic fermentation in winemaking, supporting or even replacing the use of sulphur dioxide (Gerbaux *et al,.* 1997 and 1999; Sonni *et al,.* 2011; Garland *et al*., 2006).

The use of lysozyme in musts and wines was authorized by the OIV in 1997. In 2005 the European Commission decided to permanently include lysozyme on the list of ingredients that must be indicated on the wine labels as it is egg derived.

1.4.2 The properties of Lysozyme

The most common type of lysozyme (muramidase E.C. 3.2.1.17) is the c-type, which is the lysozyme present in hen egg-white. This lysozyme has a lytic activity on the cell wall of Gram-positive bacteria, and has been successfully used in the pharmaceutical and food industry as an antimicrobial agent for prolonging product shelf-life since the 1950's (Delfini *et al.,* 2004).

Lysozyme's lytic activity is based on the hydrolisis of the β-(1-4) linkage between *N*-acetylmuramic acid (NAM) and *N*-acetylglucoseamine (NAG), which constitutes the peptoglycan layer of the bacteria cell wall. This chitinolytic activity changes the cell's solidity and permeability, causing degradation of the bacterial cell wall and thus accelerating cell lysis (Cunningham *et al.,* 1991).

The maximum stability and activity for lysozyme is found at pH in the range of 2.8-4.2, which is the pH range of most wines (Pitotti *et al*., 1991). Its efficacy towards Gram-negative bacteria (i.e., acetic bacteria) is much less than Gram-positive bacteria (i.e., lactic acid bacteria) and could be defined as bacteriostatic rather than bactericidal (Cunningham *et al.,* 1991), presumably because the outer membrane acts as a barrier. It is inactive against the eukaryotic cell wall as is the case of yeast.

1.4.3 Use of lysozyme in oenology

Four different usages of lysozyme in oenology have been described (Gerbaux *et al.,* 1997; Gerland *et al.,* 1999):

- Blockage of malolactic fermentation in white wines. It allows the reduction of SO_2 additions without negative impact on sensory profile of wines (Gerbaux *et al.*, 1999). Nevertheless, as traces of lysozyme in white wine are usually present in finished wines it can increase turbidity due to denaturation of the enzyme in extreme temperatures (<5°C reversible turbidity, >40°C irreversible turbidity, Gerbaux *et al.,* 2004).
- Slowing the malolactic fermentation in red wines. Lysozyme is used to avoid overlapping between the ALF and MLF during carbonic macerations (Beaujolais type of wine) or must with high pH.
- Treatment of difficult ALF. When alcoholic fermentation does not complete well, a risk of advanced MLF appears there with the consequent heterofementative metabolism of bacteria in presence of sugars allowing *piqure lactique* phenomenon. The application of lysozyme acting only on bacteria allowed the reestablishment of alcoholic fermentation by reducing the LAB population (Gerbaux *et al.,* 1999).

- Stabilization of wine after the MLF. The reduction of indigenous bacteria after the MLF is very important to reduce the risk of development of volatile acidity and biogenic amines. The application of lysozyme allows reducing the LAB population at this stage and the color seems to be better stabilized with respect to the use of SO_2 as the sulfitation can be delayed to the first raking. This was observed to be key for the consolidation of color in red wines (Gerland *et al*., 1999; Gerbaux *et al.,* 2003).

1.4.4 Interaction between lysozyme and polyphenols

The primary factor that has been observed to affect the bacteriolitic action of lysozyme in wines is believed to be polyphenolic components, present at higher concentration in musts and red wines, which can quickly bind proteins (Gerbaux *et al*., 2003). It was observed that lysozyme was more active in white wines than in red ones, which is attributed to difference in polyphenolic content (Bartowsky *et al*., 2004).

Guzzo *et al.* (2011) studied the inhibitory effect of wine phenolics on lysozyme activity against LAB *in-vitro* test. *Lactobacillus uvarum, Pediococcus parvulus* and *Oenococcus oeni w*ere found to be more sensible to lysozyme than species of *L. plantarum* and *L. hilgardii.* The amount of low molecular weight proanthocyanidins that are released during grapes maceration affected the lysozyme efficiency against LAB.

1.4.5 Resistance to lysozyme

The inhibitory minimal concentration of lysozyme on *O. oeni* strains was studied by Pillate *et al.* (2000). Different malolactic starter for direct inoculation (Chr. Hansen) were evaluated regarding their resistance to lysozyme. It was found that the three studied starters presented, low, moderated and high sensibility measured by their survival after treatment with the enzyme. This might indicate different cell wall composition. In fact, for other bacteria species the resistance to lysozyme was associated with more β(1-4) links between N-acethyl muramique acid and N-acethyl glucosamine acids than when β (1-6) and β (1-3) links type occurred more frequently.

Some *Pediococcus parvulus* strains synthesize a β-glucan, which can decrease the wine quality as it confers a ropy texture to the wine that can no longer be commercialized. Ropy *Pediococcu*s still remain difficult to remove from wine, because of their natural resistance to traditional wine stabilizing treatments. Coulon *et al*. (2012)

concluded that ropy *P. parvulus* are resistant to lysozyme. This resistance may be linked to the presence of the β-glucan that forms around the cell a protective barrier against anti-bacteria agents and this property increases during bacterial growth. The use of lysozyme with β-glucanase can strongly improve the treatment against ropy strains, in model media as well as red and white wine based media.

1.4.6 Lysozyme and implantation of LAB starter

Tenorio *et al.* (2005ab) studied the impact of the use of lysozyme in Tempranillo must and wine the implantation level of the malolactic starter (Uvaferm Alpha). Lysozyme was added in must and at end of ALF. The lysozyme reduced the bacterial population and the MLF of wines treated with lysozyme were faster than the wines without lysozyme. This might be due to the fact that the LAB starter found less competence with the indigenous bacteria, the authors explained. The implantation of the starter seems not to be affected by the use of lysozyme as it was about 100% in contrast with MLF without lysozyme where the implantation was 83%. They found negative impact of lysozyme on color for the studied wines (Tempranillo).

López *et al.* (2011) studied the impact of the use of lysozyme on implantation of malolactic starter and histamine generation during MLF and aging of the wine. At the end of ALF, the wines treated with lysozyme showed a higher survival rate of *L. plantarum* than *O. oeni* (sensitivity already described by Guzzo *et al*., 2011). Uvaferm Alpha was used as malolactic starter, its implantation was 100% when lysozyme was used and 80% without lysozyme. The use of lysozyme post MLF for stabilization of the wine, rise to very different LAB populations a month after MLF. The presence of the starter was insignificant given place for the development of other *O. oeni* strains. Lysozyme-treated and inoculated wines, showed lower volatile acidity, color intensity, total phenols and histamine after MLF. The pH was 3.8 which is higher than the pH described as critical for high biogenic amines production by Landete *et al*.(2005d). This result may be linked to the full implantation of the inoculated bacteria. The impact of the use of lysozyme was also noticed 1 and 2 months after the MLF. Wines treated with lysozyme had lower histamine content than the other wines treated only for stabilization with lysozyme or with metabisulfite. The higher histamine scenario in this study was the wine with spontaneous MLF, no lysozyme at any stage and 2 months after end of MLF, raising the histamine level to almost 30 mg/L. This study suggests that the different histamine content is not related with the management of the size of the LAB population,

which is the main property attributable to the lysozyme, and the possible regulation of *hdc* gene expression or enzyme activity by the lysozyme should be further investigated, said the authors (López *et al.,* 2011).

1.5. INTRODUCTION: NUTRIENTS

1.5.1 Addition of nutrients in must and wine

The must usually contains all the necessary nutrients for yeast development during the ALF, but sometimes low concentrations of nitrogen compounds in the must might be a limiting factor for the growth of the yeast populations. To avoid this problem, the addition of activators rich in amino acids and fatty acids in the must have been recommended (Gonzalez-Marco *et al*., 2005).

The addition of nutrients can be contradictory with the aim of controlling biogenic amines as these nutrients contain amino acids which are the precursors of biogenic amines (Batch *et al*., 2010) as previously shown in Table 4:

- González-Marco *et al*. (2005) studied the synthesis of biogenic amines in inoculated MLF (Uvaferm Alpha) with addition of fermentation activator (L2133, Lallemand) in the must. This blend consists in inactive yeast, rich in fatty acids (C16 and C18) which are growth activators for the yeast, and amino acids assimilable for the yeasts. The biogenic amines content at the end of ALF was not impacted by the addition of fermentation activators while tyramine and cadaverine were higher after the MLF in enriched wines.
- Corzani (2008) added Sangiovese must from Italy with isolated amino acids and performed MLF using strains with capability to synthesize biogenic amines. Higher biogenic amines content was observed in added wines after the MLF (45,5 mg/L histamine; 50,9 mg/L putrescine, 16,7 mg/L cadaverine per example); no differences were found after the ALF.
- Marques *et al.* (2008) studied the impact of fermentation activators for yeast and bacteria in ALF and MLF. A significant increase of biogenic amines due to the addition of these mixtures was not observed.

- Batch *et al*. (2010) studied the effect of addition of mineral nitrogen, yeast hulls and inactivated yeasts in must from Rhône Valley (France) made of Syrah and Grenache varieties. The addition of nitrogen at the end of MLF leads to a significant increase of histamine; this was not observed for other amines. They pointed out that when the must lacks of nitrogen and the yeast are on deficit of it, they might synthesize higher alcohols responsible for heavy flavors, or short chain fatty acids which are known for their property to inhibit LAB and therefore, jeopardize the completion of MLF. Addition of nitrogen must be reasonable in order to find a balance between fermentation and reduce the risk of producing biogenic amines.
- Garcia-Marino *et al*. (2010) found that the increase of biogenic amines was more significant in the period from the end of MLF and first racking. During aging, the addition of yeast mannoproteins was linked with an increase of biogenic amines probably due to the presence of free decarboxylase enzymes in the wine.
- Smit *et al*. (2012) studied the biogenic amines generated during the MLF in wines made of Pinotage and Shiraz varieties from South Africa, added with nutrients in must and end of ALF. Different types of complex nutrients were tested: a) blend containing inactivated yeast, di-ammonium phosphate (DAP), and ammonium sulfate; b) blend containing inactivated yeast, DAP, and one specific vitamin; c) mixture containing inactivated yeast, DAP, vitamins, minerals, unsaturated fatty acids, and sterols; d) nutrients containing inactivated yeast and cellulose; e) mixture containing inactivated yeast, cellulose, and casein. Histamine was the only biogenic amine showing treatment differences potentially attributable to the presence of complex nutrients. The MLF were inoculated with malolactic starters. The highest levels of histamine (3,5 mg/L) were obtained for both wines after the MLF, in wines enriched with mixture containing inactivated yeast, cellulose, and casein.

As the addition of nutrients has been related with the increase of biogenic amines in several cases, the impact of the level of implantation of the LAB starter needs to be determined to understand whether this increase in BA can be minimized with full implantation of malolactic starters.

1.6 Aim of the study: level of implantation and biogenic amines

The aim of this first part of the thesis was to make a fingerprint of the bacterial population during the malolactic fermentations and to analyze the impact of the bacterial population characteristics on the biogenic amines synthesis during the MLF process. The techniques to manage the MLF as the use of different inoculation methods, the addition of nutrients and processing aids as the lysozyme were also studied in this context because it is logical to think that these techniques might influence the LAB starter implantation.

The main objectives were 1) to apply molecular techniques for the typification of *O. oeni* strains during the malolactic fermentation in order to determine the level of implantation of the inoculated starter and 2) to evaluate how the level of implantation affects the biogenic amines production during the malolactic fermentation.

The specific objectives were:

a. To adapt and fine tune existing RAPD-PCR method for the typification of *O. oeni* strains to a technique able to follow-up malolactic starters during MLF and determine its level of implantation.
b. To compare inoculated and non-inoculated wines regarding biogenic amines, wine characteristics and sensorial properties.
c. To determine the level of implantation of the LAB starter in inoculated wines.
d. To determine the wine parameters that influence the level of implantation
e. To analyze the relationship between the level of implantation and the biogenic amines content.
f. To analyze the impact of types of inoculation: direct inoculation and mother tank (*pied de cuve*) method on the level of implantation and biogenic amines.
g. To analyze the impact of inoculation time (co-inoculation with yeast, inoculation at different stages of ALF and end of ALF) on implantation and biogenic amines.
h. To analyze the impact of the use of nutrients in must on the level of implantation and biogenic amines.
i. To analyze the impact of the lysozyme on the level of implantation and biogenic amines.

MATERIALS AND METHODS
TRIALS

Different sets of trials were performed with the objective to analyze the level of implantation of malolactic starters by typification of LAB strains using molecular tools, and understand its impact on biogenic amines generation, as follows:

1 Comparison of spontaneous and inoculated MLF (pilot plant scale).
2 Comparison of direct inoculation and mother tank method seeding methods (industrial scale).
3 Use of lysozyme (pilot plant scale).
4 Inoculation time or co-inoculation (pilot plant scale).
5 Use of nutrients in must (pilot plant scale).

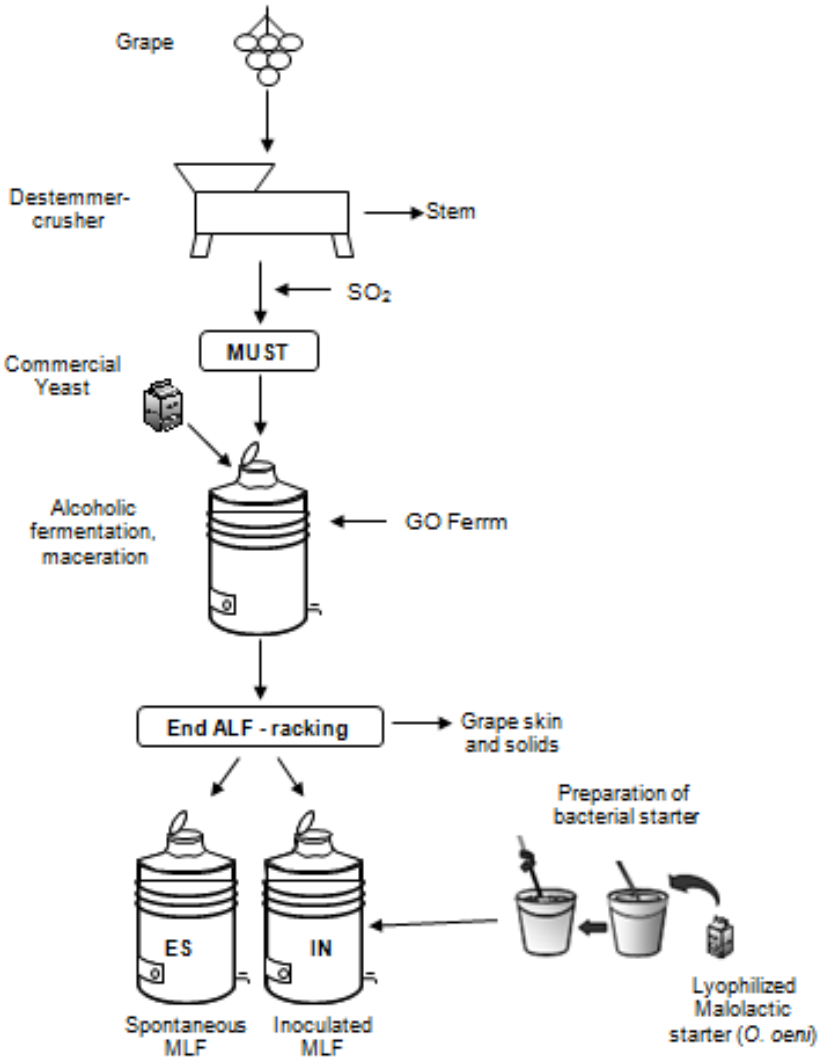
1.7.1 Trials: spontaneous and inoculated MLF

Malolactic fermentations (MLF) were carried out in 25 L batch, using ten wines of Tempranillo and Garnacha varieties from different Catalan regions elaborated in the oenological pilot plant of INCAVI Vilafranca del Penedés during harvest of 2004 (Table 18). As these vinifications were part of another ongoing R&D project, the Tempranillo followed different maceration procedure during the alcoholic fermentation (traditional-group 1 and carbonic-group 2). In consequence Tempranillo wines are treated in 2 groups because of their elaboration.

Table 18. Oenological parameters of wines at the end of alcoholic fermentation

	Code of Wines	Volumetric alcoholic degree %	Volatile acidity (g/L)	pH	Sugars g/L
Tempranillo Group 1	19	10,3	0,46	3,71	1
	38	11,9	0,46	3,94	0,5
	134	12,65	0,36	3,81	1,4
	average ± SD	11,6±1,2	0,4±0,05	3,82±0,1	0,96±0,4
Tempranillo Group 2	20	10,75	0,77	3,67	0,5
	37	11,85	0,72	3,7	0,1
	137	12,5	0,25	3,63	0,2
	average ± SD	11,7±0,8	0,58±0,2	3,6±0,03	0,2±0,2
Garnacha	44	12,2	0,22	3,19	1,7
	60	12,9	0,29	3,23	2,3
	62	13,1	0,4	3,25	4,3
	118	10,7	0,32	3,1	0,2
	average ± SD	12,2±1,0	0,30±0,07	3,19±0,06	2,1±1,6

Figure 8. Spontaneous and inoculated MLF: scheme of trials for Tempranillo group 1 and Garnacha wines.



Preparation of malolactic starter: The inoculation of the malolactic starters for direct inoculation can be prepared by a re hydration step or can be inoculated directly in the wine; the first option is recommended by the supplier. Re hydration step was carried-out: lyophilized bacteria were rehydrated 1 g in 50 mL water; the suspension was stored at ambient temperature for 20 minutes without stirring; batches were inoculated with the suspension to obtain 2 x 10^6 CFU/mL of wine.

Analysis: The typification of bacterial population during the MLF was done according to the method described in Table 22. The sampling was done at the beginning (1/3), during exponential L-malic acid consumption phase (2/3) and at the end (3/3) of the malolactic fermentation. The sampling points were determined by daily measurement of L-malic acid (Table 24). Sensory analysis and wines characterization were done in bottled wine 3 months after the end of MLF (see further in Methodology - analytics section).

1.7.2 Trials: direct and mother tank techniques

For the study of the different seeding techniques, direct inoculation and mother tank methods, a collaboration with a wine cellar from Rioja zone was set up. The entire production process was carried out at industrial scale in concordance with the procedures used at the wine cellar during the campaign 2003. The malolactic starter used was *Oenococcus oeni* Uvaferm Alpha (Lallemand S. A). Tempranillo wines performed the malolactic fermentations in oak barrels (225 L) and stainless-steel tanks (50 HL):

Direct Inoculation. In both series, malolactic starters were inoculated by direct inoculation following supplier instructions:

a) Series A: Alcoholic fermentation was performed in stainless-steel tanks and then, the wine was split in 6 oak barrels. Five barrels (A1, A2, A3, A4 and A5) were inoculated with the commercial malolactic starter strain by direct inoculation. As a control, one of the barrels performed MLF spontaneously (A-ES).
b) Series B: Alcoholic fermentation was performed in stainless-steel tanks and then, the wine was split in 6 oak barrels. Five barrels (B1, B2, B3, B4 and B5) were inoculated with the commercial malolactic starter strain by direct inoculation. One of the barrels performed MLF spontaneously (B-ES).

Mother tank technique:

a) Series T: Five alcoholic fermentations were performed in stainless-steel tanks and then, they performed the malolactic fermentation also in tanks. Inoculation using mother tank method (*pied de cuve*) was performed using a volume of tank T13 as starter: tank T13 was inoculated by direct inoculation using the dosage required to obtain $1x10^6$ CFU/mL following supplier instructions. When MLF started and the amount of L-malic acid decreased until 1/3, 15 HL was taken

from this tank. This inoculums-wine was divided in three parts and used as inoculums for the other tanks (T17, T19 and T20). The inoculums-wine represented 10% of the final volume of the tanks. In one tank the spontaneous fermentation was allowed (T-ES).

Analysis: Samples at 2/3 L-malic acid consumption was taken for the analysis of implantation. Samples were sent from the cellar to the lab in 24 h in a cold box. Wines at end of alcoholic fermentation and end of malolactic fermentation were sent to the lab for determination of biogenic amines and characterization of the wines.

1.7.3 Trials: oenological practices

In order to determine how certain oenological practices would affect the level of implantation and biogenic amines generation, micro-vinifications were performed in oenological pilot plant of INCAVI (Vilafranca del Penedés) using Tempranillo grapes harvested in 2005 from Costers del Segre region (Catalunya). 900 Kg of grapes were processed as explained in Figure 8. Twelve batches of must were additioned with SO_2 (4 g/HL) and 10 batches of them were enriched using Go-ferm (nutrients for yeast, 30 g/HL). From this point, all the batches followed alcoholic and malolactic fermentation independently. Impact of the use of lysozyme, co-inoculation and use of nutrients were performed using a replicate batch, following the schema of trials explained below (Table 19, 20 and 21)

Analytics: lactic acid bacteria count was performed before and after the application of lysozyme and before each inoculation of LAB starter. Implantation analysis with samples taken at 2/3 L-malic acid consumption was carried out. Wine characterization of samples at end of ALF and end of MLF were performed.

1.7.3.a Lysozyme

The lysozyme was applied before the malolactic fermentation as indicated in Table 19.

Table 19. Lysozyme: schema of wine-making trials

Batches	Treatment	Application time	Dosage	MLF
D1 – D2	Lysozyme	must	200 mg/L	IN*
E1 - E2	Lysozyme	Middle of ALF	200 mg/L	IN
F1 - F2	Lysozyme	in must middle of ALF	100 mg/L 100 mg/L	IN
G1 - G2	No lysozyme	-	-	IN
H1 - H2	No lysozyme	-	-	ES*

IN*: seeding with malolactic starter by direct inoculation at end of ALF; ES*: spontaneous

1.7.3.b Inoculation Time or co-inoculation

Malolactic bacterial starters were inoculated at different stages during the ALF as can be seen in Table 20.

Table 20. Inoculation time: scheme of wine- making trials

Batches	Inoculation time	Temperature
A1 - A2	12h from beginning of ALF	Room (25-30°C)
B1 - B2	12h from beginning of ALF	Room temperature until middle of ALF, then in chamber at 15°C
C1 - C2	In ALF, with residual sugars (target 10g/L)	Room (25-30°C)
G1 - G2	End of ALF (standard)	Room (25-30°C)
H1 - H2	No inoculation	Room (25-30°C)

1.7.3.c Nutrients

In order to analyze how the nutrients would affect the implantation level and biogenic amines generation, inoculated and spontaneous MLF were conducted with and without nutrients Go-ferm in the must.

Table 21. Nutrients: scheme of wine-making trials

Batches	Treatment	MLF
G1 - G2	nutrients in must (30g/HL)	IN
H1 - H2	nutrients in must (30g/HL)	ES
I	no nutrients	IN
J	no nutrients	ES

IN: inoculated MLF; ES: spontaneous MLF

The used blend of nutrients, named Go-ferm (Lallemand) is composed of special inactive yeast, produced through a specific autolysis process on yeast biomass in order to obtain high levels of certain essential vitamins (i.e. pantothenate, biotine), minerals (i.e. magnesium, zinc and manganese) and amino acids (Go-ferm, data sheet in www.lallemandwine.com).

MATERIALS AND METHODS
ANALYTICS

1.8.1 Typification of *Oenococcus oeni* during MLF

A specific protocol based on the methodology described by Rodas *et al.* (2005) was developed with the objective of analyzing the lactic acid bacteria population during malolactic fermentation and be able to trace the presence and implantation of the inoculated malolactic starter. This methodology was adapted in order to shorten the time of analysis and to allow the monitoring of the starter by previous typification of the malolactic starter and typification of colonies isolated from MLF samples. Matching of both profiles allow determining the level of implantation of the starter. Protocols are described in Table 22 and 23.

Table 22. Analytical procedure for typification of the bacterial population in malolactic fermentations and determination of level of implantation.

Stage	Description
1	Sampling of wine at 2/3 MLF (or as indicated in the set of trials)
2	Samples were spread on plates in synthetic MLO agar medium (Zuñiga *et al.,* 1993) with 0.003% cycloheximide and then they were incubated in CO_2 atmosphere at 28 ºC for 48-72 hours. Approximately 28 colonies per plate were analyzed.
3	Template DNA was prepared suspending a colony in 20 μl of bi-distilled sterilized water (Sigma).
4	The reaction mixture (20 μl of total volume) was: 1μM of M13 primer (5'-GAG GGT GGC GGT TCT-3') described by Stendid *et al*. (1994), 1μl of cellular suspension, 12,3 μl of bi-distilled sterilized H_2O, 200μM of dNTP, Taq 1X buffer, 2mM of $MgCl_2$ and 1,5 U of ecoTaq polymerase (Ecogen).
5	Initial denaturalization was performed at 94 ºC 5' in a thermocyclator (Eppendorff Mastercycler Gradient) with the cellular suspension, the primer and the bi-distilled sterilized water.
6	Then, the rest of the components of the reaction mixture were added and amplification was carried out with the following temperature cycles: cycles 1 to 15: 94ºC 30", 35ºC 30", 72ºC 1'; cycles 16 to 40: 94ºC 30", 35ºC 30" 72ºC 1', increasing 25" in each cycle (Topkins *et al,.* 1993).
7	The band profiles were obtained by electrophoresis in 1% agarose gel in TBE 1x buffer solution, after staining with 0.01% ethidium bromide and viewing with a Gel Doc 1000-Bio Rad image capturer and Diversity Database 2.2.0 software.
8	The obtained profiles were compared with the band profile of the malolactic starter (see Table 23). Ratio of matching profiles between isolates from wine and the starter are expressed in percentage.

In order to characterize the LAB starter, the following methodology was carried-out:

Table 23. Typification of lyophilized malolactic starter procedure

Stage	Description
1	A small amount of the strain lyophilized directly from the commercially-prepared strain was hydrated in 2 ml of sterilized water and then incubated at 30ºC for 15 minutes on a shaking plate (Eppendorff Compact Thermomixer).
2	An aliquot was spread in MLO agar medium (Zuñiga *et al.,* 1993) with cycloheximide 0.003%. The plate was incubated in a CO_2 atmosphere at 28 ºC for 48-72 hours.
3	Fifteen colonies were randomly selected and identified by *Oenococcus oeni*-specific PCR in accordance with the protocols described by Zapparoli *et al*. (1998).
4	Then, those strains were typified using the protocol described in Table 22.

1.8.2 Lactic acid bacteria count

The lactic acid bacteria count was done when necessary by sampling the must, must/wine or wine from the batches of fermentation. Immediately after the sampling, a series of dilutions in sterile Ringer solution were performed and an aliquot was disposed on a Petri plate containing solidified agar-culture media (all elements being sterilized). Then, the aliquot was spread by using a Digralsky loop; plates were incubated at 30°C for 10 days in CO_2 (5%) atmosphere, until the development of colonies. Results were expressed as CFU/mL.

The culture media was MLO (Zuñiga *et al.,* 1993) with 0.003% cicloheximide for wine samples to avoid the development of yeast.

For bottled wine, 100 ml of wine were filtered using a membrane of pore size of 0,45 µm. The filter was put on the MLO agar with 0.003% cicloheximide surface. Plates were incubated at 30°C for 10 days in CO_2 (5%) atmosphere.

1.8.3 Biogenic Amines determination

In order to determine the production of biogenic amines during the MLF, histamine, tyramine, putrescine and cadaverine contents were measured at the end of alcoholic fermentation (ALF), at the end of malolactic fermentation (MLF) and in must when indicated. The harmonized method established by the Ministery of Agricultural, Fish and Food, Spain (Pons and Rodriguez, 2004) according to Mafra *et al*. (1999) was used.

This method consists in filtration of wine sample using a membrane of 0,45 µm, then derivatization with ortoaftaldehyd in presence of mercaptoetanol in acid media and then injection to a HPLC (High-Performance Liquid Chromatography) system in reverse phase and detection in fluorescence at 340 nm of excitation and 420 nm emission. Results are expressed in mg/L.

The LOD (limit of detection) of the methods was 0,1 mg/L and the LOQ (limit of quantification) was 0,2 mg/L.

1.8.4 Oenologic parameters

Classical parameters were used to characterize the wines and correlate possible incidents during alcoholic and malolactic fermentations. Methods are described by the Organization International de la Vigne el du Vin (2013).

Table 24. Methodology to characterize wines (OIV, 2013)

Metabolite (unit)	Method
Alcoholic degree (%vol.)	Distillation and pycnometry
pH	potencyometric
L-malic acid (g/L)	Enzymatic (Boehringer-Mannheim - Roche)
L-lactic acid (g/L)	Enzymatic (Boehringer-Mannheim - Roche)
Citric acid (g/L)	Enzymatic (Boehringer-Mannheim - Roche)
Volatile acidity (g/L)	Steam distillation and titration
Total acidity (g/L)	Potentiometry Titration
SO_2 (E-220) total and free	Iodometry, potencyometric and titration

1.8.5 Sensory Analysis of wines

Sensory analysis was performed for the wines resulting from the first set of trials "spontaneous and inoculated MLF". The sensory analysis of wines was performed 3 months after the end of MLF, just after the bottling.

The INCAVI's panel of expert tasters on wines (6 experts) performed the test of comparison of pairs (ISO, 1983). This consisted in comparing the 2 batches of the same wine, one being fermented with the starter (IN-MLF) and the other one being fermented spontaneously (ES-MLF).

The flavor descriptors were previously defined by the panel, they were: vegetal, lactic, fruity, caramel, butter, ethanal, burnt, animal, straw, aromatic complexity, structure on mouth, visual quality and global appreciation.

The scoring was done on a continuous scale between 0 and 10.

1.8.6 Statistical analysis

The ANOVA test and coefficient of correlation were carried-out using the software Excel, Microsoft Office 2007, complement: "*statistic analysis toolPack.*"

1.9 RESULTS: SPONTANEOUS AND INOCULATED MLF

In this study, malolactic fermentations were carried-out in Tempranillo and Garnacha red wines after the alcoholic fermentation. Spontaneous and inoculated MLF were compared regarding biogenic amines, sensory properties and wines characteristics. The level of implantation of the LAB starter was determined by typification of the bacterial population during the MLF using a methodology RAPD-PCR based, adapted for such pourpose.

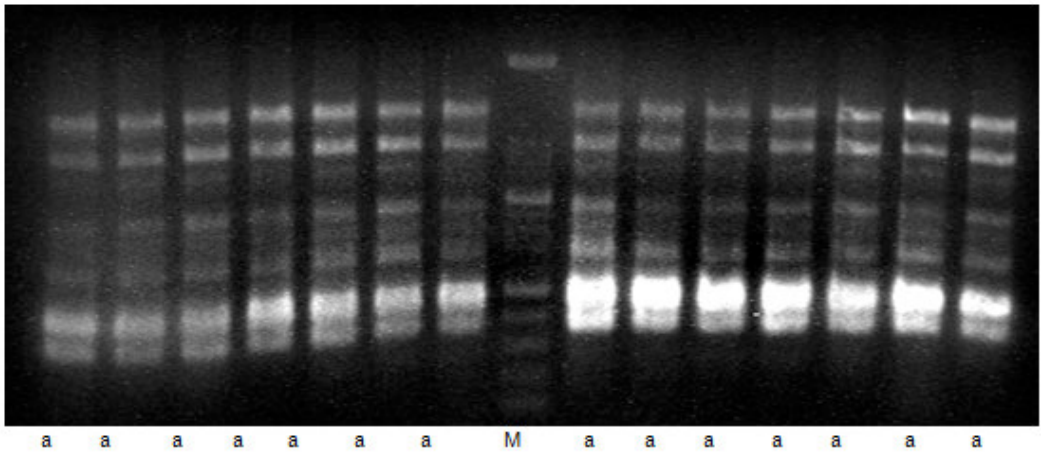
1.9.1 Results: characterization of lactic acid bacteria

All the colonies isolated from the lyophyl of commercial malolactic LAB starter amplified positively with the reaction of PCR specific to *O. oeni* (data not shown).

Figure 9 shows the RAPD-PCR profile of the isolated colonies. Each band is an isolated colony from spread plate of hydrated lyophyl.

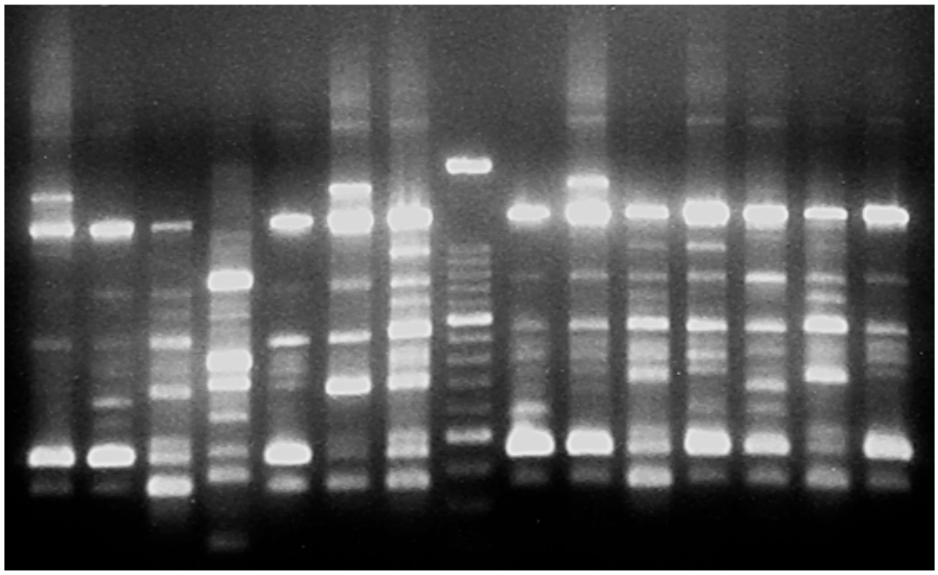
This RAPD-PCR profile was used to determine the implantation of the LAB commercial starter over the wild LAB population by comparison of profiles obtained with the same technique.

Figure 9. Malolactic starter strain profiles obtained by RAPD-PCR method.



a: RAPD-PCR of malolactic bacteria used to inoculate the wines
M: 100 bp ladder molecular weight marker.

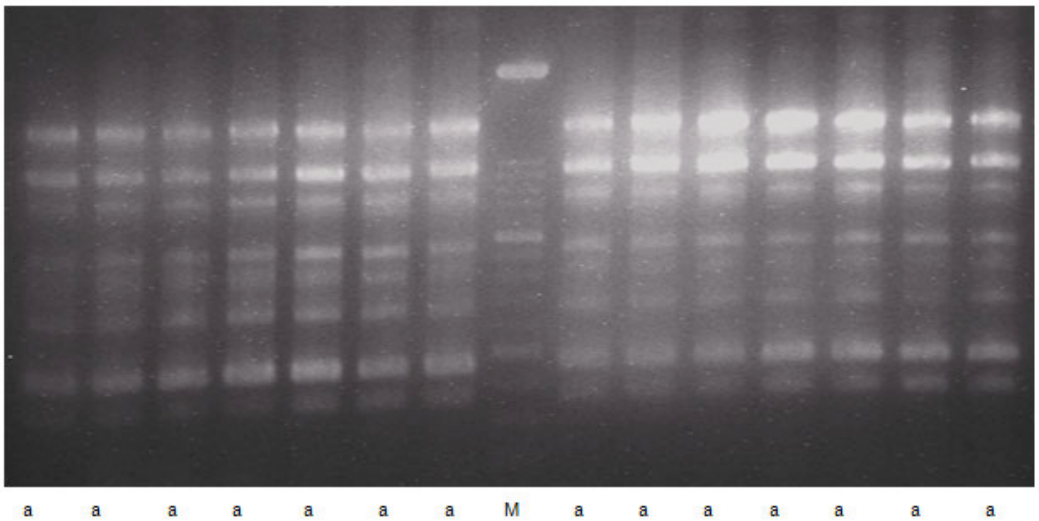
Figure 10. RAPD-PC profiles of LAB isolated from wine: example of spontaneous MLF.



M

M: 100 bp ladder molecular weight marker.

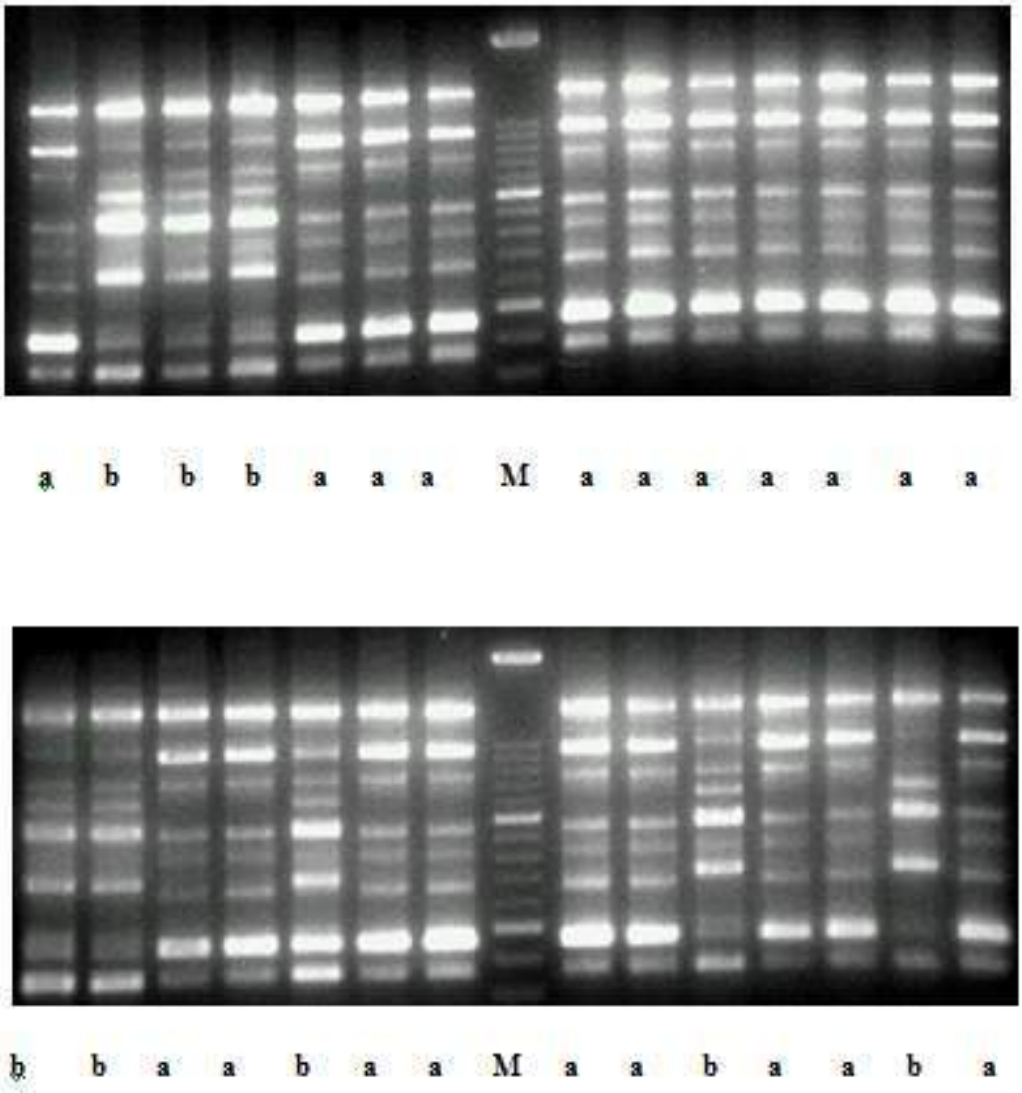
Figure 11. RAPD-PC profiles of LAB isolated from wine: example of full implantation of malolactic starter.



a a a a a a a M a a a a a a a

a: profile from commercial malolactic starter strain;
M: 100 bp ladder molecular weight marker.

Figure 12. RAPD-PC profiles of LAB isolated from wine samples during MLF: example of co-conduction of MLF between the malolactic starter and wild LAB strains.



a: profile from commercial malolactic starter strain; b: other strain;
M: 100 bp ladder molecular weight marker.

1.9.2.a Results Tempranillo group 1: Wine 19

The following figures represent the fermentative kinetics, consumption of L-malic acid (g/L) in function of time (days), of the studied MLFs.

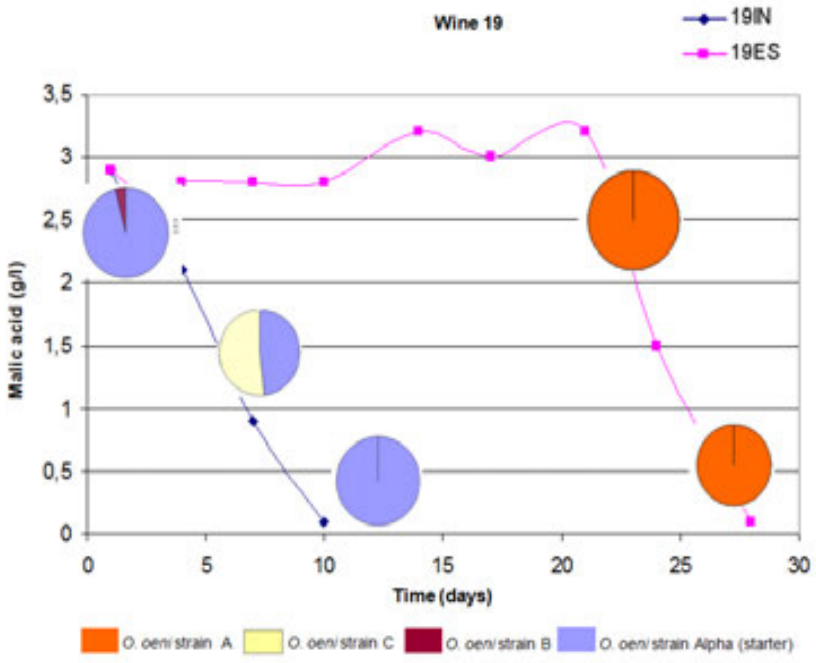
- "IN" means the MLF biologically controlled by the inoculation of the commercial LAB starter (Uvaferm Alpha).
- "ES" represents the MLF conducted by the wild LAB population.

The characterization of the LAB population by typification of isolated colonies using RAPD-PCR is represented with cake distribution, one per sampling point.

WARNING: The denomination of the strains (A, B C, etc.) was done per wine. Therefore, the strain A is the same for batch ES and for batch IN in a given wine. For example, in wine 20, the strain A is the same for 20ES and 20IN but not necessarily the same strain A than in wine 19. This is applicable for the whole set of trials.

The MLF of wine 19 (Figure 13) presented a latency phase of 23 days when performed spontaneously (19ES). This fermentation was conducted by only one autochthonous strain. The fermentation conducted by the commercial starter (19IN) did not have latency phase, consuming the malic acid in 10 days instead of the 28 days taken by MLF 19ES.

Figure 13. MLF kinetics and bacterial population profile of wine 19 (Tempranillo Group 1).



Cake graphics represent population LAB strains at that point of the MLF.

In 19IN, the LAB population at the beginning of MLF (1/3) was mainly the commercial starter with presence of small proportion of a second strain (B).
Another strain (C) was identified at 2/3 of MLF, meaning that in the critical stage of the fermentation, this one was co-conducted between the commercial and the wild strains. At the end of MLF (3/3) only the commercial starter was identified.

Regarding the oenological parameters at the end of MLF (Table 25):

- Wine 19ES had more volatile acidity and citric acid than 19IN. Presence of citric acid in 19ES compromises the microbiological long term stability as this is a fermentable substrate for some microorganisms. Increased volatile acidity suspects the presence of acetic fermentation during the stationary phase.
- The rest of oenologic parameters do not seem to be affected by the fermentative kinetics.

Table 25. Characterization of wines "Tempranillo group 1", at the end of MLF

Analytics	19IN	19ES	38IN	38ES	134IN	134ES
Alcoholic degree (% vol)	10,15	10,35	12,05	12,05	12,6	12,5
Total acidity (g/L)	5,1	4,9	4,4	4,5	4,0	4,0
Volatile acidity (g/L)	0,56	0,78	0,56	0,71	0,41	0,43
pH	3,71	3,72	3,93	3,96	3,8	3,79
Citric acid (mg/L)	23	28	38	11	101	71
L-malic acid (g/L)	0,1	0,1	0,1	0,1	0,1	0,1
L-lactic acid (g/L)	2,1	2,0	2,2	2,2	1,9	1,9

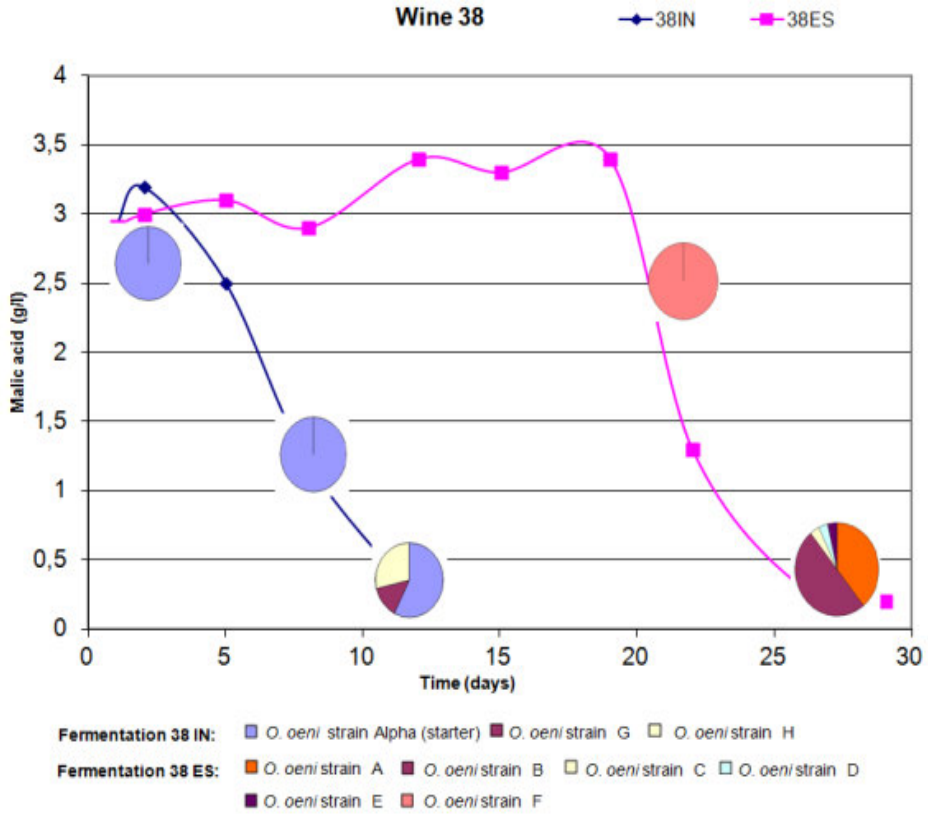
1.9.2.b Results Tempranillo group 1: Wine 38

The spontaneous fermentation of wine 38 (Figure 11), presented an extended stationary phase of 20 days. The autochthonous strain F, drove the MLF during exponential phase. At the end of MLF, a heterogeneous LAB population was identified. The inoculated wine, 38IN, presented a short stationary phase with total implantation of commercial strain Alpha during exponential phase. At the end of MLF, two other strains were observed at considerable proportion (strains H and G).

Regarding the oenological characterization of these wines at the end of the MLF (Table 25):

- Volatile acidity was increased in wine 38ES.
- Citric acid was more consumed in fermentation 38ES. It is possible that some wild strains participating in the MLF, were more adapted to the citric acid metabolic route than the commercial LAB strain. The rest of parameters are similar in both types of fermentations.

Figure 14. Fermentative kinetics and bacterial population profile of wine 38 (Tempranillo group 1)



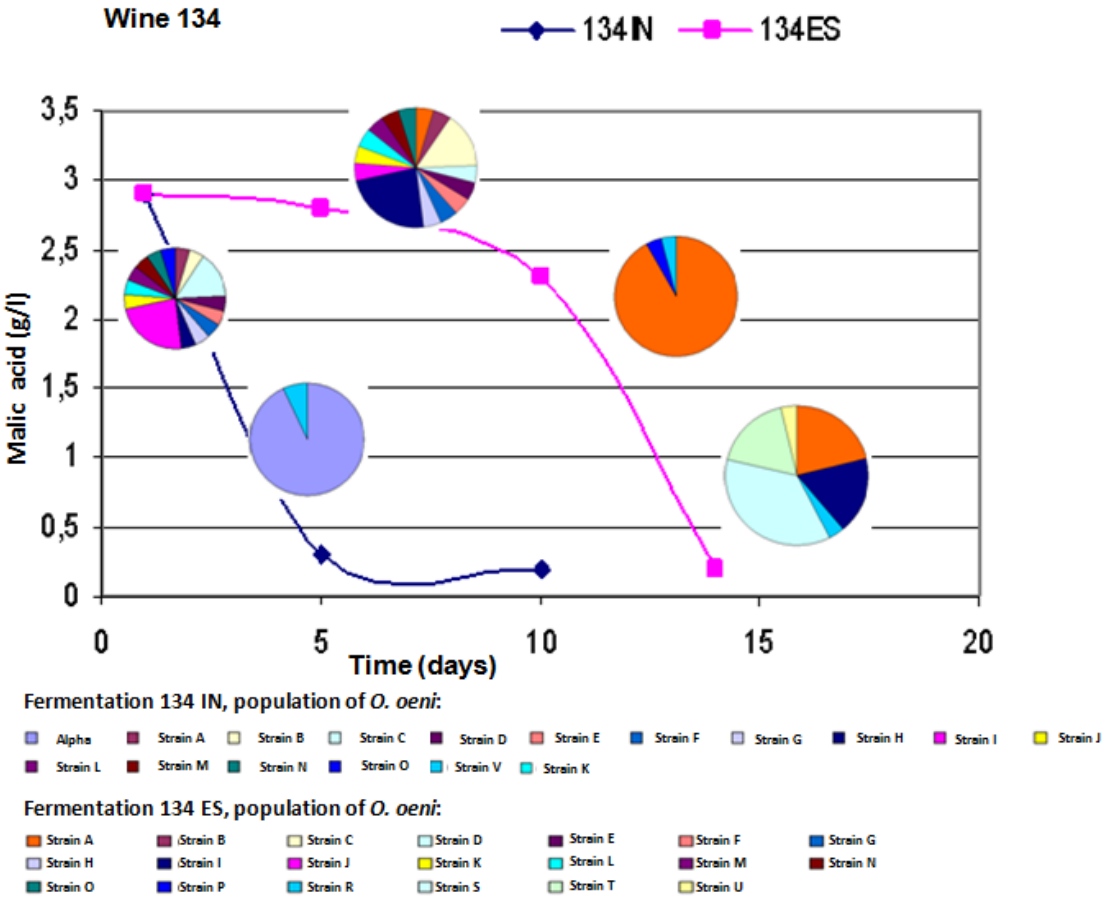
1.9.2.c Results Tempranillo group 1: Wine 134

Wine 134 contained very heterogeneous wild LAB population as 20 different strains were identified.

- Wine inoculated with commercial starter Alpha (134IN), could implant at exponential phase.
- The spontaneous MLF (134ES) was principally conducted during exponential phase by the strain A.

Regarding the resulting wines (Table 25), the wild bacterial population (134ES) was more efficient consuming citric acid than the commercial strain. Wine 134 IN, is likely to be less microbiologically stable due to the presence of fermentable substrate (citric acid). The other parameters were similar in both wines.

Figure 15. Fermentative kinetics and bacterial population profile of wine 134 (Tempranillo group 1).

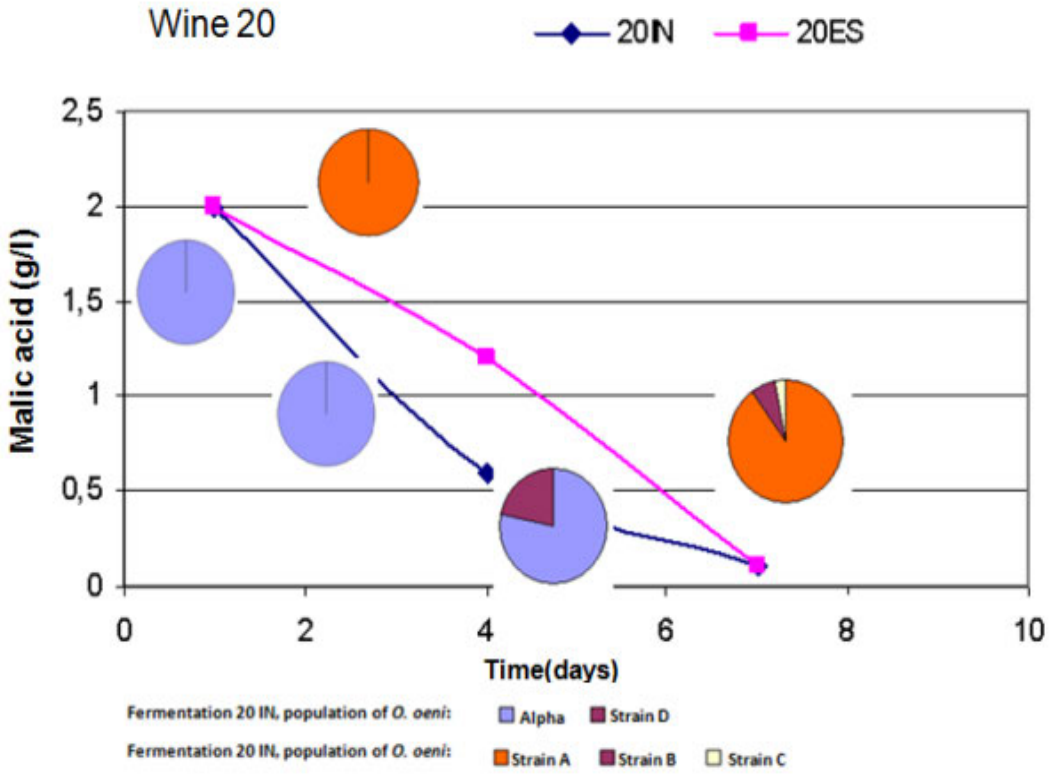


1.9.3.a Results Tempranillo group 2: Wine 20

For the Tempranillo wine 20, both types of malolactic fermentations gave similar results by consuming malic acid in 7 days (Figure 10).

- In wine 20ES, the MLF was conducted principally by strain A.
- LAB Starter strain Alpha was well implanted in this wine.
- Oenologic characteristics of wines are similar in both cases (Table 26)

- **Figure 16. Fermentative kinetics and bacterial population profile of wine 20 (Tempranillo group 2).**



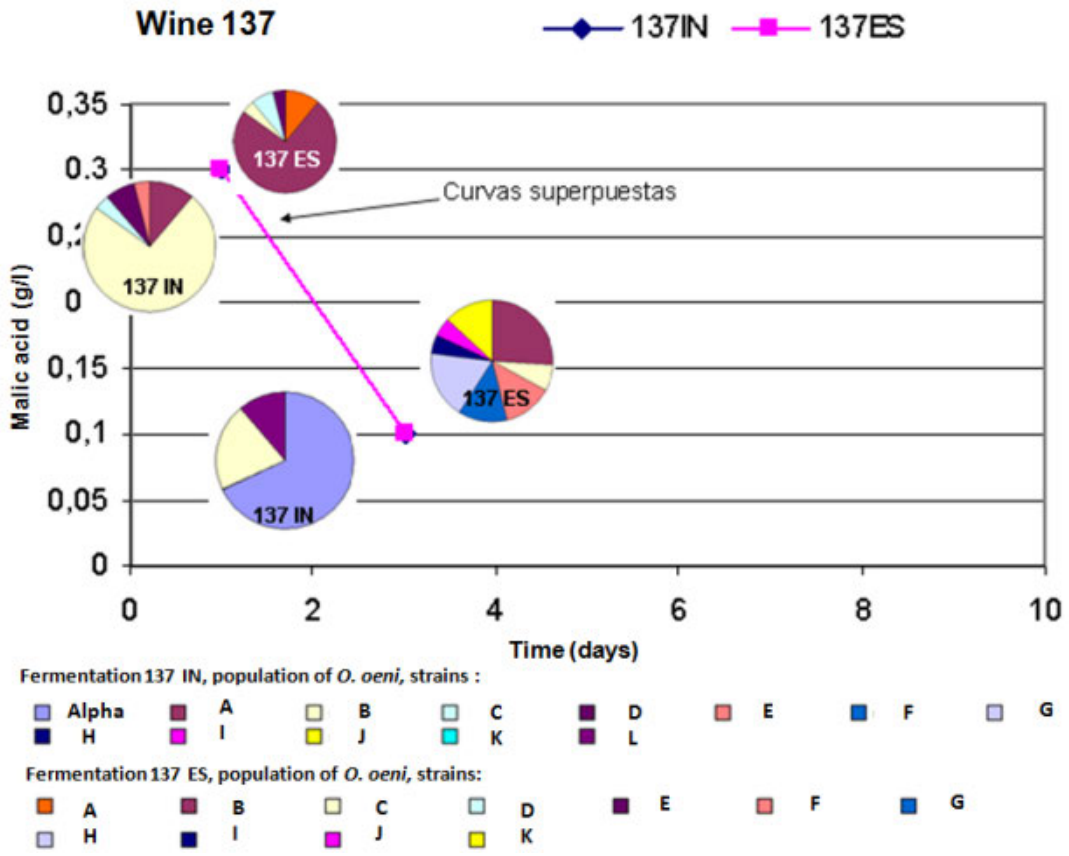
1.9.3.b Results Tempranillo group 2: Wine 137

Wine 137 (Figure 14) contained little malic acid and finalized the MLF in 3 days in both batches. It is suspected that the MLF started during the ALF as the fermentation took place under carbonic maceration. A total of 12 bacterial strains were identified in both batches; strain B (illustrated with yellow in 137 IN and in bordeaux red in 137ES) was well implanted in both fermentations.

In wine 137IN, strain Alpha was not observed at the beginning of the MLF, it achieved an implantation of 68% at the end of the MLF.

In wine 137 ES the citric acid was more consumed than 137 IN (Table 26), the other parameters are similar in both wines.

Figure 17 Fermentative kinetics and bacterial population profile of wine 137 (Tempranillo group 2).



1.9.3.c Results Tempranillo group 2: Wine 37

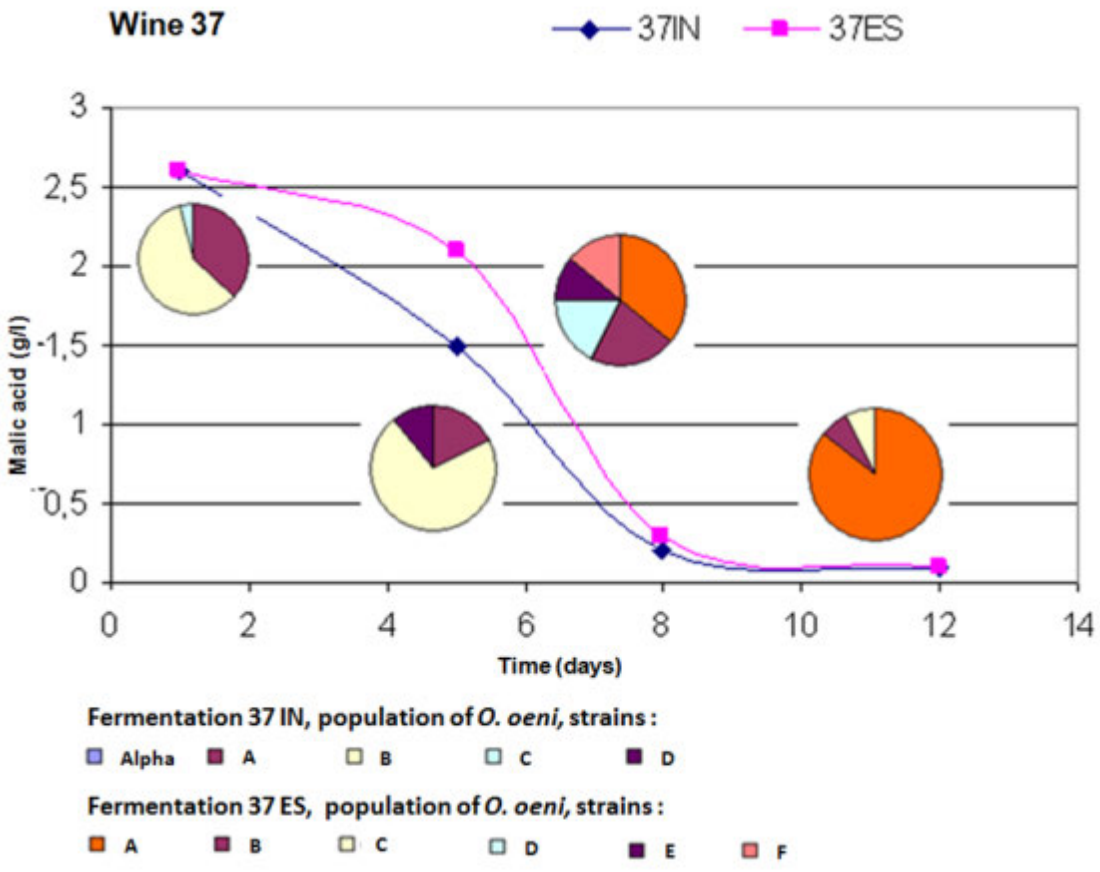
Both fermentations of wine 37 showed similar fermentative kinetics (Figure 15).

The Alpha bacterial starter was not able to impose itself over the wild bacterial population in fermentation 37IN. In fact, none of the isolated bacteria was identified as the bacterial starter in this wine. In wine 37IN, the strain B was the principal driver of the MLF. In wine 37ES, it was strain A.

Volatile acidity was increased in wine 37ES respect to 37IN (Table 26).

In 37ES the citric acid was more consumed than 37IN, but still the remaining citric acid was quite high in 37IN.

Figure 18. Fermentative kinetics and bacterial population profile of wine 37 (Tempranillo group 2).



1.9.3.d Results Tempranillo wines group 2: oenological parameters

Table 26. Characterization of Tempranillo wines (group 2) at the end of malolactic fermentation.

Analytics	20IN	20ES	137IN	137ES	37IN	37ES
Alcoholic degree (% vol)	10,85	10,85	12,5	12,55	12,1	11,9
Total acidity (g/L)	4,9	4,9	5,0	4,8	5,3	6,0
Volatile acidity (g/L)	0,78	0,82	0,29	0,28	0,72	1,39
pH	3,66	3,63	3,63	3,6	3,65	3,65
Citric acid (mg/L)	16	16	189	158	89	29
L-malic acid (g/L)	0,1	0,1	0,1	0,1	0,1	0,1
L-lactic acid (g/L)	1,7	1,6	2,3	2,3	1,7	1,7

1.9.4.a Results Garnacha wines: oenological parameters

Table 27. Characterization of Garnacha wines at the end of malolactic fermentation.

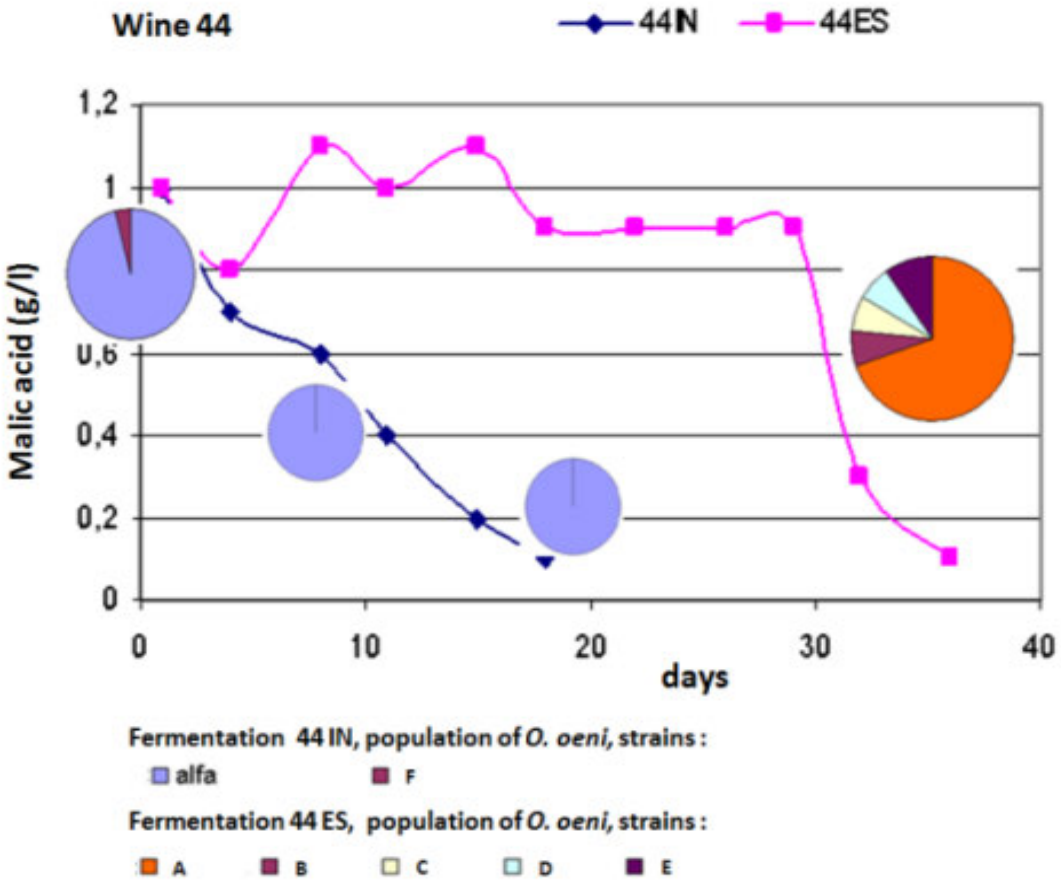
Analytics	44IN	44ES	60IN	60ES	62IN	62ES	118 IN	118 ES
Alcoholic degree (% vol)	12,15	12,1	12,85	12,75	13,05	13,05	10,8	10,75
Total acidity (g/l)	5,7	5,6	6,5	6,1	6,3	6,0	7,1	7
Volatile acidity (g/l)	0,26	0,32	0,34	0,41	0,45	0,52	0,37	0,37
pH	3,17	3,18	3,22	3,26	3,26	3,32	3,08	3,02
Citric acid (mg/l)	274	283	275	257	249	203	232	219
Malic acid (g/l)	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
L-Lactic acid (g/l)	0,7	0,7	1,2	1,1	1,1	1,1	1,8	1,8

1.9.4.b Results Garnacha: Wine 44

In wine 44 (Figure 16), spontaneous fermentation had a latency phase of almost 30 days (44ES). Once L-malic acid started to be consumed by the wild bacterial population, the MLF was finished in 6 days. Strain A was predominant in this phase.

In inoculated wine 44IN, there was no stationary phase and the L-malic acid was consumed in 18 days. In this batch, strain Alpha achieved the complete implantation during the whole MLF. Regarding the characteristics of the resulting wine (Table 27), volatile acidity was slightly higher in wine 44ES, other parameters were similar. Due to technical problems, only one sample of LAB in 44ES was taken.

Figure 19. Fermentative kinetics and bacterial population profile of wine 44 (Garnacha).

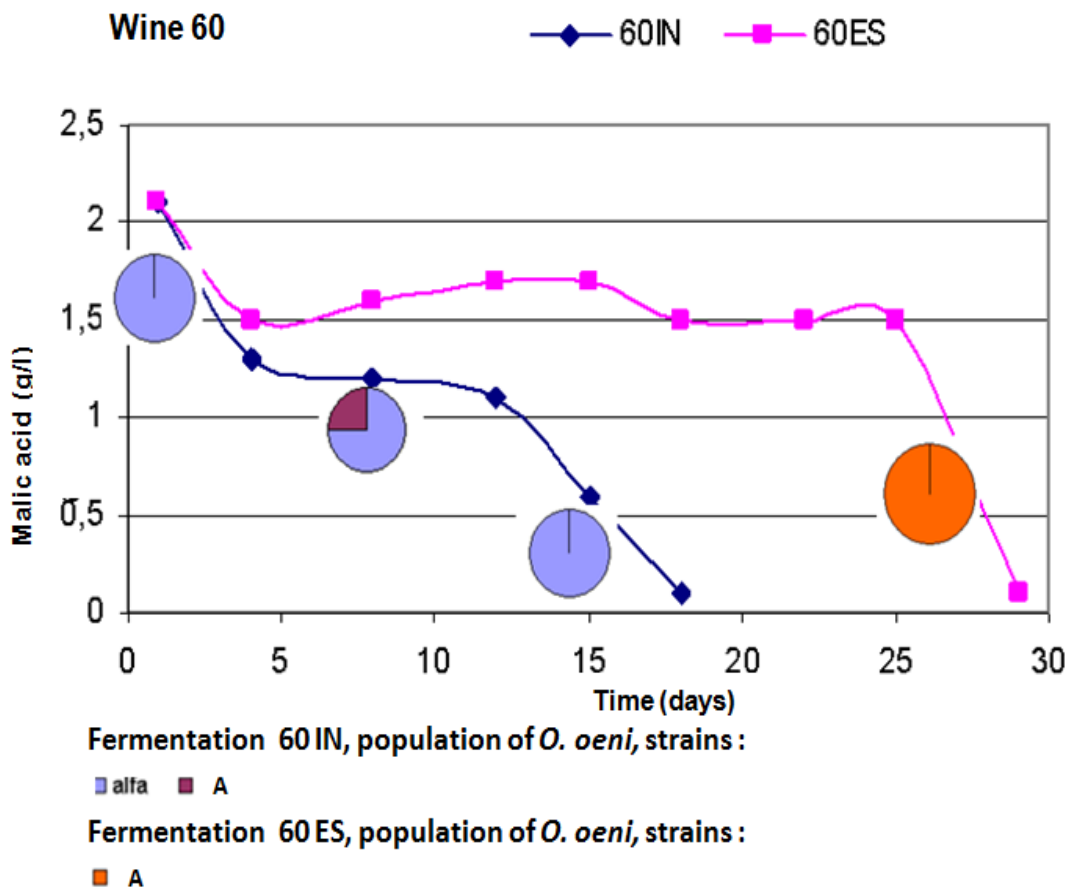


1.9.4.c Results Garnacha: Wine 60

Wine 60ES (Figure 17) presented a latency phase of 25 days; L-malic acid was principally consumed by strain A.

Strain Alpha is almost completely implanted all along the MLF in wine 60IN, with co participation of strain A at the exponential phase. Volatile acidity is slightly increased in 60 ES (Table 27).

Figure 20. Fermentative kinetics and bacterial population profile of wine 60 (Garnacha).



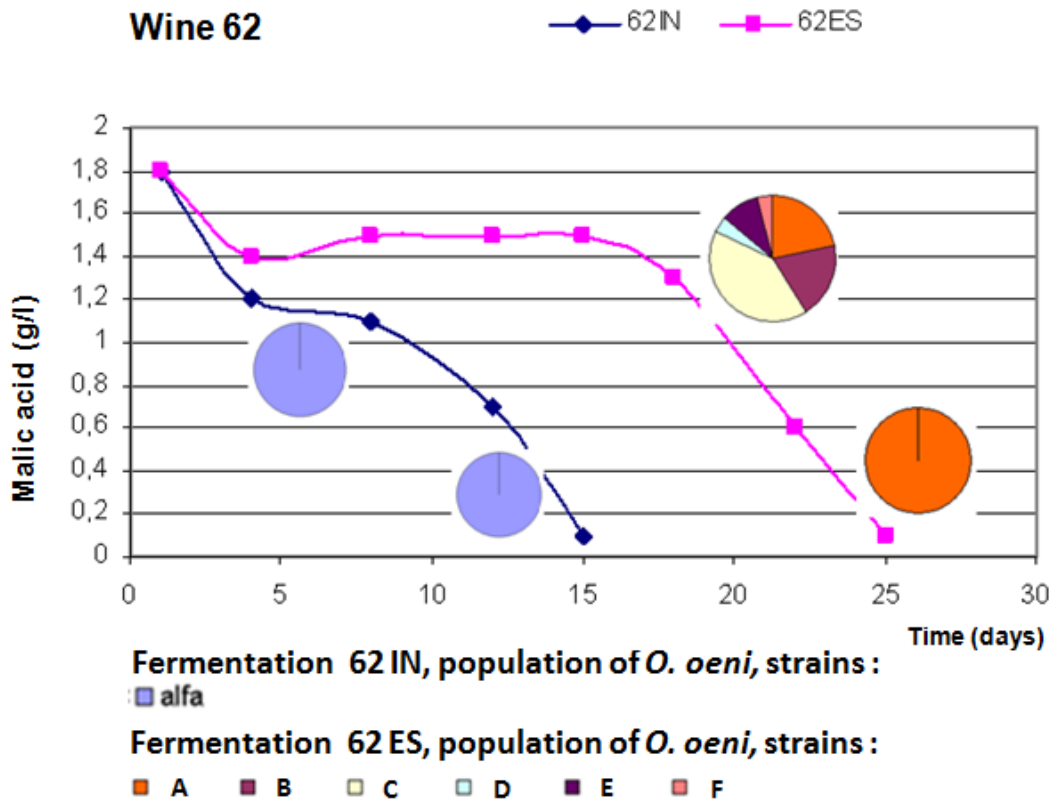
1.9.4.d Results Garnacha: Wine 62

Wine 62ES (Figure 18) had a stationary phase of 17 days; at the beginning of the MLF 6 strains were identified with A, B and C strains as predominant. At the end of MLF, only strain A was identified.

Strain Alpha (62IN) was totally implanted in 62IN, and the L-malic acid was consumed in 15 days without stationary phase.

Volatile acidity was higher and citric acid reduced in 62ES (Table 27).

Figure 21. Fermentative kinetics and bacterial population profile of wine 62 (Garnacha).



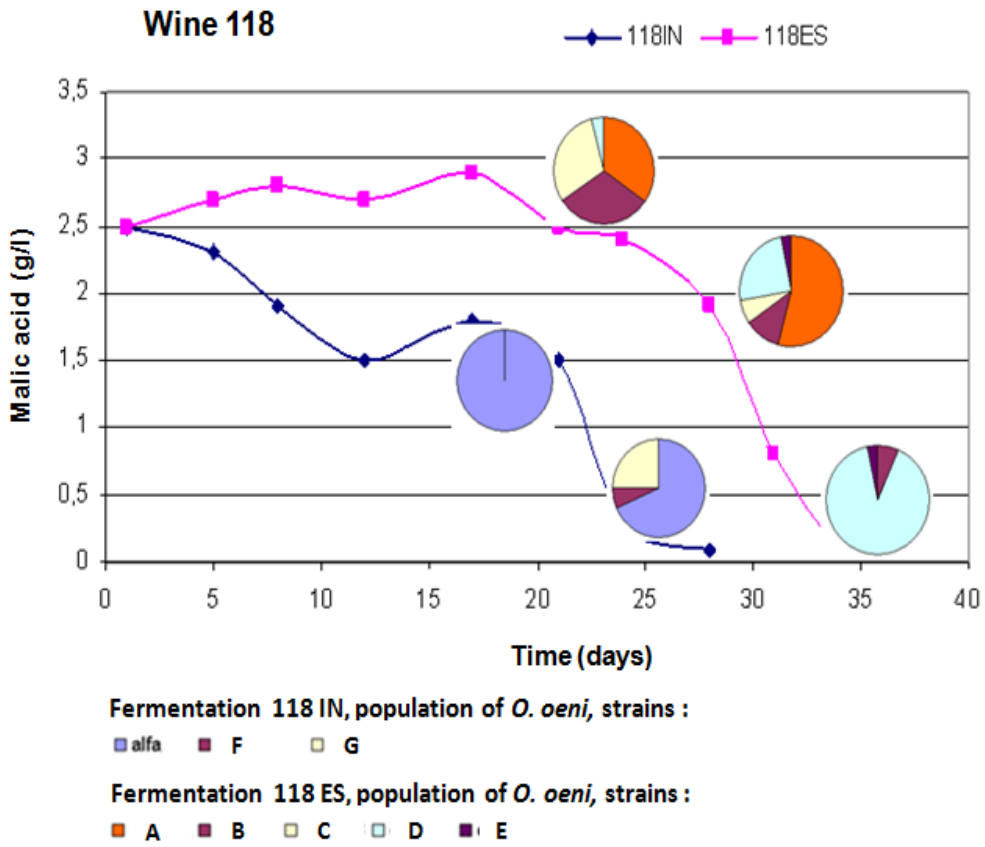
1.9.4.e Results Garnacha: Wine 118

In wine 118ES (Figure 19) strain D (light blue) started in a small proportion at 1/3 of MLF, increasing its presence all along at the end of MLF. The opposite happened with strain A (orange), where due to the competition with D its presence was reduced at the end of MLF.

The commercial bacterial strain was totally implanted during the exponential phase of MLF (118IN), and co-drove the fermentation with strain G at the end of the MLF. The fermentative kinetic in this wine was slower respect to the other wines which indicated some difficulties of adaptation of strain Alpha to this wine.

In wine 118ES citric acid was more consumed (Table 27).

Figure 22. Fermentative kinetics and bacterial population profile of wine 62 (Garnacha).



1.9.5 Results: implantation of bacterial starter on malolactic fermentations

The analysis of the LAB profiles at different sampling points of the MLF revealed that in inoculated wines (IN) the implantation of the commercial starter is quite high at the beginning of the MLF (exceptions are wines 134IN, 137IN and 37IN). This may be due to the fact that the starter is inoculated at high concentration of $1x10^8$ CFU/mL in contrast with the wild bacterial population size at this stage of the process. When consumption of L-malic acid was at the exponential phase (sampling point 2/3), the bacterial starter was in competition for the substrate with the autochthonous LAB population. This is observed in the previous figures where strain Alpha co-drove the fermentations with other strains (wines 19IN, 134IN, 137IN and 60IN). This point of fermentative kinetics is fundamental for the configuration of the LAB population driver until the end of the MLF, where the starter can be totally implanted or be dominated by the wild LAB population. The level of implantation of the inoculated starters was calculated as an average of the results obtained by the typification of the LAB population at 2/3 and 3/3 of MLF (Table 28).

The duration of MLF was variable in inoculated wines (Table 28). Surprisingly, the Garnacha wines which obtained highest levels of implantation also had the slowest MLF. The opposite was observed for the Tempranillo wines, which in average finalized the MLF quite fast, but the implantations were globally poor.

Table 28. Implantation of malolactic LAB starter and MLF duration

Group	Wines "MLF IN"	% RAPD-PCR profiles matching LAB starter profile		% implantation of commercial LAB starter [c]	MLF duration (days)
		2/3 malic acid consumption [a]	3/3 malic acid consumption [b]		
Tempranillo 1 wines	19	48	100	74	13
	38	100	57	78,5	13
	134	nd	93	93	7
Tempranillo 2 wines	20	100	78	89	5
	137	nd	68	68	3
	37	nd	0	0	12
Garnacha wines	44	100	100	100	20
	60	75	100	87,5	15
	62	100	100	100	15
	118	100	70	85	27

nd: not determined; c=average between a and b

In order to determine if the difference of average implantation between the groups of wines was significant, “ANOVA single factor” test was used (Table 29). There is not enough information to reject the null hypothesis (p-value= 0,186). Therefore the averages are not significantly different. It must be noted that the variance within the groups was quite high. Nevertheless, taking into account the % implantation of the three groups a certain trend can be observed: Garnacha wines seem to have a better and more homogeneous implantation compared to the Tempranillo wines where the response was high variable.

Table 29. Analysis of variance: level of implantation per wines groups

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Tempranillo 1 - %implantation	3	245	81,6666667	100,333333
Tempranillo 2 %implantation	3	157	52,3333333	2164,33333
Garnacha %implantation	4	372	93	66

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2913,06667	2	1456,53333	2,15676209	0,186316131	9,54657802
Within Groups	4727,33333	7	675,333333			
Total	7640,4	9				

1.9.6 Results: implantation and wines characteristics

In order to analyze the impact of the wine characteristics on the level of implantation of malolactic starter in inoculated MLF, a matrix of correlation was done crossing implantation, pH, alcoholic degree and initial L-malic acid content (Table 30).

It was found that the pH, had a negative moderated correlation (-0,32) with the implantation of the LAB starter culture (Table 30). This indicates that the more acidic the pH is, the more efficient the implantations tend to be. Even if this correlation is

moderated, this data needs to be taken into account for the technical implications in the winemaking process.

Table 30. Analysis of correlation. Implantation and wines characteristics

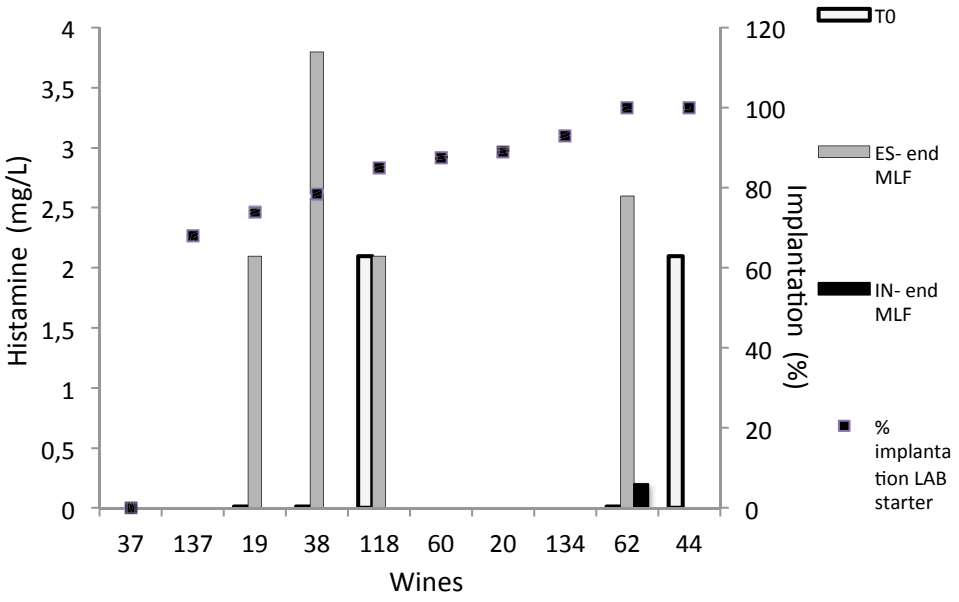
all data	pH	Implantation LAB starter	alcohol
pH	1		
Implantation LAB starter	-0,32447135	1	
alcohol	-0,12057766	0,06481227	1
L-malic initial	0,35607255	-0,13879816	-0,33195536

Regarding the characteristics of the wines (Table 27) the Garnacha wines presented more restrictive conditions for bacterial development like low pH, higher alcoholic degree and reduced L-malic acid content than the Tempranillo wines. The Garnacha wines also presented overall higher levels of implantation than Tempranillo wines.

1.9.7 Results: histamine production during MLF

Histamine was measured in all wines before inoculation (T0) and once the MLF was finalized (Figure 20). The level of implantation is also shown (%) in the second Y axis. In the X axis, the wines are plotted in increasing percentage of implantation.

Figure 23. Histamine production during MLF and implantation of LAB starter



- Histamine was not detected at T0 and remained undetectable after the MLF in wines 37, 137, 60, 20 and 134 indicating that spontaneous and inoculated LAB populations in these wines did not produce histamine during MLF. The implantations were variable: 0% (wine 37), 68% (wine 137), 87% (wine 60), 89% (wine 20) and 93% (wine 134).
- Reduction of histamine was observed in wine 44 in the two MLFs (IN and ES), as wine contained 2,2 mg/L of histamine at T0 and at end of MLF (IN and ES), histamine was not detected. The implantation here was 100%.
- Wine 118 kept the histamine level in batch ES (2 mg/L) constant and it was reduced in the inoculated MLF to 0,1 mg/L, the implantation here was roughly 90%.
- Spontaneous MLF produced more histamine than its respective inoculated batch in wines 19, 38 and 62. In wine 62, a slightly production of histamine in the inoculated MLF was observed. The implantations were 74% for wine 19, 78% for wine 38 and 100% for wine 62.

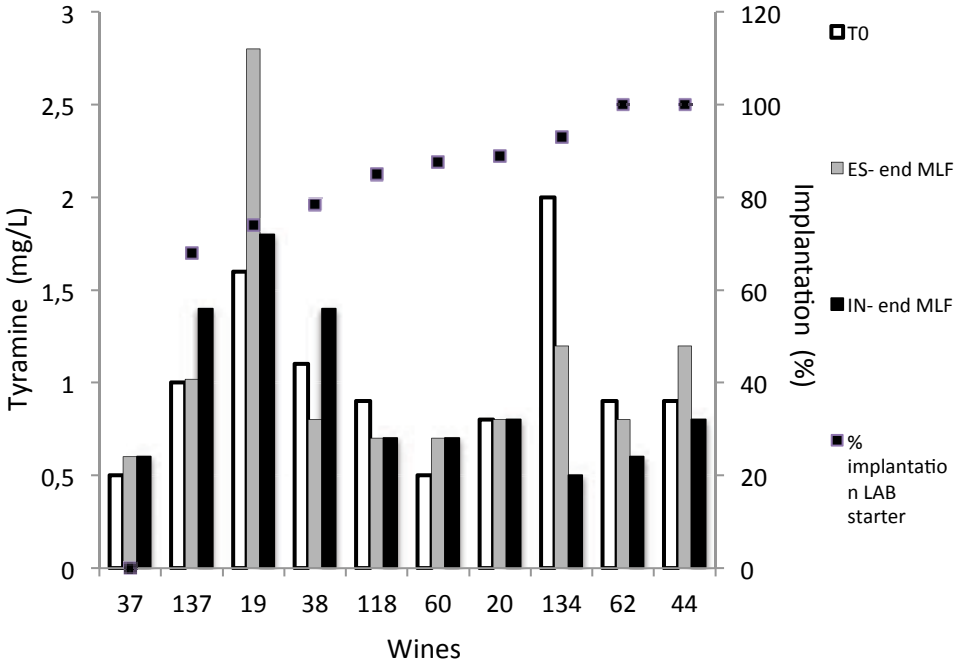
1.9.8 Results: tyramine production during MLF

The tyramine content of wines was measured at T0 and end of MLF (IN and ES) (Figure 21). All of them presented tyramine at the end of ALF (T0) with a maximum 2 mg/L (wine 134). It is to be noted that globally the tyramine content in all the MLF was not high and taking into account the LOD (0,1 mg/L) and LOQ (0,2 mg/L), different cases regarding tyramine produced during the MLF can be observed:

- More production of tyramine was observed in MLF conducted by the autochthonous LABs in wines 19 and 44 (ES). In wine 19, batch ES produced 1,2 mg/L more tyramine during MLF than its respective IN batch which produced a small amount (0,3 mg/L). In wine 44, batch IN kept its tyramine concentration constant after MLF, while ES batch produced a small amount of 0,3 mg/L. The implantations were 74% (wine 19) and 100% (wine 44).
- The tyramine was kept constant in wines 20, 37 and 60 after the MLF in both batches (ES and IN). The implantation was 89%, 0% and 87,5% respectively.

- The inoculated MLF (IN) produced more tyramine during MLF than its respective ES batch in wine 137 and wine 38. In wine 137, inoculated batch produced 0,4 mg/L while the ES MLF kept its tyramine value constant. In wine 38, The IN batch produced 0,3 mg/L of tyramine and the ES batch reduced its tyramine level to about 0,3 mg/L, thus 0,6 mg/L gap between the both batches was observed. The implantation of these wines was 68% for wine 137 and 78% for wine 38.

Figure 24. Tyramine content at T0 and end of MLF and level of implantation in inoculated MLF

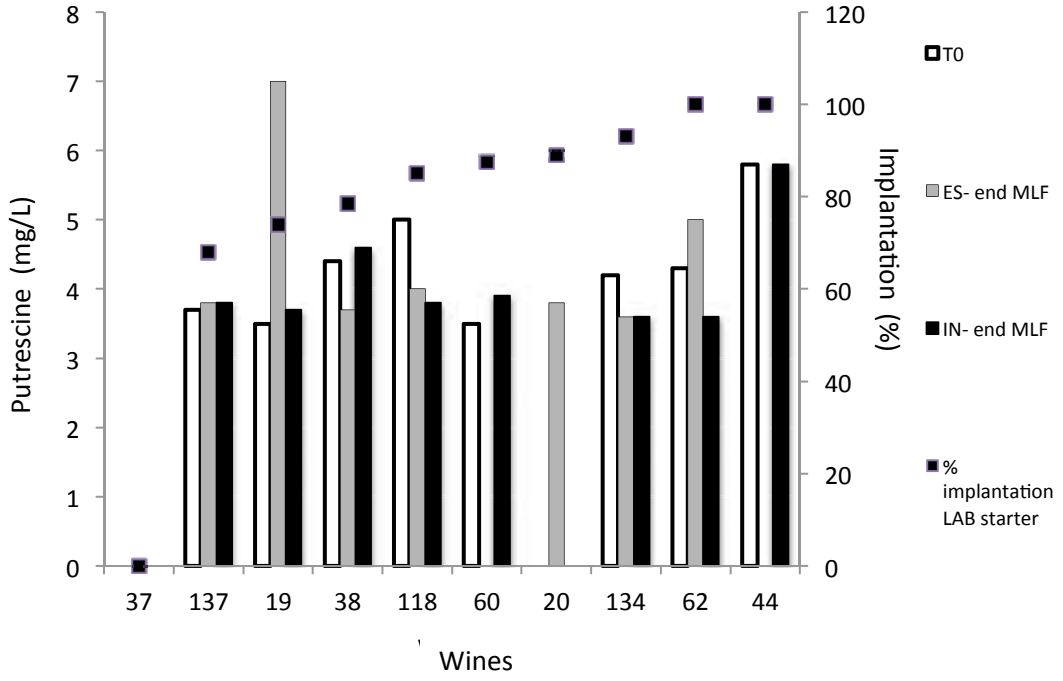


- A reduction of tyramine content from T0 to the end of MLF was observed in wines 134, 118 and 62. In wines 134 and 62 the effect was more pronounced in the batch inoculated with the LAB starter (IN) where the tyramine was degraded by 1,5 mg/L in wine 134. In wine 62 ES a slight degradation was observed (0,3 mg/L). In wine 118 reduction of tyramine was observed but because this concentration is included in the LOQ limit of the method, the tyramine level is considered constant in this wine. The implantation of the LAB starter in these cases was 93% for wine 134, 100% for wine 62 and 85% for wine 118.

1.9.9 Results: putrescine production during MLF

The putrescine was quantified in almost all wines at the end of ALF (T0), exceptions were wines 20 and 37 (Fig 22). Regarding putrescine evolution from T0 to the end of the MLF in inoculated batches (IN), different cases can be observed:

Figure 25. Putrescine content at T0 and end of MLF and level of implantation in inoculated MLF



- Putrescine was constant in IN-MLF compared to its content at T0 in wines 37 (no production), 137, 19, 38 and 44.
- A slight increase of putrescine in the inoculated MLF (IN) was observed in wine 60, where the implantation was high (87%).

For the malolactic fermentations conducted by the autochthonous bacterial populations, (ES-MLF), different scenarios were observed:

- Increase of putrescine in wines 19, 20 and 62. In wine 20 there is no putrescine prior to MLF or inoculated batch, everything is produced only in the spontaneous MLF.
- Decrease of putrescine in wines 38, 118, 60, 134 and 44. The degradation of putrescine is quite significant in wines 60 and 44 in the ES wines.
- Constant in wines 37 (no production) and 137.
- Wines 37, 137 and 134 had the same behavior independently of the type of MLF, inoculated of spontaneous.

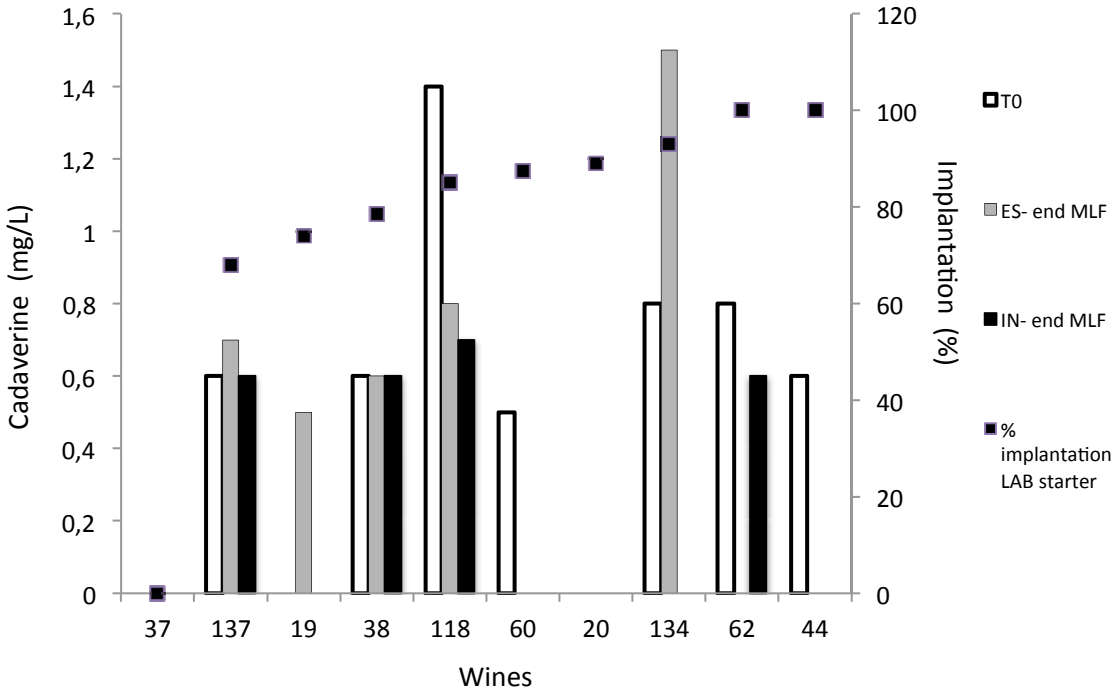
1.9.10 Results: cadaverine production during MLF

The cadaverine content of wines at T0 and end of MLF of inoculated (IN) and spontaneous (ES) wines were measured (Figure 23). Almost all of them contained cadaverine at T0, exceptions were wines 37, 19 and 20. Globally the measured cadaverine was low in all batches.

Taking into account the inoculated batches with the LAB starter (IN-MLF), the cadaverine levels produced during MLF decreased or were kept constant:

- Decrease of cadaverine from 1,4 to 0,7 mg/L was observed in wine 118IN.
- The reduction observed in wine 62IN is included in the LOQ, therefore it is also considered constant.
- Constant cadaverine from T0 to the end of MLF in IN batches were observed in wines 37 (no production), 137, 38, 20 (no production) and 62 (difference into LOQ limit).
- In summary, no production of cadaverine was observed in inoculated wines (IN-MLF) because at the end of MLF, cadaverine content is equal or lower than its level at end of ALF.

Figure 26. Cadaverine content at T0 and end of MLF and level of implantation in inoculated MLF



Regarding the spontaneous batches (ES-MLF), the cadaverine content at the end of MLF compared to the T0 was variable:

- An increased content of cadaverine was observed in ES batches of wines 19, and 134. In wine 19, there was no cadaverine at T0 or in IN MLF, everything is produced by the wild LAB population. In wine 134 cadaverine is increased from 0,8 (T0) to 1,6 mg/L (end MLF).
- Decreased content of cadaverine is observed in wines 118, 62 and 44 in ES-MLFs.
- Constant levels of cadaverine from T0 to end of MLF were observed in wines 37 and 20 (no production), 137 and 38.

1.9.11 Results: biogenic amines content in inoculated and spontaneous MLF

In order to determine if the average value of biogenic amines content (histamine + tyramine + putrescine + cadaverine) in inoculated MLF against the spontaneous MLF were significantly different, an ANOVA test "Two-Factor without Replication" was performed (Table 31).

Table 31. ANOVA: Biogenic amines in inoculated and spontaneous MLF

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
37	2	1,2	0,6	0
137	2	11,22	5,61	0,0722
19	2	14,7	7,35	6,845
38	2	18,8	9,4	15,68
118	2	12,5	6,25	2,205
60	2	9,3	4,65	0,005
20	2	1,6	0,8	0
134	2	10,6	5,3	2,88
62	2	12,6	6,3	3,38
44	2	12,8	6,4	0,08
IN-total biogenic amines	10	44,8	4,48	4,59066667
ES-total biogenic amines	10	60,52	6,052	12,3799289

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows (wines)	133,94408	9	14,8826756	7,12799128	0,0036783	3,1788931
Columns (IN vs. ES)	12,35592	1	12,35592	5,91781294	0,03781748	5,11735501
Error	18,79128	9	2,08792			

With a p-value (columns) of 0,037 and a significant level of 0,05, the null hypothesis is not confirmed, therefore the biogenic amines content in IN and ES malolactic fermentations are different. The group of spontaneous MLF (ES) contains higher biogenic amines (average: 6,05 mg/L) than the inoculated MLFs (IN) (average: 4,48 mg/L).

1.9.12 Results: correlation between implantation and biogenic

In order to analyze whether the implantation of the LAB starter has an impact on the production of biogenic amines during the MLF, a correlation analysis was performed (Table 32). To analyze the biogenic amines only generated during the MLF, a subtraction was calculated. For example, "tyramine produced during MLF= tyramine at the end of MLF- tyramine at the T0 of MLF".

The correlation with histamine was not done due to insufficient data.

Table 32. Correlation matrix for implantation level and biogenic amines produced during MLF

Tempranillo 1 (end-T0)	implantation	tyramine	putrescine	cadaverine
implantation	1			
tyramine	-0,96	1		
putrescine	-0,97	0,99	1	
cadaverine	-0,97	0,99	1	1

Tempranillo 2 (end-T0)	implantation	tyramine	putrescine
implantation	1		
tyramine	0,05	1	
putrescine	0,29	0,97	1

Garnacha (end-T0)	implantation	tyramine	putrescine	cadaverine
implantation	1			
tyramine	-0,45	1		
putrescine	0,13	0,82	1	
cadaverine	0,560	-0,28	0,064	1

In Tempranillo group 1, wines 19, 38 and 134, there is a negative correlation between tyramine, putrescine and cadaverine content and the level of implantation of the LAB starter.

With a higher level of implantation, less biogenic amines are produced during the MLF for this group of wines.

In Tempranillo group 2, wines 20, 137 and 37, no correlation was observed; this can be due to the high dispersion of data obtained for implantation.

For Garnacha wines 44, 60, 62 and 118, there is a moderated negative correlation between the tyramine produced during the MLF and level of implantation as also observed in Tempranillo group 1. In Garnacha wines, the cadaverine is moderately correlated with the level of implantation meaning that with a higher implantation, more cadaverine is produced during the MLF. Regarding raw data, the cadaverine is not produced at high levels in this group of wines (<0,1 – 0,7 mg/L).

Finally, a high correlation between the putrescine and the tyramine content was found in the three groups of wines.

1.9.13 Results: impact of implantation on volatile acidity

In order to analyze the impact of the type of malolactic fermentations on the development of volatile acidity, ANOVA test "2 factors without replication" was performed (factors: wines/ MLT type). Analyzing all data together, there is no significant difference between the 2 types of MLF indicating that inoculated and spontaneous MLF are not significantly different (ANOVA not shown). The same ANOVA test was done per group of wines. In Garnacha wines which had higher implantation levels, the difference between IN and ES is significant (p-value< 0,05, Table 33).

Table 33. Garnacha wines. ANOVA for volatile acidity; factors wines and type MLF.

Anova: Two-Factor Without Replication
Volatile acidity - Garnacha wines

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
44	2	0,58	0,29	0,0018
60	2	0,75	0,375	0,00245
62	2	0,97	0,485	0,00245
118	2	0,74	0,37	0
MLF-IN	4	1,42	0,355	0,00616667
MLF-ES	4	1,62	0,405	0,00723333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows (wines)	0,0385	3	0,01283333	22,6470588	0,01457456	9,27662815
Columns (Type MLF)	0,005	1	0,005	8,82352941	0,04904717	10,1279645
Error	0,0017	3	0,00056667			
Total	0,0452	7				

For both groups of Tempranillo wines, the difference on volatile acidity is not significantly different between inoculated and spontaneous MLF (Tempranillo 1 p-value=0,15;

Tempranillo 2 p-value=0,39, ANOVA test not shown). Nevertheless, regarding the characterization of Tempranillo wines (Table 25 and 26), with few exceptions the spontaneous MLF contained higher volatile acidity than IN-MLF.

Analyzing these data, it is logical to think that the level of implantation affects the volatile acidity development during the MLF. A correlation analysis between both variables (three groups of wines, IN-MLF data) was done (Table 34). A moderately negative correlation was found between implantation and volatile acidity indicating that a higher implantation results in a lower volatile acidity in wines.

Table 34. Correlation analysis between implantation level and volatile acidity.

	Implantation	Volatile acidity
Implantation	1	
Volatile acidity	-0,483	1

1.9.14 Results: sensory analysis

Sensory analysis was performed to determine if the use of LAB starters during the MLF changes the sensory properties of the wines. Each wine was tasted individually, comparing simultaneously, a wine with spontaneous MLF (ES) and a wine with inoculated MLF (IN). A pre-tasting was done to define the descriptors to be evaluated for each pair of wines as they were perceived as very different. This is why the descriptors ethanol, straw and animal are only evaluated for some wines. Wine 37 was not tasted due to technical issues.

The evaluation of wines as average of the scores attributed by the panel of expert tasters is presented in the Figures 24 and 25. Wines 20, 38 and 44 presented some descriptors with significant differences between IN and ES, as color, ethanal and mouth structure.

With the objective to determine if a common descriptor can be linked to the type of MLF, a “two way with replication” ANOVA test was run. The factors were “wine” (9 levels) and “MLF type” (2 levels, IN and ES). The response or dependent variable were the scores of sensory descriptors. An individual analysis was done for each sensory descriptor (Table 35).

For factor “wines”, the results showed that wines were significant different (p≤0,1) for the descriptors vegetal, fruity, color intensity, mouth structure, flavor complexity and in global appreciation.

For the factor “type of MLF” (inoculated or spontaneous), results showed significant differences for ethanal, being the spontaneous MLF with highest average. This observation only concerns wines 20 and 44 because this descriptor was only evaluated in this pair of wines.

Figure 27. Sensory analysis of wines with differencies in some descriptors between the treatments (ES and IN). Average value among the taster panel

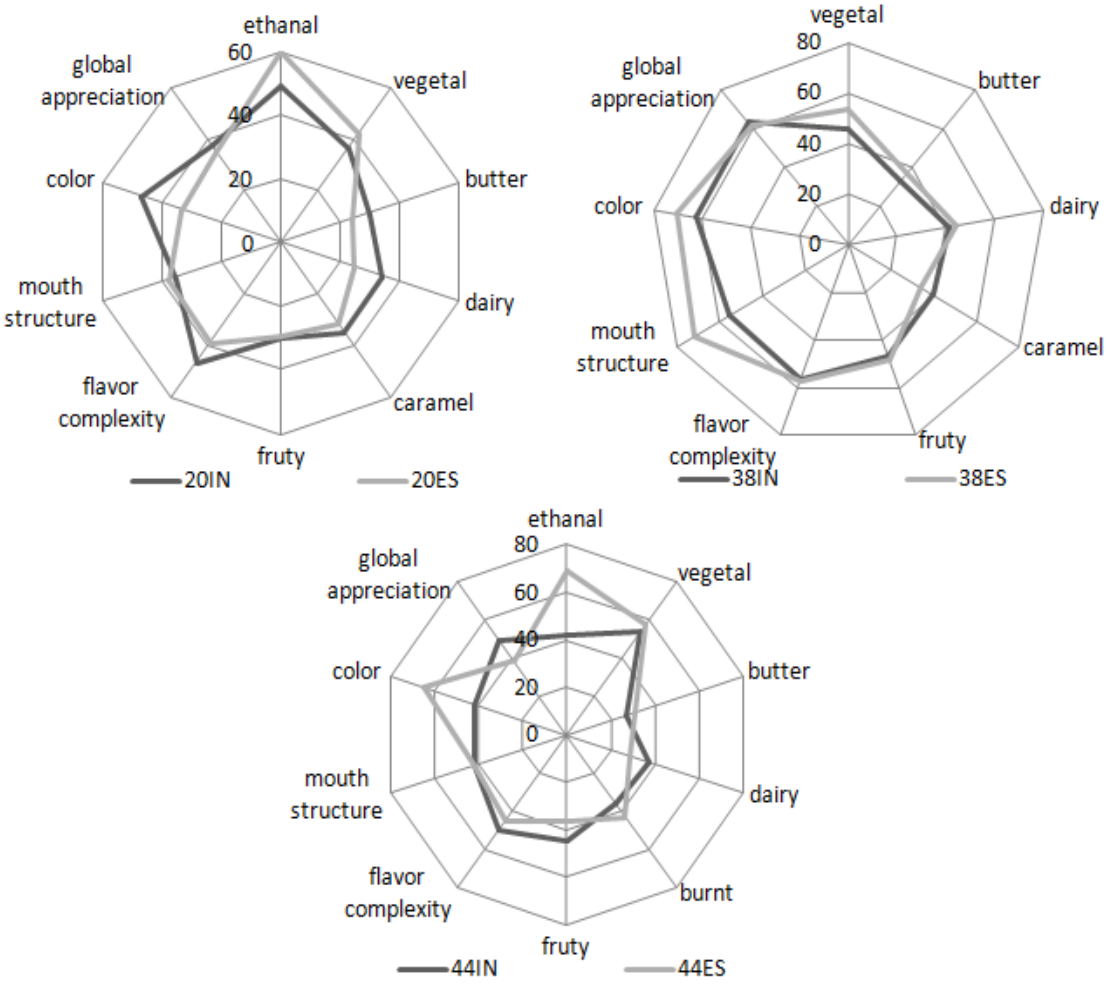
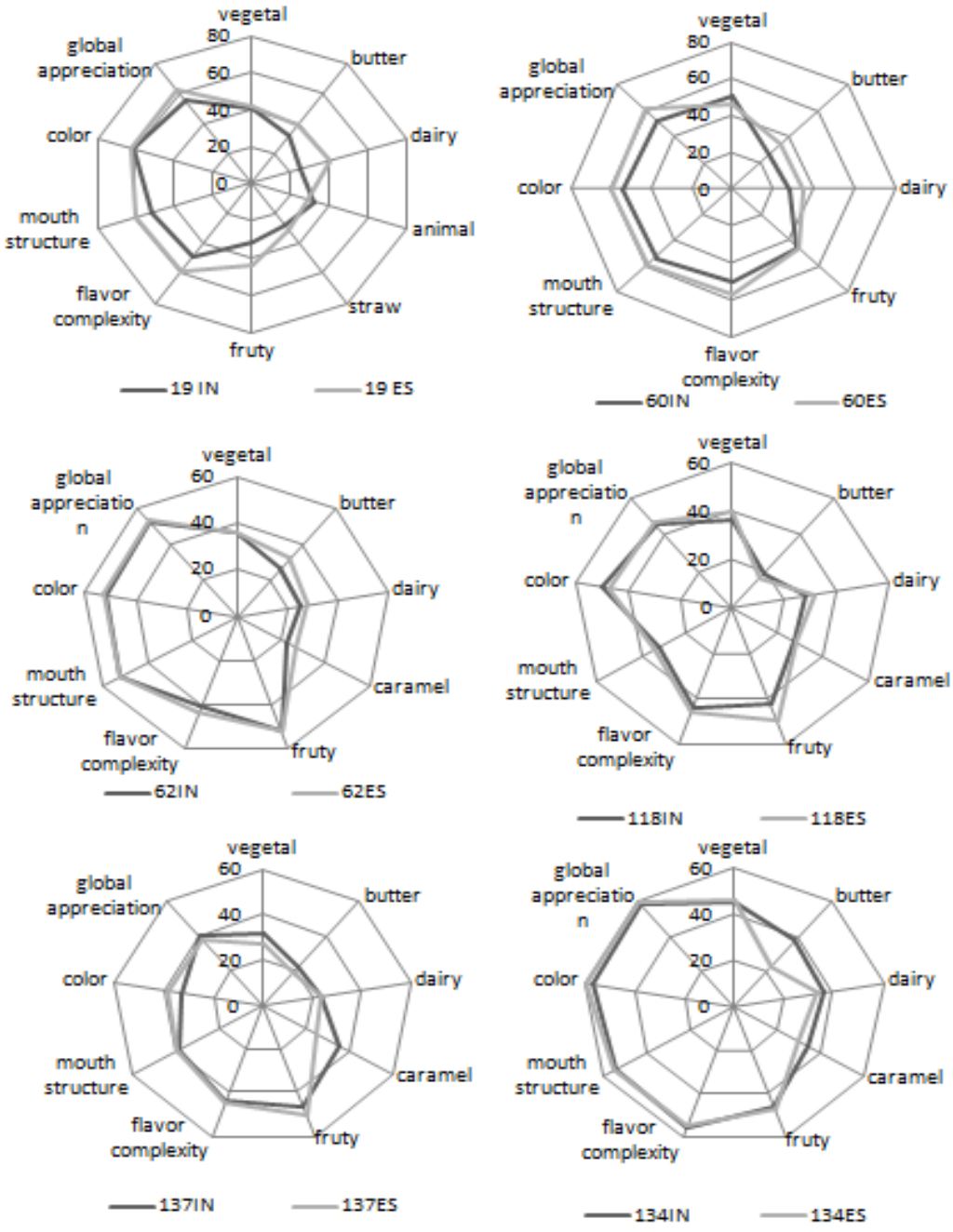


Figure 28. Sensory analysis of wines with slight differences between the treatments (ES and IN). Average values among the tasters panel



Color intensity and mouth structure were significant different according to the MLF type, being in both cases the spontaneous MLF with higher average (Table 35).

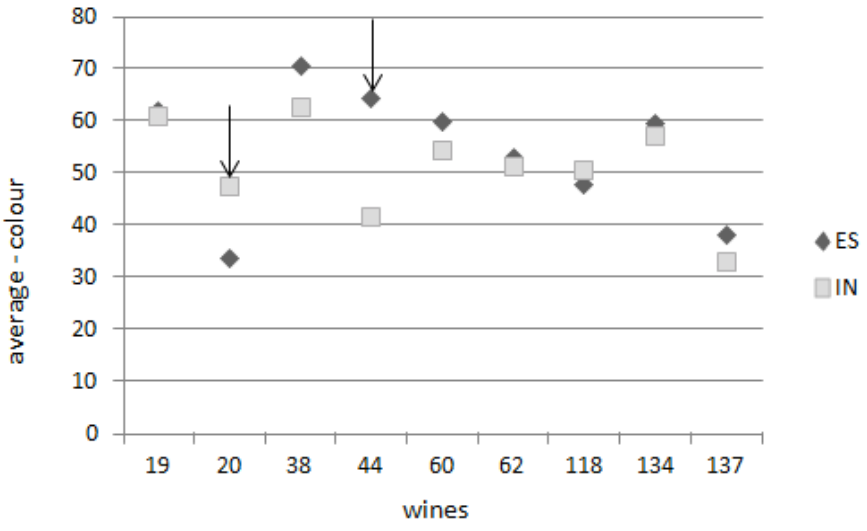
Table 35. Sensory analysis. Anova results, factors wines and MLF type

P-values	Wine	MLF Type	Interaction wine/MLF Type
Ethanal (wines 20 and 44)	0,93	0,01(ES)* *	0,27
Vegetal	0,006	0,64	0,98
Butter	0,15	0,88	0,90
Dairy	0,32	0,78	0,87
Animal (wine 19)	n.a.*	0,77	n.a.
Straw (wine 19)	n.a.	0,78	n.a.
Fruity	0,01	0,46	0,89
Color	3,8E-08	0,11(ES)	0,09
Flavour Complexity	0,004	0,55	0,85
Structure in mouth	7,0E-08	0,11(ES)	0,92
Global Appreciation	1,0E-06	0,91	0,86

n.a*.: not applicable; (ES)* *: higher score for spontaneous MLF

As the descriptor color intensity has an interaction with the factor wine, therefore, this information needs to be taken carefully as in one case the average is higher for IN MLF and in the other case is for ES MLF. In conclusion, it is difficult to conclude that color is a descriptor characteristic of the type of MLF (Figure 26).

Figure 29. Scoring for the descriptor color



Arrows indicate where significant differences were found.

1.10 DISCUSSION: SPONTANEOUS AND INOCULATED MLF

RAPD-PCR for monitoring of starter implantation during MLF

The use of molecular techniques for the characterization of the bacterial population during the malolactic fermentation was of fundamental importance for the monitoring of the malolactic starter. The adapted methodology RAPD-PCR based, allowed the typification of the starter and its recognition between the bacterial populations in order to determine its level of implantation during the process. Analyzing isolated colonies developed in MLO media allowed the semi-quantification (%) of the level of implantation which was not possible by the methodologies that proposed the extraction of DNA directly from a wine sample during the MLF (Gindreau *et al.,* 1997 and 2003). Other methodologies, PFGE based, demonstrated their efficiency with similar purpose as described by López *et al.* (2008, 2011, 2012) and Tenorio *et al.* (2005). Finally both methodologies are adapted for the monitoring of LAB starter during the MLF. Thus, the choice of the technique will be mainly based on availability of equipment and team expertise.

Diversity of *O. oeni* in spontaneous MLF

The typification of the autochthonous bacterial population in Garnacha and Tempranillo wines revealed interesting polymorphism of *O. oeni* in the studied malolactic fermentations. Several strains were identified that co-drove the MLFs in spontaneous batches; heterogeneity of bacterial populations seems to be the natural state of these malolactic fermentations. In some cases, strains that were indentified in the spontaneous batch were also found in the inoculated wine indicating that some strains were able to survive and compete with the inoculated malolactic starter. In these cases, the level of implantation was compromised.

Implantation of LAB starter

The typification of the bacterial population by RAPD-PCR method and the determination of the level of implantation of LAB starter reveal that the starters are present in the malolactic fermentation in different proportions, varying from no implantation (0%) to full implantation (100%) with all the range of possibilities in between. Different levels of implantation of malolactic starters were also reported by López *et al.* (2008, 2011, 2012), Masqué *et al*. (2007) and Romero (2010).

These different levels of implantations are due to the presence of autochthonous bacteria in the inoculated wines during the MLF.

More stressful wine conditions as low pH and high ethanol content were correlated with higher implantation of the malolactic starter as observed in the case of Garnacha wines which obtained higher implantations than the Tempranillo wines. In Tempranillo wines there is a high variability of level of implantation and the consequences from the technical point of view are not predictable.

The relation between higher implantation levels and hard wine conditions might be due to the fact that the wild bacterial populations were more stressed and were less prompt for competition when the LAB starter was inoculated. LAB starters are selected to conduct MLF in hard wine environments, hence both bacterial populations present different adaptation to stress. Probably due to the same mechanism, wines with less alcohol content and higher pH as was the case with Tempranillo wines, obtained moderated levels of implantation in the inoculated MLF because they were co-conducted with the autochthonous bacteria while the non-combined SO_2 decreased. Moreover, with this work we have demonstrated that higher pH makes the implantation of the used malolactic starter more difficult due to competition against wild bacteria population which may not be stressed in gentle wine conditions such as high pH (>3.6). The same relationship between pH and starter implantation was found by López *et al.* (2012) in Tempranillo wines.

Another aspect to be taken into account regarding starters' implantation is the timing of the MLF process. In wine cellars, the term "good implantation" usually refers to the fast completion of the MLF process after the inoculation of the LAB starter. In this work, we observed that a fast MLF is not necessarily linked to a good implantation of the LAB starter (analyzed by RAPD-PCR). Therefore, in wines with high pH (>3.6), a fast MLF should raise doubts on the implantation of LAB starter due to the active participation of wild bacterial population.

This study supports the fact that the uncertainty regarding the implantation of the LAB starters is real and wine cellars and research groups should monitor their malolactic fermentations by molecular techniques to ensure the process is under control.

Biogenic amines

Histamine, tyramine, putrescine and cadaverine were detected in wines at the end of alcoholic fermentation. Part of the putrescine might come from the grapes (Brodequis *et al*., 1989; Bauza *et al,.* 1995) and other amines were certainly synthesized via

decarboxylating metabolism (Landete *et al.,* 2005c; Pramateftaki *et al.,* 2012) before the malolactic fermentation in these wines. This indicates that the responsible of their production might be the presence microorganisms with amino decarboxylase capabilities at this stage.

Different scenarios were observed with respect to the development of biogenic amines during the MLF. In some cases biogenic amines increased during the MLF, in other cases, they were kept constant or even reduced. The latter case is particularly interesting as few works are available where a reduction of biogenic amines during MLF was observed. Coton *et al.* (1999) reported degradation of ethylamine and putrescine during MLF and González-Marco *et al.* (2005) found a variety of biogenic amines at the end of ALF which concentration decreased during the MLF, principally for phenyl ethylamine and spermidine. Garcia-Marino *et al.* (2010) observed on the other side a decrease of ethylamine and cadaverine during winemaking process in organic wines and quality press wines. However, these results were overlooked and not discussed. Marques *et al.* (2008) explained the reduction of isoamylamine and tyramine present in the must during the ALF and MLF by co-precipitation of biogenic amines with fine lees. Additionally, the fact that reduction of putrescine and ethylamine initially present in must strongly decreases during AF was explained as a consequence of the normal metabolic processes of yeast and bacteria (Del Prete *et al.,* 2009). Smit *et al*. (2012) observed reduction of putrescine during the MLF in wines using different malolactic starters of *O. oeni, L. hilgardii, L. plantarum* and in spontaneous MLF. Years before, Lonvaud-Funel *et al.* (2001) proposed that starters can degrade undesirable amines in wines because the levels of biogenic amines were very low compared with non-inoculated wines. But this hypothesis was downplayed by her because another study proved that microorganisms able to produce amines were unable to degrade tyrosine in synthetic media (Moreno-Arribas *et al*., 2000).

Although the degradation of biogenic amines was previously described in other food matrices, only recent works provided an insight on the phenomenon of degradation of biogenic amines in wines. In 2011, it was found that strains of *Lactobacillus spp. Pediococcus spp. and O. oeni* from wine were able to degrade histamine, tyramine and putrescine in culture media. This property was less pronounced in wines as ethanol and polyphenols dramatically decreased amines degradation properties of cell and cell –free suspensions (Garcia-Ruiz *et al.*, 2011). Capozzi *et al.* (2012) found *Lactobacillus plantarum* strains able to degrade putrescine and tyramine in wine-like media and the presence of amine oxidase enzymes was suggested. In a very recent article from March

2013 (Callejon *et al.,* 2013), the enzyme responsible of amines degradation belonging to two wine strains of *L. plantarum* and *P. acidilactici* was isolated. The gene that code for this enzyme was also identified in the DNA of these amine-degrading strains. A single enzyme, the amine degrading or multicopper oxidase (MCO) was pointed as the enzyme responsible of amines degradation in wines. Moreover, degradation of histamine, tyramine and putrescine by *Lactobacillus spp. Pediococcus acidilactici* in synthetic media and red wines were demonstrated. Amines degrading property seems to be strain dependent except for *Lactobacillus plantarum* which appear to be a general trait for this species. The existence of mediator compounds in wine that collaborate in amine degradation was suggested as higher levels of degradation were obtained in wine than in synthetic media. Regarding our results, the reduction of histamine, tyramine, putrescine and cadaverine were produced with more or less effect on four wines (Garnacha wines 44, 62, 118 and Tempranillo wine 134) and a trend cannot be defined as amines degradation was observed in inoculated and spontaneous MLF. These results might suggest the presence of wine bacteria able to synthesize MCO enzymes during the MLF in the studied wines. On the other hand, one can wonder if the presence of fermentable substrates at this stage, such as residual sugars, L-malic acid, citric acid and amino acids should be the preferred metabolic routes for wine bacteria instead of biogenic amines degradation. Thus other mechanism that explains biogenic amines reduction during MLF should also be considered. The influence of wine components on the conditions for MCO genetic expression and activity in wines remains to be understood.

Biogenic amines and implantations

Wines produced with spontaneous MLF contain significantly more total biogenic amines at the end of the MLF than wines inoculated with malolactic starters as confirmed by the ANOVA test. These results confirm that the use of LAB starters to manage the malolactic fermentation is a valid strategy to control the levels of biogenic amines in wines as spontaneous MLF usually results in more biogenic amines (Gerbaux and Monamy, 2000; Gindreau *et al*., 2003; Izquierdo-Pulido *et al*., 1999; Vidal and Bover, 2001; Landete *et al*., 2005d; Hernández-Orte *et al,.* 2008; Marques *et al.,* 2008; Pramateftaki *et al.,* 2012).

Moreover, the level of implantation influenced the biogenic amines content as a negative correlation was found between the level of implantation and tyramine and cadaverine in

Garnacha and Tempranillo wines. The same was observed for putrescine in Tempranillo wines. This indicates that the degree of participation of wild bacteria during the MLF drives the increase of biogenic amines in inoculated MLF with low or moderated level of implantation. The starter used in this study (Uvaferm Alpha) is a low biogenic amines producer as determined by Moreno-Arribas *et al.* (2003); consequently the wild bacteria population with amino acids decarboxylating metabolism is pointed as the responsible for the development of these metabolites in the studied inoculated wines as described by other authors (Landete *et al.*, 2007a-b-c).

Higher pH has been linked with biogenic amines development in several works (Gerbaux and Monamy, 2000; Coton *et al,.* 1999; Moreno- Arribas *et al,.* 2003; Landete *et al*., 2005d) and it is generally accepted that this effect is mostly due to a faster growth of microorganism (Lonvaud-Funel and Joyeux, 1994). In consequence high pH increases the probability of having strains able to form amines (Landete *et al*., 2005d; Wibowo *et al.,* 1985). Moreover, with this work we have demonstrated that higher pH gives place for the development of autochthonous bacteria which compete with the malolactic starter complicating its implantation, delivering inoculated wines with biogenic amines levels comparable to non inoculated wines.

Impact of implantation on volatile acidity

Special interest was put on the volatile acidity because of the negative impact it has on the sensorial quality of red wines. Volatile acidity is composed of ethyl acetate and acetic acid in a ratio of 1/10, the first smells like acetone and the second one like vinegar (Noble *et al.,* 1987). Therefore, volatile acidity must be minimal in red wines. Acetic acid can be produced by LAB as a result of the heterofermentative metabolism in presence of remaining sugars after the ALF (Palacios, 2005a). On the other hand, acetic acid and acetoinic (C4) compounds developed during the MLF also result from the citric acid metabolism but citric acid is consumed slower than malic acid is degraded (Lonvaud-Funel, 1999). Rozes *et al.* (2003) observed that in presence of phenolic compounds it seems that strains of *O. oeni* consume citric acid and thehalose (in absence of hexoses) yielding acetate, molecules that count for volatile acidity in wines.

Therefore, the production of volatile acidity during MLF depends on the bacterial metabolism. The composition of the LAB population and its metabolic state should play an important role. In our study, the analysis of the impact of levels of implantation of the LAB starter on the volatile acidity revealed that moderated to poor implantations in inoculated wines deliver levels of volatile acidity as high as spontaneous MLF.

Having in mind that malolactic starters are selected for their reduced metabolism of hexoses and pentose sugars (1st order criteria) and for their limited formation of volatile acids (3rd order criteria), the development of volatile acidity in wines during the MLF can be managed by using malolactic starters if they obtain high levels of implantation. Otherwise, moderated or poor implantations lead to volatile acidity in wines that might be similar to those where the spontaneous MLF was allowed without using LAB starters. Other authors also observed an increased development of volatile acidity with LAB starters not fully implanted (López *et al.,* 2011).

Impact of MLF on sensory properties of wines

As malolactic fermentation impacts the flavor characteristics of wine (Davis *et al*., 1985; Lonvaud-Funel, 1999; Palacios, 2005a), the impact of the use of starters in this regard was studied. Structure in mouth, was found to be significantly higher in spontaneous MLF. This descriptor is defined as overall balance of acidity, residual sugars, tannins and body. Body being described as tactile impression of thickness or relative weight, viscosity due to alcohol, glycerol or sugar on the palate (Robinson, 2006). On the other hand, glycerol, which contributes to the wine's structure, is known to be a molecule produced principally by yeast during fermentation (Jackson, 2008). As the wines were tested in pairs coming from the same alcoholic fermentation and with different MLF (IN compared to ES), it is suspected that the modifications on the structure of the wines might come from the MLF step. Polyols and sugar alcohols can be produced by spoilage bacteria, and both may have a slight effect on the sensation of body of the wine (Jackson, 2008). Polysaccharides can be produced for LAB increasing the sensation of volume or body by themselves or they can be polymerized with tannins reducing sensation of roughness (Dols-Lafargue *et al.,* 2007). Thus, regarding our results, wild bacteria populations present in the non-inoculated wines were probably able to produce metabolites affecting the structure in mouth of the wines in enough quantity to modify wine characteristics and thus be perceived during the tasting.

Significant loss of color intensity in inoculated wines was observed, even though this impacted only two wines. In general, the change in color of red wines after MLF corresponds to a reduced intensity with less blue tones, mainly due to the possible adsorption of anthocyanins, specially the methoxylated ones, by the bacterial cell walls. This process is supported by the rise in pH during depletion of malic acid and the decrease of free sulphurous anhydride (Suarez-Lepe and Iñigo-Leal 2003). The effect of the type of LAB strains on color is less known. López *et al*. (2011) concluded that

inoculation did not affect perceived color in wines. Gerbaux and Briffox (2002) found a reduction of analytic color intensity in inoculated Pinot Noir wines, but this difference was not observed during the sensory analysis. They proposed that this difference might be linked with the timing of the malolactic fermentation, the faster (inoculated MLF) being the one that experienced higher reduction in color intensity.

Ethanal descriptor was also significantly higher in spontaneous fermentations in the two wines where it was evaluated. This metabolite (also named acetaldehyde) which is highly volatile and imparts and undesirable green, grassy, apple-like aroma in wines (Zoecklein *et al,.* 1995) is formed by yeasts, acetic acid bacteria, and coupled auto-oxidation of ethanol and phenolic compounds in wines (Liu and Pilone, 2000). Osborne *et al*. (2000) studied the capability of wine bacteria to degrade ethanal and they have found that strains of *O. oeni, Lactobacillus hilgardii, L. delbrueckii, L. buchneri* (some of them commercially available), are able to completely remove this metabolite from the wines and with less success the SO_2-bound acetaldehyde form. The products of acetaldehyde catabolism by LAB are ethanol and acetic acid but to low concentrations that should not impact sensory properties of wines (Osborne *et al.,* 2000). In wines 20 and 44, the ethanal was probably developed during the alcoholic fermentation which followed carbonic maceration. Probably during the MLF the acetaldehyde of these wines was degraded in the inoculated MLF resulting in wines with less ethanal that their respective wines that performed the MLF spontaneously. The level of implantations was high for these 2 wines (100% and 90%); the capability of the used malolactic starter to degrade ethanal need to be studied to confirm this hypothesis.

Regarding the interaction between ethanal and color, it is worth noticing that the wines with significant difference on perceived color, had higher perceived ethanal as well. Acetaldehyde also plays a role in the color development of red wines by promoting rapid polymerization between anthocyanins and catechins or tannins. This polymerization forms stable pigments resistant to SO_2 bleaching (Timerlake and Bridle, 1976; Sommers and Wescombe, 1987; Osborne *et al.,* 2000). As a consequence the interaction of acetaldehyde with phenolics improves wine color (Liu and Pilone, 2000). This might explain the better scoring on color intensity for wine 44ES in which ethanal was also perceived. On the other hand, the opposite occurred for the wine 20, so this cannot be explained by the interaction acetaldehyde-anthocyans.

Biogenic amines present in the wine do not affect the perception of wines as the few descriptors that had significant impact on the sensory evaluation in this study are not linked to sensory descriptors for amines: chlorine, mousy or rotten flavors as has been described for the putrescine at 60 mg/L (Gerbaux and Monamy, 2000) or vegetable in putrefaction or fish in wine at 10 mg/L (Palacios *et al.,* 2005b). Unpleasant meat notes for the cadaverine at 1 mg/L in wines (Palacios *et al.,* 2005b). Therefore, the biogenic amines in this study did not affect the sensory properties of the studied wines, even in those wines where cadaverine was present at ≥ 1 mg/L.

Flavor complexity is often used to describe the contribution of microorganisms during processing and aging (Versari *et al.,* 1999). Therefore, increased flavor complexity is logically expected when the bacterial population during MLF is more heterogeneous as can be the case of spontaneous MLF. Our results show that flavor complexity is neither affected by the use of malolactic starter nor by its level of implantation.

1.11 RESULTS: DIRECT INOCULATION AND MOTHER TANK

1.11.1 Results: implantation of the LAB starter culture in MLF

The bacterial populations of industrial malolactic fermentations inoculated by direct inoculation (DI), series A and B (oak barrels) and via mother tank (MT) method, series T (50 HL steel tanks) were analyzed. The characterization of LAB population at exponential phase of MLF was performed via typification of LAB strains using RAPD-PCR (Table 36).

Table 36. Implantation *of Oenococcus oeni* starter in malolactic fermentations seeded by direct inoculation and mother tank methods

Samples	Inoculating method	Analyzed Isolates	LAB starter implantation (%)	Other strains	
				Main strain (%)	Different profiles
A-ES*	n.a.	24	n.a.	83	3
A1	DI*	28	100		
A2	DI	28	100		
A3	DI	28	100		
A4	DI	28	100		
A5	DI	28	100		
B-ES	n.a.	28	n.a.	79	3
B1	DI	15	80	20	1
B2	DI	28	79	18	3
B3	DI	28	100		
B4	DI	28	100		
B5	DI	27	96	4	1
B-ES*	n.a.	28	n.a.	36	8
T13	MT*	28	70	21	2
T17	MT	28	46	25	7
T19	MT	28	70	25	2
T20	MT	28	55	30	2

ES: spontaneous MLF; DI: direct inoculation; MT: mother tank (pied de cuve); n.a.: not applicable*

Inoculated malolactic fermentations displayed different levels of implantation of malolactic starter strain. These values ranged between 46% and 100 % implantation.

The MLF using the mother tank (MT) inoculation method (Series T) displayed lower degrees of implantation of the LAB starter strain, in contrast with the MLF inoculated by direct inoculation (DI) (Series A and B). The DI method yielded the higher percentage of strain implantation:

- in Series A (Table 36), the implantation of malolactic starter strain was complete in all samples in which it was inoculated. In Series B, the implantation was total in barrels B3, B4 and B5 and 80% in barrels B1 and B2.
- in Series T (Table 36), tanks T13, T17, T19 and T20 displayed a low implantation of the inoculated strain (70%, 46%, 70% and 55%, respectively). It is worth noting that tank T13, which was the mother tank of the starters used to inoculate the other tanks in the series, presented a slightly higher implantation level together with batch T19.

An analysis of variance was performed to determine whether the differences on implantation between the two inoculating methods are significant (Table 37). A significant difference was found (p≤ 0,001) between the two methods. The average of implantation was highest by direct inoculation (p-value= 0,00003).

Table 37. ANOVA. Factor: type of inoculation (direct and mother tank)

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
DI-implantation	10	954,9	95,49	74,2498889
MT-implantation	4	240,82	60,205	134,402767

ANOVA

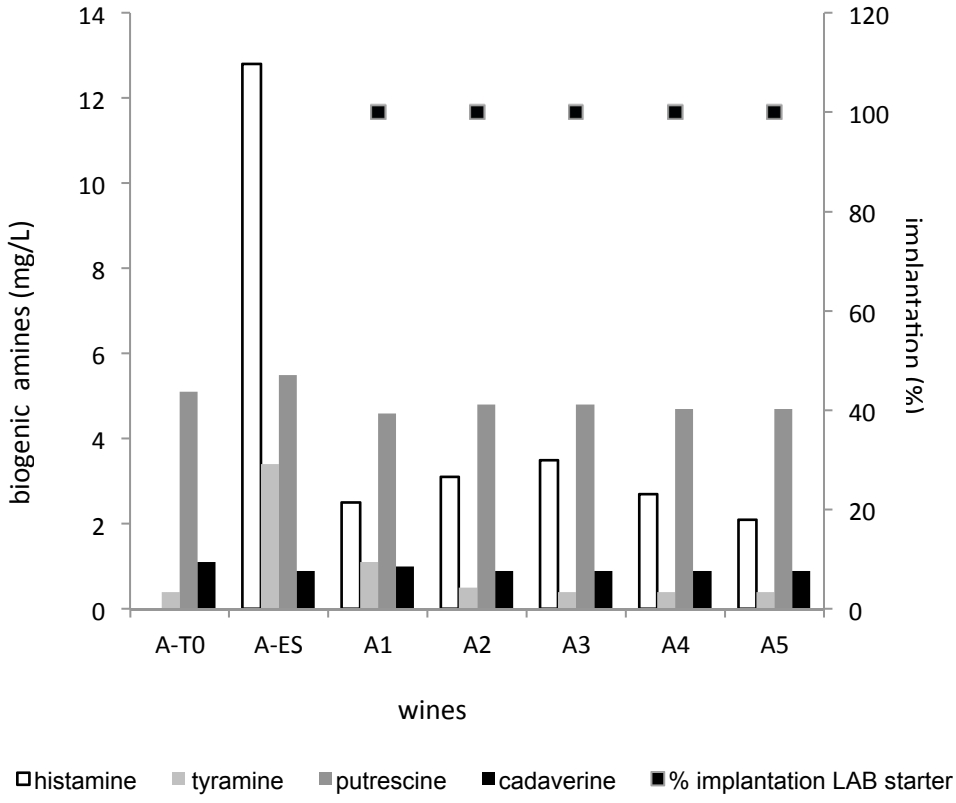
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3557,23207	1	3557,23207	39,839931	3,8783E-05	4,74722534
Within Groups	1071,4573	12	89,2881083			
Total	4628,6893	13				

1.11.2 Results: biogenic amines

Direct inoculation (series A and B) and mother tank method (series T) were compared measuring the biogenic amines content (histamine, tyramine, putrescine and cadaverine) before inoculation (T0) and once finalized the MLF.

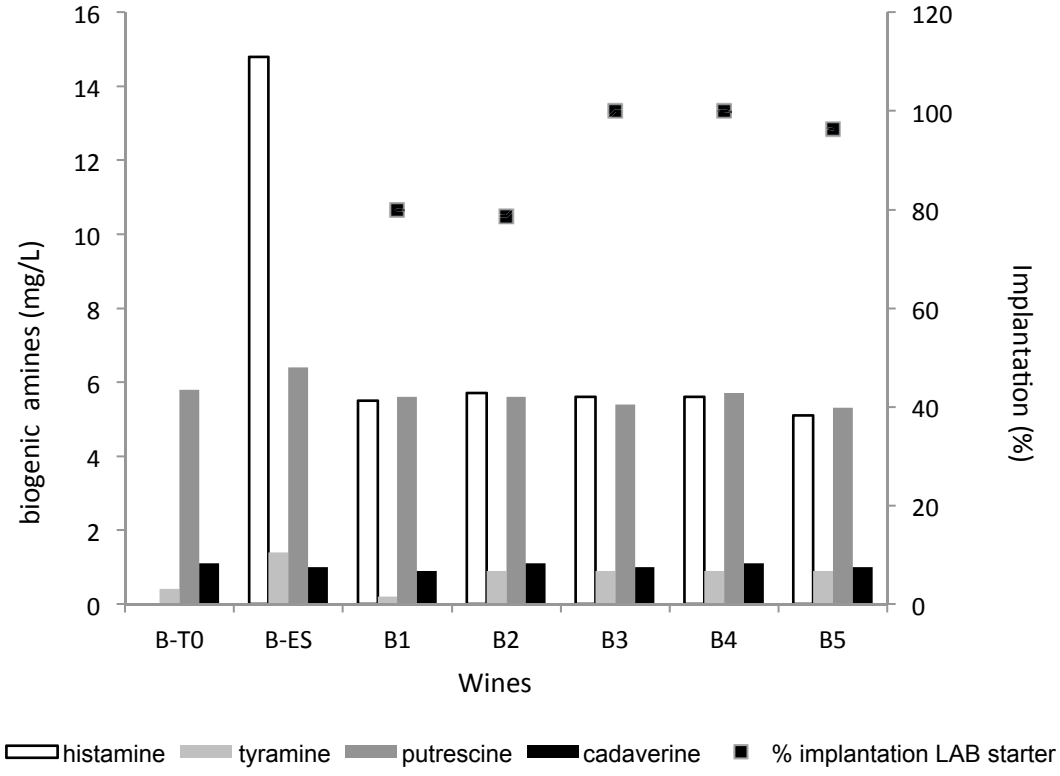
In Series A (Fig. 30), no histamine was detected in the wine prior to inoculation (sample A-T0). The samples from inoculated barrels contained histamine, between 2,1 and 3,5 mg/L. Prior to MLF, the wine contained 0,4 mg/L tyramine (sample A-T0) and this value was kept constant after the MLF in inoculated barrels. Spontaneous MLF (A-ES), produced high levels of histamine (12,8 mg/L) and tyramine (3,4 mg/L). No putrescine or cadaverine was produced during MLF since the levels observed were similar to those in the wine before MLF (A-T0).

Figure 30. Biogenic amines content of industrial MLF carried out in oak barrels (225L), series A.



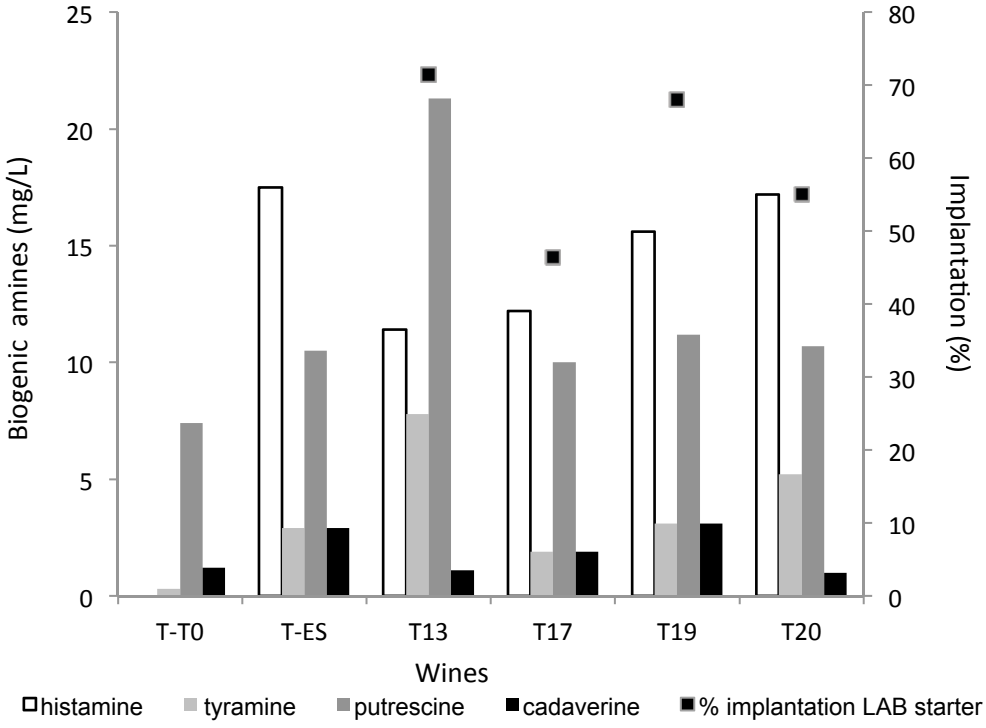
In Series B (Fig. 31), the levels of histamine in the inoculated barrels were around 5,6 mg/L. As in Series A, no histamine was detected prior to MLF (sample B-T0). A slight production of tyramine was observed in the inoculated barrels (0,9 mg/L) compared with the sample B-ES (0,4 mg/L). As in Series A, the barrel with the highest levels of histamine (14,8 mg/L) and tyramine (1,4 mg/L) was the one with MLF without inoculation of the starter strain (B-ES). No putrescine or cadaverine was produced during MLF since the levels obtained were similar to those in the wine prior to MLF (B-T0).

Figure 31 Biogenic amines content of industrial MLF carried out in oak barrels (225L), series B.



In Series T (Fig. 32), all tanks presented high levels of histamine produced during MLF. The concentration of tyramine increased during MLF, reaching a maximum of 7,8 mg/L in the case of T13. The levels of tyramine reached in the tanks inoculated via mother tank method were also higher than those detected in the barrels (Series A and B).

Figure 32. Biogenic amines content of industrial MLF carried out in steel tanks (50HL), series T.



In all tanks, the concentration of putrescine was high with respect to the other series, particularly in tank T13 (21,3 mg/L). Although, in all cases, this amine was already present before the start of MLF (T-T0), the increase in these values suggests that this metabolite was synthesized during the malolactic fermentations.

A slight increase in cadaverine was observed in tanks T17, T19 and T20, in contrast to the other series in which no increase in this anime was observed.

1.11.3 Results: impact of MLF type (ES, DI, MT) on biogenic amines

Looking at the relationship between the type of MLF (spontaneous, inoculated by direct inoculation method, and inoculated by mother tank method) and the production of biogenic amines (histamine + tyramine + putrescine + cadaverine), a "single factor " ANOVA test was run.

There are significant differences on the total biogenic amines content depending on the type of MLF with a p-value= 4,3E-07 (Table 38).

Table 38. Analysis of variance for total biogenic amines, single factor: MLF type

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
BA total- ES	3	80	26,6666667	38,4133333
BA total- IN DI	10	108,9	10,89	4,33211111
BA total- IN MT	4	134,7	33,675	40,7825

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1695,45566	2	847,727828	49,8321792	4,3006E-07	3,73889183
Within Groups	238,163167	14	17,0116548			
Total	1933,61882	16				

BA: biogenic amines; IN DI: inoculated by direct inoculation method: IN MT: inoculated by mother tank (pied de cuve) method.

In order to determine the differences between seeding methods regarding biogenic amines content, a single factor ANOVA was run confronting all methods (Table 39). In parenthesis is indicated the treatment with the highest average.

Table 39. ANOVA per pairs. Total biogenic amines

P-values	ES	DI	MT
ES	1	0,000013 (ES)	0,205
DI		1	0,0000002 (MT)
MT			1

ES: spontaneous MLF; DI: direct inoculation: MT: mother tank (pied de cuve)

There are significant differences in total biogenic amines content between spontaneous MLF and direct inoculation methods (p-value=0,000013), ES MLF giving the highest average. Significant differences were also found (p-value=0,00000002) between direct inoculation and mother tank methods and MT displayed the highest biogenic amines content average.

Surprisingly, there is no significant differences (p-value= 0,205) between spontaneous MLF (ES) and inoculated MLF when mother tank method (MT) was used. This indicates that poor implantations of LAB starters lead to similarities on biogenic amines content with MLF where no starters were used and the wild bacterial population conducts the process.

Analyzing each biogenic amine individually, significant differences were found (p≤0,001) in the three MLF types on the histamine, tyramine and putrescine produced during the MLF (end-T0), Table 40.

Table 40. ANOVA. Biogenic amines produced during MLF. Factor: MLF type.

p-values	Histamine	Tyramine	Putrescine	Cadaverine
Factors: ES / DI / MT	3,3E-05	0,0005	0,003	0,195
Factors: ES / DI	2,4E-08 (ES)	0,00017 (ES)	0,002 (ES)	0,127
Factors: DI / MT	0,0002 (MT)	0,0003 (MT)	0,002 (MT)	0,057
Factors: ES / MT	0,62	0,29	0,222	0,893

ES: spontaneous MLF; DI: direct inoculation; MT: mother tank

The differences between the treatments for each amine (Table 40) followed the same trend than for the total biogenic amines (Table 39).

- Significant differences between ES and DI, highest average for ES.
- Significant differences between DI and MT, highest average for MT.
- No significant differences between ES and MT for all amines.
- No significant differences for the cadaverine.

1.11.4 Results: influence of implantation of LAB starter on biogenic amines production

In order to determine the correlation (r) between the levels of implantation of the LAB starter in inoculated MLFs, a correlation matrix was done (Table 41). It was found that the level of implantation % was negatively correlated with the histamine and cadaverine, and more moderated for tyramine and putrescine. This indicates that the higher the implantation of LAB starters, the lower the biogenic amines content produced during the MLF.

Table 41. Matrix of correlation for implantation and biogenic amines

Coefficient of correlation (r)	Implantation	Histamine	Tyramine	Putrescine	Cadaverine
Implantation	1				
Histamine	-0,86	1			
Tyramine	-0,486	0,708	1		
Putrescine	-0,526	0,688	0,951	1	
Cadaverine	-0,730	0,733	0,269	0,373	1

1.11.5 Results: oenological parameters and their influence on MLF and biogenic amines

In series of wines A (directly inoculated barrels) can be highlighted the L-lactic acid (Table 42) in sample A-T0 (0,72 g/L). This sample was taken before starting MLF; therefore the expected maximum levels of this metabolite would be 0,3 g/L. This L-lactic acid is formed by the metabolism of the malic acid of the yeasts during alcoholic fermentation. A value of 0,72 g/L could indicate that MLF had started before the inoculation of the LAB starter strain, thus compromising the development of the starter. Despite this situation, all the barrels in the series displayed complete implantation. This was also noticed for the barrels of series B with a relatively high L-lactic acid detected in sample B-T0 (0,67 g/L)

The volatile acidity in series of barrels A and B was a little higher in all spontaneous MLF than in the inoculated barrels (Table 42).

Table 42. Oenological parameters of wines at T0 and end of MLF

Code of wine	MLF type	Implantation (%)	Alcoholic degree (vol.%)	Total sugars (g/L)	Total acidity in tartaric acid (g/L)	Volatile acidity in acetic acid (g/L)	pH	Free sulphur Dióxid (mg/L)	Total sulphur Dióxid (mg/L)	L-malic acid (g/L)	L-lactic acid (g/L)
A-T0		na	14,05	0,6	4,7	0,2	3,75	0	4	1,61	0,72
A-ES	ES	na	13,9	0,6	4,4	0,46	3,85	0	3	0,09	1,43
A1	IN	100	13,9	0,9	4,3	0,32	3,85	0	0	0,09	1,42
A2	IN	100	13,9	0,8	4,3	0,33	3,85	0	6	0,09	1,47
A3	IN	100	13,95	0,9	4,3	0,33	3,86	0	0	0,08	1,41
A4	IN	100	13,95	0,6	4,3	0,3	3,85	4	8	0,07	1,48
A5	IN	100	13,95	0,6	4,3	0,31	3,85	4	6	0,09	1,47
B-T0		na	13,95	0,8	5,1	0,22	3,72	0	0	1,56	0,67
B-ES	ES	na	13,75	0,9	4,5	0,36	3,83	0	0	0,08	1,46
B1	IN	80	13,8	0,7	4,5	0,32	3,81	0	0	0,1	1,45
B2	IN	78,6	13,8	0,9	4,5	0,32	3,82	0	3	0,1	1,45
B3	IN	100	13,8	0,9	4,5	0,32	3,82	0	0,01	0,11	1,47
B4	IN	100	13,8	0,9	4,5	0,33	3,82	0	5	0,1	1,42
B5	IN	96,3	13,8	1	4,5	0,34	3,82	0	5	0,11	1,47
T-T0		na	13,65	2	5,2	0,22	3,76	7	38	2,17	0,22
T-ES	ES	na	13,6	2	4,9	0,25	3,71	13	58	0,1	1,41
T13-T0			13,8	1,7	5,2	0,22	3,79	9	37	2,2	0,2
T13	IN	71,4	13,75	1,9	4,3	0,35	3,9	0	28	0,09	1,43
T17- T0			13,8	1,7	5,2	0,19	3,78	4	30	2,1	0,27
T17	IN	46,42	13,8	1,8	4,6	0,34	3,8	8	39	0,09	1,45
T19-T0			13,8	1,8	5,2	0,22	3,78	5	29	2,1	0,26
T19	IN	68	13,7	1,2	5,2	0,35	3,84	13	43	0,07	1,44
T20-T0			13,75	1,9	5,2	0,22	3,77	3	30	2,18	0,23
T20	IN	55	13,7	2,2	4,6	0,28	3,8	11	50	0,09	1,46

na: not applicable

Series T: In this series, the levels of L-lactic acid were low prior to MLF (0,22 g/L). Therefore, and in contrast with what happened in Series A and B, we did not consider that MLF started before the tanks were inoculated. These data would rule out the possibility of the low implantation of the LAB starter being attributable to MLF starting spontaneously before inoculation in MLF performed in the tanks (series T).

1.12 DISCUSSION: DIRECT INOCULATION AND MOTHER TANK

The direct inoculation of malolactic starter is part of the recommended protocols given by the supplier to ensure the implantation of the strain to drive the MLF. Nevertheless, in wine cellars, the practice to inoculate a wine with an ongoing MLF is a common practice even before the commercial starters became available. In this work, we tried to understand the impact of such practice on the level of implantation of the LAB starter and to evaluate its impact on biogenic amines content in wines. Samples were taken from industrial MLF of Tempranillo wines performed in 50 HL stainless-steel tanks and 225 L oak barrels from the North of Spain.

Implantation of malolactic starter

The malolactic fermentations that were inoculated directly with the LAB starter presented significantly higher levels of implantations (average 95%) than the wines inoculated following the mother tank method (average 60%). The mother tank presented poor implantation of 70% and when part of the wine was transferred to the other tanks the implantation evaluated at the exponential consumption of malic acid was kept constant or decreased. To our knowledge, no previous works demonstrated the decreasing performance of the malolactic starter inoculated in these conditions. Therefore, our results demonstrated the reduction of implantation, measured by typification of the bacterial population during the MLF, when following *pied de cuve* seeding technique.

Biogenic amines

Different behavior on biogenic amines production during the MLF was found to be dependent on the use of starters and on the choice of inoculation technique. Direct inoculation produced significantly less biogenic amines during the MLF (10 mg/L) than the malolactic starter inoculated following mother tank procedure (33 mg/L) or spontaneous MLF (27 mg/L). The same trend was observed for histamine, tyramine, and putrescine evaluated individually. Surprisingly, there is no significant difference on biogenic amines production between spontaneous MLF (ES) and inoculated MLF when mother tank method (MT) was used. This indicates that poor implantations of LAB starters bring similarities on biogenic amines content with spontaneous malolactic fermentations where no starters were used and the wild bacterial population conducted the process. The analysis of correlation between the level of implantation and

biogenic amines production during the MLF, highlighted a highly negative correlation between the level of implantation and the histamine and cadaverine content, and a moderately negative correlation for tyramine and putrescine. This indicates that the higher the implantation of LAB starters is, the lower biogenic amines content is produced in wines during the MLF. These results are in concordance with López *et al.* (2008) who also found increased histamine in MLF where starters were not totally implanted.

Oenological parameters

As observed in the first trials of this thesis and by other authors, the pH affects the clonal distribution of bacterial population during the MLF. In this set of trials to evaluate the implantation levels of starters inoculated by direct inoculation and mother tank methods, all wines were at pH between 3.72 and 3.78 before the seeding of LAB starters and the alcoholic degree was between 13,65% and 14,05% (Table 42). Both parameters are quite homogeneous in the 17 batches of Tempranillo wines harvested from the same zone and period. Therefore, the differences in implantation levels are certainly linked to the methods of inoculation and an interaction between the pH and alcoholic degree of wines is not considered.

On the other hand, the high pH (3.72 -3.78) that these wines had at the end of ALF is usually linked with high biogenic amines development. Indeed some authors defined a threshold of pH of 3.6 for wines with high biogenic amines content (Landete *et al,.* 2005d). In our study, we proved that high levels of implantation in wines with high pH reduced the biogenic amines generation during MLF.

1.13 RESULTS: LYSOZYME

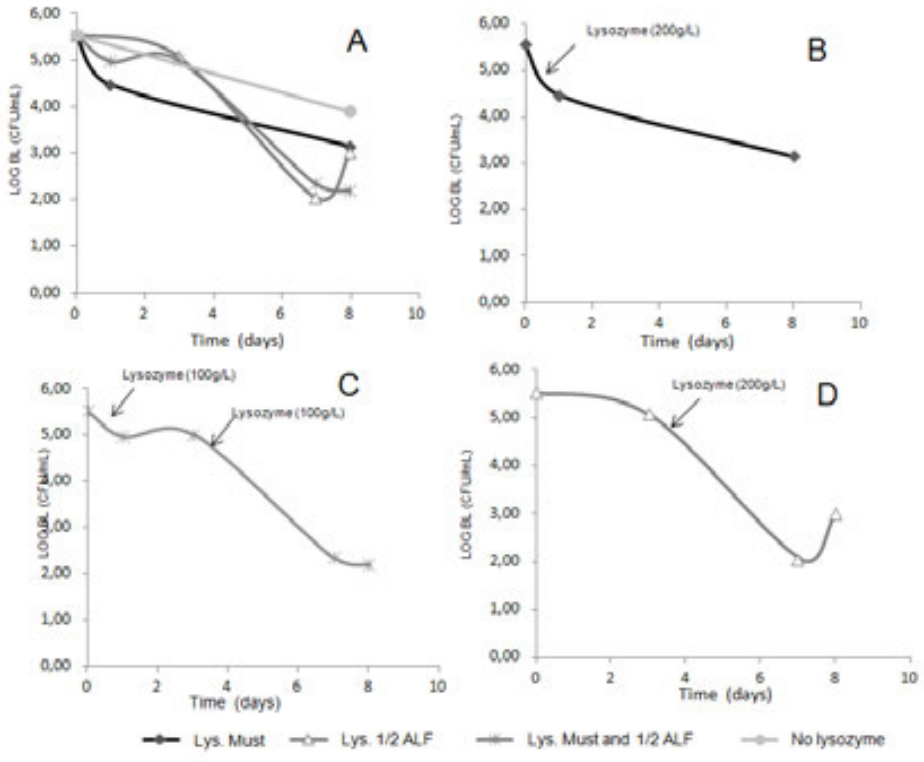
1.13.1 Results Lysozyme: lactic acid bacteria dynamics during ALF

The application of lysozyme aims at reducing Gram-positive bacteria population by acting on the peptidoglycan layer of the bacteria cell wall. Its use in oenology responds to the need to replace in part the use of SO_2. In these trials we evaluated the impact of the use of lysozyme at different stages of the winemaking process on the level of implantation of malolactic starter and biogenic amines generation. Trials were performed in duplicate as follows:

- Lysozyme added in the must
- Lysozyme added at middle of alcoholic fermentation
- Lysozyme added in must and middle of ALF
- No lysozyme was added (control batch).

In order to analyze the changes to the LAB population from the use of lysozyme at different stages, plate count of LAB was performed before the application of lysozyme and at the end of alcoholic fermentation followed by racking and inoculation of malolactic starter (Figure 33).

Figure 33. Lactic acid bacteria population dynamics during ALF as function of application of lysozyme.



A: comparison of LAB dynamics; B: LAB population when lysozyme added in must,
C: lysozyme added in must and middle ALF, D: lysozyme added middle of ALF

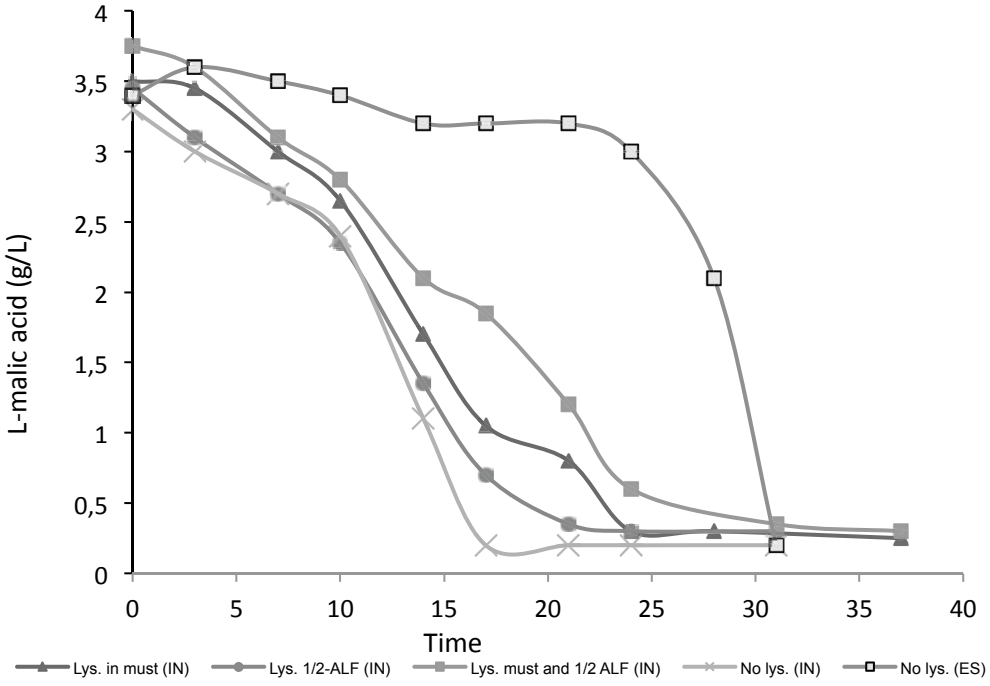
As shown in Figure 33, the LAB population naturally decreases during the ALF (Fig. 33, A) as observed in the control batch without lysozyme. In the must, the LAB population was of a size of 5,5 log, while this decreased to 4 log at the end of ALF (no lysozyme batch). When lysozyme was added in the must after the sulphitation (Fig. 33, B) the LAB population decreased to 3 log. Therefore, the presence of lysozyme in must, made a reduction of LAB during the ALF of 1 log. A similar reduction was observed when lysozyme was added in the middle of ALF (Fig. 31, D). It must be noted that on day 7 the population decreased followed by a fast increase until the end of ALF. Reduction of 3 log was obtained when part of the lysozyme was added in the must, and part in the middle of ALF (Fig. 31, C). This last treatment was the most efficient treatment for reduction of LAB.

Alcoholic fermentation was not affected by the use of lysozyme as the density dynamics were identical in all batches (data not shown).

1.13.2 Results Lysozyme: MLF Kinetics

Figure 34 illustrates the L-malic acid consumption during the MLF of the set of trials with lysozyme. As the experiments were run in duplicate, the graph plots the average value of each sampling point.

Figure 34. MLF kinetics of trials with lysozyme



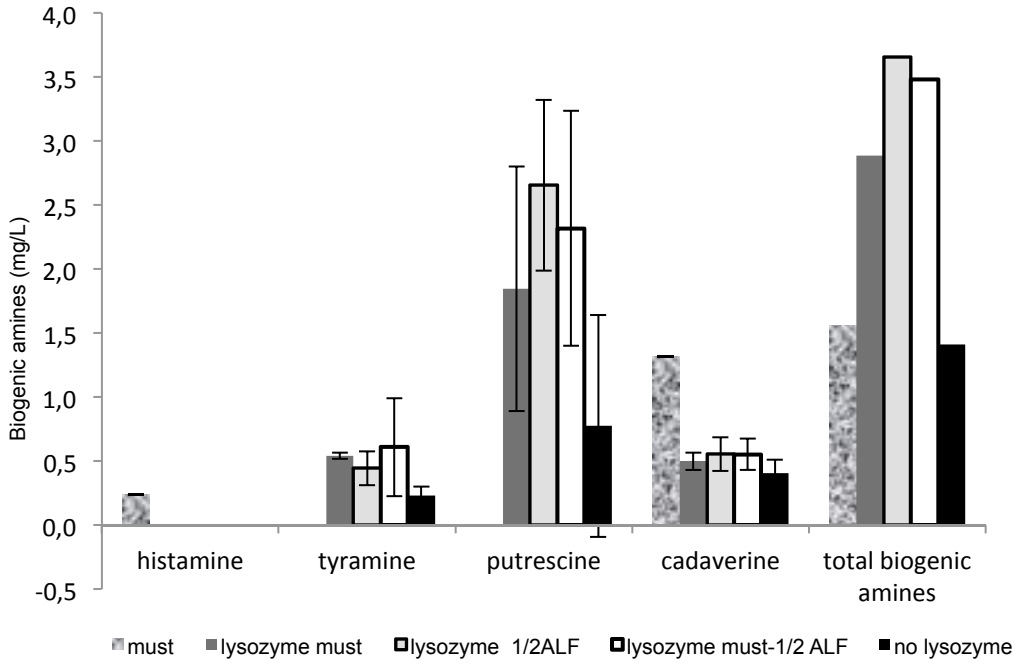
The malolactic fermentation kinetics reveals that the latency phase was longer in the spontaneous MFL (No lys. ES) with a duration of 31 days. The inoculated batches did not experience latency phase during the MLF.

The MLF inoculated with LAB starter without lysozyme (No lys. IN) lasted 17 days. The use of lysozyme seems to slow the L-malic acid degradation during the MLF as all batches with lysozyme completed the MLF between 21 and 31 days.

1.13.3 Results Lysozyme: biogenic amines before inoculation

Figure 35 shows the results of biogenic amines content before the inoculation of the malolactic starter and the original biogenic amines contained in the must (results are represented as average of the two batches). As the batches were treated with lysozyme during the alcoholic fermentation, all wines have different storical in terms of microbial population, this is reflected by the different levels of biogenic amines obtained at the end of ALF (Figure 35).

Figure 35. Biogenic amines content at the end of alcoholic fermentation



The must contained traces of histamine (0,3 mg/L), it was not detected during ALF. Cadaverine was also present in the must and it was reduced in all batches. Putrescine was not detected in the must, it was generated during the ALF in all the studied conditions. However, even if some differences in average can be observed, the high internal variability (see standard deviation bars) is quite important. Comparing the

biogenic amines content at the end of ALF, all 3 batches added with lysozyme had similar behavior: similar synthesis of tyramine and putrescine and slight reduction of cadaverine

1.13.4 Results Lysozyme: Implantation and biogenic amines production during MLF

Biogenic amines at the end of MLF as average of the two batches are illustrated in Figure 36. The inoculation of the LAB starter was done in all cases (IN-MLF) at the end of the alcoholic fermentation and one batch performed the MLF spontaneously.

The lysozyme added in the must and the batch without lysozyme presented the highest implantations of strain Uvaferm Alpha (93% and 100%), Fig. 37.

When the lysozyme was added at the middle of alcoholic (ALF) fermentation or in must and the middle of ALF, the implantation decreased to 76% and 78% respectively. However, these differences are not significant (single way ANOVA, p-value=0,531), this is due to the high internal variability of implantation in wines treated with lysozyme during the alcoholic fermentation ("Lys. 1/2 ALF, IN-MLF" and "Lys. in must and ½ ALF, IN-MLF").

Globally the batches treated with lysozyme produced more biogenic amines (Fig. 37). The ES-MLF degraded the putrescine and the cadaverine formed during the ALF and produced less tyramine than the IN-MLF (also without lysozyme).

Figure 36. Lysozyme: Biogenic amines at the end of MLF

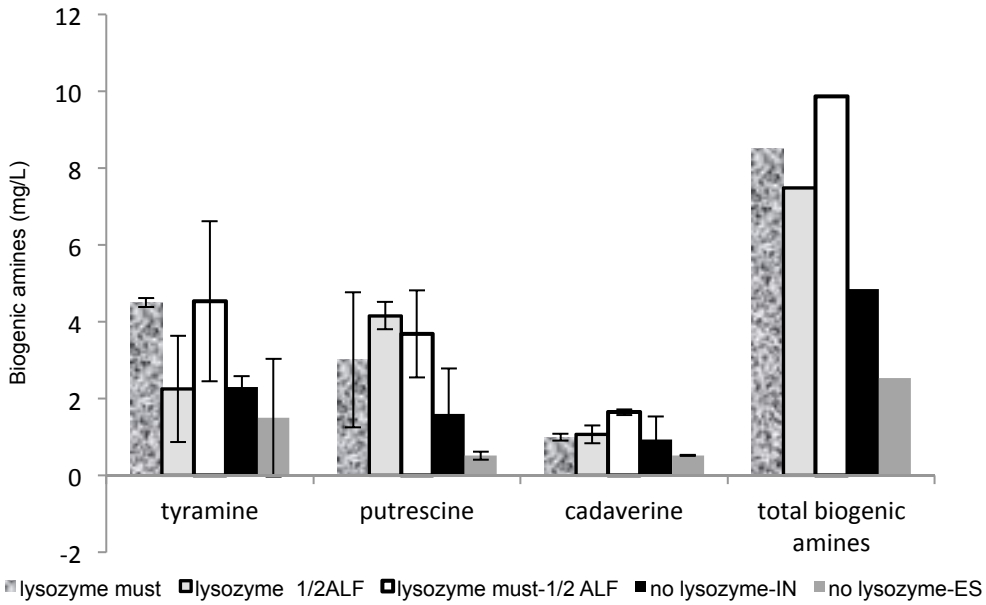
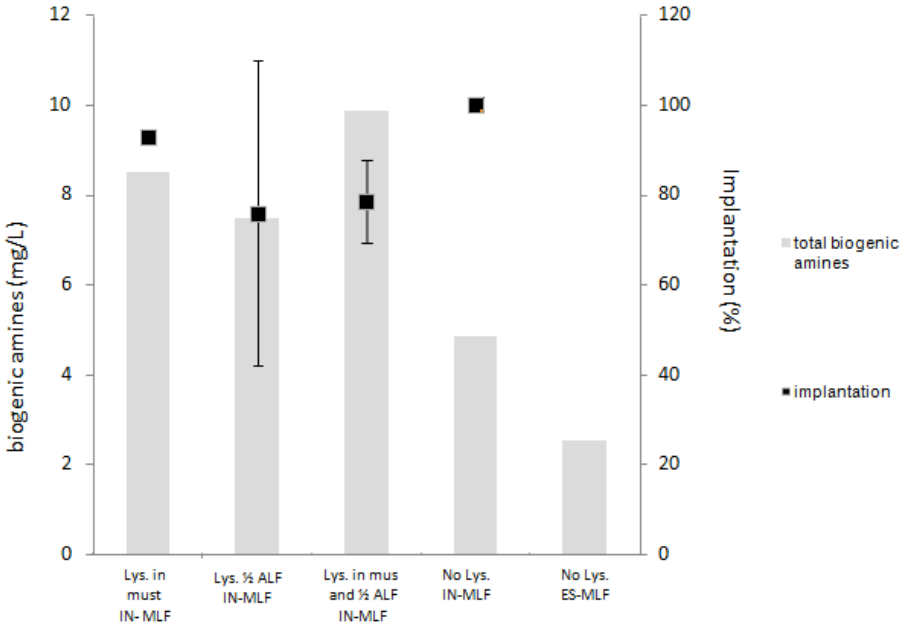


Figure 37. Lysozyme: Implantation of malolactic starter and total biogenic amines



A single way ANOVA test was run to determine if the total BA per treatment was significantly different. A p-value= 0,124 was obtained indicating that the treatments are not significantly different (ANOVA analysis not shown).

A single way ANOVA per pair was also done (Table 43). A significant difference was found between the both batches without lysozyme, the inoculated batched presenting the higher BA of the both.

Table 43. ANOVA per pairs. Total biogenic amines produced during MLF, factor treatments.

MLF		IN				ES
	Lysozyme	Must	½ ALF	Must and ½ ALF	no	No
IN	Must	1	0,780	0,471	0,549	0,237
	½ ALF		1	0,205	0,351	0,149
	Must and ½ ALF			1	0,345	0,063
	No				1	0,002
ES	No					1

MLF: malolactic fermentation; IN: inoculated MLF; ES: spontaneous MLF; ALF: alcoholic fermentation

1.13.5 Results Lysozyme: Oenological parameters

The wines were analyzed after bottling regarding their oenological characteristics (Table 44). Volatile acidity of spontaneous MLF was higher than the others treatments.

Table 44. Lysozyme: oenological characterization of wines after bottling

	Alcoholic degree %	Total acidity	Volatile acidity	Total sugars (g/L)	pH	L-malic acid	L-lactic acid
lysozyme must *	14,00	3,40	0,28	0,60	4,06	0,20	2,10
SD	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
lysozyme 1/2ALF	14,08	3,30	0,30	0,50	4,09	0,20	2,05
SD	0,46	0,28	0,01	0,00	0,13	0,00	0,07
lysozyme must-1/2 ALF	14,30	3,45	0,30	0,55	4,18	0,20	2,15
SD	0,07	0,03	0,07	0,02	0,00	0,07	0,00
No lysozyme IN	13,55	3,80	0,36	0,55	3,98	0,20	2,15
SD	0,00	0,00	0,02	0,07	0,03	0,00	0,07
No lysozyme ES	13,65	3,80	0,52	0,95	4,04	0,20	2,05
SD	0,07	0,00	0,22	0,49	0,01	0,00	0,07

**Only one batch was analyzed due to technical issues. n.a.: not applicable*

1.14 DISCUSSION: LYSOZYME

Lysozyme and implantation

The lysozyme combines with the polyphenols of red wines and precipitates during the fermentation. It is finally removed during racking after the MLF (Gerbaux *et al.,* 1999). Therefore, during the MLF, the lysozyme can remain in suspension in the wine for a certain time while being active against gram positive bacteria. This can be the reason for the decrease of the level of implantation of the starter when the addition of lysozyme and inoculation of LAB starter were closer. Additionally, the L-malic acid consumption kinetics was slowed down in wines where inoculation of LAB starter and addition of lysozyme were closer, thus probably indicating a decrease of LAB population. Unfortunately there is no data available on population size during the MLF, but having in mind the action of the lysozyme, one can suspect that a reduction of LAB population justifies the desacceleration of L-malic acid consumption kinetics. Tenorio *et al.* (2005) also found slower MLF kinetics when lysozyme was added at the middle of ALF compared with the lysozyme added in the must. Having in mind that malolactic starter might have different levels of sensibility against the lysozyme (Pillate *et al.*, 2000), the addition of lysozyme in the must, gives probably enough time for its precipitation before the inoculation of commercial *O. oeni* at the end of ALF without compromising LAB starter performance. Our results indicate that the use of lysozyme can enhance the level of implantation of the starter if the addition of lysozyme and inoculation of the LAB starter are separated in the process. This avoids negative impact of lysozyme on the selected bacteria, as was also concluded by Tenorio *et al.* (2005) in similar trials evaluating Uvaferm Alpha. Using the same LAB starter and taking care of separating both operations, López *et al*. (2011) obtained better implantation results when lysozyme was added in the must and at the end of ALF. However, in our trials, the size of the LAB population of must treated with lysozyme was higher compared with the lysozyme treated in must and during ALF and the same as the batch treated with lysozyme during ALF. Therefore, in our trials the level of implantation does not seem to be linked with the reduction of wild LAB population and the hypothesis of the sensibility of the malolactic starters to the lysozyme appears reinforced.

Lysozyme and biogenic amines

Regarding the biogenic amines of these trials, the ES MLF was the one that released the lowest biogenic amines content by active degradation of putrescine. This is a quite rare event as usually the spontaneous MLF contains higher levels of BA than the inoculated wines (Izquierdo-Pulido *et al*., 1999; Gerbaux and Monamy, 2000; Vidal and Bover, 2001; Gindreau *et al*., 2003; Landete *et al*., 2005; Hernández-Orte *et al.,* 2008; Marques *et al.,* 2008; Pramateftaki *et al,.* 2012). One of the explanations could be the presence of wild LAB strains with a capability to degrade amines via synthesis of amine oxidase enzymes. Degradation of putrescine by wine LAB was recently described by some authors (Garcia-Ruiz *et al.,* 2011; Capozzi *et al.,* 2012; Callejon *et al.,* 2013). In the case of the cadaverine, the reduction in ES MLF is included in the LOQ of the method, therefore, it is considered unchanged during the MLF.

In the wines inoculated with malolactic starter, the batch without lysozyme, which obtained the highest implantation, was the one with less biogenic amines content (3 mg/L). In the inoculated MLF with lysozyme the total biogenic amines cannot be neglected (8 to 10 mg/L). Opposite results were obtained by López *et al.* (2011), they studied the impact of lysozyme on the level of implantation of LAB starter and histamine generation and concluded that the histamine production was lower during the MLF in fully implanted LAB starters when lysozyme was used. Our results are aligned with the point that higher implantation of LAB starter generated fewer biogenic amines, but the impact of lysozyme on biogenic amines production is contradictory so the mechanism underlying should be different in both cases. As wine LAB species and strains might exhibit different sensibility to lysozyme (Pillate *et al.,* 2000; Guzzo *et al.,* 2011; Coulon *et al,.* 2012) one can wonder if the application of lysozyme might act as a selective agent in the bacterial population allowing the development of some resistant bacteria on detriment of other ones. If the strains resistant to lysozyme also are able to decarboxylate amino acids, then the use of lysozyme will lead to the increase of biogenic amines in wines depending on the composition of the autochthonous LAB population.

Regarding the impact of lysozyme on biogenic amines, as results present high internal variability within the treatments, from a scientific point of view it is difficult to conclude. Having a more practical approach, these results shown to some extent that the results of combining lysozyme with the use of malolactic starters in wines is unpredictable and

there is a risk to lose control of the process. Therefore, we agree with the conclusion of López *et al.* (2011) in the point that more investigation is need to determine the effect of the lysozyme on the possible regulation of *hdc* gene expression (and other genes coding for amine decarboxylase enzymes) or decarboxylase enzymes activity. Indeed the results suggest that the different biogenic amines content are not related with the regulation of the size of the LAB population, which is the main property attributable to the lysozyme.

1.15 RESULTS: INOCULATION TIME

1.15.1 Results Inoculation Time: LAB population before MLF

In order to analyze the impact of the inoculation time of malolactic starters on its implantation and the repercussions on biogenic amines production, the following set of trials was carried-out in duplicate:

- Inoculation of LAB starter at 12 h from the beginning of the ALF. Process run at room temperature (25-30°C) until the middle of the ALF, then batch moved to chamber at 15°C.
- Inoculation of LAB starter at 12 h from the beginning of the ALF, batch in chamber at 25-30°C.
- Inoculation of LAB starter in the last part of the ALF when some remaining fermentable sugars (10g/L) are still not consumed (batch in chamber at 25-30°C).
- As control, one batch was inoculated at the end of ALF after racking (standard procedure) and in another batch the spontaneous MLF was allowed (both batches in chamber at 25-30°C).

Table 45. Inoculation time: Lactic acid bacteria population at the end of ALF.

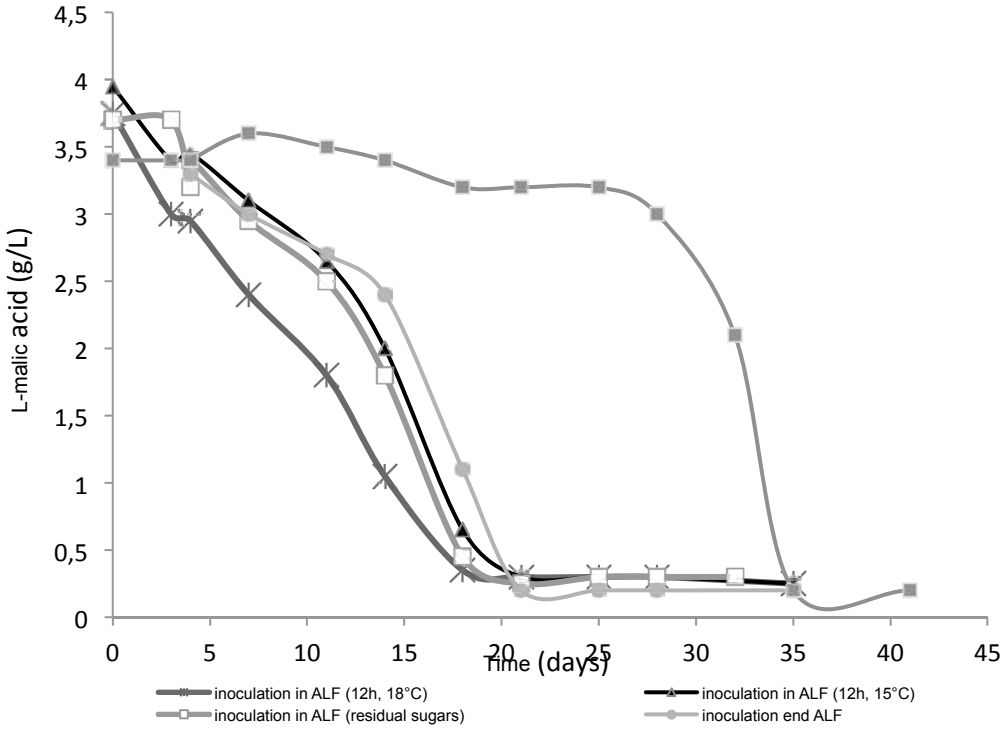
Log LAB in must (CFU/mL)	Treatments	Average Log LAB End of ALF (CFU/mL) ±SD
5,54	Inoculation 12 after start ALF (15°C)	5,83±0,0
	Inoculation 12 after start ALF (25-30°C)	5,82±0,0
	Inoculation remaining sugars (25-30°C)	2,84±0,5
	Inoculation end ALF (standard) (25-30°C)	3,04±0,05
	Spontaneous MLF(25-30°C)	3,45±0,2

The batches inoculated at the beginning of ALF show an important population of LAB at the end of ALF (Table 45). The wine inoculated when some sugars were still remaining decreased the LAB population. The density dinamics during the alcoholic fermentation were similar in all batches (data not shown).

1.15.2 Results Inoculation Time: MLF kinetics

Figure 38 shows the L-malic acid consumption of wines inoculated at different stages and conditions.

Figure 38. MLF kinetics of wines inoculated with malolactic starter at different stages



Regarding both treatments where the LAB starter was inoculated 12 h after the beginning of the ALF, the batch in a chamber at 15°C experienced a longer latency phase than the batch at 25-30°C. The MLF at 15°C finished L-malic acid consumption 3 days after the MLF at 25-30°C. This difference in timing can be attributed to the temperature of the process.

Comparing wines inoculated with LAB starter at different stages (all of them in chamber at 25-30°C):

- inoculation at 12 h after the beginning of the ALF.
- inoculation moving to the end of ALF with remaining sugars.
- inoculation at the end of ALF (standard inoculation).

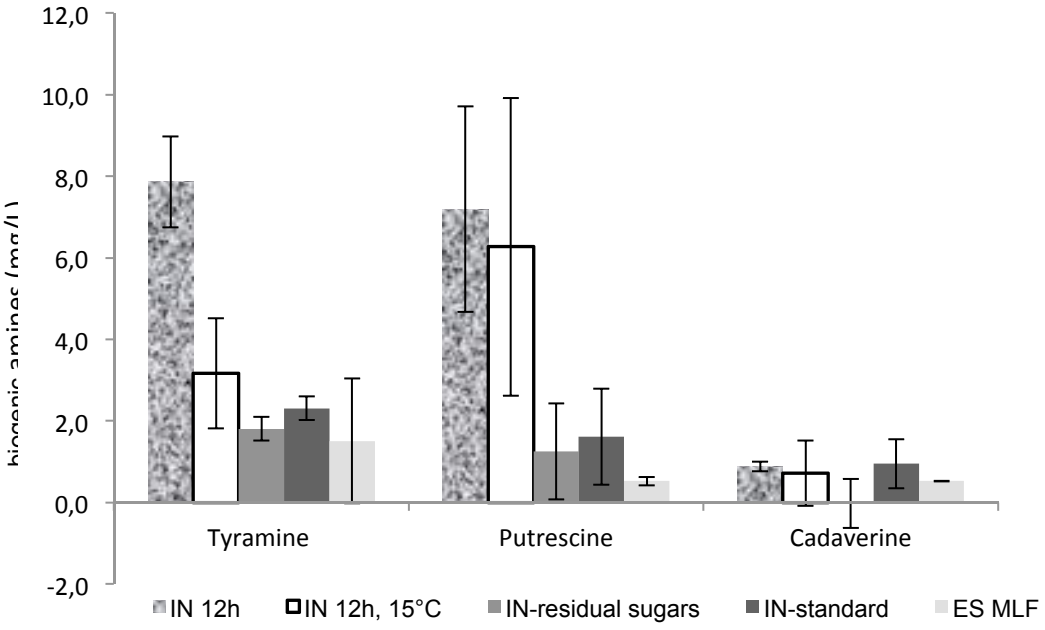
The first two, finished the MLF at day 18 and the batch with standard inoculation finished at day 21. Therefore, from a logistical point of view, the fact to inoculate during the ALF improves the timing of the MLF process. But regarding only the duration of the MLF process, the standard inoculation completed the MLF in 17 days. The longest MLF was

the wine without inoculation (ES) with a long latency phase of 24 days and a MLF process lasting 35 days.

1.15.3 Results Inoculation Time: Implantation and biogenic amines

Figure 39 shows the biogenic amines production from the inoculation to the end of L-malic acid consumption (average of the two replicates). Some treatments experienced high variation as shown with the standard deviation bars. Tyramine was more produced by the wine inoculated at the beginning of ALF (In 12h) and putrescine by the 2 wines inoculated at the beginning of ALF. No histamine was detected in the studied wines.

Figure 39. Inoculation time: biogenic amines produced from inoculation until end of malolactic fermentation.



The inoculations performed at 12 h from beginning of ALF showed poor implantations (Figure 40), even worst for the MLF performed at 15°C (76 and 46% respectively), which coincides with higher biogenic amines levels. Inoculations close to the end of ALF or at the end of it had better implantations (80 and 100%). These implantations are not significantly different (p-value=0,3) due to the high variability within the treatments (Table 46). Nevertheless, this variability needs to be taken into account because it jeopardizes the stability of the process as reflected by regarding the biogenic amines content (Figure 39, 40).

Figure 40. Inoculation time: Total biogenic amines at the end of malolactic fermentation and implantation of malolactic starter

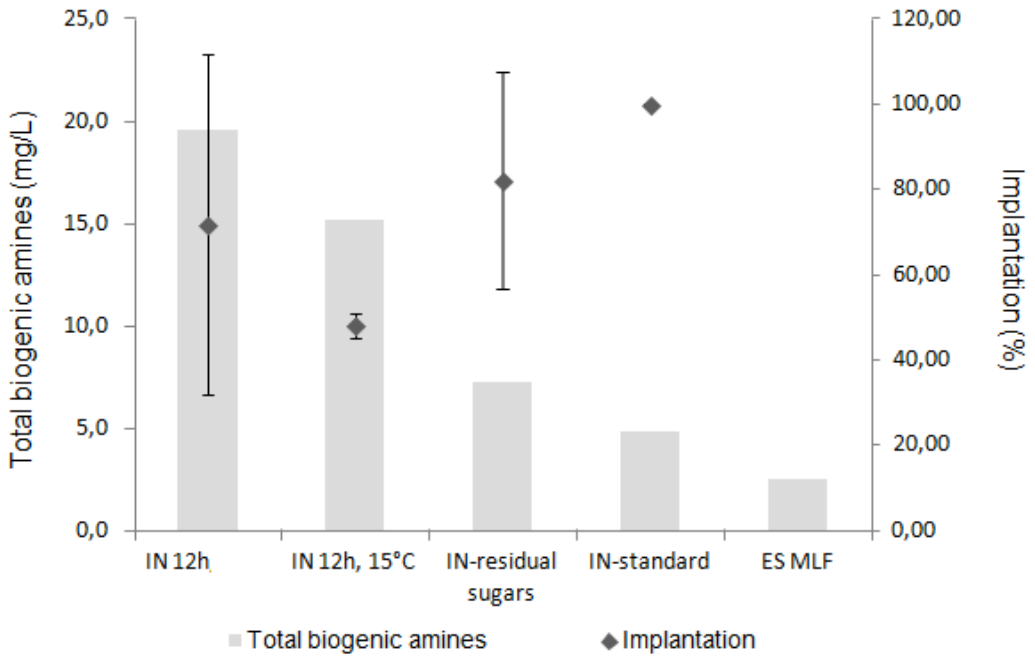


Table 46. ANOVA of starter implantation, factor: treatments

Anova: Single Factor

implantation %

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
12h	2	143,5	71,75	1596,125
12h (15°C)	2	96	48	8
remaining sugars	2	164	82	648
end ALF	2	200	100	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2825,59375	3	941,864583	1,67284602	0,30872136	6,59138212
Within Groups	2252,125	4	563,03125			
Total	5077,71875	7				

1.15.4 Results Inoculation Time: ANOVA biogenic amines

Globally important differences on the biogenic amines generated during the MLF can be observed. This is particularly true for treatments where the inoculation was performed at the beginning of the ALF in contrast with standard inoculation (end of ALF) or close to the end of ALF. Surprisingly, the spontaneous MLF experienced a decrease of putrescine and cadaverine.

In order to determine the significance of these differences, the production of biogenic amines during the MLF (BA end MLF – BA before inoculation) was analyzed using ANOVA single way. Significant differences were observed between the averages of tyramine and putrescine (Table 47). No histamine was produced in these trials.

Table 47. ANOVA of total biogenic amines, factor: treatments

Anova: Single Factor Biogenic amines (=end MLF-and ALF)	*P-value*
Total biogenic amines	0,053
Tyramine	0,048
Putrescine	0,012
Cadaverine	0,151

Despite the fact that no significant differences were found for total biogenic amines (Table 47), an ANOVA single way per pairs was done (Table 48) to determine significance of the differences among the treatments.

Table 48. ANOVA single way per pairs for total biogenic amines, factor: inoculation treatments

MLF		IN				ES
	Inoculation	ALF (12h)	ALF (12h, 15°C)	ALF (residual sugars)	End ALF (standard)	No
IN	ALF (12h)	1	0,52	0,07	0,93	0,04
	ALF (12h, 15°C)		1	0,24	0,51	0,16
	ALF (residual sugars)			1	0,086	0,47
	End ALF (standard)				1	0,002
ES	No					1

MLF: malolactic fermentation; IN: inoculated MLF; ES: spontaneous MLF; ALF: alcoholic fermentation

According to the ANOVA test (Table 48) the difference on total biogenic amines produced during MLF:

- Is not significant between the wines inoculated at beginning of ALF and MLF performed at different temperatures (15° and 25-30°C).
- Is not significant between the wines inoculated with residual sugars and end of ALF (standard).
- Surprisingly, is not significant among all the wines that carried out the MLF at 25-30°C and inoculated at different stages.
- Is significant between the ES-MLF and the wine where the inoculation was performed at 12 h (25-30º), this last treatment being surprisingly the one with highest total biogenic amines of the both.
- It is significant between the ES-MLF and the wine inoculated at end of ALF (standard), having this last one the higher biogenic amines content of the both.

1.15.5 Results Inoculation Time: ANOVA tyramine

To determine which treatments show significant differences regarding the tyramine, an ANOVA single way per pairs was performed (Table 49).

Table 49. Tyramine. ANOVA per pairs. Factor: inoculation treatments

MLF		IN				ES
	Inoculation	ALF (12h)	ALF (12h, 15°C)	ALF (residual sugars)	End ALF (standard)	No
IN	ALF (12h)	1	0,06	0,13	0,016	0,013
	ALF (12h, 15°C)		1	0,64	0,27	0,15
	ALF (residual sugars)			1	0,98	0,78
	End ALF (standard)				1	0,22
ES	No					1

Significant differences on tyramine production during the MLF were found between the wine inoculated at beginning of ALF (ALF 12h) and the standard inoculation, and the spontaneous MLF (p-value ≤ 0,05). The treatment “inoculation ALF (12h)” displayed the highest tyramine content.

1.15.6 Results Inoculation Time: ANOVA putrescine

In order to analyze in which treatment the putrescine production during the MLF was significantly different according to the inoculation time, an ANOVA test single way per pairs was performed (Table 50).

Table 50. Putrescine. ANOVA per pairs. Factor: inoculation treatments

MLF	IN					ES
	Inoculation	ALF (12h,)	ALF (12h, 15°C)	ALF (residual sugars)	End ALF (standard)	No
IN	ALF (12h)	1	0,796	0,088	0,056	0,025
	ALF (12h, 15°C)		1	0,200	0,136	0,059
	ALF (residual sugars)			1	0,189	0,015
	End ALF (standard)				1	1,21696E-07
ES	No					1

Significant differences were found on putrescine level in the spontaneous MLF compared with all treatments ($p \leq 0.05$) except for the one inoculated at beginning of ALF at room temperature (ALF-12h). The spontaneous MLF, resulted in an important reduction of putrescine while in other ones, it was produced in variable amounts.

1.15.7 Results Inoculation Time: oenological parameters

Regarding the characteristics of wines, the higher volatile acidity of the spontaneous MLF compared with the other treatments can be highlighted (Table 51).

Table 51. Inoculation Time: characterization of wines after bottling

Wines	Alcoholic degree %	Total acidity	Volatile acidity	Total sugars (g/L)	pH	L-malic acid	L-lactic acid
IN 12, 25-30°C	14,45	3,55	0,30	0,50	4,12	0,20	2,10
SD	0,35	0,21	0,02	0,00	0,01	0,00	0,14
IN 12, 15°C	14,25	3,35	0,31	0,75	4,12	0,20	2,15
SD	0,07	0,07	0,01	0,21	0,02	0,00	0,07
IN –remaining sugars	14,00	3,40	0,29	0,50	4,03	0,20	1,95
SD	0,21	0,14	0,01	0,00	0,01	0,00	0,07
IN-standard	13,55	3,80	0,36	0,55	3,98	0,20	2,15
SD	0,00	0,00	0,02	0,07	0,03	0,00	0,07
ES-MLF	13,65	3,80	0,52	0,95	4,04	0,20	2,05
SD	0,07	0,00	0,22	0,49	0,01	0,00	0,07

1.16 DISCUSSION: INOCULATION TIME

Implantation of starter at different inoculation time

The yeast/bacteria co-inoculation during winemaking process has been thoroughly discussed and challenged: the detractors of this practice highlight the risk of lactic disease (*piqure lactique*) and the supporters of this practice claim a better adaptation of starters in an increasing ethanol media and more efficient timing of process. Few works are available where the level of implantation of the malolactic starter has been measured by molecular means to really evaluate starter survival against the active yeast population or its better adaptation because of the low ethanol content. Results of Masqué *et al.* (2007) and Romero (2010) converge in the fact that high level of implantation of the LAB starter is achieved when the inoculation was done at the beginning of the ALF in Tempranillo wines. Nevertheless, the implantations at middle of ALF, with remaining sugars or end of ALF were strain dependent in the trials performed by Romero (2010): the implantation of strain (Elios 1) at all the inoculation time conditions was good while for VP41, no implantation was observed when inoculation was done moving to the end of ALF or sequential. In Merlot wines Masqué *et al.* (2007) observed that the best inoculation time regarding level of implantation was variable and strain dependent.

In our study, Uvaferm Alpha strain was tested, and it obtained better levels of implantation when it was inoculated with remaining sugars in the ALF, or sequential. In contrast with the observations of Masqué *et al.* (2007) and Romero (2010), in our study the inoculation at middle of ALF or at the beginning of ALF delivered poor implantations, even worst for the batch that conducted the MLF in colder conditions (15°C). Therefore, variations on level of implantation according to inoculation time seem to be strain dependent.

On the other hand, as the interaction yeast/bacteria plays a role in the adaptation of bacteria, it can be assumed that yeast also impacts LAB implantation. No difference on alcoholic fermentation kinetics was observed by other authors and in this work. But typification of yeast (with mitochondrial DNA digestion, per example) and bacteria all along the process would be necessary to better understand the possible interactions that might be hidden behind a global good performance of the alcoholic fermentation.

Regarding the timing of the process the longest MLF was for the wine without inoculation (ES) with a long latency phase of 24 days and an MLF process of 35 days. On the side of inoculated wines, the MLF conducted at 15°C was slower in L-malic acid consumption as is logically expected. In which concerns to the inoculated MLFs in chamber at room temperature, the inoculated MLFs at different stages were similar (17-18 days). Therefore, from a logistical point of view, the fact to inoculate during the ALF improved the global timing of the process as the wine was ready for racking sooner. But regarding the duration of the MLF process, the standard inoculation gave a shorter MLF of 17 days instead of 18, but this difference however might not be relevant.

Biogenic amines and inoculation time

Globally important differences on the tyramine and putrescine generated during the MLF were observed. It was particularly true for treatments where the inoculation was performed at the beginning of ALF in contrast with the other wines with standard inoculation, or close to the end of ALF. The spontaneous MLF batches, which are the same for lysozyme, inoculation time and nutrients trials, experienced a decrease of putrescine which is an event rarely described in wines as previously discussed in the trials with lysozyme. The highest biogenic amines were obtained in treatments with lower level of implantation in concordance with previous results in this thesis. However, some of these differences are not significant due to a high variability within the treatments. Some authors found reduced biogenic amines when co-inoculation was practiced (Smit and DuToit, 2011). Others found biogenic amines content to be *V. vinifera* variety dependent when co-inoculation was performed, as in the study performed by Smit *et al.* (2012) where co-inoculation reduced the biogenic amines in Shiraz wine while the Pinotage wine obtained lower biogenic amines with sequential inoculation. On the other hand, Massera *et al.* (2009) did not observe any impact of inoculation time on biogenic amines produced during the MLF in Merlot wines. All this contradictory and uncertain results might be explained by the level of implantation of the starter, but it was not evaluated in these works. Masqué *et al.* (2007) analyzed the level of implantation at different inoculation time in function of biogenic amines, but the latter were produced in little quantities and it was difficult to draw conclusions in this point. Our results indicate that inoculation time impacts the level of implantation and this seems to be linked with higher biogenic amines content.

Probably this is related to the wild microorganism population and its metabolic state at the moment of inoculation, the compatibility yeast/bacteria and characteristics of must/wine; the performance of the malolactic starter being dependent on all these variables. This suggests that the co-inoculation practice remains controversial and that optimal inoculation time for a given starter might need to be determined case by case which might be difficult to do in an industrial context.

1.17 RESULTS: NUTRIENTS

1.17.1 Results nutrients: LAB population before MLF

The impact of the addition of nutrients in the must on the implantation of malolactic starter and biogenic amines production was studied. Trials were performed in duplicate as follows:

- Must with *Go-ferm* was divided in two batches to perform separately the entire winemaking process, one batch was inoculated with malolactic starter in a standard way (direct inoculation at the end of ALF) and one batch was left without inoculation as control.
- Must without additions were also divided in two batches for inoculation and spontaneous MLF.

Surprisingly, the batch that performed the alcoholic fermentation without nutrients and destined to run the malolactic fermentation spontaneously counted with 2 log more of LAB at the end of the ALF than the other batch in equal conditions but that was destined for inoculation (Table 52). Both batches with nutrients counted with similar reduced LAB at this stage.

Table 52. Nutrients: Lactic acid bacteria population at end of alcoholic fermentation

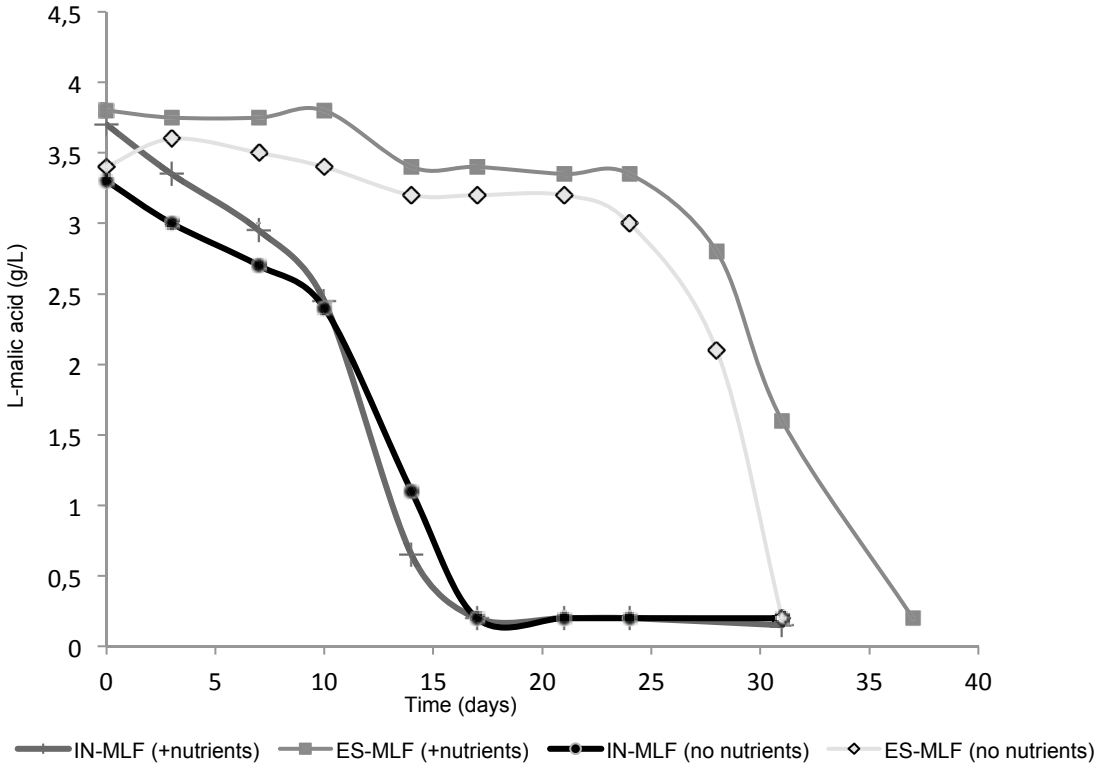
Log LAB (CFU/mL) in must	Average Log LAN (CFU/mL) ±SD End of ALF	
5,54	+ nutrients (batch to be Inoculated for MLF)	3,04±0,05
	+ nutrients (batch to run spontaneous MLF)	3,45±0,2
	no nutrients (batch to be Inoculated for MLF)	3,9±0,0
	no nutrients (batch to run spontaneous MLF)	5,8±0,0

1.17.2 Results nutrients: MLF kinetics

Malolactic fermentation kinetics was analyzed in the four batches (Figure 41):

- Inoculated MLF batches (with and without nutrients) had similar kinetics, both consuming the malic acid in 17 days.
- The spontaneous MLF with nutrients finished the MLF in 37 days instead of 31 days for the ES-MLF no-nutrients. The ES-MLF no-nutrients had 2 log more of LAB population at the end of ALF (Table 52). Differences on population size can explain the different L-malic acid consumption rates.
- A reduction of L-malic acid of 0,5 g/L was observed during the alcoholic fermentation in wines without nutrients. This is in concordance with the higher LAB population size recorded at the end of ALF which might indicate that the MLF started during the ALF.

Figure 41. MLF kinetics of trials using nutrients



1.17.3 Results nutrients: biogenic amines and implantation

Figure 42 shows the biogenic amines produced during the MLF:

- The addition of nutrients in the must increased the production of tyramine in the inoculated MLF (IN-MLF) and in the spontaneous MLF (ES-MLF) compared with the non-nutrients wines. In all these batches, the tyramine at the end of ALF was 0,2 mg/L.
- The putrescine was produced in both batches with nutrients (IN and ES). In ES-MLF no nutrients, the putrescine was degraded from 4,1mg/L at the end of ALF to 0,5 mg/L after the MLF.
- The cadaverine was synthesized in all batches except for ES-MLF with nutrient, where it was degraded; because at the end of ALF, cadaverine content was 1,3 mg/L.
- Histamine was not detected
- The implantations (Figure 43) were 100% in both inoculated batches, with and without nutrients and their repetitions.

Figure 42. Nutrients: Biogenic amines produced during MLF and implantation of malolactic starter

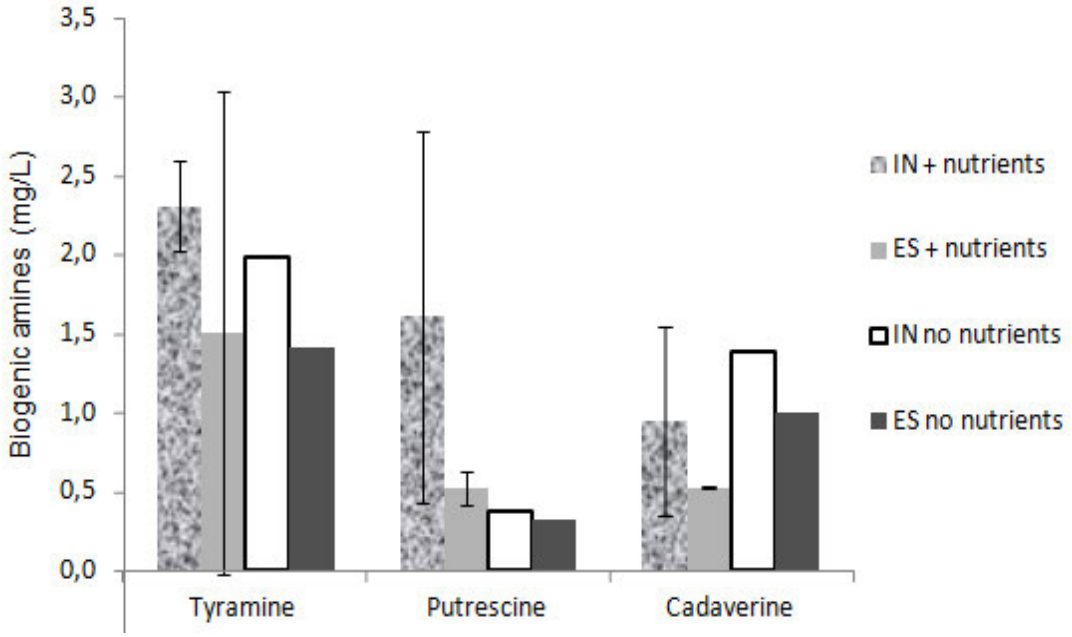
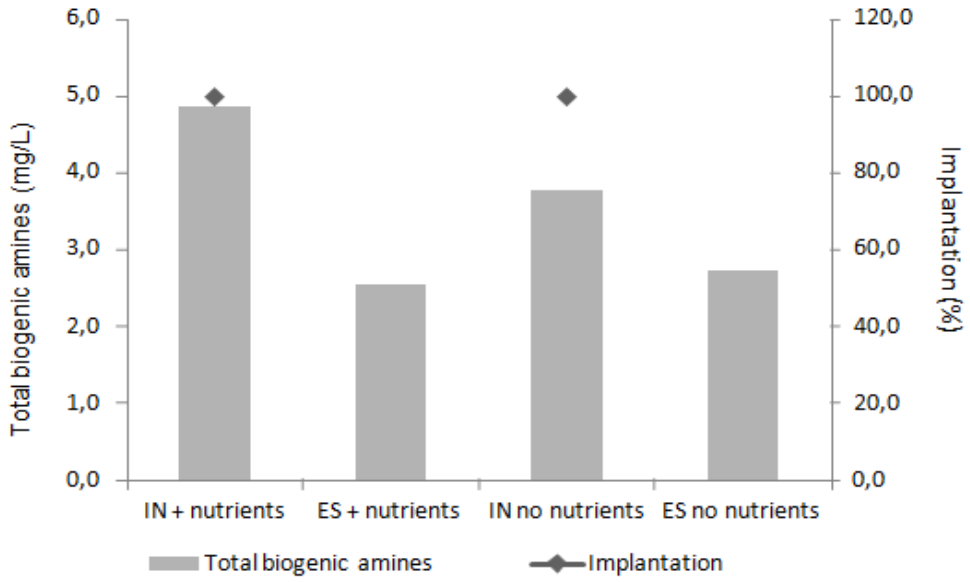


Figure 43. Nutrients: total biogenic amines at the end of MLF and level of implantation of malolactic starter.



Implantation: SD is zero in both cases as all batches had 100%.

In order to understand if the differences observed on the biogenic amines produced during the MLF are significant, an "ANOVA- 2 factors with replication" test was done (Table 53).

Table 53. ANOVA- 2 factors with replication for total biogenic amines, tyramine, putrescine and cadaverine, factors: type of MLF and nutrients

Anova: Two-Factor With Replication	Total biogenic amines	tyramine	putrescine	cadaverine
	P-values			
Sample (treatments: nutrients / no nutrients)	0,007	0,61	8,3E-08	0,68
Columns (MLF: IN/ES)	0,002	0,22	1,2E-07	0,009
Interaction	0,010	0,92	1,7E-07	0,03

There are significant differences between treatments (nutrients/no nutrients) for the total biogenic amines ($p \leq 0,01$) and putrescine ($p \leq 0,001$) produced during the MLF. The wines with nutrient presenting higher total BA and putrescine contents.

Regarding the type of MLF (IN or ES) significant differences were found in total biogenic amines ($p \leq 0,01$), putrescine ($p \leq 0,001$) and cadaverine ($p \leq 0,01$) produced during the MLF. The inoculated MLF displayed higher BA content.

Nevertheless, interaction was observed for total BA, putrescine and cadaverine, therefore the significance of the average differences need to be taken carefully as factors are not independent.

1.18 DISCUSSION: NUTRIENTS

Addition of nutrients and implantation

Addition of nutrients in the must, which is a common oenological practice, aims at enhancing the metabolism of yeast and alcoholic fermentation by increasing the concentration of essential compounds for yeast growth and metabolism, such as amino acids among another. Increased amino acids concentrations have been linked with higher biogenic amines in several works (General Introduction, Table 4) but its impact on the level of implantation of malolactic starters remains unknown. Our results showed that the addition of nutrients does not affect the level of implantation or MLF kinetics in inoculated wines. On the other hand, in spontaneous MLF, the addition of nutrients in the must slowed down the process as non-inoculated MLF with nutrients lasted longer. Zooming on the raw data, in fact the two replicates of spontaneous MLF + nutrients behaved very differently, one batch had similar MLF kinetics with the non-nutrient ES wine while the other one was very slow. Therefore, the conclusion that the addition of nutrients slows down spontaneous MLF should not be generalized.

Biogenic amines

The addition of nutrients in must or must-wine is often related to higher biogenic amines in the wine triggered by the increase of amino acids in the media (Gonzalez-Marco *et al*., 2005; Corzani, 2008; Batch *et al*., 2010; García-Marino *et al*., 2010; Smit *et al*., 2012). Our results confirm this observation as the wines enriched with nutrients had higher total biogenic amines content and putrescine. Nevertheless, the biogenic amines levels generated during these trials were low.

The inoculated wines also contained higher biogenic amines content but this might be due to the reduction of putrescine occurred in the spontaneous MLF batch, phenomenon that might suggest the presence of amines-degrading LAB or enzymes, although it is rarely observed and generalizations should be taken carefully.

1.19 CONCLUSIONS CHAPTER 1

1 **Malolactic starter might have different levels of implantation during the MLF.** Based on the results of this study, it is demonstrated that the malolactic starter might have different levels of implantation varying its performance from 0% to 100% as determined via strain typification by RAPD-PCR. Apart from full implantations, autochthonous LAB strains can develop all along the MLF together with the malolactic starter in inoculated wines and co-conduct the process.

2 **RAPD –PCR fingerprinting is a key tool to understand the MLF.** The developed methodology for monitoring the malolactic starter via the typification of LAB strains during the MLF allows the follow–up of the inoculated malolactic strain and to determine its efficiency in terms of implantation over the wild LAB population. This knowledge was key to understand the contribution of the different strains on the MLF and on the aminogenesis process. Due to the fact that a fast MLF process was not necessarily associated with a good implantation, the wine cellars should incorporate this type of analysis to verify that the malolactic fermentation is under control when malolactic starters are used.

3 **Low pH favors the implantation of the malolactic starter.** The pH was identified as a key factor to influence the LAB population during the MLF. At low pH, the level of implantation of the LAB starter is higher. This is probably due to the fact that the wild LAB population is more stressed and the commercial LAB starter has more opportunities to conduct the malolactic fermentation. At higher pH, the malolactic fermentation was conducted simultaneously by the LAB starter and the wild bacterial population.

4 **High implantation required to manage biogenic amines and volatile acidity.** The level of implantation of the LAB starters is negatively correlated with the development of biogenic amines during the MLF indicating that apart from the inoculation of bacteria which do not produce amines, its implantation needs to be guaranteed to reach low biogenic amines content. Probably for the same reasons, the volatile acidity was lower with a higher level of implantation of the malolactic starter.

5 **Reduction of biogenic amines during MLF.** Reduction of biogenic amines content was observed during the MLF probably due to the presence of amino oxidase enzymes and strains able to produce these enzymes.

6 Spontaneous MLF increased perceived structure in mouth but the biogenic amines content was not high enough to be perceived by the panelists.

7 **Direct inoculation method obtained higher implantations of malolactic starter than mother tank seeding method and it released less biogenic amines than spontaneous MLF**. Therefore, direct inoculation of malolactic starter should be the technique of choice to ensure the process is under control.

8 **Inoculation of malolactic starter by mother tank seeding method is inefficient** to ensure starter implantation and manage the biogenic amines produced during MLF. The latter were produced in similar levels as with spontaneous MLF, where malolactic starters were not used.

9 **Lysozyme affects the level of implantation of LAB starter.** Application of lysozyme and inoculation of malolactic starter need to be separated in time to avoid jeopardizing the performance of the malolactic starter.

10 **Using lysozyme, the production of biogenic amines during the MLF was not linked with the level of implantation of LAB starter.** Further studies are needed to understand the possible interaction that might exists between lysozyme and regulation of amine-decarboxylase enzyme genes or their activity.

11 **Co-inoculation improved timing of wine-making process but reduced the implantation of LAB starter which was linked to high biogenic amines content.** Therefore the inoculation of LAB starter in these conditions was not beneficial to manage biogenic amines synthesis during MLF. Even if the process timing is improved which can be of interest from an industrial point of view, the minimization of biogenic amines production should be privileged.

12 **The addition of nutrients in must does not affect the implantation of LAB starter but increases the biogenic amines in wines.** Full implantations were obtained in these trials where the malolactic starter was inoculated in standard conditions (direct inoculation at the end of ALF) but enriching the wine with amino acids coming from the blend of nutrients increased the biogenic amines.

13 **Overall, the biogenic amines and level of implantation of the malolactic starter are negatively correlated.** Nevertheless, the use of lysozyme or nutrients might deliver different results in certain conditions. Therefore, choosing the right oenological practices and monitoring the MLF using typification of LAB population is needed to ensure the process is under control and to minimize the risk of producing wines containing metabolites harmful to the health or wine commercialization.

CHAPTER 2

CHAPTER 2

Evolution of biogenic amines in bottled red wines during storage

2.1 INTRODUCTION

2.1.1 Uncertainty about final biogenic amines content in bottled wines

During the winemaking process, full implementation of a prevention program for biogenic amines control helps to manage the levels of these metabolites in the final product. Nevertheless, once the wine has been stabilized biogenic amines content do not always stay constant. On the contrary, biogenic amines content can increase or even decrease during aging or storage (Table 54). Globally there is no general rule about the evolution of biogenic amines after the MLF (Table 54) and this has been related to pH (Coton *et al*., 1999; Gerbaux and Monamy, 2000; Landete *et al*., 2005d; López *et al.,* 2012), *Vitis vinifera* variety (Hernández-Orte *et al.,* 2008), other oenological conditions (Vidal-Carou et al., 1990; Bauza *et al,.* 1995; Gerbaux and Monamy, 2000; Alcaide-Hidalgo *et al*., 2007) and to some extent, to storage temperature (Vidal-Carou *et al*., 1991; Gonzales-Marco *et al.,* 2006).

Particularly disconcerting is the increase of biogenic amines in bottled wines because once the wine is sulphited and bottled, there is not much that the oenologue can do to avoid the development of biogenic amines.

Basically, two mechanisms have been described as responsible for the evolution of biogenic amines: decarboxylation of amino acids as occurs during fermentative process which leads to increase of biogenic amines and degradation of biogenic amines mediated by amine oxidase enzymes.

Table 54. Evolution of biogenic amines during aging or storage

Biogenic Amine	Behavior	Time	Conditions	Reference
Histamine, tyramine	constant	during winemaking till a year after		Vidal –Carou *et al.* 1990
Histamine, tyramine	Increase and decrease	121 days	opened containers	Vidal –Carou *et al.* 1991
a-Histamine, Tyramine putrescine b-Phenyl ethylamine	a-increase b-decrease	Between end MLF and end winter	oak barrels	Bauza *et al*. 1995
Histamine, tyramine, putrescine, phenyl ethylamine	increase	1 month after MLF	Unsulphited wines in tanks	Coton *et al.* 1999
Histamine, Tyramine, Putrescine	increase	14 months	oak barrels	Gerbaux and Monamy 2000
a-Histamine, tyramine , cadaverine and others b-Putrescine and others	a-decrease b-increase	250 days	oak barrels,	Jimenez-Moreno *et al*. 2003
a-Histamine b-Tyramine, putrescine, phenyl ethylamine	a-decrease b-constant	12 months	Bottled wine	Landete et al. 2005d
Histamine, Tyramine Spermine, diethyl amine	decrease	120 days	Bottled wine	Gonzales-Marco *et al.* 2006
Histamine, Tyramine, Putrescine	decrease	14 months	oak barrels	Alcaide-Hidalgo *et al.* 2007
Histamine, putrescine	increase	6 months	Bottled wine	Hernandez-Orte *et al*. 2008
a-Histamine, tyramine, putrescine and others b-Cadaverine and others	a-increase b-constant	18 months	Bottles wine	Pramateftaki *et al*. 2012
a-Histamine, tyramine, putrescine and others b-cadaverine	a-increase b-constant	7 months after MLF	Bottled wine	López *et al*. 2012

2.1.2 Amino-decarboxylase activity

Decarboxylation of amino acids has been related to the presence of residual bacterial populations or exocellular enzymes present in the wine (Rollan *et al.,* 1995). Bottled wine is often an exhausted media, lacking fermentable substrates such as sugars, L-malic and citric acids. In these conditions, bacteria will obtain energy from decarboxylation of amino acids to survive generating biogenic amines (Lonvaud-Funel, 2001). Therefore, the stabilization of the wine after the MLF to control the viable bacteria populations is of paramount importance, and it can be done by sulphite addition or application of lysozyme (Gerbaux and Monamy, 2000). Effectiveness of sulphite decreases with the pH and additionally the maximum level allowed in wines is regulated. Lysozyme on the contrary has been efficient to reduce bacterial population in bottled wine at high pH (Gerbaux and Monamy, 2000). On the other hand, Landete *et al.* (2005a) demonstrated that SO_2 could prevent bacterial growth, but does not diminish the histidine decarboxylase enzyme (HDC) activity. Moreover, HDC remains stable over time, even after the extinction of the viable bacteria cells (Coton *et al.,* 1999). Cell-free extracts showed higher HDC activity in low substrates and high ethanol media, confirming that free enzymes in the bottled wine also find optimal conditions for histamine formation (Landete *et al.* 2008). With respect to the temperature, maximum HDC activity was observed between 28 - 37 °C and no HDC activity was detected at temperatures above 40°C in *in-vitro* conditions (Farias *et al.,* 1993; Landete *et al.,* 2005a). However, more histamine production was found in bottled wines stored at room temperature than in extreme temperatures (4°C and 35°C) (Gonzales-Marco *et al.,* 2006).

2.1.3 Degradation of biogenic amines

Biogenic amines degradation is a phenomenon less studied in wines. In 1971, Ough suggested that the resolution of the problem of histamine in wines will be resolved by the re-fermentation of wines using histaminolitic germs, but at that time such microorganisms were not identified (Vidal-Carou and Mariné-Font, 1985). In 1998, Leuschner *et al.* isolated lactic acid bacteria with amino-degrading ability from food. Later on, other lactic acid bacteria with this ability were isolated from meat (Fadda *et al.,* 2001), mackerel fish paste (Dapkevicius *et al.,* 2000) and anchovy fish sauce (Tapingkae *et al.,* 2010).

Jimenez-Moreno *et al.* (2003) suggested that acid pH would hinder the activity of amino oxidase enzymes in wines as these enzymes are more active at neutral or basic pH.

On the other hand, the limitation of O_2 should not be a problem for the activity of these enzymes during aging in oak barrels.

The first report on wine amino-degrading bacteria was published in 2011. Garcia-Ruiz *et al*. (2011), proved the ability to degrade histamine, tyramine and putrescine in culture media of some wine strains belonging to the species *Lactobacillus casei, L. hilgardii, L. plantarum, Pediococcus parvulus, P. pentosaceus* and *Oenococcus oeni.* This property was less pronounced in wines as ethanol and polyphenols dramatically decreased amines degradation properties of cell and cell–free suspensions.

Capozzi *et al.* (2012) found that a LAB population of 26 *Lactobacillus plantarum* strains randomly isolated from ongoing spontaneous MLF in wines, where able to degrade amines as follows: 30.8% of them degraded putrescine, 26.9% tyramine, 19.2% histamine, and 19.2% cadaverine. Two strains were selected and tested in wine and synthetic media conditions; they were able to reach high BA reductions while unable to produce biogenic amines because genes for coding amino decarboxylase were missing in these strains.

The results of these works (Garcia-Ruiz *et al*. 2011; Capozzi et al. 2012) suggested the synthesis of amino-degrading enzymes but these enzymes were not isolated or characterized.

Very recently, Callejon *et al.* (2013) finally proved that the ability to degrade biogenic amines in at least two strains of *L. plantarum* and *P. acidilactici* is linked to the presence of a single enzyme, the amine degrading or multicopper oxidase (MCO). These strains were able to degrade histamine, tyramine and putrescine in synthetic media and red wines. The amines degradation property seems to be strain dependent except for *Lactobacillus plantarum* for which this ability apparently is a general trait of the species. Higher levels of degradation were obtained in wine than in synthetic media pointing to the existence of mediator compounds in wine that collaborate in amine degradation.

Although the amine oxidase enzyme from wine LAB strains has been isolated, more work on the enzyme is needed such as definition of the optimal conditions for its activity, impact of the presence of other fermentable substrates and gene regulation. Nevertheless, amine oxidase produced by wine LAB strains might be the explanation of the reduction of biogenic amines observed during aging but this has not yet been investigated.

2.2 Aim of the study: Evolution of biogenic amines in bottled red wine

Degradation or synthesis of biogenic amines can occur in bottled wine all along the storage. This creates incertitude regarding the levels of biogenic amines that the wine might have at the moment of its commercialization and/or consumption. In this frame, the need to develop tools that can anticipate the biogenic amines that a wine will develop during the storage has become imperative.

In this investigation, we pretend to determine the causes of histaminogenesis in bottled wines with the end goal to identify key indicators of this activity that will allow in the future the development of methodologies for its early diagnosis in wines.

The main objectives of this work were 1) to accelerate the amine decarboxylation reaction in order to estimate the potential of biogenic amines production in wines and 2) to evaluate decarboxylase activity from microorganisms and cell-free wines as indicators of histaminogenesis in wines.

The specific objectives were:

a. To evaluate the evolution of biogenic amines during storage.

b. To determine the aminogenic capability of wines by their incubation at different temperatures.

c. To determine the correlation and linearity between the biogenic amines content in wines incubated at different temperatures and biogenic amines content after 12 months of storage.

d. To determine the presence of microorganisms in bottled wines at T0 with histidine decarboxylase capability.

e. To determine the presence of exocellular HDC enzymes in cell-free wines.

f. To understand the link between the presence of HDC activity with the development of histamine during storage.

2.3 MATERIALS AND METHODS

In these experiments, the biogenic amines content of finished wines coming from wine cellars were analyzed at T0, then they were stored for a year in controlled conditions, and biogenic amines were analyzed again to observe their evolution. In order to estimate whether the finished wine faces the risk of an increase of histamine, those wines were investigated at T0 for research of histidine decarboxylase activity from microflore and enzymes. At the same time, in order to accelerate the reactions wines were incubated at different conditions.

2.3.1 Wines

Tempranillo bottled wines from the North of Spain produced during campaign 2004 were taken for this study in the summer of 2005. The oenologic characteristics of these wines are shown on Table 55.

Table 55. Oenological characteristics of the Tempranillo wines.

Code of Wines	Alcoholic degree (% v/v)	Total acidity (g/L)	Volatile acidity (g/L)	Free SO_2 (mg/L)	pH	Sugars (g/L)	Yeasts (CFU/mL)	Lactic acid bacteria (CFU/mL)
36	14,25	4,6	0,38	13	4,03	3,2	0,0E+00	7,0E+06
37	13,24	5,3	0,24	16	3,75	1,1	4,0E+06	n.d.*
38	14,27	5,6	0,3	24	3,55	2,1	5,0E+04	2,6E+04
39	13,99	5,6	0,24	16	3,63	2,4	3,0E+05	9,0E+04
40	14,17	5,7	0,34	13	3,54	2,9	n.d.	n.d.
41	13,57	5,8	0,26	13	3,6	2,9	1,0E+04	n.d.

n.d.: not detected.*

Bottled wines closed at the wine cellars using cork closure were stored in a cave at 18°C ±1. At the same time, samples of these wines were split in 50 mL tubes with closure minimizing as much as possible the headspace and then treated with the methods described in Table 56.

2.3.2 Trials

Table 56. Experimental treatments

Code	Objective	Treatment	Incubation	Sampling (days)
M1	Reference sample	Bottled wine was stored	18°C ±1 in cave.	T0-T360
M2	Determine the presence of exocellular histidine decarboxylase in the wine	Sterilization of wine by microfiltration (0,2 µm) and addition of histidine (100 mg/L).	30°C in oven	T15, T30
M3	Determine histidine decarboxylase activity of microflore present in the wine	Filtration of 35 mL of wine (membrane with pores of 0,2 µm), retentate and filter was incubated in HMDBmod culture	30°C in oven	T15
M4	Accelerate decarboxylation	Wine as such was incubated	30°C in oven	T15, T30
M5	Stimulate histidine decarboxylation by presence of precursors	Wine as such + histidine (100mg/L) was incubated	30°C in oven	T15
M6	Accelerate decarboxylation	Wine as such was incubated	55°C in oven	T3, T7, T17

HMDA : histidine decarboxylating culture media

2.3.2.a Treatment M1

M1 is the reference sample. The bottled wine was stored in a cave at 18°C during 12 months (in figures, T360).

Biogenic amines histamine, tyramine, putrescine and cadaverine were measured at T0 and T360 by HPLC (method described in chapter 1) Microbiological and oenologic characteristics were also analyzed (methods described in chapter 1).

2.3.2.b Treatment M2

The objective of this treatment was to identify exocellular histidine decarboxylase activity in the wine. In fact, its presence, will announce the capability of the wine to increase the histamine content during storage even if the microbial activity is under control. This enzyme is very stable over time and is the result of the lysis of microorganisms in the wine.

The method consisted in the following steps:

- Wine sample was filtered in order to remove the microorganisms using a membrane of 0,2 µm in sterile conditions. This impedes the synthesis of histamine coming from bacterial activity and histamine formation can be attributable to the free enzymes present in the wine.
- Histidine was added to the sample (100 mg/L) in order to promote the enzyme activity.

The sample was incubated at optimal temperature for the enzyme histidine decarboxylase, 30°C (Farias *et al.* 1993, Landete *et al*. 2008)

Histamine was quantified after 15 and 30 days of incubation by HPLC as described in Chapter 1.

2.3.2.c Treatment M3

The objective of this treatment was to determine if the microorganisms present in the bottled wine have the capability to decarboxylase the histidine into histamine, and in consequence, determine if there is the risk to increase the histamine levels during storage due to bacterial activity.

The following procedure was carried-out:

- 350 mL of wine was filtered using a membrane of 0,2 µm and vacuum pump.
- The filter containing the retained solids was incubated in 10 mL of liquid H-MDBmod medium (Landete *et al.,* 2005c) which only contains histidine as fermentable substrate.
- The culture was incubated at 30°C in CO_2 (5%) atmosphere.
- The histamine was measured after 15 days incubation by HPLC, as described in chapter 1.
- After 15 days of incubation, an aliquot of the culture was seeding with a loop in HMDA agar. After incubation in anaerobic conditions, the colonies were examined by microscopy, Gram and oxidase test.

2.3.2.d Treatment M4

The objective of this treatment is to accelerate the decarboxylation of histidine in order to anticipate the formation of histamine in long term.
The method was:

- A sample of wine in a closed tube with minimal headspace was incubated in an oven at 30°C.
- Histamine was measured after 15 and 30 days of incubation by HPLC.

In case no histamine is produced, one can wonder if this is due to the lack of substrate, the histidine. To test this hypothesis, treatment M5 was also carried out.

2.3.2.e Treatment M5

The objective of this treatment was to determine if potentially a lack of histamine formation is due to unavailable substrate histidine.

The method was:

- 100 mg/L of histidine was added to the wine and inoculated in equal conditions as treatment M4.

2.3.2.f Treatment M6

The objective of this treatment was to accelerate the histidine decarboxylation and to have the results in a shorter period of time. The test was as follows:

- Wine samples were incubated in closed essay tubes of 50 mL with minimal head space at 55°C in an oven.
- Sampling was done at 3, 7 and 17 days of incubation.
- Histamine was measured by HLPC as described before.

2.3.3 Study of autochthonous micro flora present in the bottled wine

Quantification of microorganisms present in the wine was done following plate incorporation method: 1ml of wine was put in an empty Preti plate and then, melted agar culture media (Table 57) was incorporated and gently mixed. When the agar was solidified, plates were incubated in the conditions described in Table 57. This was done at T0 and T360. Results were expressed as CFU/mL.

Table 57. Culture media and incubation conditions used for investigation of microorganisms in bottled wine.

Target	Culture Media	Incubation conditions	References
Lactic acid bacteria	MLO + 50 mg/L nystatin	30°C, atmosphere at 5% CO_2	Zuñiga *et al.* 1993
Acetic acid bacteria	GYC + 50 mg/L nystatin + 50 mg/L pencillin	30°C aerobic	Merck, 2000
Yeast and moulds	Sabouread + 0.05 g/L chloramphenicol	30°C aerobic	Merck, 2000
Aerobic mesophilic bacteria	PCA	30°C aerobic	Merck, 2000

2.4 RESULTS

2.4.1 Results: treatments to accelerate biogenic amines formation

In order to accelerate the amine-decarboxylation process wines were incubated at 30°C and 55°C. Tables 58, 59, 60 and 61 show the biogenic amines content obtained in wines stored at these temperatures and sampling at different timing.

Table 58. Histamine (mg/L ± uncertainty)

wines	M1-T0 18°C-T0	M1-T360 18°C-360 days	M4-T15 30°C-15 days	M4-T30 30°C-30 days	M6-T3 55°C-3 days	M6-T7 55°C-7 days	M6-T17 55°C-17 days
36	15,6±0,8	9,5±0,8	20,4±0,8	16,5±0,8	19,4±0,8	25,8±0,8	7,2±0,3
37	2,5±0,2	8,5±0,3	8±0,3	8±0,3	4,2±0,2	3,3±0,2	2,5±0,2
38	2,2±0,2	2,5±0,2	6,8±0,3	6,4±0,3	2,8±0,2	3,6±0,2	2,7±0,2
39	7,4±0,3	10,3±0,8	12,2±0,8	6,3±0,3	3,4±0,2	5,1±0,3	3,4±0,2
40	4±0,2	0,8±0,2	2,6±0,2	1,9±0,2	3,5±0,2	3,4±0,2	2,3±0,2
41	8±0,3	4,8±0,2	9,1±0,3	8,4±0,3	7,7±0,3	9±0,3	7,3±0,3

Table 59. Tyramine (mg/L ± uncertainty)

wines	M1-T0 18°C-T0	M1-T360 18°C-360 days	M4-T15 30°C-15 days	M4-T30 30°C-30 days	M6-T3 55°C-3 days	M6-T7 55°C-7 days	M6-T17 55°C-17 days
36	4,8±0,2	6,7±1	5,4±0,2	5,4±0,2	5,4±0,2	9,1±1	4,8±0,2
37	1±0,2	5,6±0,2	0±0,2	0±0,2	1±0,2	0,9±0,2	1,7±0,2
38	0,8±0,2	1,3±0,2	1±0,2	0,7±0,2	0,8±0,2	0,8±0,2	1,1±0,2
39	1,2±0,2	1,5±0,2	1±0,2	1,1±0,2	1,1±0,2	0,8±0,2	0,9±0,2
40	0,8±0,2	1,6±0,2	0,8±0,2	0,9±0,2	0,8±0,2	0,9±0,2	1,1±0,2
41	0,7±0,2	3,3±0,2	0,8±0,2	0,8±0,2	1,2±0,2	0,8±0,2	0,9±0,2

Table 60. Putrescine (mg/L ± uncertainty)

wines	M1-T0 18°C-T0	M1-T360 18°C-360 days	M4-T15 30°C-15 days	M4-T30 30°C-30 days	M6-T3 55°C-3 days	M6-T7 55°C-7 days	M6-T17 55°C-17 days
36	30,4±1	7,9±0,2	24,3±1	25,3±1	22,3±1	26,4±1	20,4±1
37	17,7±0,2	17,3±0,2	21,4±1	20,4±1	20,4±1	17,8±0,2	15,6±0,2
38	15,4±0,2	5,9±0,2	21±0,2	28,4±1	16,4±0,2	18,9±1	17,3±0,2
39	64,4±1	15,6±0,2	75,9±1	85,8±1	45,2±1	66,2±1	43,5±1
40	30,8±1	7,3±0,2	25,9±1	32,8±1	27,7±1	29±1	24,6±1
41	77,2±1	5,2±0,2	83,7±1	78,6±1	51,4±1	66,7±1	54,9±1

Table 61. Cadaverine (mg/L ± uncertainty)

wines	M1-T0 18°C-T0	M1-T360 18°C-360 days	M4-T15 30°C-15 days	M4-T30 30°C-30 days	M6-T3 55°C-3 days	M6-T7 55°C-7 days	M6-T17 55°C-17 days
36	0,8±0,1	3,5±0,1	1,1±0,1	1,3±0,1	1,2±0,1	1,3±0,1	1,2±0,1
37	1,2±0,1	0, 1 ±0,1	1,4±0,1	1,3±0,1	1,3±0,1	1,2±0,1	1,1±0,1
38	0,6±0,1	0,7±0,1	0,6±0,1	0,5±0,1	0,6±0,1	0,6±0,1	0,5±0,1
39	1,6±0,1	1,3±0,1	1,7±0,1	1,5±0,1	1,5±0,1	1,2±0,1	1±0,1
40	0,6±0,1	0,8±0,1	0,6±0,1	0,6±0,1	0,6±0,1	0,5±0,1	0,5±0,1
41	1,3±0,1	0,7±0,1	1,4±0,1	1,3±0,1	1,4±0,1	1,2±0,1	1±0,1

2.4.2 Correlation between BA content in wine stored for a year and treatments

Analysis of correlation (r) was performed between the accelerating treatments (M4 and M6) and biogenic amines content after a year of storage (M1-T360) (Table 62).

Table 62. Matrix of correlation between the biogenic amines content after a year of storage and those obtained with the different treatments

Coefficient of Correlation (r)	Histamine M1-T360	Tyramine M1-T360	Putrescine M1-T360	Cadaverine M1-T360
M4-T15 (30°C)	0,767	0,574	0,007	-0,010
M4-T30 (30°C	0,626	0,582	0,040	0,244
M6- T3 (55°C)	0,418	0,734	-0,026	0,124
M6- T7 (55°C)	0,435	0,718	-0,002	0,350
M6- T17 (55°C)	0,300	0,805	-0,151	0,403

Histamine and tyramine were well correlated with accelerating treatments (Table 62):

- Histamine after a year (M1-T360) is highly correlated with treatment M4-T15 (incubation of wine at 30°C for 15 days).
- Tyramine after a year (M1-T360) is highly correlated with treatment M6-T17 (incubation of wine at 55°C for 17 days).
- Moderated correlation between cadaverine M1-T360 and M6-T17 (incubation of wine at 55°C for 17 days) was observed.
- Treatments are not correlated with putrescine content at T360.

Globally there is an important reduction of putrescine (Table 60) when comparing the its initial content and that one at the end of storage (M1-T0 and M1-T360) while the accelerating treatments generated more putrescine. A notable case is wine 41 (Table 60) where the initial putrescine was 77 mg/L, it was reduced to 5,2 mg/L after a year and all the treatments generated between 51 and 83 mg/L. This explains the poor correlation between the treatments and putrescine content after the storage.

2.4.3 Linearity of results

In order to determine the model that fits the pool of data of the observed correlations between biogenic amines content obtained by the accelerating treatments and the biogenic amines at the end of the storage (r values, Table 62), scatter diagrams and regression analysis were performed (Figures 44, 45 and 46).

Figure 44. Scatter diagram for histamine, treatments M1-T360 and M4-T15.

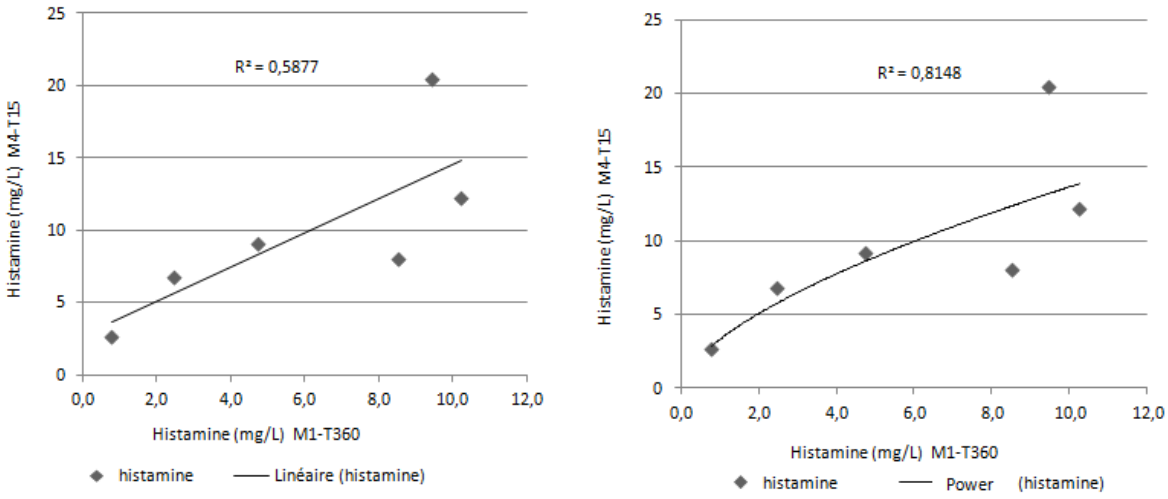


Figure 45. Scatter diagram for tyramine, treatments M1-T360 and M6-T17

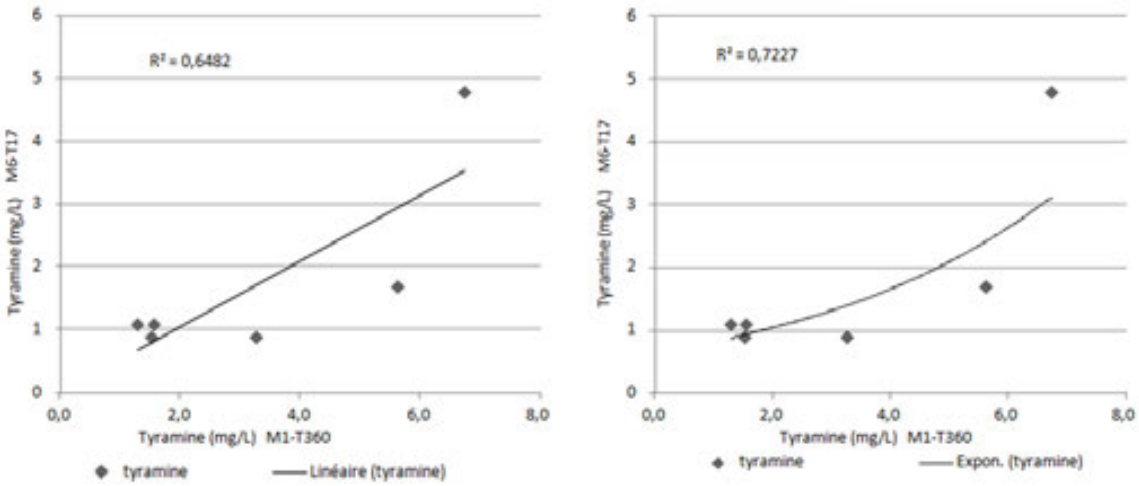


Figure 46. Scatter diagram for cadaverine, treatments M1-T360 and M6-T17

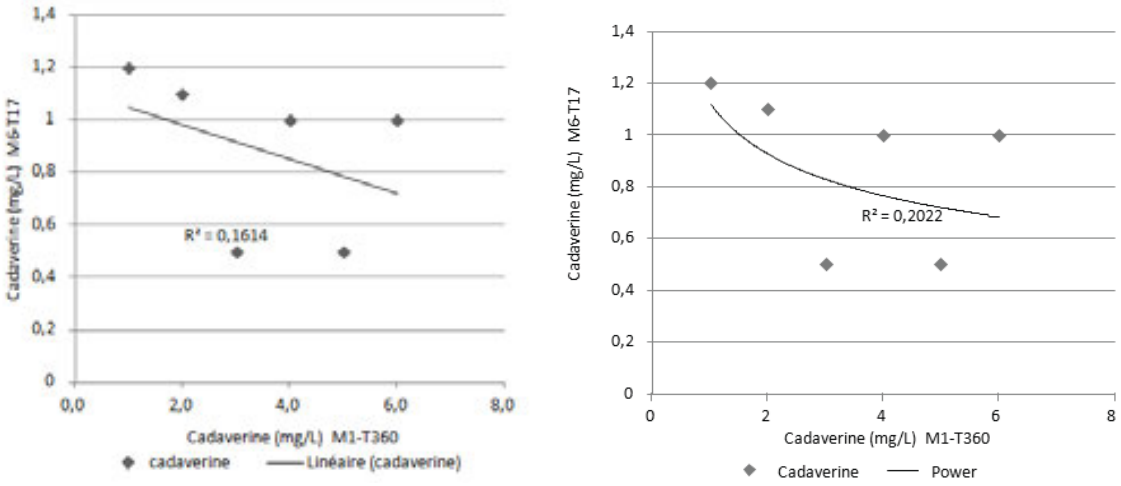


Figure 44 shows the concordance of linear and power function with the data on histamine. Power function reveals better fit between the histamine values of treatment M41 and histamine levels after a year of storage (M1-T360) with R^2 of 0,81.

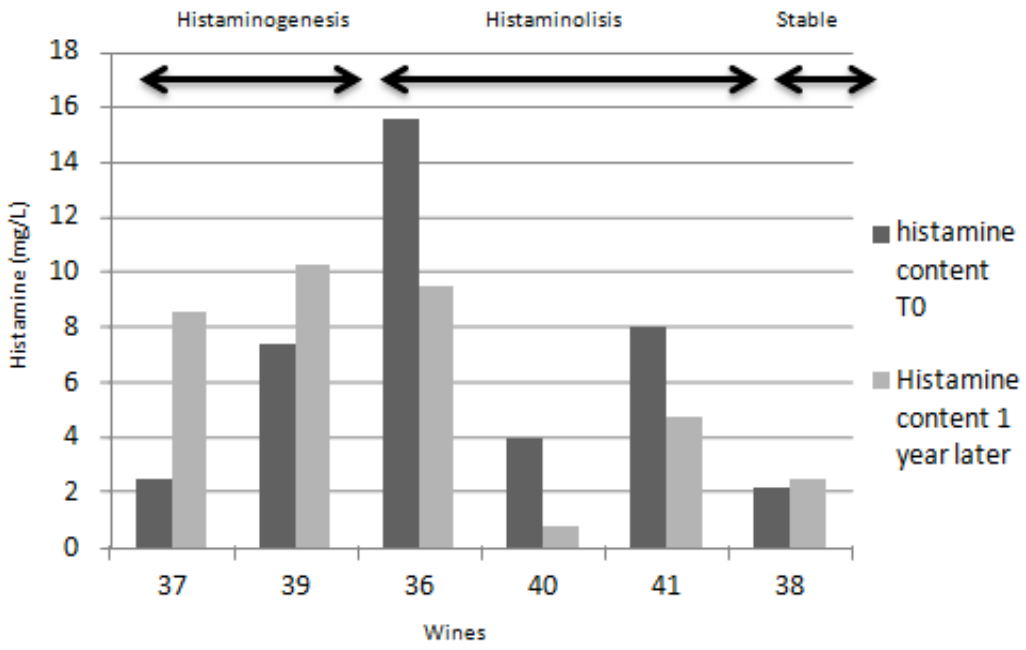
For the tyramine (Fig. 45), the concordance of linear function with the data is R^2=0,64 while that for exponential model is R^2= 0,72.

For the cadaverine (Fig. 46), the correlation between the indicator (M6-T17) and the cadaverine content after a year of storage does not seem to follow a linear (R^2=0,16) or exponential (R^2=0,20) model. Regarding the scatter diagram it is difficult to see the model that explains the moderated correlation between M6-T173 and M1-T360 for cadaverine, therefore, the correlation r=0,4 (Table 62) might not be relevant.

2.4.4 Evolution of histamine content after a year of storage

Since histamine is the most controlled biogenic amine in wine trade transactions in certain countries, its behavior was analyzed in the studied wines. Regarding histamine evolution from T0 to end of storage, 3 clusters were identified (Figure 47). Two wines had a histaminogenic profile (histamine increased during storage), three wines had histaminolitic profile (histamine reduced during storage) and one wine had a stable profile (histamine was constant, or balance between synthesis and degradation).

Figure 47. Histamine-profiles of the studied bottled wines according to the evolution of histamine during a year of storage



To understand the mechanism that drove these differences, the wines were investigated for their histamine formation capacity due to the presence of exocellular HDC enzymes (treatment M2) or/and metabolism of microorganisms present in the bottled wine (treatment M3). Additionally, histidine was added to the wine to stimulate HDC activity (treatment M5). The latter treatment will help to understand whether a lack of histamine formation is due to unavailability of substrates.

2.4.4.a Wines with Histaminogenic profile

The following Table 63 shows the results of histamine content of wines presenting histaminogenic profile, wines 37 and 39.

Wines 37 and 39 experienced an increase of histamine during storage for one year (Table 63):

- Exocellular histidine decarboxylase (HDC) enzymes were probably present in the wine as the sample of treatment M2 developed histamine where microorganisms were not present.
- When microorganisms were isolated from the wine and cultivated in HMDB culture media, the microorganisms of both wines showed HDC activity, as histamine was quantified by HPLC in the culture (M3). This indicates that the microorganisms of these bottled wines had the ability to synthesize histamine. Surprisingly, the histamine obtained of microflora of wine 39 was modest (1mg/L).

Table 63. Wines presenting histaminogenic profile. Results of histamine content (mg/L) of the different treatments.

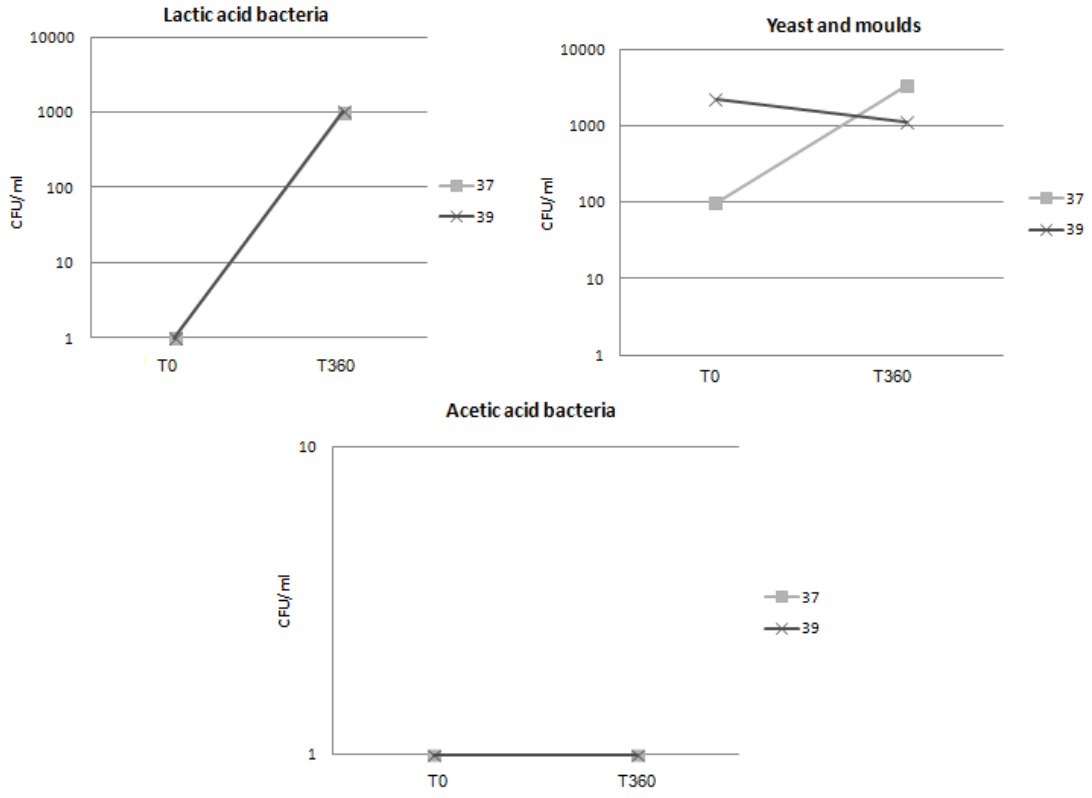
Treatments		Wine 37 Histamine (mg/L)	Wine 39 Histamine (mg/L)
Histamine evolution during storage (M1)	T0	2,5	7,4
	T360	8,5	10,3
Cell-free wines, enzymatic activity (M2)	T15	2,9	6,2
	T30	5,9	8,2
Histamine produced in H-MDBmod culture medium media by microorganisms isolated from the wine (M3)		5,7	1
Microorganisms developed in H-MDagar plates		Yeast* Moulds*	Bacteria*
Stimulation of Histidine decarboxylation by addition of substrate (M5)		2,9	4,6

**No pink halo was developed in H-MDA plates*

- The addition of histidine (M5), did not enhance histamine production in wine 37 as the measured histamine in this treatment was similar to the T0 value. In wine 39, a degradation of histamine took place, as the histamine was 4,6 mg/L (M5) and at T0 the histamine was about 7,4 mg/L. The same was observed in M2-T15.

Regarding the population of microorganism after a year of storage (Fig. 48), the wines count with a considerable population of LAB and yeast and moulds (Y-M) while acetic acid bacteria (AAB) was not present at T0 or end of storage. This is partially confirmed by the microorganisms obtained in HMDAmod plate (Y-M for wine 37 and bacteria for wine 39, Table 63).

Figure 48. Wines with histaminogenic profile. Lactic acid bacteria, yeast & moulds and acetic acid bacteria counts at T0 and end of storage



In wine 37, the HDC ability of the microorganism population seems to play an essential role in the histamine content of the wine after a year of storage.

In wine 39, the contribution of the free HDC enzymes seems to have more impact on the histamine content of the wine after a year of storage than the microorganism activity. The histaminolitic effect observed in treatment M5 finally does not seem to have a high impact as the histamine content increased after a year.

2.4.4.b Wines with Histaminolitic profile

The following Table 64 shows the histamine levels obtained upon the different treatments in the wines having an histaminolitic profile, wines 36, 40 and 41:

Table 64. Wines presenting histaminolitic profile. Results of histamine content (mg/L).

Treatments		Wine 36 Histamine (mg/L)	Wine 40 Histamine (mg/L)	Wine 41 Histamine (mg/L
Histamine evolution during storage (M1)	T0	15,6	4,0	8,0
	T360	9,5	0,8	4,8
Cell-free wines, enzymatic activity (M2)	T15	14,7	3,6	6,7
	T30	18,2	2,1	5,3
Histamine produced in H-MDBmod by microorganisms isolated from the wine (M3)		0	3,7	3,9
Microorganisms developed in H-MD agar plates		Yeast* moulds*	Bacteria*	Bacteria*
Stimulation of Histidine decarboxylation by addition of substrate (M5)		25	2,8	8,6

**No pink halo was developed in H-MDA plates or broth*

The histamine of wine 36 (Table 64) decreased by 6 mg/L in a year:

- The micro flora inoculated in culture media HMDA (M3) did not produce histamine in these conditions, indicating that the microorganism population present in the wine might not have histidine decarboxylase to synthesize histamine.
- The cell-free wines (M2) increased the histamine level after 30 days, indicating that exocellular histidine decarboxylase were present in the wine to decarboxylate the histidine.
- When histidine was added to the wine and incubated at 30°C (M5), histamine levels increased considerable after 15 days.
- The wine as such, incubated at 30°C (Table 58, treatment M4-T30) also increased its histamine content after 30 days.

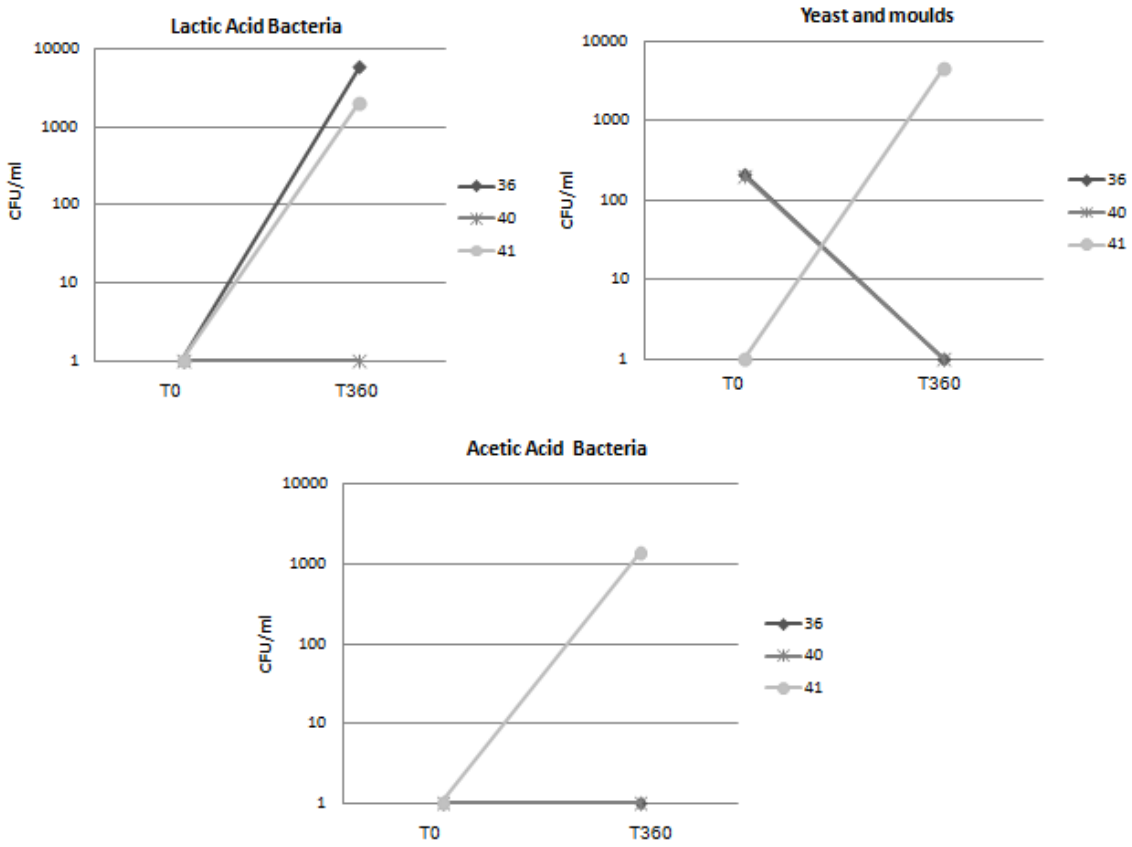
- Wine 36 increased the LAB population (Fig. 49) during storage, Y-M decrease and a lytic process of yeast cell can be envisaged. AAB remained stable (<1 CFU/mL).
- The presence of free histidine decarboxylase enzyme in wine 36 is confirmed which present a risk of histamine generation during storage. Indeed results show that a reduction of histamine occurred in wine 36 during the year of storage, a histamine degradation mechanism performed by the LAB present in the wine is envisaged. It is possible, that for these microorganisms, the histamine can be used as source of energy as they do not seem to be able to obtain energy from decarboxylation of histidine as observed in treatment M3.

The histamine of wines 40 and 41 decreased during storage after a year (Table 64):

- In wine samples without microorganisms (M2), a degradation of the histamine was observed. This might indicate that histaminolitic enzymes, like amine oxidase, might be present in the wine producing a reduction of histamine content.
- The microorganisms incubated in culture media HMDBmod synthesized histamine (M3), indicating that the risk to increase histamine in the wine by the microorganisms is real.
- The addition of histidine did not promote the synthesis of histamine as the histamine measured in M5 after 15 days of incubation at 30°C was constant in wine 41 and even decreased in wine 40, indicating histaminolitic activity in the latter case. As the histamine content did not increase with the addition of histidine, this indicates that a limitation in the synthesis of histamine by the substrate is not the mechanism that explains the absence of histamine development in these wines.
- Degradation of histamine is also observed in wine 40 stored at 30°C (M4, Table 58).
- Regarding the evolution of the microbial population (Fig 49), wine 41 increases the LAB, Y-M and AAB to considerable levels. Wine 40 kept LAB and AAB at cero, and decrease the Y-M levels. Thus, the amine-oxidase activity might come from exocellular enzymes in this wine.

Even if the microorganisms in the wine present a risk of developing histamine in the long term, it seems that the exocellular enzymes (amine oxidase type) in the wine with histaminolitic activity have a more important impact on the histamine content than the histidine decarboxylase, leading to a reduction of histamine in the wine during storage.

Figure 49. Wines with histaminolitic profile. Lactic acid bacteria yeast & moulds and acetic acid bacteria counts at T0 and end of storage



2.4.4.c Wines with Histamine-stable profile

Wine 38, presented stable histamine content during storage (M1) (Table 65):

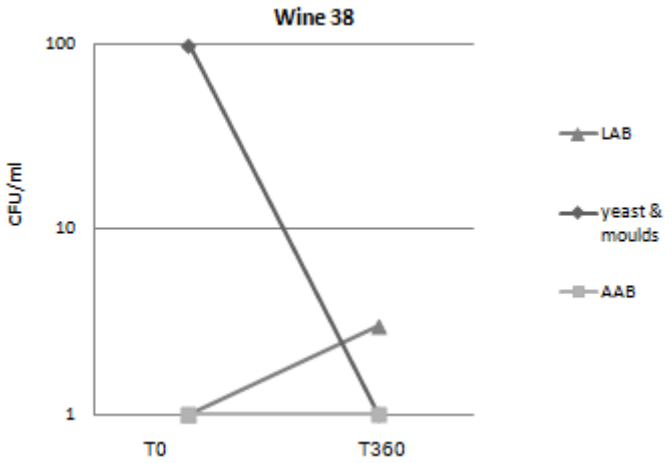
Table 65. Wine presenting histamine-stable profile. Results of histamine content (mg/L).

Treatments		Wine 38 Histamine (mg/L)
Histamine evolution during storage (M1)	T0	2,2
	T360	2,5
Cell-free wines, enzymatic activity (M2)	T15	7,7
	T30	4,2
Histamine produced in H-MDBmod culture media by microorganisms isolated from the wine (M3)		2,5
Microorganisms developed in H-MDA agar plates		Yeast*
Stimulation of Histidine decarboxylation by addition of substrate (M5)		7,3

**No pink halo was developed in H-MDA plates*

- The free HDC enzymes synthesized histamine in treatment M2 during the 15 first days and then, histaminolitic process was in place and the histamine content was reduced at T30.
- The microorganisms isolated from the wine (M3) were able to produce histamine, indicating that the microorganisms in the bottled wine present a risk to increase histamine levels during the storage.
- When histidine was added to the wine (M5), the histamine levels increased considerably, suggesting a limitation of substrate for the HDC activity.
- Regarding the microorganism evolution during storage (Fig. 50), no important population of LAB was counted at the beginning and it slightly increased at the end of storage. Yeast & moulds and AAB decreased during storage.

Figure 50. Wine with stable histamine profile, wine 38. Lactic acid bacteria, yeast & moulds and acetic acid bacteria count at T0 and end of storage period.



2.4.5 Oenologic parameters

Table 66. Oenological parameters of the studied wines.

Wines	sample	Volatile acidity (g/l)	Free SO_2	pH
37	T0	0,24	16	3,75
	T360	0,35	0	3,74
39	T0	0,24	16	3,63
	T360	0,28	0	3,61
36	T0	0,38	13	4,03
	T360	0,44	0	3,83
40	T0	0,34	13	3,54
	T360	0,35	0	3,49
41	T0	0,26	13	3,6
	T360	0,29	0	3,56
38	T0	0,3	24	3,55
	T360	0,32	0	3,5

2.5 DISCUSSION

Evolution of biogenic amines during storage

Bottled red wines were stored at constant conditions (18°C±1) for a year and biogenic amines were measured at T0 and end of storage. Effectively, the content of biogenic amines followed different behaviors, histamine and cadaverine increased, decreased or stayed constant depending on the wine/sample analyzed while tyramine increased and putrescine decreased with one exception. As previous works showed, there is no consensus on the evolution that the amines would have after a certain period of storage. This might be function of the type of microorganisms that survive in the wine, their metabolism and availability of substrates. These results put in evidence the complex nature of the subject.

Effect of temperature

Incubation of wines at 30°C, which is the optimal temperature of HDC enzymes (Farias *et al.,* 1993, Landete *et al.,* 2005a), accelerated the aminogenesis of the studied wines, increasing histamine and putrescine while tyramine and cadaverine was mostly unchanged. Moreno-Arribas and Lonvaud-Funel (1999) determined that the tyrosine decarboxylase (TDC) loses its activity at 25°C (pH 5.0) when stored for 15 and 30 days. This is close to the conditions tested in this work which in part might explain the non development of tyrosine. On the other hand, ornithin decarboxylase (ODC) optimum temperature is 35°C and pH 5.5 (Bonnin-Jusserand *et al,.* 2011) which can explain the development of putrescine in the studied conditions. Romano *et al.* (2013), recently described new enzymes responsible for cadaverine (named KDO enzyme) and putrescine synthesis (ODC enzyme) by a strain of *Lactobacillus spp.* with increased relative activity at 37°C and pH 5.5 for KDC and 47°C and pH 6.0 for ODC.

Extreme temperatures as 55°C increased histamine content in some wines when incubation was about 3 and 7 days. Tyramine levels were constant, confirming that this temperature is inhibitory for TDC enzyme. Nevertheless, in one wine the tyramine doubled its initial content; therefore, the presence of another type of enzyme active at higher temperatures should not be discarded. Putrescine experienced a decrease after 3 days incubated at 55°C, then an increase (7 days) to finally decrease again (17) days. This fluctuation can be explained by the action of different enzymes, amino acids-decarboxylase and amine-oxidase but there is not available data on the optimal

temperature for amino-oxidase activity in wines. In the case of cadaverine, fluctuation was observed in some wines while in others it increased or they were not altered by heating. In most of the cases, for the four biogenic amines, a longer incubation (17 days at 55°C) led to a reduction of them. One can wonder if traces of clarification treatments or natural macromolecules of the wine might precipitate at these conditions and because of their affinity with the biogenic amines, make them precipitate with consequent reduction of biogenic amines in wines where reduction was observed in extreme conditions.

Having in mind that the objective of these treatments is to accelerate biogenic amines formation by optimal and extreme temperatures conditions, we cannot avoid thinking that in other context, for example, during storage and transportation, these temperatures might have serious consequences on the development of biogenic amines, mostly in countries where high temperatures are common.

Correlation and linearity of results

Accelerating treatments that have high correlation with histamine and tyramine production during the storage were identified. Nevertheless, when observing values at T0 and after 12 months of storage, the responses are not linear as biogenic amines increase, decrease or stay constant. The reason could be that incubation of wines at 30°C and 55°C seems to accelerate enzymatic reactions of for example amino acids-decarboxylase but at the same time the amine-degrading enzymes. Thus, a balance between the both mechanisms will deliver the final biogenic amines. Therefore, given the fact that the evolution of biogenic amines is not linear, establishing a reliable correlation between the treatments and the biogenic amines values at the end of storage was a challenge when all wines were analyzed. Clustering of wines according to their histamine-profile gave a hint on the matter.

Histamine-profile of wines

According to the behavior of histamine during the storage, the studied wines where classified as histaminogenic, when histamine increased (2 wines), histaminolitic when histamine decreased (3 wines) and histamine-stable when changes on histamine content were not observed (one wine).

Histaminogenic wines

The wines that experienced an increase of histamine during storage already contained at T0 some microorganisms able to decarboxylase histidine in culture media and free HDC activity. A slight reduction of histamine was observed in cell-free wines inferring the presence of degrading enzymes in those wines. Viable lactic acid bacteria, yeast and moulds were detected in these wines at the end of storage confirming metabolic activity in bottled wine. In these wines, HDC ability of the microorganism together with the free HDC seem to play an essential role in the histamine content overcoming the histaminolitic effect as the histamine content increased after a year.

When the wines were analyzed at T0, the treatments indicated that the wines counted with the necessary base to increase the histamine over time and this was confirmed by measurement of histamine at the end of storage.

Histaminolitic wines

Histaminolitic wines showed two different scenarios, one where the microorganisms analyzed at T0 were unable to synthesize histamine and the other where microorganisms produced histamine. In both cases, active histamine degradation was observed in wine with and without viable microorganisms at the end of storage. This indicates that histaminolitic enzymes, might be part of metabolically active cells or they might be exocellular resulting from the lysis of microorganisms.

When wines were analyzed at T0, the active degradation of histamine indicated that histamine will potentially decrease during storage, mostly in the wine where microorganisms were not able to produce histamine. Nevertheless, these results help to understand the evolution of histamine but they can be confusing for giving a diagnosis at T0. Extensive research is needed to extend current limited database to find uncontestable indicators of histaminolitic process in wines.

Histamine-stable wine

Only one wine kept histamine constant after a year of storage. Microorganisms isolated at T0 were able to produce histamine. HDC activity in cell-free wines was evidenced as well, but it seems that a lack of substrate inhibited histamine production in this wine. Reduction of histamine was observed and at the same time the population of microorganism decreased. This probably means that the balance between the synthesis of histamine by microorganisms and free enzymes until consumption of substrates, and then the degradation of histamine led to a stable histamine content after a year. It is

possible that a peak of histamine occurred in between, but this was not measured as sampling was done at T0 and 1 year after storage.

Degradation of biogenic amines

Degradation of biogenic amines in wines has been recently linked to amino-oxidase enzymes produced by wine lactic acid bacteria (Gonzalez-Ruiz *et al,.* 2011; Corzanni *et al*., 2012; Callejon *et al*., 2013). Nevertheless, the multicopper amine-oxidase would be limited because of the reduced oxygen content in bottled wines. However, in our work, the property to degrade histamine was observed in all the studied wines, indicating that bottled wine should be the perfect ecological environment where to look for microorganisms with histamine degrading capabilities. In these conditions, the wine contains little fermentable substrates and to survive the microorganisms might need to activate metabolic routes to obtain energy from the available molecules, as in the case of biogenic amines. The presence of other microorganism than bacteria with histamine degrading capabilities should be considered as in some wines, yeast & moulds and acetic acid bacteria also were viable at the end of storage. For them as well, fermentable substrates are also limited. This idea is supported by the fact that fungi from vineyard ecosystem with ability to degrade biogenic amines were described by Cueva *et al.* (2012). *Acetobacter aceti* and *A. pasteurianus* were able to degrade up to 25% of histamine and tyramine in synthetic media and wine as described by Landete *et al.* (2005b). Moreover, biogenic amines can be used by yeast as a source of nitrogen (van Dijken and Bos, 1981; Torrea-Goñi and Ancín Azpilicueta, 2001) and the copper amine oxidase in yeast is currently well known (Cai and Klinman, 1994) but this type of enzyme might find a limitation of oxygen for its reaction in bottled wines. In fact, the oxygen level in bottled wines is function of filling operations, as design of the lines, the use of inert gases in the headspace (Jung *et al,.* 2009) and the permeability of closures types (Silva *et al,.* 2011). During aging, the O_2 combines with wine molecules untill free O_2 is not available and the bottled wine becomes an anaerobic media. If amine-oxydases are synthesized in wines, these should be active during the period where oxygen molecules are still dissolved in the wine. Jung *et al.* (2009) said that total O_2 consumption might happen between 10 days and 4 months after bottling depending on the mentioned above.

On the other hand, out of amine-oxidase, the amine-deshydrogenase is oxygen independent and has been described for other types of bacteria (Eedy and Large, 1968; Takagi *et al.,* 1999; Hacisalihoglu *et al.,* 1997). This indicates that other type of

enzymes than the oxygen dependant should not be discarded as possible amines catabolic pathways in reduced oxygen environments as the bottled wines.

Although this study focuses on histamine, it is important to mention that putrescine decreased in almost all wines during storage. Del Prete *et al.* (2009) found similar results during the alcoholic fermentations. They postulated that this could be due to the fact that putrescine is a polyamine and yeasts incorporate it in their metabolism, for example, as spermidine and spermine precursor.

Our results and the ones obtained by other authors as mentioned before suggest that presence of amine-oxidase might not completely explain the phenomenon of histamine reduction in bottled wines and the entire ecosystem in this conditions needs to be taken into account as the interactions seem to be quite complex.

HDC activity in cell-free wines

Histamine was produced in almost all the cell-free wines suggesting the presence of exocellular HDC enzymes. This confirms that HDC enzymes are quite stable (Rollon *et al*., 1995, Coton *et al.,* 1999, Lonvaud-Funel, 2008) and can be present in the wine thus increasing the histamine levels during the storage (Landete *et al.,* 2005d).

Limitation of substrate, case of histidine

In one case, histamine formation was increased substantially when histidine was added to the wine. This suggests that a lack of substrate limited histamine formation in this wine. However, amino acids are usually in excess in wines as a result of bacteria and yeast autolysis (Alcaide-Hidalgo *et al,* 2007). Certainly the HDC activity is stimulated by the presence of histidine (Lonvaud-Funel and Joyeux, 1994; Landete *et al.,* 2005a, Landete *et al.,* 2008) but analysis of amino acids should be done to confirm the lack of substrate in this specific wine.

Microorganisms with biogenic capacity

Wines at T0 were sterilized by filtration and filters were incubated in synthetic medium containing histidine followed by histamine detection by HPLC. Having in mind the objective of the study, it makes sense to analyze all the micro-ecosystem together as is wanted an assessment of the risk of histaminogenesis in the wines despite the group of microorganisms responsible for this. Landete *et al.* (2007b) reported that only lactic acid bacteria were able to synthesize histamine while acetic acid bacteria and yeast were not able in wines. It is risky to take this generalization in the context of our work because the

strains evaluated by Landete *et al.* (2007b) comes from collections and the environment of a bottled wine during long storage periods might force the microorganisms to develop adaptive metabolic routes. For example, in wine 40, the micro-ecosystem at T0 was able to produce histamine in culture media and regarding plate count results at T0, the wine contained only with yeast (110CFU/mL; LAB<1CFU/mL; acetic acid bacteria <1CFU/mL). This might suggest that the yeast had the capability to contribute to histamine production in this wine.

On the other hand, when specific histidine decarboxylating media was used (HMD), pink halo was not observed, but histamine was produced as confirmed by HPLC analysis. This might be due to the fact that no huge amount of histamine was produced in these cultures to induce a change of pH. This was also observed by other authors (Rosi *et al.,* 2009; Landete *et al.* 2005c).

Feasibility of predictive methods

The results of this study suggest that a methodology to determine the risk of histaminogenesis that integrates analysis of wine micro-ecosystem and enzymatic reactions as key indicators is feasible, provided that histaminogenic and histaminolitic profiles are taken into account.

The advantage to analyze the whole wine micro-ecosystem together as an entity is that the complexity of the interactions is treated as a “black box approach”. In this way, the main outcome of the incubation treatments will help to establish the level of risk of histamine development in a given batch of wine.

Biochemistry of amines-degradations in bottled wines during storage should be further studied to get a better understanding of the phenomenon and to develop key indicators of histamine degradation in wines.

Ideally, the increase of the data base will allow developing accurate modellization tools to make even a prediction of the exact histamine level after storage. This can sound a dream, but such tools are currently available in food industry for other purposes, provided that the model is built based on a consequent number of samples.

Fine-tuning of the treatments presented here, and the incorporation of the analysis for TDC and ODC activity detection will help to have the entire picture about the biogenic amines evolution in wines.

2.6 CONCLUSIONS CHAPTER 2

1 **Biogenic amines in bottled wine present different evolution during storage**. Histamine, tyramine, putrescine and cadaverine in wines stored at constant conditions, showed variable evolution: increase, decrease or stable after a year.

2 **Temperature accelerated enzymatic reactions** leading to an increase of histamine and putrescine while tyramine and cadaverine was mostly unchanged.

3 **Reduction of histamine was observed in almost all the studied wines**, indicating that bottled wine should be the perfect ecological environment to investigate microorganisms with histamine degrading capabilities.

4 **The mechanism of amines- degradation in bottled wine might not be completely explained by the presence of amine-oxidase** which would be limited due to the reduced O_2 content of bottled wine during storage. The biochemistry associated to degradation of amines in bottled wines needs to be further investigated to better understand the phenomenon.

5 **Exocellular enzymes seem to remain active in wines.** Bottled wines should contain HDC and amine-degrading enzymes as biogenic amines synthesis and degradation was observed in cell-free wines.

6 **Microorganisms with capability to produce biogenic amines were detected in bottled wines.** Synthesis of histamine was observed in some wines containing yeast and containing no lactic acid bacteria. Therefore, the research on decarboxylase activity in the environment of bottled wines associated also to yeast should be further investigated.

7 **Potential indicators of histaminogenesis in bottled wine were identified.** The assessment of the wine micro-ecosystem and the cell-free wines for their capability to produce or catabolize histamine could be good indicators to determine the level of risk to develop histamine in a given batch of wine.

CHAPTER 3

CHAPTER 3

Role of malolactic starters in reduction of ochratoxin A in wines

3.1 INTRODUCTION

3.1.1 Presence of OTA during winemaking process

As presented in the general introduction, the ochratoxin A (OTA) in wines comes from the contamination of grapes with OTA-producers fungi. The highest levels of OTA are generally observed in the grape and it decreases during the wine-making process (Abrunhosa *et al.,* 2005; Ponsone *et al.,* 2009, Fernandes *et al.,* 2007; Csutoras *et al,.* 2013).

The significant reduction of OTA during the vinification process could be explained by the partition of the toxin between the liquid and the solid phase, due to an extensive adsorption of OTA onto the solid parts of the grapes and yeast lees (Fernandes *et al,.* 2007; Gambuti *et al.,* 2005; Leong *et al,.* 2006 Abrunhosa *et al,.* 2005; Ponsone *et al,.* 2009). An adsorption mechanism onto biomass surface could be explained by the overall negative charge in the cell walls and the acidic nature of OTA (Castellari *et al,.* 2001). Solfrizzo *et al*. (2010) and Visconti *et al.* (2008) reported that, on average, between 70-95% of OTA is retained in pressed grape pomace during micro vinification trials. Similar results were obtained by Leong *et al*. (2006) during micro-vinifications of grapes with an initial OTA concentration ranging from 2 to 114 µg/kg.

Ponsone *et al.* (2009) observed that the OTA reduction was dependent on the initial OTA level in the must. Also, it was observed that during fermentation (either alcoholic or malolactic) the OTA content decreased in the liquid fraction.

3.1.2 Role of wine lees in removal of OTA

The reduction in OTA occurs during vinification mainly due to OTA adsorption onto suspended solids (Abrunhosa *et al,* 2005; Fernandes *et al.,* 2007).

The reduction observed in the wine were (Abrunhosa *et al,.* 2005) :

- After alcoholic fermentation 31,8%
- After racking 10,9%
- After malolactic fermentation 8,1%

They concluded that the reduction is associated with the mycotoxins removal by adsorption into solids wastes or fining agents, and not due to any degradation of ochratoxin A into other compounds.

The reductions obtained in the lees were (Fernandes *et al.,* 2007):

- Lees recovered after alcoholic fermentation had an OTA content of 50.4%;
- Sediment obtained after natural settling of the wine 17.6%;
- Lees obtained after malolactic fermentation 3%.

They also justify the lower contamination with OTA of white wines as compared with red wines by the separation of the pomace from must after crushing, a step characteristic of white wine vinification. This also explains why juices are usually more contaminated than wines. From these investigations the capability of lees to remove OTA from wines can be concluded.

3.1.3 Microorganisms removing OTA

The ability to reduce OTA content has been observed in some microorganisms: *Acinetobacter calcoaceticus* (Hwang & Draughon, 1994), *Phenylobacterium immobile* (Wegst and Lingens, 1983), some *Lactobacillus* (Fuchs *et al.,* 2008; Piotrowska and Zakowska, 2005), *Saccharomyces cerevisiae* (Bejaoui *et al.,* 2004; Patharajan *et al.*, 2010), and some *Aspergillus species* (Bejaoui *et al.,* 2006; Varga *et al.,* 2000).

3.1.3.a Yeast cells and adsorption of OTA

The potential of yeast cells to adsorb mycotoxins was first reported by Yiannikouris *et al.* (2003), and supported by researches focused on demonstrating the utility of lees for natural removal of OTA from wines, thus avoiding the use of fining agents.

García-Moruno *et al.* (2005) added white and red lees to red wine samples spiked with OTA. A significant reduction of OTA was observed after only 90 min of lees–wine contact with a very small amount of wet lees (20 g/L). After contact for 7 days the OTA reduction was greater than 70% with white lees, and around 50% with red lees. Longer contact duration did not improve the results. The authors justified the better results obtained with white lees, as compared with red lees, by the competition between polyphenols and OTA for the same binding sites on the surface of the yeast cells (Feuillat *et al.*, 2000; Ummarino *et al.,* 2001).

Blateyron *et al.* (2005) studied the use of dry active yeast and dry inactivated yeast in function of biomass, and contact time of wine with lees. They observed that the biomass and type of yeast impact the levels of reduction of OTA. The adsorption was quite quick and the mechanism is reversible when the autolysis of yeast occurs. Using a dose of 1g/L of yeast, the reduction of OTA obtained was 44%, this increased up to 55% when dosage was 5g/L of yeast.

3.1.3.b Adsorption of mycotoxins by lactic acid bacteria

Initial studies showed that different strains of LAB inhibited aflatoxin biosynthesis but were not effective enough in aflatoxin removal from media (Coallier-Ascah and Idziak, 1985; Thyagaraja and Hosono, 1994). Later, it was found that strains of *Lactobacillus rhamnosus*, effectively removed aflatoxin B1 up to 80% in contaminated culture media (El–Nezami *et al*., 1998a).

Similar observations were also made earlier with *Flavobacterium aurantiacum* (now named *Nocardia corynebacterioides*); although there was a small amount of toxin which was degraded in live cells (Line and Brackett, 1995). Later, many other strains of LAB were shown to bind aflatoxin in a strain specific manner (Peltonen *et al.*, 2001; Shah and Wu, 1999).

Two strains of *L. rhamnosus* bound aflatoxin B1 more effectively than aflatoxin B2 (El–Nezami *et al.*, 2002a). In addition, both strains showed similar aflatoxin B1 binding, even though they showed differences with respect to other metabolites. *L. rhamnosus* strains and *Propionibacterium freudenreichii* were later shown to effectively bind some of common *Fusarium* toxins (El–Nezami *et al.,* 2002b). The strains showed considerable differences in binding. Similarly, *L. rhamnosus* strains are shown to effectively bind zearalenone and its derivative up to 55% (El–Nezami *et al.,* 2002c).

Shetty and Jespersen (2006) found strains of *Lactobacillus plantarum* and *Pediococcus acidilactici* which are species commonly found during wine-making process, with capability to reduce aflatoxin B1 in spiked culture media.

3.1.3.c Removal of ochratoxin A by lactic acid bacteria

Fuchs *et al.* (2008) studied the removal of OTA and patulin in synthetic media by lactic acid bacteria. They found that the strongest decline of OTA (97%) was detected with a *Lactobacillus acidophilus* strain and two *Bifidobacterium* (50%). OTA reduction was observed from pH 5.0 to 8.0 with the maximum removal observed at pH 5.0. No reduction was observed in experiments with lower pH. The initial OTA concentration influenced the reduction observed. The lower the OTA concentration, the more reduction was obtained. The viability of the cells played an important role as with heat inactivated cells only a moderate reduction of patulin (16%) and OTA (11%) was observed. Rodriguez *et al.* (2011) studied OTA reduction capability of bacteria species able to transform aromatic compounds as *Rhodoccus, Pseudomonas and Brevibacterium* species, the last one was able to degrade 100% OTA in synthetic media.

3.1.3.d Capacity of *Oenococcus oeni* to reduce OTA

Few studies about the capability of *Oenococcus oeni* to reduce OTA are currently available:

- Silva *et al.* (2003) studied the impact of the inoculation of must and wine with *Lactobacillus plantarum* V22 and *Oenococcus oeni* strains (Lallemand), starters in liquid form, on the reduction of OTA. *L. plantarum* V22 (55%) and *O. oeni* R1101 (45,9%) showed the highest OTA reductions in wines containing 4,3 µg OTA /L.

- Del Prete *et al.* (2007) determined the ability of *O. oeni* to reduce OTA in the exponential growth phase obtaining a reduction rate of 8 to 28% in medium spiked with 5 µg OTA /L. Also OTA was recovered from the bacterial pellet. In experiments with cell-free extracts (obtained by disrupting *O. oeni* cells) no reduction of OTA was obtained, no degradation products of OTA in the media or bacterial pellet was found. It was concluded that the reduction of OTA by LAB is a cell-binding phenomenon.

- Mateo *et al.* (2010a) studied the capacity of *Oenococcus oeni* to eliminate ochratoxin A from synthetic media at pH 4.8 and other different conditions. Ten tested *O. oeni* strains removed OTA from the medium but with significant differences depending on the strain, incubation period, and initial OTA level in the medium. OTA reductions higher than 60% were recorded in 14-day cultures spiked with 2 µg OTA/L. Toxin removal was independent of bacterial viability (living or heat-inactivated cells of *O. oeni*) and culture medium composition. The OTA removal process was partly reversible in some of the cultures and upon culture prolongation a small part of the toxin was released back into the medium. They postulated that the OTA reduction mechanism may consist of physical binding of the toxin to the cell wall, although bacterial integrity appears to be necessary for this binding. Therefore, application of selected strains of *O. oeni* as starters of MLF or the use of heat-treated *O. oeni* cells in the winemaking and other processes might be a promising tool to reduce OTA contamination in some wines and other beverages and food.

- Mateo *et al.* (2010b) studied the effect of ethanol and OTA concentration on OTA reduction properties of *Oenococcus oeni* in synthetic media. The highest OTA reduction was obtained with higher LAB population at low alcoholic content (5%). In synthetic media with 15% ethanol, no reduction of OTA was observed. A relation with the initial OTA concentration was also observed as to lower OTA content, higher was the reduction obtained (%).They postulated that under acidic conditions, OTA has affinity towards hydrophobic environments, such as those provided by the teichoic acids in bacterial cell walls. However, at pH around 4.0 OTA solubility increases with the ethanol percentage in the aqueous medium, because alcohol reduces the polarity. The enhanced solubility of OTA in

ethanol-containing medium would decrease the amount of toxin adsorbed by cell walls. Therefore, the presence of ethanol in acidic wine-like medium negatively affects detoxification of OTA by *O. oeni*, probably because ethanol enhances the solubility of OTA at the acidic pH of the medium.

3.2 Aim of the study: role of malolactic starters in reduction of ochratoxin A in wines

The detoxifying mechanism of ochratoxin A (OTA) during the wine-making process has been linked to the adsorption of OTA into the cell wall of yeast and lactic acid bacteria performing alcoholic and malolactic fermentations. The mechanism of biosorption by physical binding has not yet been described. The precipitation of the yeast and lactic acid bacteria biomass, named lees, followed by successive raking steps and clarifications, removes the OTA from the wine together with the lees, resulting in a reduction of OTA content in the wine.

The technological impact of these discoveries is very important for the wine industry as it might imply that the reduction of the toxins can be partially done during the standard process reducing the use of chemicals and physical curative post-treatments. This would save money reducing the use of coadyuvants while delivering a healthier wine, not only by the OTA reduction, but also by reducing the use of chemicals. This seems to be possible with the use of selected OTA-reducers yeast and malolactic bacterial starters.

The use of yeast starters to reduce OTA levels has been well studied, but there is no available information regarding the OTA reduction property of lyophilized malolactic starters during the malolactic fermentation in wine. The few studies available on *Oenococcus oeni* used liquid starters forms and OTA reduction property was assessed in synthetic media (Del Prete *et al.,* 2007, Mateo *et al.* 2010a-b) or in must and wine (Silva *et al*., 2003).

The evaluation of the OTA reduction capability of lyophilized malolactic starters for direct inoculation is important because these types of starters are used by wine cellars to control the malolactic fermentations and they are key elements of safe process standards. Moreover, the lyophylization step that malolactic starters are submitted to for conservation purposes modify the bacteria cell wall and the adsorption properties might be different from liquid cultures.

The main objectives of this study were:

1) to evaluate the OTA reduction property of lyophilized malolactic starters for direct inoculation in microvinifications;

2) to assess the impact of wine pH and initial OTA concentration on OTA reduction properties of malolactic bacterial starters.

3.3 MATERIALS AND METHODS

Lyophilized malolactic bacterial starters used to conduct malolactic fermentations (MLF) in wines were evaluated on their capacity to reduce ochratoxin A (OTA) during the fermentative process. Wines containing OTA were inoculated by those strains and the OTA was measured at the end of MLF, before the racking. The experiments were carried out in ITV France (Beaune) during autumn-spring 2006. Two set of trials were carried-out:

- Screening of malolactic starters for their OTA reduction capabilities
- Evaluation of the impact of pH and initial OTA content on OTA reduction

3.3.1 Biologic Material

Malolactic bacteria starters for direct inoculation were provided by Lallemand SA. These strains belong to the species *Oenococcus oeni* and have been processed upon lyophilization for their conservation and transportation. In these trials, they were used in their commercial presentation. A new bag was open for each inoculation.

The strains evaluated in this study were:

- Alpha
- Beta
- Lalvin 31
- Elios 1
- FML Expertise S
- VP41
- 49A1*
- H3*

* not commercially available when trials were performed.

Inoculation was performed following re hydration step as recommended by the supplier.

3.3.2.a Analytics: Monitoring of MLF

The monitoring of the MLF was done by measuring the L-malic acid concentration after the inoculation of bacteria using enzymatic method (see chapter 1). The evolution of bacterial population was done by plate count method using MBL- culture media (Oxoid) every 3 days.

3.3.2.b Analytics: Determination of OTA content

The analysis of OTA in wines was done before the inoculation of bacteria starters and 15 days after the end of MLF (before the racking).

The analyses were performed by an external laboratory following the method recommended by the OIV (Resolution OENO 16/2001) using immune affinity column and HPLC in inverse state with fluorimetric detection. The limit of detection (LOD) was 0,22 µg/L and the limit of quantification (LOQ) was 0,77 µg/L.

3.3.3.a Trials: Screening of LAB starters

For this first set of trials, wines produced in the South of France naturally rich in OTA were used. Each batch of wine was treated with 20g/L of nutrients for lactic acid bacteria based on nitrogen and polysaccharides, the Opti'Malo Plus (Lallemand) and 2,5 g/L of L-malic acid. The pH was adjusted according to trial requirement using NaOH 10N. The microfermentations were carried-out in batches of 3 liters in chamber at 18°C , following the schema of Table 67.

Table 67. Screening of malolactic starters on their OTA reduction properties: Schema of trials.

Red Wine 1	Red Wine 2	White wine	
pH 3.6	pH 3.6	pH 3.2	pH 3.4
Alpha	Alpha	49A1	49A1
Alpha repet.	Lalvin 31	Control[2]	Control[2]
Alpha-stirring[1]	FML Expertise S		
Beta	Control[2]		
Lalvin 31			
Elios 1			
FML Expertise S			
VP41			
49A1			
H3			
Control[2]			

[1] *once per week;* [2]*not-inoculated*

The oenological characteristics of the wines (Table 68) were determined by standard methods recommended by the OIV (see chapter 1).

Table 68. Oenologic Parameters of the wines used for the experiments

Wine	Red wine 1	Red wine 2	White Wine 3
Alcoholic degree (%vol)	13,2	12,7	12,5
SO_2 free (mg/L)	<10	<10	<10
original pH	3,35	3,37	3,33
Adjusted pH	3,57	3,58	3,2 3,4
Total acidity (g/L Sulfuric acid)	5,39	4,557	3,577
Lactic acid Bacteria (CFU/mL)	<1	74	$1x10^3$
L-Malic acid (g/L)	2,2	2,5	2,18
OTA (µg/L)	1,8	2,3	0,2

3.3.3.b Trials: Impact of pH and OTA content on reduction

In this second set of trials, the impact of pH (3.4 / 3.6 / 3.8) was evaluated and initial OTA concentration (2 µg/L / 6 µg/L / 10 µg/L) on the reduction of OTA during the MLF. Some strains were selected from the previous screening (see 3.3.3.a) for their OTA reduction capabilities (Lalvin 31 and Expertise S) and others as negative controls (Alpha and 49 A1); this is further explained in the Results section. Red wine 1 and red wine produced in ITV France (Beaune) pilot plant during campaign 2005 were used (PN05).

To perform the MLF, wines were treated with 20 g/L of nutrients for lactic acid bacteria based on nitrogen and polysaccharides, the Opti'Malo Plus (Lallemand) and 2,5 g/L of L-malic acid. The pH was adjusted using NaOH 10N and OTA from *Aspergillus ochraceus* (Sigma) was added according to trials requirements. The microfermentations were carried-out in batches of 3 liters in chamber at 18°C following the schema of Table 69.

Table 69. Impact of pH and initial OTA concentration: trials

Wine	pH	Initial OTA content (µg/L)	Malolactic starter
Red Wine 1	3,42	6,3	L31
			Exp
	3,6	2,2	L31
			Exp
		6,1	L31
			Exp
			Alpha 49A1
			Control
		10	L31
			Exp
	3,8	6,5	L31
			Exp
Pinot noir	3,6	6,4	Alpha
			L31
			Control*

L31: Lalvin 31; Exp: Expertise S; Control: not-inoculated*

3.4 RESULTS

3.4.1.a Results Screening LAB starters: MLF kinetics Red Wine 1

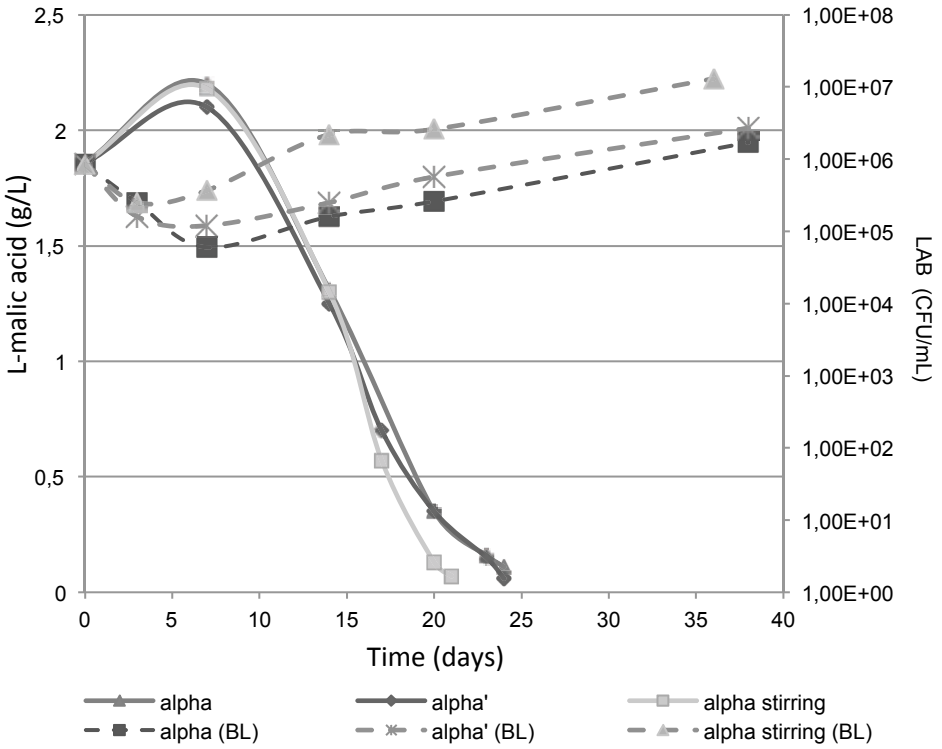
The screening of malolactic starters for their ability to reduce OTA in wines was studied by inoculating the wines with different starters and allowing them to perform the MLF. The monitoring of the MLF was done by measuring consumption of L-malic acid and LAB viable count (Figure 51, Figure 52 and Figure 53, for red wine 1). Regarding MLF kinetics, in red wine 1 all the strains with the exception of strain H3, experienced a decrease of the bacterial population after 7 days of inoculation. Afterwards, all of them increased the population almost one logarithm until the end of the MLF.

The batches inoculated with strain Alpha (Figure 51) showed similar dynamic population during MLF. Stirring the wine once per week (batch “Alpha stirring”) increased the bacterial population in almost 1 log until the end of the MLF in comparison with the other wines inoculated with the same strain and without stirring. This increase in LAB population of Alpha, leads to a faster L-malic acid consumption. In the case of “Alpha stirring”, the MLF finalized 4 days before the other batches inoculated with Alpha (without stirring).

Figure 52 shows the fermentative kinetics of the wines inoculated with the strains Beta, Expertise S (Exp), Lalvin 31 (L31) and Elios. The strain Beta shows a decrease in the population of 1,3-1,4 log reaching 5 log. But this reduction in bacterial population does not affect the timing of the process as the MLF finalizes more or less on the same day as the other batches. The strain Expertise S had the highest population and logically the fastest process as well.

The population of batch control (Figure 51) which performed the MLF spontaneously started from <1 CFU/mL at the beginning of the experiments and about day 23 its population increased to 4 log. The beginning of the L-malic acid consumption corresponds with an increase in LAB population to 5-6 log.

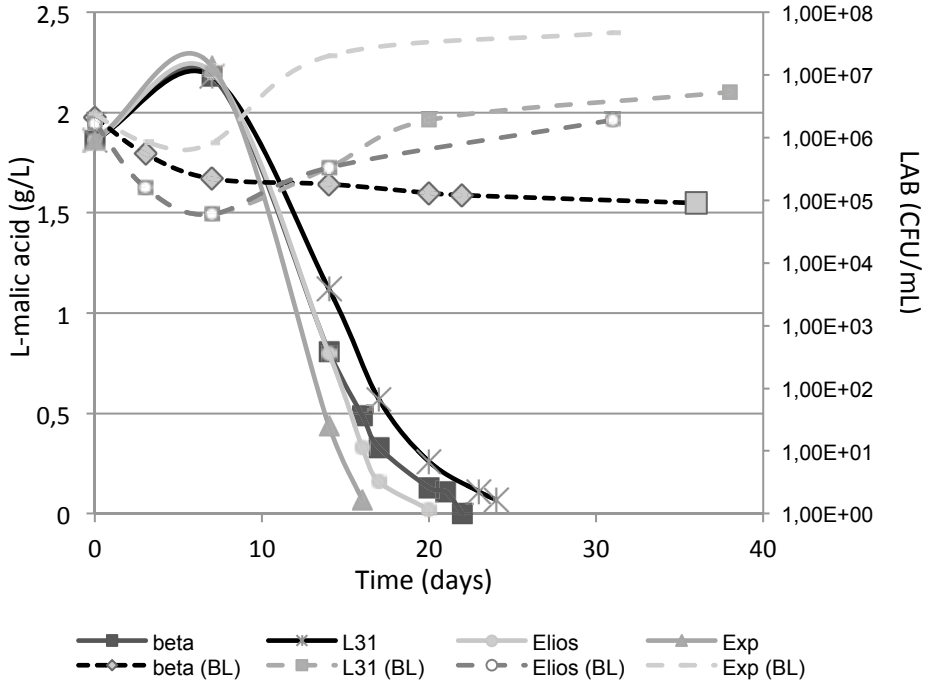
Figure 51. MLF kinetics, L-malic acid consumption and evolution of LAB population in red wines 1, strain Alpha



The last point of each curve represents the CFU/mL 15 days after finalizing the MLF, before racking

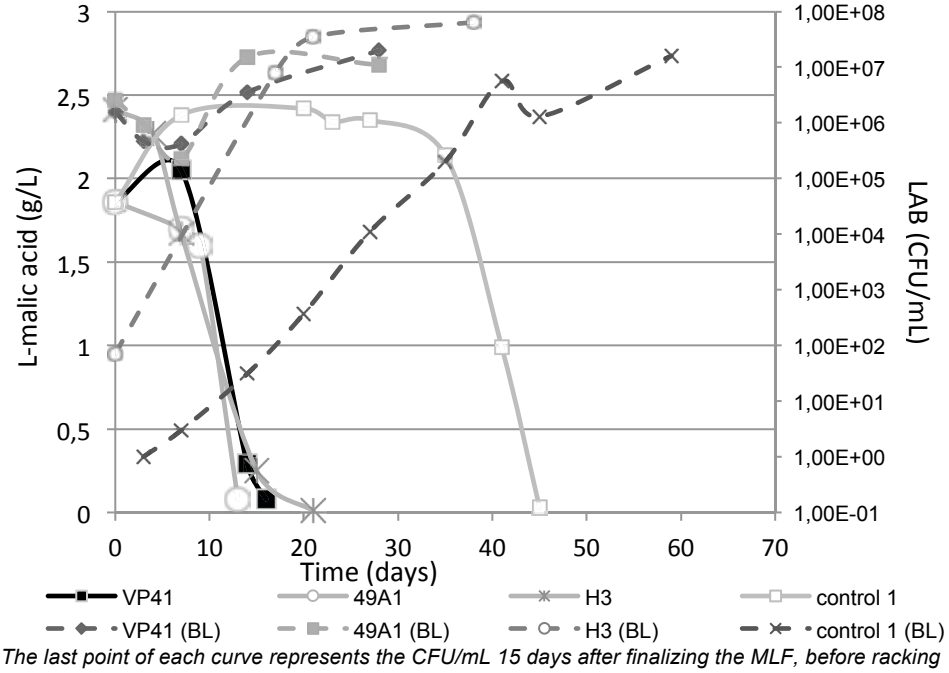
In the case of strain H3 (Figure 53), the inoculation was done at 2 log, much lower than for the other strains (6 - 6,5 log). This was due to the lack of experience on this strain which was experimental at that time, and the survival rate of the lyophilized preparation was unknown. Nevertheless, the L-malic acid consumption was run at the same time than as other batches, the LAB population adapted to the wine quite fast, obtaining higher LAB population at the end of MLF (8 log).

Figure 52. MLF kinetics, L-malic acid consumption and evolution of LAB population in red wines 1, strains Beta, Expetise S, Elios, Lalvin 31



The last point of each curve represents the CFU/mL 15 days after finalizing the MLF, before racking

Figure 53. MLF kinetics, L-malic acid consumption and evolution of LAB population in red wines 1, strains VP41, 49A1, H3 and control 1 (spontaneous MLF)

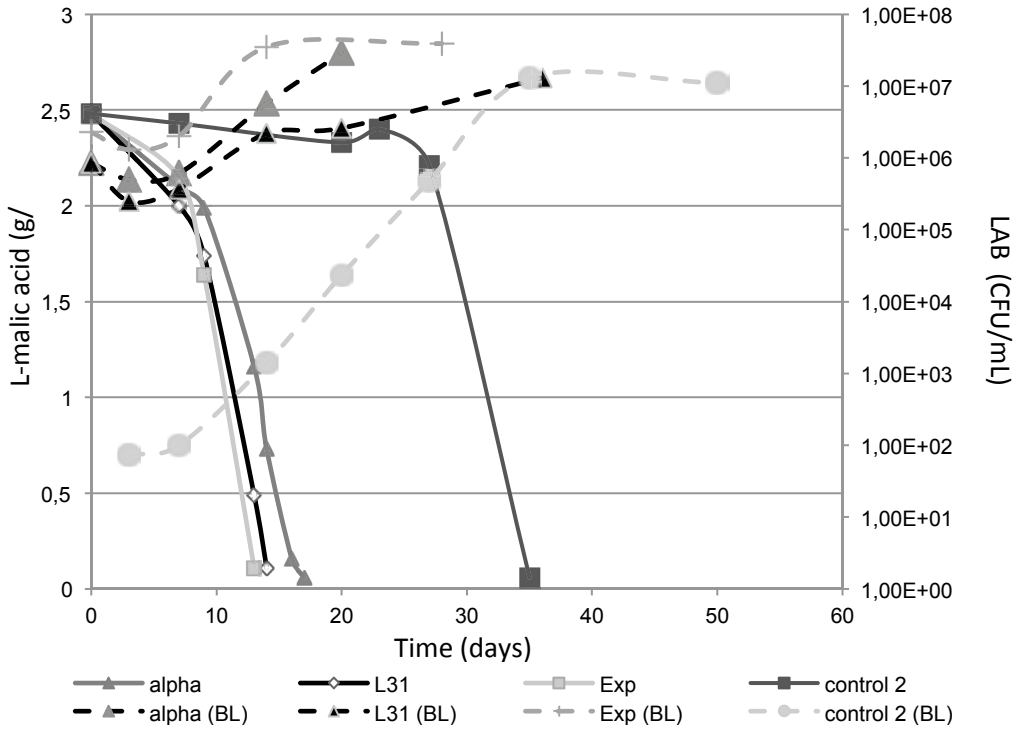


The last point of each curve represents the CFU/mL 15 days after finalizing the MLF, before racking

3.4.1.b Results Screening LAB starters: MLF kinetics Red Wine 2

In Red Wine 2 (Figure 54) the L-malic acid consumption and population dynamics are similar for the three starters (Alpha, Lalvin 31and Expertise S).The control batch, which started the MLF spontaneously by the wild bacterial population present in the wine, counted at the beginning of the MLF with 2 log of bacteria and finalized on day 41 with 7 log. This MLF took 11 days more than the wines inoculated with commercial *O. oeni*.

Figure 54. MLF kinetics, L-malic acid consumption and evolution of LAB population in red wines 2



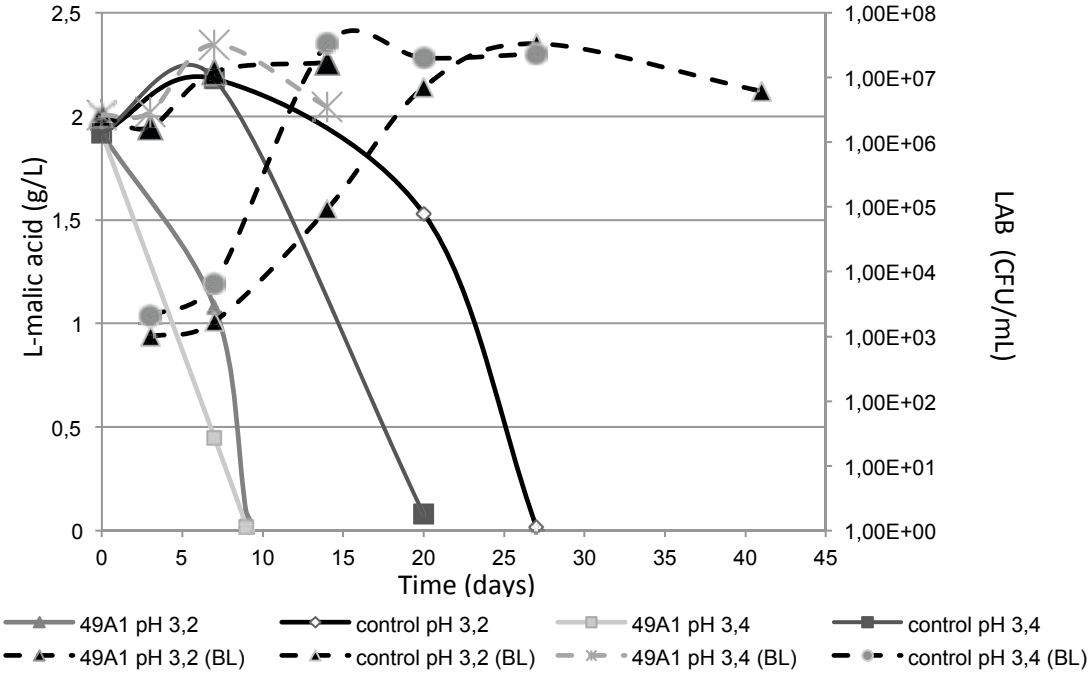
The last point of each curve represents the CFU/mL 15 days after finalizing the MLF, before racking

3.4.1.c Results Screening LAB starters: MLF kinetics White Wine 3

In white wine 3 (Figure 55), wines inoculated with strain 49A1 had similar L-malic acid consumption rate and population dynamics both at pH 3.2 and 3.4.

In the case of spontaneous MLF (control batches) the pH influenced the kinetics, the MLF at pH 3.2 lasted longer compared with the batch at pH 3.4. The LAB population at pH 3,4 grew faster until day 15.

Figure 55. MLF kinetics, L-malic acid consumption and evolution of LAB population in white wine.



The last point of each curve represents the CFU/mL 15 days after finalizing the MLF, before racking

3.4.1.d Results Screening LAB starters: Reduction of OTA in red wine 1

In order to screen the capability of malolactic starters to reduce OTA during the MLF, malolactic fermentations were performed by inoculating the wines after the alcoholic fermentation with LAB starters. One batch was left without inoculation to allow a spontaneous MLF. Table 70 shows the ochratoxin A content of the wines 15 days after finalizing the MLF. In the case of "control" batch, as the MLF did not even start, the sampling was done around day 40 (Figure 53) when the population was about 5 log.

The reduction of OTA (R%) was calculated by difference between the OTA recorded at T0 (b) and the OTA measured before racking (a):

$$a-b = c$$

$$R\ \% = (cx100) / a$$

Taking into account the LOQ limit (0,77µg/L) of the OTA measurement, the difference between the OTA content at T0 and before racking were not relevant in several cases because the measured OTA was into the LOQ. R% values <38,8% in Table 70 should

be considered not relevant as the reduction can be incriminated to the measurement method and not to the treatment. Only 3 batches had a relevant difference among the samples (T0 and end MLF+15 days), (Table 70).

Table 70. Ochratoxin A content on Red Wine 1.

Batch	sampling	OTA (µg/L)	R%
T0	Before inoculation of starters	1,8 (a)	
Lalvin 31	End MLF + 15 days	0,7 (b)	61
Expertise S	End MLF + 15 days	0,8	55,5
VP41	End MLF + 15 days	0,8	55,5
Control	End trials (MLF did not start)	1,6	11*
Alpha	End MLF + 15 days	1,6	11*
Alpha	End MLF + 15 days	1,5	16,6*
Alpha stirring	End MLF + 15 days	1,7	5,5*
Beta	End MLF + 15 days	1,6	11*
Elios 1	End MLF + 15 days	1,5	16,6*
49A1	End MLF + 15 days	1,5	16,6*
H3	End MLF + 15 days	1,7	5,5*

**No relevant OTA reduction according to LOQ of analytical method.*

The malolactic bacterial starters cultures that showed similar interesting results regarding reduction of OTA in red wine 1, were Lalvin 31 (61 %), Expertise S (55,5 %) and VP41 (55,5 %). The net reduction in these cases was about 1µg/L.

3.4.1.e Results Screening LAB starters: Reduction OTA red wine 2

Table 71 shows the OTA content of MLF performed with red wine 2, with a sampling done 15 days after finalizing the MLF. In the “control” batch the measurement was done at the same time as the other batches, as in contrast with red wine1, red wine 2 contained a certain population of bacteria (74 CFU/mL, Table 68, *Materials & Methods section)* at the beginning of the experiments and the MLF started spontaneously.

The OTA values obtained before racking are close to the initial OTA content, and they are included in the LOQ. Therefore, the differences observed can be incriminated to the method of measurement and not to the malolactic strain used. In consequence the R% values less than 30% in Table 71 should not be considered relevant.

Table 71. Ochratoxin A content in red wine 2.

Batch	sampling	OTA (µg/L)	R%
T0	Before inoculation	2,3	
Control	End MLF + 15 days	2,2	4,3*
Alpha	End MLF + 15 days	2	15*
Lalvin 31	End MLF + 15 days	1,9	17,4*
Expertise S	End MLF + 15 days	2	13*

**No relevant OTA reduction according to LOQ of analytical method*

3.4.1.f Results Screening LAB starters: Reduction OTA white wine 3

In white wine 3 (Table 72), a reduction of OTA 15 days after finalizing the MLF was not observed. To take into account that in this wine, the initial OTA content was very low (0,2 µg/L) and within the limit of detection of the method (0,2 µg/L), therefore if a reduction of OTA took place, it could not be measured by the used method.

Table 72. Ochratoxin A content in white wine 3.

Wine 3	Before MLF	pH 3,2		pH 3,4	
		49A1	Control	49A1	Control
OTA (µg/L)	0,2 (a)	0,2 (b)	0,2	0,2	0,2

3.4.1.g Results Screening LAB starters: contact with lees

In order to analyze if the reduction of OTA (R%) obtained with the different MLF are function of the contact with lees, the duration that the wine was in contact with the starter, from the inoculation until the racking, was evaluated (Table 73).

In the case of the spontaneous MLF, this timing was difficult to establish as the LAB population slowly increased with the time. Therefore, in those cases there might be the simultaneous effect of concentration of LAB population and timing. Additionally, for red wine1, the sampling was done before the MLF started (around day 40, Figure 53). Nevertheless, at the point of sampling, the control 1 counted with important LAB population (5,5 log), but was not constant during all the process, as is usually the case for spontaneous MLF.

For the inoculated MLFs, the LAB populations were almost constant from the inoculation to racking (6 -8 log). Therefore the time of contact with more or less equal LAB concentrations can be evaluated in a fair way (Table 73).

Table 73. Duration (days) of contact of bacterial strain with wine containing OTA

Wine	Malolactic starters	Duration of MLF (days)	Contact of bacteria with wine, Total duration (days)	OTA reduction R%
Red Wine 1 (OTA 1,8µg/L)	Alpha	24	39	11
	Alpha'	24	39	16,6
	Alpha stirring	21	36	5,5
	Beta	22	37	11
	Lalvin 31	24	39	61
	Elios 1	20	35	16,6
	Expertise S	16	31	55,5
	VP41	16	31	55,5
	49A1	13	28	16,6
	H3	14	29	5,5
Red Wine 2 (OTA 2,3µg/L)	Alpha	17	32	15
	Lalvin 31	14	29	17,4
	Expertise S	13	28	13

Taking into account the reduction of OTA in the wine inoculated with strain Lalvin 31, in red wine 1, the lees were in contact with the wine 39 days, and 29 days in the case of red wine 2 because the MLF was faster. It is to be noted that in case the wine was in contact with the lees for 39 days, the reduction of OTA was about 61%, in the case of 29 days, the reduction of OTA was about 17,4% for strain Lalvin 31.

The lees of strains Expertise S were in contact with wine during 31 and 28 days in red wine 1 and 2 respectively. There is no a significant difference in the contact time of the wine with the lees that explain the difference of reduction observed in the two batches (55% and 13%).

Strains VP41 which reduced the OTA by about 55%, were in contact with the wine for 31 days in red wine 1, this strain was not tested in the other wines.

Focusing on red wine 1, some batches have been with lees for the same time but the R% observed was quite different; this suggests that the property to reduce OTA in wines might be LAB strain dependent.

3.4.2 Results Impact of pH and OTA content on R%: MLF kinetics red wine1

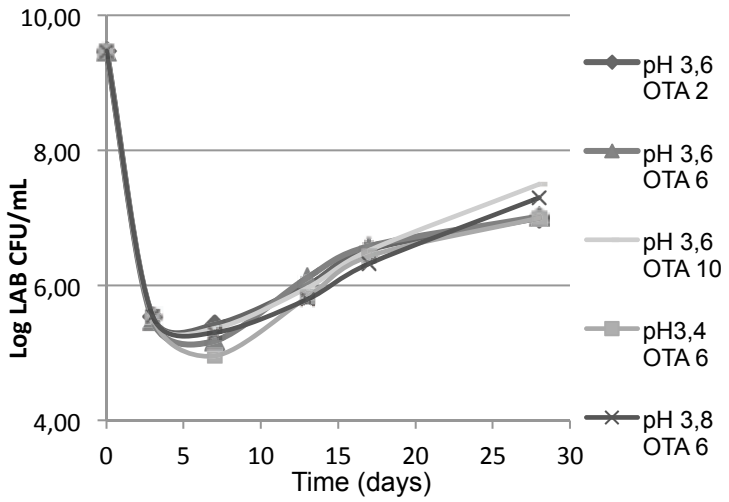
3.4.2.a Strain Lalvin 31, red wine 1

For this second set of trials, where the impact of pH and OTA content was studied, the strains reducing OTA effectively in the previous screening, Lalvin 31 and Expertise S were used. Negative controls were strains Alpha and 49A1. These strains were inoculated in wines with different pH and OTA content.

Figure 56 shows the bacterial population growth of strain Lalvin 31 at pH 3.6 with 3 OTA levels (2, 6 and 10 µg/L) and at pH 3.4 and 3.8, both containing 2 µg/L of OTA.

An abrupt decrease of bacterial population is observed until the day 5, and then, all batches recover the bacterial population size until the end of the MLF which finalized after 27 days. pH 3.8 allows a slight increase in the population after day 20, the same is observed for pH 3.6 and OTA 10µg/L. pH and OTA content do not seem to have a strong impact on the development of strain Lalvin 31 in these trials.

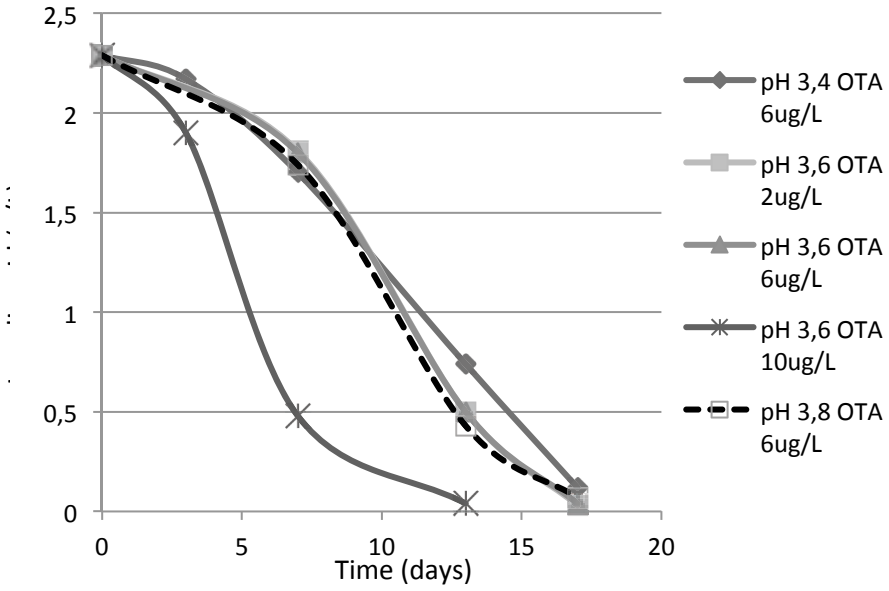
Figure 56. LAB population dynamics of strain Lalvin 31 (wine 1).



The last point of the curves represents the populations before racking (15 days after end of MLF).

Figure **57** 57 plots the L-malic acid consumption during the MLF of strain Lalvin 31. The batch at pH 3.6 containing 10 µg/L of OTA experienced a faster MLF. The other conditions presented similar kinetics.

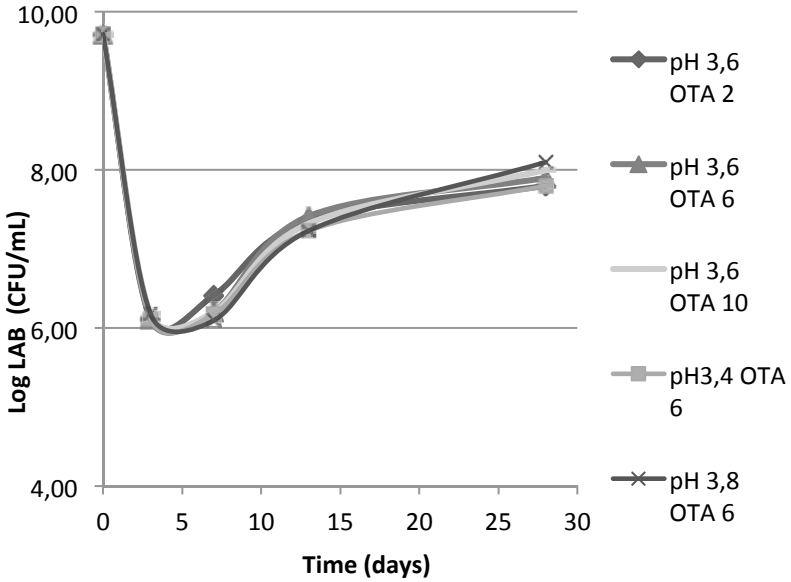
Figure 57. L-malic acid consumption, strain Lalvin 31 at different pH and OTA concentrations (red wine 1)



3.4.2.b Expertise S, red wine 1

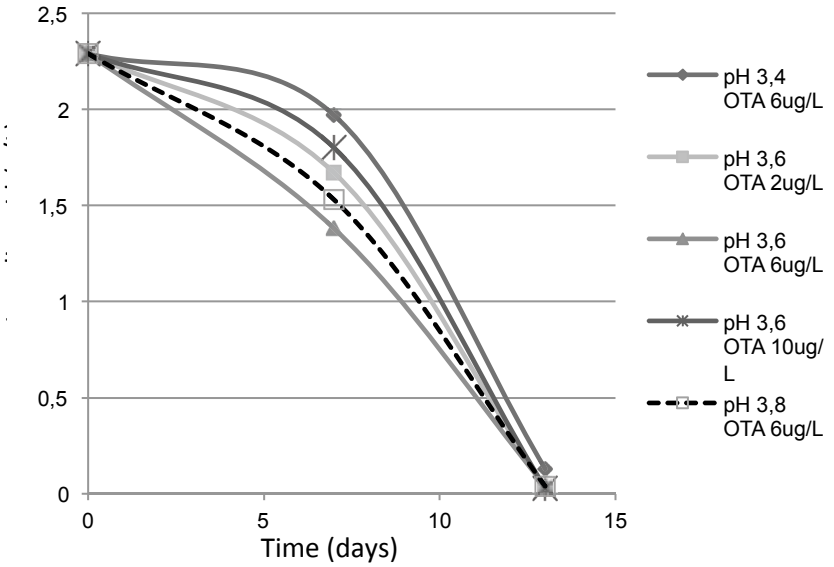
Figure 58 shows the bacterial population development of strain Expertise S during the MLF at different conditions of pH and OTA concentrations. All the studied conditions have similar behavior, ending the MLF in 14 days.

Figure 58. Population dynamics of strain Expertise S (wine 1).



The last point of the curves represents the populations before racking (15 days after end of MLF).

Figure 59. L-malic acid consumption, strain Expertise S (wine 1)



Regarding the L-malic acid consumption (Figure 59), the exponential phase experienced different behaviors the most acidic (pH 3.4) being the slowest one. For the other batches, the trend cannot be explained by pH or OTA concentration.

3.4.2.c Strains Alpha and 49A1, red wine 1

Figure 60 shows the population dynamics of strains Alpha, 49A1 and control batch. The pH was 3.6 and OTA content was 6 µg/L. Strain 49A1 increased its population from day 5, while Alpha kept population size more or less constant. This coincides with the L-malic acid consumption rate which was faster in the case of 49A1 (Fig. 61). Control batch counted with poor population and the MLF did not start in the time of the experiments.

Figure 60. Population dynamics of strains Alpha, 49A1 and control (red wine 1, pH 3,6 OTA 6µg/L)

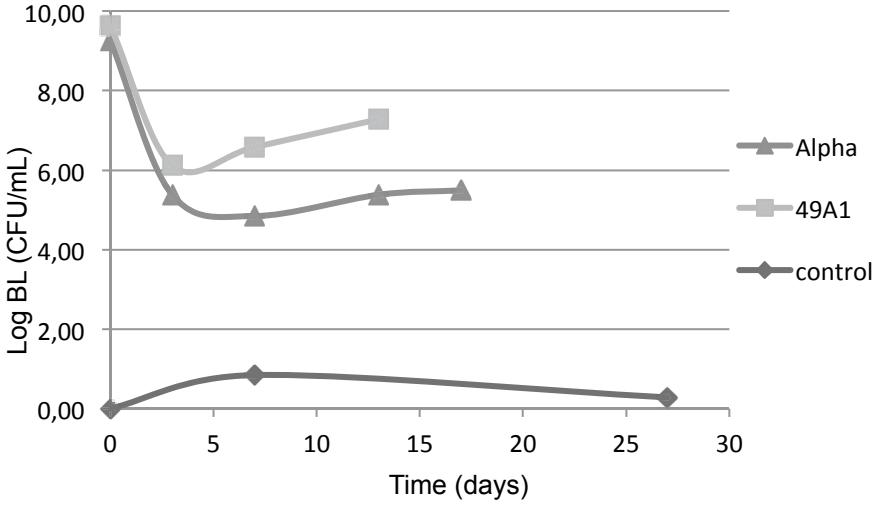
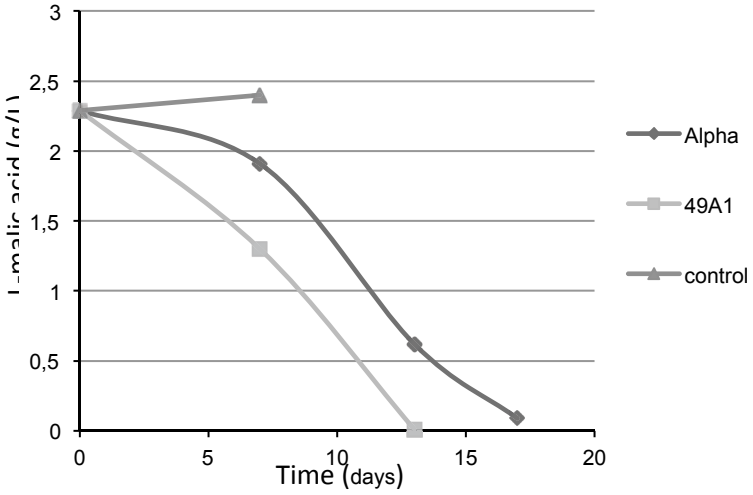


Figure 61. L-malic acid consumption, strains Alpha, 49A1 and control (red wine 1, pH 3,6 OTA 6µg/L)



For the L-malic acid consumption, strain 49A1 experienced a faster process (Figure 61).

3.4.2.d Results Impact of pH and OTA content on R%: MLF kinetics PN05 wine

Strains Alpha, Lalvin 31 and a control batch non-inoculated were tested in Pinot Noir wine (PN05). Both strains followed similar population dynamics (Fig. 62) but the strain Lalvin 31 presented faster L-malic consumption rate (Fig. 63).Control batch increased the LAB population slowly and the malic started to be consumed around day 27.

Figure 62 Population dynamics of strains Alpha, Lalvin 31 and control (wine PN05, pH 3,6 OTA 6µg/L)

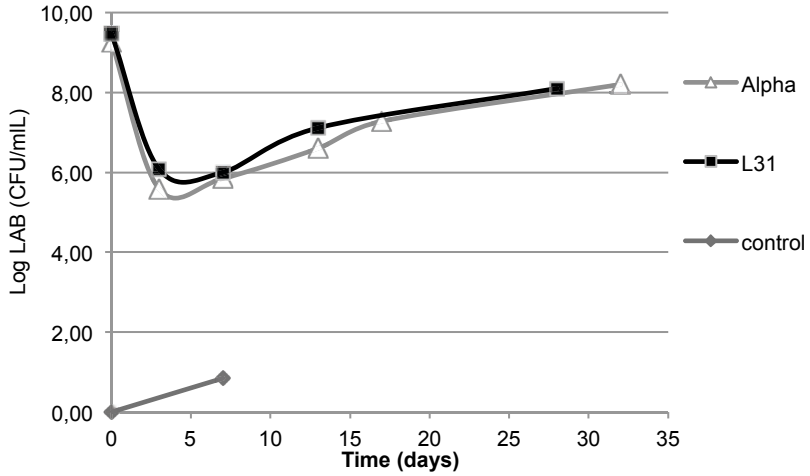
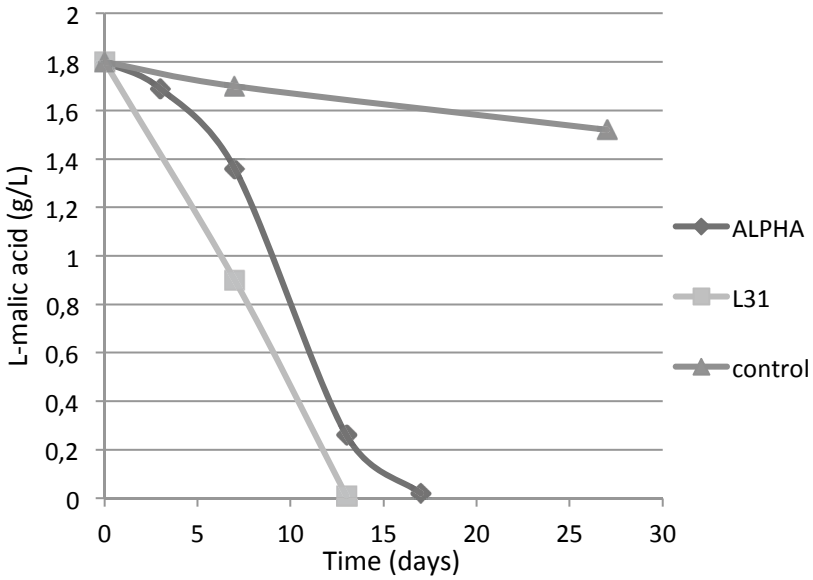


Figure 63. Malic acid consumption, strains Alpha, Lalvin 31 and control (wine PN05, pH 3,6 OTA 6µg/L)



3.4.2.e Results Impact of pH and OTA content on R%: reduction of OTA

Table 74 shows the reduction of OTA (R%) obtained in red wine 1 and PN05 wines (15 days after completion of MLF, before racking), using the different malolactic starters, at different pH and initial OTA content (Table 74).

Table 74. Results of R%, MLF duration and time contact with lees at different pH and initial OTA content (red wine 1 and PN05-second set of trials).

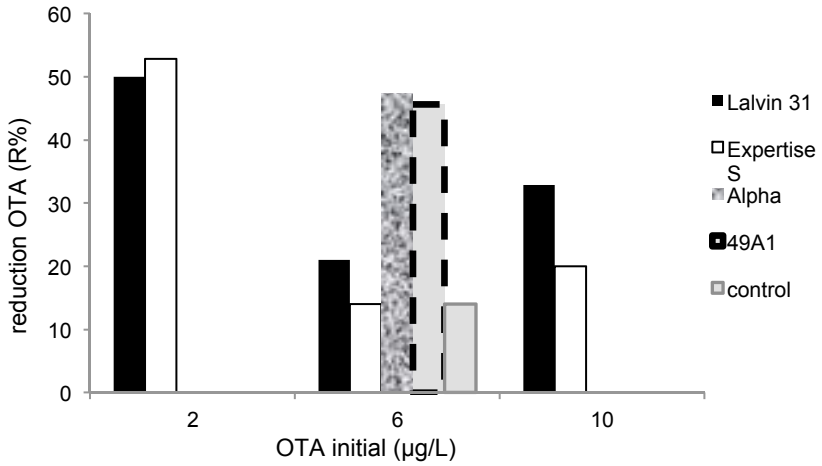
	pH	Initial OTA content (µg/l)	Malolactic starter	MLF duration (days)	Contact of bacteria with wine (days)	R%
Red Wine 1	3,42	6,3	L31	17	32	21,1
			Exp	13	28	22,8
	3,6	2,2	L31	17	32	50
			Exp	13	28	52,8
		6,1	L31	17	32	21,1
			Exp	13	28	14
			Alpha	17	32	47,4
			49A1	13	28	45,6
			Control	No started		14
		10	L31	17	32	32,9
			Exp	13	28	20
	3,8	6,5	L31	17	32	45,6
			Exp	13	28	35,1
Pinot noir	3,6	6,4	Alpha	17	32	18,8
			L31	13	28	4*
			Control	No started		10*

**R% not relevant taking into account LOQ*

3.4.2.f Results Impact of pH and OTA content on R%: initial concentration of OTA

The reduction (R%) of OTA observed in trials performed with red wine 1 and pH 3.6 before racking (15 days after MLF) as function of initial OTA concentrations are shown in Figure 64. As the control batch did not perform the MLF in the time of the experiments, and the bacterial population was low (Fig. 62) the degradation of OTA can hardly be linked to the bacterial population or its metabolism. This reduction was about R% 14. LAB starters Alpha and 49A1 which were selected for being poor OTA reducers (first set of trials), surprisingly performed better a OTA reduction achieving 45-50% when the wine contained higher levels of OTA (6 µg/L) compared with the trials done with the same wine in the first part of the study (initial OTA 1,8 µg/L).

Figure 64. Reduction of OTA in Wine 1 pH 3.6



In all three studied concentrations, a reduction by LAB strains Lalvin 31 and Expertise S was observed. This effect was more pronounced for small quantities of initial OTA (2 µg/L).

In order to understand if the differences observed in R% for Lalvin 31 and Expertise S are significantly different depending on the initial OTA concentration, an ANOVA test "two factor without replication" was done (Table 75):

The p-value for strains (rows, in Table 75) reveals that the differences observed between LAB starters Lalvin 31 and Expertise S are not significant. On the contrary, the differences observed between the different initial OTA concentrations are significant ($p \leq 0,05$)

Table 75. ANOVA 2-factor without replication

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
L31	3	103,909774	34,6365915	211,862363
EXS	3	86,8128655	28,9376218	435,159821
OTA initial 2	2	102,777778	51,3888889	3,85802469
OTA initial 6	2	35,0877193	17,5438596	24,6229609
OTA initial 10	2	52,8571429	26,4285714	82,6530612

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	48,7173825	1	48,7173825	1,56103768	0,33790789	18,5128205
Columns	1231,6277	2	615,813852	19,7323538	0,04823379	19
Error	62,4166643	2	31,2083321			
Total	1342,76175	5				

In order to determine which are the treatments significantly different, ANOVA single way per pairs was done (Table 76):

Table 76. ANOVA single way per pairs for concentration of OTA

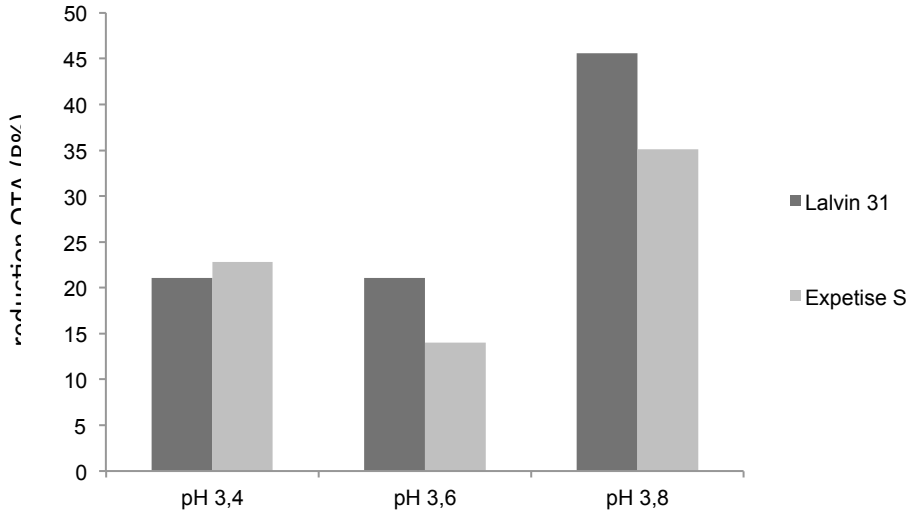
Treatments Initial OTA (µg/L)	P-value
2 and 6	0,012
6 and 10	0,348
2 and 10	0,062

According to ANOVA results, there are significant differences between the reductions (R%) observed in wines containing 2 µg/L OTA and 6ug/L OTA ($p \leq 0,05$), the reduction of OTA being more important for the wine containing 2 µg/L.

3.4.2.g Results Impact of pH and OTA content on R%: effect of pH of wine

Figure 65 shows the results of R% in the set of trials where the initial OTA content was 6 µg/L and the variable was pH in red wine 1.

Figure 65. Reduction of OTA for red wine1 containing OTA 6µg/L



In these experiments it is observed that both stains reduced more OTA when the pH was higher (pH 3.8). There is not a strong difference in R% between both LAB starters. An ANOVA two factor without replication test was carried-out (Table 77):

Table 77. ANOVA 2-factors without replication, factors: strains and pH

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
L31	3	87,7192982	29,2397661	201,087514
EXS	3	71,9298246	23,9766082	111,829281
pH 3,4	2	43,8596491	21,9298246	1,53893506
pH 3,6	2	35,0877193	17,5438596	24,6229609
pH 3,8	2	80,7017544	40,3508772	55,401662

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	41,5512465	1	41,5512465	2,07692308	0,28625357	18,5128205
Columns	585,821278	2	292,910639	14,6410256	0,06393443	19
Error	40,0123115	2	20,0061557			
Total	667,384836	5				

Surprisingly, according to ANOVA test, there is no significant difference between the R% obtained by the different pHs. There is no significant difference either between Lalvin 31 and Expertise S as observed in Figure 65.

In order to determine whether the impact of pH on R% is hidden in the pool of data, an ANOVA single way test per pairs was done (Table 78):

Table 78. ANOVA single way test per pairs for R%, factor: OTA concentration

ANOVA single way	P-value
pH 3.4 and pH 3.6	0,349
pH 3.4 and pH 3.8	0,074
pH 3.6 and pH 3.8	0,069

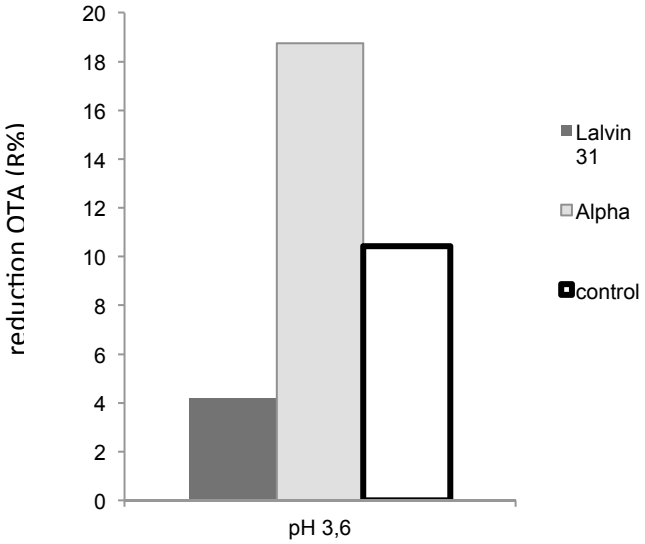
The lack of difference on R% by the pH is confirmed with the ANOVA test per pairs.

3.4.2.h Results Impact of pH and OTA content on R%: OTA reduction obtained with wines (PN05)

The results of OTA reduction (R%) of MLFs performed using pinot noir wine (PN05), where the MLF was realized at pH 3.6 and initial OTA of 6,4 µg/L are shown in Figure 66. In this set of trials, R% obtained with strain Lalvin 31 and control batch are included in the LOQ of the methods. Thus, only the R% obtained with strain Alpha can be considered.

For Lalvin 31, the results are lower than the R% obtained with wine1: in red wine 1 R% =20 for Lalvin 31 while in pinot noir wine the R% obtained is 4% at same conditions of pH and initial OTA content. These contradictory results might indicate that the reduction of OTA within the same strain should be affected by other parameters than pH or OTA content.

Figure 66. Reduction of OTA (R%) in wines with OTA 6,4 (µg/l) and pH 3,6.



3.4.2.i Results Impact of pH and OTA content on R%: Influence of contact with lees on R%

The total time (days) that the wine was in contact with the lees, which is function of how long was the MLF plus 15 days, is shown in Table 74.

Lalvin 31 took 17 days and Expertise S 13 days to carry out the MLF in all the studied conditions of pH and initial OTA content indicating that these two factors did not affect the development of the LAB starters. Nevertheless, the R% was variable and dependent on the pH and initial OTA content. This means that the duration of contact between the bacterial starter and the wine might not have a high impact on the reduction of OTA.

3.5 DISCUSSION

O. oeni with OTA reduction properties

The property to reduce OTA was observed in wines inoculated with commercial *O. oeni* starters to drive the MLF. During the screening of malolactic starters for the OTA reduction property, some strains showed this ability in wines naturally containing OTA (1,8 µg/L) and they were selected to follow up the investigation in function of pH and OTA concentration. Strains Lalvin 31, Expertise S and VP41 reduced the OTA content of wines from 21% to 61 %, 14% to 55% and 55% respectively, depending on the pH and initial OTA content of the wine. Similar levels of OTA reductions were also obtained previously in *O. oeni* liquid starter form in wines containing 4,3 µg OTA/L (Silva *et al.,* 2003).

Contradictory results of strains Alpha and 49A1

Strains Alpha and 49A1 show different behavior depending on the wine conditions. In the second set of trials, these strains reduced the OTA content to considerable levels (47% and 45% respectively) at pH 3,6 and initial 6 µg OTA /L. These results are in opposition with those observed during the screening trials where these strains did not show OTA reduction in the same wine containing 1,8 µgOTA/L. Therefore more investigation on these strains is needed to conclude on their property to reduce OTA.

Sensibility of the OTA measurement method

Due to the high LOQ of the method (0,77µg/L) the differences in reduction of OTA observed respect to the initial OTA in the wines cannot be attributable to real changes in OTA concentrations in some trials.

De-adsorption mechanism not observed

Mateo *et al.* (2010b) observed fluctuations in the OTA adsorption during MLF. Such fluctuation couldn't be observed in our trials as the sampling was done only in two points, at T0 and end of MLF + 15 days (before racking).

Fermentative kinetics

No significant impact of OTA concentration and pH in population dynamics was observed. Regarding L-malic acid consumption, in some cases it seems that OTA and/or pH affected the development of the process, but a clear tendency could not be established.

Autochthonous *O. oeni*

In some control batches, the MLF couldn't be performed during the timing of the trials because of the slow LAB development. To conclude about the wild LAB on the R% property, it might be necessary to compare with batches inoculated with an important population of wild LAB, harvested from other ongoing spontaneous MLF. In this condition, with equal biomass, it will be possible to analyze the property of wild LAB regarding reduction of OTA. This study does not allow concluding on this point.

Regarding the contact time with the lees, the time to reach a critical biomass for the reduction of L-malic acid was more important than for the inoculated MLF. To compare the spontaneous MLF in a fair way, the biomass at the beginning of the MLF should be similar for spontaneous and inoculated malolactic fermentations.

Effect of ethanol on OTA reduction

The OTA reduction occurred in wines containing 13% ethanol. This fact is in contradiction with the study performed by Mateo *et al.* (2010b) where the OTA reduction was not observed in synthetic media containing more that 5% ethanol. Therefore, the hypothesis postulated by these authors, indicating that in acidic media and ethanol, the solubility of OTA increases not favoring the adsorption to the cell wall by its loss of polarity is not that obvious.

Influence of the pH

Although the ANOVA test did not find significant different the R% obtained at different pH, an interesting trend was observed. For strains Lalvin 31 and Expertise S, it was observed that pH had an influence on the reduction of OTA (R%) in wines enriched with 6µg/L OTA, at pH 3.8 being the maximum reduction obtained at this OTA concentration (Fig. 65).

As the growth of bacterial populations was not affected by the pH, the OTA reduction observed should not be linked to a critical biomass effect. This means that the size of biomass modulated by the pH and the level of the reduction of OTA do not seem to be

linked as the bacterial populations were about the same order of magnitude in fermentations at different pH.

Fuchs *et al.* (2008) observed OTA reduction in synthetic media at pH 5.0 to 8.0, the maximum removal being observed at pH 5.0 by strains of *Lactobacillus acidophilus* and *Bifidobacterium*. No reduction was observed in experiments with lower pH; this is in contradiction with our results as all the trials were performed at lower pH (pH 3.4; 3.6; 3.8), but it is true that the tendency was to obtain higher OTA reduction with more basic pH (pH 3.8). The microorganisms used differ as well, as in our study the focus was on *O. oeni* and an interaction might exist between the strain and pH.

Mateo *et al.* (2010a) studied the capacity of *Oenococcus oeni* to eliminate ochratoxin A from synthetic media at pH 4.8. They observed strains able to reduce the OTA by 60% in 14-day cultures spiked with 2 µg OTA/L. Here the pH was higher than in our study which is in concordance with the observation that when moving to more basic pH, the R% tends to be higher.

Impact of pH and ethanol

The fact that the pH seems to affects the reduction of OTA in wines, lead to think about the structural changes of proteins and polyphenols from the wine, their interaction with lees and the consequent impact on OTA adsorption.

Siebert *et al.* (1996) studied the agglomerates of proteins and polyphenols formed by hydrophobic bonding in wines. A modellization of the haze formation as function of pH and ethanol was developed. The optimal haze protein-polyphenols formation in wines occurs around pH 4.0 at high or low ethanol content, the maximum ethanol tested in that work being at 12%. Regarding Siebert's haze formation model and the pH evaluated in our study, at pH 3.4 the wine should have less proteins-polyphenols agglomerates formation than at pH 3.8. On the other hand, polyphenols are usually adsorbed by the yeast lees (Mazauric & Salmon, 2005) and bacterial lees (Koren *et al.,* 2009). García-Moruno *et al.* (2005) suggested that the use of lees for reduction of OTA is more efficient in white wine than in red wines because of the competition between OTA and polyphenols for the adsorption sites of the cell wall (Feuillat *et al,* 2000; Ummarino *et al.,* 2001). Therefore, the adsorption of OTA to the cell wall might be increased at optimal conditions of pH and ethanol for haze protein-polyphenols formation because these structures capture the polyphenols that might compete with OTA for the cell wall. In other words, when polyphenols are associated to proteins, they cannot compete with

OTA for the cell wall, which in turns, allows more quantity of OTA to be removed from the wine via adsorption by the lees. This hypothesis is supported by our results as higher OTA reduction levels were observed at the optimal conditions for haze proteins-polyphenols formation. This hypothesis needs to be confirmed experimentally.

The ethanol also plays a role on OTA solubility as in fact, OTA is more soluble in ethanol (50mgOTA/mL ethanol) than in water (1mgOTA/mL water). This might suggest that a solution containing 10 or 15% ethanol, or 13% as it is the case of the studied wines, the structure of OTA molecules should adapt a polar configuration with hydrophilic and hydrophobic sites but not turning in a soluble structure blocking the possibility to be adsorbed by cell wall. No previous investigation about molecular changes on the structure of OTA in alcohol/water solutions has been found in the literature.

Mateo *et al.* (2010b) incriminated the low reduction of OTA they found in synthetic media containing 10 and 15% ethanol by the fact that in acidic conditions and in presence of ethanol, the OTA increases its solubility because of changes in its polarity. However, the ethanol content is not that high to justify an increase of solubility that inhibits adsorption of OTA to the cell wall. In fact our study demonstrates that OTA can be reduced in presence of 13% ethanol in wines.

The interaction of ethanol with protein-polyphenols particles also has an influence on its generation. Haze protein-polyphenols formation in a model system was inhibited by 25% dioxane, a no polar solvent, and it was concluded that dioxane interferes with hydrophobic bonding between proteins and polyphenols (Asano *et al.,* 1982). Dioxane also dissolves freshly formed model system haze to a significant extent. It is likely that ethanol, which is intermediate in polarity between water and dioxane, can reduce haze formation to some extent (Siebert *et al*., 1996). Therefore, balance between solubility of OTA in presence of ethanol and haze protein-polyphenols formation is found in the studied wines as OTA reductions of about 60% can be reached in wines at pH 3.8 and 13% ethanol.

Impact of initial OTA content

For strains Lalvin 31 and Expertise S, the initial OTA concentration influenced the reduction of OTA: a lower OTA content lead to a higher R % value. For strains Alpha and 49A1, the trend was opposite, a higher OTA concentration (second part of trials), led to higher R%.

Several authors reported that higher OTA reductions were obtained with low initial OTA concentrations (Fuchs *et al.,* 2008; Mateo *et al.,* 2010a; Del Petre *et al.,* 2007; Silva *et al.,* 2003). More trials with replication are needed to confirm or reject this observation.
On the other hand, some authors observed that for similar OTA concentration, decontaminating treatments tend to be more efficient in wines enriched with OTA than in wines naturally containing OTA (Mínguez, 2013). Therefore, the R% obtained in this work using LAB starters in OTA spiked wines, might give different results in wines naturally containing OTA.

Low OTA/ high pH scenario

The influence of pH needs to be investigated at low OTA concentration, as with 2µg OTA/L the bacterial strains reached the maximum OTA reduction at pH 3.6. But this concentration was not tested at pH 3.8. In order to determine an optimal scenario for R%, low OTA concentration together with high pH needs to be investigated.

Time of contact between strains and wine

As the MLF duration was very similar in all cases, the effect of time of contact between the lees and the wine on the reduction of OTA was not that evident because the wine cellar timing was respected in order to evaluate the use of bacteria starters in realistic conditions. Therefore, the variable time was function of the natural timing of the MLF but they were similar in all cases where malolactic starters were used.

Mateo *et al.* (2010a) found that maximum OTA reduction occurred at 14 days of the cultivation and then the OTA reduction was constant. Piotroswka and Zakoswka (2005) obtained the maximum reduction of OTA at 15 hours with *Lactobacillus* in synthetic media and also a part of it was released to the media again in the following days. These works suggest that biosorption of OTA to the cell wall might be a quite fast reaction and the risk to increase OTA is present if the lees are not removed from the wine on time. As the main objective of the starters used in our work is to perform the malolactic fermentation, which duration cannot be managed, the effort of time optimization should be focused on the time between end of MLF and racking.

This work does not allow concluding on the effect of time of contact with the lees. In order to dark conclusions in vitro test submitting the starters to different timings would be necessary.

Impact of lyophylization

The lyophylization process, which is a sublimation of water from frozen preparations, is known to change the density, porosity and structure of the yeast and bacterial cell wall (Scherrer *et al*., 1977) to ensure the conservation of the cell in dry conditions. After re-hydration of the lyophilized culture, a portion of the cells becomes metabolically active recovering their cellular functions. The aim of the process is that the portion that survives the drying process is as high as possible. One can wonder if the modifications performed in the cell wall by lyophylization treatment, such as reduction of porosity, lack of closest packing of macromolecules (teichoic acids and peptidoglycans) and collapses in amorphous forms (Scherrer *et al.,* 1977) enhance or reduce the adsorption properties of the cell wall of the used malolactic bacterial starters.

Biosorption properties of the cell wall for the decontamination of heavy metals from the environment using bacteria, yeast, fungi and algae have been of great interest in recent years (Wang and Chen, 2009). This field of research is specially focused on the modification of the cell wall by technological means, including the lyophylization, to enhance the adsorption properties. One can think therefore that lyophylization process would enhance OTA adsorption but works in this sense are not currently available. A close collaboration with the starter's supplier would be needed to study a given strain in fresh and lyophilized form to understand the impact of this technology on the OTA decontamination of wines. On the other hand, usually each culture counts with a specific lyophylization process developed to optimize the culture viability. So, one can wonder if different lyophylization variables for instance, flow rate, culture density, temperature, pressure and shape of noodle, affect the way that the cell wall is modified and in consequence, the adsorption property of the bacterial cultures. Taking into account all these open questions, it is difficult to conclude from the studied lyophilized malolactic starters if the differences observed on OTA reduction are due to the type of strain, or to the specific lyophylization protocols applied for each strain.

Nevertheless, a positive effect of reduction of OTA in wines was observed when lyophilized malolactic starters were used, which is encouraging to pursue the investigation in this field. Eventually this will allow offering to the winemakers "more value for money" when they use bacterial starters not only to manage the malolactic fermentations but also to decontaminate the wine from ochratoxin A.

3.6 CONCLUSIONS CHAPTER 3

1 **Malolactic starters were able to reduce OTA in wines during the MLF.** The potential of *Oenococcus oeni* starters to naturally reduce the OTA content of wines with 13% ethanol during malolactic fermentations is confirmed.

2 **The pH enhanced the OTA reduction in certain conditions.** A tendency was observed to obtain higher OTA reduction in more basic pH conditions.

3 **OTA reduction is affected by wine initial OTA content** as reduction was more pronounced in the case of initial lower concentrations.

OVERALL CONCLUSIONS

OVERALL CONCLUSIONS

Chapter 1. Malolactic fermentation in wines: influence of the level of implantation of lactic acid bacteria starter on the production of biogenic amines during malolactic fermentation

Globally, the investigation performed in the first part of the thesis demonstrated that the use of starters is not a binomial operation “inoculation or no inoculation” as different degrees of implantation are possible. The level of implantation is negatively correlated with the production of biogenic amines during the MLF and at the same time, the level of implantation of LAB starter is affected by pH, inoculation technique, inoculation time and addition of lysozyme with the concomitant impact on biogenic amines. Therefore, the proper choice of oenological practices followed by the monitoring of the MLF using typification of LAB population is needed to ensure the process is under control and the risk to produce wines containing metabolites for risk to the health or wine commercialization is minimized.

Chapter 2. Evolution of biogenic amines in bottled red wines during storage

To our knowledge, this is the first investigation that envisaged the development of methodologies to measure the risk of histamine development during storage of bottled wines. The results are encouraging as it was possible to establish some connections between the analysis of the wine microorganisms and cell-free wines with the development of biogenic amines in the long term. Ideally, the fine-tuning of accelerating treatments together with the information provided by microorganisms and enzymes should give a reasonable hint on the level of risk that the wine has of developing histamine during storage. Nevertheless, the understanding of physical-chemical and biological mechanisms that take place in a tough environment as the bottled wines is far from being completely understood, principally with respect to amines-degradation. These exciting results should encourage continuing the investigation with the end goal of developing accurate predictive models.

OVERALL CONCLUSIONS

Chapter 3. Role of malolactic starters in reduction of ochratoxin A in wines

Based on the results presented in the third part of the thesis, the potential of *Oenococcus oeni* starters to naturally reduce the OTA content in wines with 13% ethanol during malolactic fermentations is confirmed. More investigation is needed to understand the underlying mechanism and to determine the field of application based on wines characteristics with the end objective to maximize the reduction of OTA by using specific strains.

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