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ORIGINAL ARTICLE

# Adaptive changes in geranylgeranyl pyrophosphate synthase gene expression level under ethanol stress conditions in *Oenococcus oeni*

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**Keywords**
ethanol stress response, geranylgeranyl pyrophosphate synthase, level expression changes, *Oenococcus oeni*, qPCR.

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## Abstract

**Aims:** The aim of this study was to investigate the effect of ethanol exposure on the expression level of geranylgeranyl pyrophosphate synthase gene involved in the metabolism of *Oenococcus oeni* to probe the mechanisms of ethanol tolerance correlated with adaptive changes.
**Methods and Results:** The evaluation of ten potential internal control genes and the comparative study of their stability were performed to select the most stable internal controls for the normalization of expression data. The expression level analysis by qPCR and changes after exposure to ethanol stresses highlighted a significant increase in the presence of higher ethanol concentrations.
**Conclusions:** The analysis of results suggest that *O. oeni* adjusts the expression of genes to adapt to stress conditions and the high expression level of *ggpps* would allow a flow of isoprenoid precursors towards the carotenoids and related pathways to stabilize bacterial cell membranes, improving the cell membrane disturbances and preventing cell death induced by ethanol.
**Significance and Impact of the Study:** The involvement of *ggpps* gene in physiological changes of bacterial behaviour confirmed the exposure to stress requires the activation of defence mechanism to be more tolerant to adverse conditions. Improving the knowledge of stress tolerance and adaptation mechanisms of *O. oeni* is essential to enhance the efficiency of the malolactic starter in wine and to obtain the development of starters able to survive to direct inoculation with a large benefit for wine technology.

## Introduction

The production of wine involves a variety of microbial transformations comprising a complex succession of various yeast and bacterial species. *Oenococcus oeni* has long been reported as the main lactic acid bacteria (LAB) species associated with malolactic fermentation (MLF), crucial step for winemaking process that can occur spontaneously. However, this step can start randomly, and any delay could lead to an alteration of wine quality. These delays are due to very harsh environmental conditions in the wine for bacterial survival and growth and various physical/chemical factors, such as ethanol, pH and temperature, are known to affect the growth of the LAB responsible for the MLF of wine. *O. oeni* is the LAB species most resistant to the presence of ethanol in wine and able to grow in the hostile environment of wine by the activation of several mechanisms (G-Alegrìa *et al.* 2004).

A better knowledge of stress physiology may be useful to optimize survival of starter cultures of *O. oeni* that the winery practices recommend to use for direct inoculation into wines to improve the control of MLF (Nielsen *et al.* 1996). However, induction of MLF by inoculation with malolactic starters is not effective in 'difficult wines' (e.g. in wines having a pH below to 3·2) because of significant cell mortality (da Silveira *et al.* 2002). To overcome this problem, adaptation processes have been shown to

enhance the survival of *O. oeni* cells to stress conditions in wine, and the increase in cell survival is linked to stress response mechanisms (da Silveira *et al.* 2002).

The complete genome sequence of *O. oeni* strain PSU-1 was previously studied to advance the study of *O. oeni* and to provide fundamental information on the genetic endowment of this micro-organism (Zé-Zé *et al.* 1998, 2000; Mills *et al.* 2005).

Moreover, the differential expression of genome in *O. oeni* strains under stress conditions was previously studied by the development, and the optimization of the fluorescent differential display (FDD) technique that allows the identification of gene expression changes, associated with differential microbial behaviour under different stress conditions with a better stress response definition and a better discrimination of starter cultures (Sico *et al.* 2009).

The genome analysis reveals potential survival strategies, as well as metabolic properties that enable *O. oeni* to effectively compete in the wine environment. The existence of such unique features can be viewed as evolutionary adaptation to the wine environment (Beltramo *et al.* 2006). So a combined knowledge of genome features and specific gene expression is required for understanding the adaptive mechanisms of *O. oeni* to the wine environment (Beltramo *et al.* 2006). Although several studies (Carreté *et al.* 2002; Bourdineaud *et al.* 2003; da Silveira *et al.* 2003; Grandvalet *et al.* 2005, 2008; Olguìn *et al.* 2009) analysed few mechanisms that enable *O. oeni* to withstand stress conditions, more information about the mechanisms involved in the adaptation of *O. oeni* to stress conditions is required.

Quantitative real-time PCR (qPCR) has become a routine technique for gene expression analysis and a better understanding of these patterns is expected to provide insights into complex regulatory networks to obtain the identification of genes relevant in adaptation processes (Vandesompele *et al.* 2002). There is still no consensus about appropriate normalization of qPCR raw data, essential to compensate for experimental error that can be introduced at various stages throughout the procedure. The most commonly used strategy is the use of an internal reference gene, a so-called housekeeping gene. Although several studies have used only one gene for normalization, recent studies compared the use of a single and multiple reference genes for normalization of qPCR expression data finding that the use of a single reference gene was unreliable and revealed a risk of misinterpretation of expression data (Vaudano *et al.* 2011; Sumby *et al.* 2012). Therefore, these studies validated the use of multiple internal control genes, but revealed no ideal reference genes because the expression of many genes, which have been used as internal controls in qPCR experiments, is influenced by metabolic conditions, growth phase or experimental conditions (Theis *et al.* 2007). Therefore, each internal control gene needs to be validated within a given experimental set-up before it can be used for the normalization of qPCR data to avoid the achievement of erroneous results (Theis *et al.* 2007).

In this study, ten potential internal control genes were tested under different culture and stress conditions. A comparative study of their stability has been performed to select the most adapted internal control for further studies. These internal controls were then used to study the expression level of geranylgeranyl pyrophophate synthase (*ggpps*) gene in *O. oeni* under ethanol stress conditions.

## Materials and methods

### Bacterial strain, growth and stress conditions

The *O. oeni* S12 strain, previously isolated from Aglianico wines (Basilicata region, Southern Italy), molecularly identified and characterized on the basis of technological features and then investigated for the differential expression of its genome (Sico *et al.* 2008, 2009), was used in this study. The strain was maintained as freeze-dried stocks in reconstituted (11% w/v) skim milk, containing 0·1% (w/v) ascorbic acid in the culture collection of the Dipartimento di Scienze, Università degli Studi della Basilicata (Potenza, Italy) and routinely propagated in MRS broth supplemented with 2% (v/v) Tomato Juice (MRS-TJ) and adjusted to pH 4·8 at 30°C for 72–96 h, before the analyses.

To evaluate the effect of alcoholic stress on gene expression, late-exponential phase cells were harvested and resuspended, to a final OD600 = 1·0, in MRS-TJ containing different ethanol concentrations (7, 12, 13 and 15%). Cell suspensions were incubated at 30°C for 1 h; bacterial cells incubated at 30°C for 1 h in MRS-TJ pH 4·8 were used as control.

### Fluorescent Differential Display-PCR and bands identification

Total RNA was extracted, and cDNA was synthesized and used as template for FDD-PCR amplification as described in the study described by Sico *et al.* (2009). The PCR products of FDD analysis were separated by electrophoresis on 2% (w/v) agarose gels (EuroClone, Pero, Italy) in 1× TBE at 100 V for 4 h. Gels were stained in 1× TBE buffer containing 0·5 $\mu$g ml$^{-1}$ ethidium bromide (Serva Electrophoresis GmbH, Heidelberg, Germany) for 30 min. A 1-kb DNA ladder (EuroClone) was used as molecular weight and normalization gel standard. The banding patterns were visualized by UV transillumination

and captured with GelDoc 2000 Apparatus (Bio-Rad, Watford, Herts, UK). Gel images were digitized in Diversity Database™ software (Bio-Rad Laboratories Ltd.) and processed for analysis and selection of the fragments. In this study, a transcript, 279 bp long, was identified and selected from band pattern obtained after stress treatment with 12% ethanol.

### Excision and re-amplification of cDNA fragment

The selected band was excised from the agarose gel, and the DNA was purified into 50 μl of Tris-EDTA (TE) by heating at 60°C for at least 1 h. Five microlitres of the eluent was reamplified with the same random primer used for the FDD-PCR, the 6-carboxyfluorescein labeled 5′-anchored M13 primer (5′-6-carboxyfluorescein (FAM)-GAGGGTGGCGGTTCT-3′), and the PCR amplification was carried out as described by Sico *et al.* (2009).

### Sequencing analysis

The reamplified product was purified and then sequenced by Capillary Sequencer ABI 3730. Sequences obtained were compared with those available in the GenBank database (http://www.ncbi.nml.nih.gov/BLAST) and those of the Comprehensive Microbial Resource (JCVI-CMR: http://cmr.jcvi.org/cgi-bin/CMR) to determine the closest known relative species on the basis of sequence homology. Sequence was aligned using the ClustalW program implemented in BioEdit 7.0.5.2 software (Ibis Biosciences, Carlsbad, CA, USA). Sequence analysis identified a significant similarity between the transcript examined and a geranylgeranyl pyrophosphate synthase (*ggpps*) (NCBI no. ABJ56986), which was originally found to be expressed in *O. oeni* PSU-1 complete genome (NCBI no. CP000411).

### Real-time PCR

To evaluate changes of the expression level of the *ggpps* gene in response to alcoholic stresses in *O. oeni*, qPCR assay was carried out in all samples (control and cells treated with 7, 12, 13 and 15%). From previous experiments (Sumby *et al.* 2012), use of a single reference gene for a relative quantification of the gene expression was not sufficient, so 10 candidate reference genes (*rpoA*, *gyrB*, *gapA*, *gmk*, *recA*, *rpoB*, *ftsZ*, *pta*, *ldhD*, *rrs*) were selected based on the literature (Seta *et al.* 1997; Desroche *et al.* 2005; Theis *et al.* 2007; Fiocco *et al.* 2008; Ritz *et al.* 2009; Duary *et al.* 2010; Duquenne *et al.* 2010; Turroni *et al.* 2010; Costantini *et al.* 2011). Primers for this real-time PCR study had been designed in previous works (Desroche *et al.* 2005; Sumby *et al.* 2012) and have a length of about 20–25 bases, a G/C content of over 50% and a Tm of about 60°C. Moreover, in this work, potential primers were checked for gene-specific binding using NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) as regards 16S RNA ribosomal subunit (*rrs*) and *ggpps* genes. *ggpp*-for (5′-AACAGTTCGGACAAAGTACAG-3′) and *ggpp*-rev (5′-CGACCATCTGTGTCATTTCA-3′) were, respectively, the forward and the reverse primers designed for *ggpps* gene. Primers were purchased from Sigma-Aldrich (Milan, Italy). Nucleotide sequences for primer design were obtained from NCBI, and the sequence references of the genes from *O. oeni* PSU-1 (NCBI no. CP000411) are the following: OEOE_1085 (geranylgeranyl pyrophosphate synthase) and OEOE_r1373 (16S ribosomal RNA).

Real-time PCRs were carried out on a Chromo4™ System (Bio-Rad) with Power SYBR® Green PCR Master Mix (Applied Biosystems, Monza, Italy) in 96-well plate. After dilution of cDNA, 1 μl was added to 19 μl of PCR mixture (10 μl of Power SYBR® Green PCR Master Mix, 1 μl of each primer (10 μmol $l^{-1}$) and 7 μl of Rnase-free water). In each run, a negative control was included. Thermal cycling conditions were designated as follows: initial denaturation at 95°C for 10 min, followed by 38 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s. A further step for the performing of a melt curve was included at the end of the run from 90 to 60°C (0·05°C $s^{-1}$), to verify the specificity of the real-time PCR reaction for each primer pair.

### Statistical analysis

For each measurement, a cycle threshold ($C_t$) value was determined. Efficiency ($e$) of amplifications was taking into account for every reaction; it was calculated as $e = 10^{-1/\text{slope}}$ for each primer pair, where the slope was determined through a linear regression model over $\log_{10}$-trasformed Ct values of cDNA dilution series (Pfaffl 2001). The performance of internal control genes was analysed under all conditions using qbase_plus 2.4 software (Zwijnaarde, Belgium) that provides the geNorm expression stability value of the reference gene ($M$) and the coefficient of variation of the normalized reference genes relative quantities ($V$) (Vandesompele *et al.* 2002; Hellemans *et al.* 2007). As an optimal normalization is reaching when $V_{n/n+1}$ shows a minimal value with a particular panel of reference genes, Vandesompele *et al.* (2002) proposed a $V_{n/n+1}$ value of 0·15 as a flexible cut-off value below which the inclusion of an additional reference gene is not required. The normalization factor was calculated using the geometric mean of the quantities of the best reference genes, and target gene expressions were normalized by dividing the target gene quantities with the normalization factor (Vandesompele *et al.* 2002).

Once stable internal control genes were identified, the expression of *ggpps* gene was calculated using the five most stable internal control genes for normalization. Results were analysed using the comparative critical threshold ($\Delta\Delta C_t$) method in which the amount of target RNA was adjusted to reference (internal target RNA). This was performed using qbase_plus 2.4 software.

Statistical analysis of the expression data was performed using qbase_plus 2.4 software, and the level of statistical significance was set at $P < 0{\cdot}05$.

## Results

Fluorescent differential display profiles were produced carrying out an assay system based on FDD method, by using a single fluorescently labelled random primer for detecting and isolating differentially expressed fragments under different stress situations. Distinct bands in response to different ethanol concentrations were obtained and only the fragments present in the profiles of stress exposed samples and absent in all control profiles were considered indicating a specific response. The resulting fingerprints showed the presence and the absence of specific products between each stressed sample and the control (data not shown).

To identify genes within the resulting *O. oeni* genome database that are differentially expressed in response to various changes, differentially amplified FDD products were sequenced and results of the early sequence analysis confirmed the effectiveness of the system at identifying differentially expressed transcripts on FDD fingerprints correlated with microbial tolerance to stress situations and revealed that several of the products can be referred to specific genes already known in literature under stress conditions (Guzzo *et al.* 2000; Beltramo *et al.* 2004; Bourdineaud *et al.* 2004; Desroche *et al.* 2005). In particular, this study was focused on a transcript of 279 bp identified in the response of *O. oeni* S12 strain to 12% ethanol stress. The sequence alignment analyses of bands revealed a significant similarity to a gene coding a *ggpps* (NCBI no. ABJ56986) which was originally found to be present in *O. oeni* PSU-1 complete genome (NCBI no. CP000411).

For the quantification of *ggpps* gene expression in the different stress conditions, 10 reference genes commonly used as internal control were employed (*rpoA*, *gyrB*, *gapA*, *gmk*, *recA*, *rpoB*, *ftsZ*, *pta*, *ldhD*, *rrs*) involved in different metabolic aspects. All ten genes were evaluated for their potential as internal control for *O. oeni* gene expression experiments. qPCR assays were carried out using each primer pair for the ten selected reference genes on each sample. To identify the most stable genes to use for the normalization of the expression level of the target gene *ggpps*, geNorm, provided by qbase_plus 2.4 software, was used. It was able to supply a gene-stability measure ($M$) defined as the average pairwise variation of a particular gene with all other potential reference genes (Vandesompele *et al.* 2002). The genes with the lowest $M$ value were considered the most stable, and they were *pta* and *rpoB*, with a $M$ value of 0·38–0·40 (Fig. 1). Instead, the genes with the highest $M$ values were *rpoA* and *rrs*, with a $M$ value of about 1·18 and 1·85, respectively (Fig. 1).

To find the optimal number of reference genes to use for an accurate normalization of the expression level of a target gene, the value $V_{n/n+1}$ was determined (Vandesompele *et al.* 2002); the lowest $V_{n/n+1}$ represented the optimal number of reference genes to use to obtain the most accurate data normalization that is possible. Based on these criteria, the best genes identified by geNorm, in order of stability, were *pta*, *rpoB*, *gapA*, *recA* and *gyrB* (Figs 1 and 2). Using the five most stable reference genes, an accurate and reliable normalization of qPCR data was achieved.

Once stable internal control genes were identified, the expression level of the target *ggpps* gene was analysed by using the geometric mean of copy numbers as normalization factor.

Using cells grown in absence of ethanol as calibration (control) condition, the expression gene analysis produced various changes in the transcription level of the gene in response to different ethanol concentrations.

Figure 3 shows results of the relative expression levels of *ggpps* gene in the presence of different concentrations of ethanol. It can be observed that transcription was activated in all the media after 1 h of incubation, and statistically significant differences ($P < 0{\cdot}05$) were found comparing transcription in the control condition (0·40 relative quantity) with that in different concentrations tested. The increase in ethanol concentration in the growth medium resulted in an increase in the *ggpps* gene expression (Fig. 3).

The transcript level of *ggpps* increased significantly in the presence of higher ethanol concentrations (12, 13 and 15%), showing relative quantities of 1·1, 1·7 and 2·9, respectively, while *ggpps* showed no significant expression increase in the presence of 7% ethanol, with a relative amount (0·45) very similar to that in the control (0·40) (Fig. 3). The 12% ethanol concentration caused an increase in transcription level of 2·50 times compared with control, while in the presence of 13% of ethanol, the transcription level was higher of almost 4·25 times than that found in the control condition. Finally, the transcription reached the maximum relative quantity (2·9) observed after the stronger stress, due to 15% ethanol presence, that led to a transcription level increase in 7·25 times compared with the control condition (Fig. 3).


**Figure 1** Candidate reference genes classified according to their average expression stability values (*M*) by geNorm analysis. The highest *M* values characterize the least stable genes.


**Figure 2** Determination of the optimal number of internal control genes showing pairwise variation values ($V_{n/n+1}$) by geNorm analysis. Each bar represents the change in normalization accuracy through a stepwise addition of reference genes according to the classification showed in Fig. 1. The lower value of $V_{n/n+1}$ represents the better normalization reachable using this particular set of reference genes.

## Discussion

The previous study of Sico *et al.* (2009) provided an innovative FDD method, with a high level of reproducibility and quality for studying and probing the knowledge of the relationship between differential genome expression and different stresses tolerance. It proved *O. oeni* strains respond to stimuli through the differential expression of transcripts in order to survive and adjust to the stresses. In this study, the identification and the sequence analysis of a transcript, differentially expressed in response to ethanol presence, revealed the ethanol

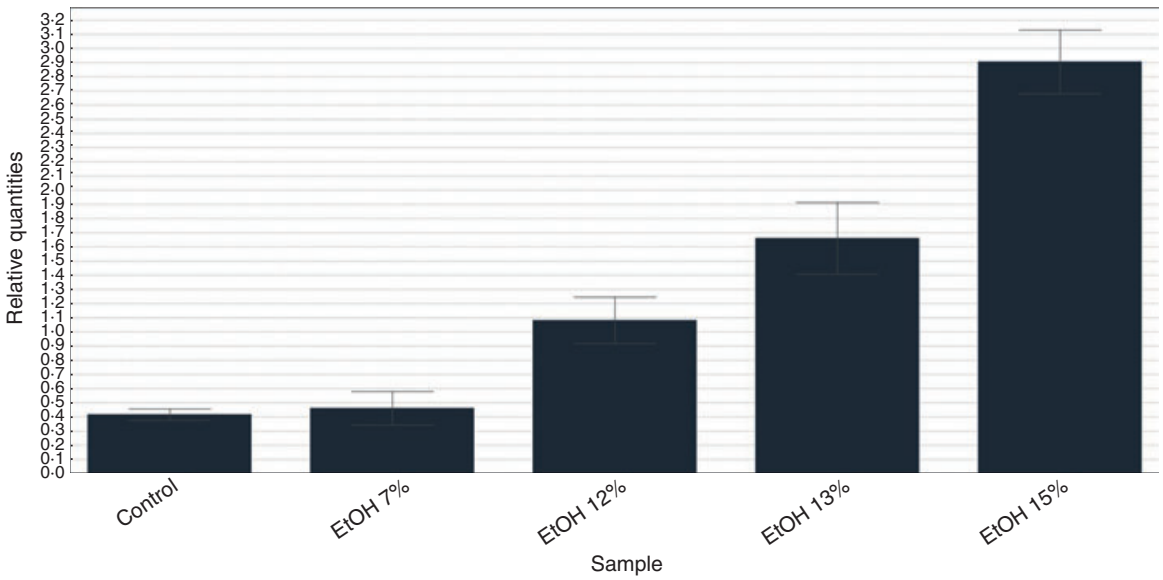


**Figure 3** Relative expression levels of the geranylgeranyl pyrophosphate synthase (*ggpps*) gene in S12 *O. oeni* cells treated with different ethanol concentrations: in absence of ethanol (control); ethanol 7% (EtOH 7%); ethanol 12% (EtOH 12%); ethanol 13% (EtOH 13%); ethanol 15% (EtOH 15%).

effect on the physicochemical state and biological functions of cells and the activation of different devices of cell response by involving various genes, such as that coding a *ggpps* (NCBI no. ABJ56986) which was originally identified in *O. oeni* PSU-1 complete genome (NCBI no. CP000411).

Geranylgeranyl pyrophosphates (GGPPs) is an enzyme that belongs to the family of *E*-prenyl diphosphate (prenyl PP) synthases and catalyses the condensation of isopentenyl diphosphate (IPP) with its allylic isomer, dimethylallyl diphosphate (DMAPP), to produce GGPP, an essential isoprenoid involved in several biosynthetic pathways such as the biosynthesis of terpenoids, carotenoids and membrane stabilizers such as hopanoids, but also the synthesis of quinones and chlorophylls and the prenylation of proteins (Velayos *et al.* 2003).

Two distinct pathways occur for the biosynthesis of isoprenoids precursors, IPP and DMAPP. Eukaryotes, except plants, perform the mevalonate (MEV) pathway to convert acetyl coenzyme A (acetyl-CoA) to IPP, that is, transformed subsequently in its isomer DMAPP. In mammals, it has been demonstrated that MEV cascade is involved in many biological phenomena and cellular functions in which mevalonate act as a precursor of cholesterol and also of isoprenoids for farnesyl and geranylgeranyl molecules, which have an important signalling function (Tanaka *et al.* 2000).

In prokaryotes, with a few exceptions, IPP and DMAPP are produced by a mevalonate-independent pathway, the deoxyxylulose-5-phosphate (DXP) pathway, while plants use both MEV and DXP pathways (Wang and Ohnuma 2000). In plants, isoprenoids are important secondary metabolites, highly specific and synthesized in particular development phases that represent chemical substances for adaptation to stresses and defence products (Wang and Ohnuma 2000).

Synthesis of isoprenoids is intrinsic to all organisms and leads to a vast array of metabolites with diverse functions for cell survival. In humans and other mammals, the products of this pathway include essential molecules such as cholesterol, haeme A, ubiquinone, dolichol and farnesoids. The latter products include farnesyl pyrophosphate (FPP) and GGPP, which are precursors for protein prenylation and might serve as nuclear receptor ligands (Kavanagh *et al.* 2006). The study of Kavanagh *et al.* (2006) described the structure of human GGPP, and, by carrying out a sequence alignment with GGPP of other organisms (including a bacterial species), shared sequence identity, the presence of conserved regions so a possible common catalytic mechanism.

Eubacteria, like plants, have the type II synthase: in particular, in *O. oeni*, this enzyme is implicated in the biosynthesis of secondary metabolites, mainly in terpenoids, steroids and membrane lipids biosynthesis (KEGG PATHWAY Database www.genome.jp/kegg/pathway.html).

Isoprenoids, one of the largest groups of natural compounds, have a variety of roles in respiration, photosynthesis, membrane structure, allelochemical interactions and growth regulation (Sangari *et al.* 2010). All free-living organisms synthesize isoprenoids from the five carbon

precursors IPP and its double-bond isomer dimethylallyl diphosphate (DMAPP). Recently, besides the usual pathways for the isoprenoids precursors biosynthesis, alternative pathways and metabolic intermediates have been proposed to be used for the biosynthesis of isoprenoid precursors in the cyanobacterium *Synechocystis* PCC 6803 illustrating how limited is still our knowledge of the alternative pathways that can be used by bacteria to synthesize their isoprenoids (Sangari *et al.* 2010).

Carotenoids are widely produced by plants and microorganisms in which they accomplish important biological functions (Hagi *et al.* 2013). Among LAB, some carotenoid-producing enterococci and lactobacilli have been isolated from various origins such as food, plants and human clinical specimens (Garrido-Fernandez *et al.* 2010; Maraccini *et al.* 2012; Hagi *et al.* 2013).

In general, carotenoids contribute to tolerance to stresses, such as oxidative stress, because of their antioxidant ability derived from their conjugated double bonds (Hagi *et al.* 2013). In Gram-positive bacteria, the effects of carotenoids produced by *Staphylococcus aureus* on oxidative stress tolerance have been reported (Clauditz *et al.* 2006), and the heterologous expression of staphylococcal carotenoid biosynthesis genes improves $H_2O_2$ stress tolerance in *Bacillus subtilis* (Yoshida *et al.* 2009). Carotenoid production in LAB, which is one of the antioxidant mechanisms, is considered to play a role in the elimination of oxygen radicals. Carotenoids are lipophilic agents that are incorporated into the bacterial membrane. Chamberlain *et al.* (1991) reported that carotenoids could influence cell membrane fluidity and decrease the sensitivity of *S. aureus* to oleic acid. This finding implies that carotenoid production in LAB also may lead to changes in membrane fluidity. So, it is possible that the change in cell membrane fluidity caused by carotenoids could aid to prevent cell membrane disturbances and cell death induced by ethanol. However, the mechanism of tolerance to ethanol remains unknown.

LAB have been widely used for starters of food fermentation and probiotics; therefore, investigation of the stress tolerance mechanism of LAB is important to optimize its application in food fermentations and probiotics (Hagi *et al.* 2013). Antioxidant enzymes in LAB, such as glutathione, superoxide dismutase and catalase, have been studied and used to improve oxidative stress tolerance of LAB (Hagi *et al.* 2013).

A previous work (Garrido-Fernandez *et al.* 2010) observed that different *Lactobacillus plantarum* strains, widely used as probiotics in dairy products and dietary supplements, were able to produce high carotenoids amounts in particular environmental conditions. Therefore, carotenoid production should be considered as an important feature for the selection of novel probiotic *L. plantarum* strains; the use of selected high-carotenoid-producing strains could contribute to increase the total amount of antioxidants supplied in the human and animal diet (Garrido-Fernandez *et al.* 2010). In all cases, these effects have been related to either the antioxidant properties of carotenoids or their ability to stabilize bacterial cell membranes.

The presence of GGPP in secondary metabolism processes of *O. oeni* was confirmed by the following investigations, revealing the presence of *ggpps* gene in S12 *O. oeni* strain under both stress and optimal conditions. A quantitative analysis was carried out by qPCR technique in order to analyse the expression level of *ggpps* gene and its changes after exposure to ethanol stresses. The specific gene expression patterns reflect mechanisms involved in adaptation to environmental conditions, and the qPCR method is the best approach to obtain a better understanding of these patterns to provide insights into complex regulatory networks leading to the identification of genes relevant in adaptation processes (Vandesompele *et al.* 2002).

As an universal reference gene of which the expression level remains constant does not exist because the expression of many internal control genes is influenced by metabolic conditions, growth phase or experimental conditions (Dheda *et al.* 2005; Theis *et al.* 2007; Bustin *et al.* 2009; Ritz *et al.* 2009; Duary *et al.* 2010; Duquenne *et al.* 2010; Costantini *et al.* 2011; Vaudano *et al.* 2011; Sumby *et al.* 2012), we used multiple internal control genes applying the statistical algorithm geNorm (Vandesompele *et al.* 2002).

In this study, the choice of reference genes was based on historical precedents; Desroche *et al.* (2005) suggested that *ldhD* (D-lactate dehydrogenase) was the best reference gene among the others that are usually used for qPCR normalization in *O. oeni*, because of its ability to keep a stable transcriptional levels in different kind of stresses and in different growth stages of the cells, but our study demonstrated an intermediate stability of *ldhD*, so in this case, it cannot be used for the normalization.

In this work, results showed that, based on $V_{n/n+1}$ value obtained from geNorm analysis, the five most stable genes must be used to quantify the expression of *ggpps* gene, that are *pta*, *rpoB* (subunit $\beta$ of DNA-direct RNA polymerase), *gapA*, *recA* (recombinase A) and *gyrB* in accordance with Sumby *et al.* (2012) that validated the use of multiple internal control genes to study esterase gene expression in *O. oeni,* suggesting that the most stable genes were *ftsZ* (filamentous temperature-sensitive mutant Z), *pta* (phosphotransacetylase), *gapA* (D-glyceraldehyde-3-phosphate dehydrogenase) and *rpoA* (sigma $\alpha$ factor). Moreover, Ritz *et al.* (2009) proved that *rpoA* was the most suitable internal control gene to study the stress response in *C. jejuni*,

in contrast with our results that demonstrated *rpoA* had the lowest stability, and finally, Costantini *et al.* (2011), established that *gyrB* (gyrase subunit B) was the most stable gene in absolute to be used as a reference gene to analyse the expression level of *mleP* gene and two genes involved in the ABC transport system in *O. oeni* strains. These literature references confirm further that there is not an ideal universal reference gene.

After the characterization of a suitable reference system, the transcription levels of the *ggpps* gene were analysed and the data obtained suggested that *ggpps* could play an essential means that *O. oeni* uses to withstand and adapt to ethanol stresses. The results proved changes for the adaption to a particular stress condition; the ethanol is an agent affecting the physicochemical state and biological functions of cells and leading numerous modifications faced by different devices of cell response. Ethanol acts as a disordering cause of the *O. oeni* cytoplasmic membrane and the metabolic activities (da Silveira *et al.* 2003). It interacts with membranes at the lipid–water interface, weakening the hydrophobic barrier to the free exchange of polar molecules, thereby perturbing membrane structure and function (Chu-Ky *et al.* 2005) with fluidity reduction and changes of cell functionality as membrane rigidity obstructs structural variations indispensable to metabolic activities development (da Silveira *et al.* 2003).

Our work was effectuated to investigate the effect of ethanol exposure on the expression level of a gene involved in the metabolism of *O. oeni* to probe the mechanisms of ethanol tolerance correlated with adaptive changes. The analysis of results suggest that *O. oeni* adjusts the expression of genes to adapt to stress conditions and the high expression level of *ggpps* would allow a flow of isoprenoid precursors towards the carotenoids and related pathways to stabilize bacterial cell membranes, improving the cell membrane disturbances and preventing cell death induced by ethanol.

In conclusion, the performance of micro-organisms under ethanol stress conditions, such as those prevailing in wine, requires specific cellular features, including modification of metabolic activities to allow survival under such adverse conditions. Improving the knowledge of stress tolerance and adaptation mechanisms of the malolactic bacterium *O. oeni* is essential to enhance the efficiency of the malolactic starter in wine and to obtain the development of starters able to survive to direct inoculation with a large benefit for wine technology.

The analysis and the investigation of the involvement of *ggpps* gene in physiological changes of bacterial behaviour confirmed and clarified that the exposure to stress requires the activation of defence mechanism so that bacteria become more tolerant to adverse conditions. qPCR turned out to be an efficient approach to obtain a better understanding of the expression level patterns to provide insights into complex regulatory networks leading to the identification of genes relevant in adaptation processes.

## Conflict of interest

The authors declare no conflict of interest.

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