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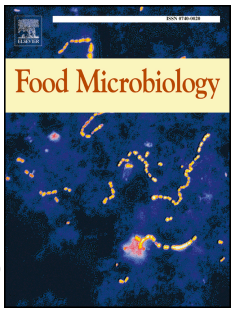

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Transcriptomic and proteomic analysis of *Oenococcus oeni* PSU-1 response to ethanol shock

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Abstract

The correct development of malolactic fermentation depends on the capacity of *Oenococcus oeni* to survive under harsh wine conditions. The presence of ethanol is one of the most stressful factors affecting *O. oeni* performance. In this study, the effect of ethanol addition (12% vol/vol) on *O. oeni* PSU-1 has been evaluated using a transcriptomic and proteomic approach. Transcriptomic analysis revealed that the main functional categories of the genes affected by ethanol were metabolite transport and cell wall and membrane biogenesis. It was also observed that some genes were over-expressed in response to ethanol stress (for example, the heat shock protein Hsp20 and a dipeptidase). Proteomic analysis showed that several proteins are affected by the presence of ethanol. Functions related to protein synthesis and stability are the main target of ethanol damage. In some cases the decrease in protein concentration could be due to the relocation of cytosolic proteins in the membrane, as a protective mechanism. The *omic* approach used to study the response of *O. oeni* to ethanol highlights the importance of the cell membrane in the global stress response and opens the door to future studies on this issue.

Keywords

Oenococcus oeni - Malolactic fermentation -Transcriptomic - Microarray analysis - Proteomic – Ethanol

1. Introduction

Oenococcus oeni is the most important of the lactic acid bacteria involved in malolactic fermentation (MLF) in wine. However, bacterial growth and MLF are not always successful due to the harsh environmental conditions of wine (Davis et al., 1985;

Malherbe et al., 2007). Several studies have been made of how *O. oeni* responds under stress conditions such as pH, temperature, sulfite concentration and ethanol content (Versari et al., 1999). However, ethanol seems to be one of the parameters that most limits *O. oeni* survival in wine. Therefore, if control over MLF in the wine industry is to be improved, it is essential to understand the mechanisms involved in ethanol stress and tolerance in *O. oeni*.

The toxicity of ethanol is generally attributed to its interaction with membranes at the aqueous interface, resulting in perturbed membrane structure and function (Weber and Bont, 1996; Beney and Gervais, 2001). Studies on *O. oeni* have shown that exposing cells to ethanol increases the permeability of the cytoplasmic membrane and enhances passive proton influx and the concomitant loss of intracellular material (Da Silveira et al., 2003). The permeability of the membrane to protons dissipates the proton motive force and affects ATP synthesis, which is no longer available for growth (Capucho and San Romão, 1994; Salema et al., 1996; Weber and Bont, 1996). This may explain the high mortality when *O. oeni* cells were directly inoculated into a wine-like medium supplemented with 12-16% ethanol (Da Silveira et al., 2003; Chu-Ky et al., 2005).

Nonetheless, in concentrations up to 12%, ethanol has no significant effect on malolactic activity, but, according to Capucho and San Romão (1994), it does strongly inhibit cell growth. These authors suggest that the mechanisms regulating cell growth are more sensitive to ethanol than the malolactic enzyme itself. On the other hand, we have also found that a number of *O. oeni* citrate pathway genes are over-expressed in the presence of ethanol, suggesting that the citrate metabolism takes part in the response to this stress (Olguín et al., 2009).

Two-dimensional gel electrophoresis (2-DE) has provided invaluable information on the adaptive response of microorganisms to changes in external conditions (Champomier-Vergès et al., 2002). For instance, Silveira and co-workers (2004) found that ethanol triggers alterations in the protein patterns of *O. oeni* cells that are directly stressed with 12% ethanol for 1 hour and cells pre-adapted in 8% ethanol. It has also been shown that cell cultures acclimated with 10% ethanol survived better in wine, probably due to the differential expression of certain proteins (Cecconi et al., 2009). Functional analysis of gene expression using comparative transcriptomics is also providing insight into stress responses and regulation mechanisms in lactic acid bacteria (LAB). Preliminary microarray analysis of the *Lactobacillus plantarum* response to several stress conditions revealed unanticipated stress response profiles that correlate specifically with lactate- and pH-induced stress (Siezen et al., 2004; Pieterse et al., 2005). However, no current studies use microarray analysis of *O. oeni*.

The aim of the present study was to evaluate the cell response of *O. oeni* PSU-1 after 12% ethanol shock using transcriptomic and proteomic approaches. In order to study the effect of ethanol alone on *O. oeni* cells, the assays were performed in rich medium (MRS) at pH 5.0 (De Man et al., 1960) with the addition of ethanol.

2. Materials and Methods

2.1. Growth conditions

O. oeni PSU-1 was cultured at 30ºC in a two-liter flask containing MRS broth medium supplemented with L-malic acid (4 g l^{-1}) and fructose (5 g l^{-1}) at pH 5.0. When cultures reached the late exponential phase ($OD_{600nm} \approx 1$) they were divided and put into two sterile flasks. Immediately, 12% (v/v) of ethanol was added to one flask and 12% (v/v) of water was added to the other (control). The latter was used as a control assay to

evaluate the possible effect of culture dilution on the proteome. At this moment, the quantities of L-malic acid and fructose remaining in the medium were 0.03 g l^{-1} and 0.28 g l^{-1} respectively. The pH of the medium was 4.35. Both flasks were incubated at 28ºC. All assays were performed in triplicate using independent cultures and the growth was monitored by counting colonies on plates of MRS medium (De Man et al., 1960), supplemented as described above. Samples were taken at time zero just before water/ethanol was added, and then at one, three and five hours after addition.

L-malic acid and fructose contents were measured using Boehringer enzymatic kits (Mannheim) on culture supernatants stored at -20ºC until use. pH measurements were taken using a GLP31 pH-meter (Crison Instruments, Barcelona, Spain).

2.2. Transcriptome analysis

O. oeni cells were harvested by centrifugation, frozen in liquid nitrogen and kept at -80ºC until RNA extraction. Total RNA extractions were performed using the Roche RNeasy kit following the manufacturer's instructions (Mannheim, Germany). RNA concentrations were calculated by measuring absorbance at 260 nm using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific SL, Alcobendas, Spain).

Arrays (090324_*Oenococcus oeni* expression 4-plex array) were developed by Roche NimbleGen (Madison, WI, USA) and samples were analyzed at the Functional Genomics Core of the Institute for Research in Biomedicine (IRB, Barcelona, Spain). cDNA library preparation and amplification were performed from 25 ng total RNA using WTA2 (Sigma-Aldrich, Madrid, Spain) with 17 cycles of amplification. Labeling, hybridization and washing were performed according to the Roche Nimblegen expression guide v5.1. For each sample, 1 µg cDNA was labeled by Cy3 nonamer primers and Klenow polymerization. A hybridization mixture with 2 µg Cy3-labeled

cDNA was subsequently prepared. Samples were hybridized to the arrays for 18 hours at 42ºC. After washing, the arrays were scanned in a Roche Nimblegen MS 200 scanner. Raw data files (Pair and XYS files) were obtained from images using Nimblescan v2.6 software (Roche Nimblegen). Normalized gene expression values were obtained with Nimblescan software using the robust multichip average (RMA) algorithm as described by Irizarry et al. (2003a; 2003b).

Data univariate (ANOVA) analyses of transcriptomic data were conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Variable means showing statistical significance were compared using Bonferroni post-test comparisons at a significance level of 0.05, after testing the homogeneity of variance assumption between the various groups. The results were submitted to GEO (Gene Expression Omnibus Database, NCBI) under accession number GSE62036.

2.3. Array validation by real-time qPCR

Several genes were selected by real-time qPCR for validation of the microarray data. The primers used for these analyses are shown in Table 1. Genes OEOE_0289, OEOE_0422 and OEOE_0665 were selected because they have been studied by qPCR before (Beltramo et al. 2006, Olguín et al. 2009, Olguín et al. 2010). Genes OEOE_1325 and OEOE_1565 were selected due to their involvement in malolactic fermentation. The other genes (OEOE_0258, OEOE_0394, OEOE_0411, OEOE_1325, OEOE_0008, OEOE_0238 and OEOE_1290) were randomly selected with the sole objective of validating the methodology. Real-time qPCR was performed on the same RNA samples used for the microarray analysis. Reverse transcription and real-time qPCR were performed as previously described (Olguín et al., 2010). Primers were designed to be about 18-23 bases long, to contain over 50% G/C and to have a melting

temperature (Tm) above 60ºC. The length of the PCR products ranged from 92 to 130 bp. Clone Manager Professional Suite software was used to select primer sequences and analyze secondary structures and dimer formation. In this work four genes were assayed as internal controls for qPCR - *ldhD, dpoIII*, *gyrA* and *gyrB* - using the primers described by Desroche et al. (2005) and Constantini et al. (2011). Of these, the *ldhD* gene, coding for lactate dehydrogenase, was the one that showed the least variation under the experimental conditions used (data not shown). For this reason *ldhD* was used as the internal control. Real-time PCR was performed in 25 µl final volume containing 5 µl of diluted cDNA, 1.5 µl of each primer at 5 µM, 4.5 µl of RNAse free water and 12.5 µl of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Amplifications were carried out using a Real Time PCR System 7300 (Applied Biosystems). The threshold value used in this study was automatically determined by the instrument. Results were analyzed using the comparative critical threshold (ΔΔCT) method, in which the amount of target RNA was adjusted to a reference (internal target RNA) as previously described (Livak Schmittgen, 2001).

2.4. Proteome analysis

Protein extract preparation. Cells cultured in the presence or absence of ethanol were harvested by centrifugation, washed with 10 mM of Tris-HCl buffer (pH 8.0) and frozen at -80ºC until analysis. Pellets were then resuspended to a final $OD_{600nm} \approx 60$ in 0.1 M of Tris-HCl buffer (pH 7.5) and cellular extracts obtained using a cell disrupter (BASIC Z; Constant Systems Ltd., Daventry, United Kingdom) at a pressure of 2.5 kbar. The suspension was first centrifuged at 4,500 x *g* for 15 min at 4ºC to remove unbroken cells and cellular debris. The supernatant was ultra-centrifuged at 50,000 x *g* for 30 min at 4ºC to remove cell envelope components. Protein concentration was estimated using the

Bradford method following the manufacturer's instructions (Coomassie Protein Assay Reagent; Pierce Biotechnology, Rockford, IL, USA).

Sample preparation and protein electrophoresis. Protein samples and 2-DE were performed as described by Sánchez et al. (2005), with some modifications. The extract was treated with 1 µl of Benzonase (Merck KGaA, Darmstadt, Germany) and 1 µl of 1M $MgCl_2$ to remove nucleic acids. The mixture was vortexed and centrifuged at 3,500 x *g* for 2 min at 4ºC. After the addition of four volumes of deionized water and vigorous vortexing, samples were centrifuged at 3,500 x *g* for 10 min at 4ºC. The upper phase was removed and proteins precipitated by adding 3 volumes of methanol and centrifuging at 3,500 x *g* for 4 min. The pellets were then resuspended in solubilization buffer. The second dimension electrophoresis was run at 11 mA/gel for 15 h at 4ºC.

Image analysis. Spots were detected and their volume quantified with Prodigy SameSpots software (Nonlinear Dynamics), analyzing images of at least three gels for each time and condition. A sample taken at time zero (just before water/ethanol addition) was chosen as the . Protein expression was deemed to have changed if the mean normalized spot volume varied at least twofold and was confirmed by analysis of variance at a significant level of $P < 0.05$. Reproducibility was assessed by performing three independent experiments, and sets of five gels were analyzed.

Identification of proteins by peptide mass fingerprinting. Individual spots were excised from the gels and submitted to tryptic digestion, and mass spectrometry analyses were performed as previously described (Guillot et al., 2003). The mass of the peptides was determined by MALDI-TOF MS on a Voyager DE STR instrument (Applied Biosystems) at the PAPSSO platform of the INRA Center in Jouy-en-Josas. Proteins were identified against the *O. oeni* NC_008528 database.

3. Results and Discussion

Functional analysis using comparative transcriptomics and proteomics could provide insight into stress responses and regulation mechanisms in *O. oeni*. Our main aim was to evaluate which genes and proteins are most affected by ethanol shock.

The growth of *O. oeni* PSU-1 in MRS medium and the effect of ethanol on population development were monitored by counting plates (data not shown). In all conditions, the population remained constant during the 3-hour assay, and cell populations of more than 10^7 CFU ml^{-1} were detected.

3.1. Transcriptional profiling after ethanol shock

Transcriptional analysis was carried out using mRNA from the control (water addition) at t=0h or at t=1h, and mRNA from ethanol treated samples at t=1h and t=3h. The rough data were first analyzed to get an indication of reproducibility, and spot intensities were compared between pairs of filters. Scatter plots of normalized spot intensities (arbitrary units) from 1611 individual spots were generated and showed good reproducibility between filters (Figure 1A is a representative example). Averaged spot intensities from a sample taken at t =0h versus t=1h after water addition (control) show a better correlation (Figure 1B) than the ethanol-treated samples (Figure 1, C and D).

In order to validate the results obtained from the microarray analysis, real-time qPCR was performed with the same RNA from the original microarray experiment. Eleven genes, some related to stress response, were selected: *nadE, canH, pyrB, hsp18, trhD, amt, citE, atpB, qnnR, mleA-2* and *mleR*. There was a general accordance between microarray and real-time qPCR data for all the genes tested (Table 2). Of the eleven genes, eight were clearly correlated using both techniques. The three remaining genes (*canH, citE, mleR*) displayed lower numerical values by microarray, indicating no

significant changes through this technique. Overall, the correlation between real-time qPCR and microarray was good, suggesting that the microarray gene expression measurements were valid.

Transcriptomic data were grouped by functional categories in order to identify biological processes influenced by ethanol shock. Time zero, just before water/ethanol addition, was used as the reference condition to normalize data. In the control condition (water addition) some genes decreased their expression, probably due to changes in nutrient concentration (data not shown). However, the greatest changes in gene expression were observed for ethanol addition. Table 3 shows the number of genes from each functional category with altered expression in samples obtained one hour after the addition of 12% ethanol (t=1h) . The presence of ethanol appeared to influence gene expression in a wide range of functional classes. A total of 1611 genes were detected by the microarray. Of these, 170 genes decreased their expression after ethanol shock and 30 genes increased their expression in the presence of ethanol. Some groups seemed to be less affected by ethanol (cell mobility and secretion, coenzyme metabolism, secondary metabolites and signal transduction mechanisms), while others were more affected (amino acid transport and metabolism, cell envelope biogenesis in the outer membrane and transcription). Transcriptomic data analysis was also performed in samples obtained 3 hours after ethanol addition (t=3h), but no significant difference was observed in comparison with t=1h samples (data not shown).

Table 4 shows the transcriptomic analysis of the relative expression of the genes between time zero and 1 hour after the addition of 12% ethanol. The table shows all up-regulated genes with known functions. A selection of the most inhibited genes has also been included for each functional category. Microarray data revealed that transport systems were widely inhibited in response to ethanol shock. In particular, permeases

involved in metabolite transport, such as amino acids and carbohydrates, and inorganic ions were down-regulated.

As far as amino acid transport is concerned, it should be pointed out that five of the seven genes encoding for amino acid permeases that were down-regulated are related to glutamate and/or gamma-aminobutyrate transport (GABA). The other two genes are generic amino acid transporters. Gene OEOE_1747 encoding a possible GABA permease showed one of the strongest inhibitions (sevenfold). The glutamate/GABA antiporter (OEOE_0883) was also down-regulated in response to ethanol. Other inhibited amino acid transporter genes (OEOE_1806, OEOE_1427, OEOE_0388) showed high homology with orthologue glutamate/GABA transport genes in other LAB species (data not shown). It has been reported that the conversion of glutamate into GABA may confer resistance to bacterial cells, including some LAB species, under acidic conditions because of the consumption of an intracellular proton in the reaction (Cotter and Hill, 2003). However, the gene of glutamate decarboxylase, the enzyme responsible for GABA production from glutamate, has not been found in *O. oeni*. However, an aminotransferase gene that transforms GABA into succinate semialdehyde and L-glutamate is present in the *O. oeni* PSU-1 genome (OEOE_0387). GABA can be assimilated as a nitrogen and/or carbon source in bacteria such as *Escherichia coli* (Bartsch et al., 1990) and *Corynebacterium glutamicum* (Zhao et al., 2012), but no information is available about LAB in this respect. The inhibition of glutamate and GABA transport after ethanol shock observed in this study may account for the cell growth arrest due to stress. Vasserot et al. (2003) described the inability of *O. oeni* to uptake L-glutamate in non-energy generating cells (membrane potential). Similar findings were described for *Lactobacillus casei* (Strobel et al., 1989) and *Lactococcus lactis* (Smid et al., 1989). Two genes involved in the transport of

spermidine/putrescine were down-regulated. Like glutamate transport, the uptake of these two polyamines has been associated with an energy-producing state/membrane potential of the cell in *E. coli* (Kashiwagi et al., 1997). Both putrescine and spermidine protect against oxidative stress (Tkachenko et al., 2001). This protective mechanism may also be a target of ethanol damage, which inhibits the uptake of these polyamines. In contrast to the previously mentioned down-regulated functions, a dipeptidase *A* gene (OEOE_1783) was over-expressed in response to ethanol. This is in line with the increase in protease or peptidase activity in response to stress reported by other authors (Manca de Nadra et al., 1999; Ritt et al., 2008).

Multiple genes involved in carbohydrate transport were negatively affected, which may partly explain the decrease in energy production that led to the arrest of the nitrogenated-compound transport mentioned above. Only an ATPase (OEOE_1456) related to sugar transport was induced after ethanol shock. However, other ATPase components related to defense mechanisms were inhibited (OEOE_0722 and OEOE_0735).

Some genes related to cell wall and membrane biogenesis were also significantly affected. The most inhibited were two genes with acetyl transferase function. The gene encoding for a rod shape-determining protein (MreB) was inhibited threefold. This protein has been reported to have a cytoskeletal, actin-like role in bacterial cell morphogenesis and seems to be essential for cell survival since its deletion causes inflated morphology and, finally, cell lysis (Jones et al., 2001). These transcriptional changes are indicative of cell envelope damage due to ethanol action. However, the down-regulation of several N-acetylmuramidase genes (OEOE_0735, OEOE_0588, OEOE_1734) is the cell's protective response against ethanol, which prevents cell wall

299 weakening since these genes encode for proteins with autolysin activity (Delcour et al.,
300 1999; Govindasamy-Lucey et al., 2000).

301 Gene expression related to defense mechanisms, DNA replication,
302 recombination, repair and transcription was widely affected after ethanol shock (Table
303 4). Among the genes inhibited under these functional categories were multidrug and
304 antimicrobial ATPase and transport systems. Several transcriptional regulators were
305 also significantly down-regulated – for example, various members of the xenobiotic
306 responsive element (xre) family (OEOE_0047) – but no information is available
307 regarding their activator/repressor role.

308 Altogether, it seems that ethanol shock triggers the transcriptional inhibition of
309 several cell defense mechanisms in response to the immediate effect of an external
310 threat to the cell, such as the presence of ethanol. Presumably the ability to recover and
311 reactivate these cell protection mechanisms is part of the adaptation response that allows
312 *O. oeni* strains to survive under wine conditions (Beltramo et al., 2006; Olguín et al.,
313 2009). Meanwhile, the activation of the widely studied stress protein Hsp20 in *O. oeni*
314 (Guzzo et al., 1997; 2000) confirms the importance of this gene as a marker of stress
315 response in *O. oeni*.

316

317 *3.2. Changes in proteins of* O. oeni *in response to ethanol shock*

318 Changes in *O. oeni* soluble proteome were followed for the first five hours after
319 12% ethanol shock. The protein profile of *O. oeni* PSU-1 at t=0h was characterized to
320 generate a standard grid, which was used for subsequent comparative studies of samples
321 obtained after water/ethanol addition. The high-resolution map that was obtained
322 revealed approximately 215 spots (Fig. 2A), indicating a better resolution than in a

previous study (Silveira et al., 2004). A larger quantity of spots was detected by Cecconi et al. (2009), even though they used a larger gradient (pH 3-10).

A comparison of proteome profiles from 12% ethanol-treated or control cultures for each sampling time revealed quantitative and qualitative modifications of the spot patterns that could be recognized by simple visual comparison of the two conditions. Further analysis of the gels confirmed that ethanol-treated and control populations (12% water addition) had different responses. Two spots decreased their intensity in the control condition. One of these spots could not be identified; the other was spot 7, which also diminished in the ethanol-treated samples (Fig. 2A). After ethanol treatment, intensity increased in one spot (spot 36) and decreased in 44. This is in agreement with a previous report in which most proteins decreased in concentration after ethanol shock (Silveira et al., 2004).

Of the 45 spots found in different quantities when compared to the proteome reference gel (t=0h), 35 were identified, corresponding to 31 different proteins (Table 5). Unfortunately spot 36 (Fig. 2B), the only one detected that increased in response to ethanol shock, could not be identified.

As shown in Table 5, the proteins identified are involved mainly in nucleotide transport and metabolism (22.86%), translation, ribosomal structure and biogenesis (17.14%), cell envelope biogenesis (14.26%) and posttranslational modification, protein turnover and chaperone functions (11.43%). Seven spots were classified in five other functional categories and for the last five spots (14.29%) no function could be predicted.

Three proteins (PyrG, PyrE and Zwf) matched two different spots (spots 1 and 2, 7 and 8, and 26 and 27 respectively). This may be due to the presence of co- and posttranslational modifications that affect their *pI* and/or mass.

As mentioned above, image analysis showed only subtle changes in the protein kinetics in the control assay with added water. Therefore all further analysis focused on the gels obtained from the ethanol-treated cultures. The fold-change value was derived from the mean normalized volumes of four groups of three gels, each group corresponding to t=0h (reference gel), 1, 3 and 5 h. Fold-changes between control at t=0h and ethanol-treated samples at t=5 h are indicated in Table 5. On the basis of their predicted function, proteins were classified into ten different functional categories.

The kinetic changes were also analyzed and seven different patterns observed. Some representative proteins are depicted in Fig. 2B. Within a given functional category, several kinetics were observed; for instance, spots 2 (PyrG) and 7 (PyrE) both belong to the pyrimidine biosynthetic pathway. These patterns of expression suggest a complex response to ethanol during the first 5 hours of exposure (Fig. 2B). Indeed, one protein (spot 36) increased after one hour and then remained stable, whereas other spots decreased after one hour and remained stable (spots 7 and 23), or decreased only after 3 hours (spots 2, 11, 27), or showed a gradual decrease over time (spots 11 and 21). However, we were unable to find a clear-cut link between functional categories and the type of kinetics.

Our results suggest that protein synthesis and stability decrease when cells are directly submitted to 12% ethanol since more than half the proteins (53.13%) that decrease in concentration are related to these protective functions. The concentration of the molecular chaperone DnaK, a stress-induced protein in several lactic acid bacteria (Kilstrup et al., 1997; Lim et al., 2000), decreases after ethanol shock (Table 5). This decrease in DnaK concentration in the cytosolic fraction may be related to the recruitment of this chaperone to the membrane, as described for *Bacillus subtilis* DnaK after short-term ethanol stress (Seydlova et al., 2012). A similar phenomenon of

membrane association has been described for heat shock protein Lo18 in *O. oeni* (Weidmann et al., 2010). It has been widely reported that *O. oeni* cells respond to the presence of ethanol by decreasing the fluidity of their membranes (Da Silveira et al., 2003; Chu-Ky et al., 2005). It has also been suggested that this decrease in fluidity stems from the changed lipid-to-protein ratio, which plays an important role in regulating fluidity. The recruitment of several stress proteins to the membrane could play a protective role of protein stabilization and membrane fluidity regulation, as described for Lo18 (Weidmann et al., 2010).

Other stress proteins such as ClpC and ClpE also diminish in the presence of ethanol. These two proteins have ATPase activity and can function either as molecular chaperones or as regulating components of a proteolytic complex by associating to ClpP protease (Beltramo et al., 2004). Therefore this association of each of the ATPases with ClpP to form a two-subunit complex in response to ethanol stress may account for the decrease in the cytosolic fraction of these proteins in the single form.

About 14% of the proteins that decrease in concentration are related to cell envelope biogenesis. One of them, identified as dTDP-D-glucose 4,6-dehydratase (RmlB), is involved in cell wall lipopolysaccharide biosynthesis. In the proteomic study by Silveira et al. (2004), RmlB was detected in the membrane-associated protein extract of cells pre-adapted in 8% ethanol but not in the control condition or after 1 hour of 12% ethanol shock. In our study RmlB was present in all the samples, although it decreased over time in the presence of ethanol. This suggests that *rmlB* is initially down-regulated after ethanol shock, which decreases the RmlB protein level, but is subsequently over-expressed when cells are adapted and so may be an indicator of cell acclimation. We have also observed that in some *O. oeni* strains there is a correlation between high levels of *rmlB* transcripts and a better malolactic performance (Olguín et

al., 2010). The present study also shows that proteins such as MurC, MurD and GlmS, which are involved in murein biosynthesis, are down-regulated after a 12% ethanol shock (Table 5). Altogether, these results suggest that ethanol stress causes important changes in cell wall composition. It is clear that resistance to stress depends on the genes involved in peptidoglycan and teichoic acid biosynthesis (Delcour et al., 1999). Nonetheless, there is little biochemical or genetic data available on the biosynthesis pathways of the cell wall constituents in lactic acid bacteria. Further study is required in this area.

3.3 Global evaluation of transcriptomic and proteomic changes

Little correlation has been found among the 31 identified proteins changing in abundance and with their gene expression analyzed by microarray. Only two genes, lactoylglutathione lyase (OEOE_0531) and glucosamine 6-phosphate aminotransferase (OEOE_0635), showed the same behavior at protein and gene level; in both cases their expression decreased after ethanol addition. These two genes could therefore be useful molecular reporters of the metabolic state of cells in response to ethanol stress. Cecconi et al. (2009) reported a similar result for one of these proteins, glucosamine 6-phosphate aminotransferase (OEOE_0635), which was less abundant in cells not acclimated to ethanol (as in the present study) than in acclimated cells. Thus we might suggest that the non-activation of these genes indicate cellular metabolic robustness against the induced stress.

The other proteins that decreased in concentration showed no changes in gene expression. These differences may be accounted for by posttranscriptional regulation, changes in protein localization and, most probably, protein degradation due to ethanol damage. However, a considerable number of the genes that are differentially expressed,

422 according to transcriptomic analysis, encode for membrane-associated proteins (e.g.
423 permeases), whereas in this work only the soluble proteome was analyzed. Nonetheless,
424 the combination of both transcriptomic and proteomic approaches confirmed the
425 functions that are mainly affected by short-term ethanol stress in *O. oeni*.

426 In conclusion, the transport of metabolites and cell wall and membrane
427 biogenesis are the main functional categories affected by ethanol shock. These results
428 highlight the importance of the membrane as a barrier to stress and as a key element for
429 cell protection. One of the mechanisms of response to cell damage is the recruitment of
430 several stress proteins to the membrane. This is the first study to present a
431 transcriptomic analysis of *O. oeni* and to combine this data with a proteomic analysis.
432 This dual approach opens the door to future studies on the behavior of *O. oeni* under
433 wine-related conditions.

434

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442

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592

593 **Figure legends**

594

595 **Figure 1.** Scatter plots of normalized spot intensities (arbitrary units) from 1611
596 individual spots. A) Typical replicates showing the intensity of each spot versus the
597 equivalent spot in a replicate filter. B), C) and D) Averaged spot intensities from a
598 sample taken at t = 0h (*X*-axis) versus ethanol-treated and control samples (*Y*-axis). t =
599 1h H_2O (control condition): one hour after water addition; t = 1h EtOH: one hour after
600 ethanol addition; t = 3h EtOH: three hours after ethanol addition. Asterisks indicate
601 significant differences (one-way analysis of variance (ANOVA) and the Bonferroni
602 post-test, $P < 0.05$).

603

604 **Figure 2. A)** Reference map of proteins extracted from *O. oeni* PSU-1 cells in the late-
605 exponential phase of growth in MRS (t=0h) before water or ethanol addition. The
606 differentially expressed spots are indicated by spot number as reported in Table 5. **B)**
607 Kinetics of expression of some spots of representative proteins at time 0, and 1, 3, and
608 5h (columns) after ethanol shock. N.Vol., normalized volumes.

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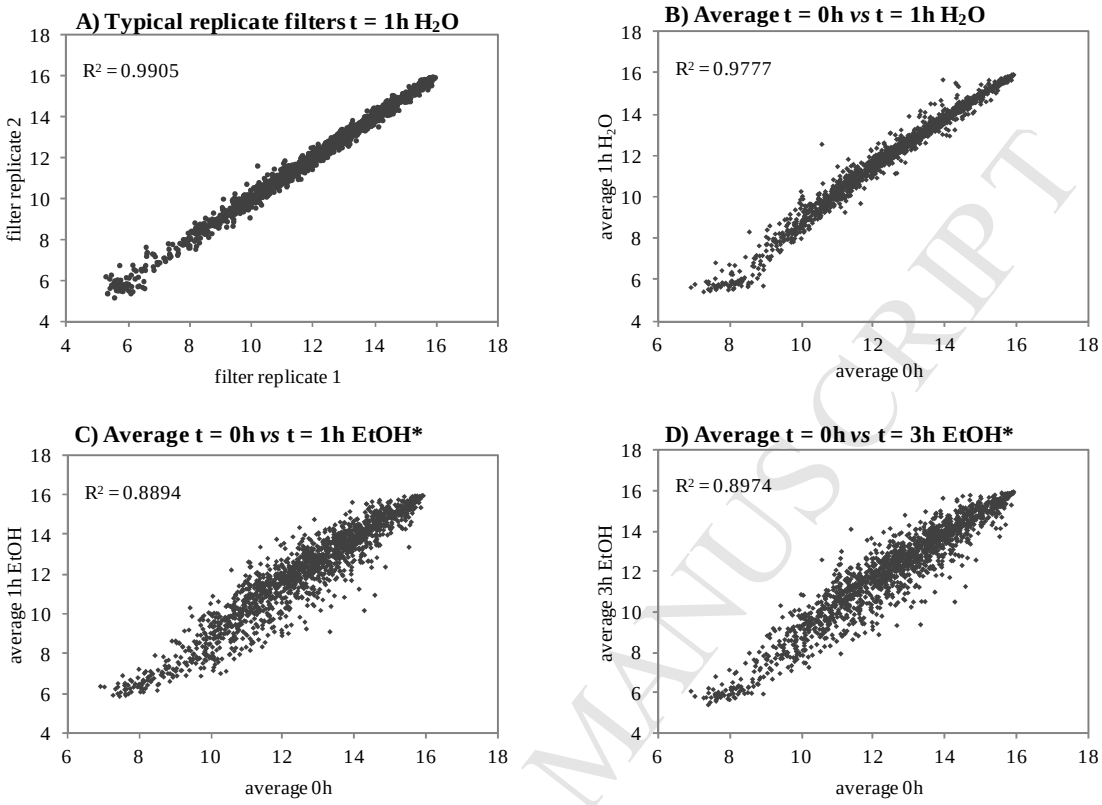

Figure 1. Scatter plots of normalized spot intensities (arbitrary units) from 1611 individual spots. A) Typical replicates showing the intensity of each spot versus the equivalent spot in a replicate filter. B), C) and D) Averaged spot intensities from a sample taken at t = 0h (*X*-axis) versus ethanol-treated and control samples (*Y*-axis); t = 1h H_2O (control condition): one hour after water addition; t = 1h EtOH: one hour after ethanol addition; t = 3h EtOH: three hours after ethanol addition. Asterisks indicate significant differences (one-way analysis of variance (ANOVA) and the Bonferroni post-test, $P < 0.05$).

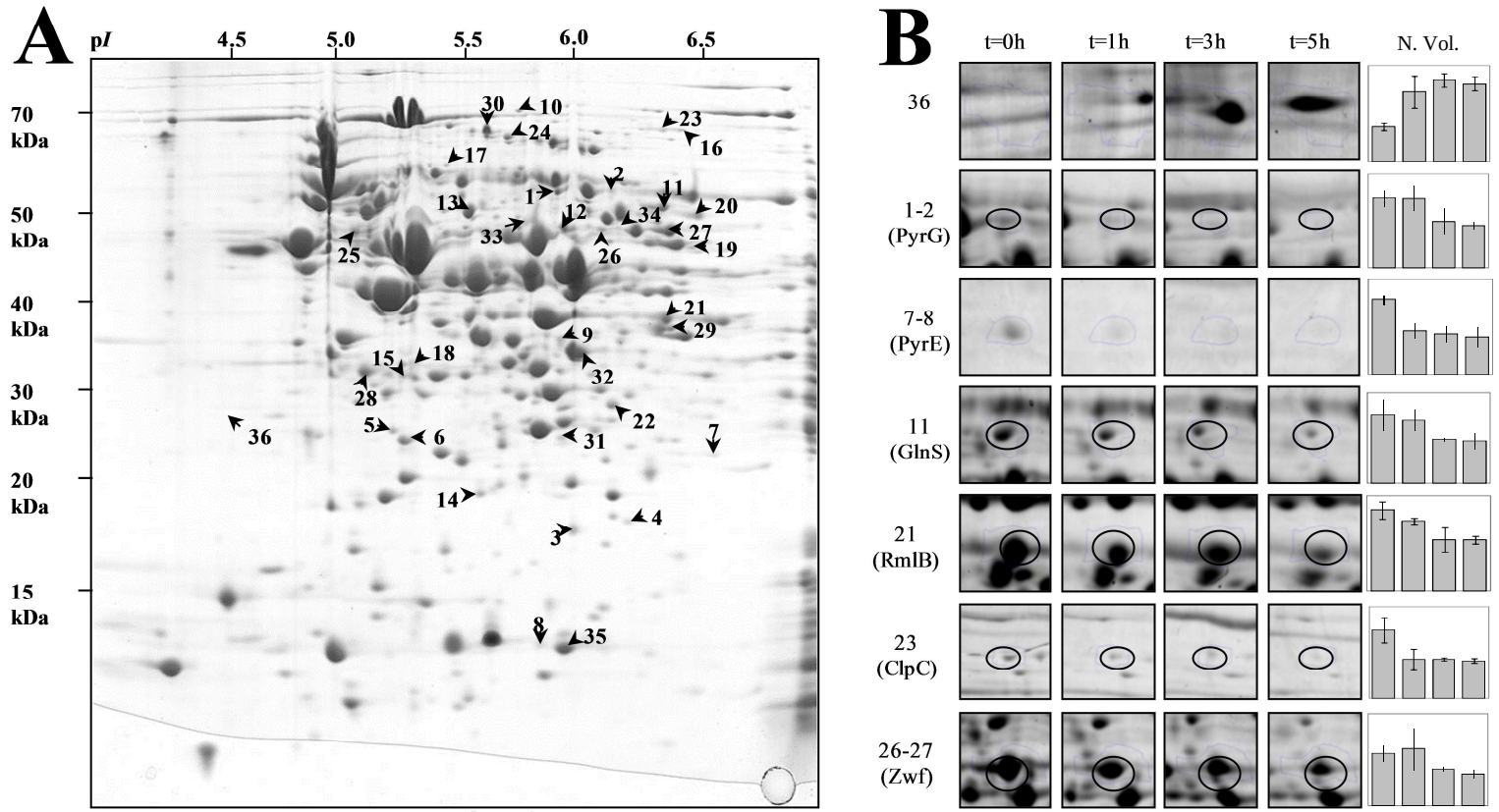

Figure 2. A) Reference map of proteins extracted from *O. oeni* PSU-1 cells in the late-exponential phase of growth in MRS before water or ethanol addition (T=0h). The differentially expressed spots are indicated by spot number as reported in Table 5. **B)** Kinetics of expression of some spots of representative proteins at time 0, and 1, 3, and 5h after ethanol shock. N.Vol., normalized volumes.

Table 1. Primers used for real-time qPCR analysis

Target gene	Description	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon length (bp)	Reference
nadE	OEOE_0008 NH(3)-dependent NAD(+) synthetase	AACATGACGGCGTTGTTC	GATCCAAATCGGTTCCTCCATC	93	This study
canH	OEOE_0238 carbonic anhydrase	CATGCTCCCAGTGAACATC	CAGCGATAACTGCTGTTCTTCC	97	This study
pyrB	OEOE_0258 pyrB aspartate carbamoyltransferase	GGCAGGTTGTTGCCAATC	TTGTTGCCGAGGACTTGTTGGG	123	This study
hsp18	OEOE_0289 heat shock protein Hsp20	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102	Beltramo *et al.* 2006
trhD	OEOE_0394 threonine dehydrogenase	AGAGTTCTTGCGCGAGAC	CCGGTGCCACTCATATTCTTAG	114	This study
amt	OEOE_0411 aminotransferase	TTGGACAGCGAAGGAAGAGT	GTTTATCTTCGGCCGTCAAC	94	This study
citE	OEOE_0422 Citrate lyase beta subunit	CCGCACGATGATGTTTGTTCC	GCTCAAAGAAACGGCATCTTCC	108	Olguín *et al.* 2009
atpB	OEOE_0665 F0F1-type ATP synthase, beta subunit	ATACTGATCCGGCTCCGGC	CAGCGGGATAAATACCTTG	93	Beltramo *et al.* 2006
qnnR	OEOE_1290 NADPH:quinone reductase	GCAGCTTGCCCTAATTCC	CCTTGATAATCGCCTGGTATCC	92	This study
mleA-2	OEOE_1325 malate dehydrogenase (NAD)	AGGCCATGTCGGATCAAC	CAAGTGCGTCCGCTTTGA	107	This study
mleR	OEOE_1565 MLF system transcription activator	GGCAACCCTGGAATTGAG	CTGATCGAAGACGCTGTTG	130	This study
ldhD	D-lactate dehydrogenase	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTTT	102	Desroche *et al.* 2005

Table 2. Validation of microarray data by real-time qPCR

Gene name (code)	Microarray[a]	qPCR[b]
*nadE (*OEOE_0008)	+1.92	+1.71
canH (OEOE_0238)	+0.08	+2.99
pyrB (OEOE_0258)	+3.12	+6.92
hsp18 (OEOE_0289)	+2.04	+1.64
trhD (OEOE_0394)	+3.18	+4.50
amt (OEOE_0411)	+2.50	+6.11
citE (OEOE_0422)	+0.99	+2.11
atpB (OEOE_0665)	+0.10	+0.52
qnnR (OEOE_1290)	+3.72	+2.91
mleA-2 (OEOE_1325)	+2.54	+3.84
mleR (OEOE_1565)	-0.40	+4.44

[a]Microarray and [b]RT-qPCR fold changes between: t=0h and t=1h after ethanol addition.

Table 3. Number of genes of *Oenococcus oeni* PSU-1 with altered expression 1h after ethanol addition according to functional group.

Functional Group	Decreased	Induced
Amino acid transport and metabolism	15	1
Carbohydrate transport and metabolism	12	1
Cell envelope biogenesis, outer membrane	12	-
Cell mobility and secretion	1	-
Coenzyme metabolism	1	1
Defense mechanisms	8	-
DNA replication, recombination and repair	6	-
Energy production and conversion	5	3
Inorganic ion transport and metabolism	8	-
Intracellular trafficking and secretion	1	-
Lipid metabolism	7	1
Nucleotide transport and metabolism	1	3
Posttranslational modification, protein turnover, chaperones	3	1
Secondary metabolites biosynthesis, transport and catabolism	2	1
Signal transduction mechanisms	1	-
Transcription	17	1
Translation, ribosomal structure and biogenesis	7	1
General function prediction only	22	7
Function unknown	41	9
Total	170	30

Table 4. Relative expression of genes differentially expressed between 0h and 1h after ethanol addition. All up-regulated genes with known functions are included (on grey background). Only a selection of the most inhibited genes are included for each functional category.

[a]Gene	[b]Description	[c]Relative Expression
Amino Acid Transport and Metabolism		
OEOE 1783	Dipeptidase A. Cysteine peptidase. MEROPS family C69	2.09
OEOE 0394	Threonine dehydrogenase or related Zn-dependent dehydrogenase	3.18
OEOE 1747	Gamma-aminobutyratepermease or related permease	-7.25
OEOE 0634	Permease of the drug/metabolite transporter (DMT) superfamily	-5.26
OEOE 1806	ABC-type amino acid transport system. permease and periplasmic component	-5.25
OEOE 1465	Spermidine/putrescine ABC transporter permeaseprotein	-2.65
OEOE 0883	Glutamate gamma-aminobutyrate antiporter	-2.60
OEOE 0633	Spermidine/putrescine-binding periplasmic protein	-2.26
OEOE 0632	Spermidine/putrescine ABC transporter permease protein	-2.05
Carbohydrate Transport and Metabolism		
OEOE 1456	ABC-type sugar transport system. ATPase component	2.39
OEOE 0021	ABC-type sugar transport system. periplasmic component	-4.47
OEOE 1777	Permease of the major facilitator superfamily	-4.18
OEOE 0023	ABC-type maltose transport system. permease component	-3.91
OEOE 1574	Permease of the major facilitator superfamily	-3.53
OEOE 0022	ABC-type sugar transport system. permease component	-3.01
Cell Envelope Biogenesis. Outer Membrane		
OEOE 1497	Predicted glycosyltransferase	-4.07
OEOE 1851	O-acetyltransferase family protein	-3.89
OEOE 0288	D-alanine-activating enzyme	-3.37
OEOE 1388	Rod shape-determining protein MreD	-3.37
OEOE 0206	Lyzozyme M1 (1.4-beta-N-acetylmuramidase)	-3.26
Defense mechanisms		
OEOE 0722	ABC-type multidrug transport system. ATPase component	-8.43
OEOE 0438	ABC-type antimicrobial peptide transport system. permease component	-3.34
OEOE 0735	ABC-type antimicrobial peptide transport system. ATPase component	-3.21
DNA Replication. Recombination and Repair		
OEOE 1020	Rossmann fold nucleotide-binding protein for DNA uptake	-6.15
OEOE 1019	RNase HII	-3.82
OEOE 0004	DNA replication and repair protein RecF	-3.58

Energy production and Conversion		
OEOE 0516	NADH:flavinoxidoreductase. Old Yellow Enzyme family	2.41
OEOE 0510	Aryl-alcohol dehydrogenase related enzyme	2.43
OEOE 0553	Malate dehydrogenase (NAD)	2.54
OEOE 0693	Acetoin reductase	2.54
OEOE 1046	NADH:flavinoxidoreductase. Old Yellow Enzyme family	-3.44
OEOE 0168	Acylphosphatase	-3.33
Inorganic Ion Transport and Metabolism		
OEOE 1355	Kef-type K+ transport system. membrane component	-4.29
OEOE 0827	Mn2+ and Fe2+ transporter of the NRAMP family	-4.25
OEOE 0172	ABC-type cobalt transport system. permease component CbiQ or related transporter	-3.96
OEOE 1087	ABC-type cobalt transport system. ATPase component	-3.43
Intracellular trafficking and secretion		
OEOE 0865	Predicted acyltransferase	-5.94
Lipid Metabolism		
OEOE 0327	Lipoate-protein ligase	2.18
OEOE 0881	Acyl carrier protein phosphodiesterase	-4.07
OEOE 1768	Esterase/lipase	-3.58
Nucleotide Transport and Metabolism		
OEOE 1543	Adenine/guanine phosphoribosyltransferase or related PRPP-binding protein	2.23
OEOE 0258	Aspartate carbamoyltransferase	3.12
OEOE 0635	Glutamine--fructose-6-phosphate transaminase	-2.82
Posttranslational Modification. Protein Turnover. Chaperones		
OEOE 0289	Heat shock protein Hsp20	2.03
OEOE 1639	Peptidyl-prolylcis-trans isomerase (rotamase) - cyclophilin family	-4.82
OEOE 1062	Cytochrome bd biosynthesis ABC-type transporter. ATPase and permease component	-3.68
Secondary metabolite biosynthesis. transport and catabolism		
OEOE 0009	Putative multicopper oxidase	2.10
OEOE 0547	Amidase	-3.67
OEOE 0287	D-alanyl transfer protein	-3.59
Transcription		
OEOE 0411	HTH containing DNA-binding domain and MocR-like aminotransferase	2.51
OEOE 0047	Transcriptional regulator. xre family	-3.87
OEOE 1830	Transcriptional regulator. AraC family	-3.49
OEOE 1685	Transcriptional regulator	-3.43

OEOE 0417	Citrate lyase regulator	-3.31
OEOE 0082	Transcriptional regulator. MarR family	-3.20
Translation. Ribosomal Structure and Biogenesis		
OEOE 1360	Sigma 54 modulation protein / SSU ribosomal protein S30P	2.43
OEOE 0950	tRNA delta(2)-isopentenylpyrophosphatetransferase	-4.37
General Function Prediction Only		
OEOE 0531	Lactoylglutahionelyase or related lyase	-2.00

[a]The gene names are taken from the NCBI database for *Oenococcus oeni* PSU-1 complete genome

[b]The information in the description column is taken from the Computational Biology and Bioinformatics Group of the Biosciences Division of Oak Ridge National Laboratory (http://compbio.ornl.gov/public/section/)

[c]The relative expression was described by the fold change value of genes after ethanol addition with respect to 0h

Table 5. Identification of the differentially expressed proteins of *O. oeni* PSU-1 growing in the presence of ethanol

Spot no[§].	Gene symbol	Gene name	**Functional category** Protein name (EC number)	Fold change*	Theoretical M_r (kDa)	Theoretical p*I*
			Nucleotide transport and metabolism			
1-2	OEOE_1786	*pyrG*	CTP synthase (UTP-ammonia lyase) (EC 6.3.4.2)	-5.6	60.11	5.52
3	OEOE_0138	*nrdL*	Ribonucleotide reduction protein	-5.4	18.05	5.52
4	OEOE_1069	*apt*	Adenine/guanine phosphoribosyltransferase or related PRPP-binding protein (EC 2.4.2.7)	-3.6	19.25	5.7
5	OEOE_1124	*hpt*	Hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8)	-2.7	20.93	4.99
6	OEOE_0437	--	Deoxynucleoside kinase (EC 2.7.1.113)	-2.1	24.74	5.07
7-8	OEOE_0263	*pyrE*	Orotate phosphoribosyltransferase (EC 2.4.2.10)	-3	23.13	5.67
			Coenzyme metabolism			
9	OEOE_1036	*pdxS*	Pyridoxine biosynthesis enzyme, SOR/SNZ family	-3.1	31.44	5.58
			Translation, ribosomal structure and biogenesis			
10	OEOE_0982	*proS*	Prolyl-tRNA synthetase (EC 6.1.1.15)	-5.2	64.33	5.41
11	OEOE_0321	*glnS*	Glutamyl- and glutaminyl-tRNA synthetase (EC 6.1.1.17 – 6.1.1.24)	-2.7	57.05	5.94
12	OEOE_0440	*serS*	Seryl-tRNA synthetase (EC 6.1.1.11)	-2.4	49.86	5.68
13	OEOE_1694	*gatA*	Asp-tRNAAsn/Glu-tRNAGln aminotransferase A subunit (EC 6.3.5.6 – 6.3.5.7)	-2.4	52.5	5.31
14	OEOE_0806	*def*	N-formylmethionyl-tRNA deformylase (EC 3.5.1.88)	-2.3	21.08	5.27
15	OEOE_1699	*map*	Methionine aminopeptidase (EC 3.4.11.18)	-2.2	29.95	5.08
			Energy production and conversion			
16	OEOE_1248	*eutG*	Iron-binding alcohol dehydrogenase / aldehyde dehydrogenase family domain (EC1.1.1.1)	-3.8	99.1	6.04
			Cell envelope biogenesis			
17	OEOE_0635	*glmS*	Glucosamine 6-phosphate synthetase, amidotransferase and phosphosugar isomerase domains (EC 2.6.1.16)	-3.7	66.17	5.28
18	OEOE_0565	*galU*	UDP-glucose pyrophosphorylase (EC 2.7.7.9)	-2.9	32.56	5.37
19	OEOE_1269	*murC*	UDP-N-acetylmuramate-alanine ligase (EC 6.3.2.8)	-2.7	48.13	6.03
20	OEOE_1147	*murD*	UDP-N-acetylmuramoylalanine-D-glutamate ligase (EC 6.3.2.9)	-2.5	48.65	6.16
21	OEOE_1447	*rmlB*	dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46)	-2.1	37.46	5.8
			Posttranslational modification, protein turnover, chaperones			
22	OEOE_1114	*sufC*	Fe-S-cluster assembly ABC-type transport system, ATPase component	-2	28.22	5.71
23	OEOE_0514	*clpC*	ATP-binding subunit of Clp protease and DnaK/ DnaJ chaperones (subunit of DnaK/J)	-3.2	91.48	5.86
24	OEOE_0640	*clpE*	ATP-binding subunit of Clp protease and DnaK/ DnaJ chaperones (subunit clpE)	-2.5	81.29	5.37
25	OEOE_1309	*dnaK*	Molecular chaperone	-2.9	66.2	4.89
			Carbohydrate metabolism			
26-27	OEOE_0135	*zwf*	Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	-2.9	55.67	6.44
28	OEOE_1523	*gnd*	6-phosphogluconate dehydrogenase (EC 1.1.1.44)	-2.1	32.86	4.83
			Amino acid transport and metabolism			
29	OEOE_0845	*appF*	ABC-type oligopeptide transport system, ATPase component	-2.7	34.49	5.74
			Signal transduction mechanisms			
30	OEOE_0807	*typA*	Stress response membrane GTPase	-3.1	68.22	5.24
			General function prediction only			
31	OEOE_1270	*arc1*	EMAP domain	-2.3	22.47	6.1
32	OEOE_0070	*ara1*	Aldo/keto reductase related enzyme	-2.1	31.68	5.6
33	OEOE_1072	*obg*	Predicted GTPase	-3	48.2	5.47

34	OEOE_0531	--	Lactoglutathione lyase or related lyase	-2.5	13.46	4.71
35	OEOE_1705	--	Methylmalonyl-CoA epimerase (EC 5.1.99.1)	-2.5	16.22	5.62
36		--	Not identified protein	+6.0		

[§]Several spot numbers for the same protein entry indicate that the protein was identified in several spots.
*Fold-change between ethanol-treated and control gels for each spot at T=5h.

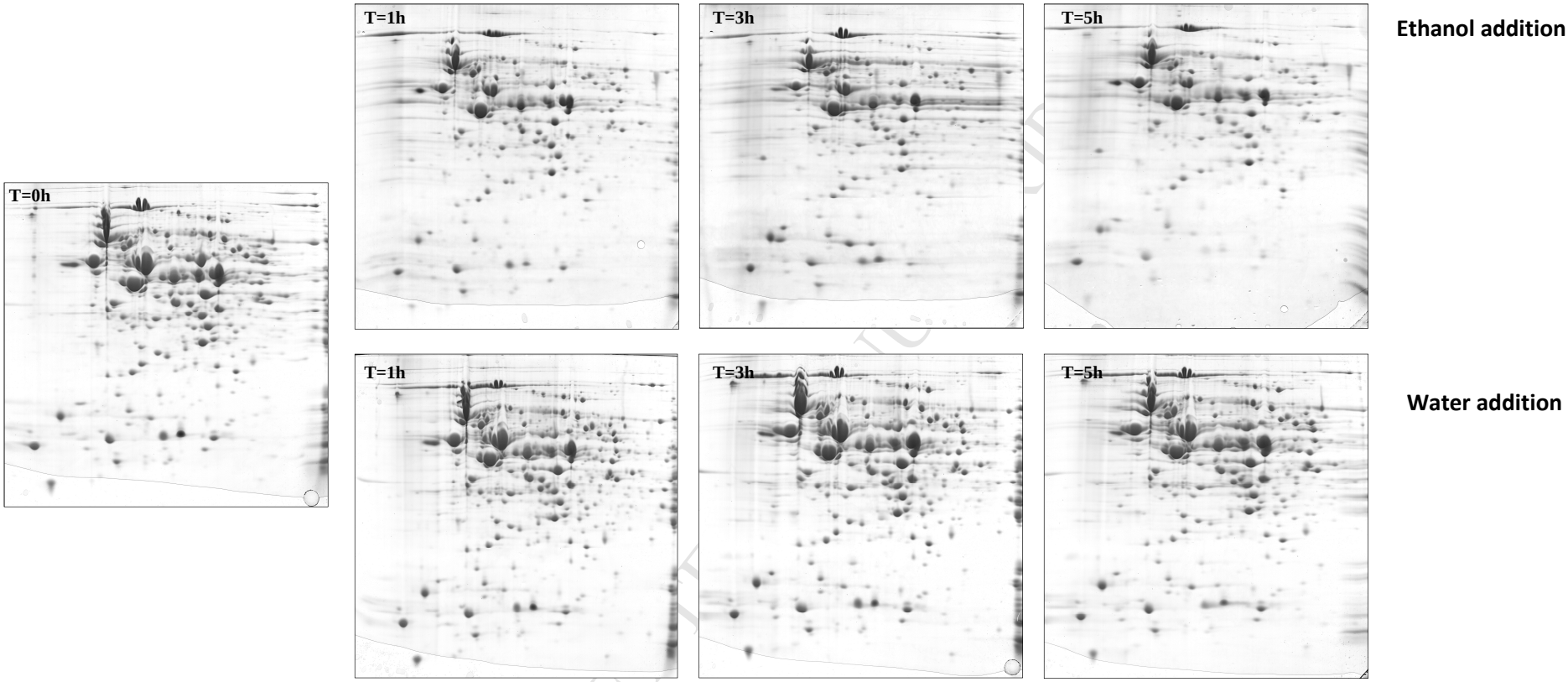

Figure S1. Pictures of 2D protein gel of the different conditions assayed: T=0h, T=1, 3 and 5 h after ethanol addition (upper side) and after water addition (lower side).

Highlights

- Transcriptomic data reveal the inhibition of transport and cell envelope biosynthesis.
- Proteomic results show a decrease in protein biosynthesis and stability.
- Global analysis confirms that the cell membrane is the main target of ethanol damage.