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DOCTORAL DISSERTATION

Study of *Oenococcus oeni* to improve wine quality

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To my parents

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PREFACE

Lactic acid bacteria (LAB) are, besides yeasts, the best adapted microbial family to wine conditions. Many genera have been isolated both from grape must and wine, and they represent an important resource in winemaking since most of them are able to perform malolactic fermentation (MLF), the conversion of L-malic acid into L-lactic acid, which is often required to obtain wines with positive flavor and taste characteristics, but has to be avoided in some cases.

Among LAB, *Oenococcus oeni* is without any doubt the best adapted species to the wine environment, and is often used as a starter to perform MLF. However, this step in winemaking is often difficult to induce and control. Moreover, this microorganism requires up to 10 days to grow and develop countable colonies on plate using classical microbiological methods to enumerate viable cells, and the control of the inoculation, as well as the evaluation of the presence or absence of *O. oeni* in a sample, requires usually a considerable amount of time. For these reasons one of the purposes of this research project was the development of a Propidium monoazide - quantitative PCR (PMA-qPCR) technique for the fast enumeration of *O. oeni* in must and wine, and the results obtained show how the developed technique is able to provide a detection limit (0.33 log CFU/mL in must and 0.69 log CFU/mL in wine) which is lower than all of the other molecular biology techniques developed until now.

Furthermore, to better understand which conditions are the most favorable for a successful MLF, a Reverse Transcription – quantitative PCR (RT-qPCR) technique has been developed to study the gene expression levels of the *mleA* gene, encoding for the malolactic enzyme, in *O. oeni*. The results obtained show that co-inoculation with *Saccharomyces cerevisiae* and high concentrations of ethanol in the medium are the best conditions to ensure high levels of transcription of the *mleA* gene.

Besides the capacity of performing MLF, LAB are capable to influence the aromatic complexity of wine thanks to the release of volatile compounds due to the activity of the β-glucosidase enzyme, which has been isolated in various strains, including *O. oeni*. For this reason, the last purpose of this work has been the development of a RT-qPCR technique to find out which winemaking practice (sequential inoculation or co-inoculation) is the best to ensure high levels of transcription of the gene encoding for the β-glucosidase enzyme. Results point out that during co-inoculation higher levels of expression are registered.

Therefore, and although winemakers try often to avoid this practice, co-inoculation can be considered the best winemaking scenario to ensure both rapid completion of MLF and expression of β-glucosidase encoding gene, which can lead to the release of positive aromatic volatile compounds in wine.

PREFAZIONE

I batteri lattici (LAB) sono, insieme ai lieviti, la famiglia microbica meglio adattata alle avverse condizioni chimiche e chimico-fisiche presenti nel vino. Molti generi sono stati isolati sia dal mosto che dal vino, ed essi rappresentano un'importante risorsa nelle pratiche enologiche, dal momento che molti sono in grado di effettuare la fermentazione malolattica (FML), la conversione dell'acido L-malico in acido L-lattico, che è spesso necessaria per ottenere vini con caratteristiche desiderate di sapore e gusto, ma che in alcuni casi deve essere evitata.
Tra i LAB, *Oenococcus oeni* è senza dubbio il meglio adattato alle condizioni del vino, ed è spesso usato come starter per eseguire la FML. Tuttavia questa fase è spesso difficile da indurre e controllare nella produzione del vino. In più questo microorganismo richiede fino a 10 giorni per crescere e dare origine a colonie visibili e contabili in piastra utilizzando metodi di microbiologia classica per contare le cellule vitali, e il controllo dell'inoculo, così come la valutazione della presenza o dell'assenza di *O. oeni* in un campione, richiede di solito una significativa quantità di tempo. Per queste ragioni uno degli scopi di questo progetto di ricerca è stato lo sviluppo di una tecnica di PCR quantitativa basata sull'uso di Propidio Monoazide (PMA-qPCR) per la rapida enumerazione di *O. oeni* in mosto e in vino, e i risultati ottenuti mostrano che tale tecnica presenta un limite di rilevabilità del microorganismo (0.33 log UFC/mL in mosto e 0.69 log UFC/mL in vino) più basso rispetto a tutte le altre tecniche di biologia molecolare finora sviluppate.
In seguito, per meglio comprendere quali condizioni siano le più favorevoli affinchè la FML venga eseguita con successo, è stata sviluppata una tecnica di trascrizione inversa seguita da PCR quantitativa (RT-qPCR) per studiare i livelli di espressione genica del gene *mleA*, codificante per l'enzima malolattico, in *O. oeni*. I risultati ottenuti indicano che il coinoculo con *Saccharomyces cerevisiae* e alte concentrazioni di etanolo nel mezzo sono le condizioni migliori per assicurare alti livelli di trascrizione del gene *mleA*.
Accanto alla capacità di eseguire la FML, i LAB sono in grado di influenzare la complessità aromatica del vino grazie al rilascio di composti volatili dovuto all'attività dell'enzima β-glucosidasi, che è stato isolato da diversi ceppi, anche appartenenti alla specie *O. oeni*. Per questa ragione l'ultimo obiettivo di questo lavoro è stato lo sviluppo di una tecnica RT-qPCR al fine di individuare quale tecnica produttiva (inoculo sequenziale o coinoculo) fosse la migliore per assicurare alti livelli di trascrizione del gene codificante per l'enzima β-glucosidasi. I risultati evidenziano che durante il coinoculo vengono registrati livelli di trascrizione più alti.
Perciò, nonostante spesso i produttori tendano ad evitare questa pratica, il coinoculo può essere considerato lo scenario migliore per assicurare una rapida conclusione della FML e alti livelli di espressione del gene codificante per l'enzima β-glucosidasi, che può condurre al rilascio di composti aromatici volatili favorevoli nel vino.

CHAPTER 1
Literature review:
Lactic Acid Bacteria and Wine

1. LACTIC ACID BACTERIA AND WINE

Although wine is a harsh environment (low pH, high content in ethanol, high concentration in SO_2) (Versari et al., 1999), it can be considered a nutrient medium in which bacteria can develop and grow because of the presence of amino acids, vitamins and fermentable compounds such as malic acid, citric acid and residual sugars (Riberéau-Gayon et al., 2006).
In this medium lactic acid bacteria (LAB) represent an important resource for winemakers since they are able to perform malolactic fermentation (MLF), which mainly consists of the conversion of the strong dicarboxylic L-malate into the softer L-lactate and CO_2 (Favier et al., 2012) and lasts from between a few days to several months depending on wine composition, temperature and LAB population (Fleet et al., 1984, Lafon-Lafourcade et al., 1983). To improve the fermentation process and enhance wine quality and safety the use of malolactic starters is becoming a common winemaking practice (Torriani et al., 2011).
The LAB genera most commonly found in wine are *Leuconostoc, Weissella, Pediococcus, Lactobacillus* and *Oenococcus* (Dicks and Endo, 2009).

***Leuconostoc* spp.**
Microorganisms belonging to this genus are ovoid cocci with a cell diameter between 0.5 and 0.7 µm and length between 0.7 and 1.2 µm. Cells are either single, arranged in pairs or form short to medium length chains (Ribéreau-Gayon et al., 2006). They are hetero-fermentative microorganisms producing D(-)-lactic acid, ethanol, carbon dioxide and acetic acid. They are also able to ferment citrate producing diacetyl, thus this genus is considered important for flavour development in dairy products (Vedamuthu, 1994).
The only species found in wine is *Leuconostoc mesenteroides*, which has been mostly isolated in grape must at the beginning of the vinification process (Lonvaud-Funel, 1999). Some strains of *Leuconostoc mesenteroides* subsp. *mesenteroides* convert L-malate to L-lactate, but only in the presence of a fermentable carbohydrate. Production of dextran from the fermentation of sucrose is possible (Dicks and Endo, 2009). Moreover, some strains can produce a heme-requiring catalase (Whittenbury, 1966). Growth is possible up to 6.5% (w/v) NaCl and they are auxotrophic for glutamic acid and valine. 90% or more of the strains are able to ferment arabinose, fructose, galactose, maltose, sucrose and trehalose (Dicks and Endo, 2009).
It has been demonstrated (Mtshali et al., 2012) that some strains possess the *bgl* gene coding for the β-glucosidase enzyme, but it is not proved if this gene is transcribed and translated into an active enzyme under oenological conditions, since most β-glucosidases in wine are regulated by winemaking parameters (Spano et al., 2005).

***Weissella* spp.**

The only species belonging to this genus isolated in wine environment is *Weissella paramesenteroides* (Petri et al., 2013), previously classified as *L. mesenteroides* (Martin et al., 2005).

The cells are normally spherical, but often ovoid and assembled in pairs and chains, non-motile, non-sporeforming. From a metabolic point of view this LAB can't produce ammonia from arginine and dextran from sucrose. The optimal growth temperature is 30°C, pH should be below 5.0 (Dicks and Endo, 2009).

***Pediococcus* spp.**

Pediococci cells are usually spherical, but may be ovoid, and they divide to form pairs or tetrads. Cell diameter is between 0.5 and 2 µm, asporogenic, catalase- and oxidase-negative. They are homo-fermentative and glucose is converted to lactic acid via the Embden-Meyerhof pathway (Holzapfel et al., 2009). *P. damnosus, P. inopinatus, P. parvulus* and *P. pentosaceus* are the species most frequently isolated from wine (Dicks and Endo, 2009).

Some strains of *P. pentosaceus* produce pseudocatalase (Simpson and Taguchi, 1995) and are able to catabolize arginine by the arginine deiminase pathway (Araque et al., 2009). When arginine is not completely catabolized to CO_2, ammonia and ATP, intermediate products of the pathway such as citrulline and carbamoyl-P can accumulate in the medium and originate ethyl carbamate, a carcinogenic compound (Vahl, 1993), after reaction with ethanol (Uthurry et al., 2006). Ornithine is also a putrescine precursor, and the accumulation of this compound could resolve in putrescine formation (Araque et al., 2013).

P. damnosus , which can grow at temperature values between 8 and 30 °C and produce acetoin or diacetyl, has been described as able to synthetize exopolysaccharide (Carr, 1970; Llaubères et al., 1990) and thus lead to the formation of ropyness in wines. Only some *Pediococcus damnosus* strains are able to synthesize this exopolysaccharide (Lonvaud-Funel and Joyeux 1988). Its production is linked to the presence of a specific gene in a plasmid or in the bacterial chromosome. *P. damnosus* is normally present in grape must and disappears almost completely during the winemaking process and/or develops during ageing. Nevertheless, sometimes this species is largely involved in malolactic fermentation. Therefore, as most *P. damnosus* strains are not spoilage agents, the winemaker must specifically detect the presence of ropy strains so that the wine may be treated before bottling, if necessary (Lonvaud-Funel 1999) and a Real-Time PCR technique has been developed to identify and quantify ropy strains of *P. damnosus* in wine (Delaherche et al., 2004).

***Lactobacillus* spp.**

Lactobacillus is the largest genus amongst all LAB, with approximately 100 known species and at least 16 subspecies. Their optimal growth temperature is between 30 and 40 °C, and all species are aciduric, with optimal growth between pH 5.5 and

6.2 (Dicks and Endo, 2009). *Lactobacillus* cells are usually non motile, regular elongated with diameter between 0.5 and 1.2 µm and length up to 10 µm, non-sporulating and often assembled in pairs or chains (Du toit et al., 2011).
Many *Lactobacillus* spp. are able to perform malolactic fermentation, leading to the conversion of L-malate to L-lactate and CO_2, and the species most commonly isolated in wine environment are *Lb. brevis, Lb. plantarum, Lb. collinoides, Lb. buchneri, Lb. hilgardii, Lb. fructivorans, Lb. kunkeei, Lb. nagelii, Lb. vini, Lb. mali and Lb. fermentum* (König and Fröhlich, 2009).
All species need complex and rich growth media with amino acids, peptides, nucleic acids derivates, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates (Dicks and Endo, 2009).
It is proved that *Lactobacillus* spp. can not only survive the winemaking conditions, but also perform secondary metabolic reactions which are involved in enhancement of wine aroma and flavor such as metabolism of citrate, amino acid, polysaccharides, polyols, aldehydes, glycosides, esters, phenolic acids, lipids, poteins and peptides (Liu, 2002; Matthews et al., 2004). Furthermore many species belonging to this genus can produce bacteriocins that could determine the survival of the strain in a competitive environment. For these reasons the interest in using *Lactobacillus* spp. as a starter to carry out MLF is high (Bou and Krieger, 2004; Du Toit et al., 2011), and actually it is possible to find a malolactic starter culture on the market (NT-202 co-inoculant, Anchor yeasts, Cape Town, South Africa) which consists in a blend of *Oenococcus oeni* and *Lb. plantarum*, assuring the winemaker the security deriving from O. oeni and the aromatic potential associated to the presence of Lb. plantarum.

***Oenococcus* spp.**
The genus *Oenococcus* was described for the first time by Dicks et al. (1995), and *O. oeni* is the best malolactic bacterium adapted to wine conditions, as well as the most commonly used as malolactic starter in winemaking.
This microbial group had previously been considered as belonging to the genus *Leuconostoc* (*Leuconostoc oenos*) (Garvie, 1967). Subsequent observations, however, have questioned this membership, since *L. oenos* was the only acidophilus member of the genus *Leuconostoc*. *L. oenos* strains were distinguishable from other *Leuconostoc* spp. for their ability to grow under conditions of high acidity and in a media containing high concentrations of ethanol (10% v/v of ethanol), by its request of tomato juice as growth factor (most of the strains), and by their lack of NAD-dependent glucose-6-phosphate dehydrogenase (Whitman et al., 1990).
DNA-DNA hybridisation studies (Dicks et al., 1990), confirmed by DNA-RNA hybridization and the analysis of 16S rRNA sequencing (Martinez-Murcia and Collins, 1990) revealed a distinction between genotypic *L. oenos* and the other *Leuconostoc* spp., leading to the classification of the new genus *Oenococcus*. In particular, the sequencing analysis of the 16S rRNA (Martinez-Murcia and Collins,

1990) and the 23S rRNA (Martinez-Murcia et al., 1993) have clearly shown that *O. oeni* forms a distinct line of descent, separate from *Leuconostoc* sensu strictu (including and *Leuconostoc paramesenteroides*) (Figure 1).

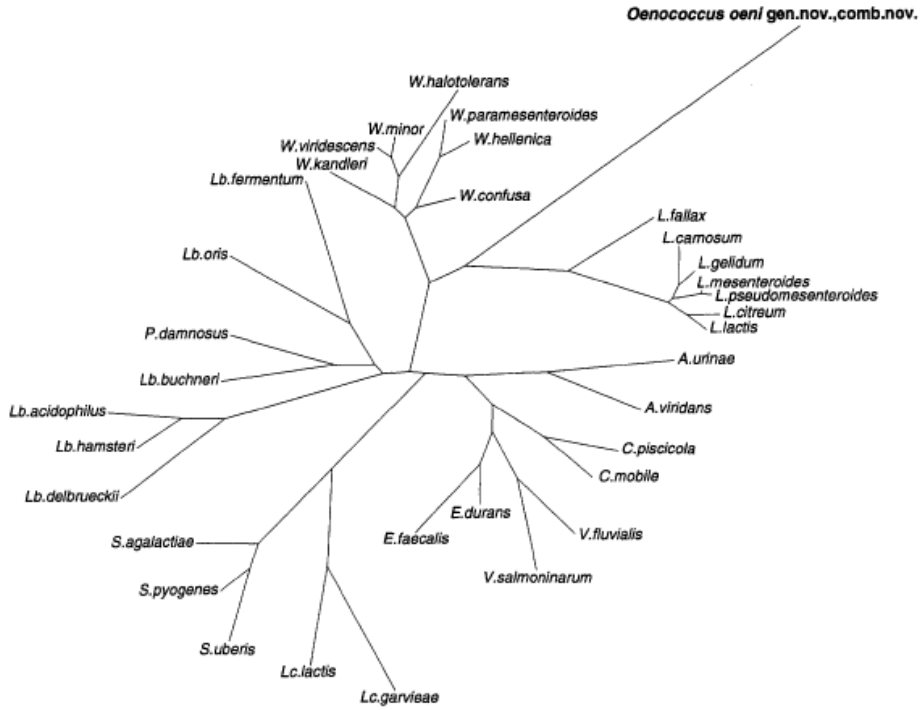

Figure 1. Unrooted phylogenetic tree showing the relationship of 0. *oeni* to *Leuconostoc* spp. and "lactic acid bacteria" based on 16s rRNA sequences. Abbreviations: *W., Weissella;L b., Lactobacillus;L ., Leuconostoc; P., Pediococcus; A., Aerococcus; C.,C arnobacterium; E., Enterococcus;* S., *Streptococcus; V., Vibrio;L c., Lactococcus* (Dicks et al., 1995).

Oenococcus cells are cocci (diameter of about 5 µm), arranged in pairs or chains; the morphology varies from strain to strain and is influenced by the growth medium. These microorganisms are hetero-fermentative and facultative anaerobes (Dicks et al., 1995).

Recently Endo and Okada (2006) isolated from the Japanese distilled product shochu a new microorganism classified as *Oenococcus kitharae*, proving that the genus *Oenococcus* was not constituted exclusively of the species *O. oeni*. Unlike *O. oeni*, this new species is not acidophilus (optimal growth at pH between 6.0 and 6.8), grows at an optimal temperature of 30°C, is not able to conduct malolactic fermentation, cannot grow in presence of 10% (v/v) of ethanol and is able to ferment maltose.

O. oeni is a non-motile, non-sporulating organism, able to grow on grapes, in must and wine (Whitman et al., 1990), and it seems to be the predominant species in South African brandy base wines, produced without sulphur dioxide (Du Plessis et al., 2004). Bacteriophages specific for *O. oeni* were isolated from sugar cane,

which could indicate that this species may be associated with habitats rich in sugar (Nel et al., 1987). However, the isolation of strains of *O. oeni* from environments different from wine and cider has never been reported (Whitman et al., 1990).
The growth of *O. oeni* is supported by a complex combination of amino acids, peptides, fermentable carbohydrates, fatty acids, nucleic acids and vitamins. In fact, biotin, nicotine, thiamine, pantothenic acid or derivatives thereof are required by most strains (Whitman et al., 1990). Amino acid auxotrophies of *O. oeni* have been well investigated, indicating that all of the strains cannot grow if glutamic acid, arginine and isoleucine are not present in the growing medium (Garvie, 1967; Fourcassie et al., 1992), and limitation of amino acid has been suggested as a possible factor for difficulties to induce MLF (Guilloux-Benatier et al., 1985). Moreover, Remize et al. (2005) found that bacterial growth yield was higher in the presence of nitrogen from peptides than that from free amino acids.
O. oeni is the main species of LAB found in wine during malolactic fermentation, it is in fact the most adapted to grow in a hostile environment such as wine, being resistant to low pH and high concentrations of ethanol and SO_2 (Bourdineaud et al., 2004; Maicas et al., 1999). Resistance of *O. oeni* to wine conditions could derive from plasmid carried genes. Favier at al. (2012) indeed proved the presence in starter and laboratory strains of *O. oeni* of two plasmids (pOENI-1 andpOENI-1v2) encoding the proteins TauE and OYE, which could be useful for wine bacteria. TauE is a membrane transporter involved in entrance or release of sulphur-containing compounds, while OYE belongs to the family of the "old yellow enzymes", which are involved in stress response mechanisms.
The malolactic activity of strains of *O. oeni* is optimal at pH values ranging from 3.0 to 3.2 and
the optimal temperature for growth of this microorganism is between 20 and 30°C. However, in winemaking conditions the most favorable temperature range is between 20 and 23°C. When the alcohol concentration of the medium increases (up to 13-14% v/v) the optimal temperature decreases and growth becomes, therefore, slower (Riberèau-Gayon et al., 2006).
Under these conditions and in the presence of fermentable carbohydrates, *O. oeni* converts L-malic acid to L (+)-lactic acid and CO_2. This species is able to ferment only a few carbohydrates: fructose and trehalose are the preferred sugars. In grape must or wine, pentose sugars (xylose and arabinose) are fermented first than glucose, and variable activities were recorded for the fermentation of arabinose, cellobiose, galactose, mannose, melibiose, salicin and xylose. Sucrose, lactose, maltose, mannitol and raffinose are not fermented. The esculin and arginine are hydrolyzed by certain strains, but only in wine or wine-related habitats. Some strains ferment citrate in the presence of a fermentable carbohydrate (Dicks et al., 1995).

2. METABOLISM OF LAB

Carbohydrates

Concerning the carbohydrate metabolism, LAB can be divided into three groups: obligatory homo-fermentative, facultative hetero-fermentative and obligatory hetero-fermentative (Moreno-Arribas and Polo, 2009).

Homo-fermentative microorganisms are able to ferment glucose with production of lactic acid and ATP through the Embden-Meyerhof-Parnas pathway (Hornsey, 2007). This group includes *Pediococcus* and some *Lactobacillus* species (Fugelsang and Edwards, 1997).

Hetero-fermentative microorganisms produce other compounds, such as acetate and ethanol, besides lactate in fermentation of glucose through the pentose phosphate (or phosphoketolase, or 6-phosphogluconate/phosphoketolase) pathway. LAB can be either obligatory hetero-fermentative or facultative hetero-fermentative (Du Toit et al., 2011). *Leuconostoc* species, some species of the *Lactobacillus* genus and *Oenococcus oeni* are obligatory hetero-fermentative. Facultative hetero-fermentative LAB like *L. casei* and *L. plantarum* are hetero-fermentative for hexose and homo-fermentative for pentose (Fugelsang and Edwards, 1997; Liu, 2002; Hornsey, 2007; Riberèau-Gayon et al., 2006).

Many LAB can ferment pentose sugars, which are metabolized by the bottom half of the pentose phosphate pathway after phosphorylation and conversion into phosphate derivates. The end-products of pentoses metabolism are equimolar emounts of lactic acid, acetic acid and CO_2 (Lerm et al., 2010).

Malic acid

The decarboxylation of malic acid to lactic acid and CO_2 is an important step in winemaking carried out by the enzyme malate decarboxylase, often referred to as the malolactic enzyme (Swiegers et al., 2005), which requires NAD^+ and Mn^{++} as cofactors (Lonvaud-Funel and Strasser de Saad, 1982). Many LAB strains, belonging to the genera *Oenococcus, Leuconostoc, Lactobacillus* and *Pediococcus* are able to perform MLF, and the rate of malate decarboxylation is correlated to the specific malolactic activity of the bacterial cell (Bartowsky, 2005).

The physiological function of MLF is to generate a proton motive force (PMF) which is important to acquire energy to perform essential cellular processes. The pathway includes the uptake of L-malate, its decarboxylation to L-lactic acid and CO_2 and excretion of the end products (including a proton). The electrochemical energy released during this reaction can be conserved via an indirect electrical potential ($\Delta\Psi$). Since a proton is consumed, the internal pH of the bacterial cell increases, leading to a pH gradient (Δ_{pH}) across the membrane. These two components make up the PMF which generates ATP via membrane ATPases (Versari et al., 1999).

In *O. oeni* the malolactic enzyme is encoded by the *mleA* gene, while the malate permease is synthetized by translation of the *mleP* gene (Labarre et al., 1996a).

These two genes are organized in an mle locus (figure 2). Upstream of the *mleA* gene, an open reading frame likely to encode a LysR-type regulatory protein was found (Labarre et al., 1996b), but the role of this regulatory protein in malolactic gene expression in *O. oeni* has not been determined yet (Galland et al., 2003). On the other hand, it has been shown that the activation of the malolactic system in *L. lactis* is mediated by *mleR* (Renault et al., 1989) .

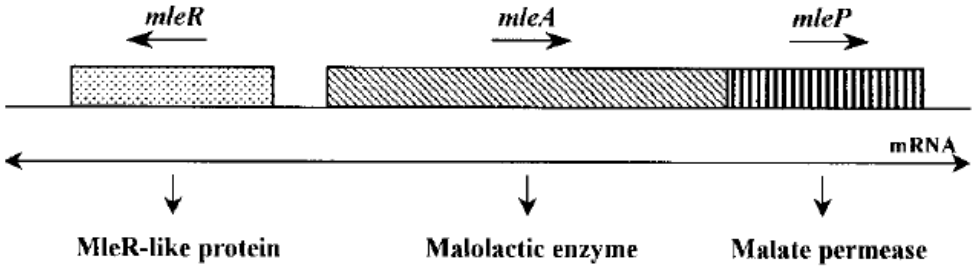

Figure 2. Genetic organization of the *mle* genes of *O. oeni*. The *mleA* and *mleP* genes encoding the MLE and the malate permease of *O. oeni*, respectively, are transcribed in an operon. Upstream of the *mle* operon, another gene encoding an MleR-like protein is transcribed divergently. This protein is related to the LysR-type regulatory protein family (Labarre et al., 1996b).

The deacidification of wine by the conversion of a dicarboxylic acid to a monocarboxylic acid, leading to an increase in pH of 0.1 to 0.3 units (Margalit, 1997) and to a less aggressive and milder taste of the final product (Lounvaud-Funel, 1999) is the main reason why MLF is generally considered as a favorable process in winemaking. Furthermore, MLF contributes to microbial stability removing malic acid as a possible carbon source available for microorganisms. Therefore MLF plays an integral role in the production of the majority of red wines, as well as some white cultivars including Chardonnay and some sparkling wines (Lerm et al., 2010). Reduction in pH could be considered an advantage for high-acid or low pH wines produced in cool climate regions, where deacidification is recommended for the production of acid-balanced wines, but it must be avoided in low-acid or high pH wines of warmer climate regions, where deacidification could have a negative impact on wine quality (Jackson, 2003).

Citric acid

Obligatory hetero-fermentative cocco-bacilli (*Leuconostoc* and *Oenococcus*) and facultative hetero-fermentative lactobacilli (*L. plantarum* and *L. casei*) are able to degrade citric acid in wine (figure 3), leading to the production of acetic acid, lactic acid, acetoin, 2,3-butanediol, diacetyl and aspartic acid (Lonvaud-Funel, 1999).

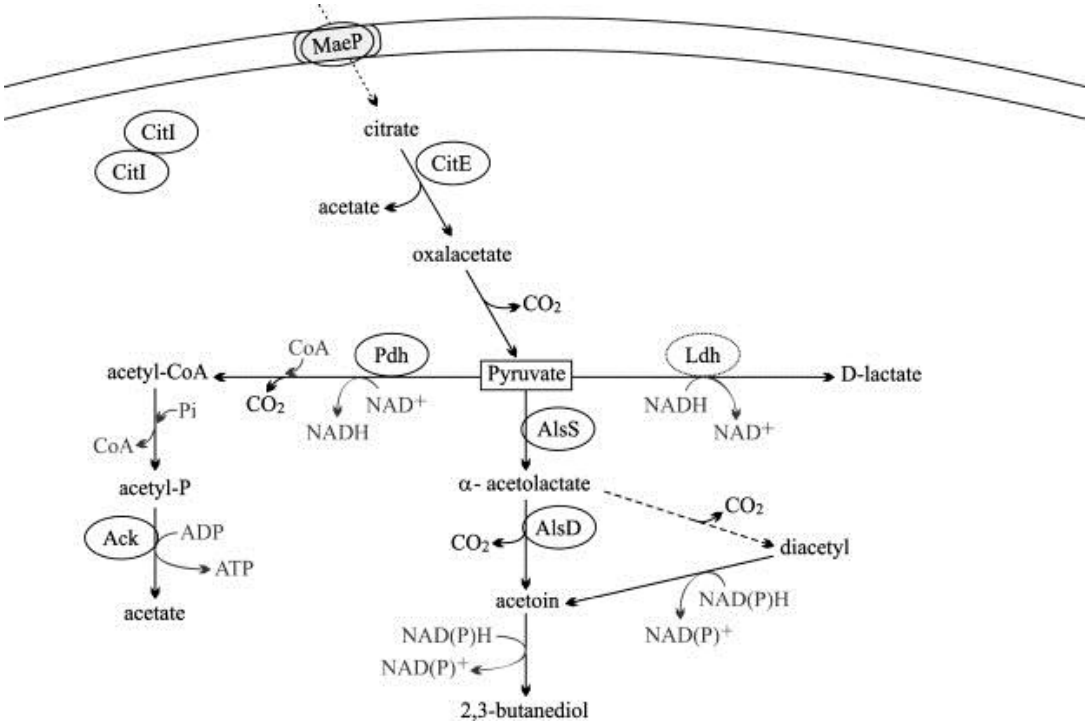

Figure 3. Main pathways for citrate/pyruvate metabolism by *O. oeni*. Genes analyzed in this study: *citI* – transcriptional activator; *maeP* – putative citrate permease; *citE* – citrate lyase; *pdh* – pyruvate dehydrogenase; *ackA* – acetate kinase;*ldh* – lactate dehydrogenase; *alsS* – α-acetolactate synthase; *alsD* – α-acetolactate decarboxylase. Dashed arrow toward diacetyl denotes a nonenzymatic reaction (Olguìn et al., 2009).

The most important meaning of citric acid metabolism carried out by LAB in a wine environment is the production of diacetyl, which is responsible for a characteristic buttery flavour note, one of the most easily recognisable changes in wine during MLF (Martineau et al., 1995). This compound is considered to contribute to wine aroma when it is present in small concentrations (1 to 4 mg/L), but in concentrations between 5 and 7 mg/L or higher it gives an undesirable rancid-like flavour (Davis et al., 1985). Several factors (wine type, inoculation rate and strain of malolactic bacteria, aeration, pH, SO_2) can influence the concentration of diacetyl in wine (Bartowsky and Henschke, 2004).

Another product of citrate metabolism is acetic acid, which contributes to the sensory perception of volatile acidity in wine (Du Toit et al., 2011). High pH values favour acetic acid formation during citric acid metabolism (Ramos et al., 1995).

Other metabolic pathways

As shown in figure 4, malolactic bacteria are able to metabolize a wide amount of compounds, and often the end products of these metabolic reactions are able to affect the sensorial quality of wine.

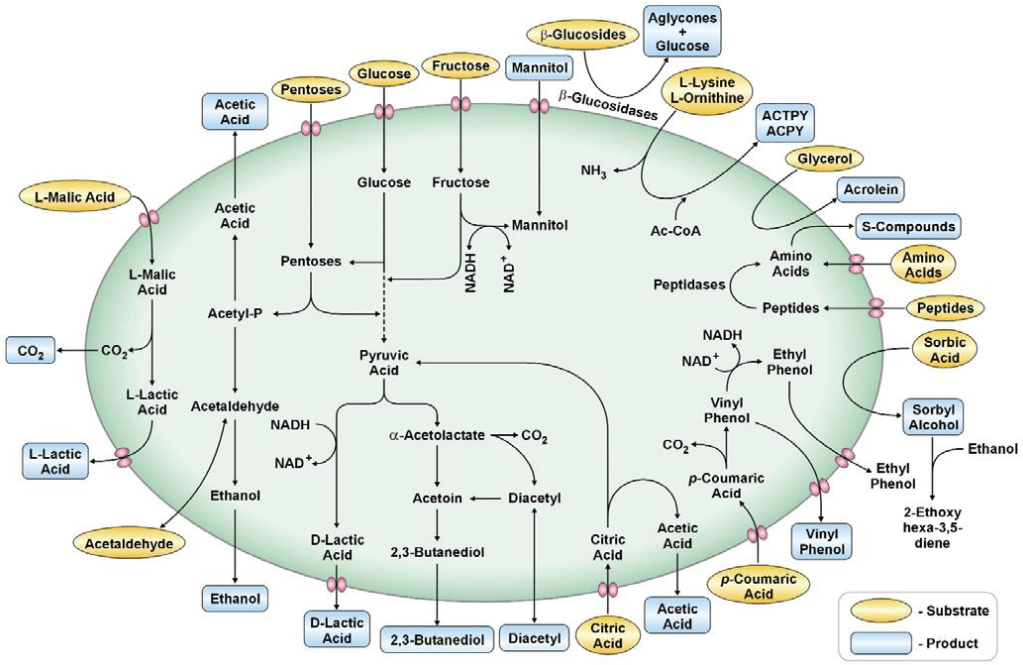

Figure 4. A schematic representation of the biosynthesis and modulation of flavour-active compounds by malolactic bacteria. (Swiegers et al., 2005)

Sorbic acid, often used as a preservative against yeasts in sweetened wines, can be reduced to sorbic alcohol by certain LAB (including *O. oeni*). Sorbic alcohol reacts then with ethanol generating 2-ethoxyhexa-3,5-diene, responsible for a geranium-like odour (Crowell and Guymon, 1975).

It is proved that wine-related LAB are able to convert L-ornithine and L-lysine to 2-ethyltetrahydropyradine (ETPY), 2-acetyl-1-pyroline (ACPY) and 2-acetyltetrahydropyradine (ACTPY), heterocyclic volatile nitrogen bases responsible for the "mousy" off-flavour in spoiled wines. This synthesis requires the availability of a fermentable carbohydrate source, ethanol and iron (Fe^{2+}) (Costello and Henschke, 2002).

Methionine and cysteine can be used by LAB as a substrate for the biosynthesis of volatile sulphur compounds, which are usually considered as a negative character in wine flavour (Pripis-Nicolau et al., 2004). Grape juice is usually deficient in these two amino acids (Landaud et al., 2008), but yeasts can synthetise them from inorganic sulphate/sulphite sources (Moreira et al., 2002) making them available for LAB metabolism.

All of the wine-related LAB genera are able to produce biogenic amines during fermentation, mainly by decarboxylation of amino acids, in response to

environmental stress factors (Spano et al., 2010). Several studies proving the presence of biogenic amines in wines originating from different countries worldwide have been published (Glòria et al., 1998; Landete et al., 2005; Soufleros et al., 2007). Putrescine is the most abundant compound, followed by histamine, tyramine and cadaverine (Romano et al., 2012). Ingestion of food containing biogenic amines, and particularly histamine, can lead to several health problems, such as headache, blushing, itching, skin irritation, impaired breathing, tachycardia, hypertension, hypotension and vomit (Ladero et al., 2010). Since biogenic amine biosynthesis is strain dependent, it is important to inoculate bacterial strains which have been selected for the absence of genes encoding for amino acid decarboxylase as MLF starters (Du Toit et al., 2011).

Glycerol, produced by yeasts during alcoholic fermentation, can be used by LAB as a carbon source through the aerobic glycerol kinase pathway, where the end product is dihidroxyacetone phosphate, or by the anaerobic glycerol dehydratase pathway (Pasteris and Strasser de Saad, 2009). In this pathway glycerol, after dehydration to 3-Hydroxypropionaldehyde (3-HPA), can be reduced to 1,3-propanediol, oxidised to 3-hydroxypropionic acid or transformed into acrolein, a toxic compound (Seaman et al., 2006) which can react with wine phenolics generating bitter compounds (Sponholz, 1993). However, acrolein can spontaneously and non-enzymatically form by dehydration of 3-HPA, especially in acidic and/or high temperature conditions (Bauer et al., 2010).

Acetaldehyde, the most important and most abundant volatile aroma compound in wine (Liu and Pilone, 2000), is usually removed by bound with SO_2 when it is present in excess after alcoholic fermentation (Du Toit et al., 2011). However it has been proved that LAB of the genera *Lactobacillus* and *Oenococcus* can metabolize acetaldehyde producing ethanol and acetic acid (Osborne et al., 2000). This metabolic ability could be considered as an advantage in white wine production, since the need to use SO_2 could be lowered, but in red winemaking it may have a negative effect on colour development, as in presence of acetaldehyde polymerisation between anthocyanins and catechin or tannins occurs, resulting in stable polymeric pigments (Du Toit et al., 2011).

3. PARAMETERS AFFECTING LAB GROWTH IN WINE

Survival and growth of LAB in wine is influenced by various factors which can be traced to three main categories: physiochemical composition of the wine, winemaking process and interactions with other organisms.

Physiochemical composition of wine

The behavior of LAB in wine is influenced in particular by 4 aspects, synergistic with each other: pH, concentration of SO_2, temperature and ethanol concentration.

pH is an important factor, acting on the selection of the better adapted strains, on the rate and efficiency of growth, on the malolactic activity (speed of degradation of malic acid) (McDonald et al., 1990; Ribéreau-Gayon et al., 2006). The highest

malolactic activity has been seen between pH 3.5 and 4.0 (Bauer and Dicks, 2004). LAB can grow actively in wine at low pH values (around 3.5); at lower pH values (up to 2.9-3.0) growth is still possible but slow, while at higher pH (3.7-3.8) is much more rapid (Ribéreau-Gayon et al., 2006). The optimum pH for the growth of *O. oeni* is between 4.3 and 4.8, but it's proved that *O. oeni* and *L. plantarum* can grow at pH 3.2 (G-Alegria et al., 2004). Low pH values have been reported as responsible for inhibition of sugar metabolism and growth in *O. oeni* (Davis et al., 1986).

Sulphur dioxide (SO_2) is used as an antimicrobial and antioxidant in wine (Fleet and Heard, 1993), since it has a strong inhibitory activity on the growth of LAB. LAB develop hardly starting from 100 mg/L of total SO_2 and 10 mg/L of free SO_2, although this depends on the value of pH (Ribéreau-Gayon, 2006). SO_2 is able to inhibit LAB by rupturing of disulphide bridges in proteins and reacting with cofactors like NAD^+ and FAD (Romano and Suzzi, 1993). SO_2 can also influence the malolactic activity (Fornachon, 1963; Lonvaud-Funel, 1999), and a total SO_2 and bound SO_2 concentration of less than 100 mg/L and 50 mg/L respectively are recommended to ensure successful MLF (Rankine et al., 1970; Powell et al., 2006).

Strains of LAB isolated from wine are mesophilic, and they are able to grow between 15 and 45°C, but the growth is optimal between 20 and 37°C. The rate of bacterial growth and the course of MLF are strongly slowed down at low temperatures, in fact, the cooling prevents the multiplication of bacteria, but does not eliminate them. For this reason an already started MLF (thanks to the bacterial biomass formed previously when conditions are favorable) continues also in case of cooling under 16°C, but with much longer times (from 5-6 days to several weeks or months) (Henick-Kling, 1993; Ribéreau-Gayon et al., 2006).

As most of the microorganisms, LAB are sensitive to ethanol; generally they are inhibited by an ethanol concentration of about 8-10% v/v (Ribéreau-Gayon et al., 2006), but G-Alegrìa et al. (2004) reported the ability of *O. oeni* and *L. plantarum* to grow at 13% v/v ethanol, and Henick-Kling (1993) stated that ethanol concentrations exceeding 14% v/v inhibit the growth of *O. oeni*, the multiplication in the wine allows bacteria to adapt to the presence of ethanol and then to have a higher tolerance (even up to 12-15% v/v). The sensitivity to alcohol varies depending on the genus, species and strain, as well as the activation steps before inoculation in wine (Britz and Tracey, 1990).

Other factors that can affect the growth of lactic acid bacteria are the presence of phenolic compounds, the concentration of oxygen, nutritional deficiencies and the concentration of malic acid.

Phenolic compounds make an important contribution to the organoleptic characteristics of wine (color, mouthfeel, bitterness and astringency), and their amount in wine is cultivar specific as well as depending on the vinification procedures implemented by the winemaker (Rozès et al., 2003). Several studies have shown that some phenolics, like free anthocyanins and gallic acid, are favorable for the growth and activity of bacteria (Vivas et al., 1997), while others,

like hydroxycinnamic acids, ρ-coumaric acid and ferulic acid appear to be unfavorable (Reguant et al., 2000; Garcìa-Ruiz et al., 2008; Vivas et al., 1997).
Oxygen can affect the multiplication of lactic acid bacteria, but different species may behave differently towards this substance. LAB can be indifferent to its presence, fit better in its absence (facultative anaerobic), tolerate oxygen partial pressure but be unable to use it (aerotolerant) or, finally, they may require small amounts of oxygen for a better growth (microaerophilic) (Ribéreau-Gayon et al., 2006).
LAB are very exigent from a nutritional point of view, therefore it is important that they have available nutrients in sufficient quantities in the medium they are growing in. If the fermentation is conducted by a yeast with high nutritional requirements, the grape must is rapidly depleted of the factors necessary to support the growth of LAB. Under these conditions it is necessary to add a specific nutritional supplement for bacteria (Ribéreau-Gayon, 2006). A recent study by Terrade and Mira de Orduña (2009) shows that 10 compounds, including carbon and phosphate sources, manganese, several amino acids and vitamins are essential for the growth of species belonging to the *Oenococcus* and *Lactobacillus* genera.
The initial concentration of malic acid may vary depending on the variety of grape and vintage year and for this reason it can happen that the duration of MLF may be different from one year to another. It is proved that with less than 1 g/L of L-malic acid in the medium MLF can hardly start, as such amount is not sufficient for the bacteria so choose L-malic acid as a carbon source (Loubser, 2004).

Winemaking process
Even the winemaking process can affect the survival and growth of LAB. The hydrostatic pressure in the tank causes the lees accumulate on the bottom, trapping bacteria and nutrients and preventing LAB from expleting their metabolic functions properly. To avoid this, it is recommended to turn over the lees regularly (at least weekly) to ensure that bacteria and nutrients remain in suspension (Loubser, 2004).
The process of clarification of must and wine can also remove a large portion of the bacteria and reduce the incidence of bacterial growth because, during this phase, some important nutrients and suspended particles important for LAB growth are removed (Ribéreau-Gayon et al., 2006).

Interactions with other microorganisms
Grape must contains a large variety of microorganisms, that are naturally selected by the changes in the conditions of the medium which occur during the fermentation and by the interactions, synergistic and antagonistic, between different micro-organisms or between micro-organisms belonging to the same species but to different strains. The most important interactions seem to be the yeast-bacteria interactions, which are the basis of winemaking (Ribèreau-Gayon, 2006).

Such interactions are generally considered negative, as yeasts deplete the medium of nutrients (Larsen et al., 2003) and are able to produce substances which are considered inhibitory to bacteria: ethanol, SO_2 and medium chain fatty acids (Capucho and San Romao, 1994; Caridi and Corte, 1997).
The ethanol produced by yeasts during alcoholic fermentation has no significant effect on the activity of malolactic LAB until it remains at a quantity of 12% v/v, but it appears to have an inhibitory effect on the ability of growth of these bacteria (Capucho and San Romao, 1994).
Under certain conditions, some yeasts are also able to produce another antibacterial metabolite: sulphur dioxide (SO_2), which at high concentrations has a negative influence on the bacterial activity (Loubser, 2004; Alexandre et al., 2004). The amount produced is generally less than 10 mg/L and only in some cases can exceed 30 mg/L (Suzzi et al., 1985), although some strains of *Saccharomyces cerevisiae* can produce up to 100 mg / L of SO_2 (Remize et al., 2005; Alexandre et al., 2004). Studies by Henick-Kling and Park (1994) suggest that the concentration of SO_2 added to the must, combined with that produced by yeasts during alcoholic fermentation, is the main factor that determines the survival of the bacteria and the success of MLF.
Other products of the yeast metabolism are the medium chain fatty acids, including decanoic acid and dodecanoic, which may be potent inhibitors of the growth of LAB (Bourdineaud et al., 2004). In particular, the decanoic acid in concentrations up to 12.5 mg/L and the dodecanoic acid in concentrations up to 2.5 mg/L appear to act as growth factors stimulating also the malolactic activity. At higher concentrations they exert an inhibitory effect by limiting the bacterial growth, as well as the capacity of the malolactic bacteria to catabolize the malic acid. It's been proved that this capacity also depends on the pH of the medium, since Capucho and San Romao (1994) proved that the toxicity of decanoic acid towards the malolactic activity of *O. oeni* is greater at pH 3.0 than at pH 6.0.
As the alcoholic fermentation goes on, the negative effects of yeast metabolism are offset by the positive effects, in fact, when the population of yeast enters the stationary phase, some cells lyse releasing vitamins, glucans nitrogenous bases, mannoproteins, peptides and amino acids in the medium, representing an important growth factor for LAB (Guilloux-Benatier et al., 2006; Alexandre et al., 2004). Yeast autolytic activity is strain dependent (Alexandre et al., 2001). Mannoproteins seem to be of significant importance as their release can stimulate bacterial growth by adsorbing medium chain fatty acids and thus detoxifying the wine medium (Guilloux-Benatier and Chassagne, 2003).

4. INDUCTION OF MALOLACTIC FERMENTATION USING COMMERCIAL STARTER CULTURES

In the past centuries, winemakers were used to let nature take its course and wait for MLF to occur spontaneously (Morenzoni 2006). This habit, which in some countries is still widespread, can sometimes lead to dangerous risks, compromising

the quality of the wine. These risks include the production of off-flavours and biogenic amines in the wine by spoilage bacteria (Davis et al., 1985), a delay in the onset or completion of MLF (Nielsen et al., 1996) and the development of bacteriophages (Bauer and Dicks, 2004). For this reasons winemakers are starting to inoculate grape must or wine with commercial starter cultures of LAB (mostly *O. oen*i or *O. oeni* and *L. plantarum*) to ensure the successful completion of MLF and also to encourage a positive flavour development (Krieger-Weber, 2009).

The selection of LAB strains to be used as starters for MLF is a crucial, complex and laborious process, and strict criteria are used in order to find the best starter cultures, including tolerance to low pH values, high ethanol and SO_2 concentrations, inability to produce biogenic amines, lack of off-flavour or off-odour production, release of aroma compounds, resistance to low temperature (Lonvaud-Funel, 2001; Marcobal et al., 2004; Volschenk et al., 2006; Guzzon et al., 2009). Coucheney et al., proposed (2005) a new approach for selection of *O. oeni* strains in order to produce malolactic starters. The expression of a small heat shock protein Lo18 was evaluated by immunoblotting and real-time PCR. These results were correlated with the performances of strains in two red wines. Physiological and molecular characteristics of the three tested strains showed significant differences for the global malolactic activity on intact cell at pH 3.0 and at the level of induction of the small heat shock protein Lo18. These two parameters appeared of interest to evaluate in the ability of *O. oeni* strains to survive into wine after direct inoculation and to perform MLF. Indeed, a tested strain that presented the highest malolactic activity on intact cells at pH 3.0 and a high level of Lo18 induction showed a high growth rate and a high specific kinetic of malate consumption.

Various types of starter cultures are present on the market. The first form, developed in the 1960's, has been the liquid suspension culture, which has a shelf life of 20 to 72 days and requires a preparation time of 3 to 7 days. During the early 1980's frozen and freeze-dried LAB starter cultures were developed. This product needs to be inoculated immediately after thawing (Lerm et al., 2010). In the 1990's Viniflora oenos was the first direct inoculation freeze-dried LAB starter culture (Nielsen et al., 1996). To survive "re-introduction" into the hostile wine environment without a decrease of viable cell numbers and a subsequent loss of malolactic activity the starter bacteria have to be acclimatised during the production. This adaptation is principally linked to the acquisition of mechanisms of resistance, which allow the microbe to regulate the intracellular pH (Alexandre et al., 2008). The adaptation also involves a modification of the membrane structure, a modification of the fluidity of the membrane and the synthesis or so-called stress proteins or HSP (heat shock proteins). The direct inoculation (MBR®) form of commercially available malolactic bacteria represents a procedure which subjects the cells to various environmental and chemical stresses which make them better able to withstand the rigors of direct addition to wines (Krieger-Weber et al.,

2011). For this reason MBR® cultures does not need special preparation and is directly added to the wine (Lerm et al., 2010).
Unlike the MBR® cultures for direct inoculation, the quick build-up 1-Step® cultures had only been exposed to a "softer" stress during production. Thus, these cultures are less preconditioned and therefore require an acclimatization step before they can be inoculated into the wine (Krieger-Weber, 2011). This requires the addition of an activator and wine to the culture 18 to 24 hours prior to the inoculation in wine (Lerm et al., 2010).
It is important to say that the use of commercial starter cultures does not always guarantee a complete and successful MLF, especially in presence of very difficult wine conditions like low pH and high ethanol (Guerzoni et al., 1995).

5. TIMING OF INOCULATION

The success or the failure of the inoculated bacterial starter culture to initiate and complete MLF is often influenced by the timing of inoculation. It is important to decide which is the most favorable time to inoculate the malolactic bacteria, taking into account the interaction they have with the yeast. The inoculum of bacteria can in fact be simultaneous with that of the yeast (early co-inoculation), performed during alcoholic fermentation (late co-inoculation), or it can be done at the end of alcoholic fermentation (sequential inoculation) (Gallander, 1979).

Early co-inoculation

The early co-inoculation consists in the contemporary addition of yeast and malolactic bacteria at the beginning of alcoholic fermentation.
The simultaneous inoculation of must with yeast and bacteria could allow for a more efficient induction of MLF, due to a gradual adaptation of bacteria to increasing concentrations of ethanol and to the benefits deriving from the higher availability of nutrients in the must instead of wine (Fugelsang and Edwards, 1997; Massera et al., 2009)
A considerable reduction in the duration of fermentation in the case of co-inoculation was also noticed. A study by Jussier et al. (2006) showed that using *O. oeni* strain Alpha, the malolactic fermentation was completed 46 days in advance when the vinification was carried out with simultaneous inoculation of yeasts and bacteria, compared to that induced by inoculation of the bacteria after the completion of the alcoholic fermentation. It does not always happen that, at the end of alcoholic fermentation, after an initial decrease, the bacterial population grows up to a cell concentration which allows the immediate start of MLF. There is in fact the risk that the decrease of the bacterial population continues until its complete disappearance. Alternatively it may happen that the antagonism between the yeast and the bacterial population will be resolved with a reduction of the alcoholic fermentation and premature growth of LAB in wine. This leads to a lower production of alcohol by yeast and to a high production of acetic acid in wine (Ribéreau-Gayon, 2006; Zapparoli et al., 2009).

Late co-inoculation

The late co-inoculation consists in the addition of the malolactic bacteria during alcoholic fermentation (approximately when the alcoholic fermentation is 1/3 completed), so that they are in a substrate less rich in sugars and MLF is promoted. However, this practice is not common, and Rosi et al. (2003) revealed a significant reduction of the bacterial viability, since bacteria are inoculated when the medium contains less nutrients, and SO_2, ethanol, other toxic metabolites and acids (with further reduction of the pH) are present in the medium. These conditions induce a strong antagonism between yeasts and bacteria and ii is not possible to guarantee that the bacteria are able to develop.

Sequential inoculation

Traditionally the inoculum of bacteria occurs after the alcoholic fermentation is complete, thus promoting the growth of LAB thanks to the presence of nutrients linked to autolysis of yeasts (Gallander, 1979; Alexandre et al., 2004). The lysis of yeasts may, in fact, change in an important way the concentration of nitrogen compounds available for the malolactic bacteria (amino acids, peptides, proteins) and also provide other macromolecules, such as glucans and mannoproteins, which influence positively their growth (Guilloux-Benatier et al., 1995).

However, the exposure of bacteria to high levels of ethanol, in combination with low levels of pH, exerts adverse effects on survival of O. oeni in wine (Zapparoli et al., 2009). It is possible that these factors damage the cytoplasmic membrane of the LAB, with a deleterious effect on the malolactic activity (Da Silveira et al., 2002). Under these conditions, the acclimatization of the bacteria plays a fundamental role in the management of MLF (Zapparoli et al., 2009).

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CHAPETR 2

Use of propidium monoazide for the enumeration of viable *Oenococcus oeni* in must and wine by quantitative PCR

ABSTRACT

Malolactic fermentation is an important step in winemaking, but it has to be avoided in some cases. It's carried out by lactic acid bacteria belonging mainly to the genus *Oenococcus*, which is known to be a slow growing bacterium. Classical microbiological methods to enumerate viable cells of *Oenococcus oeni* in must and wine take 7 to 9 days to give results.

Moreover, RT-qPCR technique gives accurate quantitative results, but it requires time consuming steps of RNA extraction and reverse transcription.

In the present work we developed a fast and reliable qantitative PCR (qPCR) method to enumerate cells of *Oenococcus oeni*, directly, in must and wine. For the first time we used a propidium monoazide treatment of samples to enumerate only *Oenococcus oeni* viable cells. The detection limit of the developed method is 0.33 log CFU/mL (2.14 CFU/mL) in must, and 0.69 log CFU/mL (4.90 CFU/mL) in wine, lower than that of the previously developed qPCR protocols.

1. Introduction

Malolactic fermentation (MLF), the enzyme-mediated decarboxylation of L-malic acid into L-lactic acid, is carried out by lactic acid bacteria (LAB) belonging to the genera *Oenococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus*. It usually occurs after alcoholic fermentation (AF) and is known to improve wine quality trough deacidification, the production of desirable flavours and aromas, and the enhancement of microbial stability (Nehme et al., 2010).

The decarboxylation of L-malic acid into L-lactic acid and carbon dioxide is catalysed by the malolactic enzyme in the presence of NAD^+ and Mg^{2+} co-factors (Wibowo et al., 1985; Lonvaud-Funel, 1999). The reaction usually results in an average reduction in total acidity of 1 to 3 g/L and an increase in pH of 0.1 to 0.3 units (Margalit, 1997). This could be considered an advantage for high-acid or low pH wines produced in cool climate regions, where deacidification is recommended for the production of acid-balanced wines, but it must be avoided in low-acid or high pH wines of warmer climate regions, where deacidification could have a negative impact on wine quality (Jackson, 2003). In addition, an increase in pH could enhance the risk of the survival and growth of spoilage microorganisms and could also cause a loss of red colour intensity in red wines (Volschenk et al., 2006). However, L-malic acid is a relatively good nutritional resource (Pilone and Kunkee, 1976), and lactic acid is a registered antibacterial agent; thus, MLF could be considered to be a process resulting in the increased resistance of wine to spoilage caused by bacterial growth because it causes a reduction of L-malic and an increase in L-lactic acid concentrations (du Toit et al., 2011). Finally, MLF also contributes to wine aroma because of the less aggressive and milder taste of lactic acid (Lonvaud-Funel, 1999).

However, this important secondary fermentation step in winemaking is often difficult to induce and control, and it can start randomly. Any delay can lead to an alteration of wine quality (Beltramo et al., 2006). These delays are due to the harsh physico-chemical conditions existing in must or wine, such as low pH (Vaillant et al., 1995), low temperature (Britz and Tracey, 1990) and SO_2 (Carretè et al., 2002).

Although research is evaluating the potential of *Lactobacillus* spp. as an MLF starter for the future (du Toit et al., 2011), *Oenococcus oeni* is the most common species of LAB associated with MLF in wine (Bon et al., 2009). From an evolutionary point of view, *O. oeni* may be considered a specialised microorganism because it has a small genome of approximately 1.8 Mb (Mills et al., 2005). This species is one of the naturally occurring LABs in grape must, and as a result of natural selection, thanks to its tolerance to must and wine conditions, *O. oeni* has become the dominant species among those able to start MLF (Spano and Massa, 2006). However, this species is highly heterogeneous, with considerable intraspecific variation in resistance to wine conditions (Henick-Kling, 1993; Kunkee, 1984), and a loss of vitality was observed when strains isolated from

wines and cultivated in the laboratory were reinoculated into wine (Henick-Kling, 1993). In addition, the viability and dominance of *O. oeni* among an indigenous LAB population can be affected by several technological factors (Arnink and Henick-Kling, 2005; Davis, 1985; Nielsen et al., 1996, Remize et al., 2005).

Finally, *O. oeni* is known to be a fastidious, slow-growing bacterium (Kunkee, 1984) and is auxotrophic for several amino acids, while other amino acids are needed for optimal growth (Henick-Kling, 1993; Kunkee, 1984). Seven to nine days are required to produce countable colonies on plates using classical microbiology methods.

For all of these reasons, the fast enumeration of viable cells of *O. oeni* in must or wine is necessary, both to verify the level of inoculum and the survival of cells in must or wine when MLF is desired and to monitor the presence and growth of malo lactic bacteria (MLB) when MLF is an unwanted process. Quantitative PCR (qPCR) techniques have already been developed (Solieri and Giudici, 2010; Pinzani et al., 2004), but none of these methods are capable of discriminating between viable and dead cells in a sample. Discrimination between viable and dead cells in a sample is possible using RNA as a target instead of DNA. In this case, a reverse transcription (RT) step is essential, and it is crucial to remember that severe and numerous complications remain associated with RT. For example, the resolving power of qPCR is limited by the efficiency of RNA-to-cDNA conversion, which depends on the enzyme used. However, the conversion efficiency is significantly (more than 3-fold) lower when target templates are rare, and it is negatively affected by nonspecific or background RNA in the RT reaction (Curry et al., 2002).

Recently, the DNA-intercalating agent propidium monoazide (PMA) has been used in conjunction with qPCR to selectively detect live cells of pathogenic and spoilage bacteria (Rawsthorne et al., 2009; Yang et al., 2011; Josefsen et al., 2010, Mamlouk et al., 2012, Elizaquìvel et al., 2012, Yokomachi and Yaquchi, 2012). This compound selectively penetrates the membranes of dead cells and forms stable DNA monoadducts upon photolysis, resulting in DNA that cannot be amplified by PCR (Nogva et al., 2003). In this manner, discrimination between live and dead cells is possible while avoiding the RT step and all of its complications.
In this work, for the first time, we developed a fast, reliable PMA-qPCR method to rapidly detect and enumerate live cells of *O. oeni* in must or wine samples, and we compared it with the enumeration of viable cells based on RT-qPCR.

2. Materials and methods

2.1. Bacterial strains and cell suspension preparation

Four different commercial strains of *O. oeni* (Viniflora CH11, Viniflora CH 16, Viniflora oenos by Christian Hansen and Amar 04 by Enologica Vason) and *O. oeni* DSMZ 20252 were used to optimise the amplification conditions. Moreover, *Lactobacillus brevis* DSMZ 20054, *L. casei* DSMZ 20111, *L. plantarum* DSMZ

20174, *L. reuteri* DSMZ 20053, *L. rhamnosus* DSA, *L. sakei* DSMZ 6333, *Lactococcus lactis* DSMZ 20481, *Leuconostoc citreum* DSMZ 5577, *Leuc. gasicomitatum* DSMZ 15947, *Leuc. mesenteroides subsp. mesenteroides* DSMZ 20343, *Pediococcus pentosaceous* DSMZ 20336, and other yeast species commonly isolated from must and wine were used in both PCR and qPCR to assess the specificity of the protocol. In particular, *Saccharomyces ludwigii* UCD 6721, *S. pastorianus* DSMZ 6580, *S. bayanus* DSMZ 70412, *S. cerevisiae* ATCC 51, *Brettanomyces bruxellensis* DSMZ 70726, *Candida etanolica* UCD 7, *C. vini* UCD 36, *Hanseniaspora guillermondii* DSA, *Pichia membranifaciens* DSA, *Metschnikovia pulcherrima* DSA, and *Kloechera apiculata* DSA were chosen.

Prior to DNA extraction, strains were cultured to obtain cell concentrations of 10^9 colony-forming units (CFU)/mL (equivalent to an optical density at 600 nm [OD_{600}] of 1) for 72 hours at 30°C in tubes containing 30 mL of *Leuc. oenos* medium (casein peptone, tryptic digest 10 g/L, yeast extract 5 g/L, glucose 10 g/L, fructose 5 g/L, $MgSO_4$ x 7 H_2O 0.20 g/L, $MnSO_4$ x H_2O 0.05 g/L, (NH_4) citrate 3.50 g/L, Tween 80 1.00 mL/L, filtered apple juice 100.00 mL/L, and cysteine-HCl x H_2O 0.50 g/L, pH adjusted to 4.8).

Ten-fold dilutions of each culture were prepared in water, grape must and red wine (ethanol concentration: 12% v/v) to obtain suspensions of *O. oeni* for standard curve construction. The final concentration of the cells in water, grape must and red wine was between 10^9 and 1 CFU/mL. The 72 hours culture of *O. oeni* used to contaminate the grape must and wine samples was enumerated on a double layer of *Leuconostoc oenos* medium mixed with 15 g/L agar technical n°3 (Oxoid, Milan, Italy) to determine the exact CFU/mLspiked into the samples. Plates were incubated under anaerobic conditions (Anaerogen, Oxoid, Milan, Italy)at 30°C for 7 days, the colonies were counted, and the numbers of viable *O. oeni* were determined from those counts.

Next, different samples of grape must and wine were inoculated with mixtures of live and dead *O. oeni* cells to evaluate the efficiency of PMA to distinguish between live and dead cells. To obtain a suspension of 10^9 CFU/mL dead cells, tubes containing 30 mL of *O. oeni* culture (OD_{600}=1) were pasteurised at 80°C for 20 minutes. Different conditions of time and temperature (60 °C for 20 minutes, 80 °C for 10 minutes) were tested to find out the best method to obtain a suspension of only dead cells. The evaluation of the presence and the enumeration of survived *O. oeni* cells, similarly to other authors (Agusti et al., 2013; Desfossés et al., 2012; Banihashemi et al., 2012) was carried out by plate count on LOM agar and by RT-qPCR. Moreover, to evaluate the presence of stressed viable-not culturable cells, 10 mL of the pasteurised suspension were added to 90 mL of LOM medium and incubated at 30 °C for 7 days. The sample was then streaked in triplicate on LOM agar plates to evaluate the growth of cells eventually survived to the heat treatment.

One millilitre of the suspension constituted only by dead cells was mixed with live cell suspensions to obtain a final concentration of the cells in the different

must and wine samples between 10^8 and <1 CFU/mL of live cells mixed with 10^9 CFU/mL dead cells. The inoculum was evaluated on a double layer of *Leuconostoc oenos* agar medium (Oxoid, Milan, Italy) at 30°C for 7 days to determine the exact CFU/mL of viable cells spiked in the samples.

All inoculations were carried out in triplicate. All samples were processed within 15 minutes after inoculation. A non-inoculated negative control was included in each experiment. Prior to the inoculation trials, the grape must and red wine used were analysed both by culture methods (double layer of *Leuconostoc oenos* agar medium at 30°C for 7 days) and qPCR to assure the absence of natural *O. oeni* contamination.

2.2. Treatment of suspensions with PMA

A 20 mM solution of PMA (Biotium, Inc., Hayward, CA, USA) in 20% (v/v) dimethyl sulfoxide (Sigma-Aldrich, Oakville, Ontario, Canada) was prepared and stored in the dark at 4°C. In total, 1 mL of each suspension previously prepared in water, grape must and red wine was centrifuged, and the cellular pellets were resuspended in 300 µL 0.1% (w/v) peptone water and mixed with 1.5 µL of the PMA solution. Each tube was incubated on ice in the dark for 5 min. Then, the tubes were inserted in the PhAST Blue instrument (GenIUL, Barcelona, Spain) for a 15-minute photo-activation process.

2.3. DNA extraction

In total, 1 mL of each suspension without PMA treatment and 300 µL of the suspensions treated with PMA were centrifuged at 10,000 rpm for 7 min to pellet the cells. DNA was extracted from the pellets using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA) according to the manufacturer's instructions for DNA purification from cell samples, except that the DNA was resuspended in 35 µL of sterile bidistilled water. After extraction, the DNA concentration was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Rodano, Italy) and standardised to 40 ng/µL by dilution with sterile DNA-free Milli-Q water.

2.4 RNA extraction

In total, 1 mL of each suspension without PMA treatment was centrifuged at 10,000 rpm for 7 min to pellet the cells. RNA was extracted from the pellets using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin) according to the manufacturer's instructions for RNA purification from cell samples, except that DNAse treatment was extended to 150 minutes to ensure the total degradation of contaminating DNA. Moreover, RNA was resuspended in 35 µL of sterile bidistilled water treated with DEPC (Sigma, Milan, Italy). The RNA concentration was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Rodano, Italy) and

standardised to 40 ng/µL by dilution with sterile DNA-free Milli-Q DEPC-treated water.

The absence of contaminating DNA in RNA samples was proved by both classical and quantitative PCR.

2.5 Reverse transcription

Standardised RNA samples were converted into cDNA. Reverse transcription reactions were carried out using the ImProm-II™ Reverse Transcription System (Promega, Milan, Italy) according to the manufacturer's instructions. cDNA samples were used as templates for qPCR.

2.6. Primer design and specificity

Using FastPCR 6.1 software (Kalendar et al., 2009), the following oligonucleotides were developed to perform real-time quantitative PCR for the gene coding for the malolactic enzyme in *O. oeni* (accession number: AY786176): forward primer, Malomar F: 5'-GTT AAT CAT GCC GAA TCG-3' (region 658-675), and reverse primer, Malomar R: 5'-GTC GGA AAG ACC CTG-3' (region 928-942), generating a PCR product of 285 bp.

The specificity of the primers was tested in both PCR and qPCR using the bacterial and yeast strains listed in section 2.1.

Conventional PCR was carried out in a final volume of 25 µL containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) dNTPs, 1 µM primer, and 1.25 U of Taq polymerase (Applied Biosystems, Milan, Italy). The amplification cycle was as follows: 95°C denaturation for 5 min; 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min in a Thermal Cycler (DNA Engine Dyad peltier Thermal Cycler, BioRad, Milan, Italy).

2.7. qPCR protocol

The primers Malomar F and Malomar R, which target the mleA gene of *O. oeni*, were used for qPCR. The qPCR mixture contained 10 µL of 2x SsoFast EvaGreen Supermix (Biorad, Milan, Italy), 400 nM of each primer, and 2 ng/µL of DNA or cDNA, and the volume was adjusted to 20 µL with sterile DNA-free Milli-Q water. qPCR was performed using a RotorGene Q system (Qiagen, Milan, Italy) with one cycle of initial denaturation of template DNA and activation of Taq DNA polymerase at 98°C for 3 minutes, followed by 45 cycles of denaturation at 95°C for 10 s, primer annealing at 60°C for 15 s and extension at 72°C for 20 s. Fluorescence signal acquisition was performed during the extension step. To determine whether non-specific products or primer dimers had formed, the dissociation curves of the final products of each PCR were analysed from 55 to 95°C at 1°C intervals.

2.8. Construction of standard curves

O. oeni was enumerated as described above in all of the artificially contaminated samples to determine the exact CFU/mL spiked in the samples. DNA and cDNA were obtained and amplified as described in section 2.3. The signals produced (threshold cycle, C_t) by the serial dilution in grape must and red wine were plotted against the $\log_{10}$ CFU/mL, and standard curves were constructed. Correlation coefficients (R^2) and the efficiency of amplification were calculated as previously described (Bustin et al., 2009). The construction of standard curves was performed using three replicates per each standard point, and each reaction was carried out in triplicate, per each standard strain (4) used.

2.9 PMA-qPCR application in real samples

At the end of the PMA-qPCR optimisation, the enumeration of *O. oeni* was performed in 10 samples of must co-inoculated with *S. cerevisiae* and 10 samples of wine, collected from wineries in Friuli Venezia Giulia region (north east of Italy). Results were also compared to the traditional plate count on LOM agar medium. For these samples, ethanol concentration was determined using the Alcolyzer Plus instrument (Anton Paar, Austria), following the manufacturer protocol.

3. Results

3.1 PCR and qPCR assay specificity

In silico PCR performed by FastPCR software using the primers Malomar F and Malomar R showed a positive result only for *O. oeni*.

The specificity of the assay was confirmed by both PCR and qPCR analysis of several strains of *O. oeni* and all of the different species of lactic acid bacteria and yeasts reported in section 2.1. Both PCR and qPCR demonstrated specificity: using PCR, only one band corresponding to the amplification of *O. oeni* strains, as expected, was obtained (Figure 1); using qPCR, a positive fluorescence signal was yielded only for *O. oeni* samples.

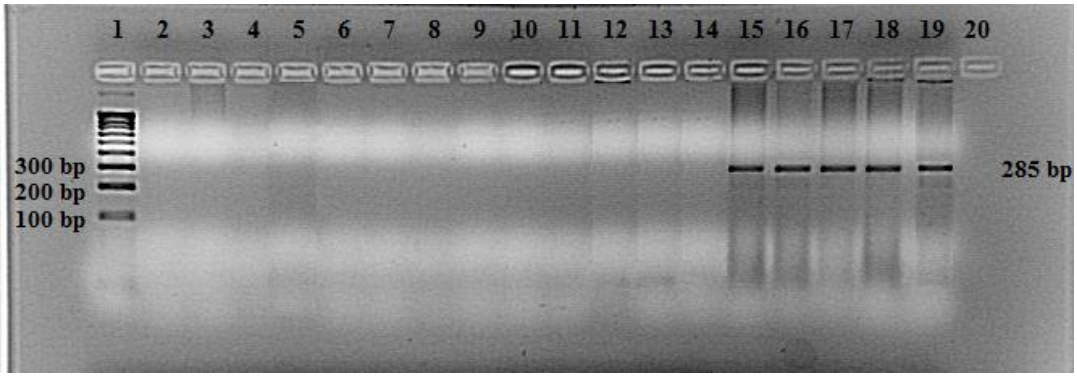

Figure 1. Results of conventional PCR reaction. Agarose gel (2%), 120V, 60'. Line 1: Molecular weight marker 100 bp (Promega, Milan, Italy); Line 2: *L. plantarum* DSMZ 20174; Line 3: *L. brevis* DSMZ20054; Line 4: *L. reuteri* DSMZ 20053; Line 5: *L. sakei* DSMZ 6333; Line 6: *Lc. Lactis* DSMZ 20481; Line 7: *Leuc. citreum* DSMZ 5577; Line 8: *Leuc. gasicomitatum* DSMZ 15947; Line 9: *P. pentosaceus* DSMZ 20336; Line 10: *Brett. bruxellensis* DSMZ 70726; Line 11: *S. cerevisiae* ATCC 51; Line 12: *S. bayanus* DSMZ 70412; Line 13: *S. ludwigii* UCD 6721; Line 14: *S. pastorianus* DSMZ 76580; Line 15: *O. oeni* DSMZ 20252; Line 16: *O. oeni* Viniflora CH oenos; Line 17: *O. oeni* Viniflora CH11; Line 18: *O. oeni* Viniflora CH16; Line 19: *O. oeni* Vason Amar 04; Line 20: Negative control.

3.2 Standard curves

Pure DNA and RNA were extracted from the suspensions of all of the *O. oeni* strains in must and wine and photometrically quantified. The absence of contaminating DNA in RNA samples was proved by both classical and quantitative PCR. No band on an agarose gel and no fluorescence signal were obtained, confirming the purity of the extracted nucleic acids.

The number of viable *O. oeni* cells was also determined by plate counts for all of the suspensions. DNA and RNA concentrations were standardised to 40 μg/mL. Standardised DNA and cDNA obtained from standardised RNA samples were used as templates for qPCR. To correlate plate count and Ct values, standard curves for each strain of *O. oeni* in must and wine were created. To construct the standard curves, the plate count values of at least four serial dilutions for each suspension in must and wine were used as standards in each qPCR. All of the *O. oeni* strains tested, which were diluted in must or in wine, behaved in a comparable manner and yielded similar results (Table 1).

Table 1. Efficiencies of amplification and correlation coefficients (R^2) value of the standard curves for the different *O. oeni* strains used.

Strain	Matrix	Method	Efficiency	R^2
Viniflora CH11	Must	qPCR[1]	0.97083	0.99621
		PMA-qPCR[2]	0.89316	0.99962
		RT-qPCR[3]	1.33185	1.00000
	Wine	qPCR	1.11059	0.98949
		PMA-qPCR	0.93728	0.99998
		RT-qPCR	0.73068	0.97614
Viniflora CH16	Must	qPCR	0.91758	1.00000
		PMA-qPCR	0.92459	0.98154
		RT-qPCR	1.04586	0.87458
	Wine	qPCR	1.04103	0.99843
		PMA-qPCR	0.99845	0.94581
		RT-qPCR	1.06894	0.96428
Viniflora CH oenos	Must	qPCR	1.03128	0.98305
		PMA-qPCR	0.84676	0.84567
		RT-qPCR	0.97215	0.94518
	Wine	qPCR	0.96654	0.97880
		PMA-qPCR	0.98214	0.94512
		RT-qPCR	1.05481	0.98451
Vason Amar 04	Must	qPCR	0.98451	0.95486
		PMA-qPCR	0.99452	0.94871
		RT-qPCR	1.06891	0.97442
	Wine	qPCR	0.91786	0.88481
		PMA-qPCR	0.98414	0.94751
		RT-qPCR	0.84124	0.91545

Legend: [1]quantitative PCR on DNA samples; [2]quantitative PCR on PMA treated DNA samples; [3]quantitative PCR on reverse transcribed RNA samples.

The linear regression analysis of average Ct values obtained for all *O. oeni* strains in must gave the following results: slope = −2.936 (qPCR), -2.4382 (PMA-qPCR), -2.5124 (RT-qPCR), y-intercept = 439.017 (qPCR), 43.758 (PMA-qPCR), 43.058 (RT-qPCR), and correlation coefficient = 0.9975 (qPCR), 0.9960 (PMA-qPCR), 0.9985 (RT-qPCR). The average efficiency of PCR, which was calculated for each reaction on the basis of the slope of the standard curve by the equation efficiency = [10(-1/slope)] − 1, was 0.97605 for qPCR, 0.9148 for PMA-qPCR and

1.1047 for RT-qPCR (Figure 2, panel A, C and E). An analysis of the average Ct values obtained for all *O. oeni* strains diluted in wine gave the following results: slope = −2.468 (qPCR), 2.4615 (PMA-qPCR), 2.8397 (RT-qPCR), y-intercept = 38.263 (qPCR), 45.309 (PMA-qPCR), 48.568 (RT-qPCR), correlation coefficient = 0.9806 (qPCR), 0.9905 (PMA-qPCR), 0.9978 (RT-qPCR), and efficiency of PCR = 1.00900 for qPCR, 0.97550 for PMA-qPCR and 0.92391 for RT-qPCR (Figure 2, panel B, D and F).

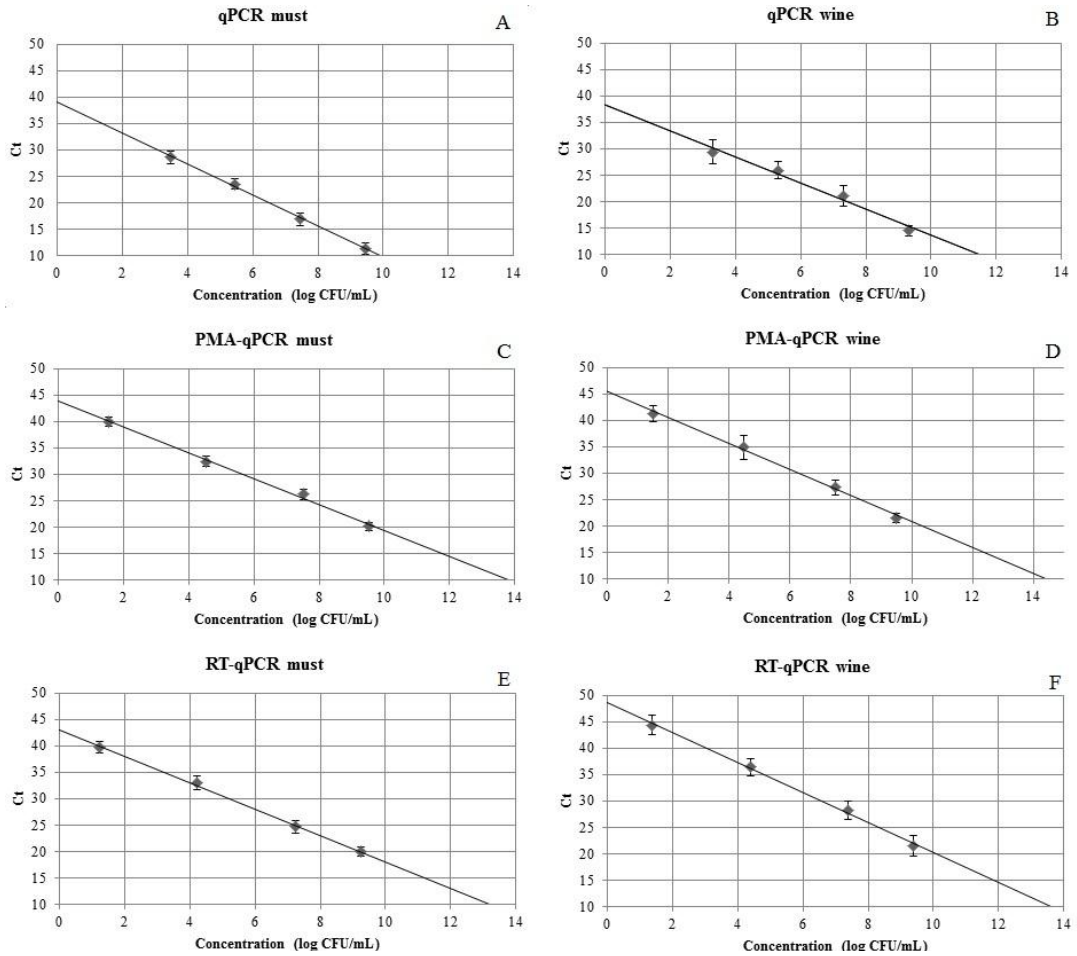

Figure 2. Standard curves (concentration vs Ct) obtained by qPCR, PMA-qPCR and RT-qPCR from 10-fold serial dilutions of four strains of *Oenococcus oeni* (Viniflora CH11, Viniflora CH16, Viniflora CH oenos, Vason Amar04) in must (panels A, C and E) and wine (panels B, D and F) samples. Correlation coefficients (R^2), slopes and efficiency of amplification are shown in Table 1. Detection limits were: 2 log CFU/mL for qPCR (in must and in wine), 0.33 log CFU/mL for PMA-qPCR in must, 0.63 log CFU/mL for PMA-qPCR in wine, 0.45 log CFU/mL for RT-qPCR in must and 0.78 log CFU/mL for RT-qPCR in wine. Ct values are the averages of 3 replicates for each strain and error bars represent Standard Deviations.

3.3 Correlation between qPCR results and plate count values in must and wine samples

The results of real-time PCR measurements targeted to the gene encoding the malolactic enzyme in samples of *O. oeni* cultures in non-sterilised must and wine were compared with those obtained by the CFU assay. The correlation (Table 2) between data obtained with qPCR and plate count techniques for *Oenococcus* Viniflora 11 in must and wine showed that the developed method provided accurate concentration results for samples with log CFU/ml values higher than 2

Table 2. Correlation between plate count values and quantification using qPCR on suspensions of *Oenococcus* Viniflora 11 strain. A serial diluted concentration of viable cells (from 0 to 10^9 CFU/mL) was inoculated in each suspension of both must and wine.

Sample	Inoculated cells (CFU/mL)	Plate count value[1] (log CFU/mL)	Ct[1]	qPCR[1] (log CFU/mL)
0 must	0	<1[2]	/[3]	No amplification
1 must	1	0.46 ± 0.12	29.87 ± 0.16	1.71 ± 0.24
2 must	10	1.46 ± 0.12	29.40 ± 0.16	1.92 ± 0.02
3 must	10^2	2.46 ± 0.12	29.37 ± 0.33	2.94 ± 0.01
4 must	10^3	3.46 ± 0.12	27.60 ± 0.23	3.86 ± 0.07
5 must	10^4	4.46 ± 0.12	26.64 ± 0.14	4.25 ± 0.06
6 must	10^5	5.46 ± 0.12	22.92 ± 0.46	5.34 ± 0.13
7 must	10^6	6.46 ± 0.12	20.25 ± 0.31	6.18 ± 0.20
8 must	10^7	7.46 ± 0.12	16.82 ± 0.13	7.24 ± 0.11
9 must	10^8	8.46 ± 0.12	13.19 ± 0.21	8.21 ± 0.06
10 must	10^9	9.46 ± 0.12	10.16 ± 0.30	9.09 ± 0.09
0 wine	0	<1[2]	/[3]	No amplification
1 wine	1	0.31 ± 0.08	30.24 ± 0.18	1.87 ± 0.21
2 wine	10	1.31 ± 0.08	30.44 ± 0.22	1.98 ± 0.10
3 wine	10^2	2.31 ± 0.08	30.52 ± 0.09	2.68 ± 0.25
4 wine	10^3	3.31 ± 0.08	28.14 ± 0.07	3.72 ± 0.18
5 wine	10^4	4.31 ± 0.08	26.98 ± 0.17	4.38 ± 0.29
6 wine	10^5	5.31 ± 0.08	24.57 ± 0.21	5.22 ± 0.19
7 wine	10^6	6.31 ± 0.08	22.48 ± 0.12	6.51 ± 0.24
8 wine	10^7	7.31 ± 0.08	19.86 ± 0.13	7.44 ± 0.14
9 wine	10^8	8.31 ± 0.08	16.59 ± 0.18	8.33 ± 0.02
10 wine	10^9	9.31 ± 0.13	13.69 ± 0.17	9.52 ± 0.07

Legend:[1]values are expressed as the mean of three replicates ± standard deviation; [2]this value is expressed in CFU/mL; /[3]no values obtained as a consequence of no amplification signal.

for both must and wine. This limit could be due to the amplification of the DNA of dead cells in the sample in variable concentrations. This drawback can be overcome using PMA treatment before DNA extraction, as described above.

The regression analysis (Figure 3, panel A) of data starting from 2 log CFU/mL demonstrated a good correlation between the results of the two analytical procedures ($R^2 = 0.99857$).

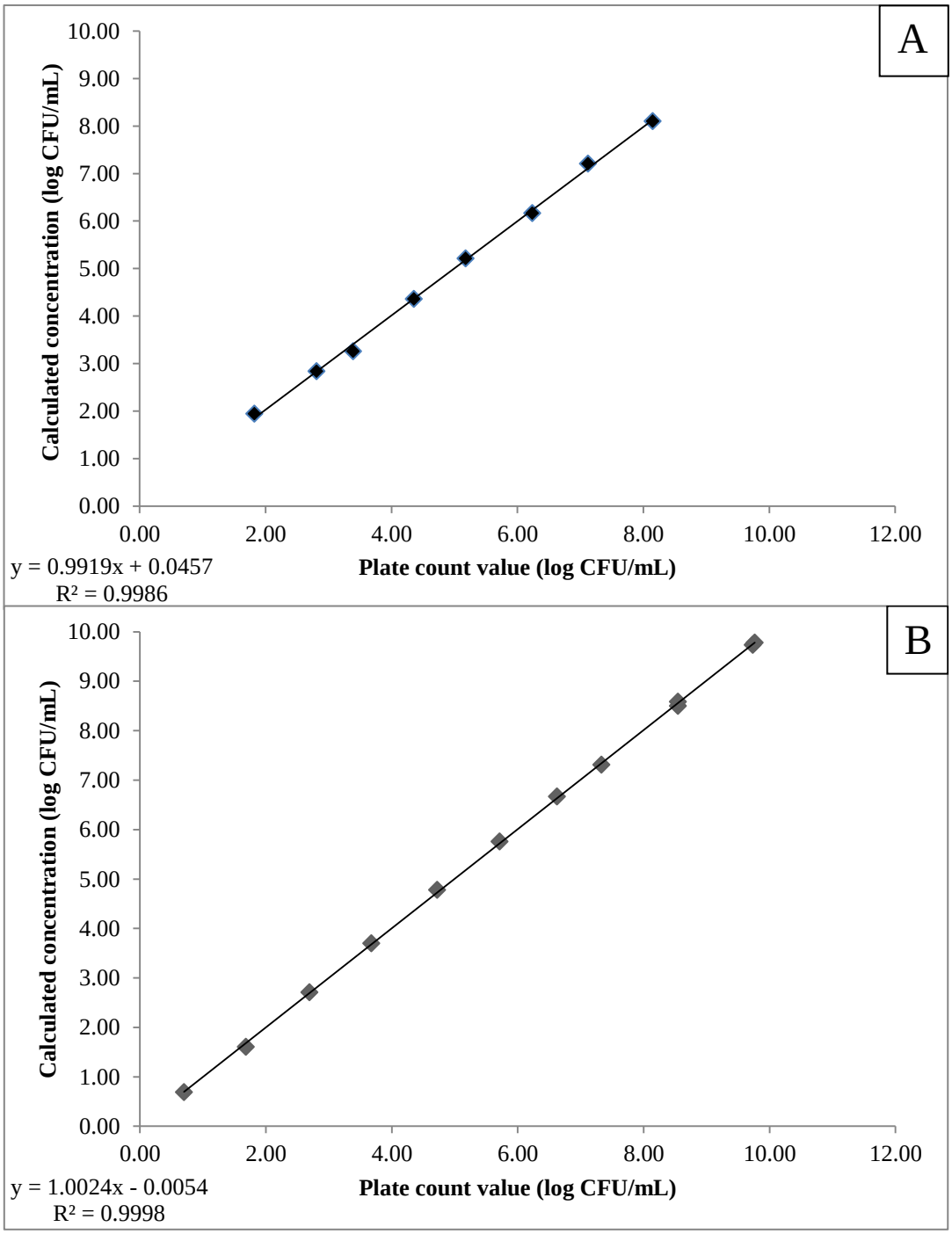

Figure 3. Correlation between plate count values and qPCR calculated concentrations (Panel A) and between plate count values and PMA-qPCR calculated concentrations (Panel B) for suspensions of *Oenococcus oeni* Viniflora CH11 in must. Results show good correlation starting from 2 log CFU/mL for qPCR and from 0.33 log CFU/mL for PMA-qPCR.

3.4 Survival of cells to heat treatments

Plate count values and RT-qPCR analysis on suspensions treated with different conditions of time and temperature revealed that only the treatment at 80 °C for 20 minutes allowed to obtain suspensions constituted only by killed cells. No growth on plate (< 1 CFU/mL), not even after enrichment in LOM broth (absent/10 mL), and no amplification signal were indeed observed analysing these samples. Conversely, plate count technique showed the survival of 2.32 (± 0.24) log CFU/mL of *O. oeni* after treatment at 60 °C for 20 minutes, and of 1.87 (± 0.13) log CFU/mL after treatment at 80°C for 10 minutes. These results were confirmed by RT-qPCR, which showed a concentration of 2.54 (± 0.18) and 1.69 (± 0.21) for treatments at 60 °C for 20 minutes and at 80 °C for 10 minutes, respectively.

3.5 PMA-qPCR analysis results

A fixed concentration of 9 log CFU/mL dead cells was mixed with must and wine suspensions of viable cells of *O. oeni*, and the cellular pellet was recovered by centrifugation and washed with sterile peptone water. The pellet was then treated with PMA before DNA extraction, and the DNA samples, whose concentration was normalised to 40 ng/µL, were used as template for qPCR. The results of the qPCR analysis of samples treated with PMA (Table 3) showed that the detection limit of the developed method decreased to 0.33 log CFU/mL in must and 0.63 log CFU/mL in wine. As we expected, no fluorescence signal was detected for DNA samples extracted from suspensions comprising only dead cells.

The regression analysis of the data demonstrated a good correlation between plate count values and qPCR calculated concentrations (R^2 = 0.99984 for must, as shown in Figure 3, panel B).

At the end of the optimisation of the method, 10 samples of must and 10 samples of red wine were subjected to classical microbiological analysis and PMA-qPCR analyses, and the results showed the same good correlation between the two methods (Table 4), confirming the reliability of the developed technique.

Table 3. Correlation between plate count values and quantification using PMA-qPCR on suspensions of *Oenococcus* Viniflora 11 strain. A serial diluted concentration of viable cells (from 0 to 10^9 CFU/mL) and a constant concentration of dead cells (10^9 CFU/mL) were inoculated in each suspension of both, must and wine.

Sample	Viable cells (CFU/mL)	Dead cells (CFU/mL)	Plate count values[1] (log CFU/mL)	Ct[1]	PMA-qPCR[1] (log CFU/mL)
0 must	0	10^9	< 1[2]	/[3]	No amplification
1 must	1	10^9	0.53 ± 0.04[1]	43.34 ± 0.24	0.33 ± 0.01
2 must	10	10^9	1.53 ± 0.04	40.02 ± 0.21	1.75 ± 0.12
3 must	10^2	10^9	2.53 ± 0.04	39.60 ± 0.42	2.02 ± 0.13
4 must	10^3	10^9	3.53 ± 0.04	34.89 ± 0.27	3.59 ± 0.11
5 must	10^4	10^9	4.53 ± 0.04	32.42 ± 0.27	4.58 ± 0.23
6 must	10^5	10^9	5.53 ± 0.04	30.20 ± 0.44	5.05 ± 0.22
7 must	10^6	10^9	6.53 ± 0.04	29.18 ± 0.35	6.69 ± 0.10
8 must	10^7	10^9	7.53 ± 0.04	27.16 ± 0.50	7.56 ± 0.26
9 must	10^8	10^9	8.53 ± 0.04	23.69 ± 0.33	8.52 ± 0.23
10 must	10^9	10^9	9.53 ± 0.04	20.06 ± 0.21	9.53 ± 0.18
0 wine	0	10^9	< 1[2]	/[3]	No amplification
1 wine	1	10^9	0.49 ± 0.02	43.78 ± 0.12	0.63 ± 0.10
2 wine	10	10^9	1.49 ± 0.02	40.35 ± 0.15	1.55 ± 0.12
3 wine	10^2	10^9	2.49 ± 0.02	37.92 ± 0.25	2.50 ± 0.16
4 wine	10^3	10^9	3.49 ± 0.02	36.29 ± 0.31	3.09 ± 0.14
5 wine	10^4	10^9	4.49 ± 0.02	31.58 ± 0.24	4.69 ± 0.08
6 wine	10^5	10^9	5.49 ± 0.02	30.20 ± 0.36	5.51 ± 0.10
7 wine	10^6	10^9	6.49 ± 0.02	28.46 ± 0.33	6.01 ± 0.08
8 wine	10^7	10^9	7.49 ± 0.02	26.00 ± 0.26	7.72 ± 0.14
9 wine	10^8	10^9	8.49 ± 0.02	22.18 ± 0.14	8.81 ± 0.03
10 wine	10^9	10^9	9.49 ± 0.02	19.44 ± 0.22	9.60 ± 0.11

Legend:[1]values are expressed as the mean of three replicates ± standard deviation; [2]this value is expressed in CFU/mL; /[3]no values obtained as a consequence of no amplification signal.

Table 4. Comparison of results obtained by plate count and PMA-qPCR techniques on real samples (must and wine samples) with unknown *O. oeni* concentration. The concentration of ethanol was also determined.

Sample	Ethanol concentration (% v/v)*	Plate count value (log CFU/mL)*	Calculated concentration (log CFU/mL)*
1 must	1.21 (± 0.21)	9.15 ± 0.12	9.19 ± 0.06
2 must	1.80 (± 0.41)	3.12 ± 0.22	3.03 ± 0.07
3 must	2.64 (± 0.10)	2.36 ± 0.04	2.47 ± 0.13
4 must	1.82 (± 0.12)	6.38 ± 0.09	6.35 ± 0.09
5 must	5.43 (± 0.36)	6.29 ± 0.15	6.30 ± 0.21
6 must	2.97 (± 0.27)	5.76 ± 0.10	5.76 ± 0.05
7 must	6.75 (± 0.11)	6.57 ± 0.18	6.59 ± 0.12
8 must	3.24 (± 0.12)	< 1+	No amplification
9 must	2.13 (± 0.23)	1.58 ± 0.11	1.67 ± 0.06
10 must	1.42 (± 0.12)	< 1+	No amplification
1 wine	11.56 (± 0.14	8.06 ± 0.04	8.11 ± 0.14
2 wine	12.01 (± 0.22)	7.36 ± 0.12	7.38 ± 0.12
3 wine	11.48 (± 0.12)	< 1+	No amplification
4 wine	12.20 (± 0.13)	3.94 ± 0.20	3.99 ± 0.07
5 wine	11.64 (± 0.21)	3.72 ± 0.08	3.58 ± 0.13
6 wine	11.55 (± 0.14)	2.45 ± 0.04	2.42 ± 0.05
7 wine	12.00 (± 0.10)	8.63 ± 0.18	8.75 ± 0.11
8 wine	11.63 (± 0.19)	< 1+	No amplification
9 wine	12.27 (± 0.27)	< 1+	No amplification
10 wine	12.29 (± 0.25)	7.17 ± 0.09	7.29 ± 0.11

Legend:*values are expressed as the mean of three replicates ± standard deviation; +these values are expressed in CFU/mL. Wines were sampled before filtration/pasteurization process.

3.6 Comparison between RT-qPCR, PMA-qPCR and qPCR calculated concentrations and plate count values

The concentrations of viable cells of *O. oeni* calculated by RT-qPCR and PMA-qPCR gave similar results in both must (Figure 4) and wine (Figure 5) suspensions, which were comparable (the same order of magnitude was obtained) with plate count values. The qPCR of non-PMA-treated DNA samples gave the same result for all samples (except for the negative controls), yielding a calculated concentration of 10^9 CFU/mL, which corresponded to the total amount of cells (viable and dead) present in the prepared suspensions.

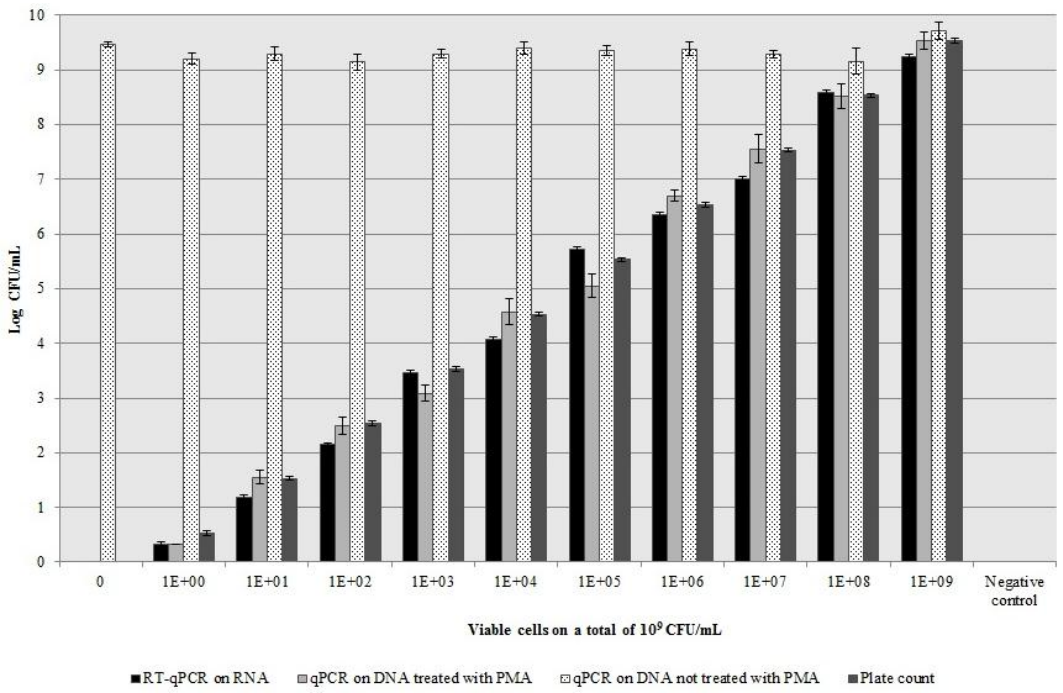

Figure 4. Comparison of the different methods used to quantify *O. oeni* (Viniflora CH11) in must.

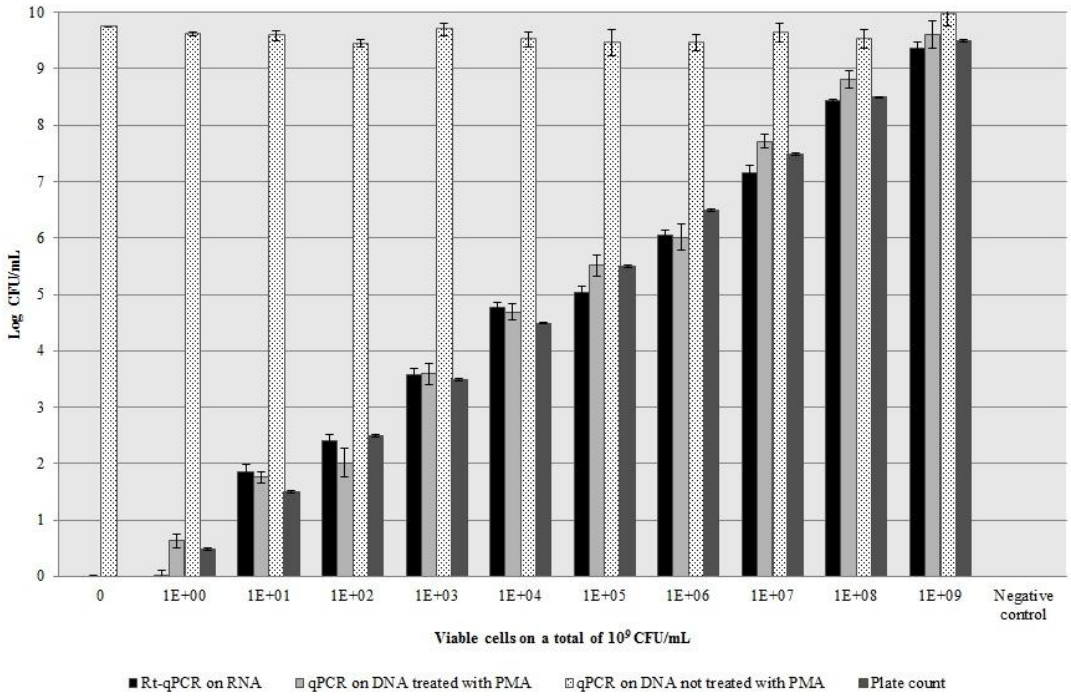

Figure 5. Comparison of the different methods used to quantify *O. oeni* (Viniflora CH11) in wine.

4. Discussion

MLF is an important step in winemaking, and it is a desirable process for some wines but must be avoided during the production of other wines. For this reason, rapid techniques for the monitoring of viable populations of *O. oeni* are a useful tool to quickly identify the right corrective measure to stimulate or avoid bacterial growth.

In this work, for the first time, a rapid and reliable PMA-qPCR technique was developed to detect and enumerate viable *O. oeni* cells in pure cultures and in must or wine samples, avoiding the time-consuming need to use plate count methods. The results showed that the DNA extraction method provided DNA samples free of PCR inhibitors, such as polyphenols and polysaccharides, which are known to copurify with nucleic acids.

The specificity of the assay was demonstrated by the absence of an amplification signal for DNA samples extracted from several bacterial and yeast strains that could possibly be found in must or wine. The results of qPCR were not affected by the presence of yeasts or bacteria in must and wine used to obtain *O. oeni* suspensions in this study.

The use of PMA, which is fundamental to only enumerate viable cells capable of fermentation, allowed for this qPCR technique to have a detection limit of 0.7 log CFU/mL, which is lower than that obtained by Pinzani et al. (2004), avoiding the amplification of DNA extracted from dead cells. For this reason, this method could be used to determine the presence/absence and number of viable cells of *O. oeni* in any must or wine samples and in pure cultures. Results obtained in this work also reach a lower detection limit than that obtained by Solieri and Giudici (2010), although all the methods developed in this study are species-specific but not strain-specific.

Treatment with deoxycholate, as suggested by many authors (Lee and Levin, 2009, Yang et al., 2011), to avoid the uncertain inactivation of DNA in cells exposed to relatively low lethal temperatures was not necessary in our study, because the absence of an amplification signal for DNA samples extracted from suspensions comprising only dead microorganisms demonstrated the permeation of PMA into all cells whose wall had been destroyed by the heat treatment.

A comparison with RT-qPCR demonstrated that the results obtained with both methods (PMA qPCR and RT-qPCR) are comparable. This finding can be explained by the use of mRNA as a target for the quantitative PCR. The use of primers targeting to the gene encoding the malolactic enzyme, therefore cDNA obtained from mRNA, resulted useful to amplify only viable cells and not dead cells. In fact, some authors (Hierro et al. 2006, Andorrà et al. 2011) suggested that the use of rRNA is not useful to quantify only viable cells, because it remains stable for a long period after the death of cells. mRNA is turned over rapidly in living bacterial cells, with most mRNA species having a half-life of only a few minutes (Alifano et al., 1994; Belasco et al., 1993; Kaberdin et al., 2011; Deutscher, 2006). Detection of mRNA might therefore be a good indicator of living cells at the time of sampling. In fact, Sheridan et al. (1998) demonstrated that the relationship between mRNA and viability may depend on the method used to inactivate cells, or the type of mRNA sought. In their work, the cells were exposed to two different stress treatments (heating at 60 °C x 20 min, 80 °C x 10 min, and 100 °C x 5 min, and using ethanol) and assayed mRNA from three different genes (*rpoH*, *groEL*, and *tufA*). Detectable mRNA disappeared more quickly from heat-killed cells than from ethanol-killed cells. The time at which mRNA became undetectable varied slightly and depended on the target. Generally, target mRNA from all three genes was detectable for up to 2 h but disappeared after 16 h. However, *groEL* mRNA was undetectable at 2 h after heating at 60 °C for 20 minutes. By contrast, 16S rRNA was detected immediately after heating and also at 16 h in all the samples. Confirming the stability of rRNA in comparison to mRNA. This data are perfectly in accordance with our data, in fact, 30 min after a treatment at 80 °C for 20 minutes no viability was detected by both, plate count and enrichment of the culture to recover also the stressed viable-but not culturable cells. Moreover, RT-qPCR on the mRNA gave a negative result, confirming the total degradation of the mRNA target in dead cells.

Bej et al. (1991, 1996) used RT-PCR to examine *Legionella pneumophila* and *Vibrio cholerae* exposed to heat or starvation, respectively, and detected specific mRNA only in samples that contained viable cells detected by culturing. Similarly, Patel et al. (1993) successfully assessed the viability of heat-killed *Mycobacterium leprae*, detecting a heat shock protein mRNA in living cells. And also more recently, other authors developed RT-qPCR methods based on mRNA to detect pathogens (Ye et al. 2012, Yang et al. 2008, McGuinness et al. 2010).

Despite RT-qPCR remains a good quantification technique, the developed PMA-qPCR method has the great advantage of being quick because it permits to obtain very low detection limits (0.33 log CFU/mL in must and 0.69 log CFU/mL in wine), avoiding the time-consuming and more expensive steps of RNA extraction and reverse transcription.

Results obtained in must and wine, with all the applied methods, were comparable and in accordance with Britz and Tracey (1990). In fact, these authors demonstrated the inhibitory effect of ethanol on the growth of *L. oenos*, also considering the combinatory effect of SO_2 and pH. As demonstrated by their data, the growth was negatively affected by ethanol after 10 days, and this inhibition resulted to be strain-specific. Notable is the fact that all of the tested strains resulted able to grow also after 10 days of staying at 13 % of ethanol and, moreover, 13 % of ethanol combined with stressful conditions of temperature, SO_2 and pH. On this basis, the fact that, in our study, the cell concentration values obtained for wine and must samples were similar after inoculation is not surprising, also considering that our *O. oeni* cells remained at 12 % of ethanol only for 30 minutes. Moreover, the strains used in this work are commercial malolactic starters, and for this reasons selected to be resistant to high concentrations of ethanol.

Finally, the reliability of the PMA-qPCR technique is confirmed by the application of the method on real must and wine samples. The obtainment of results, which are comparable with the plate count values, indicates that this new technique is useful to enumerate *O. oeni* in less than 24 hours (instead of 9 days using the traditional plate count method) after the collection of the sample. This allows the winemaker, if necessary, to take the right corrective measure to perform or avoid MLF.

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CHAPTER 3
Evaluation of gene expression of *mleA* gene in *Oenococcus oeni* under different experimental conditions

ABSTRACT

Malolactic fermentation (MLF) is a crucial step in winemaking, but little is known about the expression of *mleA*, which encodes for the malolactic enzyme in *Oenococcus oeni*. In the present study, an RT-qPCR method was developed to evaluate the expression of this gene. Seven different experimental conditions were investigated: absence of both L-malic and L-lactic acid, 0%, 12% and 14% ethanol, co-inoculation with *Saccharomyces cerevisiae*, sequential inoculum, and the presence of L-malic and L-lactic acid in equal concentrations. Two reference genes encoding lactate dehydrogenase and the 16S rRNA subunit were used to calculate Mean Normalised Expression (MNE) values. The results show that when MLF occurred, an increase in gene expression was registered in coordination with the maximum slope of curves, which describe the decrease in concentration of L-malic acid and an increase in concentration of L-lactic acid. Increased MNE levels were observed in the presence of ethanol and when MLF was carried out under coinoculation with *S. cerevisiae* and *O. oeni*. In the presence of equal concentrations of L-malic and L-lactic acid, MLF did not occur and no peaks in the expression profile were observed, suggesting that L-lactic acid could be involved in *mleA* gene regulation.

1. Introduction

Oenococcus oeni is known as the best-adapted wine-associated lactic acid bacteria (LAB) and is mainly used to induce malolactic fermentation (MLF) in red, white, and sparkling wines (Wibowo et al., 1985). MLF, the enzyme-mediated decarboxylation of L-malic acid into L-lactic acid, improves wine quality through deacidification, production of desirable flavours and aromas, and enhancement of microbial stability (Nehme et al., 2010). This reaction, which results in an average reduction in total acidity, could be considered to be an advantage of the low pH wines produced in regions with cool climate, but it must be sometimes avoided in the high pH wines of warmer climate regions (Jackson, 2003), where MLF is mainly conducted to change the aromatic profile of wine (Lerm, 2010).

Because of the harsh physicochemical conditions existing in must or wine, such as low pH (Vaillant et al., 1995), low temperature (Britz and Tracey, 1990), ethanol and SO_2 (Carretè et al., 2002), MLF is often difficult to induce and control, and it can start randomly, when fermentation is carried out by naturally contaminating bacteria (Beltramo et al., 2006).

In addition to the selection of starter culture strains of LAB to provide greater tolerance to these adverse properties of wine, there is an increasing awareness of the potentially important effects of the interaction between the yeast strain used to conduct alcoholic fermentation (AF) and the ability of the malolactic bacteria (MLB) strains to grow and carry out MLF (Alexandre et al., 2004). Such interactive effects of wine yeast and MLB have been observed for decades and can range from inhibitory to neutral and stimulatory. Some authors have affirmed that yeast metabolites such as ethanol, medium chain fatty acids (Capucho and San Romao, 1994) and SO_2 (Carretè et al., 2002) have an inhibitory effect on the MLF capacity of *O. oeni*, while yeast mannoproteins have been associated with the stimulation of bacterial growth in wine (Guilloux-Benatier et al., 1995). Therefore, the success or failure of MLF is closely related to the choice of the yeast and bacterial strain in combination and the interactions that may occur between them.
The influence of pH and ethanol on expression of the structural malolactic enzyme gene (*mle*) from *Lactobacillus plantarum* (Miller et al., 2011), which could be used as a starter MLB (du Toit et al., 2011), has been well defined. Additionally, to better understand the physiological response of *O. oeni* during rehydration, the quantification of expression levels of the malate transporter gene (*mleP*) and of two genes putatively involved in the ATP-binding cassette transport system (*oeoe_1651*, *oeoe_0550*) have also been well described (Costantini et al., 2011), but no information is available about *mle* gene expression in *O. oeni* under different environmental conditions.

An alternative inoculation strategy to the traditional sequential culture is the co-culture, in which both alcoholic and malolactic fermentations are simultaneously conducted. This technique can be used to successfully induce MLF under commercial conditions (Krieger, 2002). However, more information about

the MLF capacity and the mechanisms involved in the adaptation of *O. oeni* to stress conditions in co-culture is required. In particular, information about the expression of *mleA* in simultaneous culture with *Saccharomyces cerevisiae* and *O. oeni* in wine or wine-like medium is lacking.

Many studies have been carried out to better understand the behaviour of *O. Oeni* under stress conditions (Beltramo et al., 2006, Olguin et al., 2010) and to describe the genetic organisation of the *mle* operon in *O. oeni* (Galland et al., 2003, Labarre et al., 1996); however, it is still not clear how this locus is regulated and how various substances and metabolites affect its expression levels. For these reasons, the goal of this study was the optimisation of a Reverse Transcription quantitative PCR technique (RT-qPCR) to evaluate the expression levels of the *mle* gene in a wine-like medium under different experimental conditions: the presence or absence of L-malic and L-lactic acid, 0%, 12% and 14% ethanol, co-inoculation with *Saccharomyces cerevisiae*, and sequential inoculum.

2. Materials and methods

2.1. Preparation of O. oeni suspensions

To obtain cultures from which to prepare suspensions, 1 commercial strain of *O. oeni* (Viniflora CH11 by Christian Hansen, Copenhagen, Denmark) was grown for 72 hours at 30°C in test tubes containing 30 mL of *Leuconostoc oenos* (LO) medium, suggested by the Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ) as the best medium to grow *O. oeni* cells (casein peptone, tryptic digest 10.00 g/L, yeast extract 5.00 g/L, glucose 10.00 g/L (200 g/L in case of sequential and co-inoculation trials), fructose 5.00 g/L (50 g/L L in case of sequential and co-inoculation trials), $MgSO_4$ x 7 H_2O 0.20 g/L, $MnSO_4$ x H_2O 0.05 g/L, (NH_4) citrate 3.50 g/L, tween 80 1.00 mL/L, apple juice, filtered 100.00 mL/L, cysteine-HCl x H_2O 0.50 g/L, pH adjusted to 4.8) to obtain a cell concentration of 10^9 colony forming units (CFU)/mL (equivalent to an optical density at 600 nm [OD_{600}] of 1). Strain Viniflora CH11 was chosen for the gene expression trials because preliminary tests carried out using 4 commercial strains (Viniflora CH11, Viniflora CH16, Viniflora oenos and Amar04) showed that CH11 was the fastest to conclude MLF. Two ten-fold serial dilutions of this culture were prepared in LO medium to obtain a suspension of 10^7 CFU/mL *O. oeni*, which was used to inoculate the samples with a cellular concentration of 10^6 CFU/mL. One mL of each dilution was included in double layer of LO medium with 15 g/L Agar technical n°3 (Oxoid, Milan, Italy). The plates were incubated in anaerobic conditions at 30 °C for 7 days, then colonies were counted to determine the number of viable *O. oeni* CFU/g.

2.2 Composition of mediums used

In this study, gene expression of *mleA* of *O. oeni* in 7 different experimental conditions was investigated. In all the experimental cases 10^6 CFU/mL of *O. oeni* were inoculated in the appropriate synthetic medium.

To study the gene expression in absence of malic acid and ethanol, *O. oeni* was inoculated in LO medium, pH adjusted to 3.5.

For tests carried out in the presence of malic acid, *O. oeni* was inoculated in LO medium added with a concentration of L-malic acid (Sigma-Aldrich, Milan, Italy) of 3.5 g/L, while the study of gene expression levels in the presence of L-malic and L-lactic acids required the addition of 3.5 g/L of L- malic acid and 3.5 g/L of L-lactic acid (Sigma-Aldrich, Milan, Italy) to LO medium.

Trials in the presence of ethanol were carried out in LO medium containing 12% or 14% ethanol (Fluka, Milan, Italy).

The study of gene expression in co-inoculation with *Saccharomyces cerevisiae* required the simultaneous inoculation of *O. oeni* and 10^6 CFU/mL of a commercial oenological yeast (NT50, Anchor, Cape Town, South Africa). For the sequential inoculum test, the LO medium was inoculated with *O. oeni* after completion of alcoholic fermentation carried out by the same commercial oenological yeast (NT50, Anchor, Cape Town, South Africa).

All inoculated samples were incubated at 19 °C and samples were sterile-collected 4 times a day, every 3 hours, starting from the inoculation point until the end of the malolactic fermentation, if it occurred.

Samples were then subjected to the analysis described in the following paragraphs. Trials were conducted in triplicate for each different condition.

2.3. Plate counts

One mL of each sample, after serial ten-fold dilutions in Maximum recovery diluent (Oxoid, Milan, Italy), was enumerated on a double layer of LO medium supplemented with 15 g/L Agar technical n°3 (Oxoid, Milan, Italy) to determine the exact CFU/mL inoculated in the samples. Plates were incubated in anaerobic conditions at 30 °C for 7 days and colonies were then counted to determine the numbers of viable *O. oeni*.

To effectively enumerate yeast cells inoculated in conditions in which the effect of co-inoculation vs. sequential inoculation with *S. cerevisiae* on *mleA* expression was evaluated, 0.1 mL of each serial dilution of each sample was plated onto WL nutrient agar (Oxoid, Milan, Italy). Plates were incubated in aerobic conditions at 30 °C for 2 days, colonies were counted and the numbers of viable *S. cerevisiae* were determined from those counts.

2.4. Determination of concentration of L-malic acid

Concentration of L-malic acid was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Rodano, Italy) using the enzymatic kit L-malic acid (Roche, Milan, Italy) and following the manufacturer's instructions.

2.5. Determination of concentration of L-lactic acid

Concentration of L-lactic acid was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Rodano, Italy) using the enzymatic kit L-lactic acid (Roche, Milan, Italy) and following the manufacturer's instructions.

2.6. Determination of concentration of ethanol

In trials in which the effect of co-inoculation vs. sequential inoculation with *S. cerevisiae* on *mleA* gene expression was evaluated, concentration of ethanol was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Rodano, Italy) using the enzymatic kit Ethanol (Roche, Milan, Italy) and following the manufacturer's instructions.

2.7. pH measurement

For all the collected samples, pH was measured using the Basic20 pH instrument (Crison instruments S.A., Alella, Spain).

2.8. RNA extraction

One mL of each suspension was centrifuged at 10000 rpm for 7 min to pellet the cells. RNA was extracted from the pellets using the MasterPureTM Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin). The manufacturer's instructions for RNA purification from cell samples were followed, except that DNAse treatment was extended for 150 minutes to achieve total degradation of contaminating DNA. Moreover, RNA was resuspended in 35 μL of sterile bidistilled DEPC (Sigma-Aldrich, Milan, Italy) treated water. RNA concentration was determined using the Nanodrop 2000c spectrophotometer (Thermo Scientific, Rodano, Italy) and then standardised to 40 ng/μL by dilution with sterile DNA-free Milli-Q DEPC treated water.

The absence of contaminating DNA in RNA samples was confirmed with both classical and quantitative PCR reactions using universal primers P1V1-P4V3 (Klijn et al., 1991) as described by Iacumin et al., (2009). When positive signals were detected, the DNase treatment was repeated to eliminate the co-extracted DNA.

2.9. Reverse Transcription

Standardised RNA samples were then converted into cDNA. Reverse transcription reactions were carried out by using the ImProm-IITM Reverse Transcription System (Promega, Milan, Italy), following the manufacturer's instructions. cDNA samples were used as templates for qPCR
reactions.

2.10. Primer design and PCR conditions

Using FastPCR 6.1 software (Kalendar et al., 2009), the following oligonucleotides were developed to establish real-time quantitative PCR for the gene (Accession Number: AY786176.1) coding for the malolactic enzyme in *O. oeni*. Forward primer, Malomar F: 5'-GTT AAT CAT GCC GAA TCG-3' (region 658-675) and Malomar R: 5'-GTC GGA AAG ACC CTG-3' (region 928-942), generating a PCR product of 285 bp.

To optimise the amplification conditions and to confirm the specificity of the primers in both PCR and qPCR the following bacterial and yeast strains were used: *Oenococcus oeni* DSMZ 20252, *Lactobacillus brevis* DSMZ 20054, *L. casei* DSMZ 20111, *L. plantarum* DSMZ 20174, *L. reuteri* DSMZ 20053, *L. rhamnosus* DSA, *L. sakei* DSMZ 6333, *Lactococcus lactis* DSMZ 20481, *Leuconostoc citreum* DSMZ 5577, *Leuc. gasicomitatum* DSMZ 15947, *Leuc. mesenteroides subsp. mesenteroides* DSMZ 20343, *Pediococcus pentosaceous* DSMZ 20336, and other yeast species commonly isolated from must and wine were used in both PCR and qPCR to assess the specificity of the protocol. In particular, *Saccharomyces ludwigii* UCD 6721, *S. pastorianus* DSMZ 6580, *S. bayanus* DSMZ 70412, *S. cerevisiae* ATCC 51, *Brettanomyces bruxellensis* DSMZ 70726, *Candida etanolica* UCD 7, *C. vini* UCD 36, *Hanseniaspora guillermondii* DSA, *Pichia membranifaciens* DSA, *Metschnikovia pulcherrima* DSA, and *Kloechera apiculata* DSA.

Conventional PCR was carried out in a final volume of 25 µL containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) dNTPs, 1 µM primer, and 1.25 U of Taq polymerase (Applied Biosystems, Milan, Italy). The amplification cycle was as follows: 95°C denaturation for 5 min, 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad, Milan, Italy).

2.11. Real-time PCR (qPCR)

cDNA samples were subjected to qPCR reactions with the three couples of primers Malomar F –Malomar R, ldhD F – ldhD R (Desroche et al., 2004) and 16S-qPCR-F (5'-CCT CGG GAT TTC ACA TCA GAC T-3') – 16S-qPCR-R (5'-CCA GCA GCC GCG GTA AT-3') (Mtshali et al., 2011), as two reference genes were used as internal controls.

Real-time PCR mixtures contained 10 µl of 2x SsoFast EvaGreen Supermix (Biorad, Milan, Italy), 400 nM of each primer, 2 ng/µL of DNA and the reaction mixture's volume was adjusted to 20 µL with sterile DNA-free Milli-Q water.

qPCRs were performed using a RotorGene Q system (QIAGEN, Milan, Italy) with one cycle of initial denaturation of template DNA and activation of Taq DNA polymerase at 98 °C for 3 minutes, followed by 45 cycles of denaturation at 95 °C for 10 s, primer annealing at 60 °C for 15 s and extension at 72 °C for 20 s. Fluorescence signal acquisition was performed during the extension step. To verify

that formation of non-specific products or primer dimers had not occurred, the melting analyses of the final products for each qPCR were evaluated using a melting ramp from 55 to 95 °C at 1 °C/s.

2.12. Calculation of MNE values

According to the MIQE (Bustin et al., 2009), mean normalised expression (MNE) levels were calculated using the mathematical model proposed by Pfaffl (2001) and modified by Muller et al. (2002).

3. Results

3.1 PCR and qPCR assay specificity

In silico PCR performed by FastPCR software using the primers Malomar F and Malomar R showed a positive result only for *O. oeni*.

The specificity of the assay was confirmed by both PCR and qPCR analysis of several strains of *O. oeni* and all of the different species of lactic acid bacteria and yeasts reported in section 2.11. Both PCR and qPCR demonstrated specificity: using PCR, only one band corresponding to the amplification of *O. oeni* strains, as expected, was obtained (Figure 1); using qPCR, a positive fluorescence signal was yielded only for *O. oeni* samples.

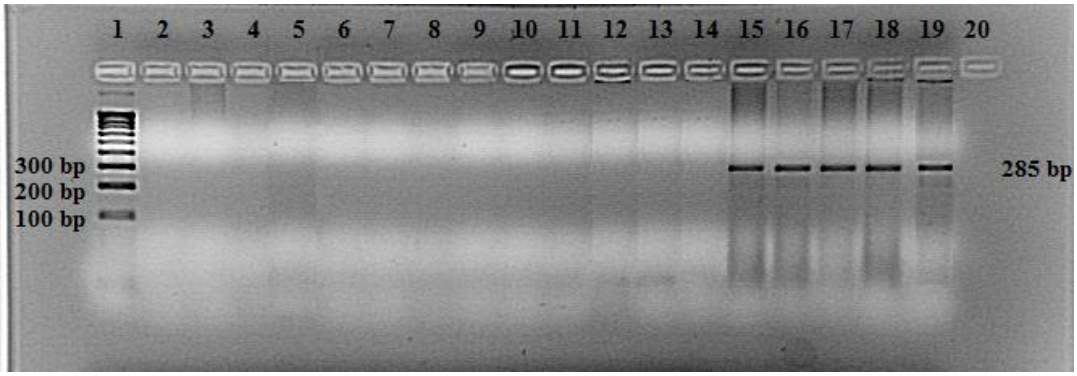

Figure 3. Results of conventional PCR reaction. Agarose gel (2%), 120V, 60'. Line 1: Molecular weight marker 100 bp (Promega, Milan, Italy); Line 2: *L. plantarum* DSMZ 20174; Line 3: *L. brevis* DSMZ20054; Line 4: *L. reuteri* DSMZ 20053; Line 5: *L. sakei* DSMZ 6333; Line 6: *Lc. Lactis* DSMZ 20481; Line 7: *Leuc. citreum* DSMZ 5577; Line 8: *Leuc. gasicomitatum* DSMZ 15947; Line 9: *P. pentosaceus* DSMZ 20336; Line 10: *Brett. bruxellensis* DSMZ 70726; Line 11: *S. cerevisiae* ATCC 51; Line 12: *S. bayanus* DSMZ 70412; Line 13: *S. ludwigii* UCD 6721; Line 14: *S. pastorianus* DSMZ 76580; Line 15: *O. oeni* DSMZ 20252; Line 16: *O. oeni* Viniflora CH oenos; Line 17: *O. oeni* Viniflora CH11; Line 18: *O. oeni* Viniflora CH16; Line 19: *O. oeni* Vason Amar 04; Line 20: Negative control.

3.2. Growth and metabolic activity of bacteria and yeasts

For each one of the assays, the inoculated population of *O. oeni* was approximately 10^6 CFU/mL, which is similar to the inoculum used for starter cultures in winemaking. Plate count values show that *O. oeni* exponential growth phase started for all of the samples approximately 20 hours after the inoculation; for the trials in which ethanol was present (12% v/v, 14% v/v and sequential inoculation), the exponential phase started at approximately 40 hours. The stationary phase was achieved in all samples within 100 hours after inoculation (Table 1).

Table 1. Table 1. Growth of *O. oeni* in the different environmental conditions tested. Values are expressed in log CFU/mL (mean ± standard deviation)

Hours after inoculation	no malic	ethanol 0%	ethanol 12%	ethanol 14%	co-inoculation	sequential	Malic + Lactic
0	6.28 ± 0.21	6.20 ± 0.25	6.73 ± 0.25	6.43 ± 0.23	6.32 ± 0.32	6.08 ± 0.24	6.53 ± 0.21
16.5	6.73 ± 0.14	6.79 ± 0.23	6.23 ± 0.14	6.23 ± 0.24	6.34 ± 0.25	6.08 ± 0.11	6.51 ± 0.24
19.5	6.85 ± 0.11	6.86 ± 0.24	6.32 ± 0.27	6.21 ± 0.22	6.73 ± 0.16	6.15 ± 0.14	6.45 ± 0.27
22.5	6.85 ± 0.24	6.92 ± 0.54	6.51 ± 0.13	6.36 ± 0.10	6.79 ±0.29	6.11 ± 0.21	6.83 ± 0.11
25.5	7.20 ± 0.15	7.12 ± 0.26	6.73 ± 0.19	6.38 ± 0.26	7.00 ± 0.12	6.15 ± 0.27	6.96 ± 0.10
40.5	7.73 ± 0.22	7.64 ± 0.44	6.32 ± 0.32	6.22 ± 0.22	7.38 ± 0.21	6.22 ± 0.15	6.87 ± 0.18
43.5	7.85 ± 0.18	7.67 ± 0.14	6.34 ± 0.19	6.54 ± 0.31	7.43 ± 0.25	6.27 ± 0.16	6.84 ± 0.14
46.5	8.71 ± 0.12	8.08 ± 0.13	6.54 ± 0.22	6.70 ± 0.24	8.08 ± 0.18	6.26 ± 0.21	6.90 ± 0.17
49.5	9.36 ± 0.29	8.96 ± 0.29	6.53 ± 0.21	6.93 ± 0.27	8.91 ± 0.16	6.74 ± 0.22	6.96 ± 0.23
64.5	11.81 ± 0.17	10.94 ± 0.22	6.98 ± 0.26	6.95 ± 0.19	10.83 ± 0.10	6.51 ± 0.16	6.97 ± 0.17
67.5	11.88 ± 0.20	11.41 ± 0.19	7.12 ± 0.25	6.98 ± 0.20	10.41 ± 0.13	6.23 ± 0.15	6.98 ± 0.28
70.5	11.91 ± 0.21	11.88 ± 0.18	7.18 ± 0.17	6.98 ± 0.56	10.44 ± 0.24	6.88 ± 0.14	6.98 ± 0.23
73.5	11.97 ± 0.28	11.93± 0.23	7.08 ± 0.21	7.04 ± 0.21	11.83 ± 0.19	7.28 ± 0.17	7.08 ± 0.21
88.5	12.36 ± 0.19	12.73 ± 0.17	7.18 ± 0.11	7.40 ± 0.29	11.65 ± 0.27	7.54 ± 0.12	7.34 ± 0.20
91.5	12.40 ± 0.45	12.76 ± 0.29	7.23 ± 0.15	7.43 ± 0.26	11.68 ± 0.24	7.57 ± 0.20	7.54 ± 0.14
94.5	12.45 ± 0.23	12.79 ± 0.51	7.28 ± 0.19	7.32 ± 0.27	11.77 ± 0.11	7.61 ± 0.14	7.53 ± 0.15
97	12.41 ± 0.27	12.79 ± 0.27	7.25 ± 0.13	7.89 ± 0.24	11.72 ± 0.17	7.94 ± 0.10	7.98 ± 0.27
114	12.43 ± 0.11	12.78 ± 0.18	7.42 ± 0.19	7.88 ± 0.14	11.61 ± 0.14	7.75 ± 0.23	7.97 ± 0.10
117	12.45 ± 0.31	12.78 ± 0.11	7.40 ± 0.17	7.88 ± 0.13	11.60 ± 0.20	7.93 ± 0.40	7.98 ± 0.19
120	12.51 ± 0.14	12.66 ± 0.27	7.34 ± 0.18	7.86 ± 0.11	11.81 ± 0.24	7.91 ± 0.21	7.96 ± 0.14

In both the co-inoculation and sequential inoculation trials, *S. cerevisiae* cell number reached 10 log CFU/mL, and alcoholic fermentation was completed within 100 hours after the inoculation (10.77% v/v and 11.04% v/v of ethanol in co-inoculated and sequential inoculated samples, respectively). According to Margalit (1997), when MLF occurred, the pH of the samples increased between 0.23 to 0.28 units.

The trends of the curves describing the decrease in concentration of L-malic acid and the increase in concentration of L-lactic acid (Figures 2 to 6, panel A) show that MLF was complete within 50 hours after inoculation in all of the samples in which ethanol was absent at the moment of *O. oeni* inoculation (co-

inoculation and 0% v/v of ethanol). Samples with ethanol (12% v/v, 14% v/v and sequential inoculation) required up to 120 hours for MLF to be completed. In each case, MLF was complete before the end of the exponential growth phase of *O. oeni*. MLF didn't occur in the samples in which L-malic acid was absent or the L-malic acid concentration was equal to the L- lactic acid concentration (Figure 8, panel A).

3.3. mleA expression levels

MNE calculation requires that all of the cDNA samples obtained after reverse transcription are amplified in qPCR reactions in which couples of primers targeting the gene of interest and a reference gene are used. In this study, because many authors (Desroche et al., 2004, Olguin et al., 2009, Beltramo et al., 2006) describe the *ldhD* gene as the ideal reference gene for gene expression studies in *O. oeni*, *ldhD* gene was used for this purpose. Conversely, because the debate on the stability of lactate dehydrogenase (*ldhD*) expression levels is not resolved, a gene encoding for 16S rRNA subunit was also used as reference gene, and MNE levels for *mleA* were calculated using both *ldhD* and 16S rRNA as reference genes.

MNE profiles of samples collected in absence of L-malic acid (Figure 7) show that *mleA* is constitutively expressed at a baseline level (1.16 to 1.31 using *ldhD* and 1.62 to 1.74 using 16S rRNA).

In all of the cases in which MLF occurred, both MNE profiles (Figures 2-6, panels B and C) show an increase in gene expression corresponding to the maximum slope of curves that describe the decrease in the concentration of L-malic acid and an increase in the concentration of L-lactic acid. MNE profiles of samples collected in the presence of equal concentrations of L-malic and L-lactic acids (Figure 8, panels B and C) denote that expression levels settle at a baseline level in the absence of positive peaks.

MNE levels obtained using the 16S rRNA reference gene are on average higher than values obtained using *ldhD*.

4. Discussion

Although the malolactic enzyme has been reported as being induced by L-malic acid (Lonvaud-Funel, 1995), and an addition of L-malic acid results in increased malolactic activity in *Lactobacillus collinoides* (Arthurs and Lloyd, 1999), MNE levels of *mleA* in the absence of L-malic acid indicate that this gene is constitutively expressed in *O. oeni* cells at a baseline level.

Comparison between trials carried out under different experimental conditions demonstrate that MNE levels of *mleA*, calculated using two different reference genes, show a positive peak in correspondence of the end of the MLF, which does not correspond to an increase in the number of viable cells in the culture. For this reason, the increase of the MLF rate could be due to the increased

expression level of *mleA*. Thus *mleA* expression, according to Lonvaud-Funel (1995), is increased in the presence of malic acid. When ethanol was present in the medium, gene expression levels of *mleA* gene were appreciably higher than under all other experimental conditions studied in this work. In particular, the highest concentration of ethanol (14% v/v) corresponded to the highest MNE values (31.84 ± 0.29) of *mleA*. These results support the findings of Beltramo et al., (2006), who reported that the presence of ethanol results in an increase of *mleA* expression; these observations also confirm the hypothesis formulated by Miller et al. (2011) because *mleA* expression seems to be enhanced in stress conditions.

Values obtained using both 16S rRNA and *ldhD* genes as reference for the calculation of MNE in trials with co-inoculation of *O. oeni* and *S. cerevisiae* show high *mleA* expression levels (29.64 ± 0.14), suggesting that the metabolic activity of *O. oeni* is enhanced. This is most likely due to mutualism between the two microorganisms growing in the same medium, which leads to an increased availability of amino acids derived from lysed yeast cells present in the starter used for the inoculation and the presence of released mannoproteins associated with the stimulation of bacterial growth in wine (Guilloux-Benatier et al., 1995).

During sequential inoculation, although ethanol was present (11.04% v/v) in the fermented medium at the moment of the inoculation of *O. oeni*, the maximum MNE level was achieved after 73.5 hours post-inoculation, 18 hours less than the peak reached by the samples collected in the presence of 12% v/v of ethanol. This increased fermentation rate could also be due to the increased metabolic activity of *O. oeni* in the presence of yeast cellular extracts in the medium.

Finally, the MNE profile of samples in trials carried out in the presence of equal concentrations of L-malic and L-lactic acids show that even if L-malic acid is present in the medium, L-lactic acid could inhibit *mleA* expression and MLF was not detected. For this reason, it is possible to presume that L-lactic acid is involved in *mleA* regulation, but further studies are needed to elucidate how this substance is able to affect the expression of the gene encoding for the malolactic enzyme.

5. Conclusions

Results obtained in this study reveal that the designed primers, targeting the *mleA* gene of *O. oeni*, are specific for this microorganism, and their use in the developed RT-qPCR method to evaluate gene expression in a wine-like medium of gene encoding for the malolactic enzyme showed that L-lactic acid is probably involved in *mleA* gene regulation.

Moreover, the study of MNE levels of *mleA* gene in *O. oeni* points out that the best conditions to obtain high levels of gene expression are co-inoculation and presence of high concentrations of ethanol.

Further gene expression studies are needed to find out which one of these two antithetic conditions is the best to ensure rapid completion of MLF in wine.

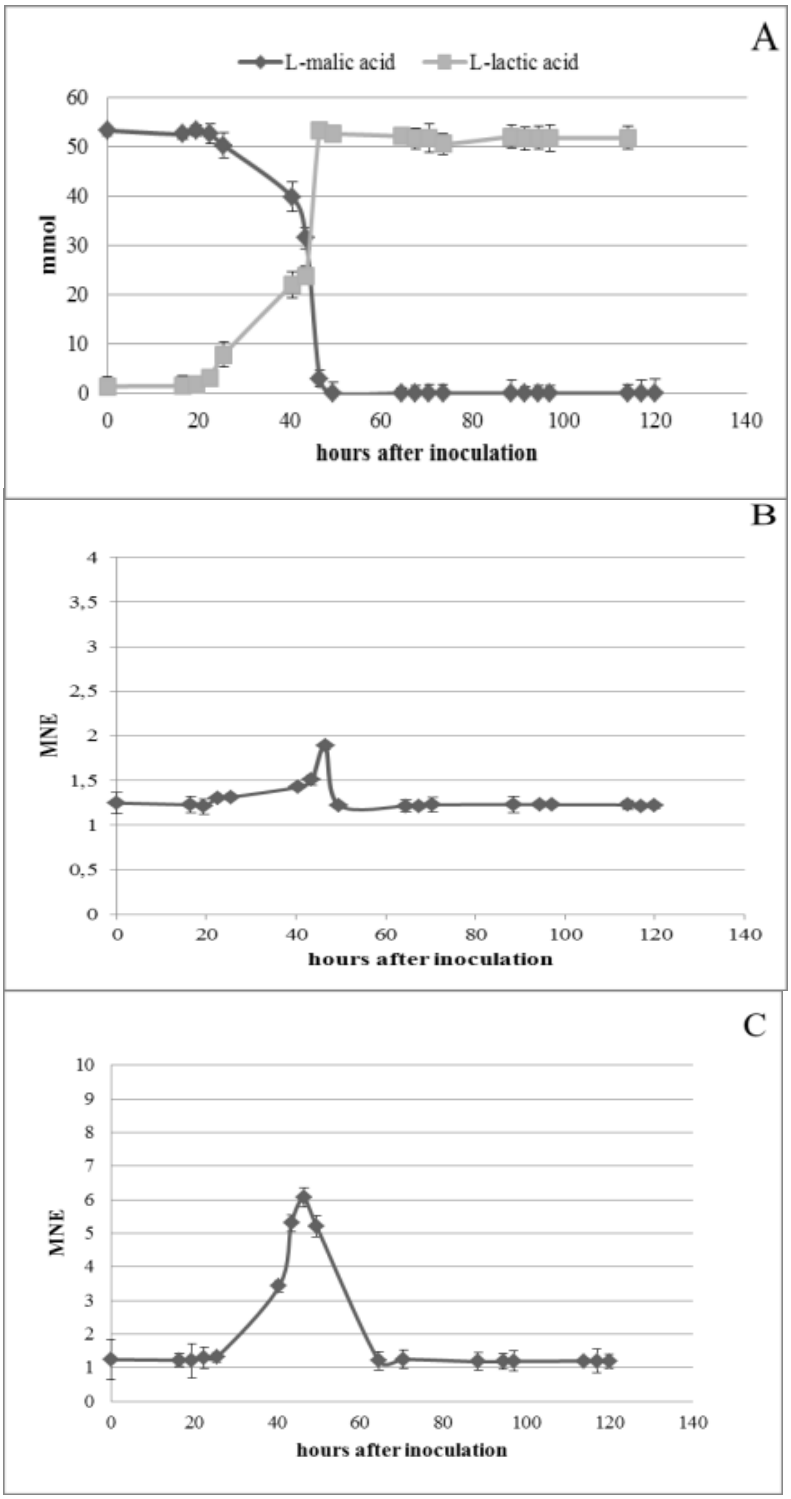

Figure 2. Evaluation of L-malic and L-lactic concentrations (panel A), MNE using *ldhD* gene (panel B) and 16S rRNA (panel C) as reference genes in samples of trial carried out in the presence of 0% v/v ethanol.

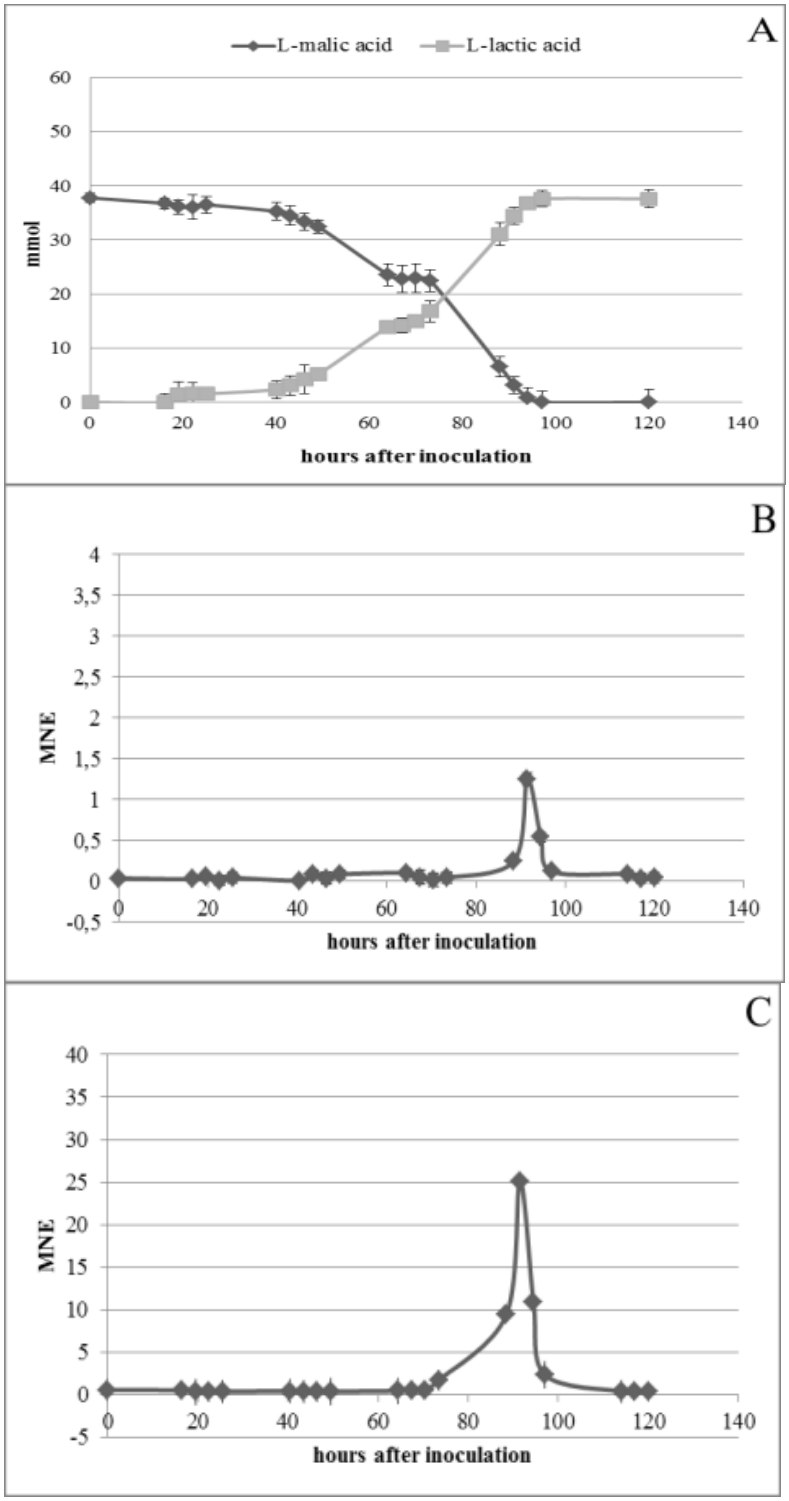

Figure 3. Evaluation of L-malic and L-lactic concentrations (panel A), MNE using *ldhD* (panel B) and 16s rRNA (panel C) as reference genes in samples of trial carried out in presence of 12% v/v ethanol.

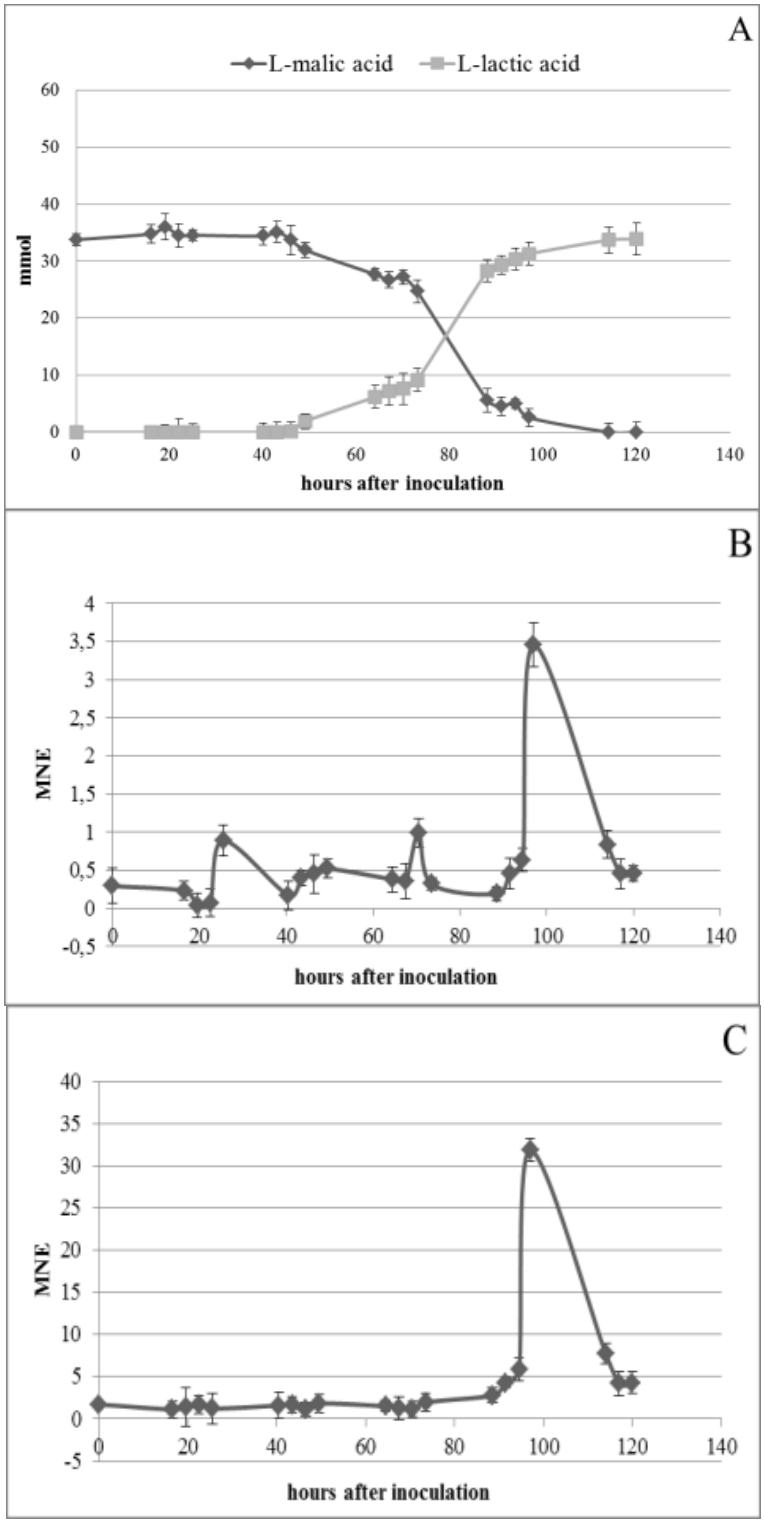

Figure 4. Evaluation of L-malic and L-lactic concentrations (panel A), MNE using *ldhD* (panel B) and 16s rRNA (panel C) as reference genes in samples of trial carried out in presence of 14% v/v ethanol.

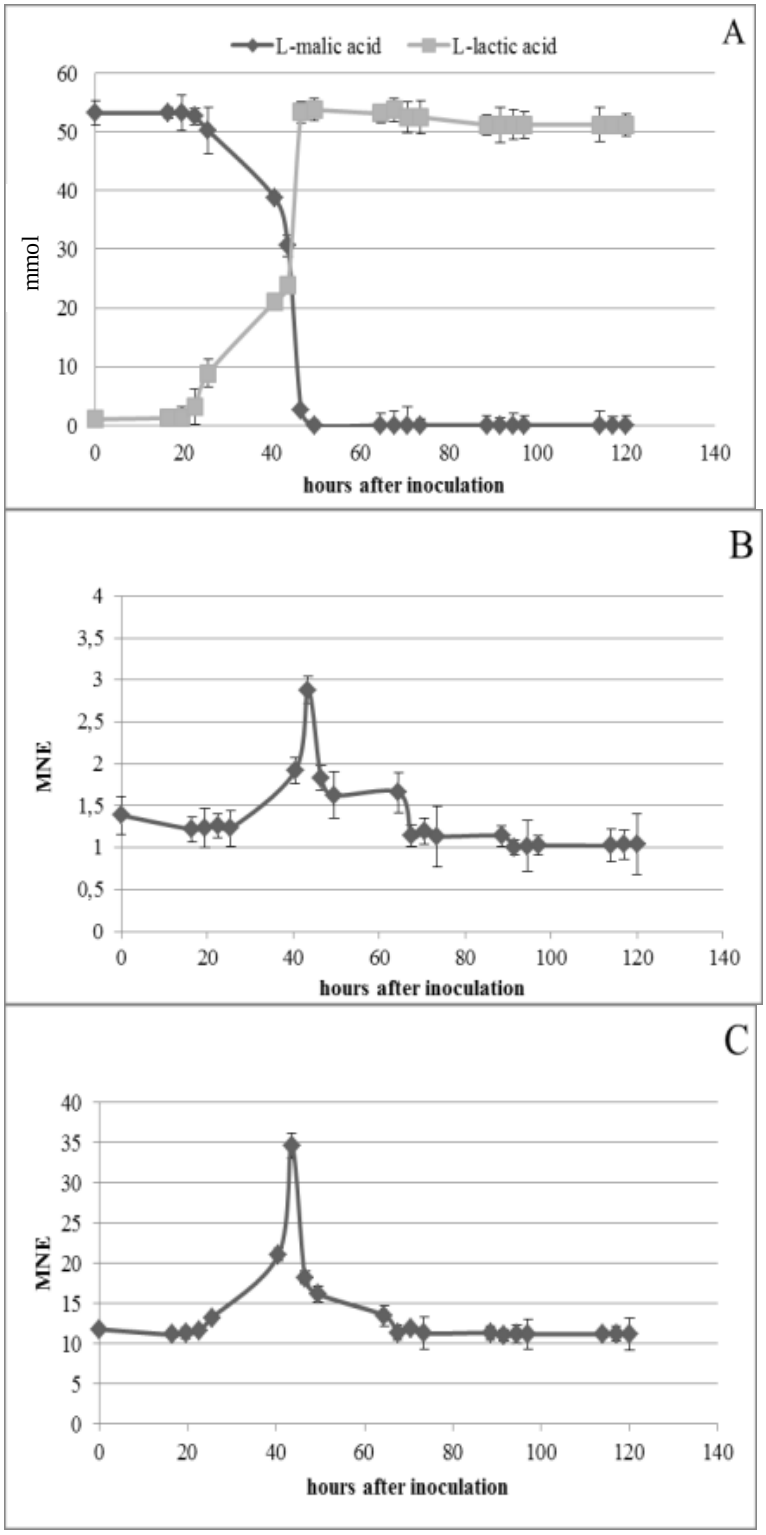

Figure 5. Evaluation of L-malic and L-lactic concentrations (panel A), MNE using *ldhD* (panel B) and 16s rRNA (panel C) as reference genes in samples of trials where MLF was conducted using co-inoculation of *S. cerevisiae* and *O. oeni*.

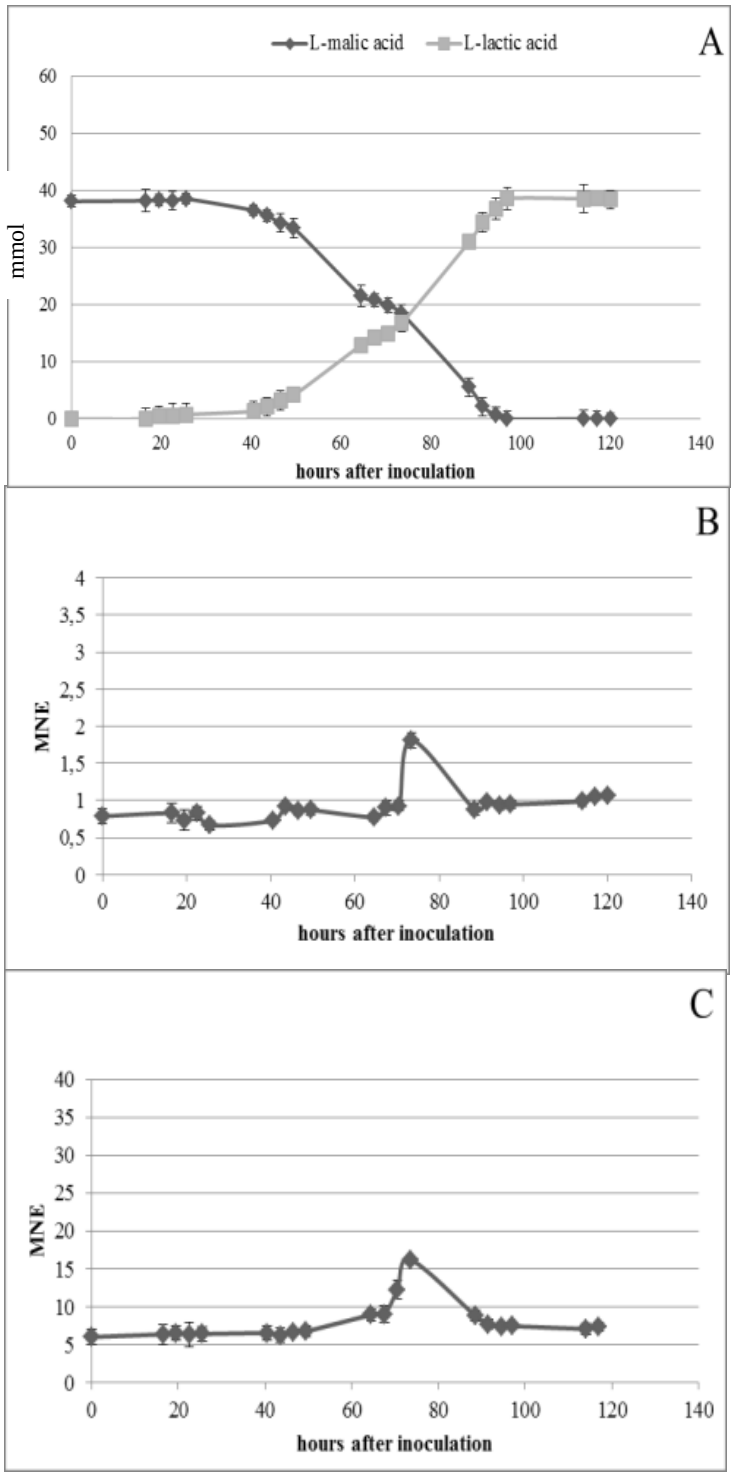

Figure 6. Evaluation of L-malic and L-lactic concentrations (panel A), MNE using *ldhD* (panel B) and 16s rRNA (panel C) as reference genes in samples where MLF was conducted using sequential inoculation of *S. cerevisiae* and then *O. oeni*.

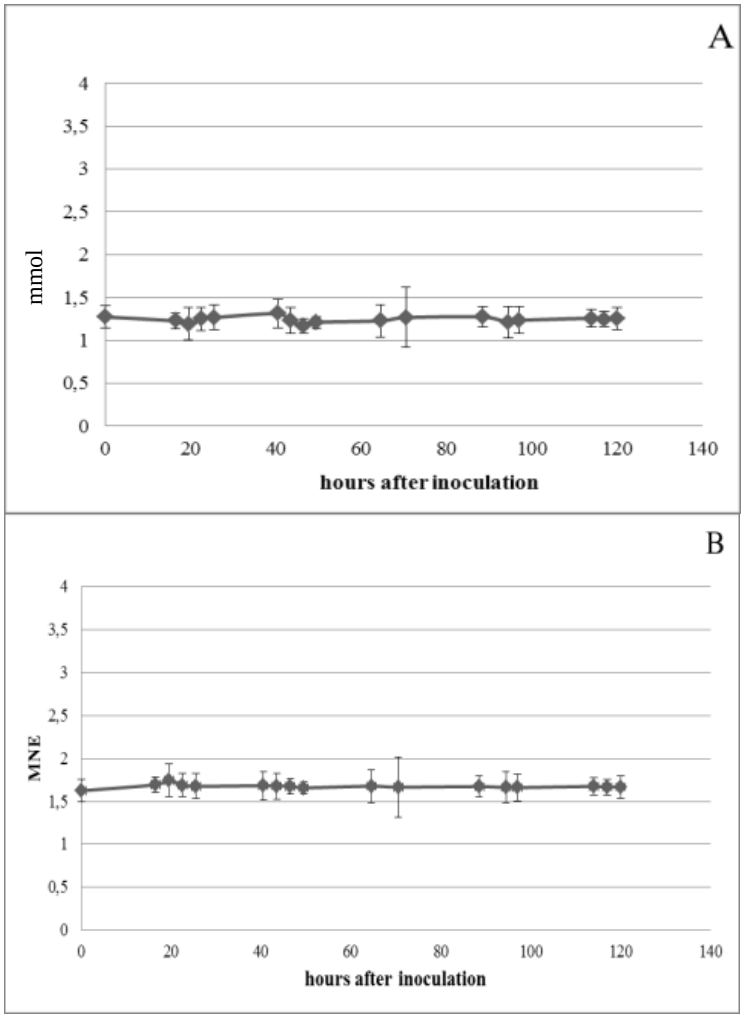

Figure 7. Evaluation of L-malic and L-lactic concentrations (panel A), MNE using *ldhD* gene (panel B) and 16s rRNA gene (panel C) as reference in trials carried out in presence of equal concentrations of L-malic and L-lactic acids.

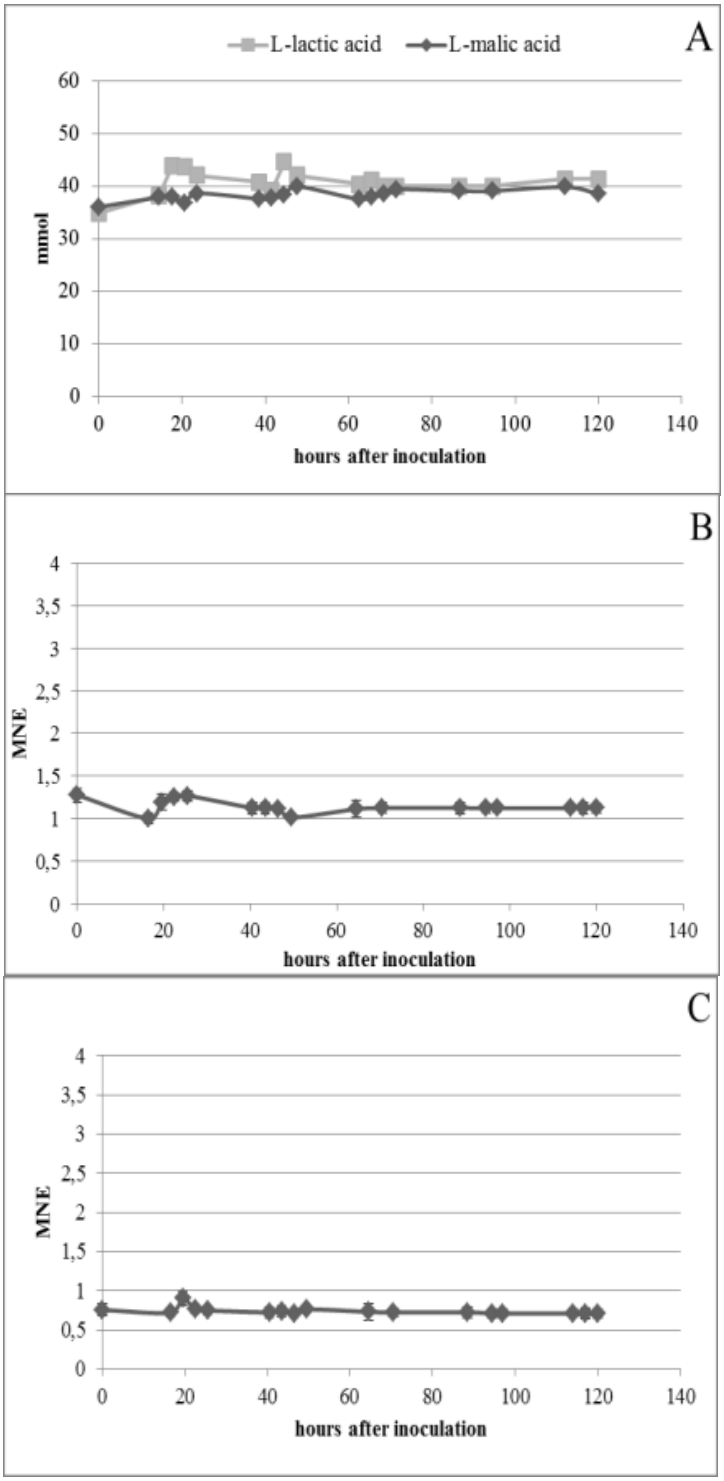

Figure 8. Evaluation of L-malic and L-lactic concentrations (panel A), MNE using *ldhD* gene (panel B) and 16s rRNA gene (panel C) as reference in trials carried out in presence of equal concentrations of L-malic and L-lactic acids.

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CHAPTER 4

Development of a Reverse Transcriptase – qPCR method for the evaluation of gene expression of the β-glucosidase encoding gene in *Oenococcus oeni* in winemaking conditions

ABSTRACT

Besides the capacity of performing malolactic fermentation, lactic acid bacteria (LAB) show the capability to influence the aromatic complexity of wine thanks to the release of volatile compounds due to the activity of the β-glucosidase enzyme, which has been isolated in various strains, including *Oenococcus oeni*. The purpose of this study was the optimization of a Reverse Transcriptase – qPCR technique to evaluate which winemaking scenario (co-inoculation or sequential inoculation) was the best to ensure the highest levels of relative expression of the gene encoding for this enzyme. Results confirm that the presence of β-glucosidase is strain specific in *O. oeni*, and show that in case of sequential inoculation the expression levels of the gene encoding for this enzyme are stable at a baseline level, while in co-inoculation conditions higher expression levels are reached, probably because of an increased availability of nitrogen compounds in the medium due to the presence of yeasts.

1. Introduction

The compounds that stimulate our senses while drinking wine are both released from grapes and synthesized, degraded or modified during vinification. Many of these sensory changes, such as changes in the intensity of floral, fruity, spicy and honey-like attributes, can be related to the release of volatile compounds through the action of enzymes, including glycosidases (Matthews et al.,2004; Ugliano and Moio, 2006).

The sensory properties of the wine are the result of a multitude of individual compounds. Four groups of these, the monoterpenes (like geraniol, nerol and linalool), the C13-norisoprenoids, benzene by-products and aliphatic compounds can be found, associated with sugars to form glycosides (Matthews et al.,2004; Maicas and Mateo, 2005), which are a heterogeneous group of natural substances formed by a sugary part (glyco) and a non-sugar part (aglycone). Depending on the sugar part, which characterizes them, they may have different names; in particular, if the glyco is a glucose, they are called glycosides (Spano et al., 2005). In general, the aromatic compounds are conjugated with glucose (monoglycosides), or with a glucose linked to another sugar (rhamnose, arabinose or apiose), constituting a diglucoside (Ribéreau-Gayon et al., 2006; Spano et al., 2005; Grimaldi et al., 2005). They are present in many fruits such as peach, apricot, yellow plum, passion fruit, kiwi, papaya, pineapple, mango, raspberry, strawberry and also grapes (Maicas and Mateo, 2005).

In wine, aromatic compounds, which would normally be detectable by human senses, are not perceptible when they are in the glycosidic form. Consequently, since up to 95% or more of such aromatic compound is present in this form, most of their aromatic potential is not realized (Mathews et al., 2004). The hydrolisis of these glycosides, which allows the release of aglycones from sugars thus releasing the aromas, can occur thorugh acid or enzymatic hydrolisis catalyzed by glycosidases (Spano et al., 2005; Gagnè et al., 2010; Mathews et al., 2004).

Glycosidases also affect the color of the wine. When the anthocyanin glycosides (glycosides whose aglycone is an anthocyanin, or a pigment) are deglycosilated, the corresponding anthocyanin is less stable and can be easily converted into a brown or colorless compound. This result may be undesirable in a red wine, but these enzymes have been suggested as a mean to reduce the intensity of color in white and rosé wines made from red grapes (Wightman et al., 1997; Mathews et al., 2004).

The β-glucosidase is a group of hydrolases, which have been isolated in species also far apart from grapes from the taxonomical point of view, such as yeast, bacteria and fungi (Maicas and Mateo, 2005). The main reaction catalyzed by this class of enzymes is the hydrolytic cleavage of the β-glycosidic bonds of glycosides, which allows the liberation of the aglycone from glucose; in the case of disaccharides glycosides, this reaction must be preceded by the action of another

hydrolase (eg. α-L-ramnosidase) separating the glucose from the terminal sugar (Spano et al., 2005; Ugliano and Moio, 2006).
Specific strains of lactic acid bacteria (LAB), suitable to perform the MLF, can be supplied with the enzyme β-glucosidase and then be exploited to influence the aromatic complexity of the wine.

The β-glucosidase activity in the LAB was highlighted about 20 years ago, and since then several studies have given controversial results on the ability of LAB to hydrolyze glycosides (Grimaldi et al., 2000; Ugliano et al., 2003; D'Inecco et al., 2004). This enzyme has been detected in different species of the wine-related LAB genera *Lactobacillus, Pediococcus* and *Oenococcus* (Ugliano et al., 2003; Michlmayr et al., 2009; Michlmayr et al., 2010). This might be considered interesting for oenological purposes, since the enzymatic hydrolysis of glucosides associated to the yeasts is limited, because of their low enzymatic activity in fermentation conditions (Mateo and Di Stefano, 1997). On the contrary, enzymes associated with bacteria are more suited to the environmental conditions of the wine, since the LAB have a lower optimum pH value than many yeasts and an increased tolerance to high levels of ethanol (up to 16%) (Capaldo et al., 2011).

Another important consideration is the temperature at which the β-glucosidase activity is expressed. Even though it is proved that the optimal temperature is around 45°C (Sestelo et al., 2004; Michlmayr et al., 2009), it has been highlighted the conservation of certain glycosidasic activities even at values below 20°C, which is of fundamental importance for the wine industry, given that the wines are usually kept in this temperature range during MLF (Grimaldi et al., 2005).

It should also be noted that the β-glucosidase enzymes are usually inhibited by pH, ethanol and sugar. Regarding the sugar, it was shown that the enzyme activity is strongly inhibited by glucose (Ribéreau-Gayon et al., 2006), but not by fructose (Sestelo et al., 2004), although, as demonstrated by Grimaldi et al. (2000), such influence is strain specific in *Oenococcus oeni* . Grimaldi et al. (2000) also evaluated the effect of the ethanol concentration on the activity of β-glucosidase, concluding that in all of the five cultures examined, a concentration of ethanol up to 10% v/v leads to an increase in the activity of this enzyme; above the 10% v/v responses of bacteria examined begin to be different, a sign that this factor also appears to have a strain specific influence. The conservation of glycosidase activity up to 10% v/v of ethanol therefore supports the possible use of these enzymes in the production of alcoholic beverages such as wine (Sestelo et al., 2004). Also the acidity conditions in the wine may have effects on β-glucosidase, as they can cause a denaturation and/or inhibition of enzymatic hydrolysis; the optimal pH appears to be around 5.5, although strains of O. oeni can save 80% of their β-glucosidase activity between pH 4.5 and 7.0 (Grimaldi et al., 2000; Spano et al., 2005; Sestelo et al., 2004).

Since the perception of positive aromas in the wine is one of the most important parameters determining of the product by the consumer, this study is

aimed to find out which winemaking scenario (co-inoculation or sequential inoculation) is the best one to ensure the highest expression levels of the β-glucosidase encoding gene in *O. oeni*.

2. Materials and methods

2.1 Primer design

The nucleotide sequence of the coding gene for the β-glucosidase enzyme of *O. oeni* was recovered using the GenBank database of the National Center for Biotechnology Information (NCBI). This gene (Accession Number AY489108.1) has a sequence of 1392 bp.

For the design of the primer, coding sequences (found in GenBank) belonging to *O. oeni* for the enzyme β-glucosidase were aligned with all the genomic sequences currently deposited for the following microorganisms: *Lactobacillus casei* (NC_008526.1), *Lactobacillus plantarum* (AL_935263.2), *Lactobacillus bulgaricus* (NC_008054.1), *Lactobacillus reuteri* (NC_009513.1), *Lactobacillus rhamnosus* (CP003094.1*), Lactobacillus sakei* (NC_007576.1) and *Pediococcus pentosaceus* (NC_008525.1), using the software "Multalin", to highlight homologous and different areas. The primers were designed manually by searching gene regions with a low similarity. Table 1 shows sequences of the primers thus obtained (BgluF and BgluR), as well as their characteristics of length, position, melting temperature, percentage by GC and the length of the expected amplicon. The in silico verification of the specificity of the primers, of the absence of formation of dimers and the prediction of the length of the amplification product was performed using the software FastPCR (Kalendar et al., 2009).

Table 1. Sequences and characteristics of primers BgluF and BgluR

	Forward primer: Bglu F	**Reverse primer: Bglu R**
Primer sequence 5'→ 3'	5'-GGACAAACAAGGCGT-3'	5'-GATTCCATGACCAACAG-3'
Position on the gene (bp)	1137- 1151	1263-1279
Primer length (bp)	15	17
Tm (°C)	50.3	46.6
% GC	53.3	47.1
Amplicon length (bp)	143	143

The specificity of the primers was then tested in both PCR and qPCR using the following bacterial and yeast strains: *Lactobacillus casei* DSMZ 20011, *Lactobacillus plantarum* DSMZ 20174 , *Lactobacillus bulgaricus* DSMZ 20081, 20481 *Lactococcus lactis, Lactobacillus reuteri* DSMZ 20053, DSMZ 20021 *Lactobacillus rhamnosus, Lactobacillus sakei* DSMZ 6333, *Pediococcus*

pentosaceus DSMZ 20336, *Saccharomyces cerevisiae* ATCC51 *and Saccharomyces bayanus* DSMZ 3774. A positive control (strain Alpha MBR, Lallemand, Verona, Italy), declared able to perform β-glucosidase activity by the producer, was used in each PCR reaction.

Conventional PCR was carried out in a final volume of 25 μL containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) dNTPs, 1 μM primer, and 1.25 U of Taq polymerase (Applied Biosystems, Milan, Italy). The amplification cycle was as follows: 95°C denaturation for 5 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min in a Thermal Cycler (DNA Engine Dyad peltier Thermal Cycler, BioRad, Milan, Italy).

2.2 Screening of O. oeni strains for the presence of β-glucosidase encoding gene

The DNA of 84 *O. oeni* strains, belonging to the strain collection of the Department of Food Science, University of Udine, Italy, where then used as templates for conventional PCR reactions using the BgluF and BgluR couple of primers in order to find out which strains were provided with the gene encoding for the β-glucosidase enzyme, and therefore suitable to be used for the subsequent gene expression analysis.

2.3 Inoculation of wine and must samples and experimental plan

The strain of *O. oeni* chosen for this study was grown in MLO broth (casein peptone, tryptic digest 10 g/L, yeast extract 5 g/L, glucose 10 g/L, fructose 5 g/L, $MgSO_4$ x 7 H_2O 0.20 g/L, $MnSO_4$ x H_2O 0.05 g/L, (NH_4) citrate 3.50 g/L, Tween 80 1.00 mL/L, filtered apple juice 100.00 mL/L, and cysteine-HCl x H_2O 0.50 g/L, pH adjusted to 4.8) at 30°C until reaching a value of optical density at 600 nm equal to 1, corresponding to 10^9 ufc/mL. In order to check the concentration of the suspension, this has been subjected to sampling on an MLO agar (15g/L Agar technical n°3, Oxoid, Milan, Italy).

For the sequential inoculation trial, 200 mL of sterile filtered Moscato wine was inoculated, into sterile bottles, with a concentration of 10^6 CFU / mL of *O. oeni*.

For the trial conducted in co-inoculation, 10^6 CFU/mL of *O. oeni* and 10^6 CFU/mL of *Saccharomyces cerevisiae* (Premium Zinfandel, Enologica Vason, Italy) were added, in sterile conditions, in 200 mL of Moscato grape must.

Inoculated samples of must and wine were maintained in an incubator at 19°C for the duration of the experimentation, during which samplings were performed, at regular intervals for a total of 17 sampling points for musts and 30 points for wine.

The following analysis were conducted for each sampling point: microbial count of *O. oeni*, pH measurement, evaluation of the expression of the β-glucosidase coding gene by Reverse Transcription – quantitative PCR (RT-qPCR), determination of L-malic acid, determination of L-lactic acid. For the samples

collected in co-inoculation the following additional analysis were performed: plate count of *S. cerevisiae* cells on WL agar medium (Oxoid, Milan, Italy), determination of D-glucose , determination of ethanol.
All trials were carried out in triplicate.

2.4 RNA extraction

In total, 1 mL of each sample was centrifuged at 10,000 rpm for 7 min to pellet the cells. RNA was extracted from the pellets using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin) according to the manufacturer's instructions for RNA purification from cell samples, except that DNAse treatment was extended to 150 minutes to ensure the total degradation of contaminating DNA. Moreover, RNA was resuspended in 35 µL of sterile bidistilled water treated with DEPC (Sigma, Milan, Italy). The RNA concentration was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Rodano, Italy) and standardised to 40 ng/µL by dilution with sterile DNA-free Milli-Q DEPC-treated water.

The absence of contaminating DNA in RNA samples was proved by both classical and quantitative PCR.

2.5 Reverse transcription

Standardised RNA samples were converted into cDNA. Reverse transcription reactions were carried out using the ImProm-II™ Reverse Transcription System (Promega, Milan, Italy) according to the manufacturer's instructions. cDNA samples were used as templates for qPCR.

2.6 qPCR protocol

The quantitative analysis of the expression of the the β-glucosidase coding gene was performed by quantitative PCR on the cDNA obtained through the reverse transcription.

The reference gene used in the following analysis was the one coding for the lactate dehydrogenase enzyme (ldhd) (Desroche et al., 2004).

Real-time PCR mixtures contained 10 µl of 2x SsoFast EvaGreen Supermix (Biorad, Milan, Italy), 400 nM of each primer, 2 ng/µL of DNA and the reaction mixture's volume was adjusted to 20 µL with sterile DNA-free Milli-Q water. Real-time PCRs were performed using a RotorGene Q system (QIAGEN, Milan, Italy) with one cycle of initial denaturation of template DNA and activation of Taq DNA polymerase at 98°C for 3 minutes, followed by 45 cycles of denaturation at 95 °C for 10 s, primer annealing at 55°C for 15 s and extension at 72 °C for 20 s. Fluorescence signal acquisition was performed during the extension step. To verify that formation of non-specific products or primer dimers had not occurred, the dissociation curves of the final products for each PCR were analyzed from 55 to 95 °C at 1 °C intervals. Each qPCR reaction was performed in triplicate for each sample, on three biological replicates.

2.7 Calculation of MNE values

According to the MIQE (Bustin et al., 2009), mean normalized expression (MNE) levels were calculated using the mathematical model proposed by Pfaffl (2001).

2.8. Plate count

1 mL of each sample was enumerated on double layer of *Leuconostoc oenos* medium added with 15 g/L Agar technical n°3 (Oxoid, Milan, Italy) to determine the exact CFU spiked in the samples. Plates were incubated in anaerobic conditions (Anaerogen, Oxoid, Milan, Italy) at 30°C for 7 days, then colonies were counted and the numbers of viable *O. oeni* were determined from those counts.

To enumerate yeast cells, 0.1 mL of each serial dilution of samples collected from the co-inoculation with *S. cerevisiae* trial was plated on WL agar (Oxoid, Milan, Italy). Plates were incubated in aerobic conditions at 30°C for 2 days, then colonies were counted and the numbers of viable *S. cerevisiae* were determined from those counts.

2.9. Determination of concentration of L-malic acid

Concentration of L-malic acid was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Rodano, Italy) by using the enzymatic kit L-malic acid (Roche, Milan, Italy), following the manufacturer's instructions.

2.10. Determination of concentration of L-lactic acid

Concentration of L-lactic acid was spectrophotometrically determined (NanoDrop 2000c, Thermo Scientific, Rodano, Italy) by using the enzymatic kit L-lactic acid (Roche, Milan, Italy), following the manufacturer's instructions.

2.11. Determination of concentration of ethanol

The determination of ethanol in wine and in co-inoculated must was carried out using the Alcolyzer Plus instrument (Anton Paar, Austria), following the manufacturer protocol.

2.12. Determination of D-glucose

For the determination of glucose the D-glucose (UV test) Kit (R-Biopharm AG, Germany) was used, following the manufacturer protocol

2.13. pH measurement

For all the collected samples, pH was measured using the Basic20 pH instrument (Crison instruments S.A., Alella, Spain).

3. Results and discussion

3.1. Specificity of primers and strain selection

Conventional PCR carried out with the primers BgluF and BgluR gave positive result only when DNA extracted from positive control sample (Alpha MBR, Lallemand) was used as template, obtaining the expected amplicon of 143 bp length. For none of the other organisms tested in the analysis to verify the specificity of the primers there was amplification (Figure 1). The primers were then shown to be highly specific, amplifying only the template represented by the DNA extracted from strains of *O. oeni* coding for the β-glucosidase enzyme.

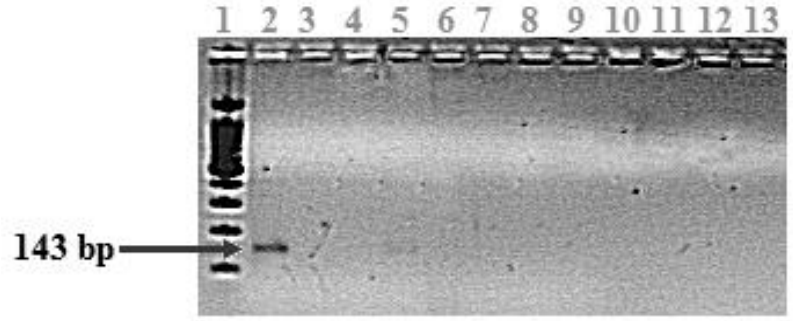

Figure 1. Results of PCR carried out using the primers BgluF and BgluR on LAB strains. Line 1: molecular weight marker, line 2: *Oenococcus oeni* Alpha MBR, line 3: *Lactobacillus casei DSMZ 20011* , line 4: *Lactobacillus plantarum* DSMZ 20174, line 5: *Lactobacillus reuteri* DSMZ, line 6: *Pediococcus pentosaceus* DSMZ 20336, line 7: *Lactobacillus bulgaricus* DSMZ 20081, line 8: *Lactobacillus rhamnosus* DSMZ 20021, line 9: *Lactococcus lactis* DSMZ 20481, line 10: *Lactobacillus sakei* DSMZ 6333, line 11: *Oenococcus oeni Viniflora* 11, line 12: *Oenococcus oeni* Viniflora 16, line 13: *Oenococcus oeni* Amar 04.

Furthermore, for none of the other strains of *O. oeni* tested there has been amplification; therefore it has been possible to confirm the presence of the coding gene for the enzyme β-glucosidase only for the strain Alpha MBR and the absence of this gene for all of the other strains belonging to the strain collection of the Department of Food Science at the University of Udine. qPCR reactions did not show increase in fluorescence for samples different from *O. oeni* Alpha MBR (data not shown). This result differs from the findings of Pérez-Martìn et al. (2012), as these authors report a percentage of 36% *O. oeni* strains, among the 180 tested, which showed β-glucosidase activity. In our study, only the 1.2% of the tested *O. oeni* strains showed positivity for the presence of the β-glucosidase coding gene.

For this reason, strain Alpha MBR was selected as a starter to carry out the fermentations and to evaluate the gene expression levels of the β-glucosidase encoding gene. Results confirm that β-glucosidase activity is strain specific for *O. oeni* (Ugliano et al., 2003)

3.2. Optimization of the RT-qPCR technique

The RT-qPCR technique, used for the evaluation of the expression of the β-glocosidase gene in *O. oeni* , was optimized in order to be able to calculate, for each sampling point, the value of mean normalized gene expression (MNE) (Bustin et al., 2009).

The optimization of the method was made starting from the optimization of the reaction efficiency. For this purpose, a series of standards of known concentration of cDNA was prepared, which has been used as template for the qPCR reaction. Standard curves were then obtained, and the values of the coefficient of determination (R^2) and efficiency (E) are 0.97 (±0.02) and 0.73 (±0.01) for the co-inoculation trial and 0.96 (±0.03) and 0.78 (±0.01) respectively for the sequential inoculation trial when cDNA samples were amplified using BgluF and BgluR as primers in the qPCR reaction.

Using the primers annealing on the reference gene the obtained values of R^2 and E are as follows: 0.99 (±0.01) and 0.77 (±0.04) respectively for the co-inoculation trial, and 0.98 (±0.02) and 0.78 (±0.05) respectively for the sequential inoculation trial. Since for the comparative CT method to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal (Bustin et a., 2009), these results were considered adequate for the prosecution of the study.

3.3. Expression levels of β-glucosidase encoding gene in case of sequential inoculation

The most common way to analyze gene expression data is the calculation of MNE levels, which requires that all of the cDNA samples obtained after reverse transcription are amplified in qPCR reactions where couples of primers targeting the gene of interest and a reference gene are used. In this study, because many authors (Desroche et al., 2004, Olguin et al., 2009, Beltramo et al., 2006) point out the ldhd gene as the ideal reference gene for gene expression studies with *O. oeni*, it was used with this purpose.

In case of inoculation of *O. oeni* in wine, the fact that the alcoholic fermentation had come to an end has determined the absence of glucose in the medium and a final level of ethanol equal to 12.05% v/v, with a pH value of 3.4.

The malolactic fermentation did not start (Figure 2, panel A), even though the time of observation was prolonged from 92 hours up to 175 hours. In these conditions the growth of *O. oeni* had an obvious decrease in the first 50 hours, and attested at relatively low levels (around 4 log CFU/mL, Figure 2, panel B).

The coding gene for the β-glucosidase was always expressed at a baseline level (<1 MNE, Figure 2, panel C) troughout the observation time. This result is

fully in agreement with those reported by Spano *et al.* (2005), who demonstrated the inhibitory effect of the expression of the coding gene for the β-glucosidase in *L. plantarum* by ethanol values of 12% vol and low pH (3.5), conditions similar to those found in the wine used in this study. The results obtained in this study may

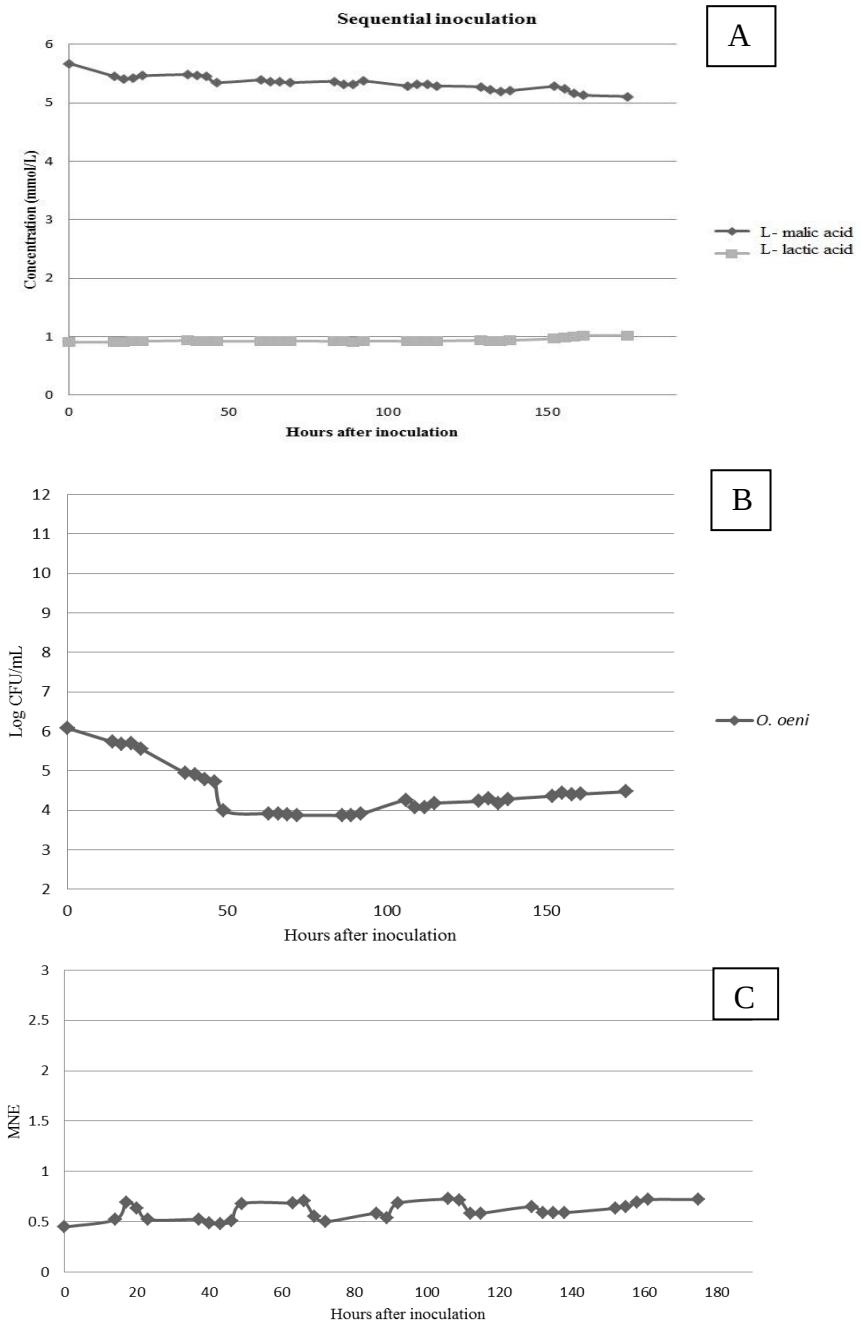

Figure 2. Results obtained in sequential inoculation scenario: Concentrations of L-malic acid and L-lactic acid in wine inoculated with *O. oeni* in the considered time period (panel A), Plate count values (MLO agar) for *O. oeni* in wine.(panel B), MNE levels of β-glucosidase encoding gene in *O. oeni* (panel C)

also confirm the work by Pilatte et al. (2003) on the inhibitory effect of ethanol on the *O. oeni* β-glucosidase, hypothesizing that the reduction in activity could be due to a limited transcription level because of the presence of ethanol, which could therefore be involved in negative regulation of the gene. It is also possible, considering the obtained results, to say that no difference was registered between gene expression of β-glucosidase encoding gene in a synthetic medium and in real wine, disproving the hypothesis formulated by Spano et al. (2005) at the end of their work.

On the other hand, the absence of glucose in the medium did not create favorable conditions for the expression of the gene, as reported by Nagaoka et al. (2008), suggesting that both ethanol and glucose are able to influence, probably in a synergic way, the regulation of this coding region.

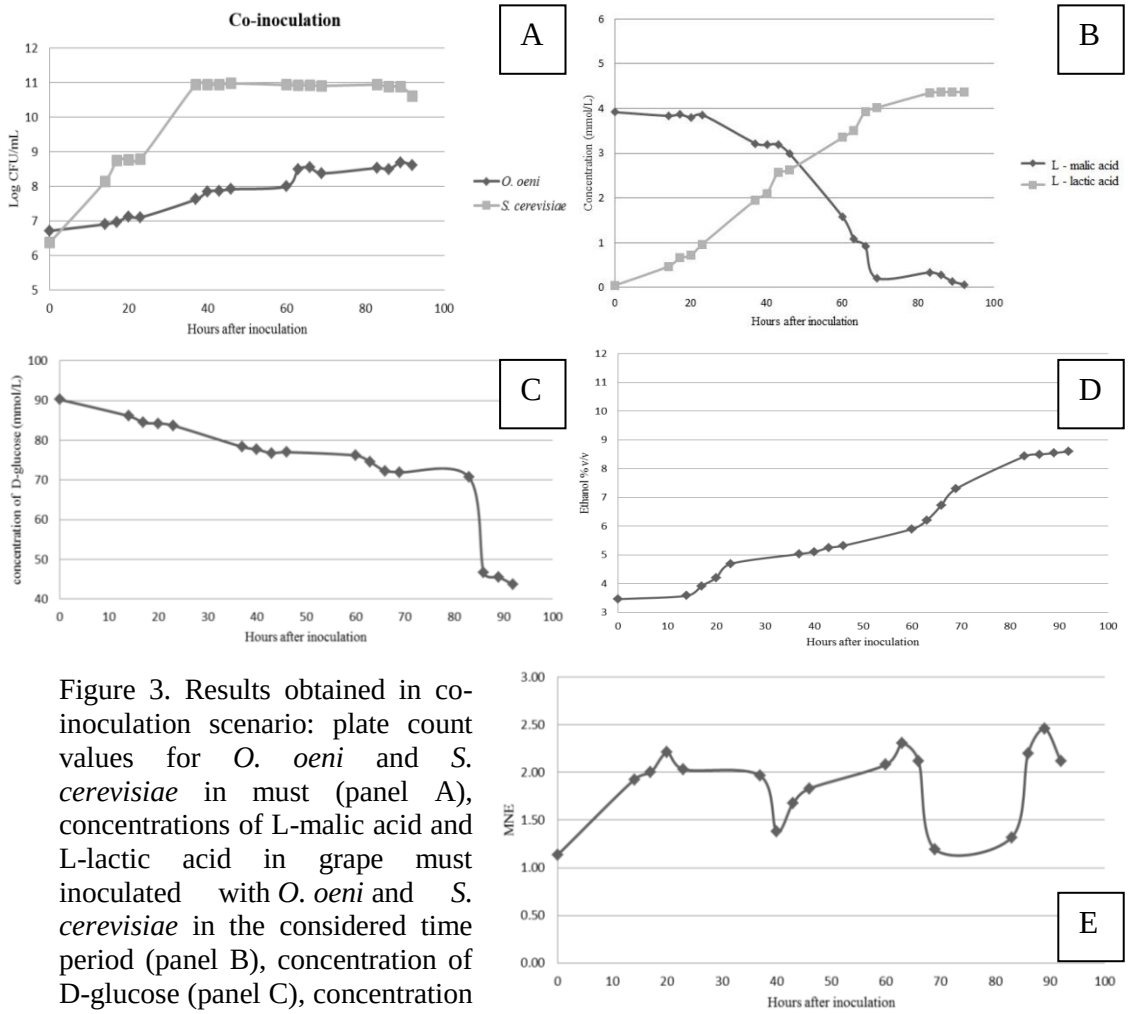

Figure 3. Results obtained in co-inoculation scenario: plate count values for *O. oeni* and *S. cerevisiae* in must (panel A), concentrations of L-malic acid and L-lactic acid in grape must inoculated with *O. oeni* and *S. cerevisiae* in the considered time period (panel B), concentration of D-glucose (panel C), concentration of ethanol (panel D), MNE levels of β-glucosidase encoding gene in *O. oeni* (panel E).

3.4 Expression levels of β-glucosidase encoding gene in case of co-inoculation

In case of co-inoculation in must with *S. cerevisiae*, there was a progressive growth of *O. oeni* (from 6.7 to 8.6 log ufc/mL) and yeasts (from 6.4 to 6.10 log CFU/mL) (Figure 3, panel A). During the time of observation, *O. oeni* completed the MLF, increasing the level of L-lactic acid and decreasing the concentration of L-malic acid (Figure 3, panel B); yeasts led the alcoholic fermentation causing a progressive increase of ethanol, which rose, at the end of the monitoring time, to 8.6% v/v, also contributing to a reduction of glucose (Figure 3, panels C and D).
The coding gene for the β-glucosidase enzyme was expressed in an intermittent way throughout the monitoring time (Figure 3, panel E), in a range between 1.3 and 2.4 MNE.

This trend may be motivated by the fact that, in presence of *S. cerevisiae* (which led to competition, as well as a high consumption of sugar), the microorganism had a greater need for glucose and therefore expressed more intensively the coding gene for the β-glucosidase. The release of glucose in the medium, on the other hand, could have led to a negative regulation of the gene, since many authors report not only the strain specific inhibition of glucose on β-glucosidase activity on *O. oeni* (Grimaldi et al., 2000), but also the suppressive activity of this sugar on the β-glucosidase encoding gene expression on LAB (Nagaoka et al., 2008).

Moreover, *S. cerevisiae* could have released nitrogenous substances in the medium, which have promoted the activity of *O. oeni* thus making it more metabolically active and, therefore, in a more favorable condition to have a higher level of expression of the coding gene for the β-glucosidase. It should be remembered, in fact, that LAB are not able to use the nitrogen and inorganic compounds (in particular ammonia) and that they do not use proteins, being devoid of proteolytic activity; therefore they must find mixtures very rich in amino acids or peptides in the nutritional media (Zambonelli, 1998). It is for this reason that the protease activity, the production of macromolecules (such as glucans and mannoproteins) and the autolytic ability of yeasts can have a stimulating effect on the growth of LAB and thus on their metabolic activity (Alexandre et al., 2004).

4. Conclusions

The β-glucosidase enzyme, responsible for the hydrolytic disruption of the β-glycosidic bonds of glycosides, and therefore the liberation of the aglycone from glucose, appears to be strain specific in *O. oeni*; the performed analyzes show in fact that only one of the tested strains (Alpha MBR) is β-glucosidase positive.

The optimization of the RT-qPCR method led to the design of primers (BgluF and BgluR) specific for the amplification of the gene coding for β-

glucosidase enzyme in *O. oeni*. Such primers, thanks to the optimization of an ad hoc protocol, resulted in a qPCR reaction with good efficiency, which was comparable with the efficiency of the reaction qPCR obtained using the protocol of amplification with the primers ldhd F and ldhd R, annealing on the reference gene. The efficiencies of the reactions for the target gene and for the reference gene were, moreover, comparable among each other and this allowed to use this method for the evaluation of gene expression of the enzyme β-glucosidase in *O. oeni*.

As emerged from this study, the β-glucosidase appears to be expressed at a low basal level in case of inoculum of *O. oeni* Alpha10 MBR in wine.

In case of co-inoculation of *O. oeni* with S. cerevisiae in grape must, the environmental conditions resulted in an increased transcription of the gene, determined by the presence of yeast, which may have improved the conditions of growth of *O. oeni* and thus the level of expression of the gene, releasing nitrogenous compounds in the medium and therefore increasing its metabolism.

Considering the obtained results, the co-inoculation scenario seems to be the most favorable condition for the expression of β-glucosidase encoding gene. Further studies are needed to clarify the reason for the variability of the expression levels reported in this work.

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