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Complementing DIGE proteomics and DNA subarray analyses to shed light on *Oenococcus oeni* adaptation to ethanol in wine-simulated conditions

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5, Deceased May 27th, 2014. This paper is dedicated to her memory

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Abstract

Direct addition of *Oenococcus oeni* starters into wine can cause viability problems. In the present study, the influence of ethanol in wine-simulated conditions on *O. oeni* has been evaluated by complementing microarray techniques and DIGE proteomics. Two different ethanol concentrations were studied. In 12% ethanol, pyrimidine anabolism was stimulated, but in 8% ethanol some energy-consuming biosynthetic pathways were limited. The most significant result was the stress response induced by alcohol that concerned both the cell-envelope and specific stress proteins. Interestingly, 8% and 12% ethanol triggered different stress responses: in mild ethanol stress (8%), chaperones with prevalent refolding activity (like HSP20) were over-expressed, whereas at higher alcohol concentration (12%), together with HSP20 and the refolding DNAJ/K, also chaperones having proteolytic activity (like ClpP) were induced. Furthermore the stress response repressor HrcA was downregulated only at 12% ethanol, suggesting that it controls stress pathways, which are different from those active at 8% alcohol. This result confirms that the HrcA system is operative in *O. oeni* where the CtrS system is prevalent.

Biological significance

The use of malolactic starter cultures has become widespread to control the MLF process and to prevent off-flavours. There is significant interest in understanding the molecular mechanisms that *O. oeni* uses to adapt to harsh wine conditions. The overall results highlight that the alcohol-induced stress response involves not only biosynthesis of stress proteins but also envelope-linked mechanisms. From a practical point of view this research underlines the importance of starters acclimation to induce responses that would allow better adaptation to the wine. As a consequence, a well adapted starter can complete malolactic fermentation and improve the final wine quality.

Key-words: *O. oeni*, stress response, cell envelope, energy metabolism, EPS, chaperones.

1. Introduction

The most important role of *Oenococcus oeni* in winemaking involves flavour development and de-acidification through the decarboxylation of malate to lactate with the production of carbon dioxide, the so-called malolactic fermentation (MLF) [1]. Besides MLF, *O. oeni* possesses genes for citrate metabolism, one of the pathways associated with the wine sensory profile. Therefore, the use of this species in oenology represents an added value for the quality of a wine. Winery practices are facilitated by commercial freeze-dried starter cultures of *O. oeni* available for direct inoculation into wines [2].

O. oeni is the lactic acid bacterium best adapted to survive in the harsh wine environment characterized by the presence of ethanol, low pH, nutritional limitation and the presence of sulphites [3,4]. However, direct inoculation of *O. oeni* starters into wine leads to significant cell mortality and, consequently, failure of MLF. Alcohol stress is for sure the biggest challenge. To improve survival, adaptation of *O. oeni* cells to the wine environment before inoculum has been proposed [5]. However, it is important to understand the mechanisms involved in *O. oeni* stress response and alcohol tolerance. It should also be considered that a starter can have a different behaviour as compared to a spontaneous/ autochthonous malolactic strains, because it undergoes industrial processes, among which lyophilisation, which are stressing “per se” .The role of adaptation to different wine stressors has been discussed in several works. Guzzo et al. [6] demonstrated that pre-incubating *O. oeni* cells in the presence of a sub-lethal concentration of sulphite (15 mg/l) enhanced tolerance to a highly inhibitory sulphite concentration, suggesting the involvement of adaptation mechanisms. Beltramo et al. [7] demonstrated that to increase survival and growth of *O. oeni* in wine, cells had to be adapted by growing them in wine-like medium at low pH (3.5) before inoculation into wine. Conversely, they did not observe any effect in terms of better survival in wine by adapting the cells in 10% ethanol.

Several genes involved in stress tolerance of *O. oeni* have been identified [7,8]. However, the link between a behavior observed under particular conditions and the genetic background of *O. oeni*

is essential to disclose the molecular regulatory mechanisms in order to pave the way to new practical applications [9].

Functional analysis of gene expression using comparative transcriptomics and proteomics may provide insight into stress responses and regulation mechanisms in lactic acid bacteria (LAB). This approach is useful also to study the behavior of bacteria in a real food system. As far as transcriptomics is concerned, the microarray technique has proven to be a well-established method. In *O. oeni* the only application of microarrays has been by Borneman et al. [10] who studied the genomic content of ten strains in order to understand the basis of phenotypic differences among them. For what concerns proteomics, several approaches are possible. The two-dimensional (2D) - DIGE technique was first described in 1997 [11]. It relies on a pre-electrophoretic labeling, allowing multiplexing of samples into the same gel. This technique was applied in the study of acidic stress response of *Lactobacillus rhamnosus* GG [12], of the exoproteome of trehalose-adapted *Lactococcus lactis* [13], and recently of the production by *Penicillium expansum* of geosmin, an earth-smelling sesquiterpine that spoils wine [14], but as far as we know, it has never been applied to *O. oeni*.

Silveira et al. [5] used 2D gel electrophoresis (without the DIGE application) and showed that both ethanol stress and adaptation changed the protein profiles of *O. oeni* cells, in particular that of membrane associated proteins. Cecconi et al. [15] observed that the different physiological behavior between acclimated and non-acclimated *O. oeni* cells was mainly ascribable to differential modulation of specific proteins involved in stress response, and in sugar and amino acid metabolism. Very recently [16] a partial proteome reference map of *O. oeni* has been produced, which underlined the importance of malate and citrate metabolism in this species.

Since commercialized LAB starters for MLF differ in their phenotypic traits and especially in the time required to adapt them to wine conditions, and the new type of starters are for the direct inoculation (MBR®) in wine [17], we sought to investigate if an adaptation phase and, in particular, the composition of the adaptation medium can influence the *O. oeni* starter behaviour. To this

purpose a rich medium was compared to wine-like media having different ethanol content (8% and 12% respectively). In the first part of the study we developed a subgenomic-array, which takes into account the main genes involved in different metabolic pathways of interest for growth and MLF under wine conditions. In the second part of the work, we applied the differential gel electrophoresis (DIGE) technique, which allowed a more accurate and sensitive quantitative proteomic analysis. To our knowledge, a part from some data concerning spoilage fungi, this is the first application of DIGE in the area of stress investigation in enology. The experimental approach used, by complementing transcriptomic and proteomic data, aimed at describing the physiological behavior of *O. oeni* when adapted to different media prior to inoculation in wine.

2 Material and Methods

2.1 Bacterial strain and growth conditions.

A commercial starter of *O. oeni* was used in this study (Elios, MBR process, Lallemand, Verona, Italy). Samples of 0.5 g of lyophilized culture were rehydrated in 50 mL of three different media: MRS (indicated as M) (DeMan Rogosa and Sharpe, Biogenetics, Milan, Italy) supplemented with 3 g/L malic acid; a wine-like medium composed of glucose 2g/L, yeast extract 0.5 g/L, peptone 1 g/L, potassium phosphate 0.2 g/L, sodium acetate 0.5 g/L, ammonium citrate 0.2 g/L, magnesium sulphate 0.02 g/L, manganese sulphate 0.005 g/L, tween80 0.1 g/L, 20% apple juice (containing about 5 g/L fructose), 3 g/L malic acid and supplemented with 8% ethanol, (indicated as et.8%); and the same wine-like medium supplemented with 12% ethanol, (indicated as et.12%). All the media used were buffered to reach pH 3.5. Samples were mixed for a few seconds, incubated at 25°C for 20 min and then inoculated into 150 ml of the same medium. Cultures were kept for 1 day at 25°C to acclimate the cells. After this acclimation period, samples were taken and used for the expression and protein profile analyses. Three and four biological replicates were analyzed for microarray and proteomic studies, respectively.

2.2 Microarray

2.2.1 Probes and design of subgenomic-array

The first step of microarray fabrication required the selection of probes to be used. The genes of interest were chosen in order to have representative enzymes of the main metabolisms. To this purpose, *O. oeni* pathways and gene function found on KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.ad.jp/kegg) [18] were analysed and gene sequences were retrieved from GenBank. Probes, 35 nucleotide long, were designed by using Array designer software (Premier Biosoft, http://www.premierbiosoft.com) and Primer3 [19], and they are deposited on NCBI with the accession numbers from Pr031952747 to Pr031952891. BLAST analysis was automatically performed by the software Array Designer. Moreover every obtained probe was further analyzed by BLAST against the genome draft of AWRI553 [20].

The microarray format used in this study was based on standard microscopic glass slides. Probes were spotted onto the activated slide surface by Centro di Ricerca Interdipartimentale per le Biotecnologie Innovative (CRIBI University of Padova, Italy). The chip was arranged in 16 blocks and genes were repeated three times.

2.2.2 RNA extraction and cDNA synthesis

RNA extraction was performed using "MasterPure TM®Complete DNA and RNA purification kit", after 1 day of adaptation for all samples. DNAse treatment was performed in all extractions using TURBO DNase (Ambion, Milan, Italy) at 37°C for 3 hours. After the DNAse treatment, RNA was analysed with Experion (Biorad, Milan, Italy) microcapillary automated electrophoresis system in order to determine its quality, integrity and suitability for microarrays analysis.

Reverse transcriptase reaction was performed using 2 µg of RNA in a 25 µl mix containing reverse transcriptase M-MLV (Promega, Milan, Italy) according to the manufacturer's instructions. cDNA synthesis and precipitation was made according to Rantsiou et al. [21]; finally the cDNA pellet was air dried. Three biological replicates were made.

2.2.3 Hybridization conditions

Slides were pre-treated in the following steps: one wash with 0.1% Triton X100 for 5 min; KCl 100 mM for 10 min; one wash for 1 min with sterile water; 1 hour with a blocking buffer (1X QI process Schott Nexterion® supplemented with 0.02% v/v HCl 37%, Jena, Germany) using an oven PersonalHyb® (Stratagene, Milan, Italy) at 50°C; one wash with sterile water. Lastly, slides were covered with SecureSeal™ hybridization chamber 22 mm (Invitrogen, Milan, Italy).

For hybridization experiment it was necessary to find the optimal conditions for the analysis. To this purpose two different temperatures of hybridization were tested, 30° and 50°C. A greater number of probes hybridized at 30°C (data not shown), therefore in the subsequent analysis the temperature of 30 °C was used.

cDNA samples were resuspended in 350 µl of hybridization buffer composed of 1X SSC and SDS 0.1%, denatured at 95 °C for 5 min and loaded in the hybridization chamber. Slides were incubated overnight at 30°C, using the Eppendorf Thermomixer at a constant velocity of 300 rpm. The detection of the slides was performed using the SilverQuant Detection Kit (Gentel Bioscience, Madison, USA), following the manufacturers' instructions.

2.2.4 Microarray data analysis

Air-dried slides were scanned at a resolution of 10 µm with a Silverquant microarray scanner (Eppendorf, Milan, Italy); the scanned images were further analysed with the software Silverquant microarray analysis (Eppendorf) in order to quantify the spots.

Background corrections, test for outliers, mean of gene replicates, normalization and data plotting were performed using the "Micro Array Data Analyzer" MADA (www.mpi-bremen.de). Data obtained from this analysis were further processed with Genesis software [22]. Differentially expressed genes were evaluated with ANOVA analysis using SPSS (SPSS 15.0 for Windows 2004; SPSS, Chicago, Ill., U.S.A.).

2.3 Proteomic

2.3.1 Protein sample preparation

Samples, taken after 1 day of adaptation in the three different media described, were pelleted and proteins were extracted. Cells were disrupted using a Bio 101 Savant FastPrep FP 120 bead beater (Savant, Farmingdale, USA) in the presence of phenol, Tris 10 mM pH 8.5 and acid washed glass beads (Sigma). The interphase was collected and proteins were precipitated with 3 volumes of ice cold 96% ethanol at -20°C overnight. Proteins were resuspended in 500 µL of rehydration buffer (Tris 10 mM, 7 M urea, 2 M thiourea, 3% CHAPS). Four biological replicates were analyzed.

Protein concentration was estimated using the 2-D Quant kit (GE Healthcare, Copenhagen, Denmark) following the manufacturer's instructions.

2.3.2 SDS-PAGE

Monodimensional electrophoresis was performed on precasted gel NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen, Copenhagen, Denmark). The molecular weight marker MARK 12^{TM} unstained standard (Invitrogen) was used. The run was performed using the XCell *SureLock*® Mini-Cell apparatus (Invitrogen). The gel was fixed and stained with Colloidal Coomassie Blue [23].

2.3.3 Sample preparation for DIGE

Proteins (50 µg) were labeled with 200 pmol of Cy2, Cy3 and Cy5 CyDye DIGE Fluors for Ettan DIGE (GE Healthcare) according to the manufacturer's instructions. An internal standard was prepared with a pool of the tested samples representing all the different biological replicates of all the studied conditions.

2.3.4 2D gel electrophoresis

Samples labeled with the different dyes were mixed, and added to the rehydration buffer (7 M Urea, 2 M thiourea, 3% CHAPS, Tris 10 mM pH 8.5, 10% glycerol, 5% isopropanol, 100 mM DTT). Each strip for the first dimension contained a mixture composed of 50 µg of internal standard labeled with Cy2, 50 µg of the sample labeled with Cy3 and 50 µg of the sample labeled with Cy5. Isoelectrofocusing (IEF) was done by in-gel rehydration on IPG strips pH 4–7, 18 cm. The IEF was carried out in IPG-Phor (GE Healthcare) at 20°C, 50 µA/strip until 60 kVh.

Gels (24 cm width) for the second dimension were cast using Gel caster for Ettan DALT*six* system (GE Healthcare). After the first dimension, the IPG strips were placed in 5 mL equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS, 0.01% bromophenol blue) supplemented with 1% DTT for 15 min and then placed in the equilibration buffer containing 2.5% iodoacetamide for 15 min. The strips were thereafter, placed on 12.5% SDS-PAGE gels and overlaid with 0.5% agarose sealing solution. Electrophoresis was performed with the Ettan DALT*six* electrophoresis unit (GE Healthcare) with the following program: 2 W per gel for 1 h, then 12 W per gel until the bromophenol blue reached the bottom of the gel.

2.3.5 Image analysis

DIGE gels were scanned using the Typhoon 9410 Variable Mode Imager (GE Healthcare). Imaging of DIGE gels was done immediately after the second dimension at excitation/emission wavelengths of 488/520 nm (Cy2), 532/580 nm (Cy3) and 633/670 nm (Cy5), respectively.

After image acquisition, the gels were fixed in the fixing solution (30% ethanol and 2% phosphoric acid) and stained overnight by colloidal CBB as described by Candiano et al. [23].

Progenesis SameSpot (version 3.3, nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) was used for spot detection. DIGE gel images were then aligned by automated calculation of twenty manually assigned alignment landmark vectors. A 1.5-fold threshold (spot volume ratio change)

and ANOVA ($p<0.05$) was chosen as criterion in the identification of differentially abundant protein candidates.

The choice of the fold-change threshold used in the current study was based on the statistical analysis and it is of significance. The 1.5-fold threshold value used was based on the Power analysis, which has a recommended value of 80%. Power analysis can be used to calculate the minimum sample size required to accept the outcome of a statistical test with a particular level of confidence. The experimental setup had enough statistical power with the four replicate gels. False discovery rate estimates the number of false positives within statistically significant changes in the experiment. The *p* value was set to < 0.05 giving a false discovery rate of 5%.

2.3.6 Trypsin digestion and mass spectrometry analysis

The selected spots were excised from the gel and treated as described by Majumder et al. [24]. One μL of trypsin digested sample was loaded on the AnchorChip target and allowed to dry, then 1 μL of matrix α-cyano-hydroxycinnamic acid (CHCA 0.5 μg/μL in 90/10 ACN/TFA) was added to the dried sample and left to dry completely. Spots were washed with 2 μL of 0.5% trifluoroacetic acid. β-lactoglobulin (5 pmol/mL) was used as standard. Mass spectrometry peptide fingerprint and MS/MS were performed with the Ultraflex II MALDI-TOF MS mass spectrometer (Bruker-Daltonics, Bremen, Germany) using Flex Control v3.0 (Bruker-Daltonics) and processed by Flex Analysis v3.0 (Bruker-Daltonics). The MS together with MS/MS spectra were searched against the NCBI nr (NBCInr 20111105) database for bacteria using the MASCOT 2.0 software integrated with BioTools v3.1 (Bruker-Daltonics). Peptide mass maps were acquired in re- flectron mode with 500 laser shots per spectrum. Spectra were calibrated externally and internally using a tryptic digest of β-lactoglobulin (5 pmol/L) and porcine trypsin autolysis products, respectively. MS/MS data were acquired with stop conditions so that 1000–1600 laser shots were accumulated for each spectrum.

Search parameters were monoisotopic peptide mass accuracy of 80 ppm, fragment mass accuracy to ±0.7 Da; maximum of one missed cleavage; carbamidomethylation of cysteine and partial oxidation of methionine; Mascot cutoff score was 40. Filtering of peaks was carried out for known keratin and autocatalytic trypsin peaks. The protein function was described using Microbial Genome Database (www. mbgd.genome.ad.jp).

3 Results and Discussion

MLF is a desirable transformation in winemaking processes both for reducing acidity and for adding flavours to wine. Inoculation with a commercial starter strains into wine increases the control of MLF with respect to endogenous bacteria performing spontaneous MLF [2]. Although some authors [25] disagree about the real contribution of added starter LAB, it is generally accepted that improvements in the quality and the performance of MLF are ascribed to exogenous starter cultures [26–29]. However, the limit of using exogenous bacteria lies in their difficulty to adapt to the harsh wine conditions (low pH and, especially, very high alcohol content). In this context, acclimation procedures can improve adaptation. In this study, as shown by two different approaches (transcriptomics and proteomics), acclimation media strongly influence gene expression and protein profiles in the malolactic bacterium *O. oeni*, through the involvement of adaptive responses that could lead to improved survival and MLF when the microorganism is inoculated into wine.

The developed subgenomic array was successfully applied and the reliability of the technique was confirmed by the comparison of the response of some genes studied in a previous investigation using reverse transcription quantitative PCR (RT-qPCR) [30]. For instance *oeoe 1651* gene, coding for an ABC-type sugar transporter, has been previously shown to be transcribed in the wine-like medium (which contains a very low sugar concentration) and not transcribed in MRS, and this was confirmed here with the subgenomic microarray approach.

Overall, several genes became up-regulated by wine-like medium acclimation. In Figure 1 the differentially expressed genes after the adaptation phase are shown. These genes could be ascribed to 9 classes referred in Table 1; the main differences in gene expression concerned the physiology of cell wall and membrane, sugar, acid, nitrogen metabolisms and stress.

In order to check which genes were significantly differentially expressed, ANOVA analysis was performed on the data after normalization. The results obtained are shown in Figure 2. It was observed that in the presence of ethanol (both 8% and 12%) two genes encoding alcohol dehydrogenase (*oeoe 0242* and *oeoe 0527*) were less expressed. In et.8%, the glucosidase gene (*oeoe 0040*), genes involved in exopolysaccharide synthesis (*oeoe 0071*), *glnQ* (*oeoe 1634*) encoding a polar amino acid transporter, lysylaminopeptidase (*oeoe 1058*), and *hrcA* were on the contrary more expressed. Finally in et.12% the transcription was higher for genes related to glycan biosynthesis (*oeoe 0144* and *oeoe 1444*), genes related to nitrogen metabolism (*oeoe 0258*) and the gene *oeoe 0455*, which was related to the signal recognition particle.

In the second part of the study, comparative proteome analysis was performed by DIGE. The pH region 4–7 was chosen since the pI values of most proteins from lactic acid bacteria lie in this pH range. Only statistically significant changes (p value <0.05) in spot intensities were considered (Suppl. Tab. 1).

Statistically significant differentially abundant proteins were found comparing the samples adapted in MRS with those adapted in et.8% (Fig. 3). The selected protein spots were excised from the gels and analyzed by MS and MS/MS, because MS data alone were not sufficient for the identification. Results are shown in Table 2A.

The comparative analysis of the gels derived from cells grown in MRS and with ethanol 12% respectively, revealed that the differentially abundant proteins were the same as found in the previous analyses of et.8%, with some differences in the fold changes (Suppl. Fig. 2). Additional proteins were also found to be differentially abundant. Thus as shown in Table 2B, four proteins (not significantly induced in the 8% ethanol cultures), identified as clpL2 protein (spot 428), heat

shock protein HSP20 (spot 994), 50S ribosomal protein L13 (spot 905) and ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones (spot 432) were found to be more abundant in samples adapted in et.12% (Fig. 4).

Taking into account the results obtained from the two different analyses, it is possible to classify the differentially abundant genes/proteins into 6 main functional and metabolic families: 1) stress response, 2) surface modifications, 3) MLF and citrate metabolism, 4) sugar metabolism, 5) nitrogen metabolism (amino acids, purines and pyrimidines), 6) diacetyl biosynthetic route.

The overall, most significant response of *O. oeni* in media containing ethanol, was an increased transcription of stress related genes and an enhanced production of stress related proteins. In the literature data concerning *O. oeni* proteomics are scarce, nevertheless, all agree about the capability of this species to biosynthesize a variety of stress proteins [5,15]. Similarly, expression of genes encoding stress response proteins have been reported by several authors [31–34].

Interestingly, some stress proteins/genes (*trxA*, HSP20, ribosomal-associated HSP) were more induced in mild (et.8%) than in strong (et.12%) ethanol stress. On the other hand, the stress response repressor HrcA is down-regulated only at higher alcohol content (12%) where a strong stress response is needed (figure 2). These data suggest that different stress responses are operative at different stress degrees and also underline the importance of a gradual and progressive acclimation to alcohol for better inducing concerted phenotypic responses useful for wine adaptation. No differences were found for other stress related genes included in the array such as *hsp33*, *ftsH*, *clpX*, c*spE*, *oeoe 0513* (Transcriptional repressor of class III stress) and *oeoe 0807* (stress response membrane GTPase).

TrxA (thioredoxin-encoding gene) is induced by both oxidative stress [35] and by heat [36]. This is the first time that this gene is associated with alcohol stress response. The heat-shock response is a ubiquitous phenomenon that enables cells to survive to a variety of environmental stresses [37] and heat shock proteins (HSP) are referred to contribute to the ethanol-stress response in bacteria

[38,39]. In particular, HSP20 family protein is strongly induced by several kinds of stresses (temperature, ethanol, sulphites and acidic pH) and during stationary growth phase in *O. oeni* cells [6,39]. In the present investigation we observed an increased level of protein HSP20 in cells adapted in media containing 8% (spot 995, figure 3) and 12% ethanol (spot 994, figure 4). This is an additional evidence that HSP20 has a role in the ethanol stress response, probably refolding moderately damaged proteins, and that this response in *O. oeni* occurs after 1 day of acclimation.

Many important *hsp* genes are controlled by a negative modulation mediated by specific repressor proteins such as CtsR and HrcA. Grandvalet et al. [8] demonstrated that the CtsR repressor is the major regulator of chaperone gene expression in *O. oeni*. These authors showed that under optimal growth conditions, CtsR would prevent the synthesis of unnecessary stress proteins until environmental changes (ethanol, acid, nutritional and oxidative stresses) occur. In this condition CtsR coordinates the synthesis of HSPs strongly inducing transcription of stress genes, thus enhancing the adaptability of this LAB under adverse conditions. A second negative heat shock control mechanism is the CIRCE/HrcA system; it was found in several bacilli and it has been best characterized in *Bacillus subtilis* [40]. The increased transcription of *hrcA* gene due to ethanol 8% proves that this regulative system is operative also in *O. oeni*. Furthermore, the evidence that a negative modulator is active at 8%, but less expressed at 12% ethanol, is consistent with the cell's need of triggering different stress responses at higher alcohol concentration, as discussed above. Actually, 12% ethanol enhanced the expression of stress proteins such as ClpL2 (spot 428) Hsp20 (spot 994) and ClpP and DNAJ/DNAK chaperones (spot 432). Beltramo et al. [7] observed, by RT-qPCR, that the expression level of the genes *clpL1* and *clpL2*, also increased in *O. oeni* cells adapted at low pH, and they concluded that these genes confer to the cell ability to adapt to acidic stress. The present finding of enhanced transcription of ClpP in 12% ethanol medium by *O. oeni* suggests that this system is involved in the alcohol stress response. Although the refolding/proteolytic cycle set-up by chaperones and proteases is far from being fully elucidated (especially in prokaryotes) and new paradigms are continuously reported in the literature [41] it is

generally accepted that ClpP displays chiefly protease activity, suggesting that it cannot restore protein functionality, but only eliminate irreversibly damaged proteins in order to recover and recycle its amino acids. Actually, ClpP is a serine protease: alone it is able to degrade short peptides; when associated with ClpA, it can degrade larger peptides, whereas the degradation of larger proteins requires the ClpA-ClpP complex plus energy supplied by ATP hydrolysis. Conversely, other stress proteins, such as DnaJ/K are induced for both refolding moderately denatured proteins or irreversibly degrading protein aggregates [43]. The present findings, highlighting over-expression of proteolytic chaperones in 12%, but not in 8% ethanol, are consistent with the higher degree of protein damage in the former condition. Moreover, the expression of *clp* genes seems to be regulated by HrcA, as observed in several *Lactobacillus* species, where direct interaction between HrcA and the promoter of a *clp* gene has been reported [44]. It is generally recognized that *clp* genes are mainly regulated by the CtsR repressor in low G+C content Gram-positive bacteria: hence the present result suggests that HrcA plays an important role in such negative modulation not only in lactobacilli, but also in oenococci.

Additionally, it is worth noticing that some stress chaperones (e.g. GroEL, spot 1043) were less abundant in ethanol, probably due to a possible more stressing event than alcohol being present in the control medium. We speculate that the high MRS sugar content could represent a stressor, generating a high osmotic stress.

A second interesting modification induced by ethanol concerns the bacterial envelope structures such as membrane, cell-wall and surface layer. Genes for exopolysaccharides (EPS) and glycan biosynthesis, as well as for membrane stability, were up-regulated by alcohol exposure. The gene *oeoe 0071,* described as gene related to EPS production, was significantly induced in both 8% and 12% ethanol. According to Ciezack et al. [45], most *O. oeni* strains produce significant amounts of EPS. This production is independent from their genotype and not directly connected with growth. Conversely, it could be stimulated by changing the growth medium composition. Very recently production of EPS by *O. oeni* ATCC BAA-1163 was hypothesized based on detection of enzyme-

encoding genes. Nonetheless, no proteins directly involved in their biosynthesis were found in the partial proteomic reference map determined by 2DE MS-MS [16]. Hence our finding can provide further suggestive evidence that in *O. oeni* EPS production is not constitutive but stress-related. Generally LAB produce EPS by diverting a certain amount of glucose from catabolic routes, to protect the cell internal environment from several stressors (toxic metal ions, ethanol, and sulphur dioxide). However, although a role in protecting the bacterial cell from phage attack was initially suggested [46], but more recent experiments failed to reveal a significant phage resistant phenotype in EPS producing strains [47]. Nevertheless, due to their high water-binding properties, EPS constitute a protection factor against dehydration and osmotic stress [48]. It is thus possible that the EPS may enhance bacterial survival in wine resulting in an advantage for malolactic starters. Recently Dimopoulou et al.[49] showed that in *O.oeni* many genes are involved in EPS production. Among them there is also *oeoe 1737,* which, in our subarray, proved to be upregulated in ethanol 8%. This result strongly supports the hypothesis that EPS production can be a response to stressing environmental conditions.

The genes encoding enzymes involved in cell-wall biosynthesis and degradation were strongly induced by ethanol: some of them (*oeoe 1444* and *oeoe 0144*) only at 12% ethanol, other (*oeoe 0719*) also at 8%. This finding is consistent with the need of cell-wall renewal after contact with stressing alcohol concentrations: probably some damage occurs and hence both degradation and *ex novo* synthesis are required. Repair can be achieved by enhancing the amount of cell wall processing enzymes,, Increased transcription of the gene *oeoe 0455,* coding for a signal recognition particle protein (SRP), was also observed in both ethanol conditions. The SRP pathway is considered crucial for the viability of all organisms and conserved in all life domains [50,51]. In the literature, no studies focusing on *O. oeni* are available to this regard. Gutierrez et al. [52] studied the Ffh protein, homologue of the eukaryotic SRP, in the lactic acid bacterium *Streptococcus mutans*. This protein is a chaperone, also found in *Escherichia coli,* involved in protein translocation and membrane biogenesis [53]. These authors suggested that Ffh may participate in the maintenance of

a functional membrane protein composition during adaptation of *S. mutans* to changing environmental conditions. Tourdot-Maréchal et al. [54] suggest that the capacity of *O. oeni* to regulate its membrane fluidity represents a stress tolerance mechanism, related with its ability to regulate membrane composition with respect to both lipids and proteins. Silveira et al. [5,55] observed that membranes of *O. oeni* cells exposed to 12% ethanol showed a decreased permeability and increased disordering. At molecular level, they demonstrated that *O. oeni* was able to respond to the fluidizing effect of ethanol by increasing the degree of unsaturation of the membrane's fatty acids. In our experiments, genes related with membrane stability and glycan biosynthesis, were up-regulated in the presence of ethanol 12% in agreement with what described by these authors. Moreover, Hasona et al. [56] observed that SRP were involved in many cellular mechanisms including ATPase activity, protein synthesis machinery and biofilm formation. These SRP could have similar functions also in *O. oeni* and hence they need further attention to better elucidate their real role.

Remarkably, Delmas et al. [57] and Maitre et al. [58] suggested that the expression level of stress proteins could be regulated by the degree of membrane fluidity: the combined transcriptomics and proteomics data obtained in the present research seem to confirm this hypothesis, revealing modifications in both stress proteins and membrane stability-related enzymes. On the other hand, the bacterial "envelope stress response" is a very common mechanism for counteracting environmental stresses and this was also demonstrated with proteomic studies [59]. All these data underline the importance of studying the functional properties of the cell envelope, which will include investigating surface sub-proteomes, to understand microbial interactions with the environment and their adaptation mechanisms, as previously suggested by Konings [60].

Malate permease (*mleP*), MLF system transcription activator (*oeoe 1565*), and malolactic regulator (*mleR*) genes were also up-regulated by ethanol, especially et.12%, and *mleA* was slightly up-regulated in both ethanol conditions. Mills et al. [61] proved that the oenococcal *mle* operon (*mleA, mleP* and *mleR*) is linked to malate metabolism, however, the same authors, observed that it

remains to be determined if solely the three genes contained in the operon, are responsible for malate conversion. Actually, in *O. oeni* PSU-1, in the upstream region of *citCDEFG* operon for citrate metabolism, there is also an open reading frame encoding an NAD-dependent malic enzyme, which is a putative malate oxidoreductase (*mae* gene). This gene is co-transcribed with the genes of the *cit* operon [62]. In the present investigation the citrate operon was found to be down-regulated by ethanol at gene expression level (gene *oeoe 0419* encoding a citrate transporter and *oeoe 0423* encoding citrate lyase alpha subunit) and this was confirmed at protein level where citrate lyase alpha and beta subunits (spots 546 and 993, respectively), and malate oxidoreductase (spot 629) showed reduced abundancy. Apparently, alcohol seems to positively control MLF, but negatively acts on other malate conversion enzymes, linked to the citrate operon. The present observed down-regulation of the citrate lyase reaction in ethanol-containing media is in contrast with what reported by Olguín et al. [63] who showed that, in *O. oeni,* citrate pathway genes were over-expressed in the presence of ethanol. It should be considered that the conditions used by these authors are different from the ones of this work, since they used ft80 medium supplemented with citrate. Actually, citrate can act as an inducer of the citrate catabolic pathway. Furthermore, the same authors in a more recent work [34] also demonstrated many differences among different strains regarding the expression level of the citrate related genes. A possible explanation for the present observed down-regulation lies on the evidence that the cells need to convert ethanol in some way, to neutralize its toxicity. This can be achieved by ethanol oxidation to acetate. However, since acetate is also one of the end-products of the citrate lyase reaction, probably some regulatory mechanism occur, to prevent excess of acetate accumulation and hence pH lowering. Furthermore, the stimulation of MLF main pathway also underlines the need of the cells to cope with the stressing ethanol environment: it is worth noting that alcohol leads to the dissipation of the proton gradient across the cytoplasmic membrane [60,64]. Actually, since MLF is coupled with an electrogenic antiport system, the overall result of the presence of ethanol is an increase of energy availability.

Two studies revealed that LAB can hydrolyze glycosylated aroma precursors in the course of MLF [65,66]. Enzymatic hydrolysis of these glycosides can enhance the sensory profile of wine or fruit juices, therefore it has been often of considerable interest in wine research [67]. It has also been observed that pH, temperature, sugars and ethanol can influence this enzyme [65,68]. In this study it was shown that the gene *oeoe 0040* encoding for a glucosidase, was more expressed in et.8%. This gene is involved in sugar metabolism and organism specific pathways for flavor production. These data agree with Li et al. [69] who showed higher glycosidase enzyme activity at low ethanol concentrations. Hence the acclimation procedure in 8% ethanol seems to induce a pathway of interest for wine quality. This was opposed by a second glycosidase enzyme (gene *oeoe 1779)* that was down-regulated by et.12%.

ButA is a gene encoding acetoin reductase, enzyme involved in diacetyl/acetoin biosynthetic pathway. This protein catalyzes the formation of 2,3-butanediol from pyruvate with acetolactate and diacetyl as intermediates. This last compound acts on the sensory properties of wine, since it confers "butter" aroma. The sensory perception of diacetyl depends on many factors such as origin of wine, fermentation temperature, SO_2, duration of MLF [70]. In this study it was shown that *butA* gene was down-regulated in presence of ethanol 12%. Garcia-Quintas et al. [71] demonstrated that in *Lactococcus lactis*, this biosynthetic pathway is induced at the transcriptional level by acidic growth conditions. Olguin et al. [63] observed that in presence of ethanol transcription of the gene *aslD* (acetolactate decarboxylase), also involved in diacetyl production, was significantly reduced; it seems from the present results that also *butA* gene follows the same behavior of *aslD* gene.

For what concerns protein metabolism, shikimate kinase *(oeoe 0153*) was down-regulated. It is well known that the shikimate pathway is essential for the synthesis of all aromatic amino acids, as well as other metabolites, such as folic acid and ubiquinone [72]. This pathway is essential for parasites, bacteria, and fungi [73–75]. Shikimate kinase is the fifth enzyme in the shikimate pathway that catalyzes the phosphate transfer from ATP to shikimate to generate shikimate 3-phosphate and ADP [76]. Therefore in this anabolic route, ATP is consumed. The finding that in

media containing 8% ethanol shikimate kinase gene was less expressed, can be due to the fact that in these conditions bacteria try to limit ATP consumption (and, in general, ATP-consuming anabolic routes) to better cope with alcohol-induced stress, as suggested by Henick-Kling et al. [77]. On the other hand, it is well known that *O. oeni*, as most LAB, displays limited amino acid biosynthetic pathways. Remize et al. [78] found that amino acid requirement was strain-dependent and some strains appeared to be particularly demanding. Since LAB cannot synthesize glutamic acid [79], it was found to be essential for all strains. The results presented here underline that 8% ethanol stimulated the biosynthesis of a glutamate transporter (gene *oeoe 1634*). It has to be considered that glutamate can also be used as source of energy by decarboxylation to γ-amine butyrate (GABA) coupled to extrusion of GABA in the environment by a glutamate-GABA antiport system [80]. This system both maintains pH homeostasis and allows proton motive force generation. Taken together, these results suggest that the cells react to the ethanol stress by both conserving ATP and enhancing potential energy availability.

As far as purine metabolism is concerned no significant differences in the ethanol-stimulated and the control condition were observed. On the contrary aspartate carbamoyl transferase, the first enzyme of the anabolic pathway for pyrimidine biosynthesis is up-regulated (gene *oeoe 0258*). This enzyme is negatively affected, at catalytic level, by both feed-back mechanisms generated by end-products of the pathway and by allosteric systems acting on protein subunits different from those bearing the active sites [81]. This finding suggests that also biosynthetic regulation plays a role besides catalytic mechanisms. Furthermore, this stress-linked up-regulation is in agreement with what observed in *Pseudomonas putida* under saline stress conditions using 2-DE and MALDI TOF MS [82].

With respect to sugar metabolism the transcriptomic and proteomic results seem contradictory. In particular, the phosphogluconate dehydrogenase-encoding gene proved to be up-regulated whereas the corresponding enzyme was shown to be less abundant in ethanol (both in et.8% and et.12%). Furthermore, genes encoding the heterofermentative enzymes, trans- and phospho-

ketolase, as well as alcohol dehydrogenase isoforms were down-regulated. On the other hand a flow direction of glucose towards biosynthesis of EPS and cell-wall sugars is supported as it has already been discussed above.

4 Conclusions

The present combined transcriptomic/proteomic approach was useful to obtain integrated information regarding the physiological response of *O. oeni* adapted in different media prior to inoculation into wine. The results obtained demonstrated that composition of the medium strongly affects starter responses, as expected. Following ethanol acclimation a significant expression increase of genes related to MLF and stress response occurs. The stress response is mainly focused on the control of envelope composition (membrane, cell wall and EPS), but also concerns general stress proteins such as chaperones and proteases. In particular, we found that 8% or 12% ethanol differentially modulate bacterial responses. For instance adaptation to the lower ethanol content induces surface modifications such as EPS layer production, whereas adaptation to the higher ethanol concentrations induce structural changes such as membrane composition modification. Furthermore, the 8% and 12% ethanol conditions can control different stress response pathways by activating refolding or degrading chaperones, respectively. Further investigations, includes analysis of a higher number of *O oeni* strains, are necessary to clarify the role of the rehydration medium on the survival and performance of *O. oeni* during wine malolactic fermentation. Nevertheless, based on the results obtained in this study, we suggest that even with a commercial strain for a direct inoculation in wine, an acclimation step can improve performance of the selected starter through induction of different defence mechanisms and MLF stimulation prior to transfer into wine.

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REFERENCES

[1] Malherbe S, Tredoux AGJ, Nieuwoudt HH, du Toit M. Comparative metabolic profiling to investigate the contribution of *O. oeni* MLF starter cultures to red wine composition. J Ind Microbiol Biotechnol 2012;39:477–94.

[2] Nielsen JC, Prahl C, Lonvaud-Funel A. Malolactic Fermentation in Wine by Direct Inoculation with Freeze-Dried *Leuconostoc oenos* Cultures. Am J Enol Vitic 1996;47:42–8.

[3] Pfeiler EA, Klaenhammer TR. The genomics of lactic acid bacteria. Trends Microbiol 2007;15:546–53..

[4] G-Alegrìa E, Lopez I, Ruiz JI, Sa¡enz J, Fernandez E, Zarazaga M, et al. High tolerance of wild Lactobacillus plantarum and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol Lett 2004;230:53–61.

[5] Silveira MG, Baumgartner M, Rombouts FM, Abee T. Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. Appl Environ Microbiol 2004;70:2748–55. doi:10.1128/AEM.70.5.2748-2755.2004.

[6] Guzzo J, Jobin M-P, Diviès C. Increase of sulfite tolerance in *Oenococcus oeni* by means of acidic adaptation. FEMS Microbiol Lett 1998;160:43–7.

[7] Beltramo C, Desroche N, Tourdot-Maréchal R, Grandvalet C, Guzzo J. Real-time PCR for characterizing the stress response of *Oenococcus oeni* in a wine-like medium. Res Microbiol 2006;157:267–74.

[8] Grandvalet C, Coucheney F, Beltramo C, Guzzo J. CtsR is the master regulator of stress response gene expression in *Oenococcus oen*i. J Bacteriol 2005;187:5614–23. doi:10.1128/JB.187.16.5614-5623.2005.

[9] Torriani S, Felis G, Fracchetti F. Selection criteria and tools for malolactic starters development: an update. Ann Microbiol 2011;61:33–9.

[10] Borneman AR, Bartowsky EJ, McCarthy J, Chambers PJ. Genotypic diversity in *Oenococcus oeni* by high-density microarray comparative genome hybridization and whole genome sequencing. Appl Microbiol Biotechnol 2010;86:681–91.

[11] Unlü M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 1997;18:2071–7.

[12] Koponen J, Laakso K, Koskenniemi K, Kankainen M, Savijoki K, Nyman TA, et al. Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG. J Proteomics 2012;75:1357–74.

[13] Genovese F, Coïsson JD, Majumder A, Pessione A, Svensson B, Jacobsen S, et al. An exoproteome approach to monitor safety of a cheese-isolated *Lactococcus lactis*. Food Res Int 2013;54:1072–9.

[14] Behr M, Serchi T, Cocco E, Guignard C, Sergeant K, Renaut J, et al. Description of the mechanisms underlying geosmin production in *Penicillium expansum* using proteomics. J Proteomics 2014;96:13–28.

[15] Cecconi D, Milli A, Rinalducci S, Zolla L, Zapparoli G. Proteomic analysis of *Oenococcus oeni* freeze-dried culture to assess the importance of cell acclimation to conduct malolactic fermentation in wine. Electrophoresis 2009;30:2988–95.

[16] Mohedano M de la L, Russo P, de Los Ríos V, Capozzi V, Fernández de Palencia P, Spano G, et al. A partial proteome reference map of the wine lactic acid bacterium *Oenococcus oeni* ATCC BAA-1163. Open Biol 2014;4:130154.

[17] Lerm E, Engelbrecht L, Du Toit M. Malolactic fermentation: the ABC's of MLF. S Afr J Enol Vitic 2010;31:186–212.

[18] Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, et al. KEGG for linking genomes to life and the environment. Nucleic Acids Res 2008;36:D480–4.

[19] Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–86.

[20] Borneman AR, McCarthy JM, Chambers PJ, Bartowsky EJ. Comparative analysis of the *Oenococcus oeni* pan genome reveals genetic diversity in industrially-relevant pathways. BMC Genomics 2012;13:373.

[21] Rantsiou K, Greppi A, Garosi M, Acquadro A, Mataragas M, Cocolin L. Strain dependent expression of stress response and virulence genes of *Listeria monocytogenes* in meat juices as determined by microarray. Int J Food Microbiol 2012;152:116–22.

[22] Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. Bioinformatics 2002;18:207–8.

[23] Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, et al. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 2004;25:1327–33.

[24] Majumder A, Sultan A, Jersie-Christensen RR, Ejby M, Schmidt BG, Lahtinen SJ, et al. Proteome reference map of *Lactobacillus acidophilus* NCFM and quantitative proteomics towards understanding the prebiotic action of lactitol. Proteomics 2011;11:3470–81.

[25] Lopez I., Lòpez R., Santamaría P., Torres C. R-LF. Performance of malolactic fermentation by inoculation of selected Lactobacillus plantarum and *Oenococcus oeni* strains isolated from Rioja red wines. Vitis 2008;47:123–9.

[26] Pilatte E. PC. Biological deacidification of acid grape varieties by inoculation on must with a freeze-dried culture of *Lactobacillus plantarum*. 48th Annu. Meet. ASEV, San Diego, USA: 1997.

[27] Carbó R., Soler B., Gordón E., De Castro J. SJ. Adaptación de las bacterias lácticas para la fermentación maloláctica del vino. Riv Vitic Enol 1998;4:39–45.

[28] Nielsen JC, Richelieu M. Control of flavor development in wine during and after malolactic fermentation by *Oenococcus oeni*. Appl Environ Microbiol 1999;65:740–5.

[29] Delaquis P, Cliff M, King M, Girard B, Hall J, Reynolds A. Effect of Two Commercial Malolactic Cultures on the Chemical and Sensory Properties of Chancellor Wines Vinified with Different Yeasts and Fermentation Temperatures. Am J Enol Vitic 2000;51:42–8.

[30] Costantini A, Vaudano E, Rantsiou K, Cocolin L, Garcia-Moruno E. Quantitative expression analysis of mleP gene and two genes involved in the ABC transport system in *Oenococcus oeni* during rehydration. Appl Microbiol Biotechnol 2011;91:1601–9.

[31] Coucheney F, Gal L, Beney L, Lherminier J, Gervais P, Guzzo J. A small HSP, Lo18, interacts with the cell membrane and modulates lipid physical state under heat shock conditions in a lactic acid bacterium. Biochim Biophys Acta 2005;1720:92–8.

[32] Desroche N, Beltramo C, Guzzo J. Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. J Microbiol Methods 2005;60:325–33.

[33] Capozzi V, Russo P, Beneduce L, Weidmann S, Grieco F, Guzzo J, et al. Technological properties of *Oenococcus oen*i strains isolated from typical southern Italian wines. Lett Appl Microbiol 2010;50:327–34.

[34] Olguín N, Bordons A, Reguant C. Multigenic expression analysis as an approach to understanding the behaviour of *Oenococcus oeni* in wine-like conditions. Int J Food Microbiol 2010;144:88–95.

[35] Reott MA, Parker AC, Rocha ER, Smith CJ. Thioredoxins in redox maintenance and survival during oxidative stress of *Bacteroides fragilis*. J Bacteriol 2009;191:3384–91.

[36] Jobin M-P, Garmyn D, Diviès C, Guzzo J. Expression of the *Oenococcus oeni* trxA gene is induced by hydrogen peroxide and heat shock. Microbiology 1999;145:1245–51.

[37] Moritomo R.I., Tissières A. GC. Stress proteins in biology and medicine. New York: Cold Spring Harbor Laboratory Press; 1990.

[38] Guzzo J, Cavin JF, Divies C. Induction of stress proteins in *Leuconostoc oenos* to perform direct inoculation of wine. Biotechnol Lett 1994;16:1189–94.

[39] Guzzo J, Delmas F, Pierre F, Jobin M-P, Samyn B, Van Beeumen J, et al. A small heat shock protein from *Leuconostoc oenos* induced by multiple stresses and during stationary growth phase. Lett Appl Microbiol 1997;24:393–6.

[40] Schumann W., Hecker M. MT. Regulation and function of heat-inducible genes in Bacillus subtilis. In: Sonenshein, A.L., Hoch, J.A, Losick R, editor. *Bacillus subtili*s its Closest Relat. From Genes to Cells, Washington: American Society for Microbiology; 2002, p. 359–68.

[41] Frees D, Gerth U, Ingmer H. Clp chaperones and proteases are central in stress survival, virulence and antibiotic resistance of *Staphylococcus aureus*. Int J Med Microbiol 2014;304:142–9.

[42] Suzuki CK, Rep M, van Dijl JM, Suda K, Grivell LA, Schatz G. ATP-dependent proteases that also chaperone protein biogenesis. Trends Biochem Sci 1997;22:118–23.

[43] Herman C, D'Ari R. Proteolysis and chaperones: the destruction/reconstruction dilemma. Curr Opin Microbiol 1998;1:204–9.

[44] Suokko A, Savijoki K, Malinen E, Palva A, Varmanen P. Characterization of a mobile clpL gene from *Lactobacillus rhamnosus*. Appl Environ Microbiol 2005;71:2061–9.

[45] Ciezack G, Hazo L, Chambat G, Heyraud A, Lonvaud-Funel A, Dols-Lafargue M. Evidence for exopolysaccharide production by *Oenococcus oeni* strains isolated from non-ropy wines. J Appl Microbiol 2010;108:499–509.

[46] Moineau S, Borkaev M, Holler BJ, Walker SA, Kondo JK, Vedamuthu ER, et al. Isolation and characterization of *Lactococcal* bacteriophages from cultured buttermilk plants in the United States. J Dairy Sci 1996;79:2104–11.

[47] Deveau H, Van Calsteren M-R, Moineau S. Effect of exopolysaccharides on phage-host interactions in *Lactococcus lactis*. Appl Environ Microbiol 2002;68:4364–9.

[48] Kumar AS, Mody K, Jha B. Bacterial exopolysaccharides--a perception. J Basic Microbiol 2007;47:103–17.

[49] Dimopoulou M, Hazo L, Dols-Lafargue M. Exploration of phenomena contributing to the diversity of *Oenococcus oeni* exopolysaccharides. Int J Food Microbiol 2012;153:114–22.

[50] Herskovits AA, Bochkareva ES, Bibi E. New prospects in studying the bacterial signal recognition particle pathway. Mol Microbiol 2000;38:927–39.

[51] Cao TB, Saier MH. The general protein secretory pathway: phylogenetic analyses leading to evolutionary conclusions. Biochim Biophys Acta 2003;1609:115–25.

[52] Gutierrez JA, Crowley PJ, Cvitkovitch DG, Brady LJ, Hamilton IR, Hillman JD, et al. *Streptococcus mutans* ffh, a gene encoding a homologue of the 54 kDa subunit of the signal recognition particle, is involved in resistance to acid stress. Microbiology 1999;145:357–66.

[53] Luirink J, Dobberstein B. Mammalian and *Escherichia coli* signal recognition particles. Mol Microbiol 1994;11:9–13.

[54] Tourdot-Maréchal R, Gaboriau D, Beney L, Diviès C. Membrane fluidity of stressed cells of *Oenococcus oeni.* Int J Food Microbiol 2000;55:269–73.

[55] Silveira MG da, Romão MVS, Loureiro-Dias MC, Rombouts FM, Abee T. Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. Appl Environ Microbiol 2002;68:6087–93.

[56] Hasona A, Zuobi-Hasona K, Crowley PJ, Abranches J, Ruelf MA, Bleiweis AS, et al. Membrane composition changes and physiological adaptation by *Streptococcus mutans* signal recognition particle pathway mutants. J Bacteriol 2007;189:1219–30.

[57] Delmas F, Pierre F, Coucheney F, Divies C, Guzzo J. Biochemical and physiological studies of the small heat shock protein Lo18 from the lactic acid bacterium *Oenococcus oeni*. J Mol Microbiol Biotechnol 2001;3:601–10.

[58] Maitre M, Weidmann S, Rieu A, Fenel D, Schoehn G, Ebel C, et al. The oligomer plasticity of the small heat-shock protein Lo18 from *Oenococcus oeni* influences its role in both membrane stabilization and protein protection. Biochem J 2012;444:97–104.

[59] Mazzoli R, Fattori P, Lamberti C, Giuffrida MG, Zapponi M, Giunta C, et al. High isoelectric point sub-proteome analysis of *Acinetobacter radioresistens* S13 reveals envelope stress responses induced by aromatic compounds. Mol Biosyst 2011;7:598–607.

[60] Konings WN. Microbial transport: Adaptations to natural environments. Antonie Van Leeuwenhoek 2006;90:325–42.

[61] Mills DA, Rawsthorne H, Parker C, Tamir D, Makarova K. Genomic analysis of *Oenococcus oeni* PSU-1 and its relevance to winemaking. FEMS Microbiol Rev 2005;29:465–75.

[62] Bekal-Si Ali S, Diviès C, Prévost H. Genetic organization of the citCDEF locus and identification of mae and clyR genes from *Leuconostoc mesenteroides.* J Bacteriol 1999;181:4411–6.

[63] Olguín N, Bordons A, Reguant C. Influence of ethanol and pH on the gene expression of the citrate pathway in *Oenococcus oeni.* Food Microbiol 2009;26:197–203.

[64] Chu-Ky S, Tourdot-Marechal R, Marechal P-A, Guzzo J. Combined cold, acid, ethanol shocks in *Oenococcus oeni*: effects on membrane fluidity and cell viability. Biochim Biophys Acta 2005;1717:118–24.

[65] Grimaldi A, Bartowsky E, Jiranek V. A survey of glycosidase activities of commercial wine strains of *Oenococcus oen*i. Int J Food Microbiol 2005;105:233–44.

[66] Ugliano M, Genovese A, Moio L. Hydrolysis of Wine Aroma Precursors during Malolactic Fermentation with Four Commercial Starter Cultures of *Oenococcus oeni.* J Agric Food Chem 2003;51:5073–8.

[67] Mateo J., Jiménez M. Monoterpenes in grape juice and wines. J Chromatogr A 2000;881:557–67.

[68] Spagna G, Barbagallo RN, Palmeri R, Restuccia C, Giudici P. Properties of endogenous β-glucosidase of a *Saccharomyces cerevisiae* strain isolated from Sicilian musts and wines. Enzyme Microb Technol 2002;31:1030–5.

[69] Li, Y H, Fan, M T, Zhang, G Q, Liu YL. Assessment of B-D-Glucosidase activity from two typical strains of the lactic acid bacteria *Oenococus oeni* in China. S Afr J EnoL Vitic 2012;33:144–9.

[70] Bartowsky EJ, Henschke PA. The "buttery" attribute of wine--diacetyl--desirability, spoilage and beyond. Int J Food Microbiol 2004;96:235–52.

[71] García-Quintáns N, Repizo G, Martín M, Magni C, López P. Activation of the diacetyl/acetoin pathway in *Lactococcus lactis* subsp. lactis bv. diacetylactis CRL264 by acidic growth. Appl Environ Microbiol 2008;74:1988–96.

[72] Parish T, Stoker NG. The common aromatic amino acid biosynthesis pathway is essential in *Mycobacterium tuberculosis*. Microbiology 2002;148:3069–77.

[73] Kishore GM, Shah DM. Amino acid biosynthesis inhibitors as herbicides. Annu Rev Biochem 1988;57:627–63.

[74] Roberts F, Roberts CW, Johnson JJ, Kyle DE, Krell T, Coggins JR, et al. Evidence for the shikimate pathway in apicomplexan parasites. Nature 1998;393:801–5.

[75] Herrmann KM, Weaver LM. The shikimate pathway. Annu Rev Plant Physiol Plant Mol Biol 1999;50:473–503.

[76] Berlyn MB, Giles NH. Organization of enzymes in the polyaromatic synthetic pathway: separability in bacteria. J Bacteriol 1969;99:222–30.

[77] Henick-Kling, T., Cox, D.J. and Olsen E. Prodution de l'énergie durant la fermentation malolactique. Rev Enol 1991;132:63–6.

[78] Remize F, Gaudin A, Kong Y, Guzzo J, Alexandre H, Krieger S, et al. *Oenococcus oeni* preference for peptides: qualitative and quantitative analysis of nitrogen assimilation. Arch Microbiol 2006;185:459–69.

[79] Hebert EM, Raya RR, De Giori GS. Nutritional Requirements and Nitrogen-Dependent Regulation of Proteinase Activity of *Lactobacillus helveticus* CRL 1062. Appl Environ Microbiol 2000;66:5316–21.

[80] Mazzoli R, Pessione E, Dufour M, Laroute V, Giuffrida MG, Giunta C, et al. Glutamate-induced metabolic changes in *Lactococcus lactis* NCDO 2118 during GABA production: combined transcriptomic and proteomic analysis. Amino Acids 2010;39:727–37.

[81] Gerhart J. From feedback inhibition to allostery: the enduring example of aspartate transcarbamoylase. FEBS J 2014;281:612–20.

[82] Rangeshwaran R, Ashwitha K, Sivakumar G, Jalali SK. Analysis of proteins expressed by an abiotic stress tolerant *Pseudomonas putida* (NBAII-RPF9) isolate under saline and high temperature conditions. Curr Microbiol 2013;67:659–67.

Table 1: Up- and down-regulated genes, grouped into functional categories, in media containing 8% (indicated as et.8%) and 12% ethanol (indicated as et.12%) tested with respect to medium MRS (M).

Functional group	gene	Protein encoded	Expression in et.8%	Expression in et.12%
Envelope	oeoe1444	Peptidoglycal interpeptide bridge formation protein	-	↑
	oeoe0144	N-acetylmuramoyl-L-alanine amidase	-	↑
	oeoe0719	D-alanyl D-alanine carboxypeptidase	↑	↑
	oeoe0040	Glucosidase	↑	-
	oeoe1779	Glycosidase	-	↓
	oeoe0071	Exopolysaccharide biosynthesis protein	↑	↑
	oeoe1737	UDP glucose 6- dehydrogenase	↑	-
Cell membrane	oeoe0455	Signal recognition particle	↑	↑
	oeoe0457	Signal recognition particle	↓	-
	oeoe1333	Signal peptidase	↓	-
Stress response	*trxA*	thioredoxin	↑	↓
	oeoe0289	Heat shock protein HSP20	↑	↓
	oeoe0184	Heat shock protein, ribosome associated	↑	↓
	hrcA	Heat inducible transcription repressor HrcA	↑	↓
	clpP	ATPase protein as HSP100	-	↑
	oeoe1191	Glutathione reductase	↑	↑
Sugar metabolism	oeoe1044	lacZ (galactosidase/ glycosyl hydrolase)	-	↑
	oeoe1651	ABC-type sugar transport system, ATPase	↑	↑
	oeoe0249	Phosphomannose isomerase	↑	↑
	oeoe0367	*pgm* (Phosphoglycerate mutase)	-	↑
	oeoe1650	Enolase	↓	↓
	oeoe0243	Aryl alcohol dehydrogenase	↑	↑
	oeoe0527	Alcohol dehydrogenase	↓	↓
	butA	Acetoin dehydrogenase/reductase	-	↓
	oeoe0135	Glucose -6 phosphate 1-dehydrogenase	↑	↑
	oeoe0892	6-Phosphogluconate dehydrogenase	↑	↑
	oeoe1480	Transketolase	↓	↓
	oeoe1183	Putative phosphoketolase	↓	↓
Nitrogen metabolism	oeoe1634	glnQ polar amino acid transport	↑	-
	oeoe0952	Glutamine synthase	↑	↓
	oeoe0630-oeoe0631	ABC-type spermidine/putrescine transport system	↓	↓
	oeoe0632-oeoe0634	Spermidine-putrescine ABC transporter permease protein	↓	↓
	oeoe0153	Shikimate kinase	↓	-
	oeoe1058	lysylaminopeptidase	↑	-
	oeoe1857	Aromatic compounds catabolism protein	↑	↑
	purK	Purine biosynthesis phosphoribosylaminoimidazole carboxylase	-	-
	oeoe0258	Aspartate carbamoyltransferase	-	↑
	oeoe0766	Cystathione β-lyase	↑	↓
MLF/citrate metabolism	oeoe1565	MLF system transcription activator	-	↑
	mleP	Malate permease	↑	↑
	mleR	Malolactic regulator	↑	↑
	mleA	Malolactic enzyme gene	↑	↑
	oeoe0419	Putative citrate transporter	↓	↓
	oeoe0423	Citrate lyase, α-subunit	↓	-
Secondary metabolism	oeoe0242	Short chain alcohol dehydrogenase	↓	↓

Functional group	gene	Protein encoded	Expression in et.8%	Expression in et.12%
Energy metabolism	oeoe1435	Phosphotransacetylase	-	↑
	oeoe1629	Polyphosphate kinase	↓	↓
	oeoe0659	ATP synthase, subunit α	↑	↑
Metals	oeoe0623	Cobalt transport	↓	↓
	oeoe1054	Metal ion transport	↑	-
Transcription/ Translation/ Protein synthesis/regulation	oeoe0005	DNA gyrase β subunit	-	-
	oeoe0673	DNA polymerase I	↑	-
	rpoB	RNA polymerase β subunit	↑	-
	oeoe0976	Elongation factor	-	-
	oeoe0105	*vicR* DNA-binding response regulator	↑	↑

Table 2 Differentially abundant proteins obtained comparing cells adapted in MRS (M) towards cells adapted in 8% (et.8%) (A) and 12% (et.12%) (B) ethanol, and the identification by MS/MS analysis.

A)

spot n.	ANOVA p value	Fold Change	Average normalised volumes		Mw	pI	Mascot Score	Protein ID	NCBI Ac No
546	<0.001	1.6	MRS: 1.223±0.08	et.8%: 0.788±0.09	55434	5.81	113	citrate lyase, alpha subunit *[O. oeni PSU-1]*	gi|116490503
993	<0.001	1.7	MRS: 1.325±0.21	et.8%: 0.797±0.07	33293	4.8	670	citrate lyase beta subunit *[O. oeni PSU-1]*	gi|116490502
946	<0.001	3.4	MRS: 2.208±0.94	et.8%: 0.657±0.2	15459	5.3	154	metylmalonil coA epimerase *[O. oeni PSU-1]*	gi|116491672
629	<0.001	1.8	MRS: 1.444±0.35	et.8%: 0.793±0.15	40943	5.1	262	malate oxidoreductase *[O. oeni PSU-1]*	gi|116490498
736	<0.001	4.7	MRS: 2.173±1	et.8%: 0.459±0.23	32898	4.83	60	6-phosphogluconate dehydrogenase-like protein *[O. oeni PSU-1]*	gi|116491501
763	0.001	1.5	MRS: 1.289±0.24	et.8%: 0.873±0.1	28154	5.65	170	Short-chain alcohol dehydrogenase *[O. oeni PSU-1]*	gi|116490347
985	0.009	1.5	et.8%: 1.390±0.29	MRS: 0.935±0.19	14000	4.7		hypotetical protein	
995	0.013	1.7	et.8%: 1.105±0.3	MRS: 0.634±0.14	16927	5.1	80	heat shock protein Hsp20 *[O.oeni PSU-1]*	gi|116490389
1043	0.007	1.7	MRS: 1.363±0.24	et.8%: 0.809±0.29	57253	4.84	337	chaperonin GROEL *[O.oeni PSU-1]*	gi|116491379

Table 2 Continued

B)

spot n.	ANOVA p value	Fold Change	Average normalised volumes		Mw	pI	Mascot Score	Protein ID	NCBI Ac No
905	0.003	1.7	et.12%: 1.171±0.5	MRS: 0.672±0.3	16256	9	111	50S ribosomal protein L13 *[O. oeni PSU-1]*	gi|116490691
994	<0.001	4	et.12%: 1.843±0.6	MRS: 0.465±0.32	16927	5.1	75	heat shock protein Hsp20 *[O. oeni PSU-1]*	gi|116490389
428	<0.001	1.7	et.12%: 1.276±0.25	MRS: 0.740±0.13	79275	5.4	112	clpL2 protein *[O. oeni]*	gi|71466871
432	<0.001	1.8	et12%: 1.314±0.35	MRS: 0.737±0.09	81243	5.37	204	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones *[O. oeni PSU-1]*	gi|116490703

Figure legend

Figure 1. Heatmap of changes in gene expression of *O. oeni* after 24 hour of adaptation in ethanol 8% and ethanol 12%. Data presented were obtained by normalization using the strain adapted in MRS (M) as reference condition: green color represents down-regulated genes in comparison with normalizing condition, red color indicates up-regulated genes.

Figure 2. Differentially expressed genes in the media used in this study ($p < 0.05$) after microarray analysis, showing the mRNA values (M indicates cell adapted in MRS; et.8% indicated cells adapted in a medium containing ethanol 8%; et.12% indicated cells adapted in a medium containing ethanol 12%).

Figure 3. a) Representative 2D DIGE image of whole-cell extract soluble proteins of *Oenococcus oeni* **b)** 2DE map. Numbers indicate the spots differentially abundant comparing *O. oeni* adapted in MRS (M) with cells adapted in medium with 8% (et.8%) and 12% ethanol (et.12%); Asterisks indicate the spots differentially abundant protein in medium with 12% ethanol (et.12%) compared with MRS medium (M).

Figure 4. Differentially abundant protein in medium with 12% ethanol (et.12%) (right side) compared with MRS medium (M) (left side). Spot 428: clpL2 protein; spot 432: hsp20; spot 994: ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones.

Supplementary Figure 1. 2D-DIGE proteome maps. The extracted proteins from *O. oeni* after adaptation in different media (M, MRS; et. 8%, ethanol 8%; et. 12%, ethanol 12%) were labelled with fluorescent dyes as shown in Suppl. Table 1 and separated by 2D.DIGE. Panel 1, M + et. 8%; panel 2, M + et. 12%; panel 3, et. 8% + et. 12%; panel 4, et. 12% + M; panel 5, et. 12% + et. 8%; panel 6, et. 8% + et. 12%.

Figure 1

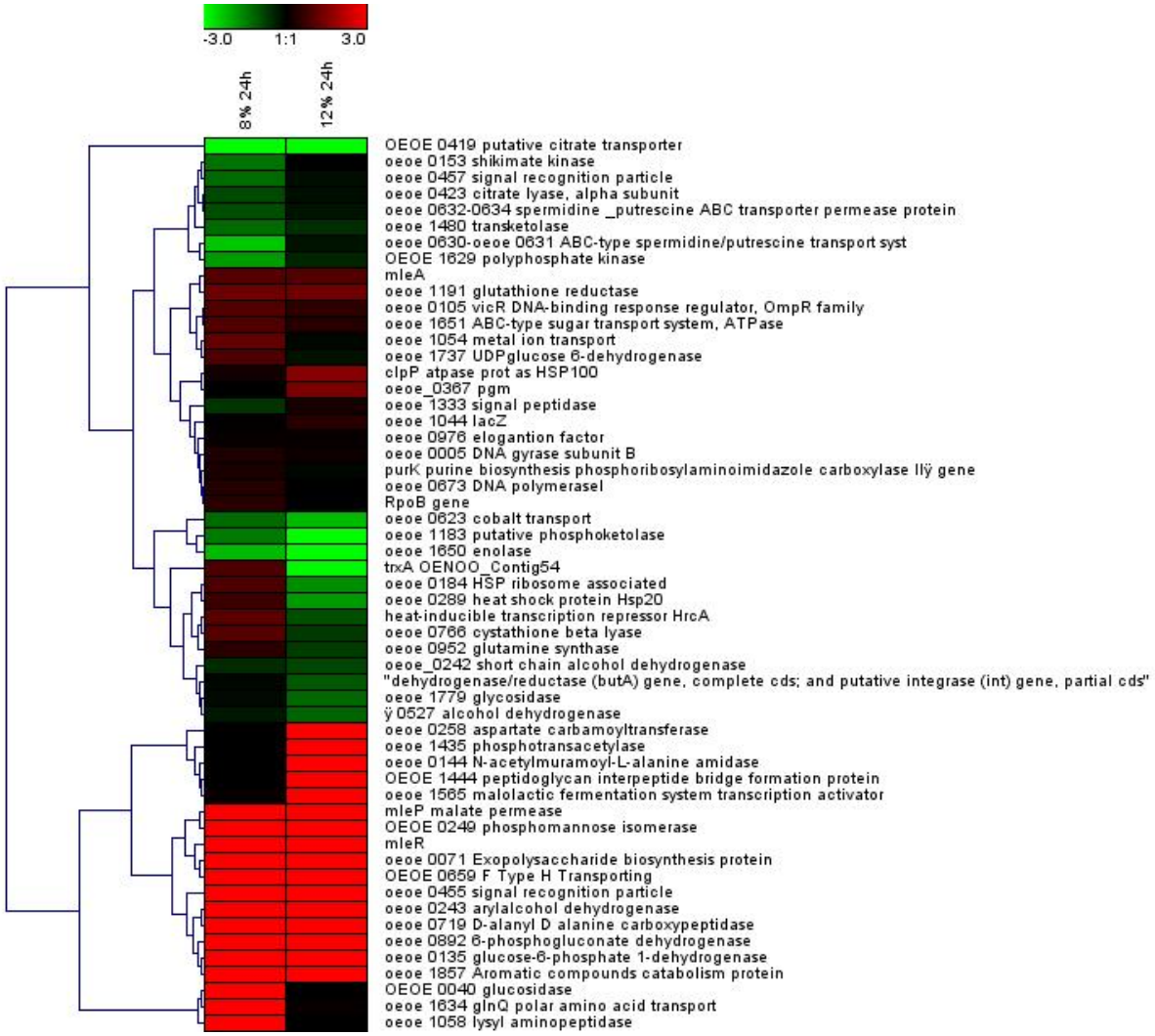
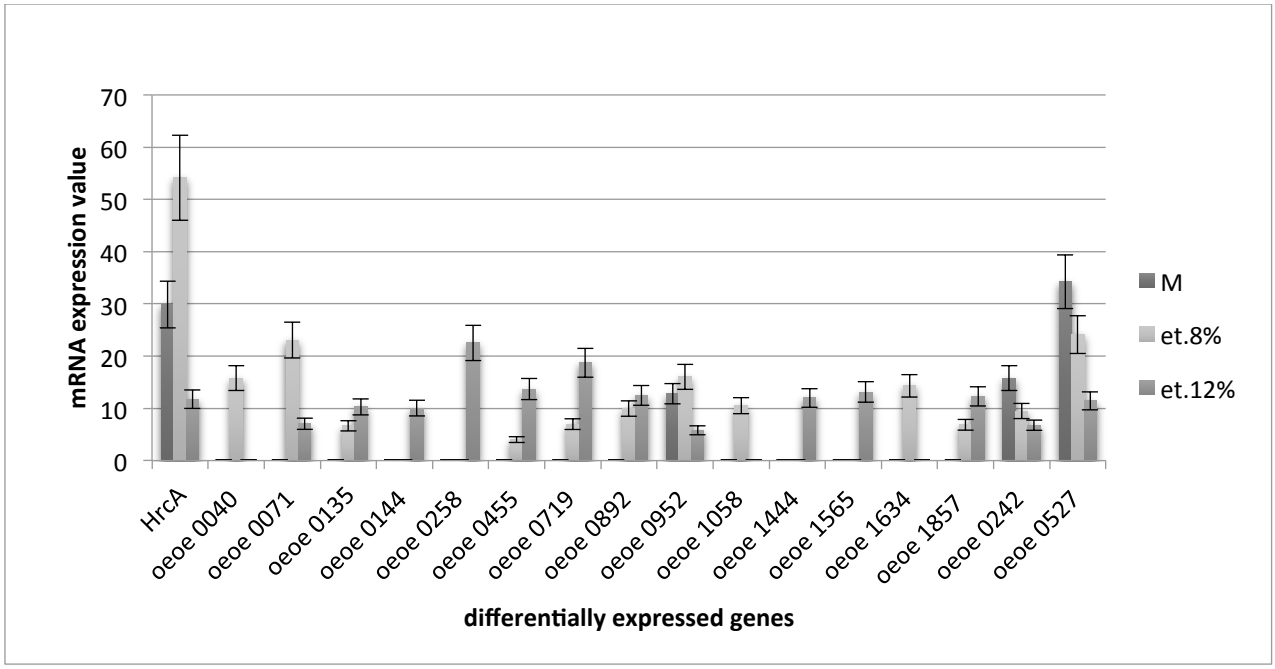

-3.0
1:1
3.0
8% 24h
12% 24h
OEOE 0419 putative citrate transporter
oeoe 0153 shikimate kinase
oeoe 0457 signal recognition particle
oeoe 0423 citrate lyase, alpha subunit
oeoe 0632-0634 spermidine _putrescine ABC transporter permease protein
oeoe 1480 transketolase
oeoe 0630-oeoe 0631 ABC-type spermidine/putrescine transport syst
OEOE 1629 polyphosphate kinase
mleA
oeoe 1191 glutathione reductase
oeoe 0105 vicR DNA-binding response regulator, OmpR family
oeoe 1651 ABC-type sugar transport system, ATPase
oeoe 1054 metal ion transport
oeoe 1737 UDPglucose 6-dehydrogenase
clpP atpase prot as HSP100
oeoe_0367 pgm
oeoe 1333 signal peptidase
oeoe 1044 lacZ
oeoe 0976 elogantion factor
oeoe 0005 DNA gyrase subunit B
purK purine biosynthesis phosphoribosylaminoimidazole carboxylase IIÿ gene
oeoe 0673 DNA polymerasel
RpoB gene
oeoe 0623 cobalt transport
oeoe 1183 putative phosphoketolase
oeoe 1650 enolase
trxA OENOO_Contig54
oeoe 0184 HSP ribosome associated
oeoe 0289 heat shock protein Hsp20
heat-inducible transcription repressor HrcA
oeoe 0766 cystathione beta lyase
oeoe 0952 glutamine synthase
oeoe_0242 short chain alcohol dehydrogenase
"dehydrogenase/reductase (butA) gene, complete cds; and putative integrase (int) gene, partial cds"
oeoe 1779 glycosidase
ÿ 0527 alcohol dehydrogenase
oeoe 0258 aspartate carbamoyltransferase
oeoe 1435 phosphotransacetylase
oeoe 0144 N-acetylmuramoyl-L-alanine amidase
OEOE 1444 peptidoglycan interpeptide bridge formation protein
oeoe 1565 malolactic fermentation system transcription activator
mleP malate permease
OEOE 0249 phosphomannose isomerase
mleR
oeoe 0071 Exopolysaccharide biosynthesis protein
OEOE 0659 F Type H Transporting
oeoe 0455 signal recognition particle
oeoe 0243 arylalcohol dehydrogenase
oeoe 0719 D-alanyl D alanine carboxypeptidase
oeoe 0892 6-phosphogluconate dehydrogenase
oeoe 0135 glucose-6-phosphate 1-dehydrogenase
oeoe 1857 Aromatic compounds catabolism protein
OEOE 0040 glucosidase
oeoe 1634 glnQ polar amino acid transport
oeoe 1058 lysyl aminopeptidase

Figure 2


70
60
50
40
30
20
10
0
mRNA expression value
M
et.8%
et.12%
HrcA
oeoe 0040
oeoe 0071
oeoe 0135
oeoe 0144
oeoe 0258
oeoe 0455
oeoe 0719
oeoe 0892
oeoe 0952
oeoe 1058
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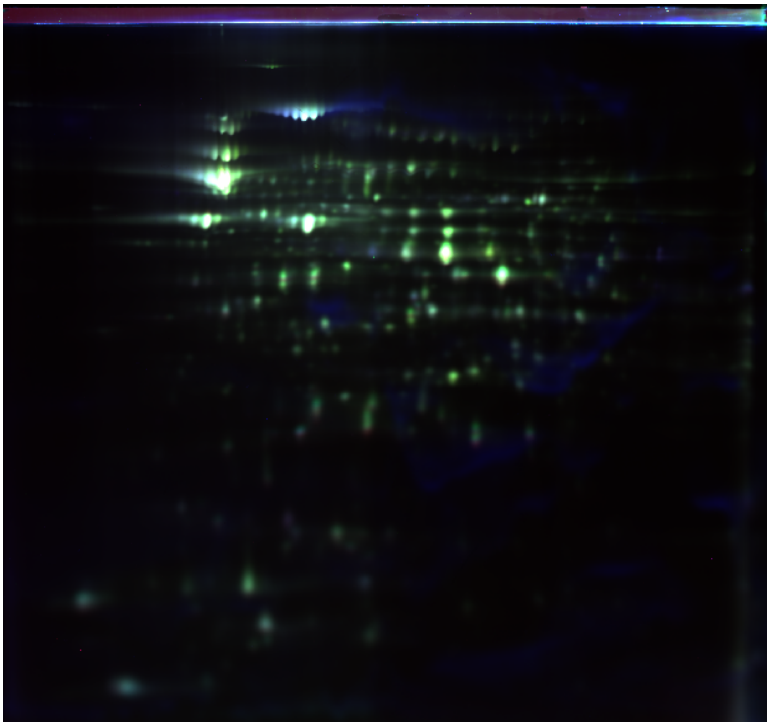
oeoe 1565
oeoe 1634
oeoe 1857
oeoe 0242
oeoe 0527
differentially expressed genes

Figure 3

a)



b)

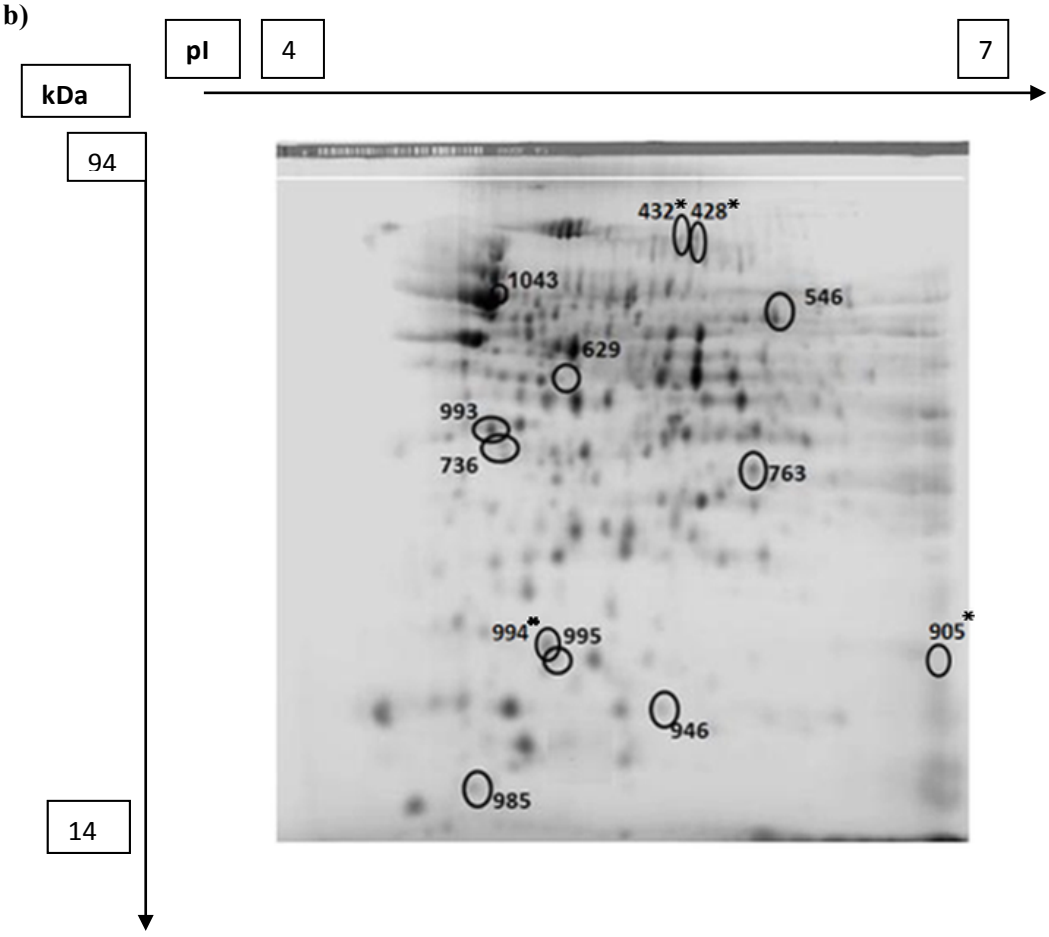
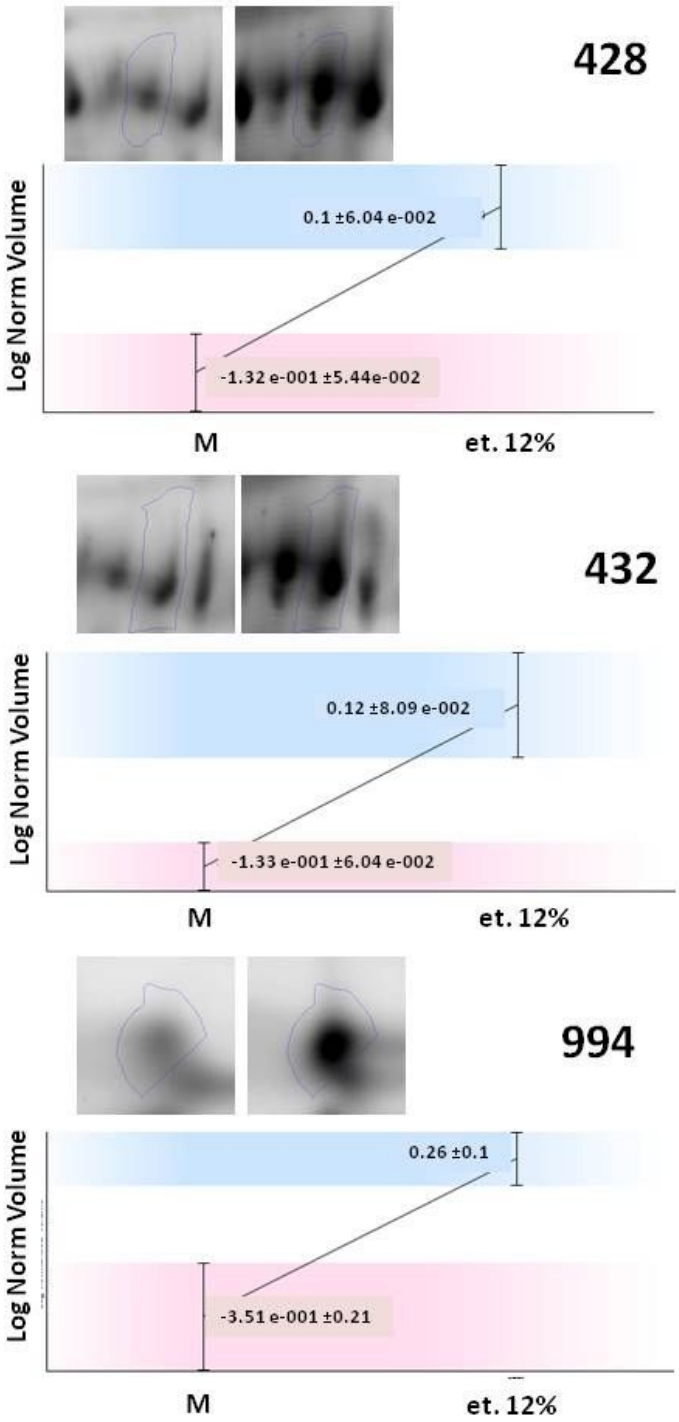
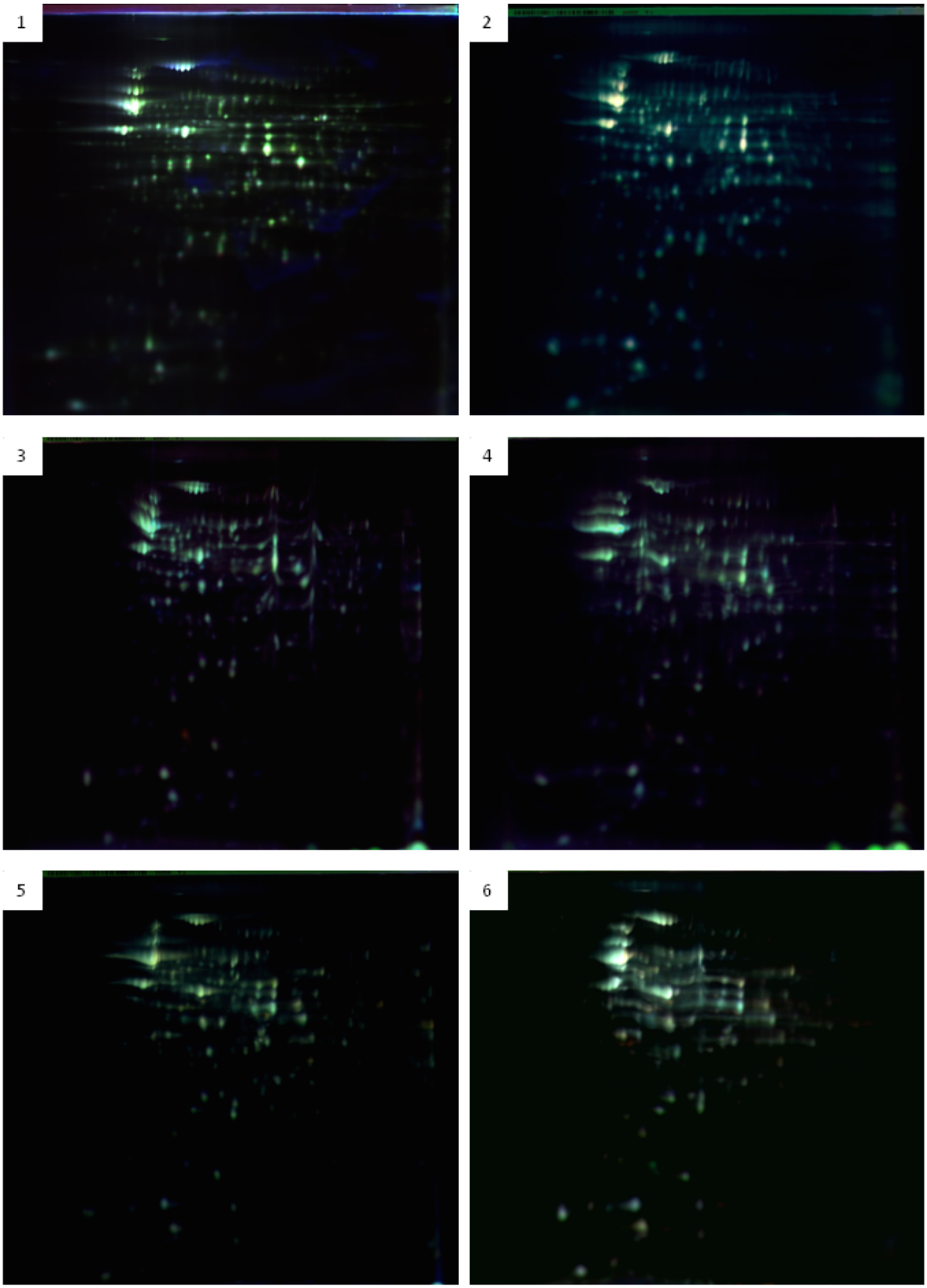

pI
4
7
kDa
94
14
432*
428*
1043
546
629
993
736
763
994*
995
905*
946
985

Figure 4


428
Log Norm Volume
0.1 ±6.04 e-002
-1.32 e-001 ±5.44e-002
M
et. 12%
432
Log Norm Volume
0.12 ±8.09 e-002
-1.33 e-001 ±6.04 e-002
M
et. 12%
994
Log Norm Volume
0.26 ±0.1
-3.51 e-001 ±0.21
M
et. 12%

Supplementary Figure 1



Supplementary Table 1. DiGE experimental design: gels and corresponding labeled samples. M indicates medium MRS; et.8% indicates wine like medium containing 8% ethanol and et.12% indicates wine like medium containing 12% ethanol. A,B,C,D corresponds to the biological replicates

gel	Cy2	Cy3	Cy5
1	Int std	M A	et.8% A
2	Int std	M B	et.12% A
3	Int std	et.8% B	M C
4	Int std	et.12% B	M D
5	Int std	et.12% C	et.8% C
6	Int std	et.8% D	et.12% B

Supplementary Table 2. Identification of the differentially abundant protein by MS/MS analysis

Spot	ID protein	NCBI entry	Mw/pI	Theor Mw/pI	Mascot Score	Match	Coverage	Start	End	Mr	Peptides	Miss cleavage	COG function category
546	citrate lyase, alpha subunit *[Oenococcus oeni PSU-1]*	gi|116490503	55434/5.8	55468/5.75	113	13	34%	492	499	963.5345	K.TVALIEYR.D	1	carbohydrate metabolism
								476	484	967.5901	K.AQQIVGKPK.A	0	
								407	416	1081.7353	K.LTIISAPLVR.G	0	
								33	47	1610.9705	K.QRVAPIVTVTNGDNK.V	0	
								256	271	1892.0813	K.IAQMVNEVITHSPYFK.D + Oxidation (M)	1	
								344	359	1906.9910	R.KQQEIDASWYADPDNK.A	0	
								203	221	2053.2058	R.VVLLTDNLVPYPNTPASIK.Q	0	
								93	111	2079.0817	K.DLTLAPSSLTNVMNDMVVK.A + 2 Oxidation (M)	0	
								74	92	2118.0823	R.EGDQIFNDVMQAIIDLGIK.D	1	
								74	92	2134.0930	R.EGDQIFNDVMQAIIDLGIK.D + Oxidation (M)	0	
								360	379	2159.1590	K.AAMVDQLDVVILSALEIDTK.F + Oxidation (M)	0	
								202	221	2209.1712	R.RVVLLTDNLVPYPNTPASIK.Q	0	
								302	324	2371.1981	K.ASFALGGITKPTVDLLEEGLVNK.I	0	
993	citrate lyase beta subunit *[Oenococcus oeni PSU-1]*	gi|116490502	33293/4.8	33314/4.6	670	18	65%	182	189	910.4754	R.NMVLHAAR.A	0	carbohydrate metabolism
								182	189	926.4931	R.NMVLHAAR.A + Oxidation (M)	1	
								100	107	953.3966	K.TESAEMMR.Q	1	
								90	99	1066.6654	K.AGVDVIRLPK.T	0	
								72	81	1136.6246	R.VNGLDTPFFK.N	0	
								171	181	1266.6336	R.YPDGAELEFAR.N	1	
								138	151	1382.7688	K.GVLNAPEIAAASDR.M	1	
								11	24	1565.8040	R.TMMFVPGNNPAMLK.D + Oxidation (M)	0	
								291	304	1615.8460	K.ASHLIDQEGNYIEK.-	0	
								168	181	1660.8914	K.THRYPDGAELEFAR.N	0	
								152	167	1757.9066	R.MIGIALSAEDYTTDMK.T	0	
								120	137	1937.9570	K.FSIEVGTTHMMAAIESAK.G + Oxidation (M)	1	
								263	281	1996.0754	K.GSGVISLNGQMVDRPVVLR.A	0	
								263	281	2012.1557	K.GSGVISLNGQMVDRPVVLR.A + Oxidation (M)	0	
								72	89	2057.0813	R.VNGLDTPFFKNDVYAMVK.A	0	
								52	71	2236.2219	R.ILVFNALTTQDYGDAELVVR.V	0	
								25	51	2858.1714	K.DAGIYGADSIMFDLEDAVSLSEKDAAR.I	0	
								25	51	2874.1942	K.DAGIYGADSIMFDLEDAVSLSEKDAAR.I + Oxidation (M)	0	
946	methylmalonyl-CoA epimerase *[Oenococcus oeni PSU-1]*	gi|116491672	15459/5.3	16219/5.5	154	5	53%	43	53	1196.6816	K.SLGFEEAGLFK.N	0	amino acid transport and metabolism
								81	96	1696.0014	K.AGAINHISLNTTDIEK.A	0	
								108	122	1817.9609	R.LVNDEIQSIPSFWDR.G	0	
								64	80	1907.0284	R.FGNLTIETWEGDPAVMK.A	0	
								19	36	1958.0719	K.NFFTGIQHVGIPSADLDK.T	0	
629	malate oxidoreductase *[Oenococcus oeni PSU-1]*	gi|116490498	40943/5.1	40910/5.10	262	4	19%	191	205	1381.8447	R.IVINGIGAAGVATAR.L	0	energy production and conversion
								2	17	1779.9947	M.ALNNTELDEILAIHSK.N	0	
								126	143	1972.0161	K.NFSNTFAGIHLEDIAAPR.C	0	
								101	125	2693.2853	K.DLANVNAIPVAIDQVPVDEFVQTVK.N	0	
736	6-phosphogluconate dehydrogenase like protein	gi|116491501	32898/4.83	32861/4.8	60	6	23%	240	247	847.2932	R.MHSSGEGK.W + Oxidation (M)	1	lipid transport and metabolism
								208	216	1080.5805	K.LWNHGSVIR.G	1	

	[Oenococcus oeni PSU-1]							248	257	1279.5890	K.WTLEEAMNMR.I	0	
								248	257	1295.5906	K.WTLEEAMNMR.I + Oxidation (M)	0	
								18	38	2365.1143	K.NMIDNKIEVVGFDLNSDSIDK.A	0	
								39	61	2420.1602	K.AASYGAQTAVDTQDLVDKLDSPR.I	0	
763	Short-chain alcohol dehydrogenase	gi|116490347	28154/5.65	27510/5.6	170	7	33%	184	193	1154.6571	R.INTIHPGYIK.T	0	secondary metabolites biosynthesis, transport and catabolism
	[Oenococcus oeni PSU-1]							73	83	1208.6512	K.LFADVIAEFGK.V	1	
								194	205	1379.6534	K.TPLVEDMDGFEK.A	0	
								59	71	1516.6944	K.FVVHDASNEEGWK.K	0	
								59	72	1644.7851	K.FVVHDASNEEGWKK.L	0	
								170	183	1657.7590	K.SAAIYCAQNDYNLR.I	0	
								84	108	2737.2918	K.VDILVNNAGIGVPGDVEHTDYAQWR.Q	0	
995	heat shock protein Hsp20	gi|116490389	16928/5.1	16937/5.1	80	11	62%	2	8	847,3212	M.ANELMDR.N	0	stress response
	[Oenococcus oeni PSU-1]							9	30	2474,0705	R.NDGLMDVSDMMGNLMNNFFGPR.D	0	
								9	30	2490,0607	R.NDGLMDVSDMMGNLMNNFFGPR.D + Oxidation (M)	0	
								9	30	2506,0475	R.NDGLMDVSDMMGNLMNNFFGPR.D + 2 Oxidation (M)	0	
								9	30	2522,0636	R.NDGLMDVSDMMGNLMNNFFGPR.D + 3 Oxidation (M)	0	
								31	38	932,4174	R.DGLWESAR.H	0	
								39	45	870,4021	R.HNNSIMR.T	0	
								46	58	1510,7651	R.TDISENDKEYGLK.I	1	
								71	87	1810,9768	K.IDYSNDNLTVSGVLSSK.A	0	
								103	109	914,441	R.RYGNYSR.S	1	
								137	148	1422,6992	K.SDESQTHHIEIQ.-	0	
1043	chaperonin GroEL	gi|116491379	57253/4.84	57287/4.85	337	11	24%	370	378	840.5543	K.LAGGVAVVR.V	0	Cellular processes and signaling
	[Oenococcus oeni PSU-1]							105	116	1167.5994	K.NVTAGANPVGIR.S	0	
								392	402	1320.6419	K.YRIEDALNATR.A	0	
								427	440	1358.6389	K.VADSLQGDVATGAR.I	0	
								426	440	1486.7281	K.KVADSLQGDVATGAR.I	1	
								349	362	1669.6729	R.QEIDSTTSDFDREK.L	0	
								452	467	1682.8089	R.QIVENAGLEGSVIAQR.A	1	
								180	195	1836.7556	K.GIETELDVVEGMQFDR.G	0	
								180	195	1852.8252	K.GIETELDVVEGMQFDR.G + Oxidation (M)	1	
								225	243	2077.0149	K.IGNIQDILPVLQSVVEQGR.S	0	
								403	425	2274.1097	R.AAVEEGFVAGGGTAFVNIIPEVK.K	0	
432	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones	gi|116490703	81243/5.37	81293/5.37	204	19	35%	101	107	855.5295	R.VIEILNR.R	0	Cellular processes and signaling; chaperones
	[Oenococcus oeni PSU-1]							388	396	969.5129	K.TKIPVGDLK.K	1	
								101	108	1011.5965	R.VIEILNRR.T	0	
								299	310	1286.6781	K.AIDLIDEAGSRK.N	1	
								412	423	1321.6595	R.AHVIGQNEAVER.V	1	
								595	604	1326.6409	K.NYFKPEFLNR.F	1	
								476	489	1690.8399	R.FDMSEYMEPQSISK.L	1	
								216	230	1724.9184	R.GEFQLIGATTLKEYR.N	1	
								677	691	1771.8129	K.VADFYLDNPNAHQLR.A	1	
								438	455	1775.0068	K.SGRPIGSFLFVGPTGVGK.T	0	
								69	84	1801.9452	K.NGLLEQFGINITEQAR.K	0	
								461	475	1802.8916	K.QLSKEMFGSEESMIR.F + 2 Oxidation (M)	1	
								69	85	1929.9651	K.NGLLEQFGINITEQARK.G	0	
								242	260	2261.1096	R.FQEVMVEEPSRIEAVEILK.G + Oxidation (M)	0	
								677	696	2299.2052	K.VADFYLDNPNAHQLRATLNK.D	0	
								490	511	2320.1443	K.LIGAPAGYVGYEEAGQLTEQVR.R	0	

								266	288	2762.3611	K.YEDFHHVKYPDDVLETAVDLSDR.Y	1	
								668	691	2840.4538	R.VVQEQIEDKVADFYLDNPNAHQLR.A	0	
								555	588	3371.7381	K.DTIVIATSNAGSGDVGNNPVGFAAETAEDSAEHR.L	1	
428	clpL2 protein *[Oenococcus oeni PSU-1]*	gi|71466871	79275/5.4	79323/5.4	112	15	30%	86	92	855.5295	R.VIEILNR.R	0	Cellular processes and signaling; chaperones
								373	381	969.5129	K.TKIPVGDLK.K	0	
								86	93	1011.5965	R.VIEILNRR.T	1	
								284	295	1286.6781	K.AIDLIDEAGSRK.N	0	
								397	408	1321.6595	R.AHVIGQNEAVER.V	1	
								580	589	1326.6409	K.NYFKPEFLNR.F	1	
								461	474	1690.8399	R.FDMSEYMEPQSISK.L	1	
								201	215	1724.9184	R.GEFQLIGATTLKEYR.N	1	
								423	440	1775.0068	K.SGRPIGSFLFVGPTGVGK.T	1	
								54	69	1801.9452	K.NGLLEQFGINITEQAR.K	0	
								54	70	1929.9651	K.NGLLEQFGINITEQARK.G	0	
								475	496	2320.1443	K.LIGAPAGYVGYEEAGQLTEQVR.R	0	
								2	21	2379.0603	M.ADYNDDPFVFGNFNVDDIFR.Q	0	
								251	273	2762.3611	K.YEDFHHVKYPDDVLETAVDLSDR.Y	0	
								540	573	3371.7381	K.DTIVIATSNAGSGDVGNNPVGFAAETAEDSAEHR.L	0	
994	heat shock protein Hsp20 *[Oenococcus oeni PSU-1]*	gi|116490389	16927/5.1	16937/5.1	75	8	70%	9	30	2506,0569	R.NDGLMDVSDMMGNLMNNFFGPR.D + 2 Oxidation (M)	0	stress response
								31	38	932,431	R.DGLWESAR.H	0	
								39	45	870,4365	R.HNNSIMR.T	0	
								46	58	1510,7319	R.TDISENDKEYGLK.I	1	
								68	87	2167,1478	K.DIKIDYSNDNLTVSGVLSSK.A	1	
								103	109	914,4486	R.RYGNYSR.S	1	
								110	124	1668,923	R.SYYVPGVDENKISAK.Y	1	
								137	148	1422,659	K.SDESQTHHIEIQ.-	0	
905	50S ribosomal protein L13 *[Oenococcus oeni PSU-1]*	gi|116490691	16256/9	16265/9.9	111	5	48%	3	14	1427,7538	R.STFLAKPHEIQR.N	0	Information processing ; Translation, ribosomal structure and biogenesis
								15	28	1617,7982			
											R.NWYIVDATDVPLGR.L	0	
								29	38	1043,5875	R.LSSVVATVLR.G	0	
								74	86	1478,7143	K.TYYHHSGYPGGLK.A	0	
								123	145	2494,3669	K.LHVYASGEEVGQSAQKPQVLNIK.D	0	