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Marie-Christine Champomier-Verges, Patricia Anglade, Fabienne F. Baraige, Ricardo Cordero-Otero, Albert Bordons, Monique Zagorec, Cristina Reguant

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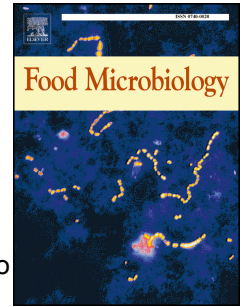
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# Accepted Manuscript

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

**Nair Olgún<sup>1</sup>, Marie Champomier-Vergès<sup>2</sup>, Patricia Anglade<sup>2</sup>, Fabienne Baraige<sup>2</sup>,  
Ricardo Cordero-Otero<sup>1</sup>, Albert Bordons<sup>1</sup>, Monique Zagorec<sup>2</sup>, Cristina Reguant<sup>\*1</sup>**

<sup>1</sup> Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, c/ Marcel·lí Domingo 1, 43007, Tarragona, Catalonia, Spain.

<sup>2</sup> Unité MICALIS (UMR1319) équipe FLEC, INRA, Domaine de Vilvert, 78350 Jouy-en-Josas, France

\*Corresponding author: Cristina Reguant

Tel: +34 977 558043; Fax: +34 977 558232; E-mail address: [cristina.reguant@urv.cat](mailto:cristina.reguant@urv.cat)

**26 Abstract**

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

42

**43 Keywords**

44 *Oenococcus oeni* - Malolactic fermentation -Transcriptomic - Microarray analysis -  
45 Proteomic – Ethanol

46

**47 1. Introduction**

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

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

57         The toxicity of ethanol is generally attributed to its interaction with membranes  
58 at the aqueous interface, resulting in perturbed membrane structure and function (Weber  
59 and Bont, 1996; Beney and Gervais, 2001). Studies on *O. oeni* have shown that  
60 exposing cells to ethanol increases the permeability of the cytoplasmic membrane and  
61 enhances passive proton influx and the concomitant loss of intracellular material (Da  
62 Silveira et al., 2003). The permeability of the membrane to protons dissipates the proton  
63 motive force and affects ATP synthesis, which is no longer available for growth  
64 (Capucho and San Romão, 1994; Salema et al., 1996; Weber and Bont, 1996). This may  
65 explain the high mortality when *O. oeni* cells were directly inoculated into a wine-like  
66 medium supplemented with 12-16% ethanol (Da Silveira et al., 2003; Chu-Ky et al.,  
67 2005).

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

75 Two-dimensional gel electrophoresis (2-DE) has provided invaluable  
76 information on the adaptive response of microorganisms to changes in external  
77 conditions (Champomier-Vergès et al., 2002). For instance, Silveira and co-workers  
78 (2004) found that ethanol triggers alterations in the protein patterns of *O. oeni* cells that  
79 are directly stressed with 12% ethanol for 1 hour and cells pre-adapted in 8% ethanol. It  
80 has also been shown that cell cultures acclimated with 10% ethanol survived better in  
81 wine, probably due to the differential expression of certain proteins (Cecconi et al.,  
82 2009). Functional analysis of gene expression using comparative transcriptomics is also  
83 providing insight into stress responses and regulation mechanisms in lactic acid bacteria  
84 (LAB). Preliminary microarray analysis of the *Lactobacillus plantarum* response to  
85 several stress conditions revealed unanticipated stress response profiles that correlate  
86 specifically with lactate- and pH-induced stress (Siezen et al., 2004; Pieterse et al.,  
87 2005). However, no current studies use microarray analysis of *O. oeni*.

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

92

## 93 **2. Materials and Methods**

### 94 *2.1. Growth conditions*

95 *O. oeni* PSU-1 was cultured at 30°C in a two-liter flask containing MRS broth  
96 medium supplemented with L-malic acid (4 g l<sup>-1</sup>) and fructose (5 g l<sup>-1</sup>) at pH 5.0. When  
97 cultures reached the late exponential phase (OD<sub>600nm</sub> ≈ 1) they were divided and put into  
98 two sterile flasks. Immediately, 12% (v/v) of ethanol was added to one flask and 12%  
99 (v/v) of water was added to the other (control). The latter was used as a control assay to

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

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

110

## 111 2.2. Transcriptome analysis

112 *O. oeni* cells were harvested by centrifugation, frozen in liquid nitrogen and kept  
113 at -80°C until RNA extraction. Total RNA extractions were performed using the Roche  
114 RNeasy kit following the manufacturer's instructions (Mannheim, Germany). RNA  
115 concentrations were calculated by measuring absorbance at 260 nm using a NanoDrop  
116 1000 Spectrophotometer (Thermo Fisher Scientific SL, Alcobendas, Spain).

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

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

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

### 137 138 *2.3. Array validation by real-time qPCR*

139 Several genes were selected by real-time qPCR for validation of the microarray  
140 data. The primers used for these analyses are shown in Table 1. Genes OE0E\_0289,  
141 OE0E\_0422 and OE0E\_0665 were selected because they have been studied by qPCR  
142 before (Beltramo et al. 2006, Olguín et al. 2009, Olguín et al. 2010). Genes  
143 OE0E\_1325 and OE0E\_1565 were selected due to their involvement in malolactic  
144 fermentation. The other genes (OE0E\_0258, OE0E\_0394, OE0E\_0411, OE0E\_1325,  
145 OE0E\_0008, OE0E\_0238 and OE0E\_1290) were randomly selected with the sole  
146 objective of validating the methodology. Real-time qPCR was performed on the same  
147 RNA samples used for the microarray analysis. Reverse transcription and real-time  
148 qPCR were performed as previously described (Olguín et al., 2010). Primers were  
149 designed to be about 18-23 bases long, to contain over 50% G/C and to have a melting



150 temperature ( $T_m$ ) above 60°C. The length of the PCR products ranged from 92 to 130  
151 bp. Clone Manager Professional Suite software was used to select primer sequences and  
152 analyze secondary structures and dimer formation. In this work four genes were assayed  
153 as internal controls for qPCR - *ldhD*, *dpoIII*, *gyrA* and *gyrB* - using the primers  
154 described by Desroche et al. (2005) and Constantini et al. (2011). Of these, the *ldhD*  
155 gene, coding for lactate dehydrogenase, was the one that showed the least variation  
156 under the experimental conditions used (data not shown). For this reason *ldhD* was used  
157 as the internal control. Real-time PCR was performed in 25  $\mu$ l final volume containing  
158 5  $\mu$ l of diluted cDNA, 1.5  $\mu$ l of each primer at 5  $\mu$ M, 4.5  $\mu$ l of RNase free water and  
159 12.5  $\mu$ l of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA).  
160 Amplifications were carried out using a Real Time PCR System 7300 (Applied  
161 Biosystems). The threshold value used in this study was automatically determined by  
162 the instrument. Results were analyzed using the comparative critical threshold ( $\Delta\Delta CT$ )  
163 method, in which the amount of target RNA was adjusted to a reference (internal target  
164 RNA) as previously described (Livak Schmittgen, 2001).

165

#### 166 2.4. Proteome analysis

167 *Protein extract preparation.* Cells cultured in the presence or absence of ethanol were  
168 harvested by centrifugation, washed with 10 mM of Tris-HCl buffer (pH 8.0) and frozen  
169 at -80°C until analysis. Pellets were then resuspended to a final  $OD_{600nm} \approx 60$  in 0.1 M of  
170 Tris-HCl buffer (pH 7.5) and cellular extracts obtained using a cell disrupter (BASIC Z;  
171 Constant Systems Ltd., Daventry, United Kingdom) at a pressure of 2.5 kbar. The  
172 suspension was first centrifuged at 4,500 x  $g$  for 15 min at 4°C to remove unbroken cells  
173 and cellular debris. The supernatant was ultra-centrifuged at 50,000 x  $g$  for 30 min at  
174 4°C to remove cell envelope components. Protein concentration was estimated using the

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

177 *Sample preparation and protein electrophoresis.* Protein samples and 2-DE were  
178 performed as described by Sánchez et al. (2005), with some modifications. The extract  
179 was treated with 1  $\mu$ l of Benzonase (Merck KGaA, Darmstadt, Germany) and 1  $\mu$ l of  
180 1M MgCl<sub>2</sub> to remove nucleic acids. The mixture was vortexed and centrifuged at 3,500  
181 x g for 2 min at 4°C. After the addition of four volumes of deionized water and vigorous  
182 vortexing, samples were centrifuged at 3,500 x g for 10 min at 4°C. The upper phase  
183 was removed and proteins precipitated by adding 3 volumes of methanol and  
184 centrifuging at 3,500 x g for 4 min. The pellets were then resuspended in solubilization  
185 buffer. The second dimension electrophoresis was run at 11 mA/gel for 15 h at 4°C.

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

193 *Identification of proteins by peptide mass fingerprinting.* Individual spots were excised  
194 from the gels and submitted to tryptic digestion, and mass spectrometry analyses were  
195 performed as previously described (Guillot et al., 2003). The mass of the peptides was  
196 determined by MALDI-TOF MS on a Voyager DE STR instrument (Applied  
197 Biosystems) at the PAPSSO platform of the INRA Center in Jouy-en-Josas. Proteins  
198 were identified against the *O. oeni* NC\_008528 database.

199

### 200 3. Results and Discussion

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

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

208

#### 209 3.1. Transcriptional profiling after ethanol shock

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

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

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

228 Transcriptomic data were grouped by functional categories in order to identify  
229 biological processes influenced by ethanol shock. Time zero, just before water/ethanol  
230 addition, was used as the reference condition to normalize data. In the control condition  
231 (water addition) some genes decreased their expression, probably due to changes in  
232 nutrient concentration (data not shown). However, the greatest changes in gene  
233 expression were observed for ethanol addition. Table 3 shows the number of genes from  
234 each functional category with altered expression in samples obtained one hour after the  
235 addition of 12% ethanol ( $t=1h$ ). The presence of ethanol appeared to influence gene  
236 expression in a wide range of functional classes. A total of 1611 genes were detected by  
237 the microarray. Of these, 170 genes decreased their expression after ethanol shock and  
238 30 genes increased their expression in the presence of ethanol. Some groups seemed to  
239 be less affected by ethanol (cell mobility and secretion, coenzyme metabolism,  
240 secondary metabolites and signal transduction mechanisms), while others were more  
241 affected (amino acid transport and metabolism, cell envelope biogenesis in the outer  
242 membrane and transcription). Transcriptomic data analysis was also performed in  
243 samples obtained 3 hours after ethanol addition ( $t=3h$ ), but no significant difference was  
244 observed in comparison with  $t=1h$  samples (data not shown).

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

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

252 As far as amino acid transport is concerned, it should be pointed out that five of  
253 the seven genes encoding for amino acid permeases that were down-regulated are  
254 related to glutamate and/or gamma-aminobutyrate transport (GABA). The other two  
255 genes are generic amino acid transporters. Gene OE0E\_1747 encoding a possible  
256 GABA permease showed one of the strongest inhibitions (sevenfold). The  
257 glutamate/GABA antiporter (OE0E\_0883) was also down-regulated in response to  
258 ethanol. Other inhibited amino acid transporter genes (OE0E\_1806, OE0E\_1427,  
259 OE0E\_0388) showed high homology with orthologue glutamate/GABA transport genes  
260 in other LAB species (data not shown). It has been reported that the conversion of  
261 glutamate into GABA may confer resistance to bacterial cells, including some LAB  
262 species, under acidic conditions because of the consumption of an intracellular proton in  
263 the reaction (Cotter and Hill, 2003). However, the gene of glutamate decarboxylase, the  
264 enzyme responsible for GABA production from glutamate, has not been found in *O.*  
265 *oeni*. However, an aminotransferase gene that transforms GABA into succinate  
266 semialdehyde and L-glutamate is present in the *O. oeni* PSU-1 genome (OE0E\_0387).  
267 GABA can be assimilated as a nitrogen and/or carbon source in bacteria such as  
268 *Escherichia coli* (Bartsch et al., 1990) and *Corynebacterium glutamicum* (Zhao et al.,  
269 2012), but no information is available about LAB in this respect. The inhibition of  
270 glutamate and GABA transport after ethanol shock observed in this study may account  
271 for the cell growth arrest due to stress. Vasserot et al. (2003) described the inability of  
272 *O. oeni* to uptake L-glutamate in non-energy generating cells (membrane potential).  
273 Similar findings were described for *Lactobacillus casei* (Strobel et al., 1989) and  
274 *Lactococcus lactis* (Smid et al., 1989). Two genes involved in the transport of

275 spermidine/putrescine were down-regulated. Like glutamate transport, the uptake of  
276 these two polyamines has been associated with an energy-producing state/membrane  
277 potential of the cell in *E. coli* (Kashiwagi et al., 1997). Both putrescine and spermidine  
278 protect against oxidative stress (Tkachenko et al., 2001). This protective mechanism  
279 may also be a target of ethanol damage, which inhibits the uptake of these polyamines.  
280 In contrast to the previously mentioned down-regulated functions, a dipeptidase A gene  
281 (OEOE\_1783) was over-expressed in response to ethanol. This is in line with the  
282 increase in protease or peptidase activity in response to stress reported by other authors  
283 (Manca de Nadra et al., 1999; Ritt et al., 2008).

284 Multiple genes involved in carbohydrate transport were negatively affected,  
285 which may partly explain the decrease in energy production that led to the arrest of the  
286 nitrogenated-compound transport mentioned above. Only an ATPase (OEOE\_1456)  
287 related to sugar transport was induced after ethanol shock. However, other ATPase  
288 components related to defense mechanisms were inhibited (OEOE\_0722 and  
289 OEOE\_0735).

290 Some genes related to cell wall and membrane biogenesis were also significantly  
291 affected. The most inhibited were two genes with acetyl transferase function. The gene  
292 encoding for a rod shape-determining protein (MreB) was inhibited threefold. This  
293 protein has been reported to have a cytoskeletal, actin-like role in bacterial cell  
294 morphogenesis and seems to be essential for cell survival since its deletion causes  
295 inflated morphology and, finally, cell lysis (Jones et al., 2001). These transcriptional  
296 changes are indicative of cell envelope damage due to ethanol action. However, the  
297 down-regulation of several N-acetylmuramidase genes (OEOE\_0735, OEOE\_0588,  
298 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

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

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

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

339 As shown in Table 5, the proteins identified are involved mainly in nucleotide  
340 transport and metabolism (22.86%), translation, ribosomal structure and biogenesis  
341 (17.14%), cell envelope biogenesis (14.26%) and posttranslational modification, protein  
342 turnover and chaperone functions (11.43%). Seven spots were classified in five other  
343 functional categories and for the last five spots (14.29%) no function could be predicted.

344 Three proteins (PyrG, PyrE and Zwf) matched two different spots (spots 1 and 2,  
345 7 and 8, and 26 and 27 respectively). This may be due to the presence of co- and  
346 posttranslational modifications that affect their *pI* and/or mass.



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

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

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

372 membrane association has been described for heat shock protein Lo18 in *O. oeni*  
373 (Weidmann et al., 2010). It has been widely reported that *O. oeni* cells respond to the  
374 presence of ethanol by decreasing the fluidity of their membranes (Da Silveira et al.,  
375 2003; Chu-Ky et al., 2005). It has also been suggested that this decrease in fluidity  
376 stems from the changed lipid-to-protein ratio, which plays an important role in  
377 regulating fluidity. The recruitment of several stress proteins to the membrane could  
378 play a protective role of protein stabilization and membrane fluidity regulation, as  
379 described for Lo18 (Weidmann et al., 2010).

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

386 About 14% of the proteins that decrease in concentration are related to cell  
387 envelope biogenesis. One of them, identified as dTDP-D-glucose 4,6-dehydratase  
388 (RmlB), is involved in cell wall lipopolysaccharide biosynthesis. In the proteomic study  
389 by Silveira et al. (2004), RmlB was detected in the membrane-associated protein extract  
390 of cells pre-adapted in 8% ethanol but not in the control condition or after 1 hour of  
391 12% ethanol shock. In our study RmlB was present in all the samples, although it  
392 decreased over time in the presence of ethanol. This suggests that *rmlB* is initially  
393 down-regulated after ethanol shock, which decreases the RmlB protein level, but is  
394 subsequently over-expressed when cells are adapted and so may be an indicator of cell  
395 acclimation. We have also observed that in some *O. oeni* strains there is a correlation  
396 between high levels of *rmlB* transcripts and a better malolactic performance (Olguín et

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

405

### 406 *3.3 Global evaluation of transcriptomic and proteomic changes*

407 Little correlation has been found among the 31 identified proteins changing in  
408 abundance and with their gene expression analyzed by microarray. Only two genes,  
409 lactoylglutathione lyase (OE0E\_0531) and glucosamine 6-phosphate aminotransferase  
410 (OE0E\_0635), showed the same behavior at protein and gene level; in both cases their  
411 expression decreased after ethanol addition. These two genes could therefore be useful  
412 molecular reporters of the metabolic state of cells in response to ethanol stress. Cecconi  
413 et al. (2009) reported a similar result for one of these proteins, glucosamine 6-phosphate  
414 aminotransferase (OE0E\_0635), which was less abundant in cells not acclimated to  
415 ethanol (as in the present study) than in acclimated cells. Thus we might suggest that the  
416 non-activation of these genes indicate cellular metabolic robustness against the induced  
417 stress.

418 The other proteins that decreased in concentration showed no changes in gene  
419 expression. These differences may be accounted for by posttranscriptional regulation,  
420 changes in protein localization and, most probably, protein degradation due to ethanol  
421 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  $t = 0\text{h}$  ( $X$ -axis) versus ethanol-treated and control samples ( $Y$ -axis).  $t =$   
599 1h H<sub>2</sub>O (control condition): one hour after water addition;  $t = 1\text{h EtOH}$ : one hour after  
600 ethanol addition;  $t = 3\text{h EtOH}$ : 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

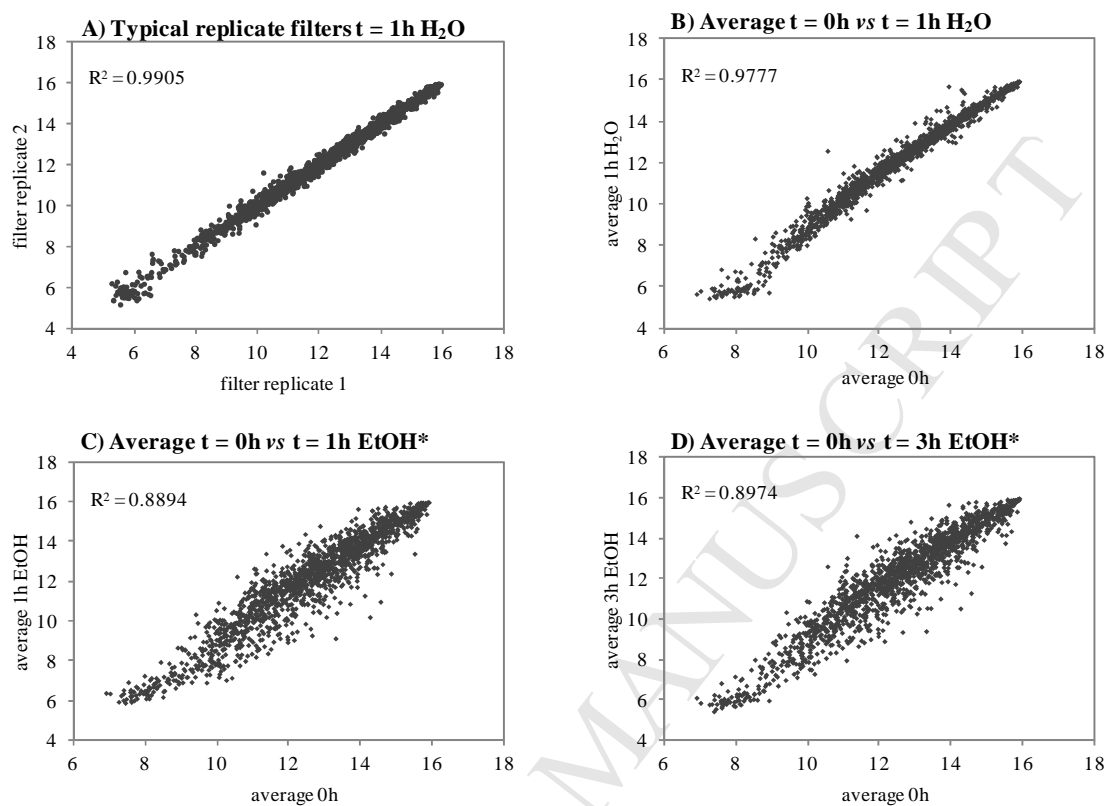
604 **Figure 2. A)** Reference map of proteins extracted from *O. oeni* PSU-1 cells in the late-  
605 exponential phase of growth in MRS ( $t=0\text{h}$ ) before water or ethanol addition. The  
606 differentially expressed spots are indicated by spot number as reported in Table 5. **B)**  
607 Kinetics 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.

609

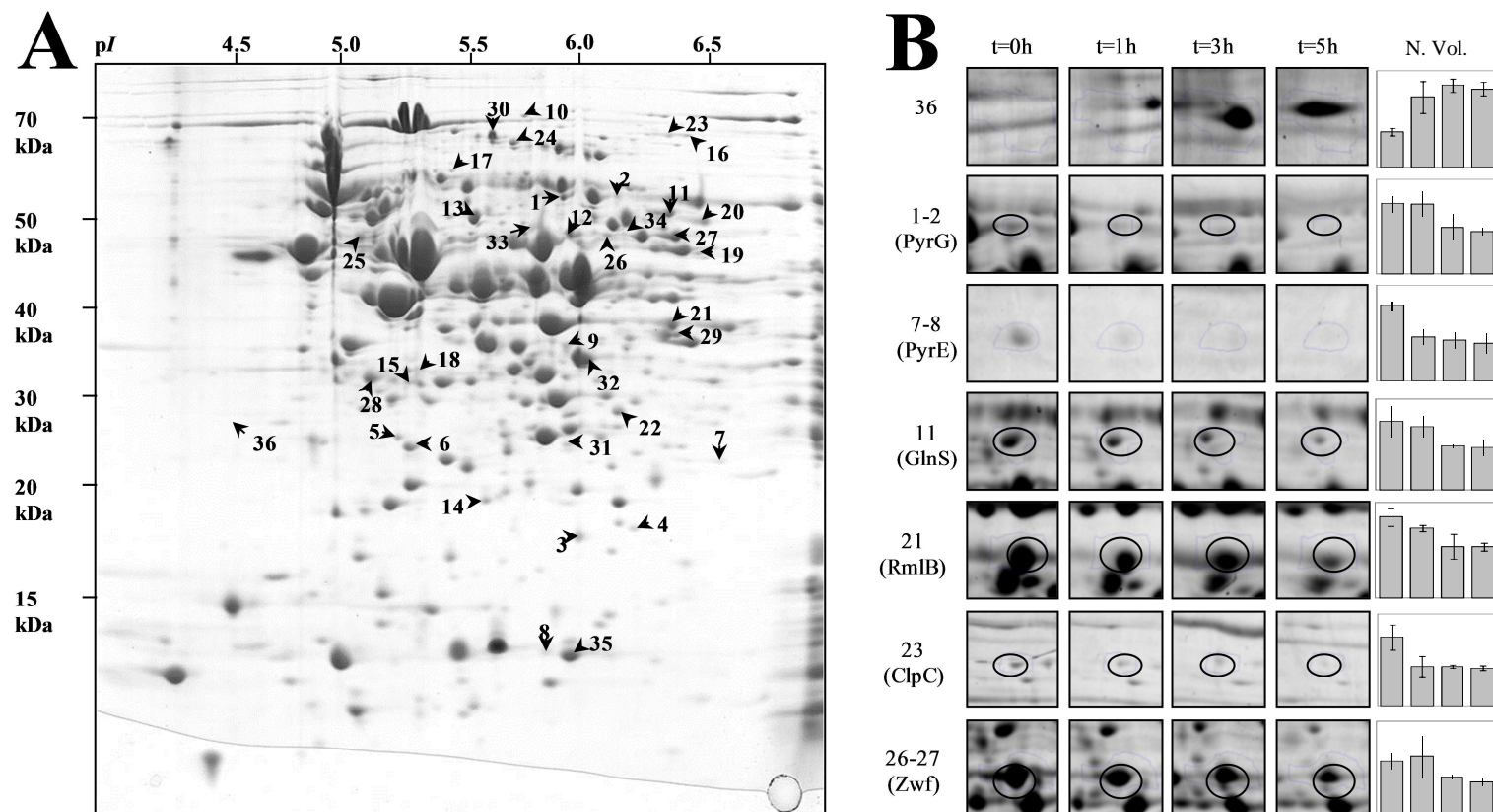
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**Figure 1.** Scatter plots of normalized spot intensities (arbitrary units) from 1611 individual spots. A) Typical replicates showing the intensity of each spot versus the equivalent spot in a replicate filter. B), C) and D) Averaged spot intensities from a sample taken at  $t = 0h$  (X-axis) versus ethanol-treated and control samples (Y-axis);  $t = 1h$  H<sub>2</sub>O (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
<i>nadE</i>	OEOE_0008 NH(3)-dependent NAD(+) synthetase	AACATGACGGCGTTGTTTC	GATCCAAATCGGTTCCCTCCATC	93	This study
<i>canH</i>	OEOE_0238 carbonic anhydrase	CATGCTCCCAGTGAACATC	CAGCGATAACTGCTGTTCTTCC	97	This study
<i>pyrB</i>	OEOE_0258 pyrB aspartate carbamoyltransferase	GGCAGGTTGTTGCCAATC	TTGTTGCCGAGGACTTGTGGG	123	This study
<i>hsp18</i>	OEOE_0289 heat shock protein Hsp20	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102	Beltramo <i>et al.</i> 2006
<i>trhD</i>	OEOE_0394 threonine dehydrogenase	AGAGTTCCTGCGCGAGAC	CCGGTGCCACTCATATTCTTAG	114	This study
<i>amt</i>	OEOE_0411 aminotransferase	TTGGACAGCGAAGGAAGAGT	GTTTATCTTCGGCCGTCAAC	94	This study
<i>citE</i>	OEOE_0422 Citrate lyase beta subunit	CCGCACGATGATGTTTGTTC	GCTCAAAGAAACGGCATCTTCC	108	Olguín <i>et al.</i> 2009
<i>atpB</i>	OEOE_0665 F0F1-type ATP synthase, beta subunit	ATACTGATCCGGCTCCGGC	CAGCGGGATAAATACCTTG	93	Beltramo <i>et al.</i> 2006
<i>qnnR</i>	OEOE_1290 NADPH:quinone reductase	GCAGCTTGCCCTAATTCC	CCTTGATAATCGCCTGGTATCC	92	This study
<i>mleA-2</i>	OEOE_1325 malate dehydrogenase (NAD)	AGGCCATGTCCGATCAAC	CAAGTGCGTCCGCTTTGA	107	This study
<i>mleR</i>	OEOE_1565 MLF system transcription activator	GGCAACCCTGGAATTGAG	CTGATCGAAGACGCTGTTG	130	This study
<i>ldhD</i>	D-lactate dehydrogenase	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTTT	102	Desroche <i>et al.</i> 2005

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

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

<sup>a</sup>Microarray and <sup>b</sup>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.

<sup>a</sup> Gene	<sup>b</sup> Description	<sup>c</sup> Relative Expression
<b>Amino Acid Transport and Metabolism</b>		
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-aminobutyrate permease 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 permease protein	-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
<b>Carbohydrate Transport and Metabolism</b>		
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
<b>Cell Envelope Biogenesis. Outer Membrane</b>		
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	Lysozyme M1 (1.4-beta-N-acetylmuramidase)	-3.26
<b>Defense mechanisms</b>		
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
<b>DNA Replication. Recombination and Repair</b>		
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

<b>Energy production and Conversion</b>		
OEOE 0516	NADH:flavinooxidoreductase. 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:flavinooxidoreductase. Old Yellow Enzyme family	-3.44
OEOE 0168	Acylphosphatase	-3.33
<b>Inorganic Ion Transport and Metabolism</b>		
OEOE 1355	Kef-type K <sup>+</sup> transport system. membrane component	-4.29
OEOE 0827	Mn <sup>2+</sup> and Fe <sup>2+</sup> 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
<b>Intracellular trafficking and secretion</b>		
OEOE 0865	Predicted acyltransferase	-5.94
<b>Lipid Metabolism</b>		
OEOE 0327	Lipoate-protein ligase	2.18
OEOE 0881	Acyl carrier protein phosphodiesterase	-4.07
OEOE 1768	Esterase/lipase	-3.58
<b>Nucleotide Transport and Metabolism</b>		
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
<b>Posttranslational Modification. Protein Turnover. Chaperones</b>		
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
<b>Secondary metabolite biosynthesis. transport and catabolism</b>		
OEOE 0009	Putative multicopper oxidase	2.10
OEOE 0547	Amidase	-3.67
OEOE 0287	D-alanyl transfer protein	-3.59
<b>Transcription</b>		
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

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**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

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**General Function Prediction Only**

OEOE 0531	Lactoylglutahionelyase or related lyase	-2.00
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<sup>a</sup>The gene names are taken from the NCBI database for *Oenococcus oeni* PSU-1 complete genome

<sup>b</sup>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/>)

<sup>c</sup>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 <sup>s</sup> .	Gene symbol	Gene name	Functional category Protein name (EC number)	Fold change*	Theoretical M <sub>r</sub> (kDa)	Theoretical pI
<b>Nucleotide transport and metabolism</b>						
1-2	OEOE_1786	<i>pyrG</i>	CTP synthase (UTP-ammonia lyase) (EC 6.3.4.2)	-5.6	60.11	5.52
3	OEOE_0138	<i>nrdL</i>	Ribonucleotide reduction protein	-5.4	18.05	5.52
4	OEOE_1069	<i>apt</i>	Adenine/guanine phosphoribosyltransferase or related PRPP-binding protein (EC 2.4.2.7)	-3.6	19.25	5.7
5	OEOE_1124	<i>hpt</i>	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	<i>pyrE</i>	Orotate phosphoribosyltransferase (EC 2.4.2.10)	-3	23.13	5.67
<b>Coenzyme metabolism</b>						
9	OEOE_1036	<i>pxs</i>	Pyridoxine biosynthesis enzyme, SOR/SNZ family	-3.1	31.44	5.58
<b>Translation, ribosomal structure and biogenesis</b>						
10	OEOE_0982	<i>proS</i>	Prolyl-tRNA synthetase (EC 6.1.1.15)	-5.2	64.33	5.41
11	OEOE_0321	<i>glnS</i>	Glutamyl- and glutamyl-tRNA synthetase (EC 6.1.1.17 – 6.1.1.24)	-2.7	57.05	5.94
12	OEOE_0440	<i>serS</i>	Seryl-tRNA synthetase (EC 6.1.1.11)	-2.4	49.86	5.68
13	OEOE_1694	<i>gatA</i>	Asp-tRNA <sup>Asn</sup> /Glu-tRNA <sup>Gln</sup> aminotransferase A subunit (EC 6.3.5.6 – 6.3.5.7)	-2.4	52.5	5.31
14	OEOE_0806	<i>def</i>	N-formylmethionyl-tRNA deformylase (EC 3.5.1.88)	-2.3	21.08	5.27
15	OEOE_1699	<i>map</i>	Methionine aminopeptidase (EC 3.4.11.18)	-2.2	29.95	5.08
<b>Energy production and conversion</b>						
16	OEOE_1248	<i>eutG</i>	Iron-binding alcohol dehydrogenase / aldehyde dehydrogenase family domain (EC1.1.1.1)	-3.8	99.1	6.04
<b>Cell envelope biogenesis</b>						
17	OEOE_0635	<i>glmS</i>	Glucosamine 6-phosphate synthetase, amidotransferase and phosphosugar isomerase domains (EC 2.6.1.16)	-3.7	66.17	5.28
18	OEOE_0565	<i>galU</i>	UDP-glucose pyrophosphorylase (EC 2.7.7.9)	-2.9	32.56	5.37
19	OEOE_1269	<i>murC</i>	UDP-N-acetylmuramate-alanine ligase (EC 6.3.2.8)	-2.7	48.13	6.03
20	OEOE_1147	<i>murD</i>	UDP-N-acetylmuramoylalanine-D-glutamate ligase (EC 6.3.2.9)	-2.5	48.65	6.16
21	OEOE_1447	<i>rmlB</i>	dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46)	-2.1	37.46	5.8
<b>Posttranslational modification, protein turnover, chaperones</b>						
22	OEOE_1114	<i>sufC</i>	Fe-S-cluster assembly ABC-type transport system, ATPase component	-2	28.22	5.71
23	OEOE_0514	<i>clpC</i>	ATP-binding subunit of Clp protease and DnaK/ DnaJ chaperones (subunit of DnaK/J)	-3.2	91.48	5.86
24	OEOE_0640	<i>clpE</i>	ATP-binding subunit of Clp protease and DnaK/ DnaJ chaperones (subunit clpE)	-2.5	81.29	5.37
25	OEOE_1309	<i>dnaK</i>	Molecular chaperone	-2.9	66.2	4.89
<b>Carbohydrate metabolism</b>						
26-27	OEOE_0135	<i>zwf</i>	Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	-2.9	55.67	6.44
28	OEOE_1523	<i>gnd</i>	6-phosphogluconate dehydrogenase (EC 1.1.1.44)	-2.1	32.86	4.83
<b>Amino acid transport and metabolism</b>						
29	OEOE_0845	<i>appF</i>	ABC-type oligopeptide transport system, ATPase component	-2.7	34.49	5.74
<b>Signal transduction mechanisms</b>						
30	OEOE_0807	<i>typA</i>	Stress response membrane GTPase	-3.1	68.22	5.24
<b>General function prediction only</b>						
31	OEOE_1270	<i>arcI</i>	EMAP domain	-2.3	22.47	6.1
32	OEOE_0070	<i>araI</i>	Aldo/keto reductase related enzyme	-2.1	31.68	5.6
33	OEOE_1072	<i>obg</i>	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

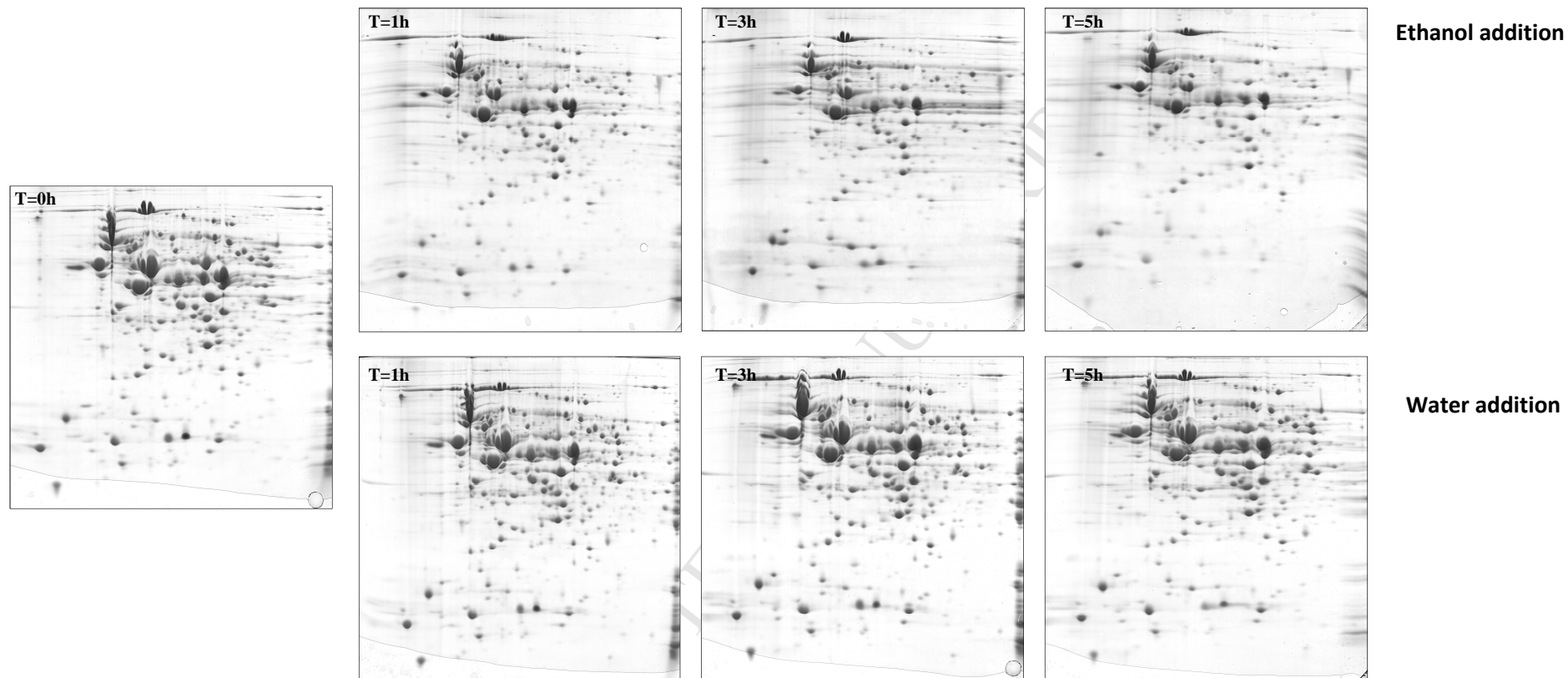
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36		--	Not identified protein	+6.0		
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<sup>§</sup>Several spot numbers for the same protein entry indicate that the protein was identified in several spots.

\*Fold-change between ethanol-treated and control gels for each spot at T=5h.

ACCEPTED MANUSCRIPT



**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.