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Optimization of oenological practices to reduce biological contaminants in wine

Coordinator: Ch.mo Prof. Romeo Astorri

Candidate: Alessandro Moncalvo

Matriculation n. : 3810664

Tutor: Prof. ssa Angela Silva

Co-tutor: Dott.ssa Maria Daria Fumi

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Those who dream by day are cognizant of many things which escape those who dream only by night

Edgar Allan Poe

Il faut être toujours ivre.
Mais de quoi? De vin, de poésie ou de vertu,
à votre guise. Mais enivrez-vous.

Charles Baudelaire

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Preface and aim of PhD thesis

Wine is an alcoholic fermentation made from fermented juice of grape with appropriate processing. During the processes, the grapes, the must and the wine are susceptible to various safety and quality hazards. The quality hazards are usually related to products appearance, acceptability, taste, flavour, colour and components. As far as safety, physical, chemical and microbiological hazards can affect consumer health. Hazards introduced to the wine may come from the environment, the processes equipment and the processing operations and treatments in the winery. Quality and safety are significant variables for a product as the wine; the quality is important for the consumer acceptability, while the assurance of the safety is obligatory for protection of human health. Safety of the wine product may only be assured by taking care of the relative hazards throughout the whole winemaking process.

Normally the wine is considered a safe product, the low pH, the ethanol and the presence of SO_2 ensure a microbiological stability to the product during winemaking and the ageing. The possible risks may be due to the presence of chemical residues derived from pesticides used in vineyard, and biological metabolites products by microorganisms in the vineyard or during winemaking.

Two of the major biological metabolites present in wine are the ochratoxin and the biogenic amines. The first of these contaminants was studied in recent decades because of its toxicity in humans, although its presence is not frequent in wines.

The biogenic amines are present in every types of wine in different concentration, and some of them, in high concentrations, can cause allergenic reactions in humans.

The research managed in this PhD is focused on the use of the selected starter for malolactic fermentation (MLF) to reduce these contaminants at guarantee of wine quality and safety assurance for the consumers.

The objectives regard three different aspects. In the first part, it was investigated the biological methods to reduce ochratoxin A in wine during winemaking; in particular the study is focused to use a *Lactobacillus plantarum* strain as malolactic starter. Previous studies have described different methods to reduce ochratoxin in wine; in particular, adsorbent material (carcoal, bentonite, and yeast cell wall) and enzymes (proteases).

The ochratoxin A is usually present in must and wine in particular regions of Europe as South Italy, Greece and Spain. These wines have different characteristics respect to the wines original of colder regions; a relevant condition that affects the MLF is the high pH values. The *L. plantarum* used in

this study was already tested in winemaking trials that have demonstrated the ability of this strain to carry out MLF especially in wine with high pH levels.
A problem related to the high pH is the formation of biogenic amines as histamine, tyramine and putrescine. Some bacteria are able to produce these compounds in winemaking, in particular during MLF, or can be already present in the grapes before crushing.
The second part of this work has the objective to investigate the presence of *Lactobacillus* spp., isolated from must and wine, able to produce the amines, using molecular techniques as polymerase chain reaction (PCR) to detect the genes that encode for the enzymes responsible of the synthesis of these compounds. This study is important to identify the real risk related to malolactic bacteria normally present in wine.
In the last part of this PhD a malolactic starter, unable to produce biogenic amines, is tested to investigate the ability to perform MLF in relationship with inoculation time to shorter overall process of winemaking and to have a final product that maintains the varietal characteristics and has a good quality. Furthermore, the trend of biogenic amines already present in must was investigated to assess the relationship between the *L. plantarum* and the amines naturally present on the grapes that are affected by vintage, cultivar and other variables not related with the lactic acid bacteria.

Introduction

Chapter 1- Ochratoxin A

1.1 Chemical characters of ochratoxins

The ochratoxins are a group of mycotoxins that contain a dihydro-isocumarin moiety linked to L-β-phenylalanine by an amide bond. Ochratoxin A (OTA), (R) N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzo-pyran-7-yl) carbonyl]-L-phenylalanine, and its ethyl ester (ochratoxin C) are the most toxic compounds. The isocoumarin moiety is known as ochratoxin α (OTα), and is commonly reported to be less toxic than OTA. Strong acidic conditions cause hydrolysis of the amide bond, and strong bases open the lactone ring, reformed by acidification (Pohland *et al.*, 1992). OTA can be converted into OTα and L-β-phenylalanine by heating under reflux for 48 h in 6 M hydrochloric acid (van der Merwe *et al.*, 1965) or by hydrolysis with carboxypeptidase A (Pitout, 1969). In fact the amide bond mimics a peptide bond and is therefore susceptible to the action of hydrolytic proteases.

1.2 Toxicity of ochratoxin A

OTA is a well-known nephrotoxin and it is classified by International Agency for Research on Cancer (IARC) as possible human carcinogen (group 2B) (IARC, 2003). A tolerable weekly intake of 120 ng/kg of body weight (bw) for humans was fixed on the basis of the lowest observed adverse effect level of 8 μg/kg (bw) per day for early markers of renal toxicity in pigs, the most sensitive animal species (EFSA, 2006). Long-term exposure to OTA has been implicated in Balkan Endemic Nephropathy and associated with urinary tract tumours because of rather high OTA levels detected in food samples and in blood or urine from affected persons (EFSA, 2006).

The toxicity of OTA involves several mechanisms. OTA inhibits protein synthesis by competing with the phenylalanine amino acylation reaction catalysed by Phe-tRNA synthase (Creppy *et al.*, 1984). This involves the inhibition of protein as well as DNA and RNA synthesis. OTA also disrupts hepatic microsomal calcium homeostasis by impairing the endoplasmic reticulum membrane via lipid peroxidation (Omar and Rahimtula, 1991).

Based on animal studies (Kumagai, 1988), OTA is easily absorbed through the gastrointestinal tract mainly in the duodenum and jejunum. There are no studies on skin or inhalational absorption of

OTA. When absorbed, OTA has a high binding affinity for plasma protein. OTA was found in decreasing order of concentrations in kidney, liver, fat, and muscle tissues (Krogh *et al.*, 1974); excretion is mainly via renal elimination (Chang and Chu, 1977).

The half-life of OTA in humans is about 35 days; blood concentration is considered to represent a convenient biomarker of exposure and has been used in epidemiological studies. The human blood levels of OTA, in geographical areas with relatively high dietary exposure, are at least two order of magnitude below the mean concentration of OTA in the blood of rats that is known to cause nephrotoxicity and kidney tumours with long-term treatment (Mally *et al.*, 2007).

There is a positive correlation between plasma levels of OTA and the consumption of cereals, processed meat, animal fat, mutton/lamb meat, wine, beer, and jam/honey, Di Giuseppe *et al.* (2011) found a strong positive association between OTA intake, cardiovascular risk, and C-reactive protein.

Recently, it was detected the presence of OTα in human plasma and urine suggesting the OTA-detoxification process in humans; in the same study the differences of OTα levels, especially in urine, between males and females (Muñoz *et al.*, 2010a) were revealed. Another study investigated the presence of OTA and OTα in human milk; OTA was detected with an average concentration of 106±45 ng/L, while levels of OTα were 40±30 ng/L, but increased upon enzymatic hydrolysis with β-glucuronidase/sulfatase, indicating that the majority of OTα in milk is excreted as glucuronide or sulphate (Muñoz *et al.*, 2010b).

These new data provide further insight into the fate of OTA in the human organism, documenting partial detoxification of OTA to OTα and conjugation of this metabolite.

1.3 Ochratoxin A occurrence in food and wine

OTA has been found in many food and raw materials such as cereals, coffee, cocoa and other crops in warmer and tropical regions.

In a study on coffee OTA was detected in almost half of the samples analyzed (299 positive samples out of 633) and high OTA values (>10 ng/g) were very rare (Stegen *et al.*, 1997).

In the cocoa Bonvehí (2004) reported that the average of OTA was 11 μg/kg (range of 2.9-23.1 μg/kg) (100% positive samples) for the roasted cocoa shells and 2.60 μg/kg in raw cocoa shells.

Ariño *et al.* (2007) investigated the effect of the sorting, washing and peeling of fresh liquorice on the concentration of OTA and the transfer of OTA from dry liquorice roots to derived products; in

this case the 21.4% of the OTA present in dry roots was transferred to the corresponding liquorice extract.

Kabak (2009) reported the presence of OTA in cereal-derived products in Turkey, the occurrence of OTA has been determined in 24 breakfast cereals, 24 cereal-based baby foods and 35 beers. Fortunately none of the cereal-derived products tested contained OTA at levels above the recommended dose by EU for safe consumption.

The presence of OTA in grape juices and wines was reported for the first time in 1996 (Zimmerli and Dick, 1996). Recently, overviews of OTA contamination in wine were reported by Battilani and Silva (2010). The range of OTA content in wine produced in Europe varied between 0.01 and 3.4 µg/L. The highest values were reported in some samples of dessert wines and in wines made from dehydrated grapes. Since the vintage of 2005, with the adoption of Regulation CE 123/05, the level of OTA in commercial wines cannot exceed 2 µg/kg.

Wine, particularly red wine, is considerate, after cereals, the second source of OTA intake for humans (15%) (Pietri *et al.*, 2001). The exposure of Italian population to OTA ingestion from wine was evaluated. The results indicated a daily intake for wine consumers from 0.59-1.24 ng/kg bw/day and from 0.33 to 0.90 ng/kg bw/day for the total population, in the worst case accounting for 9.8% of tolerable daily intake (Brera *et al.*, 2008). Other data on contribution of wine in human exposure to OTA are showed by Battilani and Silva (2010). The estimated exposure to OTA due to wine consumption was obtained by combining the OTA occurrence with data sets of wine consumption and consequent OTA ingestion per week was computed. The exposure values indicate that wine contribution does not represent a serious risk factor, even if data confirmed a major exposure in Mediterranean areas.

1.4 Ochratoxin A production in the vineyard

OTA is a mycotoxin produced by several species belonging to *Aspergillus* and *Penicillium* genera (Battilani *et al.*, 2001). OTA is produced in the vineyard and *Aspergillus* section *Nigri*, the so-called black Aspergilli, includes all fungi responsible for OTA presence in grapes (Battilani and Pietri, 2002; Da Rocha Rosa *et al.*, 2002; Sage *et al.*, 2002).

Black Aspergilli usually overwinter in soil (Leong *et al.*, 2006), but they are present on berries from fruit-set and their incidence increases as the fruit grows and matures, with their numbers peaking at ripening; the incidence of berries infected by black Aspergilli at harvesting is significantly related to

latitude and longitude, the infection incidence is highest in the hottest and driest years (Battilani *et al.*, 2006a).
Fungi development is strongly related to environmental conditions, mainly air temperature and relative humidity, but a relevant role is played by available water of the medium. Meteorological conditions and region of origin are crucial for OTA production in grape (Serra *et al.*, 2006). High variability in OTA contamination was observed in clusters grown on the same vine in the same vineyard (Battilani *et al.*, 2006b); also the cropping system has an effect, but is difficult to quantify the role of each variable (Battilani *et al.*, 2006c).
Black Aspergilli are considered saprophytes, responsible for secondary rots, and wounds of both mechanical and biological origin are important entry sites (Bellí *et al.*, 2007a). *Lobesia botrana* (*Lepidoptera: Tortricidae*) is the major grape berries moth in vineyard of Southern Europe; first-generation larvae damage flowers, while the succeeding larval generations feed on berries at different stages of maturity. OTA content in berries and pest damage are related, probably due both to the increase of entry points for fungi and the role of larvae as spore vectors (Cozzi *et al.*, 2006).
Grapes originating from south Europe are more affected that those from temperate region of central Europe (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000; Pietri *et al.*, 2001; Valero *et al.*, 2008); in wine the concentration decreases from the southern regions compared to northern regions and in red wines compared to white ones. In some countries of the Mediterranean basin the percentage of wines in which OTA is detected is very high, but only a few wines contained concentrations exceeding the legal limit.

1.5 Ochratoxin A prevention in vineyard

The application of good agriculture practices (GAP) can minimize the amount of OTA in final product; controlling of pests and diseases that can damage the berries is of particular importance. The use of chemical compounds is a very attractive strategy to prevent mycotoxin production, in fact, some fungicides have been found to be effective against fungal colonization and OTA production (Varga and Kozakiewicz, 2006).
The following chemicals have been shown to be active in reducing, to varying degrees, both fungal growth and OTA levels in grape bunch: mepanipyrim, pyrimethanil, fluazinam, iprodione and the mixture cyprodinil/fludioxonil (Visconti *et al*., 2008). The latter mixture was confirmed as effective in a number of field trials carried out in several Mediterranean countries, including France, Spain, Greece and Italy (Tjamos *et al*., 2004; Kappes *et al*., 2005; Bellí *et al*., 2007b).

This mixture of active ingredients applied against black Aspergilli at the same combination and schedule, both in dosage and timing, is effective against grey mould caused by *Botrytis cinerea*. Moreover, the insecticide treatment against *L. botrana* in combination with the fungicide contributed significantly to a reduction of OTA levels in the field, particularly in crop-years at high contamination risk (Kappes *et al*., 2005).

Promising results were also obtained using yeast as a biological control agent isolated from grapes, in particular, good results were obtained in Argentina with two strains of *Kluyveromyces thermotolerans*, (Ponsone *et al*., 2011).

To reduce the risk of the presence of OTA, harvesting must be at ripening and over-ripening must be avoided, especially if damaged berries with visible black mould are present; the interval between harvesting and processing should be minimized to prevent further fungal growth and OTA biosynthesis and the normal procedures for sanitizing materials and machines must be followed.

1.6 The fate of ochratoxin A during winemaking

Grape processing has a significant impact on the behaviour of OTA during the main stages of winemaking (crushing, maceration, alcoholic and malolactic fermentation, ageing stabilisation and filtration). OTA in grapes is transferred to wine during vinification. The fate of OTA during winemaking and the role of unit operations have been studied by several authors. Fernandes *et al*. (2003) observed an increase of OTA concentration in must during maceration of crushed grapes and a consistent reduction of the OTA in wine after pomaces and lees separations. Grazioli *et al*. (2005, 2006) focused on the red winemaking, carrying out full-scale fermentations in different wineries in the south of Italy with naturally OTA-contaminated grape varieties (Negroamaro and Primitivo). The results showed that no OTA is produced during winemaking. The OTA present in grapes is released to the juice during crushing; maceration increases OTA content, while alcoholic and malolactic fermentations (MLF) and solid-liquid separation reduce OTA in wine. As far as identifying winemaking OTA Critical Control Points, the process can minimize the hazard, but it cannot assure the absence of the toxin. Lasram *et al*. (2008) conducted two assays of red and rosé microvinification, with artificially and naturally contaminated grapes. The results from the different assays showed that the maceration of pomace has an effect on the increase of OTA content in red wine whereas the alcoholic fermentation (AF) has a reducing effect on OTA content. In this case the spontaneous MLF showed no effect on the OTA level in wine. Storage of red wine in tanks

followed by draining caused a decrease of OTA of about 55%, also clarification with a gelatin fining agent contributed to the removal of up to 50-60% of OTA from red wine.
In other microvinification tests only 4% of the OTA present in grapes is released in wine, whereas most of OTA is retained in pressed grape pomace and 1% is retained in the lees. The results obtained with the microvinification were confirmed at an industrial scale (Solfrizzo *et al.*, 2007).
Leong *et al.* (2006b) reported that the concentration of OTA released into wine, relative to the initial concentration present in grapes, was greater in red (9%) than in white wine (4%). This may be partly attributed to the differences in red and white winemaking processes. In red winemaking the release of OTA is seen to happen in the first stage of winemaking (crushing and maceration), while in white winemaking the OTA released into must during pressing of the grapes is reduced in must settling. The OTA level decreases during red and white winemaking considering the trend of OTA from AF end to wine bottling. Therefore, the OTA detected in the finished wine is much less than that in the grapes, as most of the toxin is removed during winemaking through binding to the marc and lees. Also the binding of OTA to the increased yeast biomass during AF appears to be an important stage in the reduction of OTA.
All studies indicate that winemaking reduce OTA during processing, although the percentage reduction varied between the studies. The results of different studies are not always in agreement, but in general confirm that no OTA is produced during winemaking and the balance of OTA in wine during winemaking is negative. Probably these differences are related to the use of artificially and naturally contaminated grapes in the researches. Unfortunately, the identification of critical control points in winemaking can minimize the OTA hazard, but cannot assure the absence of the toxin.

1.7 Removal of ochratoxin A in wine

The methods to reduce or eliminate OTA in food and feed are investigated from some years. OTA detoxification and elimination strategies are classified depending on the type of treatment; physical, chemical or microbiological, and their objective is to protect consumer health by destroying, modifying or absorbing this mycotoxin (Amézqueta *et al.*, 2009). The physical methods used for mycotoxin detoxification include cleaning, mechanical sorting, and separation, heat treatment, ultrasonic treatment, and irradiation. Chemical methods consist of the utilization of chemical compounds such as ammonium, alkaline compounds, bisulphites and ozone (Merwe *et al.*, 1965; Chelkowski *et al.*, 1982; Mendez Albores *et al.*, 2007; Amézqueta *et al.*, 2008, 2009; Jalili *et al.*,

2010). Many physical adsorbents have been extensively studied and available as commercial preparations as animal feed additives. However, many of these adsorbents can bind to only a small group of toxins while show very little or no binding to others (Huwig *et al.*, 2001). Biological methods use enzymes able to catalyse the mycotoxins degradation or microorganisms involved in decomposition, transformation or adsorption of mycotoxins.
Likewise, several studies have focused on the reduction of OTA in musts and wines in the winery and different procedures based on physical, chemical or biological removal have been proposed. The removal of OTA by adsorption with fining agents is most frequently applied. Biological decontamination of OTA is a very promising approach for the decontamination of wine, as use of chemical or physical tool may also remove, along with the mycotoxin, other organoleptic important substances. The use of microorganisms such as *Saccharomyces cerevisiae* and lactic acid bacteria (LAB) could present great advantages, since they have an historical and extensive application in the food and wine industry (Abrunhosa *et al.*, 2010).

1.7.1 Adsorbent material

Many methods to control the OTA concentration during winemaking have been proposed and the removal of the mycotoxin with fining agents has been the most frequently studied approach.
Adsorption involves the accumulation of molecules from a solvent onto the exterior and interior surfaces of an adsorbent. The surface phenomenon is a manifestation of complex interactions (van der Walls, resonance and electrostatic forces and hydrogen bonding) between the adsorbent, adsorbate and the solvent. The molecular size and the physicochemical properties of OTA as well as the physical structure of the adsorbent, including the total charge and charge distribution, the size of the pores together with the surface area, among other factors, play a significant role in the achievement of OTA-binding by adsorbent materials (Huwig *et al.*, 2001). OTA is a weak acid; it is partially dissociated at the pH of wine (3.0-3.8) and carries a negative charge that may interact with a positively charged surface. OTA may also react by means of carboxylic group and phenol moiety that could be adsorbed through hydrogen bonding and/or charge transfer complexes and interactions of two π-electron orbitals (hydrophobic adsorbent, as i.e. charcoal).
Activated carbon is an effective adsorbent having a high surface area per unit mass and its adsorption ability varies depending on the activation process. The chemically activated carbon has an irregular surface as compared to the steam activated carbon. Therefore, the former has highest adsorption surface to bind compounds from the media (Castellari *et al.*, 2001). Previous studies

performed on red wines pointed out that charcoal-based product are highly effective to reduce OTA contamination (Silva *et al.*, 2003). Gambuti *et al.* (2005) described a decrease in OTA concentration related to increase of carbon dosage; this behaviour, at increasing carbon concentration, was due to the presence of specific binding site and their gradual saturation. Competitive adsorption, between OTA and other wine components, could be one of the more important factors influencing the binding efficiency of activated carbon toward target organic contaminants. When a practical application is considered, dose and contact time are very important variables. Since carbon is a very porous non-soluble powder with a relatively unspecific adsorption capacity, it can produce substantial depletion of wine aroma and phenolic profile. Consequently, the contact time and the amount of charcoal to be used are preferred to be as low as possible (Silva *et al.*, 2007). Var *et al.* (2008) confirmed that the adsorption abilities of activated carbon vary widely and depend on both carbon and toxin concentrations; the application of 1 mg/mL activate carbon was sufficient to reduce OTA levels in white wine, with up to a 98.3% reduction in OTA being observed, when compared to control.

Other fining agents have been described by Castellari *et al.* (2001): silica gel, bentonite, egg albumin, gelatin, potassium caseinate. Silica gel positively charged shows a good affinity versus OTA, whereas the silica gel negatively charged is less effective. In fact, OTA is a weak acid partially dissociated at the pH of wine and carries a negative charge.

Bentonite showed a relative efficiency (0.16% OTA adsorbed/m^2/g) as compared to carbons (0.04% OTA adsorbed/m^2/g) and silica gel (0.07% OTA adsorbed/m^2/g). However, the total OTA removed was poor (8% adsorption) when compared to the most active fining agents. Probably the OTA is adsorbed within the inter-laminar spaces of bentonite by a cation exchange mechanism.

Egg albumin, gelatin and potassium caseinate, which are proteins positively charged at the pH of wine, showed a good affinity for OTA and removed 40%, 30% and 35% of this mycotoxin respectively.

Savino *et al.* (2007) described the reduction of OTA, in contaminated wines, by chips and powder from oak (*Quercus robur*); OTA concentration was strongly reduced with both products and the best results were obtained with powder. A maximum dose of 8 g/L chips reduced OTA by 65.4% after 30 days of contact, while using the powder the reduction was 75.7% after 15 days; unfortunately the OTA concentration increased after 30 days and this desorption indicates a relatively weak bond between OTA and wood.

Grape pomaces have a high affinity for OTA and have been shown to remove OTA from must and wine during winemaking, Solfrizzo *et al.* (2010) demonstrate that the repassage of wine from

Primitivo and Negroamaro grapes, spiked with 2-10 μg/kg OTA over uncontaminated pomaces, removed up to 65% of OTA within 24 h.

Microbiological-binding agents to remove the mycotoxin have been tested; it was demonstrated that chitosan, chitin, chitin-glucan and chitin-glucan hydrolysate of fungal origin are able to reduce OTA (26.1-83.4%) (Bornet and Teissedre, 2007).

1.7.2 Biological agents

1.7.2.1 Enzymes

Several enzymes may be involved in the microbiological degradation of OTA. However, little information is available and very few have been purified and characterized. The first reported protease able to hydrolyze OTA was carboxypeptidase A from bovine pancreas (Pitout, 1969). Subsequently, a screening study which included several commercial hydrolases verified that a crude lipase preparation from *A. niger* was able to hydrolyze OTA via the break of the amide bond (Stander *et al.*, 2000).

Several proteolytic preparations, involved in the hydrolysis of OTA to OTα, were also studied. These include protease A from *A. niger*, pancreatin from porcine pancreas and to a lesser extent, Prolyve PAC from *A. niger*. Protease A converts 87.3% of total OTA into OTα after 25 h, when incubated at pH 7.5 and 37°C. Pancreatin is able to transform 43.4% of the initial OTA into OTα under the same conditions. For Prolyve PAC, at pH 3.0, a small amount activity was detected (3% after 25 h at 37°C). The identification of the enzymes involved is complex since it is necessary to employ the appropriate pH, temperature, ionic-strength and time in order to allow the occurring of the catalytic reaction, and to measure the reaction (Abrunhosa *et al.*, 2006).

Recently, Abrunhosa and Venâncio (2007) isolated and purified from *A. niger* an enzyme hydrolyzing OTA ; this enzyme presented maximal activity at pH 7.5 and 37°C. The inhibition studies, using the metal-ion chelator EDTA, suggested that the OTA hydrolytic enzyme was a metalloenzyme. Studies on OTA degradation by enzymes are not available in wine but some trials made in our laboratory showed that proteases from microorganisms, as subtilisin and acid proteases, or from vegetal source, as papain,are effective to reduce the OTA level in must (unpublished data).

1.7.2.2 Yeast

Yeasts to reduce the OTA have been tested for the mycotoxin interaction with the cell wall. The cell wall of *S. cerevisiae* is a bi-layered structure, containing chains of β-1,3-glucan and β-1,6-glucan.

The cell wall proteins (mannoproteins) consist of a very heterogeneous class of glycoproteins. From the mannoproteins core, extend out highly branched mannose side chains and short and rigid rods like clusters of oligomannosyl chains. Another important feature is represented by phosphodiester bridges in mannosyl side chains that yield numerous negative charges on the cell surface (Shetty and Jespersen, 2006).

Yeast cell wall is known to bind sterols from the medium (Adams and Park, 1967) and binding molecule was identified as the cell wall mannan (Thompson *et al.*, 1973). Based on some of the studies reported, it is confirmed that removal of mycotoxin occurs by adhesion to cell wall components rather than by covalent binding (Baptista *et al.*, 2004; Santin *et al.*, 2003). Reported literature indicates that mannan components of cell wall play a major role in aflatoxin binding (Devegowda *et al.*, 1996), and can bind OTA (Raju and Davegowda, 2000). The binding of OTA is also enhanced when yeast cells are replaced with physically extracted cell wall (Huwig *et al.*, 2001) or heat treated cells, and the very rapid removal of toxin from liquid medium (Bejaoui *et al.*, 2004) indicates also the physical nature of ochratoxin adsorption .

The treatment of red wine with active dry yeast or with lees obtained from AF led to a strong reduction of OTA in wine. The adsorption of OTA from lees is very high, even after a short period of treatment. Cecchini *et al.* (2006) reported OTA residues in the lees after extraction with methanol.

Garcia-Moruno *et al.* (2005) showed that the percentages of OTA reduction are different using white and red lees-wine; after 90 min of lees-wine contact, with 20 g/L of less, the reduction with white lees-wine was between 50.4% and 63.0% while with red lees-wine was between 22.8% and 31.7%; after 7 days of contact the OTA reduction was greater than 70% with the white lees and ~50% with the red lees. The differences found between white and red lees are probably due to competition, between polyphenols and OTA, for the same binding site on the surface of the yeast cells (Caridi, 2007).

In some studies a significant decrease of OTA levels was observed in synthetic and natural grape juices but no degradation products were detected; OTA removal was enhanced with dead cells of yeast, indicating that the mechanism involved is adsorption and not catabolism.

Nunez *et al.* (2008) tested whole yeast cells and yeast cell walls to remove OTA in a wine model system; the yeast cell walls mannoproteins were involved in the removal of OTA through adsorption mechanisms and the contact time can affect the capacity of whole yeast cells to remove OTA from model wine. Heat treatment of whole yeast cells and yeast cell walls (85°C for 10 min) before their use as decontamination agents increased the OTA removal capacity and the efficiency of the process.

Bejaoui *et al.* (2004) showed that treatments of yeasts with heating and acidity, compared with viable cells, enhanced significantly OTA removal from liquid media (75%) but no significant difference was noted between these two treatments. Polysaccharides and peptidoglycans are both expected to be affected by heat and acid treatments. Heating may cause denaturation of proteins or formation of Maillard reaction products. Acidic conditions could affect polysaccharides by releasing monomers, further fragmented into aldehydes after the breaking of glycosidic bonds. These released products could offer more adsorption sites than viable cells, increasing surfaces for OTA binding.

Caridi *et al.* (2006) described a percentage of removed OTA of 68.45% (in a wide range, from 39.81% to 90.95%) in wines obtained from the naturally OTA-containing grape must; while, in wines obtained from the grape must spiked with OTA, the value had a mean of 78.22% with a much narrower range, from 67.89% to 83.34%.

Different yeasts by-products, yeast walls and hulls and inactivated yeast, were tested by Silva *et al.* (2007) and the maximum OTA reduction was generally obtained after eight days of contact at 20°C; a reduction of 40-50% OTA was determined by the addition of 100 g/hL of yeast walls or 40 g/hL of yeast hulls or 200 g/hL of active dry yeasts.

In general, OTA removal by *Saccharomyces* strains corresponds to an adsorption mechanism confirmed both by absence of degradation products during growing culture and ability of dead cells to reduce OTA concentration during the adsorption assays. Ionic properties of the OTA molecule, yeast membrane conditions, and biomass concentration are the major factors affecting the OTA adsorption phenomenon.

Nevertheless some authors suppose that a process to degrade OTA is involved. Angioni *et al.* (2007) carried out in vitro experiments using different strains of *S. cerevisiae* and *Kloeckera apiculata* to evaluate OTA reduction and the presence of hydrolysis products (OTα and phenylalanine). The absence of OTA residues in biomass excluded an adsorbing effect from the yeast cell walls of the strains studied. Neither phenylalanine nor OTα were found in the fermentative media or in the pellet extract. The absence of OTα and phenylalanine in the media and in the pellet suggested other degradation pathway; nevertheless, the authors did not exclude that the degradation pathway of hydrolysis of the OTA don't occurs, because phenylalanine and OTα could also react with other compounds in the media.

Péteri *et al.* (2007) examined the OTA degrading and adsorbing activities of astaxanthin-producing yeasts *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous*. Their data indicate that *P. rhodozyma* is able to convert OTA to OTα, and this conversion is possibly mediated by an enzyme related to carboxypeptidases. Chelating agents like EDTA and 1,10-phenanthroline inhibited OTA

degradation caused by *P. rhodozyma,* indicating that the carboxypeptidase is a metalloprotease, similarly to carboxypeptidase A.
In subsequent studies, it was found that carboxypeptidase Y, a vacuolar exopeptidase from *S. cerevisiae,* is able to hydrolyze OTA with optimal activity at pH 5.6 and 37°C. After five days of incubation, carboxypeptidase Y converted 52% of the OTA present in the reaction mixture into OTα. This activity could be sufficient to reduce significantly levels of OTA during wine or beer fermentation, since these processes take several days to complete (Abrunhosa *et al.*, 2010).
In perspective the literature results show the possibility of greatly reducing the OTA content of must and wine with selected wine yeasts, used like a sponge, sequestering OTA during winemaking. In this contest yeasts represent a very promising biomaterial, also considering their role in the vinification process.

1.7.2.3 Lactic Acid Bacteria

The LAB are widely used for the production of fermented foods and are also part of intestinal microflora. In winemaking the LAB are implicated in MLF. *Oenococcus oeni* was identified as the principal organism involved in the process, but different species of LAB, such as species of *Lactobacillus* and *Pediococcus*, have been isolated from wine (Davis *et al.*, 1986; Bartowsky, 2005; Rodas *et al.*, 2005). In winemaking the LAB activity can be used as method to reduce OTA in wine; unfortunately, the results reported in the literature are contradictory (Fernandes *et al.*, 2007; Grazioli *et al.*, 2006; Fumi *et al.*, 2008).
In vitro removal of OTA by some LAB species was studied by Del Prete *et al.* (2007); the amount of OTA removed during bacterial growth was shown to vary between 8% and 28%. In this study, to determine the mechanism by which LAB reduce OTA, the degradation by protein in cell-free extract and the binding to LAB cells have been investigated. OTA degradation by cell-free extracts of three different LAB strains, after 48 h of incubation, was determined, and no degradation products were detected, suggesting that OTA removal by LAB is a binding process.
In LAB the cell wall consist of a peptidoglycan matrix, which forms the major structural component of cell wall, housing other components such as teichoic and lipoteichoic acid, a proteinaceous S layer, and neutral polysaccharides (Delcour *et al.*, 1999). These components play various functions including adhesion and macromolecular binding. Probably, the peptidoglycan and polysaccharides are involved in toxin binding (Zhang and Ohta, 1991).
It has been shown that peptidoglycan or the structures closely associated might be the most likely molecules involved in aflatoxin B_1 binding process (Lahtinen *et al.*, 2004). Another mechanistic study conducted by Haskard *et al.* (2001), showed that binding of aflatoxins to cell surface is

considerably strong. Binding of aflatoxin B_1 was found to be unaffected by pH values ranging from 2.5 to 8.5, suggesting the absence of a cation exchange mechanism; when the cells were treated with organic solvents, bound toxin was quickly extracted, suggesting the main role of hydrophobic interaction in the binding (Haskard *et al.*, 2000).

Fuchs *et al.* (2008) investigated the mechanisms which account for the removal of OTA by LAB, comparing viable and heat inactivated bacteria. The adsorption of OTA depended strongly on the amount of bacteria, significant effects were seen when the cfu/mL was $\geq 10^8$/mL; moreover the elimination from the liquid medium depended also on OTA concentrations. The authors found that the viability of the cells plays an important role, as with heat inactivated cells only a moderate reduction of OTA was observed. OTA was removed by far better by viable bacteria, which can be taken as an indication that, other than binding to the cell walls, processes, as metabolic conversion mediated by the release of specific enzymes could be involved.

Piotrowska and Żakowska (2005) tested 29 species of *Lactobacillus* and *Lactococcus* genera, assessing their ability to remove OTA in liquid medium. This study show that the elimination by LAB takes place and that the ability to reduce the amount of OTA is common among LAB, but varies depending on the species and the strain of bacteria. The biggest decrease of the toxin was observed in the culture of intestinal lactobacilli *L. acidophilus* and *L. rhamnosus* (70.5% and 87.5% respectively). A somewhat smaller decrease, about 50% reduction of the OTA amount, was observed in the strains of *L. plantarum*, *L. sanfranciscensis* and *L. brevis*. To balance the eliminated OTA with the OTA bound to the cells, the authors suggested that an unknown mechanism, in addition to binding to the biomass, is involved.

The capacity of *O. oeni* to eliminate OTA from synthetic media was studied by Mateo *et al.* (2010a). The mechanism proposed was the biosorption on the cell wall; the cell-binding phenomenon was unrelated to bacteria viability and OTA removal process was partly reversible. The reductions detected at the end of the incubation period varied between 36% and 63% depending on the strain and initial OTA level. In the study it was shown a significant toxin reduction and no differences in OTA removal were found between cultures inoculated with living and dead bacteria.

Recently it was shown that 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 (Mateo *et al.*, 2010b).

Fumi *et al.* (2008) studied the OTA removal in laboratory and full-scale trials by using naturally contaminated wines having different OTA levels and *L. plantarum* and *O. oeni* selected strains. The results were species and strains dependent and a biodegradation was hypothesized. This hypothesis was supported by appearance of OTα, the isocoumarin rest of the OTA, during MLF and during the

bacteria starvation. The analysis of data available in the literature indicate that the application of bacterial strains for OTA reduction in winemaking requires further evaluation to clarify the mechanism of LAB- mycotoxin interaction.

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Chapter 2 - Biogenic amines

2.1 Chemical characteristics of biogenic amines

Biogenic amines are basic nitrogenous compounds formed mainly by the decarboxylation of amino acids or by amination and transamination of aldehydes and ketones. They are organic bases with low molecular weight and are synthesized by microbial, vegetable and animal metabolisms. The chemical structure of biogenic amines can either be: aliphatic (putrescine, cadaverine, spermine, spermidine); aromatic (tyramine, phenylethylamine); heterocyclic (histamine, tryptamine). Amines have an important metabolic role in living cells. Polyamines are essential for growth but their biological role in cell metabolism is still unclair; other amines like histamine and tyramine are involved in nervous system functions and in the control of blood pressure (Silla, 1996).

2.2 Toxicological aspects

Problems related to biogenic amines formation can affect numerous fermented food products such as cheese, beer, wine and meat (Fernández-García *et al.*, 1999; Izquierdo-Pulido *et al.*, 2000; Kaniou *et al.*, 2001).

In wine, biogenic amines have received more attention, because ethanol can increase the effects on health, by directly or indirectly inhibiting the enzymes responsible for the detoxification of these compounds (Maynard and Schenker, 1996).

The human organism easily tolerates low concentrations of amines, since there are efficiently broken down by mono- and diaminoxidase enzymes in the intestinal tract. The pharmacological reactions can take place after excess intake of these compounds with differences in individual susceptibility to intoxication by biogenic amines.

The best know reactions are those caused by histamine; some histamine-induced symptoms include rash, edema, headaches, hypotension, vomiting, palpitations, diarrhea and heart problems. Tyramine can causes hypertension and other symptoms associated with vasoconstriction caused by the release of noradrenaline. Putrescine and cadaverine are not themselves toxic, but can have negative effect on the food aroma, giving them flavors of putrefaction or rotting flesh, respectively. Furthermore,

they can interfere in detoxification reactions and increase the toxicity of histamine, tyramine and phenylethylamine.
Tyramine, spermidine, spermine, putrescine and cadaverine are possible precursors of carcinogenic nitrosamines, especially in meat products to which nitrate and nitrite salts are added as curing agents (Önal, 2007). Due to their possible association with cancer, the formation of nitrosamines in foods is of concern to researchers, consumers, food companies, and health authorities. Nitrosamines are generally formed through reactions between secondary and tertiary amines and nitrite under certain conditions (Al Bulushi *et al.*, 2009). Primary amines such as putrescine and cadaverine have been suggested to cyclize during heating to secondary amines such as pyrrolidine and piperidine, which react with nitrite to form carcinogenic nitrosamines (Lijinsky and Epstein, 1970; Warthesen *et al.*, 1975).
Normally, the biogenic amines absorbed from food are detoxified in the human blood through conjugation or by the action of amine oxidase that catalyse deamination to produce an aldehyde, hydrogen peroxide and ammonia (Gardini *et al.*, 2005).
The toxic level of biogenic amines depends on the tolerance of the individual, on the concentration and on the efficacy of the catabolic pathway of the individual (Ten Brink *et al.*, 1990). In the case of allergenic individual or if monoamine oxidase inibitors are applied or when too high levels are consumed the detoxification process is disturbed and biogenic amine accumulate in the body. The toxicological level of amines is very difficult to establish because it depends on individual characteristics and the presence of other amines (EFSA, 2011).
The toxic dose in alcoholic beverages is considered to be between 8 and 20 mg/L for histamine, 25 and 40 mg/L for tyramine and 3 mg/L for phenylethylamine (Soufleros *et al.*, 1998); nevertheless, there aren't legal limits and some countries have drawn up their own recommendations. In fact, Switzerland and Austria reject wines which contain more than 10 mg/L of histamine, while lower top limits have been recommended in Germany (2 mg/L), Holland (3 mg/L), Belgium (5-6 mg/L) and France (8 mg/L) (Lehtonen, 1996).

2.3 Biogenic amines presence in food and beverages

Biogenic amines are present in a wide range of food products including fish products, dairy products, vegetables, fruits, nuts, chocolate, beer and wine.
Fish is more likely to form biogenic amines when decomposition occurs at harvest or in the first stages of handling on the fishing vessels, rather than, later, in the distribution chain (Staruszkiewicz

et al., 2004). Scombroid fish have most commonly been associated with incidents of histamine intoxication and the formation of histamine in scombroid and other marine fish has been attributed to microbial action rather to endogenous histidine decarboxylase activity. With the exception of salmon and swordfish, most of these fish species are rich in free histidine (Hungerford, 2010).

Biogenic amines formation is possible during processes such as brining, salting, smoking, drying, fermenting, and pickling until the product is fully shelf-stable (Visciano *et al.*, 2012). Refrigeration can be used to inhibit histamine formation during these processes (FDA, 2011). Samples of fermented fish products (fish sauce, fish paste, and shrimp paste) were analyzed for histamine content (Tsai *et al.*, 2006), which was 394, 263, and 382 mg/kg, respectively. Some fish sauce products, particularly those made from sardine and mackerel, often contain large quantities of histamine, about 1000 mg/L or greater (Tsai *et al.*, 2006; Kuda and Miyawaki, 2010).

Several juices, nectars and lemonades made from oranges, raspberries, lemons, grapefruit, mandarins, strawberries, currants and grapes contain different biogenic amines in variable concentrations, putrescine being the most important (Maxa and Brandes, 1993)

Halász *et al.* (1994) have reported high amine levels in orange juice, tomato, banana, plum and spinach leaves. In chocolate, spermidine, ethanolamine, tyramine, and 2-phenylethylamine are the most abundant biogenic amines (Mayr and Schieberle, 2012).

Santiago-Silva *et al.* (2011) investigated, simultaneously in five types of tropical Brazilian fruits, the presence of biogenic amines. Mango and guava contained only spermine, spermidine and putrescine at low levels. Agmatine was detected in pineapple, papaya and passion fruit. Pineapple and passion fruit were sources of serotonin. Passion fruit was found to be a rich source of polyamines.

In cooked and uncooked ground beef and in pork meat various biogenic amines have been identified (Németh-Szerdahelyi *et al.*, 1993). Vinci and Antonelli (2002) reported that the most abundant amines in fresh beef meat samples are tryptamine and spermine and their concentration is not affected by the storage time. Conversely, in chicken meat putrescine and cadaverine have been found to represent the most abundant amines (Nadon *et al.*, 2001).

Varying concentrations of biogenic amines were found in fermented sausages. The only amines present at significant levels in fresh meat used for fermented sausage production are spermidine and spermine, and, to a lesser extent, putrescine (Hernandez-Jover *et al.*, 1997).

The data reported by many authors (Maijala *et al.*, 1995; Eerola *et al.*, 1998; Komprda *et al.*, 2001) confirmed the key role played by the raw material quality. However, other variables (such as pH, a_w, redox potential, NaCl, etc.) can have an important effect on the production of biogenic amines in

sausages. The physiological role of amine formation by microorganisms has not yet been completely elucidated (Suzzi and Gardini, 2003).

Cheese is an ideal substrate for the production of biogenic amines by microbial decarboxylation of the corresponding amino acids. The formation and presence of amines depend on a variety of factors including the presence of substrate and microbial enzymes, temperatures, pH, salt and water content, presence of enhancing substances, and catabolism of amines.

Many studies have been undertaken to determine the amine content of cheese products. A variety of amines, such as histamine, tyramine, cadaverine, putrescine, tryptamine and phenylethylamine have been found in many types of cheeses (Stratton *et al.*, 1991; Celano *et al.*, 1992; Valsamaki *et al.*, 2000; Marino *et al.*, 2000; Gardini *et al.*, 2006).

Most wine-producing countries such as Hungary, Spain, Portugal, USA, France and Italy, have investigated the presence and concentration of biogenic amines in their wine (Glòria *et al.*, 1998; Soufleros *et al.*, 1998; Mafra *et al.*, 1999; Gerbaux and Monamy, 2000; Hajós *et al.*, 2000; Guerrini *et al.*, 2002; Herbert *et al.*, 2005).

Soufleros *et al.* (1998) investigated the presence of biogenic amines in 135 wines representing a cross-section of French wine production; the study showed that Burgundy wines contain the greatest quantity of amines (mean 47.7 mg/L), followed by Champagne wines (32.5 mg/L) and red Bordeaux (32.0 mg/L); very small amounts were found in sweet white Bordeaux and white Alsatian wines (12.5 mg/L). Moreover, histamine, tyramine and putrescine were present in much larger concentration in red wines and Champagne wines. Glòria *et al.* (1998) determined the amine concentration in 59 samples of Pinot Noir and Cabernet Sauvignon wines produced in Oregon, USA, from the 1991 and 1992 vintages.

A large number of samples (209) comprising wines and musts from different varieties, Alentejo sub-regions and vintages were studied. For the majority of the samples, the amines cadaverine, tryptamine, β-phenylethylamine and isoamylamine were found to be below the limit of detection of the used method. In general, low levels of biogenic amines were found in the musts or wines studied, especially if compared with other foodstuffs, where biogenic amines can occur in much higher concentrations (Herbert *et al.*, 2005).

The presence of biogenic amines in wines (Hárslevelü, Furmint, Muscat Ottonel) and aszu-wines from Tokaj region have been investigated. Although the concentrations of phenylethylamine and histamine increased during winemaking process for all these three varieties of wine, the amines content of the Tokaj wines was well below recommended limits for any of the biogenic amines. The composition of the biogenic amines in aszu-wines highly differed from that of the other wines and

the concentration of the amines increased in parallel with the age of the aszu-wines (Hajós *et al.*, 2000; Sass-Kiss *et al.*, 2000).

Moreno-Arribas and Polo (2008) analyzed sherry wines of the Jerez region (southern Spain). They did not detect the presence of biogenic amines in any of the young wine. By contrast, histamine tyramine, putrescine and cadaverine were detected in the sobretablas and during the biological ageing process.

Patrignani *et al.* (2012) investigated the biogenic amines in 8 different Primitivo wine (Apulia region, Italy); their results confirm that putrescine is the major amine present in wine (from 5.41 to 9.51 mg/L). Tyramine was found in five wines (1.58-10.19 mg/L) and spermidine was only sporadically detected at very low concentration in four wines, while the concentration of histamine ranged from 1.49 to 16.34 mg/L.

2.4 Biogenic amines production in winemaking

The origin and evolution of the biogenic amines in wines is still a matter of controversy. Histamine, tyramine and putrescine are major amines in wine, their concentration is low in must and after alcoholic fermentation (AF), and increases in most wines during malolactic fermentation (MLF). Other amines such as ethylamine, phenylethylamine, ethanolamine, cadaverine already present in grape must are produced and degraded during winemaking (Soufleros *et al.*, 1998; Lonvaud-Funel, 2001).

The abundance of amines is strictly related to the microflora but also to the amino acid composition of the wine after AF. At high pH, biogenic amines are always produced in high amounts. This is a consequence of an easier total growth, and of the greater bacterial diversity (Lonvaud-Funel and Joyeux, 1994). White wines, which are generally more acidic, contain lower biogenic amine concentrations than red wines (Gerbaux and Monamy, 2000).

After MLF, wine is sulfited in order to eliminate yeasts and bacteria which are no longer desirable. This should normally prevent any changes in composition due to microorganisms. However, several compounds vary in their level and this is the case with biogenic amines. In Burgundy wines, histamine, tyramine and putrescine have been shown to increase in Chardonnay and Pinot noir during MLF, and also during ageing (Gerbaux and Monamy, 2000).

Has been demonstrated that the level of biogenic amines in wine depends, on the one hand, on metabolism of the microorganisms, and on the vintage, grape variety, storage of the grapes and vine

nutrition on the other (Soufleros *et al.*, 1998; Lonvaud-Funel, 2001; Herbert *et al.*, 2005; Landete *et al.*, 2007; Marques *et al.*, 2008; Del Prete *et al.*, 2009; Cecchini and Morassut, 2010).

2.4.1 Yeast

While some authors have not given much importance to AF in the formation of biogenic amines, the species of yeast that intervene in this fermentation could be responsible for the presence of biogenic amines in wine (Ancín-Azpilicueta *et al.*, 2008). Few studies have been conducted on the formation of biogenic amines by yeast and it was showed that small amounts of histamine are produced during AF (Somavilla *et al.*, 1986).

Torrea and Ancín (2002) compared different species of *Saccharomyces cerevisiae* and quantified some amines produced during winemaking; the authors assumed that yeast formed non-volatile amines during fermentation, probably due to consumption, during AF, of the precursor amino acids of these amines. In other studies it was found a slight biogenic amines production by *S. cerevisiae* depending on the strain (Torrea-Goñi and Ancín-Azpilicueta, 2001).

Caruso *et al.* (2002) showed that histamine, tyramine, cadaverine or putrescine were produced in non-detectable or low amounts in 50 yeast strains isolated from grapes and/or wine, including *S. cerevisiae* and other non-*Saccharomyces* yeasts, whereas methylamine and agmatine were formed by all the species considered. In this study the production variability was found, and some biogenic amines were produced in a significant range inside the species, resulting in a discriminating strain characteristic. This is the case for agmatine and phenylethylamine formed in wine, with considerable variability by strains of *Kloeckera apiculata*, *Brettanomyces bruxellensis* and *Metschnikowia pulcherrima*. A wide and significant variability in ethanolamine production was exhibited by *S. cerevisiae* strains. Ethanolamine is a precursor of phosphatidylcholine, the most abundant phospholipid in the membranes of eukaryotic cells and, because of its involvement in regulation phenomena in the metabolism of phospholipids; it is probably surrendered outside in the medium by yeast (Del Prete *et al.*, 2009).

Recently have been screened 36 strains of yeasts isolated from must and wine to produce biogenic amines; no amines were produced by yeasts tested under the conditions present in the screening test in synthetic medium, grape must or wine (Landete *et al.*, 2007).

In conclusion, amines are formed during AF, even from the very beginning, since it has been observed that the apiculate yeasts possess the capacity for the formation of these substances. During AF, the formation of amines will depend, among other factors which have been previously outlined, on the strain of *S. cerevisiae* that predominates during fermentation. This would probably help to

explain the fact that different authors have found such a variability of amines formation in wine with a similar vinification process (Ancín-Azpilicueta *et al.*, 2008).

2.4.2 Lactic acid bacteria

In the formation of biogenic amines in wine an important role is attributed to the lactic acid bacteria (LAB) responsible for carrying out the MLF. In fact, many authors feel that LAB are responsible for large accumulations of these compounds in wine (Soufleros *et al.*, 1998; Gerbaux and Monamy, 2000; Leitão *et al.*, 2000; Moreno-Arribas *et al.*, 2000, 2003; Lonvaud-Funel, 2001; Guerrini *et al.*, 2002; Costantini *et al.*, 2006; Landete *et al.*, 2007).

Buteau *et al.* (1984) have shown that biogenic amines, especially histamine, decrease during MLF. Ough *et al.* (1987) studied the capacity of different LAB (*Lactobacillus*, *Oenococcus* and *Pediococcus*) to produce histamine from histidine under different fermentation conditions and they did not find significant amounts of histamine from decarboxylation of histidine neither in model solutions nor in fermented juice samples.

Some strains of LAB are unique microorganisms responsible for the histamine, tyramine and phenylethylamine concentrations in wine. Landete *et al.* (2007) propose that the LAB is not responsible for putrescine levels in wine, because this is not a frequent character of the LAB. Moreover they have demonstrated that phenylethylamine production is associated with tyramine production. This correlation could be explained by the observation that phenylalanine is also a substrate for tyrosine decarboxylase, producing phenylethylamine in a secondary reaction, as demonstrated by Boeker and Snell (1972). It has been demonstrated that the ability to form tyramine and phenylethylamine is not widespread among wine LAB.

This variability in the results could be explained by the fact that the LAB of wine have a different capacity for producing amines, and this capacity is strain dependent, rather than being related to specific species (Coton *et al.*, 1998; Bover-Cid and Holzapfel, 1999; Leitão *et al.*, 2000).

Lonvaud-Funel and Joyeux (1994) isolated a strain of *O. oeni* able to produce histamine via histidine decarboxylase, from a wine of the Bordeaux area. Coton *et al.* (1998) purified and characterized this enzyme and the cloning and sequencing of the corresponding gene were also carried out.

The physiological function of amino acid decarboxylative pathways in bacteria is still not fully clear. It seems that for the LAB the accumulation of amines is a mechanism of protection against the acid media (Lonvaud-Funel 2001; Schelp *et al.*, 2001; van de Guchte *et al.*, 2002) and/or of obtaining metabolic energy through coupling amino acid decarboxylation with electrogenic amino

acid/amine antiporters (Molenaar *et al.*, 1993; Konnings *et al.*, 1997; Abe *et al.*, 2002). Furthermore, the production of polyamines such as putrescine could intervene in other physiological functions in bacteria such as osmotic stress (Schiller *et al.*, 2000) and oxidative stress (Tkachenko *et al.*, 2001) responses, and also bacterial cell cross-talk (swarming) (Sturgill and Rather, 2004). Pessione *et al.* (2005) seem to indicate that the biosynthesis of the amino acid decarboxylase enzymes could take place when the bacterial population are at the exponential growth stage and at the end of the growth stage.

Landete *et al.* (2005) screened 136 strains of LAB, belonging to different species (*Lactobacillus*, *Leuconostoc*, *Oenococcus*, and *Pediococcus*), for the presence of the *hdc* (histidine decarboxylase) gene and their ability to form histamine in a synthetic medium.

Other authors have also shown that histamine-producing strains of *O. oeni* are very frequent in wine (Guerrini *et al.*, 2002). However, in different studies no potential to form biogenic amines was observed in different *O. oeni* strains (Moreno-Arribas et al., 2003; Constantini *et al.*, 2006).

Constantini *et al.* (2006), after examining 133 strains of LAB, only found a strain of *L. hilgardii* with the *hdc* gene responsible for the histidine decarboxylase activity. Moreno-Arribas *et al.* (2003) also found histidine decarboxylase activity in *Lactobacillus* 30a.

With respect to the production of tyramine in wine, Moreno-Arribas *et al.* (2000) studied the formation of this amine by different LAB isolated from different samples of wine that had undergone MLF. They concluded that *Lactobacillus* could be the main LAB responsible for the formation of tyramine in wine, since their results showed that some strains of *L. hilgardii* and *L. brevis* were strong tyramine producers. In a later study, Moreno-Arribas and Lonvaud-Funel (2001) purified and characterized the tyrosine decarboxylase enzyme from *L. brevis* IOEB 9809.

Some authors indicate that the production of tyramine is not very extended within the *O. oeni* species (Guerrini *et al.*, 2002; Constantini *et al.*, 2006).

Putrescine, the biosynthetic precursor of polyamines, is formed by the decarboxylation of either ornithine or arginine into agmatine, which is then converted into putrescine either directly or indirectly via N-carbamoylputrescine (Tabor and Tabor, 1985). The ornithine decarboxylase activity in LAB has been described in *Lactobacillus* 30a (Gale, 1946; Tabor and Tabor, 1985).

Recently, Marcobal *et al.* (2004) isolated a putrescine producer strain of *O. oeni* from the lees of a Spanish wine, and sequenced its *odc* gene, responsible for the ornithine decarboxylase activity. The high concentration of putrescine observed in some wines after MLF cannot come only from the decarboxylation of the free ornithine in wine, since the levels of this amino acid in this product is usually low. These high concentrations of putrescine in wine could be due to some LAB showing a capacity for degrading arginine, one of the majority amino acids both in must as well as wine, to

ornithine (Ancín-Azpilicueta *et al.*, 2008). Arginine can be catabolized via the arginine deiminase (ADI) pathway, consisting of three enzymes, arginine deiminase (ADI), ornithine transcarbamoylase (OTC) and carbamate kinase (CK) (Liu *et al.*, 1996).
However, Guerrini *et al.* (2002) showed that the arginine degradation and ornithine decarboxylation do not necessarily co-exist in putrescine producer *O. oeni* strains. More recently, Mangani *et al.* (2005) showed that *O. oeni* can produce putrescine in wine through a metabiotic association, with an interchange of ornithine, between strains capable of metabolizing arginine to ornithine, but unable to form putrescine, and strains capable of producing this amine from ornithine, but unable to degrade arginine.
From these studies it may be concluded that the control of MLF is one of the most important measures to take in order to avoid important accumulations of biogenic amines in wine.

2.4.3 Factors affecting biogenic amines formation in winemaking

It was shown that there is a relationship between some amines and important factors such as variety, sub-region, vintage, safety of the grapes and oenological parameters (Herbert *et al.*, 2005; Marcobal *et al.*, 2006; Marques *et al.*, 2008; Del Prete *et al.*, 2009; Cecchini and Morassut, 2010).
Biogenic amines are formed through the action of decarboxylase enzymes produced by microorganisms (ten Brink *et al.*, 1990) from the corresponding amino acid precursors. So, as amino acids are the precursors of amines, the content of free amino acids in the must could be related to the quantity of amines in wine. Herbert *et al.* (2005) observed that the variety with the highest final concentration of biogenic amines were also the most prominent in assimilable amino acids levels.
The concentration of amino acids in must could also be modified by the addition of nutrients to the medium, to avoid fermentation problems. In this way, some authors have studied the possible relationship between the distinct factors which affect the concentration of nitrogen amino acid precursors in the must and the production of amines in the wine. Bertrand *et al.* (1991) found that the nitrogenous fertilization of vineyards of the Merlot variety produced an increase in the nitrogenous compounds of the grape, as well as in the concentration of histamine, putrescine, cadaverine and phenylethylamine in wine. Soufleros *et al.* (1998) found that, during MLF carried out by indigenous LAB, amino acid concentrations declined significantly while biogenic amines increased.
However, the data of the correlation between the presence of biogenic amines and amino acids in real vinification are contradictory; Ancín *et al.* (2004), using Garnacha have found no relationship

between the content of biogenic amines in wine and the utilization of their precursor amino acids during fermentation.

Some amines, such as ethanolamine, ethylamine, putrescine and other polyamines, may already be present in grape berries (Hálasz *et al.*, 1994; Bover-Cid *et al.*, 2006; Del Prete *et al.*, 2009). Potassium deficiencies in the soil have been linked to a rise in putrescine content in plants, while water deficiencies do not seem to influence the content of biogenic amines in grape berries and wines (Bover-Cid *et al.*, 2006). Del Prete *et al.* (2009) showed that the putrescine initially present in must strongly decrease during AF, and supposed that this could be due to the fact that putrescine is a polyamine and yeasts incorporate it in their metabolism.

Hajós *et al.* (2000) reported that, in the case of grapevine, biotic stress such as the one produced by *Botrytis cinerea* can also alter the composition of grape berries, increasing the amine content.

The obtained results from anti-fungi treatment assays clearly show that fungal metabolic activity could have some influence in biogenic amines formation; in a recently study Marques *et al.* (2008) showed that the control wines made with grapes not treated with anti-fungi products present higher content of amines than wines obtained from treated grapes.

The differences in biogenic amines in wines from different years can be due to the diversity of wine microorganisms that are naturally differently selected each year, probably due to climatic conditions (Marques *et al.*, 2008). Moreover, biogenic amines content in musts is the variable most affected by the sub-region factor (Herbert *et al.*, 2005).

A recent study shows that the grape storage time before crushing affects the amines content in must. The results show that biogenic amines in must are not necessarily those present in the grape, but the same grapes may give musts with different content of amines, depending on sanitary conditions in the winery and of the grape time before being crushed and pressed. Moreover, the grape cultivar play a significant role in the initial content of biogenic amines in grape must, but the interaction between grapes and microorganisms present in the winery cause an uncontrolled increase of these amines (Cecchini and Morassut, 2010).

Martín-Alvarez *et al.* (2006) studied the influence of certain technological practices on biogenic amines content in red wines. This study showed that a longer time period of skin maceration increased the formation of histamine, tyramine, and putrescine and that the wine aging on lees mainly increased the concentration of putrescine and methylamine.

Lees can be responsible for the presence in wines of amino acids, decarboxylase-positive microorganisms and decarboxylase enzymes (which can be released during yeast lees autolysis), which, under favourable environmental conditions, can lead to biogenic amines formation (Pérez-Serradilla and Luque de Castro, 2008).

Two recent studies have been focused on this subject. González-Marco and Ancín-Azpilicueta (2006) studied a restricted number of white wines, meanwhile Martín-Álvarez *et al.* (2006) used 224 red wines for their research. The main conclusion in both cases was that the overall concentration of biogenic amines in wines matured with lees was higher than in those elaborated without lees.

Nevertheless, concerning the behaviour of histamine and tyramine the data are contradictory: Martín-Álvarez *et al.* (2006) observed a decrease in these amines after wine lees contact, meanwhile González-Marco and Ancín-Azpilicueta (2006) found a significant increase in the concentration of these amines in wines aged in the presence of lees subjected to stirring.

Another important oenological parameter is the pH. Indeed, as pH increases, the number and variety of microbial population increase, because pH acts as a selective factor of microorganisms in wine. At high pH, biogenic amines are always produced in high amounts (Lonvaud-Funel and Joyeux, 1994). This is a consequence of an easier total growth, and of the greater bacterial diversity (Lonvaud-Funel, 2001).

After MLF, wine is sulfited in order to eliminate yeasts and bacteria which are no longer desirable. Vidal-Carou *et al.* (1990) studied the relationship between the concentration of histamine and tyramine in wine with the level of SO_2 and volatile acidity. These authors found that exist a correlation (99.9%) between total sulfur dioxide level and biogenic amines in red wines; the highest amine contents were found in wines with low total sulfur dioxide level. They also found a correlation (99.9%) between histamine and tyramine contents and volatile acidity in white and rosé wines. However, Bauza *et al.* (1995) found that using SO_2 (20-30 mg/L) to stabilize wine before bottling is insufficient to prevent the formation of biogenic amines.

Gardini *et al.* (2005) observed that an increase of the SO_2 concentration resulted in a reduction of spermine and spermidine, but this relationship was more complex in the case of the formation of tyramine, the SO_2 effect on tyramine accumulation depending also on other variables as pH values.

The influence of other wine compounds such as malic acid, citric acid, ethanol and sugar has also been studied. Rollan *et al.* (1995) found that high ethanol (12% v/v), L-lactic acid, and citric acid concentrations reduced the histidine decarboxylase activity of cell suspension of an *O. oeni* strain (*Leuconostos oenos* 9204).

Lonvaud-Funel and Joyeux (1994) showed that in the poorest growth conditions (without glucose and malic acid) the production of histamine was enhanced while Moreno-Arribas *et al.* (2000) found that tyramine formation was enhanced in a rich medium (with glucose), so that it would seem that the formation of these amines does not have the same metabolic role for microorganisms.

The effect of these factors will be different. On the one hand, they could favour the growth of microorganisms with high aminogenic activity and on the other hand they could increase the total content of amino acids in must. It should also be pointed out that, once the amines have been formed, their elimination would be difficult without changing the wine composition (Ancín-Azpilicueta *et al.*, 2008).

2.5 Prevention and reduction of biogenic amines in wine

Generally, most studies in the literature agree that there are slight variations in biogenic amine concentrations, corresponding to a slight decrease or stabilization of these compounds during wine storage. Unfortunately, the concentration of biogenic amines in wines is still a problem for many wine cellars because avoid the presence of these compounds in wine results is very complex, in particular for the amines naturally present on the grapes. Nevertheless, many studies have been carried out to reduce the biogenic amines in wine, in particular testing selected commercial starter of LAB able to made MLF (Marques *et al.*, 2008; Costantini *et al.*, 2009).

Marques *et al.* (2008) demonstrated that the application of commercial malolactic starters in wines was useful to reduce the biogenic amines amounts; in their study, in the inoculated wines amines concentrations were significantly lower when compared with those not inoculated. The authors supposed that the MLF conducted by indigenous malolactic bacteria was able to produce biogenic amines

Costantini *et al.* (2009) investigated if contaminating microorganisms, eventually present in bacteria and yeast preparations used as commercial starter in winemaking, have the ability to produce the biogenic amines. Regarding the commercial strains, none of them were able to produce biogenic amines. The study demonstrated that the risk of biogenic amines production exists, and it depends on the contaminating bacteria species; *L. brevis* can survive during the fermentation and can produce amines.

Recently, some studies on bacteria inoculation for MLF simultaneously with AF, carried out to reduce the development of biogenic amines in wine, were compared to the traditional sequential inoculation. This could be a tool to limit biogenic amines contamination. The inoculated culture may be able to dominate and inhibit the growth of the natural LAB flora and thus the chances of unwanted activities by these bacteria are lessened during the course of AF (Smit *et al.*, 2012).

However, the data present in the literature are few and contradictory. Massera *et al.* (2009) investigated the effect of simultaneous and sequential inoculation on five amines (histamine,

tyramine, phenylethylamine, putrescine and cadaverine) levels; for this test two commercial active dried *S. cerevisiae* strains and one dried commercial strain *O. oeni* were used. The authors not observed significant differences in the amine levels between the timing of bacteria inoculation.

Smit *et al.* (2012) observed a decrease in the amount of putrescine and cadaverine, while the concentrations of histamine and tyramine were not affected to the inoculum time; significant tyramine formation was observed in the samples inoculated with one strain of *L. hilgardii*.

A recently method to reduce the concentration of biogenic amines regards the enzymatic degradation of amines by microorganisms. The research on amine degrading enzymes, such as amino oxidases, for food industrial application might have useful applications for wines. Cueva *et al.* (2012) demonstrated the ability of vineyard ecosystem fungi to reduce the biogenic amines content in assay broth as well as in wines. It is thought that amine oxidases allow the fungi to degrade an amine as a source of ammonium for growth; however, the role of these enzymes has not always been well defined (Frébort *et al.*, 2000). García-Ruiz *et al.* (2011) reported novel data about the presence of histamine-, tyrosine- and putrescine-degrading enzymatic activities of wine-associated LAB. This potential for histamine, tyramine and/or putrescine degradation among wine LAB does not appear to be very frequent, since out of the 85 strains examined, only nine displayed noteworthy amine-degrading activity in culture media. The authors claim that, although the amine-degrading ability of the LAB seemed to be good at pH values close to wine pH, wine components such as ethanol and polyphenols as well as wine additives such as SO_2 might limit this ability.

Capozzi *et al.* (2012) selected two *L. plantarum* strains, from a pool of *L. plantarum* strains isolated from red wine undergoing MLF, able to degrade biogenic amines such as putrescine and tyramine. The degradation rates observed was higher than that previously reported for *L. plantarum* strains (Leuschner *et al.*, 1998; García-Ruiz *et al.*, 2011). The authors observed that, although MLF was incompletely performed, the two biotypes showed a respectable aptitude to degrade malic acid, indicating a possible application in reason of wine/must malic acid content.

Therefore, a potential application of amine-degrading strains to prevent the accumulation of biogenic amines in wine could consist in their use as starters to be inoculated or as enzymatic preparations to be added to the contaminated wines.

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PhD thesis project

Development of the research project

The research managed in this PhD is focused on the study of the starter for malolactic fermentation (MLF). Among the lactic acid bacteria used in MLF we considered the *Lactobacillus* spp. and in particular a strain of *L. plantarum* that we have isolated from wine. At present this strain is commercialized by company Lallemand Inc. (Montréal, QC, Canada) as *L. plantarum* V22.

In the last decade the interest for the population of *Lactobacillus* isolated from wine is increased to expand the availability of lactic acid bacteria, in particular strains for higher pH (3.5÷3.8). In fact, at present the wine are less acidic that in the past. Grape maturity is prolonged as far possible to increase the extractability of phenolic compounds and the concentration of aroma precursors. In addition, the change in climatic condition has changed the balance of sugars and organic acids. Therefore, total acidity is lower and pH higher. In this situation it is necessary to understand the actions of lactic acid bacteria present in wine, especially for the strains whose use will probably become more frequent in the future.

In detail this project was carried out along three lines of investigation that have the common goal to determine the best conditions for *Lactobacillus* spp. use in the ; MLF and to minimize the occurrence of OTA and biogenic amines in wine. For this purpose were considered both indigenous bacteria and selected strains. In regard to indigenous bacteria the study has focused on the identification of strains capable of developing the MLF without the ability to produce biogenic amines. Concerning to selected starter for MLF we have investigated the ability of a strain of *L. plantarum* to remove OTA as well as the technological conditions to minimize the presence of biogenic amines.

Working lines are:

- OTA reduction by *L. plantarum*;
- bacteria strains screening able to perform MLF without produce biogenic amines;
- biogenic amines in wine related to *L. plantarum* inoculation time.

Chapter 3 - Ochratoxin A reduction by *Lactobacillus plantarum*

3.1 Introduction

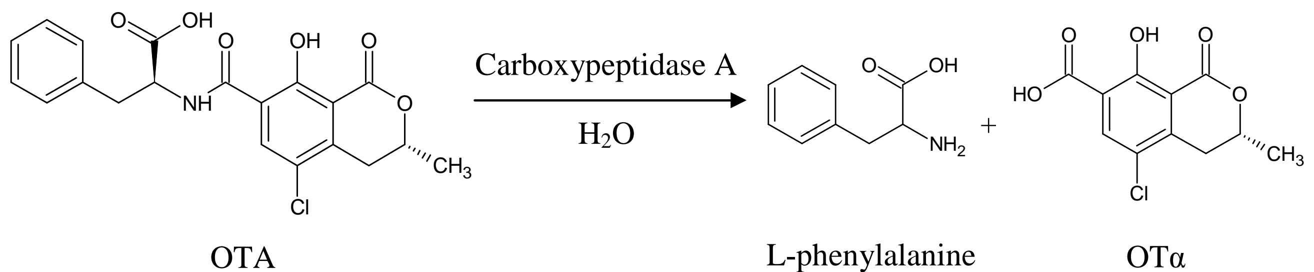
Ochratoxin A (OTA), N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzo-pyran-7-yl) carbonyl]-(R)-L-phenylalanine, is a mycotoxin produced by several species of the genus *Aspergillus* and *Penicillium* (Battilani *et al.*, 2001). OTA is a well-known nephrotoxin in various species, and it was classified by IARC as possible human carcinogen (IARC, 2003). Long-term exposure to OTA has been implicated in Balkan Endemic Nephropathy and associated with urinary tract tumours, because of rather high OTA levels detected in food samples and in blood or urine from affected persons.

OTA is a secondary metabolite of moulds and is the most common contaminating agent of food raw materials and products. The presence of OTA in grape juices and wines was reported for the first time in 1996 by Zimmerli and Dick.; since the vintage of 2005, with the adoption of Regulation EC 123/05, the level of OTA in commercial wines cannot exceed 2 μg/kg.

As previously reported in this thesis work, several approaches have been tested to reduce the occurrence of OTA in grape and to remove the toxin during winemaking. Prevention strategies to control OTA in the vineyard include the use of biocontrol agents, fungicides and the good agricultural practices. However they cannot completely prevent the OTA problem and severe contamination of wine can occur.

During red winemaking process the OTA concentration increase in maceration probably caused by prolonged contact of skins and must, but the balance of OTA in wine during winemaking is negative because operations as clarification and filtration, and processes as malolactic fermentation (MLF) cause a relevant decrease of this mycotoxin (Grazioli *et al.*, 2006). The use of bacterial strains for OTA reduction in winemaking requires further studies, because the available literature is limited. Many studies were carried out on adsorption process by yeasts during winemaking (Raju and Davegowda, 2000; Huwig *et al.*, 2001; Bejaoui *et al.*, 2004; Shetty and Jespersen, 2006; Caridi, 2007; Nunez *et al.*, 2008); while the degradation process by lactic acid bacteria (LAB) and its mechanism have been little investigated (Piotrowska and Żakowska, 2005; Fumi *et al.*, 2008).

The ochratoxin α (OTα) is a no-toxic metabolite resulting from the OTA hydrolysis in the isocoumarinic part (OTα) and L-phenylalanine; if this metabolite is found in wine, an enzymatic degradation caused by the LAB during MLF could be supported.
Some enzymes are able to degrade OTA; in particular carboxypeptidase A from bovine pancreas hydrolyses OTA in OTα (Pitout, 1969).



Abrunhosa *et al.* (2006) screened 14 commercial enzymes, among them, Protease A, Prolyve PAC and pancreatin showed OTA hydrolytic activity. In particular Protease A, an acid protease from *Aspergillus niger*, had higher degradation capacity of all commercial enzymes tested. This enzyme converted 87.3% of total OTA in OTα after 25 h.
The fungi of the genera *Aspergillus* are able to degrade OTA, in particular black aspergilli, *A. clavatus*, *A ochraceus*, *A. versicolor* and *A. wentii*; Abrunhosa *et al.* (2002) suggested that the enzyme involved in this reaction was a carboxypeptidase and the product of this degradation was OTα.
Recently, an OTA hydrolytic enzyme was isolated from *A. niger* presenting its maximum activity at pH 7.5 and 37°C; this enzyme is probably a metalloenzyme, as carboxypeptidase A is, since it is strongly inhibited by the metal-ion chelator ethylenediaminetetraacetic acid (EDTA) and it is insensitive to phenylmethanesulfonylfluoride (PMSF), a serine protease inhibitor (Abrunhosa and Venâncio, 2007).
In the last years the OTA-degradation activity were studied in some bacteria. Hwang and Draughon, (1994) showed that *Acinetobacter calcoaceticus* were able to degrade OTA in ethanol-minimal salts medium with an initial OTA concentration of 10 μg/mL both at 25 and 30°C; the end product of OTA degradation was OTα.
Recently, Rodriguez *et al.* (2011) investigated the capability to hydrolyze OTA in some *Brevibacterium* species; in their study different strains of *B. casei*, *B. linens*, *B. iodinum* and *B. epidermidis* were able to completely degrade OTA in OTα. The reduction/degradation of OTA by LAB used in winemaking has been little investigated but the data reported in the literature do not explain the mechanism of action.

The aim of this work is to investigate the ability to remove OTA by *Lactobacillus plantarum* V22, a LAB used during winemaking as malolactic starter, in wine and in synthetic substrate. In particular the study was focused on the reduction mechanism, either adsorption process or enzymatic degradation, of OTA. In our study a preliminary experiment, using *Brevibacterium linens* DSM 20425 showing the ability to degrade OTA, was carried out to assess the presence of Otα and to set up the HPLC method to analyze OTA and OTα.

3.2 Materials and methods

3.2.1 Standards, reagents and biochemicals

Methanol, isopropanol were obtained from BDH (Poole, UK); acetic acid, dichloromethane, sodium hydrogen carbonate ($NaHCO_3$), glucose, sodium chloride, hydrogen chloride, sodium hydroxide were purchased from Carlo Erba (Milano, Italy); polyethylene glycol 8000 (PEG) was obtained from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile was obtained from Merck (Darmstadt, Germany). Solvent used as mobile phase were HPLC and LC-MS grade, respectively. The OTA and OTα standards were purchased from Biopure (Waterlooville, UK). MRS (Man-Rogosa-Sharpe), tryptone and yeast extract were purchased from Oxoid (Basingstoke, UK), Agar was obtained from Bacto^{TM} (Sparks, MD, USA) and YNB (Yeast Nitrogen Base) without Amino Acids and Ammonium Sulfate was purchased from Difco^{TM} (Sparks, MD, USA).

3.2.2 Bacterial origin and growth conditions

The *Brevibacterium linens* DSM 20425 was routinely grown in Luria-Bertani (20 g/L NaCl; 5 g/L yeast extract; 10 g/L tryptone; 5 g/L glucose; pH 7-7.4) broth supplemented with 0.5% glucose and incubated at 25°C under aerobic conditions (Rodriguez *et al.*, 2011). Before the inoculation the cells were recovered by centrifugation for 15 min at 6000xg at 4°C and rinsed twice with the media used in the trials (BSM or YNB). The *B. linens* was kindly provided by Dr. Emilia Garcia-Moruno from the CRA-Centro di Ricerca per l’Enologia, Italy.

The *L. plantarum* V22 was grown in MRS broth for 48 h at 25°C under aerobic conditions. After incubation, the cells were harvested by centrifugation at 6000xg for 15 min at 4°C, washed with sterilized physiological solution and suspended in YNB/wine to yield an initial population in the media of 10^9 cfu/mL. The strain of *L. plantarum* used in this work was originally isolated from wine by Piacenza area.

3.2.3 Ochratoxin A reduction assay in wine

The preliminary investigation on the OTA reduction by LAB was carried out in wine. OTA has been added to three aliquots of 200 mL in red wine in order to obtain three different final concentrations of OTA: 0.2, 1 and 20 µg/L; 10^9 cfu/mL of *L. plantarum* V22 have been inoculated in these three aliquots. The analysis of OTA has been performed after 5, 12, 26, and 30 days. For each test analyses were carried out in duplicate. The red wine presented the following parameters: titratable acidity 6.78 g/L, pH 3.5, reducing sugars 1.44 g/L and alcohol 12.39 v/v%.

3.2.4 Ochratoxin A degradation by B. linens

To confirm the capability of *B. linens* to degrade OTA in OTα, BSM media with and without glycerol was used as reported by Rodriguez *et al*. (2011). *B. linens* was also tested in YNB media pH 6.7 and in YNB adjusted at pH 4.7 using phosphoric acid 1 M, to evaluate the influence of media composition on the OTA degradation ability. In this assays *B. linens* was inoculated in 30 mL of media with (20 µg/L) and without OTA and grown in aerobic conditions at 25°C. For the trials were prepared 4 tubes with OTA and 4 tubes without OTA for each media as control.

The OTA and OTα analysis were conducted at the inoculation time and after 1, 2 and 3 days. The culture supernatants were separated by centrifugation at 6000xg for 15 min at 4°C and further analyzed by HPLC. The supernatant was filtered (0.45 µm) and injected.

3.2.5 Ochratoxin A removal assay in YNB

The *L. plantarum* V22 was used as model to investigate the mechanisms of OTA reduction. We have conducted the test to assess the activity of *L. plantarum* V22 in a substrate poor to nitrogen and carbon source. The media was Yeast Nitrogen Base (YNB) without Amino Acids and Ammonium Sulfate (Difco) 6.7 g/L in water, the pH was adjusted at 3.8 using phosphoric acid 1 M. The solution was splitted in two parts and in one L-malic acid at the final concentration of 2 g/L was added. The two theses were labelled L-malic- and L-malic+ and were carried out at 25°C. For both (L-malic-; L-malic+) were prepared 10 tube with 30 mL of media with OTA and 5 tube without OTA as negative control.

10^9 cfu/mL of bacteria have been added to 30 mL of YNB having a concentration of OTA equal to 25 µg/L; it was fixed this concentration to better detect the OTα production by hydrolysis. The medium used for this assay was prepared by diluting 400 times the OTA working solution (10 mg/L).

The analysis has been conducted at the inoculation time and after 3, 6, 10, 17, 24 days. We controlled the bacterial viability at the inoculation and after 10 and 20 days evaluating the cfu per mL of media.

In theses, to assess the trend of bacterial cells concentration in the media, optical density (O.D.) was carried out at 630 nm, after shaking the samples, using a spectrophotometer UV-1601 UV-visible Shimadzu (Kyoto, Japan).

The HPLC analysis of OTA and OTα was carried out in supernatant and in pellet extract. The biomass and supernatant were separated by centrifugation at 6000xg for 15 min at 4°C. The fresh weight of pellet was obtained by weighing the biomass of each sample after drying at 20±2°C for three hours. The bacterial pellet was suspended twice in 2 mL of absolute methanol for 1 h to extract the OTA. After centrifugation at 6000xg for 15 min at 4°C, the methanolic supernatants were separated, collected in 5 mL vials, and evaporated to dryness with a stream of dry nitrogen gas. The dry residue was reconstituted with the mobile phase immediately before analysis for the determination of OTA concentration. The supernatants were filtered (0.45 μm) and injected. In the supernatant the L-malic acid was quantified by Megazyme kit (Wicklow, Ireland).

3.2.6 HPLC analysis

Chromatographic analysis were performed on a HPLC system including a Perkin Elmer (Norwalk, CT, USA) 200 Series pump equipped with a Perkin-Elmer 650-10S fluorescence detector, Jasco LC-Net II/ADC (Oklahoma City, OK, USA) communication module and ChromNAV Control Center software.

In the preliminary investigation it was detected only the OTA to assess the percentage of reduction. In this trial each sample was filtered before OTA analysis according to Visconti *et al.* (1999). In brief, wine was diluted with a water solution containing PEG (1%) and $NaHCO_3$ (5%), mixed, filtered and cleaned up by Ochraprep immunoaffinity columns (R-Biopharm Rhône, Lanarkshire, UK). OTA was eluted with methanol and quantified by reversed-phase HPLC with fluorescence detection (330 nm excitation wavelengths, 470 nm emission wavelengths). Chromatographic separation was performed on a column Lichrosphere (Phenomenex, Torrance, CA, USA) 100 RP-18, 250 mm×4.6 mm, 5 µm particles fitted with a pre-column with the same stationary phase. The mobile phase consisted of a mixture of acetonitrile/water/acetic acid (49:49:2 v/v) eluted at a flow rate of 1.0 mL/min. Quantification of OTA was performed by measuring peak area at OTA retention time and comparing it with calibration curve constructed by injecting eight standard solution containing OTA in mobile phase at concentrations ranging from 0.05 to 10 µg/L.

The concentration of OTA and OTα in supernatants and pellets was quantified by a Gemini C18 column 250 mm×4.6 mm, 5 µm (Phenomenex). The injection volume was 20 µL and the analysis was performed at room temperature. The mobile phases used were: phase A water/acetic acid (98:2 v/v) and phase B acetonitrile/acetic acid (98:2 v/v) eluted at a flow rate of 1.0 mL/min. The stepwise gradient was: 0-5 min 25% B, 5-10 min 25-30% B, 10-18 min 30-52% B, 18-23 min 52% B, 23-31 min 52-75% B, 31-36 min 75-100% B, 36-41 min 100% B, 41-44 min 100-25% B, 44-54 min 25% B. The fluorescence detector was set at 333 nm excitation and 460 nm emission wavelengths. Quantification of OTA and OTα was carried out by a calibration curve, which was constructed by injecting nine standard solutions containing OTA and OTα in mobile phase at concentrations ranging from 0.1 to 40 µg/L (**Figure 3.1**).

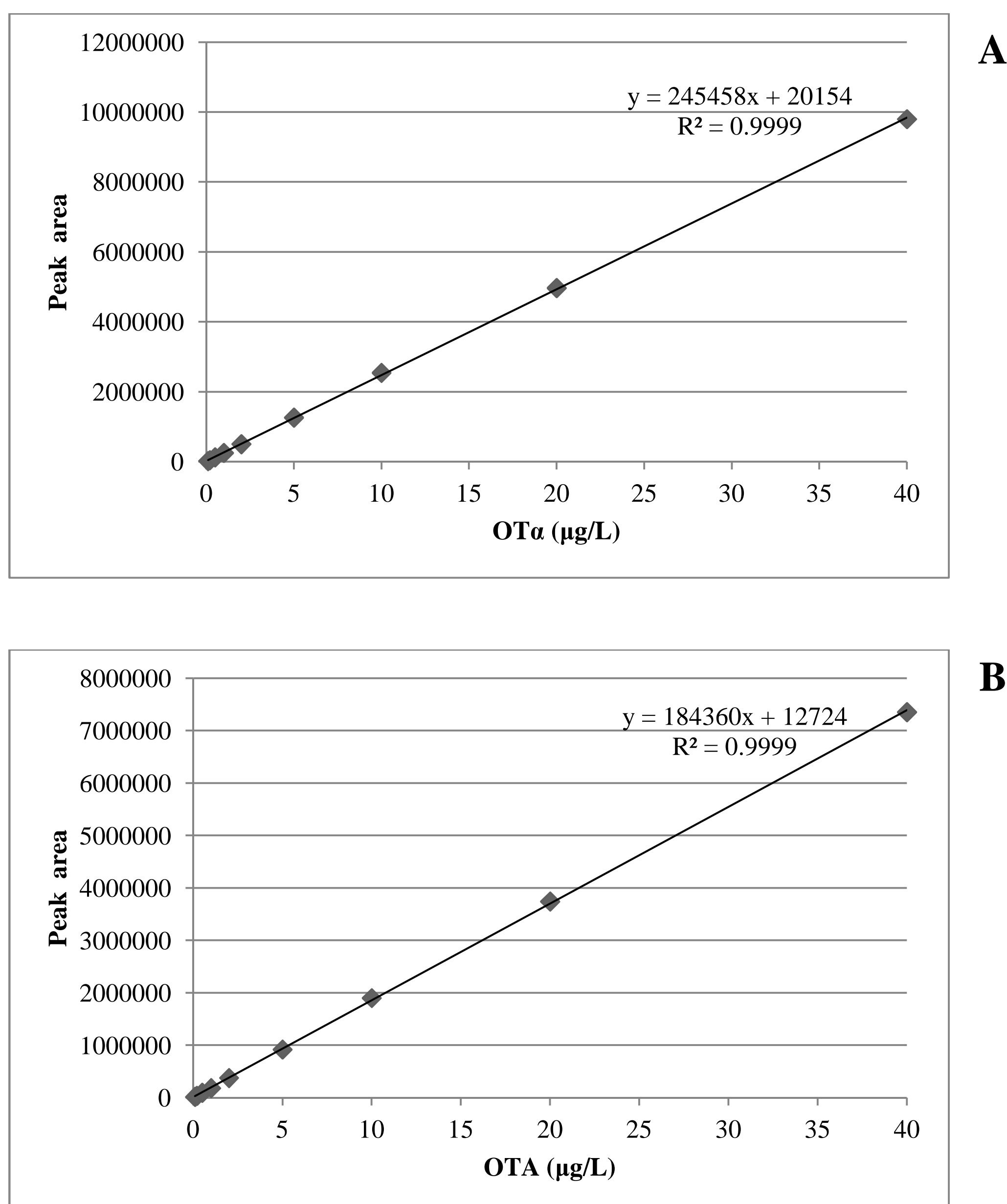


Figure 3.1 – *(A) OTα calibration curve and (B) OTA calibration curve by HPLC analysis.*

Analysis of OTA-OTα in the *Brevibacterium* degradation trials were carried out to confirm the presence of OTα using a RP-HPLC, coupled to a mass spectrometer supplied with an ion trap analyzer (Agilent 1100 LC/MSD Series, Walbronn, Germany) OTA and OTα were extracted three times with two volumes of dichloromethane, the organic extracts were pooled and evaporated; finally OTA and OTα were dissolved in HPLC mobile phase and 20 μL were used in LC-MS analysis. This work was carried out in collaboration with the Center of Excellence for Biomedical Research (CEBR) of the University of Genova.

3.2.7 Statistical analysis

The experiments were carried out on two replicates, and the analyses were performed in duplicate. IBM SPSS® 19.0 (SPSS, Chicago, IL, USA) software for Windows was used to perform statistical analyses. Differences of pellet weight at each time were evaluated by Student's *t*-test, by comparison of the results of two theses (L-malic+, L-malic-). Analysis of variance (ANOVA) followed by Tukey test were performed to evaluate the differences of O.D., pellet weight and OTA content in the samples respect to time. Significance tests were conducted at $P \leq 0.05$.

3.3 Results

3.3.1 Ochratoxin A reduction preliminary assay in wine

In the wine the reduction of OTA is related to the initial level of contamination: a decrease of 85%, 70% and 57.1% has been found after 30 days in the wines where a quantity of 0.2, 1 and 20 µg/L was respectively added (**Figure 3.2**).

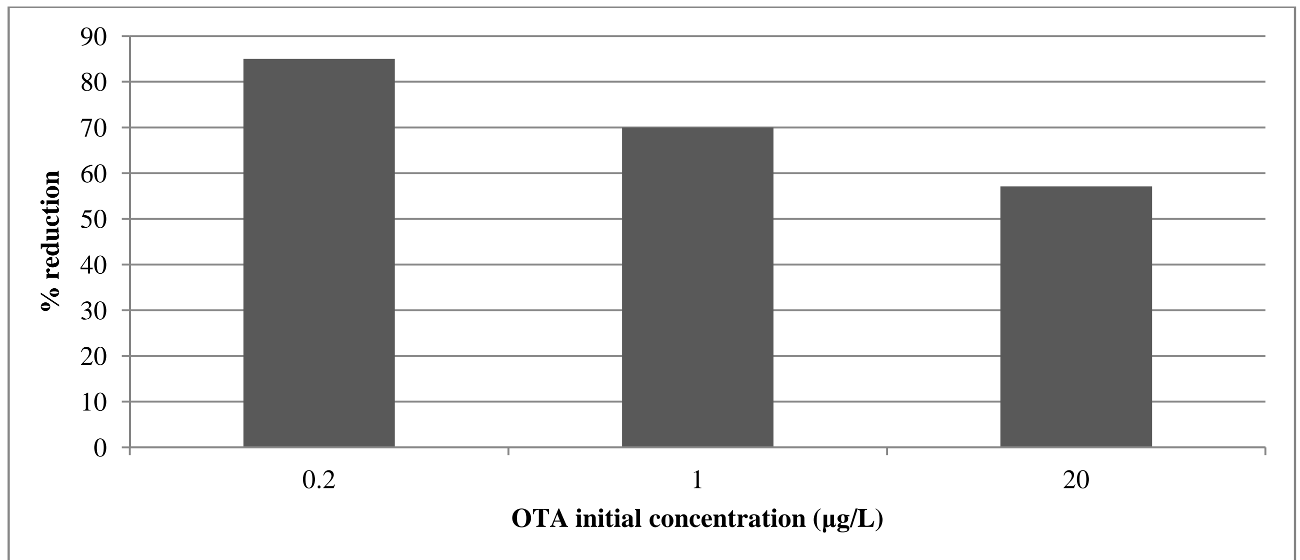


Figure 3.2 – *OTA percentage reduction in wine trial in relationship with OTA initial concentration.*

The results confirm the decontamination operated by LAB and specifically by *L. plantarum*. On the bases of these data it was performed the trial in YNB media with 25 µg/L to investigate the OTA removal mechanism, adsorption or degradation, and the metabolites formed, such as OTα.

3.3.2 Ochratoxin A degradation by B. linens

The OTA was completely degraded in OTα in 3 days both in BSM (+ and - glicerol) and in YNB media (pH 6.7 and 4.7). Analyses of the chromatograms from the supernatant showed that while the OTA peak was reduced, a new peak with a different retention time was present in the elution profile and was similar to OTα standard retention time (**Figure 3.3**). Furthermore, LC-MS confirmed the identification of OTA and OTα, since the peak showed a molecular ion $[M^{-}H]^{-}$ at m/z 402.0 and 254.7 in the MS total ion current (OTA molecular weight = 403; OTα molecular weight = 256) (**Figure 3.4**).

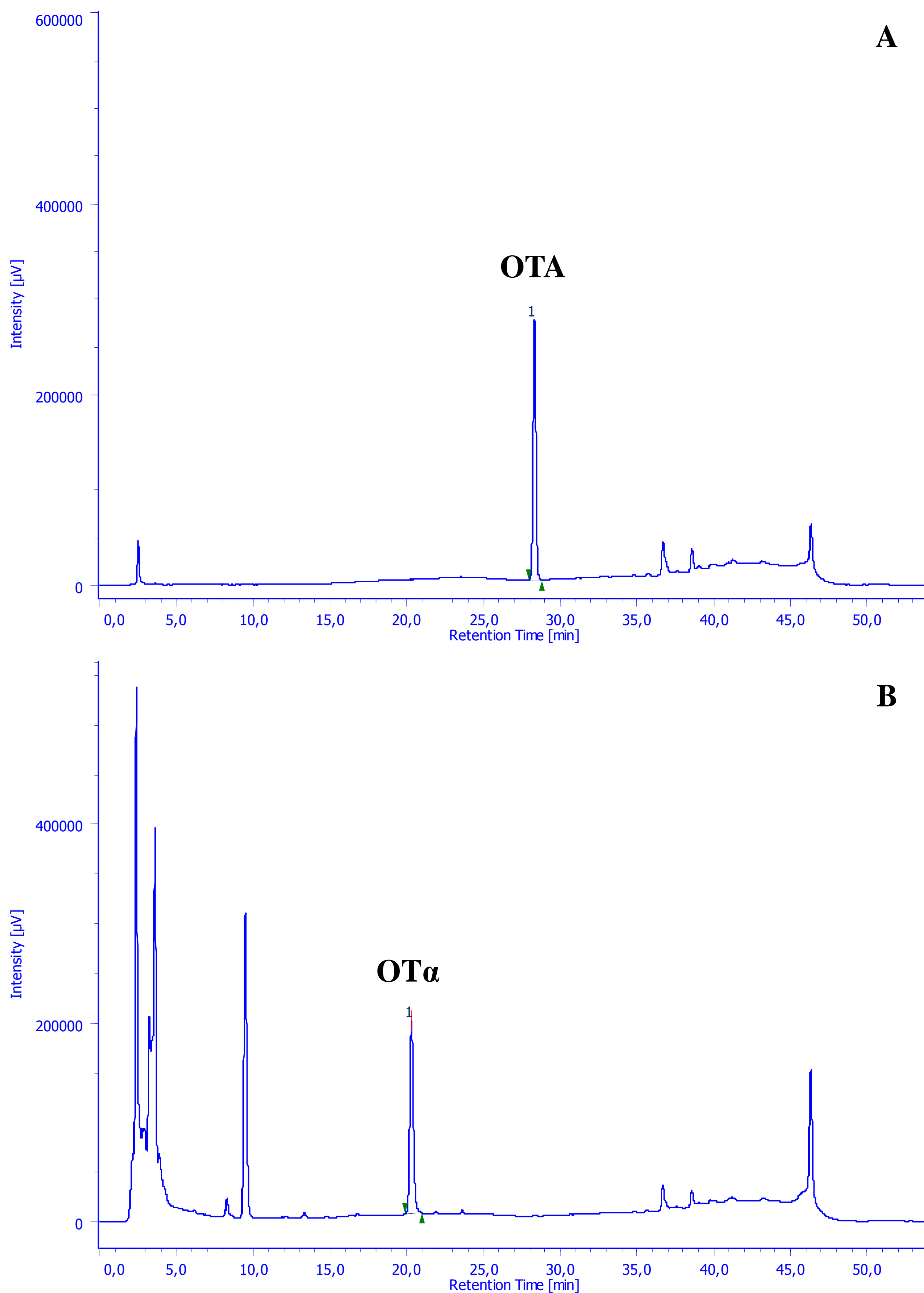


Figure 3.3 – *Chromatograms obtained from the supernatant of B. linens DSM 20425 grown in BSM – glycerol containing OTA (20 µg/L). (A) Supernatant at time 0 and (B) supernatant after 3 days of growth showing the disappearance of the OTA peak and the appearance of the OTα peak.*

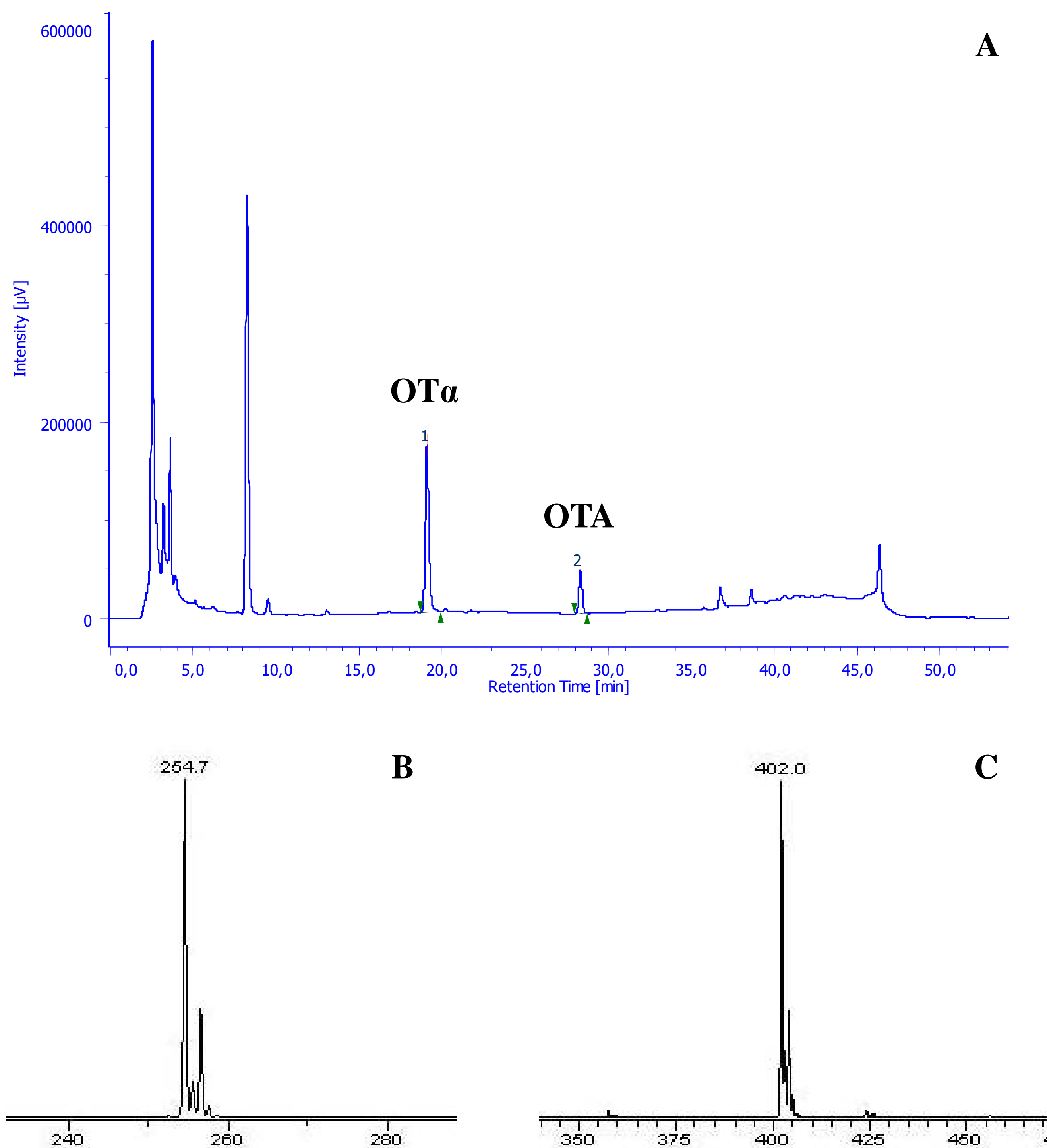


Figure 3.4 – (A) *Chromatogram obtained from the supernatant of B. linens grown in YNB pH 4.7 containing OTA (20 µg/L) after 2 days, the OTA is not completely degraded in OTα. (B) LC-MS identification of OTα. (C) LC-MS identification of OTA. Mass spectra were acquired in the negative ion mode.*

This study confirms that *B. linens* DSM 20425 is able to degrade OTA in OTα in BSM but also in YNB media. Rodriguez *et al.* (2011) suggest that these bacteria use OTA as carbon source, the same hypothesis is tested in this work using *L. plantarum* V22. Furthermore, LC-MS analysis confirms that the HPLC method developed is able to detect OTα and OTA in the same analysis.

3.3.3 Ochratoxin A removal assay by L. plantarum in YNB

The evolution of bacterial cells concentration in the media (O.D.) and pellet weight over time is shown in **table 3.1**. The bacteria concentration and the pellet weight decrease in both theses, but after the 10th day the levels in the L-malic- thesis were lower than in the L-malic+ thesis.

The data elaboration by ANOVA show that the O.D. decrease related to time is significant after 6 days and during the remaining 14 days. Regarding the pellet weight compared to time 0 there is a significant decrease after 6 days in L-malic- and 10 days in L-malic+.

Days	O.D.		mg of pellet	
	L-malic+	L-malic-	L-malic+	L-malic-
0	0.9255±0.0021a	0.9235±0.0064a	155.1±1.0a	155.1±1.0a
3	0.8025±0.0078ab	0.7895±0.0078ab	133.6±21.7ab	100.3±24.2ab
6	0.7705±0.0092bc	0.7675±0.0276bc	99.3±23.1ab	85.3±14.0b
10	0.6705±0.0403cd	0.6445±0.0290cd	82.5±2.7b	64.1±2.2b
17	0.6060±0.0693de	0.5540±0.0615d	82.8±7.5b	60.8±13.2b
24	0.4925±0.0021e	0.3195±0.0403e	78.8±13.2b	68.9±13.4b

Table 3.1 – *Data, arithmetic mean±standard deviation, of two different theses (L-malic+, L-malic-) regarding the O.D., and mg of pellet. Means followed by the same letter are not significantly different (P≤0.05).*

The changes of O.D. and pellet weight in the time are reported in **figures 3.5** and **figure 3.6**. Comparing the two curves of O.D. (**Figure 3.5**) the bacterial biomass decreases with the same kinetics until the sixth day, then the trend is different because the O.D. lowering is the higher in L-malic- thesis.

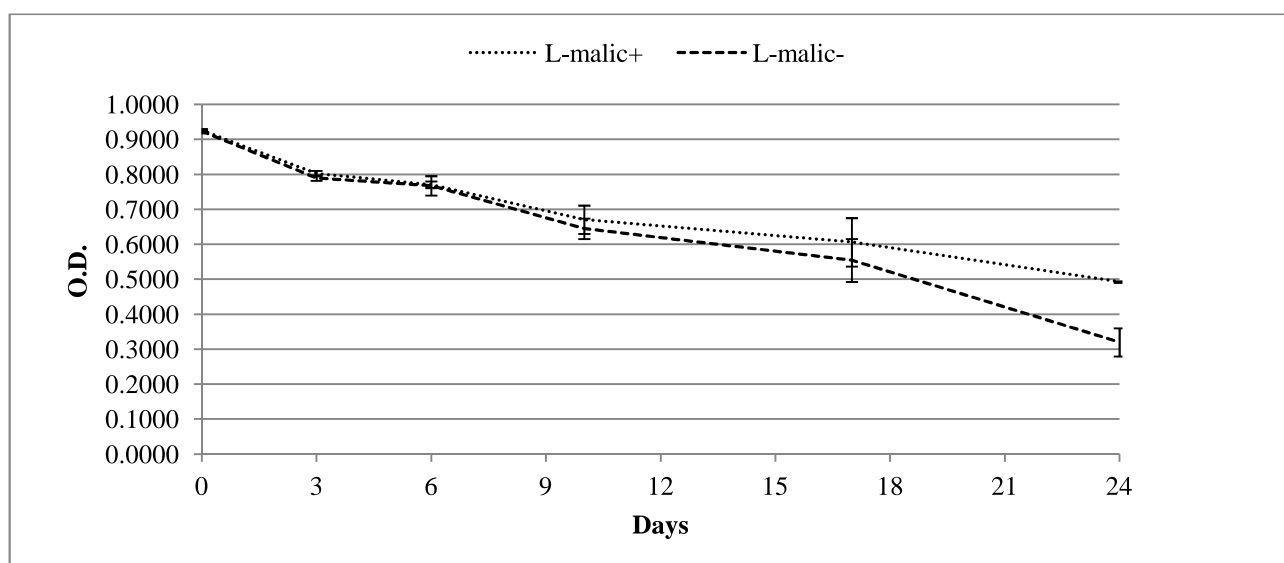


Figure 3.5 – *O.D. trend during the 24 days. Error bars denote standard deviation.*

Figure 3.6 shows the trend of the pellet fresh weight during the 24 days; the data indicate that the biomass decrease was higher in L-malic- samples that in the L-malic+ samples. In each condition the pellet weight quickly decrease until the 10th day and is stable over the next fourteen days. The *t*-test ($P \leq 0.05$) showed that the pellet average values at each time were significantly different among L-malic- and L-malic+ thesis.

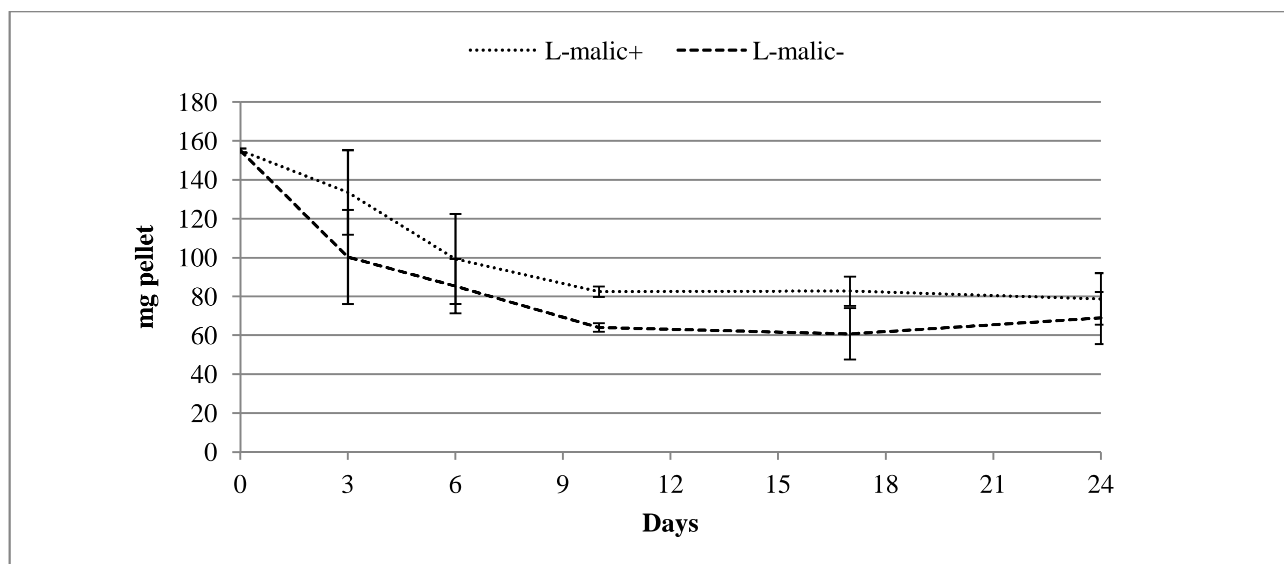


Figure 3.6 – *Trend of bacterial pellet weight during the trial. Error bars denote standard deviation.*

The control of the cell viability was carried out to check the cell condition especially in the final steps of the experiment. After 10 days in the sample L-malic+ the bacteria were more viable than in the L-malic-, this difference was the greater after 20 days (**Table 3.2**).

Days	L-malic+	L-malic-
0	1.0×10^9	1.0×10^9
10	1.3×10^6	1.2×10^3
20	5.0×10^5	7.0×10^2

Table 3.2 – *cfu/mL of L. plantarum V22 at the inoculation and after 10 and 20 days.*

The malic acid in L-malic+ thesis was almost completely degraded in three days after *L. plantarum* inoculation, subsequently the kinetics was lower. After six days the malic acid levels were very low and constant (**Figure 3.7**).

In the control thesis, without *L. plantarum* inoculation, the malic acid concentration did not change. The data of OTA in supernatants are shown in **table 3.3**. The data elaboration by ANOVA show

that in L-malic+ the OTA concentration is different compared to L-malic- and control, in fact there is OTA reduction only in L-malic+ trial.

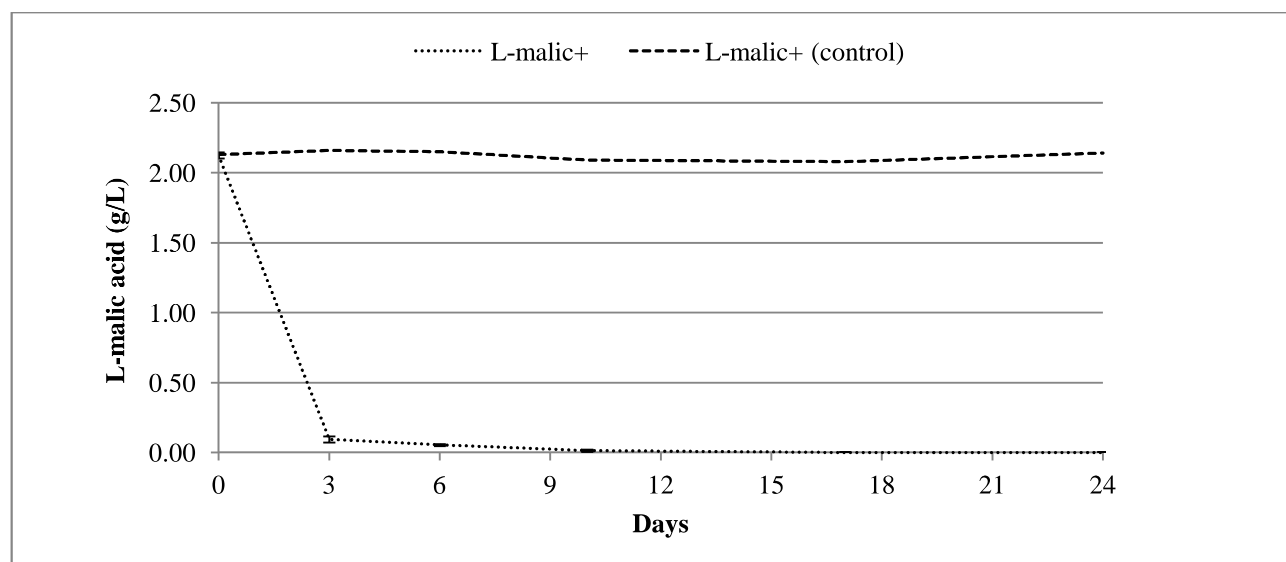


Figure 3.7 – *L-malic acid degradation trend in the thesis L-malic+ and in the control no inoculated. Error bars denote standard deviation.*

Days	µg/L OTA in supernatant		
	L-malic+	L-malic-	Control
0	23.93±1.48a	23.93±1.48a	23.93±1.48a
3	19.08±0.17b	21.67±0.73a	22.25±1.07a
6	18.82±0.24b	20.58±1.24a	23.11±0.99a
10	19.52±0.06b	22.54±2.65a	24.84±0.78a
17	17.97±0.01b	20.46±0.16a	21.37±1.11a
24	17.58±1.15b	21.09±0.01a	22.15±0.99a

Table 3.3 – *Data, arithmetic mean±standard deviation, of two different theses (L-malic+, L-malic-) regarding OTA detected in supernatant. Means followed by the same letter are not significantly different (P≤0.05). The control is the mean between control L-malic+ and control L-malic- because the data were similar.*

The analysis of the **figure 3.8** show that OTA in the L-malic+ thesis decreases in three days after inoculum and doesn't change in control and in L-malic- theses. The L-malic+ samples shown a total OTA reduction of 20.27%. The OTA levels in L-malic- samples and in the control were comparable for all the time of the test. The HPLC analysis of supernatant does not show the presence of OTα during the trial. The OTA analysis on the pellet extracts was carried out at the 17th and 24th day after *L. plantarum* inoculation, to detect the OTA bound to the bacterial cell wall. The data of OTA concentration at times 0, 17 and 24 days in L-malic+ thesis (OTA decreased and in control (OTA

unchanged) were considered to investigate the differences caused by *L. plantarum* inoculation. The OTA distribution between supernatant and pellet was investigated to perform a mass balance.

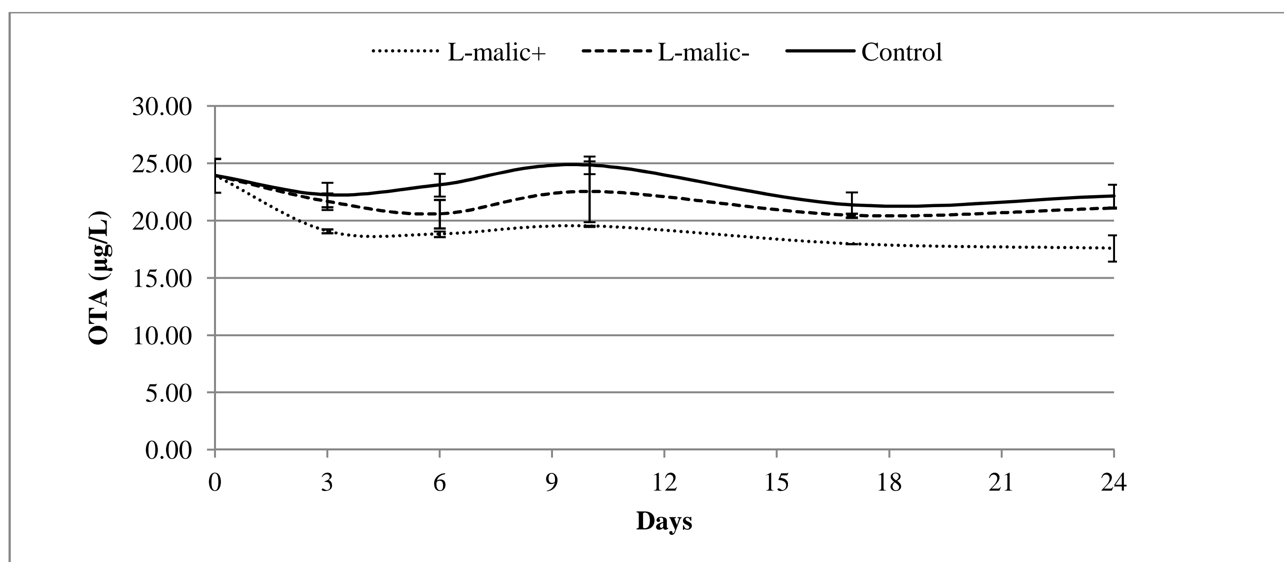


Figure 3.8 – *Trend of OTA removal in different theses and in the control no inoculated. Error bars denote standard deviation.*

The data in **table 3.4** show the highest OTA level in the supernatant. In the control the OTA variation over time is very low and it can be due at the uncertainty of the OTA analysis. The mass balance in L-malic+ thesis show a recovery of OTA lower by 15-20% compared to the control thesis. The difference from 100% could be due to *L. plantarum* both OTA binding and OTA degradation. The presence of OTA in pellet may indicate an adsorption even if the OTA levels are very low. As the mass balance is defective, we can suppose that other phenomena are involved.

Thesis	ng OTA pellet	ng OTA supernatant	ng OTA total	ng OTA difference time 0 vs. 17 and 24 days	ng OTA difference L-malic+ vs. Control
L-malic+_Time 0		686.41±0.01	686.41±0.01		
L-malic+_17 days	4.52±1.69	538.95±0.21	543.47±1.48	142.94	97.48
L-malic+_24 days	3.72±1.86	545.25±34.37	531.12±32.51	155.29	133.38
CTR_Time 0		686.41±0.01	686.41±0.01		
CTR_17 days		640.95±33.30	640.95±33.30	45.46	
CTR_24 days		664.5±29.70	664.5±29.70	21.91	

Table 3.4 – *The values are expressed in ng/supernatant volume or in ng/ pellet weight. The data are the arithmetic mean±standard deviation of two replicates of each thesis and of two analyses for each replicate. CTR = control.*

Considering the evolution of OTA concentration and degradation of malic acid in supernatant in L-malic+ and control theses (**Figure 3.9**) it is possible to observe that the OTA reduction was associated with the degradation of malic acid mainly in first step of the MLF.

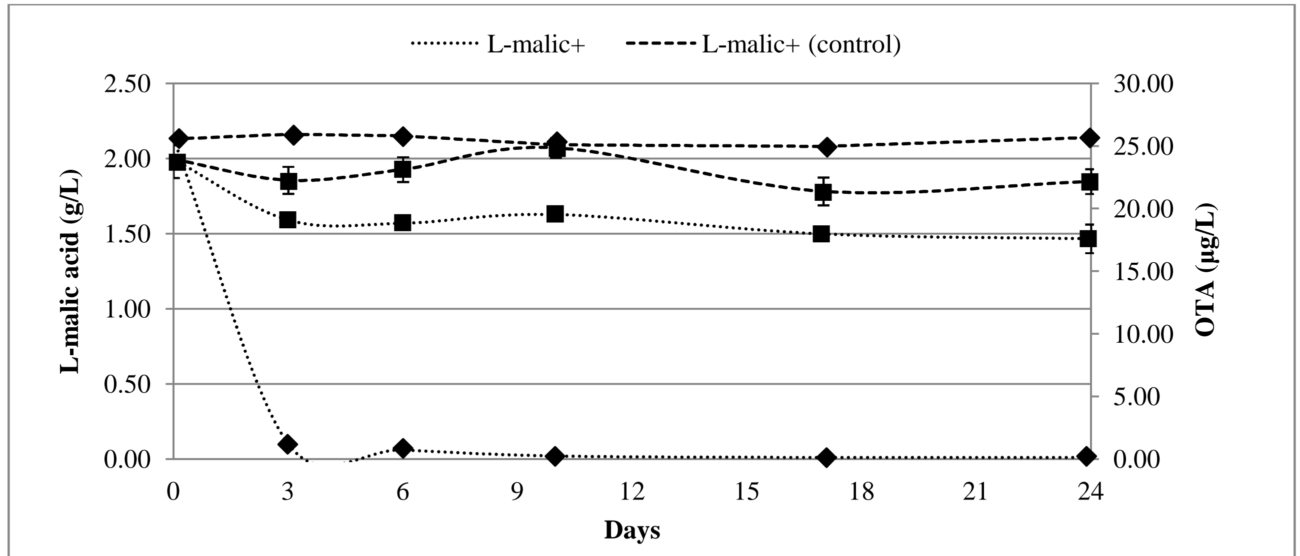


Figure 3.9 – *Trend of L-malic acid (◆) and OTA (■) concentration in supernatant in thesis L-malic+ and in control no inoculated. Error bars denote standard deviation.*

3.4 Discussion

The effect of LAB on the OTA was studied in the last years, but the data of the reduction mechanisms is still unclear. In particular, some authors suggest that the adsorption on the bacterial cell wall is the mechanism involved in the OTA reduction process (Haskard *et al.*, 2001; Del Prete *et al.*, 2007) while other studies indicate the simultaneous presence of an adsorption and an enzymatic mechanism (Piotrowska and Żakowska, 2005; Fumi *et al.*, 2008).

As mentioned above, it is known that certain LAB strains are able to detoxify other mycotoxins such as AFB_1 (Kabak *et al.*, 2006), fusarium toxins such as deoxynivalenol, nivalenol, T-2 toxin, HT-2 toxin (El Nezami *et al.*, 2002; Niderkorn *et al.*, 2006) and zearalenone (El-Nezami *et al.*, 2002, 2004). Del Prete *et al.* (2007) studied the ability of crude cell-free extracts to degrade OTA and concluded that OTA is not degraded by LAB and that the reduction mechanism is essentially an adsorption process.

In order to investigate the mechanisms which account for the removal of mycotoxins by LAB, the effects of viable and heat inactivated bacteria were compared in some studies (El-Nezami *et al.*, 1998, 2002, 2004; Haskard *et al.*, 2001; Mateo *et al.*, 2010). Fuchs *et al.* (2008) showed that the

OTA was removed by far more efficiently by viable bacteria. This can indicate that processes other than binding to the cell walls are involved, for example metabolic conversion by specific enzymes.
Piotrowska and Żakowska (2005) investigated the ability of 29 strains of LAB to eliminate OTA from a synthetic media. The highest decrease was caused by some strains of *L. acidophilus*, *L. rhamnosus*, *L. sanfraciscensis*, *L. brevis* and *L. plantarum*; this study shown that the decrease is partly reversible and a part of toxin is released back into the medium after 40 hours of incubation. The authors suggested that a part of the toxin was bound by the bacteria biomass and the remaining amount was eliminated in a different way.
In the present study we investigated the ability and the mechanism to remove OTA by *L. plantarum* V22, a LAB strain selected to perform MLF in two different conditions, with and without malic acid, a natural carbon source present in wine.
The preliminary investigation in wine demonstrated that *L. plantarum* was able to reduce OTA. The capability to remove OTA depended by initial OTA concentration, and it was better at low concentration of the toxin. To investigate the OTA reduction mechanism it was performed the trial in synthetic media (YNB w/o amino acids and ammonium sulphate); the initial concentration in YNB was higher than in wine to detect the possible presence of OTα low quantity too. The YNB was performed to create synthetic media with vitamins and mineral salts and without amino acids and ammonium salts. All theses were spiked with OTA and two theses were added with malic acid as carbon source to verify bacteria viability.
We observed some differences of OTA reduction kinetic in the two theses. In the L-malic+ after three days the malic acid was completely degraded and it was observed an OTA reduction of 20.27%, while from 3rd to 24th day was detected just a little decrease of OTA (1.5 μg/L). In the L-malic- after three days the OTA concentration in supernatant was similar to the control, from 6th day a slightly reduction, but not statistically significant, was observed and after the 17th day of incubation a low increase was detected probably due a release of OTA from cell bacteria to the medium. The different evolution of O.D. and OTA in L-malic+ thesis and mass balance compared to the control thesis suggest that the OTA was partly metabolized by bacteria.
This study confirms that the adsorption on the bacterial cell wall is one of the mechanisms to remove OTA and also an enzymatic pathway can be assumed. The OTA biodegradation process is more evident in presence of a carbon source as malic acid; this condition suggests that the OTA metabolization is related to bacteria viability. Previous studies about the OTA reducing ability in wine by *L. plantarum* and *O. oeni* strains showed a different ability in reducing OTA: best result was 45.9% of reduction. The higher decrement was registered during the first phase of MLF and after a prolonged starvation (unpublished data).

It is possible that in high stress conditions, caused to lack of nutrients, only a small quantity of OTA is adsorbed on the bacterial cell wall, because the bacteria don't growth and the cell lysis processes reduce the available surface of the adsorption and also the toxin already adsorbed could be released in the media. The OTA data in supernatant and the malic acid degradation suggested that *L. plantarum* don't use OTA as carbon source, rather, bacteria seem able to degrade OTA only with a carbon source, in our case malic acid. In general the OTA reduction in synthetic medium is very lower then in wine and this can be due to different composition of wine and YNB and to the high OTA concentration (25 µg/L) in YNB.

Some authors suggest that OTA has a negative effect on the growth of LAB, when used at high concentration (Piotrowska and Żakowska, 2005). OTα was not detected in media and in pellet in this conditions, and we can suppose that OTα produced by hydrolysis of amide bond of OTA is metabolized by bacteria in substrate poor in nutrients; moreover other mechanisms of OTA degradation, in which no OTα is produced, could be involved, as reported by Madsen *et al.* (1983) and Li *et al.* (2000).

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Chapter 4 - Bacteria strains screening able to perform the malolactic fermentation without produce biogenic amines

4.1 Introduction

Malolactic fermentation (MLF), which is the transformation of L-malic acid into L-lactic acid and CO_2 by lactic acid bacteria (LAB), is a metabolic process which occurs spontaneously in wine, generally after alcoholic fermentation.

MLF has important consequences for the quality of wine, increasing microbiological stability and enhancing the flavour. Outcome of MLF depends on several physical, chemical and biological factors widely described, including ethanol concentration, pH, the presence of SO_2 and of other antimicrobial compounds, or nutrient depletion by yeasts (Alexandre *et al.*, 2004; Rosi *et al.*, 2003; Zapparoli *et al.*, 2006). The introduction of *Oenococcus oeni* starter cultures for direct inoculation in wine has greatly simplified the management of this fermentation (Nielsen *et al.*, 1996; Krieger, 2002).

In the last years, *Lactobacillus* spp. received increasing attention as an important resource for the design of a new generation of MLF starter cultures, insomuch that we found in commerce malolactic starter formulate using *L. plantarum* (du Toit *et al.*, 2011; Miller *et al.*, 2011).

In wine several factors have an effect on the survival and development of bacteria; all these factors interact according to synergic or opposing effects (Britz and Tracey, 1990; Versari *et al.*, 1999): pH and SO_2 show opposite effect, low temperature influences tolerance of bacteria to ethanol. Modification of one of these parameters can have serious consequences on the starting and development of MLF. For example, Vaillant *et al.* (1995) in a comprehensive study found that ethanol, pH, temperature and SO_2 have the largest inhibitory effect out of the 11 factors (concentrations of L-malic acid, citric acid, L-tartaric acid, SO_2, D-glucose, D-fructose, pentoses, glycerol and ethanol, pH and temperature) investigated among the three strains of *Leuconostoc oenos* studied. Nielsen *et al.* (1996) confirmed the interaction between pH and SO_2 in wine and showed that the combination of low pH (3.2) and high SO_2 (26 mg/L) was strongly inhibitory to MLF whereas the value of pH or SO_2 individually had only a small inhibitory effect on MLF.

An important problem related to MLF is the production of biogenic amines by LAB. Biogenic amines are mainly produced by decarboxylation of the precursor amino acid through the substrate-specific enzymes of microorganisms present in food (Ten Brink *et al.*, 1990). The enzymes investigated are histidine decarboxylase (HDC), which catalyzes the formation of histamine (Coton *et al.*, 1998); tyrosine decarboxylase (TDC), which is specific for tyramine formation; and ornithine decarboxylase (ODC), which catalyzes the formation of putrescine (Guirard and Snell, 1980; Marcobal *et al.*, 2004).

HDC has been examined in *O. oeni* by Coton *et al.* (1998). The authors found that, of the 118 wines examined, half possessed bacteria that carried *hdc* gene, and they proposed that the ability to form histamine is strain-dependent and not related to the species.

TDC has been studied in *Lactobacillus* spp., particularly in *L. brevis* (Moreno-Arribas and Lanvoud-Funel, 1999). Moreno-Arribas *et al.* (2000) studied the activity of TDC and its *tdc* gene was purified and characterized; furthermore were isolated two different strains of *L. brevis* able to produce tyramine. The study showed that the occurrence and hazard levels of tyramine in wines are dependent not only on the development of bacteria able to produce this amine, but also on the presence of the available precursor.

ODC activity in LAB has been described in *Lactobacillus* 30a; the gene is activated by low pH and in enriched medium containing ornithine (Tabor and Tabor, 1985). Recently, Marcobal *et al.* (2004) isolated a putrescine-producing strain of *O. oeni* and sequenced its *odc* gene.

The objectives of this study were: to investigate the behaviour of the LAB in different growth conditions in order to isolate a strain able to make a MLF, to detect the presence of genes that encode for amino acid decarboxylases (HDC, TDC, and ODC) in LAB that have been isolated from wine and must. This work was carried out in collaboration with Dr. Eveline Bartowsky of the Australian Wine Research Institute (AWRI), Adelaide, Australia.

4.2 Materials and methods

4.2.1 Microorganisms, growth conditions and malic acid degradation tests

A total of 35 strains of *Lactobacillus* spp. signed by B (followed by three numbers) were studied. The strains were isolated from musts and wines from Adelaide Hills, Barossa Valley and McLaren Vale (South Australia, Australia). Each strain were cultured in MRSA (Man-Rogosa-Sharpe, enriched with apple juice) broth (Oxoid, Basingstoke, UK), pH 3.6, and incubated at 28°C.

The growth was performed in different conditions of pH, temperature, ethanol and SO_2 concentrations in MRSA broth:

- pH: 3.0, 3.2, 3.4, 3.5, 3.6, 3.8, 4.0, 4.2 at a constant temperature of 28°C;
- temperature: 4°C, 18°C, 22°C, 28°C, 37°C at a constant pH of 3.6;
- ethanol: 0 v/v%, 8 v/v%, 10 v/v%, 12 v/v%, 14 v/v%, 16 v/v% at 28°C and pH 3.6;
- SO2: 0 mg/L, 20 mg/L, 40 mg/L, 60 mg/L, 80 mg/L, 100 mg/L at 28°C and pH 3.6;

The media were inoculated with biomass suspended in MRSA broth derived from the logarithmic growth phase with density of 10^5 cfu/mL. The bacteria growth was monitored by optical density measure every day for 5 days in trials at different pH and temperature and for 11 days in trials at different concentrations of ethanol, and SO_2.

The bacteria more resistant to conditions investigated were tested in wine at different condition of pH and ethanol, to assess the capability to degrade the L-malic acid in wine:

- ethanol 14 v/v%, pH 3.6;
- ethanol 14 v/v%, pH 3.8;
- ethanol 12 v/v%, pH 3.6;
- ethanol 12 v/v%, pH 3.8.

In this trial the bacteria strains were pre-cultured in MRSA:wine (1:1) and after centrifugation were suspended in wine. The wine was used after filtration with filter at pore size 0.20 µm. The SO_2 in wine was 20 mg/L and the L-malic acid 1.4 g/L. All theses were inoculated and the final concentration of bacteria strains was 10^6 cfu/mL. The working temperature was maintained at 22°C. The controls of L-malic acid were carried out every 2/3 days for 32 days with enzymatic analysis by Freedom Evo (Tecan Group Ltd., Männedorf, Schweiz). All experiments were performed in quadruplicate using F96 MicroWell™ plates (Thermo Fisher Scientific Inc., Waltham, MA, USA) with working volume of 200 µL/well. The plates were gently shaken and absorbance at 630 nm measured for each well by Instant Multiskan Ascent spectrophotometer (Thermo Fisher Scientific Inc.).

4.2.2 DNA extraction

Total bacteria DNA was extracted using GenElube™ Bacterial Genomic DNA Kit (Sigma, St. Louis, MO, USA) (Sambrook and Russell, 2001). The quantification was carried out by Qubit Fluorometer (Life Technologies Australia, Mulgrave, VIC, Australia).

4.2.3 PCR conditions

Each PCR reagents were purchased by Astral Scientific (Sydney, NSW, Australia).

To identify the *L. casei* the PCR conditions were performed according to Ward *et al.* (1999).

Primers: Y2 5'-CCCACTGCTGCCTCCCGTAGGAGT-3'

Casei 5'-TGCACTGAGATTCGACTTAA-3'

PCR amplification was performed in a 25 µL mixture containing 2.5 µL of Buffer 10x (670 mM Tris-HCl, pH 8.8 a 25 °C, 166 mM $(NH_4)_2SO_4$, 4.5% Triton®-X-100, 2 mg/ml gelatine), 2 mM $MgCl_2$, 0.2 mM dntps (deoxyribonucleotide triphosphates), and 1 U of *Taq* DNA polymerase, along with 0.4 µM of the primers Y2 and casei, DNA template 20 ng.

To identify *L. plantarum* the PCR conditions were performed according to Torriani *et al.* (2001).

Primers: planF 5'-CCGTTTATGCGGAACACCTA-3'

pREV 5'-TCGGGATTACCAAACATCAC-3'

PCR amplification was performed in a 20 µL mixture containing 2.5 µL of Buffer 10x (670 mM Tris-HCl, pH 8.8 a 25 °C, 166 mM $(NH_4)_2SO_4$, 4.5% Triton®-X-100, 2 mg/ml gelatine), 3 mM $MgCl_2$, 0.2 mM dntps, and 1 U of *Taq* DNA polymerase, along with 0.12 µM of the planF and 0.25 µM of the pREV, DNA template 5 ng.

To detect the *hdc*, *tdc* and *odc* genes we followed the protocol of Costantini *et al.* (2006). The primers used were:

hdc:	PHDC1	5'-CCGTGCGGAAACAAAGAAT-3'
	PHDC2	5'-CCAAACACCAGCATCTTCA-3'
tdc:	Pt3	5'-TACACGTAGATGCTGCATATG-3'
	Pt4	5'-ATGGTTGACTATGTTTTAAAAGAA-3'
odc:	AODC1	5'-GMTCGTGAAATYGAARCKG-3'
	AODC2	5'-KGRGTTCMGCYGGRGTCAT-3'

PCR amplification was performed in a 20 µL mixture containing 2.5 µL of Buffer 10x (670 mM Tris-HCl, pH 8.8 a 25 °C, 166 mM $(NH_4)_2SO_4$, 4.5% Triton®-X-100, 2 mg/ml gelatin), 2 mM $MgCl_2$, 0.2 mM dntps, and 0.5 U of *Taq* DNA polymerase, along with 0.4 µM of the primers for *hdc* and *tdc* genes and 1 µM of primers for the *odc* gene, DNA template 12.5 ng.

4.3 Results

4.3.1 Bacteria growth and malic acid degradation trials

Among the *Lactobacillus* strains tested in MRSA we observed that 7 strains were able to growth in conditions of pH 3.4-3.6, temperature 22°C, ethanol 10-14 v/v% and SO_2 20-40 mg/L: B425, B426, B694, B750, B751, B752, B754.

In wine at 12 v/v% of ethanol and pH 3.6 three strains (B425, B426, B752) degraded the malic acid; B425 and B752 completely metabolize the malic acid in 8 days, while B426 in 18 days. In the other strains the percentage of malic acid reduction ranged between 10.34 (B694) and 28.28% (B750) after 32 days (**Figure 4.1**).

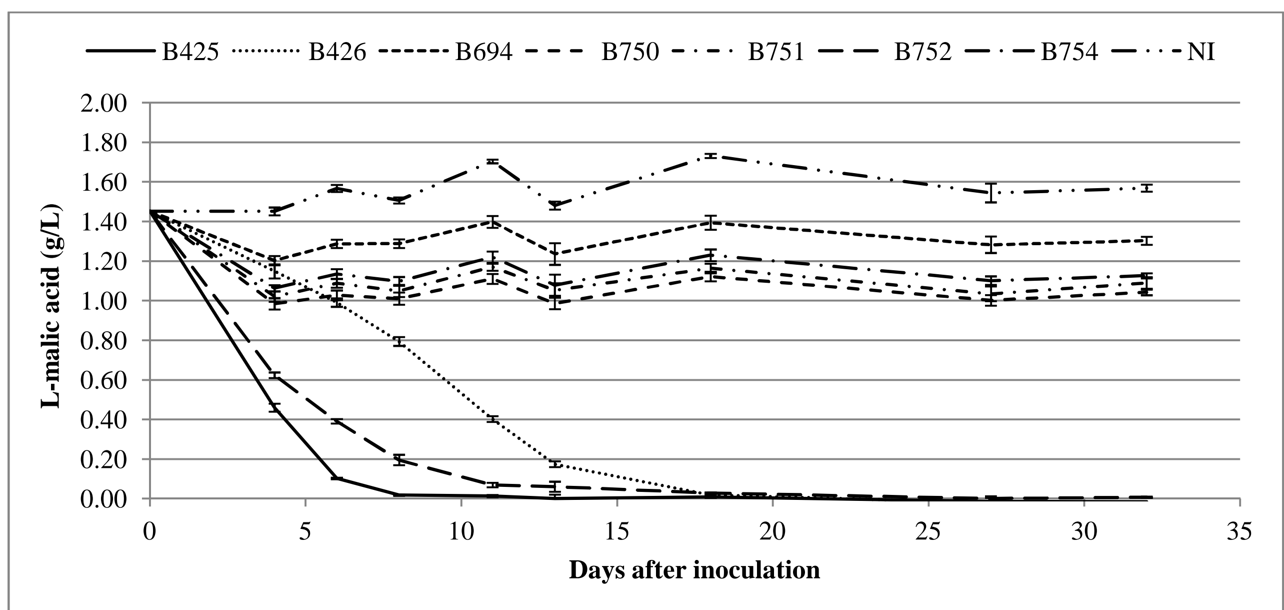

Figure 4.1 – *Malic acid degradation in wine at 12% of ethanol and pH 3.6. B = Lactobacillus spp.. NI = not inoculated. Error bars denote standard deviation.*

At pH 3.8 all strains have degraded the malic acid. The metabolism of malic acid was complete in 8 days from the inoculation in thesis with bacteria strains B426 and B694 and in six days for the other thesis (**Figure 4.2**).

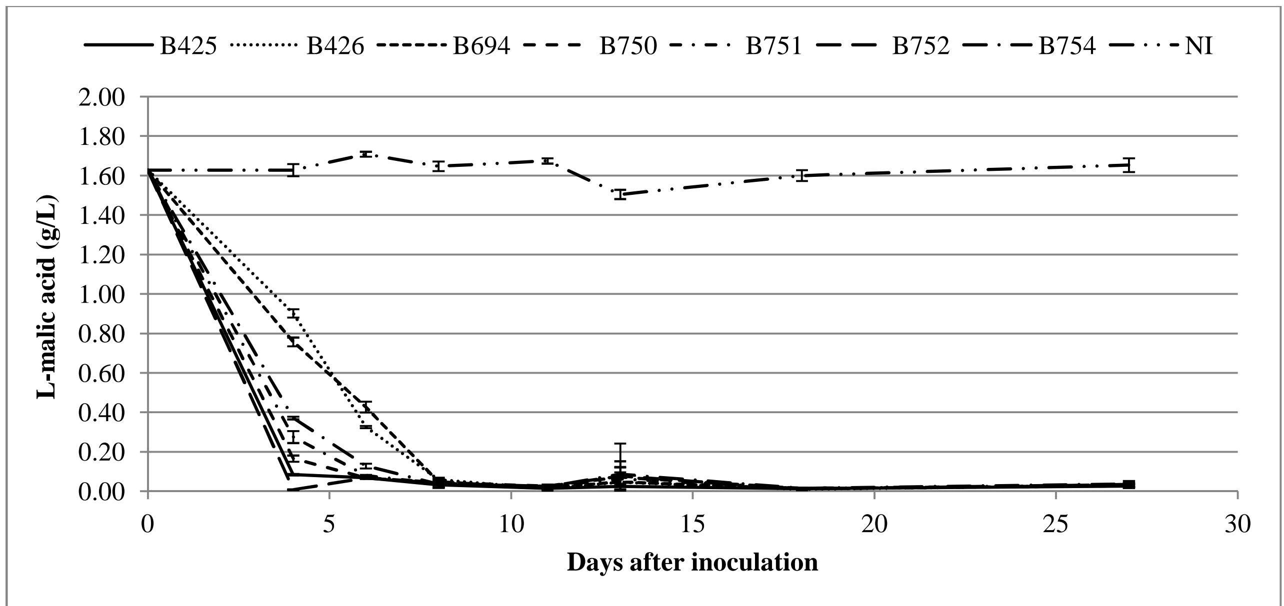

Figure 4.2 – *Malic acid degradation in wine at 12% of ethanol and pH 3.8. B = Lactobacillus spp.. NI = not inoculated. Error bars denote standard deviation.*

When the ethanol was 14 v/v% at the pH 3.6 the trend of malic acid was similar at the wine with ethanol 12 v/v%; the thesis B752 is distinguished from others because acid malic was still detectable (0.07 g/L) at the end of the test after 32 days (**Figure 4.3**).

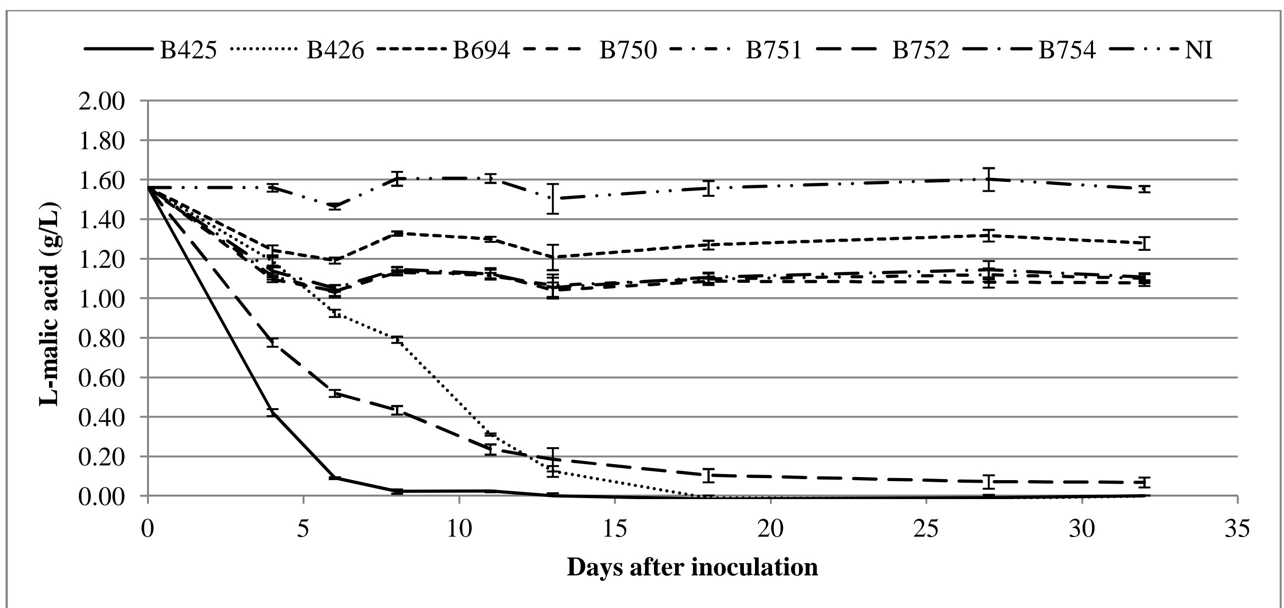

Figure 4.3 – *Malic acid degradation in wine at 14% of ethanol and pH 3.6. B = Lactobacillus spp..NI = not inoculated. Error bars denote standard deviation.*

At pH 3.8 the degradation of the malic acid was faster in B425, B426 e B752 assays; the malate degraded completely between the sixth and eighth day. The other strains did not metabolize the

malic acid completely over the 32 days of the test; the percentage of reduction ranged between 64.58% (B694) and 88.19% (B750) (**Figure 4.4**).

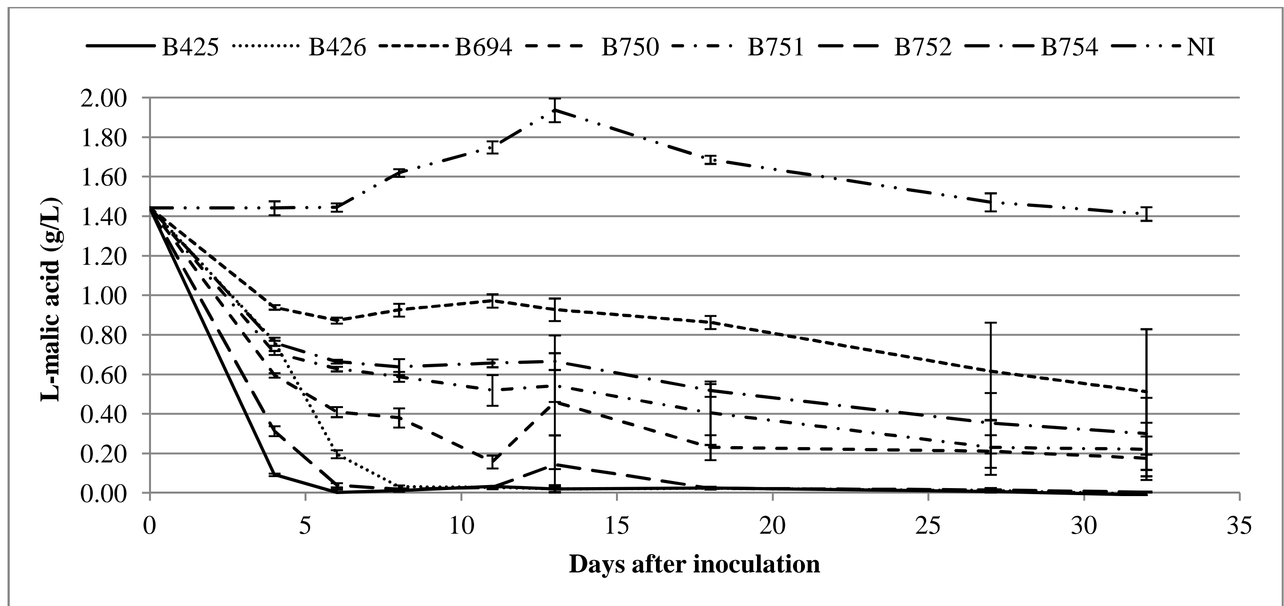

Figure 4.4 – *Malic acid degradation in wine at 14% of ethanol and pH 3.8. B = Lactobacillus spp.. NI = not inoculated. Error bars denote standard deviation.*

4.3.2 PCR detection of L. casei and L. plantarum

Of the 35 bacteria investigated only one was *L. casei* (B749) and 8 strains were *L. plantarum* (B659, B694, B698, B699, B722, B751, B752, B754) (**Figure 4.5**). The other 26 strains were not identified.

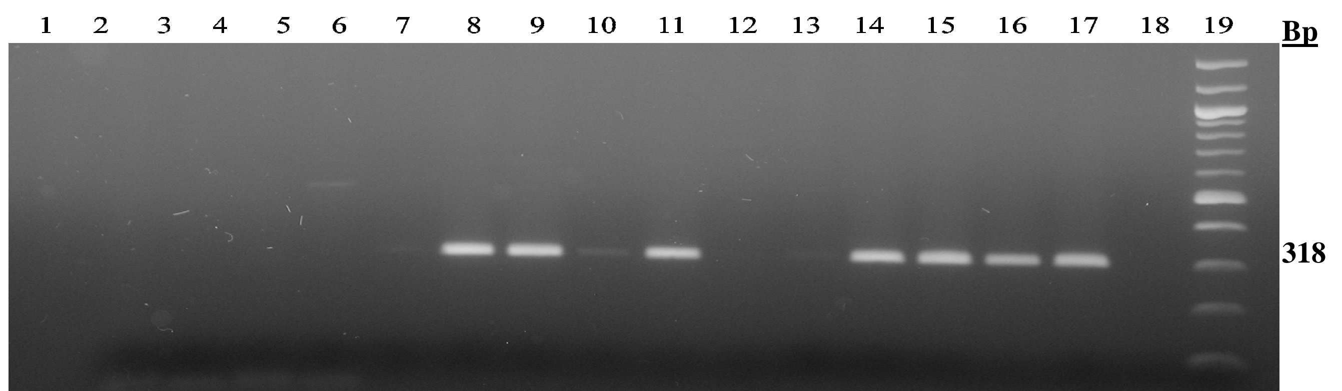

Figure 4.5 – *Electrophoresis gel depicting the result of PCR amplification performed to identify L. plantarum. PCR was performed according to Torriani et al. (2001). The amplified fragment gave an amplicon of 318 bp. Lane 19 contained a 1 kb marker (Sigma). Lane 8-9: B751-B752. Lane 10: B753. Lane 11: B754. Lane 12-13: B425-B426. Lane 14-16: B694-B698-B699. Lane 17: L. plantarum V22 (positive control). Lane 18: no template DNA (negative control). The molecular size of the marker (Marker 1Kb, Sigma, lane 1) is indicated on the right.*

4.3.3 PCR detection of hdc, tdc and odc genes

The **figure 4.6** shows an example of PCR performed on the *tdc* gene. The strain *tdc*$^{+}$ was *L. casei* B749 (lane 7), the other strains were negative. None of the bacteria investigated presented the *hdc* and *odc* genes.

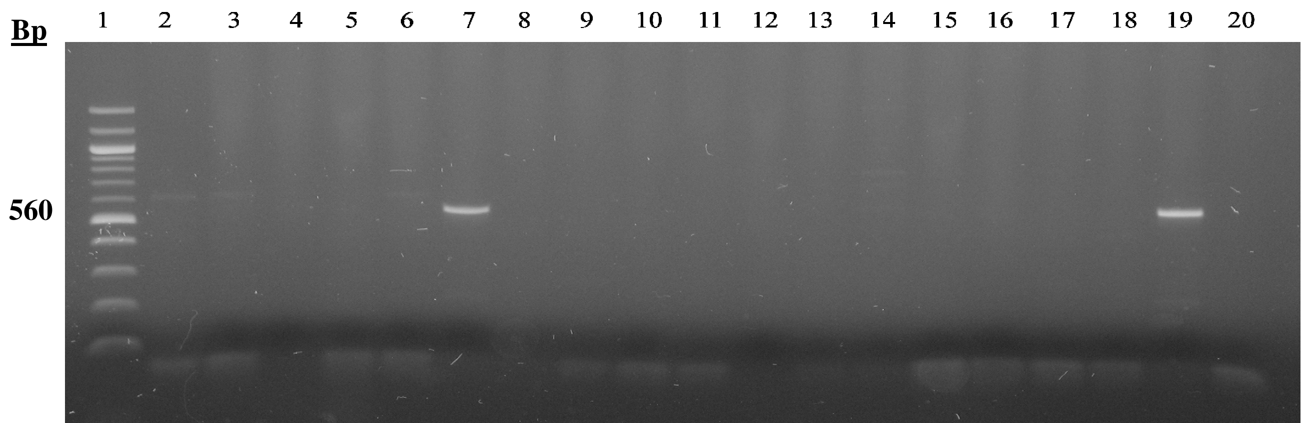

Figure 4.6 – *Example of electrophoresis gels of PCR amplification performed on the tdc gene. Lane 1 contained a 1 kb marker (Sigma). Lane 7: B749. Lane 19: L. brevis CECT 5354 (positive control), the amplified fragment of tyrosine decarboxylase gene gave an amplicon of 560 bp. The negative control that had no template DNA is in lane 20. The molecular size of the marker (Marker 1Kb, Sigma, lane 1) is indicated on the left.*

4.4 Discussion

In the last years many studies have been carried out to isolate LAB able to perform MLF without the production of biogenic amines. In this work the effects of several chemico-physical factors (pH, temperature, SO_2, and ethanol concentration) on the growth and malolactic activity of *Lactobacillus* spp. strains, isolated from different Australian musts and wines, were evaluated. The screening process in the synthetic wine medium was used as an indication of the possible survival and performance of the potential starter strains in the wine environment.
Strains were first selected after characterization in a synthetic matrix, which resulted in 7 potential MLF strains. These strains were evaluated in wine to reject the strains as they did not complete the MLF. Other studies make use of synthetic or wine-like media for the characterization of LAB strains. Capozzi *et al.* (2010) used two different wine-like media with 11 and 13% (v/v) ethanol, respectively. Lerm *et al.* (2011) investigated in synthetic media several strains of *O. oeni* and *L. plantarum*, isolated from South African wines, as possible MLF starter cultures.

On the basis of our results, few strains of LAB growth in the conditions similar to wine, the value of pH seem to be the more limiting condition, in fact in wine the malolactic activity was more influenced by the pH variation respect the ethanol.

The pH and alcohol content of wine are important oenological parameters that determine bacterial growth, and consequently the capacity of malate degradation in *Lactobacillus* spp. cultures.

The inability to produce amines is an important characteristic for any strain considered for use in a starter culture, because the biogenic amines have an impact on wine quality and healthiness.

Among the biogenic amines, histamine is most frequently found in wine. For a long time and even today, oenologists have considered that only *Pediococcus* strains were responsible for histamine (Aerny, 1985). This genus is always represented in wine microflora, in addition to *Lactobacillus*, *Leuconostoc* and *Oenococcus*, but usually at a low proportion (Lonvaud-Funel, 2001). Recently, Lucas *et al.* (2005) demonstrated that the *hdc* gene is located on an unstable plasmid and that LAB could lose the ability to produce histamine, depending on culture conditions.

Moreno-Arribas *et al.* (2000) isolated several tyramine-producing strains; in particular were identified as *L. brevis* and *L. hilgardii*, TDC activity greatly depended on the presence of the precursor, the authors suggested that tyrosine induced the TDC system. Moreno-Arribas *et al.* (2003) investigated the presence LAB able to produce biogenic amines in 78 strains isolated from Spanish musts and wines; the authors reported that the ability to produce amine might be strain-dependent and more common among strains of particular species.

In order to totally eliminate the potential for biogenic amine production, the molecular screening of possible starter cultures for biogenic amine-encoding genes is a quick and efficient method to ensure this.

In our study, 35 strains of LAB were investigated to detect the *hdc*, *tdc* and *odc* genes that encode for amino acid decarboxylases (HDC, TDC and ODC). None of the bacteria presented the *hdc* and *odc* genes, while only the *L. casei* B749 was tdc^{+}.

Costantini *et al.*, 2006 investigated 26 *Lactobacilli*, only one strain of *L. hilgardii* was hdc^{+} and four strains of *L. brevis* showed to possess the *tdc* gene, none of the bacteria presented the *odc* gene. Our results confirm that there are few LAB able to produce biogenic amine in wine.

The problem of the presence of biogenic amines in wine may be associated with other variables affecting the level of these compounds; many studies investigated the relationship between cultivar, vintage, storage of the grape, oenological practices, microorganism and other wine compounds (Herbert *et al.*, 2005; Marques *et al.*, 2008; Moreno-Arribas and Polo, 2008; Marcobal *et al.*, 2006; Del Prete *et al.*, 2009; Cecchini and Morassut, 2010). It is possible that the formation of biogenic amines is due to microorganism associated with a lack of hygiene during the winemaking process.

In conclusion, in must and wine there are few autochthonous LAB able to growth in the physic-chemical wine conditions and to perform MLF. As to the biogenic amines, we confirmed that the presence of *hdc*, *tdc* and *odc* genes *Lactobacillus* spp. is low, and that the molecular screening is a good a fast technique to detect potential amines-producing bacteria. On the basis of our results, the strains of *Lactobacillus* spp. that perform MLF are more frequently *L. plantarum* and these are not able to produce histamine, tyramine and putrescine.

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Chapter 5 - Biogenic amines in wine related to *Lactobacillus plantarum* inoculation time

5.1 Introduction

Malolactic fermentation (MLF), the de-acidification of the wine by the enzymatic decarboxylation of L-malic acid to L-lactic acid, is an important secondary fermentation carried out by lactic acid bacteria (LAB). Several factors affect the MLF process: ethanol, pH, SO_2, nutrients, interaction of bacteria with yeasts and temperature (Fugelsang and Edwards, 2007).
The use of the LAB starter cultures improved and simplified the management of MLF. In general the LAB used in winemaking are *Oenococcus oeni*, but in the last years some studies have been conducted on *Lactobacillus plantarum* (Fumi *et al.*, 2010) and in particular assays have been carried out to determine the best time for bacterial inoculation. Some results from scientific literature suggest a simultaneous fermentation (alcoholic and malolactic) by adding the LAB in the must or in different steps of the alcoholic fermentation (AF). The gradual adaptation of bacteria to increasing alcohol concentration and the higher nutrient present in must could facilitate the induction of the MLF. Different combinations of LAB and yeasts have been studied (Alexandre *et al.*, 2004; Rosi *et al.*, 2003). Jussier *et al.* (2006) observed that the simultaneous inoculation with *Saccharomyces cerevisiae* and two selected strains of *O. oeni*, led to faster and complete malic acid degradation compared with the bacteria inoculation at the end of alcoholic fermentation. Fumi *et al.* (2010) investigated the compatibility of the *L. plantarum* V22 in simultaneous inoculation with various wine yeast strains using co-inoculation as such and compared to sequential inoculation after AF in high pH conditions. However the co-inoculation practice could cause problems to the wine's quality due to the activity of LAB in must, mainly related to an excessive acetic acid production as a consequence of sugar metabolism. Another risk is related to the production of biogenic amines. The concentration of biogenic amines that can potentially be produced in wine largely depends on the abundance of amino acid precursors in the medium, the presence of decarboxylase positive microorganisms and must/wine parameters such as pH, alcohol and sulfur dioxide that will impact the growth of microbes, as demonstrated by the results reported in chapter 4.
In the literature there are few studies that investigate the level of biogenic amines related to bacteria co-inoculation time, Massera *et al*. (2009) show that no statistical differences, in the biogenic amine

level, result between the timings of inoculation in Malbec wine using commercial strains of *S. cerevisiae* and *O. oeni*.

Biogenic amines are undesirable in all foods and beverages because, if present in high concentration, they may induce headache, respiratory distress, hyper/hypotension and several allergenic disorders (Silla, 1996).

They are low molecular weight organic bases, aliphatic (putrescine, cadaverine, spermine and spermidine), heterocyclic (histamine and tryptamine) or aromatic (tyramine and phenylethylamine) (Lounvaud-Funel, 2001). Some amines are normal constituents of grapes, varying according to variety, soil type and composition, fertilization and climatic conditions, level of grape ripening and oenological parameters (Herbert *et al.*, 2005; Marques *et al.*, 2008; Moreno-Arribas and Polo, 2008; Marcobal *et al.*, 2006; Del Prete *et al.*, 2009). A recent study shows that the grape storage time before crushing affects the amines content in must (Cecchini and Morassut, 2010).

Biogenic amines are formed by decarboxylation of the precursor amino acids by microorganisms through the substrate-specific enzymes activity. This property is not linked to a microbial species, usually it is strain dependent (Leitão *et al.*, 2000). Enzymes on which most research has been focused are: histidine decarboxylase (HDC), which catalyzes the formation of histamine; tyrosine decarboxylase (TDC), which is specific for tyramine formation; and ornithine decarboxylase (ODC), which catalyzes the formation of putrescine (Costantini *et al.*, 2006).

Some studies report no remarkable increase of biogenic amines concentration during AF and show that yeasts do not seem to be responsible for the production of amines present in wine (Herbert *et al.*, 2005). In wine MLF is considered a critical step for biogenic amines production. The wild bacteria are the major responsible of the formation of amines (Lonvaud-Funel, 2001). Recent studies (Capozzi *et al.*, 2012) analyze the ability of LAB, in particular *L. plantarum*, to degrade biogenic amines commonly found during wine fermentation. Two biotypes of *L. plantarum* showed a respectable aptitude to degrade malic acid in wine-like medium and were found capable to work in synergy for tyramine and putrescine degradation.

In this work we investigated the relationship between *L. plantarum* V22 and oenological practices to explore the ability of *L. plantarum* to produce/reduce the biogenic amines. In particular the impact of different inoculation time of selected bacteria strains on the biogenic amines production was considered. The strain of *L. plantarum* used in this work was originally isolated from must grape/wine by Piacenza area. The microvinification trials were carried out at the cellar of the Institut Agricole Régional (IAR) of Aosta.

5.2 Materials and methods

5.2.1 Grape cultivar

The study was carried out with the collaboration of the IAR located in Aosta in the Valle d'Aosta region (Italy) (45°44'14"N latitude, 7°19'14"E longitude) at 583 m above sea level. The trials were made using Cornalin, an autochthonous *Vitis vinifera* cultivar of the Valle d'Aosta. The grapes were grown in the same pedoclimatic conditions and with the same training system; the grapes at harvest were in good sanitary state, without mould infections.

5.2.2 Yeast and bacteria strains

The *S. cerevisiae* strain QD145 (Montréal, QC, Canada) was used to ferment the must. The LAB *L. plantarum* V22 (Lallemand Inc.) was selected because of its good capacity to induce MLF. The use of QD145 and V22 was chosen based on a previous study that showed the good compatibility between these microorganisms in co-inoculation trials (Fumi *et al.*, 2010)

5.2.3 Standards and reagents

Methanol was obtained from BDH (Poole, UK); di-sodium hydrogen phosphate (Na_2HPO_4) and acetonitrile were purchased from Merck (Darmstadt, Germany), sodium tetraborate ($Na_2B_4O_7 \cdot 10$ H_2O), hydrogen chloride were obtained from Carlo Erba (Milano, Italy). Solvent used as mobile phase were HPLC grade. 2-mercaptoethanol, O-phthaldialdehyde, amines standards (ethanolamine, histamine, ethylamine, tyramine, phenylethylamine, cadaverine) and hexamethylenediamine (internal standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2.4 Chemical analysis of must and wine

Chemical analysis of must and wine: reducing sugars, titratable acidity, pH, alcohol, volatile acidity, total SO_2, tartaric acid, L-malic acid, L-lactic acid and citric acid were determined according to OIV methods (Compendium 2012).

5.2.5 Biogenic amines samples preparation and analysis

Biogenic amines analysis was carried out by HPLC using a Perkin-Elmer (Norwalk, CT, USA) liquid chromatography series 200 pump, Jasco (Oklahoma City, OK, USA) FP-2020 plus fluorescence detector, Jasco LC-Net II/ADC communication module and ChromNAV Control Center software. A Gemini NX-C18 column, 250 mm×4.6 mm, 5 µm (Phenomenex, Torrance, CA, USA) was used for separation of the analytes. The injection volume was 20 µL and the analysis was

performed at a column temperature of 35°C. The mobile phases for the biogenic amines were prepared as described in resolution OIV/OENO 346/2009: mobile phase A phosphate buffer, and mobile phase B acetonitrile. The stepwise gradient was: 0-15 min 20-30% B, 15-23 min 30-40% B, 23-42 min 40-50% B, 42-55 min 50-65% B, 55-60 min 65% B, 60-70 min 65-20% B, 70-95 min 20% B. The fluorescence detector was set at 356 nm excitation and 445 nm emission wavelengths. Standards and samples were submitted to derivatization with o-phthaldialdehyde according to procedure of resolution OIV/OENO 346/2009.

5.2.6 Winemaking trials

The grapes of the Cornalin variety were destemmed, crushed and separated from the skins. Skins and free run juice were sub-divided equally into the various demijohns of 20 L and SO_2 was added of 40 mg/L.

In all theses the must was inoculated by *S. cerevisiae* (30 g/hL) pre-rehydrated in presence of yeast nutrient GO-FERM (30 g/hL) (Lallemand Inc.). AF and maceration were conducted at the range temperature between 17 and 23°C, with three punching the cap to day. During AF FERMAID (30 g/hL) (Lallemand Inc.) was added; at the end of the AF the wine was decanted in glass container of 5 L and added of Opti'Malo plus (20 g/hL) (Lallemand Inc.), LAB nutrient. Each container was inoculated with *L. plantarum* V22 at 1 g/hL (1.8 x 10^6 cfu/mL) after hydration in water for 15 minute at 20°C. The theses consisted in:

thesis 1- bacteria inoculated in the must and the yeast 24 h later;

thesis 2- bacteria inoculated 24 h after yeast;

thesis 3- bacteria inoculated at 30% of AF;

thesis 4- bacteria inoculated at 60% of AF;

thesis 5- bacteria inoculated at end of AF;

thesis 6-control not inoculated with bacteria.

After MLF end the wine was cleared, filtered and bottled.

The analyses were carried out in 6 different steps: must, 50% of AF, end of AF, end of MLF, at bottling and after one year in bottle.

5.2.7 Statistical analysis

The experiments were carried out on two replicates, and the analyses were performed in duplicate (analysis of the biogenic amines after one year in bottle in triplicate). IBM SPSS® 19.0 software for Windows was used to perform statistical analyses (SPSS, Chicago, IL, USA). Statistical analysis

were done by analysis of variance (ANOVA) followed by Tukey test to evaluate the significance of variation among biogenic amines mean values. All significance tests were conducted at $P \leq 0.05$.

5.3 Results and discussion

5.3.1 Oenological parameters

The chemical characterization of the must is shown in **Table 5.1**. The reducing sugar contents were high while the pH value was low. The titratable acidity was low and principally constituted to the tartaric acid, while the levels of L-malic acid and citric acid were not very high.

Reducing sugars	pH	Titratable acidity	L-malic acid	Tartaric acid	Citric acid
217 g/L	3.27	4.8 g/L	1.04 g/L	5.7 g/L	0.12 g/L

Table 5.1 – *Chemical parameters of the must.*

In all the theses the AF finished in 5 days after yeast inoculation, no significant differences in duration of AF were observed from the different timing of LAB inoculation (**Figure 5.1**).

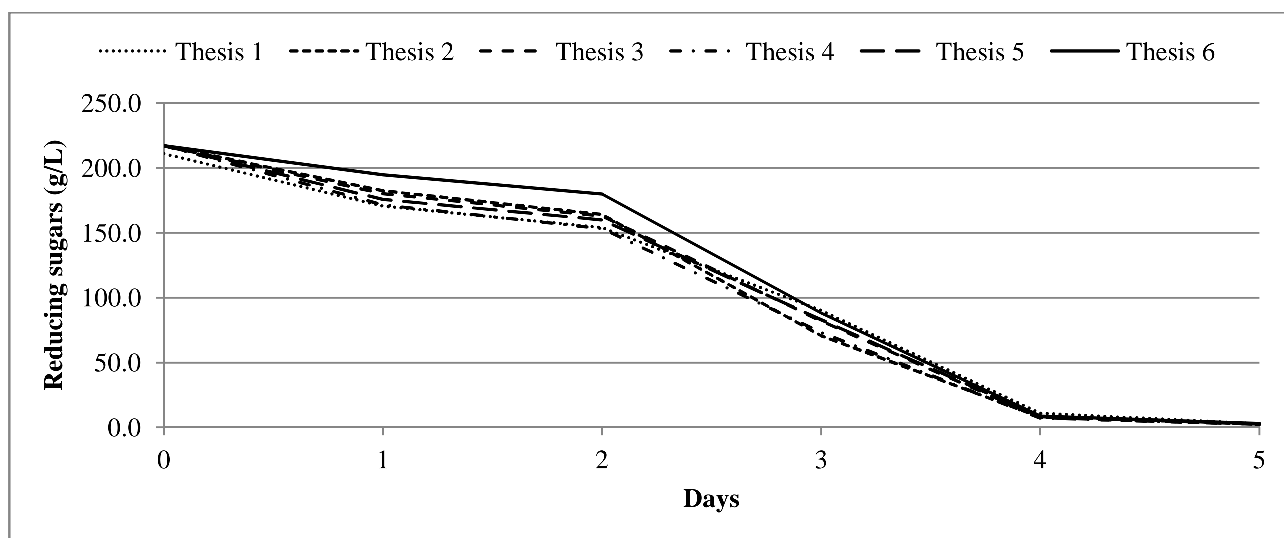

Figure 5.1 – *Trend of AF in theses with different co-inoculation time. Thesis 1- bacteria inoculated in the must and the yeast 24 h later; thesis 2- bacteria inoculated 24 h after yeast; thesis 3 - bacteria inoculated at 30% of AF; thesis 4 - bacteria inoculated at 60% of AF; thesis 5- bacteria inoculated at end of AF; thesis 6 -control not inoculated with bacteria.*

In our experiments, volatile acidity was 0.22-0.26 g/L acetic acid in all the theses (**Table 5.2**) at the end of AF; a negative impact of the presence of bacteria on the performance of AF could not be found. It can be assumed that in none of the cases studied here the bacteria produced worrisome levels of acetic acid from sugar and citric acid utilization, and that the production of acetic acid is mostly related to yeast activity. Malic acid and titratable acidity were increased in the first phase of maceration (respectively 6.47±0.19 g/L and 1.43±0.06 g/L); subsequently, a slightly reduction of malic acid was detected in the four theses inoculated in must or during AF. In these, higher levels of L-lactic acid were detected compared to theses 5 (inoculation after AF) and 6 (control).

	Thesis 1	Thesis 2	Thesis 3	Thesis 4	Thesis 5	Thesis 6
Reducing sugars g/L	2.72±0.73	2.79±0.04	2.37±0.88	2.27±0.30	2.52±0.03	3.13±0.61
pH	3.33±0.04	3.34±0.01	3.35±0.03	3.34±0.06	3.35±0.01	3.36±0.01
Titratable acidity g/L	6.66±0.03	6.47±0.03	6.46±0.11	6.43±0.03	6.29±0.50	6.51±0.03
L-malic acid g/L	1.19±0.03	1.22±0.02	1.12±0.09	1.16±0.10	1.40±0.03	1.44±0.02
L-lattic acid g/L	0.14±0.06	0.16±0.02	0.27±0.06	0.18±0.02	0.03±0.04	0.08±0.01
Tartaric acid g/L	3.53±0.04	3.33±0.05	3.31±0.18	3.26±0.11	3.14±0.14	3.46±0.23
Citric acid g/L	0.15±0.02	0.15±0.02	0.18±0.01	0.18±0.01	0.19±0.01	0.20±0.01
Total SO_2 mg/L	33.90±2.69	32.00±2.99	32.65±0.92	33.26±3.59	33.26±3.59	32.65±0.92
Ethyl alcohol % v/v	12.39±0.06	12.50±0.09	12.61±0.07	12.69±0.11	12.65±0.09	12.34±0.06
Acetic acid g/L	0.26±0.08	0.22±0.04	0.25±0.09	0.25±0.06	0.23±0.01	0.22±0.01

Table 5.2 – *Chemical parameters of wine at the end of AF (arithmetic mean±standard deviation).*

In our study the timing of the LAB inoculation do not affect on AF, in fact in all the thesis the AF finishes in five days (the short duration is related to small volumes). MLF complete the degradation of the L-malic acid in 25 days and there were no substantial differences in all theses inoculated with bacteria (Figure 2). In control (thesis 6) the malic acid concentration unchanged in the same period of time, but was metabolized in 40 days.

The MLF was ended 20 days after the LAB inoculation, the pH increase is not considerable, 0.05-0.15 units more to the must values. The data showed an increase of the acetic acid in the control (thesis 6) and in theses 3 and 5 (**Table 5.3**). In these last theses the bacteria inoculation was carried out respectively at 30% of AF and at end of AF.

In the thesis 1, with bacteria inoculated in must before of the yeasts, there was an initial increase of the acetic acid but during the fermentation its level was fairly stable; the initial increase could be

caused by wild microorganism present in must in relation with the delay of the AF. In the thesis 5, bacteria inoculated at the end of MLF, the concentration of the acetic acid increase especially during MLF but not achieved harmful level. In the other thesis the level of acetic acid not showed differences among them. Our results show the possibility of simultaneous induction of AF and MLF without excessive increase in volatile acidity.

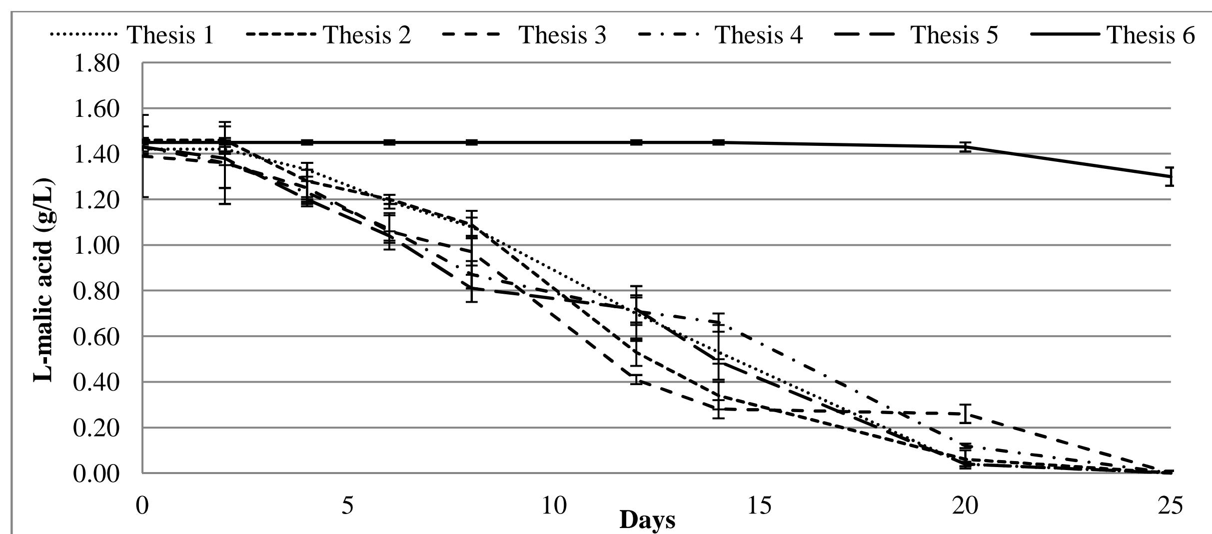

Figure 5.2 – *Trend of L-malic acid degradation in theses with different co-inoculation time. Error bars denote standard deviation. Thesis 1- bacteria inoculated in the must and the yeast 24 h later; thesis 2- bacteria inoculated 24 h after yeast; thesis 3 - bacteria inoculated at 30% of AF; thesis 4 - bacteria inoculated at 60% of AF; thesis 5- bacteria inoculated at end of AF; thesis 6 -control not inoculated with bacteria.*

	Thesis 1	Thesis 2	Thesis 3	Thesis 4	Thesis 5	Thesis 6
Reducing sugars g/L	0.73±0.13	0.79±0.01	0.71±0.33	0.77±0.10	0.68±0.23	0.82±0.05
pH	3.36±0.06	3.4±0.04	3.44±0.01	3.36±0.08	3.43±0.04	3.50±0.01
Titratable acidity g/L	4.22±0.03	3.78±0.21	4.17±0.50	3.83±0.01	4.18±0.43	3.77±0.09
L-malic acid g/L	0.05±0.01	0.09±0.04	0.02±0.03	0.11±0.01	0.03±0.04	0.10±0.05
L-lattic acid g/L	0.89±0.01	0.88±0.01	0.88±0.04	0.87±0.02	0.79±0.01	0.85±0.02
Citric acid g/L	0.15±0.01	0.01±0.01	n.d.	n.d.	0.03±0.02	n.d.
Total SO_2 mg/L	29.44±0.01	30.08±0.01	30.08±0.01	30.72±0.01	28.8±0.01	26.88±0.01
Ethyl alcohol %v/v	12.53±0.01	12.72±0.01	12.67±0.01	12.73±0.01	12.6±0.01	12.32±0.01
Acetic acid g/L	0.26±0.01	0.29±0.05	0.30±0.06	0.31±0.08	0.41±0.01	0.39±0.15

Table 5.3 – *Chemical parameters of wine at the end of MLF (arithmetic mean±standard deviation). n.d. = not detected.*

The values obtained for other wine parameters, including pH, titratable acidity, and the concentrations of ethanol and total SO_2, further confirm the similarity of the wines obtained by the different winemaking strategies, regardless of the inoculation time.

The our results show that the *L. plantarum* V22 was able to degrade the L-malic acid also to low pH (~3.3), nevertheless the MLF was complete slowly compared to pH>3.5 (Fumi *et al.*, 2010).

5.3.2 Biogenic amines evolution

The level of biogenic amines in the must is reported in **table 5.4**. The results show that the ethanolamine was the major amine present in must, ethylamine, phenylethylamine, histamine and cadaverine were detected in very low concentration and tyramine was not detected. The presence of amines in must could be caused by grapes storage time before to be crushed, necessary because the grapes temperature was very low (~12°C) for the winemaking. The grapes were stored in cellar overnight to range 16°C before crushing and yeast/bacteria inoculation.

Ethanolamine	Histamine	Ethylamine	Tyramine	Phenylethylamine	Cadaverine
10.60 mg/L	0.10 mg/L	0.50 mg/L	n.d.	0.90 mg/L	0.10 mg/L

Table 5.4 – *Level of the biogenic amines in the must. n.d. = no detected.*

Ethanolamine was the relevant amine in each step of winemaking process. The concentration of this compound decreases during AF and remains constant until the end of MLF (**Figure 5.3**).

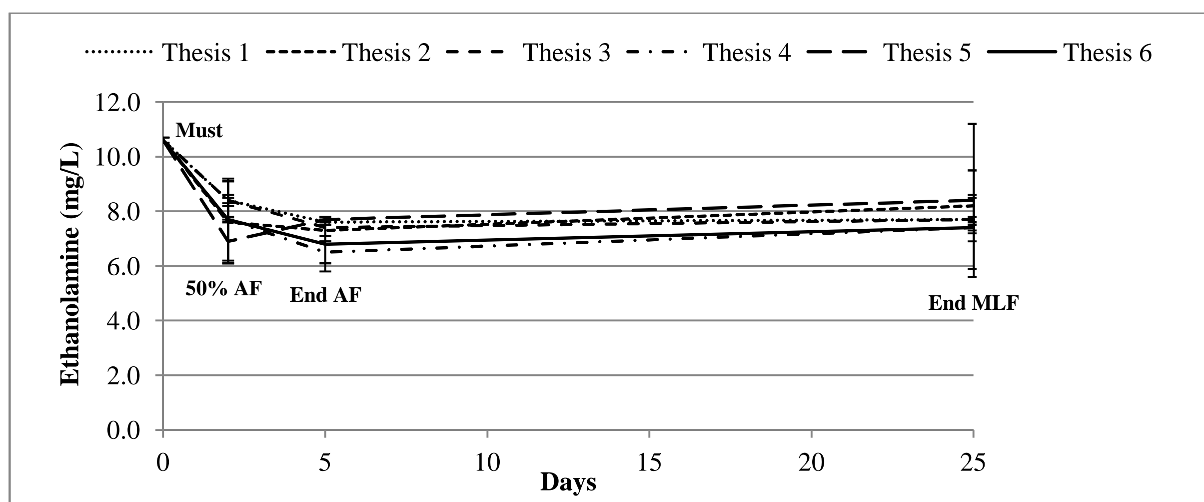

Figure 5.3 – *Ethanolamine trend during winemaking. 25th days corresponds at the end of MLF, thesis 6 termined MLF after 40 days but the concentration between 25h to 40th day remain constant. Error bars denote standard deviation.Thesis 1- bacteria inoculated in the must and the yeast 24 h later; thesis 2- bacteria inoculated 24 h after yeast; thesis 3 - bacteria inoculated at 30% of AF; thesis 4 - bacteria inoculated at 60% of AF; thesis 5- bacteria inoculated at end of AF; thesis 6 -control not inoculated with bacteria.*

The change during AF could be related to the role that this amine plays, in different pathways, as precursor of the phosphatidylethanolamine and then of the phosphatidylcholine, a phospholipid present in the membrane of the yeast (Schuiki *et al.*, 2010); in fact in Kennedy pathway exogenous ethanolamine and choline are both transported into the cell by the *HNM1*-encoded choline/ethanolamine transporter (Nikawa *et al.*, 1986).

In each thesis, during winemaking, cadaverine was detected in trace or not detected; this work confirms that this amine is not a problem for the wine.

The ethylamine and phenylethylamine slightly increase during AF and in MLF is constant as reported in **figure 5.4** and **figure 5.5**.

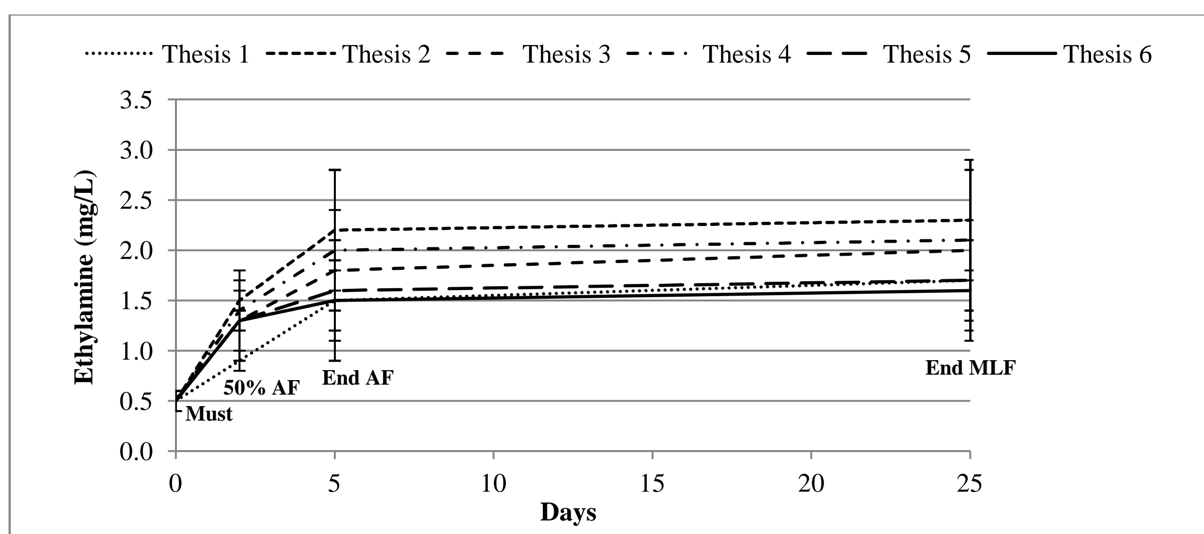

Figure 5.4 – *Ethylamine trend during winemaking. 25th days corresponds at the end of MLF, thesis 6 termined MLF after 40 days but the concentration between 25th to 40th day remain constant. Error bars denote standard deviation. Thesis 1- bacteria inoculated in the must and the yeast 24 h later; thesis 2- bacteria inoculated 24 h after yeast; thesis 3 - bacteria inoculated at 30% of AF; thesis 4 - bacteria inoculated at 60% of AF; thesis 5- bacteria inoculated at end of AF; thesis 6 -control not inoculated with bacteria.*

Related to ethylamine, literature data are not in agreement, in some studies ethylamine decreases during AF and in other instead increases. However, in all works this amine is always present in grape and in must. The data of phenylethylamine indicate that this compound can be present in the grapes but can be formed by yeast during AF too, according to literature data.

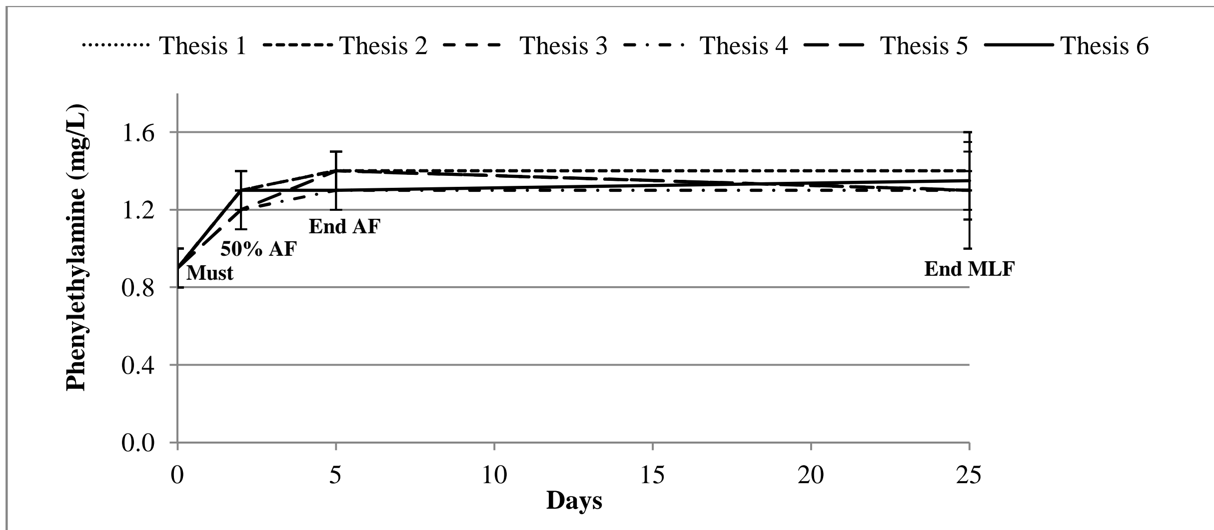

Figure 5.5 – *Phenylethylamine trend during winemaking. 25th days corresponds at the end of MLF, thesis 6 termined MLF after 40 days but the concentration between 25th to 40th day remain constant. Error bars denote standard deviation. Thesis 1- bacteria inoculated in the must and the yeast 24 h later; thesis 2- bacteria inoculated 24 h after yeast; thesis 3 - bacteria inoculated at 30% of AF; thesis 4 - bacteria inoculated at 60% of AF; thesis 5- bacteria inoculated at end of AF; thesis 6 -control not inoculated with bacteria.*

Tyramine was absent in must and not detected in all theses during AF and MLF, histamine was detected always at level very low and increase slightly during winemaking (**Figure 5.6**); these results confirm the inability of *L. plantarum* V22 to produce these amines.

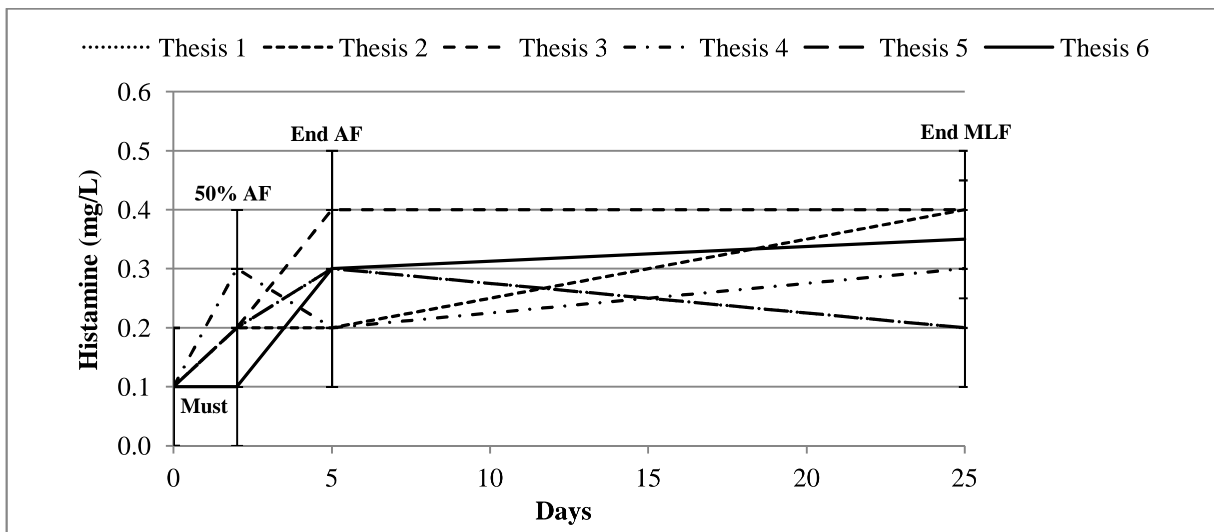

Figure 5.6 – *Histamine trend during winemaking. 25th days corresponds at the end of MLF, thesis 6 termined MLF after 40 days but the concentration between 25th to 40th day remain constant. Error bars denote standard deviation. Thesis 1- bacteria inoculated in the must and the yeast 24 h later; thesis 2- bacteria inoculated 24 h after yeast; thesis 3 - bacteria inoculated at 30% of AF; thesis 4 - bacteria inoculated at 60% of AF; thesis 5- bacteria inoculated at end of AF; thesis 6 -control not inoculated with bacteria.*

At bottling, we observed no significant differences in amine content among the six theses tyramine and cadaverine were present in trace or not detected, the histamine data are low and similar to values at the end of MLF (**Table 5.5**).

	Thesis 1	Thesis 2	Thesis 3	Thesis 4	Thesis 5	Thesis 6
Ethanolamine mg/L	7.70±1.84a	8.15±0.35a	7.65±0.78a	7.35±0.07a	8.35±2.76a	7.95±0.35a
Histamine mg/L	0.20±0.14a	0.35±0.07a	0.35±0.07a	0.30±0.01a	0.20±0.14a	0.40±0.01a
Ethylamine mg/L	1.65±0.64a	2.25±0.64a	1.95±0.78a	2.05±0.78a	1.70±0.42a	1.65±0.35a
Tyramine mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenylethylamine mg/L	1.35±0.21a	1.35±0.07a	1.25±0.07a	1.25±0.07a	1.30±0.28a	1.35±0.21a
Cadaverine mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	0.05±0.07

Table 5.5 – *Level of the biogenic amines at bottling in the different theses (arithmetic mean±standard deviation). Means followed by the same letter are not significantly different (P≤0.05). n.d. = no detected. Thesis 1- bacteria inoculated in the must and the yeast 24 h later; thesis 2- bacteria inoculated 24 h after yeast; thesis 3 - bacteria inoculated at 30% of AF; thesis 4 - bacteria inoculated at 60% of AF; thesis 5- bacteria inoculated at end of AF; thesis 6 -control not inoculated with bacteria.*

Amines content in the not inoculated thesis (thesis 6) is similar to the other theses and this suggests that LAB amino acids decaboxylases-positive were not present in grapes and/or that must conditions were not favorable to produce the biogenic amines. In this study, excluding tyramine and cadaverine (not detected), we observed that the different time of co-inoculation not significantly influences the behaviour of the biogenic amines during winemaking.

As reported in the literature (Herbert *et al.*, 2005), it's clear that grape variety, region of production and vintage can affect biogenic amines contents in must and wine, although AF and MLF can overcome these factors.

Figure 5.7 shows the concentration of each amine in different theses after one year in bottle. Histamine, ethylamine and phenylethylamine values are similar in all theses and slightly lower respect to bottling data. The theses are significantly different in ethanolamine content. In particular the thesis 1 and thesis 6 have a higher ethanolamine level than the other.

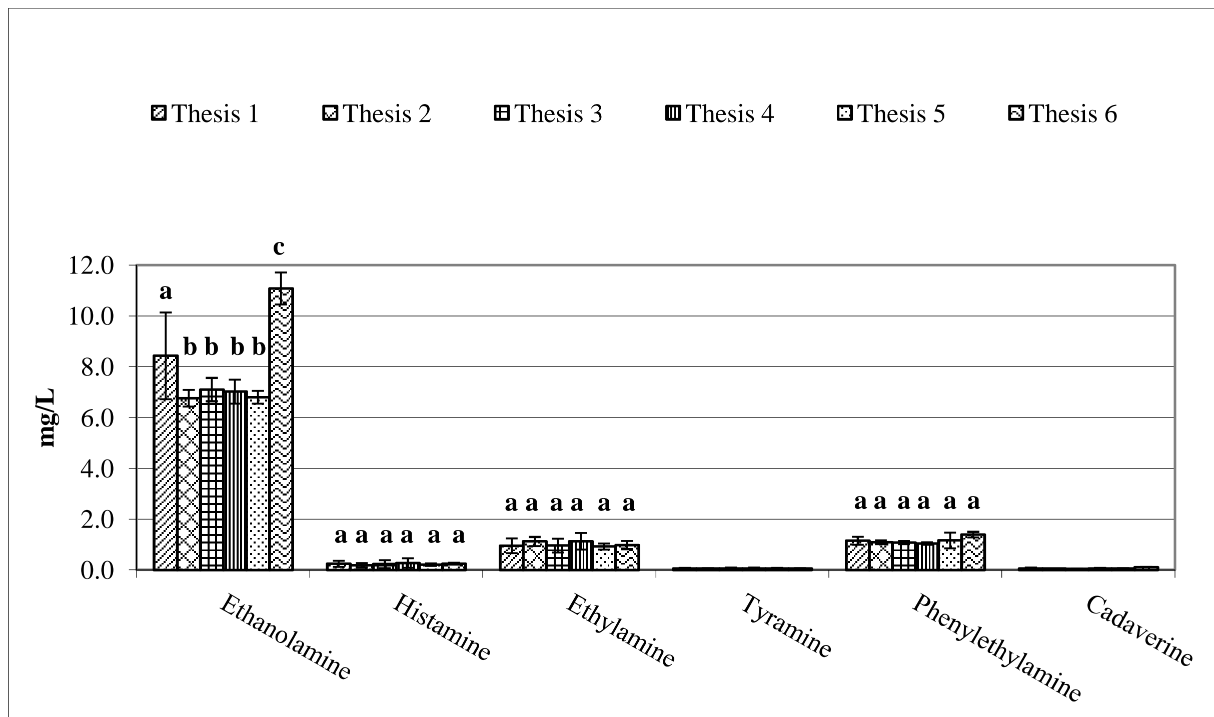

Figure 5.7 – *Level of biogenic amines of the five different inoculation time and the test in wine after1 year in bottle (wine filtered before bottling). Error bars denote standard deviation. Means followed by the same letter are not significantly different (P≤0.05). Thesis 1- bacteria inoculated in the must and the yeast 24 h later; thesis 2- bacteria inoculated 24 h after yeast; thesis 3 - bacteria inoculated at 30% of AF; thesis 4 - bacteria inoculated at 60% of AF; thesis 5- bacteria inoculated at end of AF; thesis 6 -control not inoculated with bacteria.*

Generally, most studies in the literature agree that there are slight variations in biogenic amine concentrations, corresponding to a slight decrease or stabilization of these compounds during wine storage.

The accumulation of ethanolamine in theses 1 and 6 during storage of wine in bottle is probably related to an unforeseen occurrence during bottling.

5.4 Conclusion

Simultaneous inoculation with yeast and bacteria could be an interesting winemaking practice with an easy protocol to carry out. Different studies have suggested that simultaneous inoculation of yeast and bacteria could have a negative impact on the kinetics of the AF (King and Beelman, 1986, Huang *et al.*, 1996; Jussier *et al.*, 2006).

Massera *et al.* (2009) described that a significant reduction in total fermentation time (AF and MLF) was observed when using simultaneous inoculation techniques compared to sequential inoculations.

This represents an important advantage for the wineries not only for the process efficiency, but also for safety, because reduce the presence of spoilage microorganisms and/or biogenic amines. Our results show that the co-inoculation of selected strains of *S. cerevisiae* and *L. plantarum*, does not have a negative impact on the performance of AF. No acetic acid was produced when the bacteria were inoculated in must, or during AF. Probably the successful of co-inoculation depend on the selection of suitable yeast-bacterium combinations.

Regarding to biogenic amines evolution in winemaking, our studies confirm that the major amines represented in must and wine are ethanolamine, ethylamine and phenylethylamine. These amines don't present particular risk for the human health. *L. plantarum* V22, used because is a HDC^- and TDC^-, confirms the inability to produce histamine and tyramine. These amines are not produced also in the thesis not inoculated with selected strains of bacteria. These last data confirm that the LAB rarely are able to produce biogenic amines according to the literature (Costantini *et al.*, 2006). The co-inoculation doesn't seem to influence the level of biogenic amines already present in must and their evolution is not related to the different co-inoculation time. Furthermore the use of the selected strains, without specifics amino acids decarboxylase, reduces the risk related to the presence in wine of potential allergen such as histamine and tyramine.

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Conclusions

Chapter 6 - Conclusions and future perspectives

The presence of substances which are physiologically active in the human metabolism has been extensively studied in wine and foods. Special interest has been paid to OTA and biogenic amines. These substances present different risk for the human health. OTA is a possible human carcinogen and long-term exposure has been implicated in Balkan Endemic Nephropathy and associated with urinary tract tumours (IARC, 2003; EFSA, 2006). OTA in wine is not common and, usually, is detected in wine produced in Mediterranean regions, but only a few wines have a concentration exceeding the legal limit of 2 µg/kg.

Biogenic amines are nitrogenous compound present in all wines. These substances are necessary for several critical functions in man, and they are also natural components of foods of animal or vegetable origin (Tesseidre *et al.*, 1995). In general, there is no risk for consumers. However, when high amine concentrations, in particular histamine and tyramine, are ingested, or the detoxification mechanisms of one or several amines are inhibited or generically deficient, their consumption may be problematic (Ten Brink *et al.*, 1990).

The research managed in this PhD is focused on the use of the selected starter for malolactic fermentation (MLF) to reduce these contaminants at guarantee of wine quality and safety assurance for the consumers.

The OTA reduction in wine by microorganisms used for MLF is considered. Another part of the study investigates the relationship between MLF, oenological practices, bacteria strains and presence of biogenic amines in wine. Among the lactic acid bacteria (LAB) used in MLF we considered the *Lactobacillus* spp. and in particular a strain of *Lactobacillus plantarum* that we have isolated from wine. At present this strain is commercialized by company Lallemand Inc. (Montréal, QC, Canada) as *Lactobacillus plantarum* V22.

In the detail the OTA removal by *L. plantarum* V22 is developed in chapter 3; the selection of *Lactobacillus* spp. strains, able to perform MLF without produce biogenic amines, is studied in chapter 4 and the relationship between different inoculation times of malolactic commercial starters is investigated in chapter 5.

Chapter 3 was centered to investigate the ability and the mechanism to remove OTA by *L. plantarum* V22, a lactic acid bacteria (LAB) strain selected to develop its metabolic activity in two different conditions, with and without malic acid, a natural carbon source present in wine.

The preliminary investigation in wine demonstrated that *L. plantarum* was able to reduce OTA. The capability to remove OTA is related to initial OTA concentration, and it is better at low concentration of the toxin. To study the OTA reduction mechanism it was performed the trial in synthetic media (YNB w/o amino acids and ammonium sulphate); the initial concentration of OTA in YNB was higher than in wine to detect the possible presence of OTα low quantity too. We observed some differences of OTA reduction kinetic in the two theses. In the +malic thesis the malic acid is completely degraded and a OTA reduction of 20.27% is registered. In the –malic thesis the OTA concentration in supernatant is, in the first time, similar to the control, followed by a low increase, probably due a release of OTA from cell bacteria to the medium. The contemporary O.D. decrease suggests that the bacteria cell lysis promote the OTA release. The different evolution of O.D. and OTA in + malic thesis and mass balance compared to the control thesis suggest that the OTA was partly metabolised by bacteria. This study confirms that the adsorption on the bacterial cell wall is one of the mechanisms to remove OTA and also an enzymatic pathway can be assumed. The OTA biodegradation process is aided by a carbon source. It is possible that in high stress conditions, caused to lack of nutrients, only a small quantity of OTA is adsorbed on the bacterial cell wall, because the bacteria don't growth and the cell lysis processes reduce the available adsorption surface and, also, the toxin already adsorbed could be released in the media. The OTA data in supernatant and the malic acid degradation suggest that *L. plantarum* doesn't use OTA as carbon source; rather, bacteria seem able to degrade OTA only with a carbon source, in our case malic acid. In general the OTA reduction in synthetic medium is very lower than in wine and this can be due to different composition of wine and YNB and to the high OTA concentration (25 μg/L) in YNB. High level of OTA could have a negative effect on the growth of LAB, as demonstrated by Piotrowska and Żakowska (2005).

OTα was not detected in media and in pellet in this conditions and we can suppose that Otα, produced by hydrolysis of amide bond of OTA, is metabolized by bacteria in substrate poor in nutrients and/or that other mechanisms of OTA degradation, in which no OTα is produced, could be involved, as reported by Madsen *et al.* (1983) and Li *et al.* (2000).

Chapter 4 confirms that just few strains of LAB naturally present in musts and wine are able to degrade malic acid and grow in particular conditions. In this work the effects of several chemico-physical factors (pH, temperature, SO_2, and ethanol concentration) on the growth and malolactic

activity of *Lactobacillus* spp. strains, isolated from different Australian musts and wines, were evaluated. The screening process in the synthetic wine medium was used as an indication of the possible survival and performance of the potential starter strains in the wine environment. Strains were first selected after characterization in a synthetic matrix, which resulted in 7 potential MLF strains. These strains were evaluated in wine to reject the strains as they did not complete the MLF. Other studies used synthetic or wine-like media for the characterization of LAB strains (Capozzi *et al.*, 2010; Lerm *et al.*, 2011).

On the basis of our results, few strains of LAB grow in the conditions similar to wine, the value of pH seem to be the more limiting condition; in fact in wine the malolactic activity is more influenced by the pH variation than by the ethanol. The pH and alcohol content of wine are important oenological parameters that determine bacterial growth, and consequently the capacity of malate degradation in *Lactobacillus* spp. cultures.

The inability to produce biogenic amines is an important characteristic for the strains considered for use in a starter culture, because the biogenic amines have an impact on wine quality and healthiness. In order to totally eliminate the potential of biogenic amine production, molecular screening of possible starter cultures for biogenic amine-encoding genes is a quick and efficient method to ensure this.

In our study, 35 strains of LAB were investigated to detect the *hdc*, *tdc* and *odc* genes that encode for amino acid decarboxylases (HDC, TDC and ODC). None of the bacteria has the *hdc* and *odc* genes, while only the *L. casei* B749 is tdc^{+}.

The problem of the presence of biogenic amines in wine may be associated with other variables affecting the level of these compounds; many studies investigated the relationship between cultivar, vintage, storage of the grape, oenological practices, microorganism and other wine compounds (Herbert *et al.*, 2005; Marques *et al.*, 2008; Moreno-Arribas and Polo, 2008; Marcobal *et al.*, 2006; Del Prete *et al.*, 2009; Cecchini and Morassut, 2010). It is possible that the formation of biogenic amines is due to microorganism associated with a lack of hygiene during the winemaking process.

In conclusion, in must and wine there are few autochthonous LAB able to grow in the physico-chemical wine conditions and to perform MLF. As to the biogenic amines, we confirm that the presence of *hdc*, *tdc* and *odc* genes *Lactobacillus* spp. is low, and that the molecular screening is a good rapid technique to detect bacteria potential producers of biogenic amines. On the basis of our results, the strains of *Lactobacillus* spp. who perform MLF are more frequently *L. plantarum,* and these are not able to produce tyramine, histamine and putrescine.

The aim of the study presented in chapter 5 was to assess the capability of a strain of *L. plantarum*, inoculated in different steps of winemaking process to perform MLF. The influence of co-inoculation and sequential inoculation on the biogenic amines naturally present in must and/or produced during winemaking was investigated. In spite of the low level of pH, *L. plantarum* V22 finish the MLF in 20 days and the wine doesn't show quality alteration; in fact the levels of acetic acid are not high in each thesis. The *Lactobacilli* usually perform MLF at levels of pH between 3.6 and 3.8; this work demonstrates that this commercial starter can perform MLF also at low levels of pH (~3.3) and the different inoculation time doesn't affect the performance of MLF.
Regarding to biogenic amines evolution in winemaking our studies confirm that the major amines represented in must and wine are ethanolamine, ethylamine and phenylethylamine. These amines don't present particular risk for the human health. *L. plantarum* V22, used because is a HDC^- and TDC^-, confirms the inability to produce histamine and tyramine. These amines are not present also in the thesis not inoculated with selected strains of bacteria. These last data confirm that the LAB rarely are able to produce biogenic amines according to literature (Costantini *et al.*, 2006).
The sequential inoculation doesn't seem to influence the level of biogenic amines already present in must and their evolution is not related to the different inoculation time. Furthermore the use of the selected strain, without specifics amino acids decarboxylase, reduces the risk related to the presence in wine of potential allergen such as histamine and tyramine.

In conclusion, regarding to OTA removal in wine by LAB, this PhD work shows that the *L. plantarum* V22 could be able to reduce the toxin, not only by adsorption, but also by enzymatic degradation; nevertheless, the nutrients availability and initial OTA concentration seem to be limiting factors for the OTA bacterial reduction. The research concerning the biogenic amines included the investigation of the relationship between inoculation time and amines behaviour and the molecular screening to detect bacteria able to perform MLF without produce biogenic amines. This work demonstrates that the use of co-inoculation and sequential inoculation in winemaking with commercial starter, HDC^- and TDC^-, doesn't influence the trend of amines naturally present in wine and there are not problems related to the presence of amines as histamine and tyramine. Moreover, there is not a quality decrease of the product when a strain of *L. plantarum* is used.
On the basis of these results, even if the OTA reduction ability by *L. plantarum* V22, in particular by enzymatic hydrolysis, must be confirmed, this bacteria could be used as MLF starter in winemaking because it is able to perform MLF in different conditions of inoculation and reduces the OTA concentration; moreover, the risk of the presence of unwanted biogenic amines in wine, as histamine and tyramine, is minimized.

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