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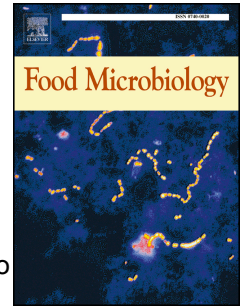


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Transcriptomic and proteomic analysis of *Oenococcus oeni* PSU-1 response to ethanol shock

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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⁻¹) and fructose (5 g l⁻¹) at pH 5.0. When
97 cultures reached the late exponential phase (OD_{600nm} ≈ 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⁻¹ and 0.28
102 g l⁻¹ 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 μ l final volume containing
158 5 μ l of diluted cDNA, 1.5 μ l of each primer at 5 μ M, 4.5 μ l of RNase free water and
159 12.5 μ 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 μ l of Benzonase (Merck KGaA, Darmstadt, Germany) and 1 μ l of
180 1M MgCl₂ 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⁻¹ 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

443 **References**

444 Bartsch, K., von Johnn-Marteville, A., Schulz, A., 1990. Molecular analysis of two
445 genes of the *Escherichia coli* *gab* cluster: nucleotide sequence of the
446 glutamate:succinic semialdehyde transaminase gene (*gabT*) and characterization of

- 447 the succinic semialdehyde dehydrogenase gene (*gabD*). *Journal of Bacteriology* 172,
448 7035–7042.
- 449 Beltramo, C., Grandvalet, C., Pierre, F., Guzzo, J., 2004. Evidence for multiple levels of
450 regulation of *Oenococcus oeni clpP-clpL* locus expression in response to stress.
451 *Journal of Bacteriology* 186, 2200-2205.
- 452 Beltramo, C., Desroche, N., Tourdot-Marechal, R., Grandvalet, C., Guzzo, J., 2006.
453 Real-time PCR for characterizing the stress response of *Oenococcus oeni* in a wine-
454 like medium. *Research Microbiology* 157, 267-274.
- 455 Beney, L., Gervais, P., 2001. Influence of the fluidity of the membrane on the response
456 of microorganisms to environmental stresses. *Applied Microbiology and*
457 *Biotechnology* 57, 34–42.
- 458 Capucho, I., San Romão, M.V., 1994. Effect of ethanol and fatty acids on malolactic
459 activity of *Leuconostoc oenos*. *Applied Microbiology and Biotechnology* 42, 391-
460 395.
- 461 Cafaro, C., Bonomo, M.G., Salzano, G., 2014. Adaptative changes in geranylgeranyl
462 pyrophosphate synthase gene expression level under ethanol stress conditions in
463 *Oenococcus oeni*. *Journal of Applied Microbiology*, 116(1), 71-80.
- 464 Cecconi, D., Milli, A., Rinalducci, S., Zolla, L., Zapparoli, G., 2009. Proteomic analysis
465 of *Oenococcus oeni* freeze-dried culture to assess the importance of cell
466 acclimatation to conduct malolactic fermentation in wine. *Electrophoresis* 30, 2988-
467 2995.
- 468 Cotter, D., Hill, C., 2003. Surviving the acid test: Responses of Gram-positive bacteria
469 to low pH. *Microbiology and Molecular Biology Reviews* 63(3), 429-453.
- 470 Constantini, A., Vaudano, E., Rantsiou, K., Cocolin, L., García-Moruno, E., 2011.
471 Quantitative expression analysis of *mleP* and two genes involved in the ABC

- 472 transport system in *Oenococcus oeni* during rehydration. Applied Microbiology and
473 Biotechnology 91, 1601-1609.
- 474 Champomier-Vergès, M.C., Maguin, E., Mistou, M.Y., Anglade, P., Chich, J.F., 2002.
475 Lactic acid bacteria and proteomics: current knowledge and perspectives. Journal of
476 Chromatography 771, 329-342.
- 477 Chu-Ky, S., Tourdot-Maréchal, R., Marechal, P.A., Guzzo, J., 2005. Combined cold,
478 acid and ethanol shocks in *Oenococcus oeni*: effects on membrane fluidity and cell
479 viability. Biochimica and Biophysica Acta 1717, 118-124.
- 480 Da Silveira, M.G., Golovina, E.A., Hoekstra, F.A., Rombouts, F.M., Abee, T., 2003.
481 Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells. Applied
482 and Environmental Microbiology 69(10), 5826-5832.
- 483 Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H., Fleet, G.H., 1985. Practical
484 implications of malolactic fermentation: a review. American Journal of Enology and
485 Viticulture 36(4), 290-301.
- 486 Delcour, J., Ferain, T., Deghorain, M., Palumbo, E., Hols, P., 1999. The biosynthesis
487 and functionality of the cell-wall of lactic acid bacteria. Antonie Van Leeuwenhoek
488 76, 159-184.
- 489 De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of
490 lactobacilli. Journal of Applied Bacteriology 23, 130-135.
- 491 Desroche, N., Beltramo, C., Guzzo, J., 2005. Determination of an internal control to
492 apply reverse transcription quantitative PCR to study stress response in the lactic
493 acid bacterium *Oenococcus oeni*. Journal of Microbiology Methods 60, 325-333.
- 494 Govindasamy-Lucey, S., Gopal, P.K., Sullivan, P.A., Pillidge, C.J., 2000. Varying
495 influence of the autolysin, N-acetyl muramidase, and the cell envelope proteinase on

- 496 the rate of autolysis of six commercial *Lactococcus lactis* cheese starter bacteria
497 grown in milk. *Journal of Dairy Research* 67, 585-96.
- 498 Guillot, A., Gitton, C., Anglade, P., Mistou, M.Y., 2003. Proteomic analysis of
499 *Lactococcus lactis*, a lactic acid bacterium. *Proteomics* 3, 337-354.
- 500 Guzzo, J., Delmas, F., Pierre, F., Jobin, M.P., Samyn, B., Van Beeumen, J., Cavin, J.F.,
501 Diviès, C., 1997. A small heat shock protein from *Leuconostoc oenos* induced by
502 multiple stresses and during stationary growth phase. *Letters in Applied*
503 *Microbiology* 24, 393-396.
- 504 Guzzo, J., Jobin, M.P., Delmas, F., Fortier, L.C., Garmyn, D., Tourdot-Marechal, R.,
505 Lee, B., Divies, C., 2000. Regulation of stress response in *Oenococcus oeni* as a
506 function of environmental changes and growth phase. *International Journal of Food*
507 *Microbiology* 55, 27-31.
- 508 Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., Speed, T.P., 2003a.
509 Summaries of Affymetrix GeneChip probe level data. *Nucleic Acid Research* 31,
510 e15.
- 511 Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y., Antonellis, K.J., Scherf, U.,
512 Speed, T.P., 2003b. Exploration, Normalization, and Summaries of High Density
513 Oligonucleotide Array Probe Level Data. *Biostatistics* 4, 249-264.
- 514 Jones, L.J., Carballido-Lopez, R., Errington, J., 2001. Control of cell shape in bacteria:
515 helical, actin-like filaments in *Bacillus subtilis*. *Cell* 104, 913-922.
- 516 Kashiwagi, K., Shibuya, S., Tomitori, H., Kuraishi, A., Igarashi, K., 1997. Excretion
517 and uptake of putrescine by the PotE protein in *Escherichia coli*. *The Journal of*
518 *Biological Chemistry* 272(10), 6318-6323.

- 519 Kilstrup, M., Jacobsen, S., Hammer, K., Vogensen, F.K., 1997. Induction of heat shock
520 proteins DnaA, GroEL, and GroES by salt stress in *Lactococcus lactis*. Applied and
521 Environmental Microbiology 63(5), 1826-1837.
- 522 Lim, E.M., Ehrlich, S.D., Maguin, E., 2000. Identification of stress-inducible proteins in
523 *Lactobacillus delbrueckii* subsp. *bulgaricus*. Electrophoresis 21, 2557-2561.
- 524 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using
525 real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. Methods 25, 402-408.
- 526 Malherbe, S., Bauer, F.F., Du Toit, M., 2007. Understanding problem fermentations – A
527 review. South African Journal of Enology and Viticulture 28(2), 169-186.
- 528 Manca de Nadra, M.C., Farias, M., Moreno-Arribas, M.V., Pueyo, E., Polo, M.C., 1999.
529 A proteolytic effect of *Oenococcus oeni* on the nitrogenous macromolecular fraction
530 of red wine. FEMS Microbiology Letters 174, 41–47.
- 531 Olguín, N., Bordons, A., Reguant, C., 2009. Influence of ethanol and pH on the gene
532 expression of the citrate pathway in *Oenococcus oeni*. Food Microbiology 26, 197-
533 203.
- 534 Olguín, N., Bordons, A., Reguant, C., 2010. Multigenic expression analysis as an
535 approach to understanding the behaviour of *Oenococcus oeni* in wine-like conditions.
536 International Journal of Food Microbiology 144, 88-95.
- 537 Pieterse, B., Leer, R.J., Schuren, F.H.J., van der Werf, J., 2005. Unravelling the multiple
538 effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling.
539 Microbiology 151, 3881-3894.
- 540 Ritt, J.F., Guilloux-Benatier, M., Guzzo, J., Alexandre, H., Remize, F., 2008.
541 Oligopeptide assimilation and transport by *Oenococcus oeni*. Journal of Applied
542 Microbiology 104, 573-580.

- 543 Salema, M., Lolkema, J., San Romão, M.V., Loureiro Dias, M.C. 1996. The proton
544 motive force generated in *Leuconostoc oenos* by L-malate fermentation. Journal of
545 Bacteriology 178(11), 3127-3132.
- 546 Sánchez, B., Champomier-Vergès, M.C., Anglade, P., Baraige, F., Reyes-Gavilán, C.G.,
547 Margolles, A., Zagorec, M., 2005. Proteomic analysis of global changes in protein
548 expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809.
549 Journal of Bacteriology 187(16), 5799- 5808.
- 550 Seydlová, G., Halada, P., Fišer, R., Toman, O., Ulrych, A., Svobodová, J., 2012. DnaK
551 and GroEL chaperones are recruited to *Bacillus subtilis* membrane after short-term
552 ethanol stress. Journal of Applied Microbiology 112, 765-774.
- 553 Siezen, R.J., van Enckevort, F.H.J., Kleerebezem, M., Teusink, B., 2004. Genome data
554 mining of lactic acid bacteria: the impact of bioinformatics. Current Opinion in
555 Biotechnology 15, 105-115.
- 556 Silveira, M.G., Baumgartner, M., Rombouts, F.M., Abee, T., 2004. Effect of adaptation
557 on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. Applied and
558 Environmental Microbiology 70 (5), 2748-2755.
- 559 Smid, E.J., Driessen, A.J., Konings, W.N., 1989. Mechanism and energetics of
560 dipeptide transport in membrane vesicles of *Lactococcus lactis*. Journal of
561 Bacteriology 171, 292–298.
- 562 Strobel, H.J., Russell, J.B., Driessen, A.J.M., Konings, W.N., 1989. Transport of amino
563 acids in *Lactobacillus casei* by proton-motive-force-dependent mechanisms.
564 American Society for Microbiology 171(1), 280-284.
- 565 Sumbly, K.M., Grbin, P.R., Jiranek, V., 2012. Validation of the use of multiple internal
566 control genes, and the application of real-time quantitative PCR, to study esterase

- 567 gene expression in *Oenococcus oeni*. *Applied Microbiology and Biotechnology*,
568 96(4), 1039-1047.
- 569 Tkachenko, A.G., Pshenichnov, M.R., Nesterova, L.Y., 2001. Putrescine as a factor
570 protecting *Escherichia coli* against oxidative stress. *Microbiology* 70(4), 422-428.
- 571 Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A.,
572 Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data
573 by geometric averaging of multiple internal control genes. *Genome Biology* 3(7), 1-
574 12.
- 575 Vasserot, Y., Dion, C. Bonnet, E., Tabary, I., Maujean, A., Jeandet, P., 2003. Transport
576 of glutamate in *Oenococcus oeni* 8403. *International Journal of Food Microbiology*
577 85, 307-311.
- 578 Versari, A., Parpinello, G.P., Cattaneo, M., 1999. *Leuconostoc oenos* and malolactic
579 fermentation in wine: a review. *Journal of Industrial Microbiology and*
580 *Biotechnology* 23, 447-455.
- 581 Weber, F.J., Bont, J.A.M., 1996. Adaptation mechanisms of microorganisms to the
582 toxic effects of organic solvents on membranes. *Biochimica and Biophysica Acta*
583 1286, 225–245.
- 584 Weidman, S., Rieu, A., Rega, M., Coucheney, F., Guzzo, J., 2010. Distinct amino acids
585 of the *Oenococcus oeni* small heat shock protein Lo18 are essential for damaged
586 protein protection and membrane stabilization. *FEMS Microbiology Letters* 309, 8-
587 15.
- 588 Zhao, Z., Ding, J-Y., Ma, W-H., Zhou, N-Y., Liu, S-J., 2012. Identification and
589 characterization of γ -aminobutyric acid uptake system GabP_{Cg} (NCgl0464) in
590 *Corynebacterium glutamicum*. *Applied and Environmental Microbiology* 78, 2596-
591 2601.

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 H₂O (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

610

611

612

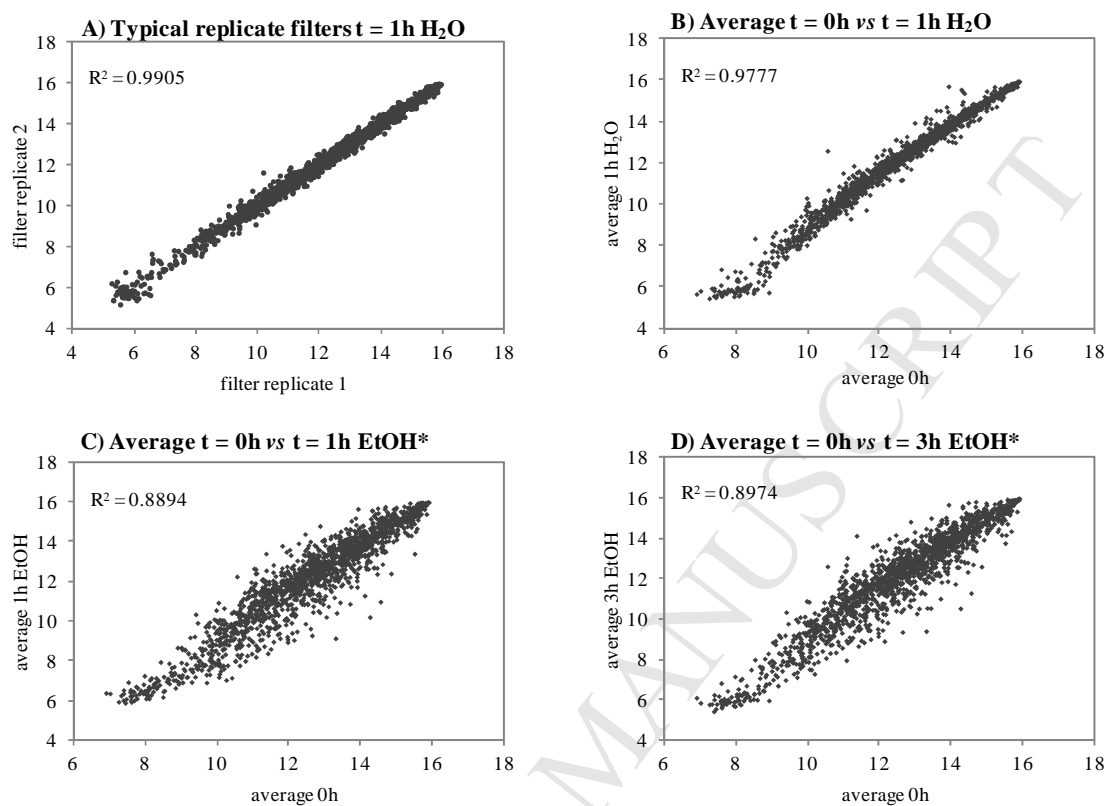


Figure 1. Scatter plots of normalized spot intensities (arbitrary units) from 1611 individual spots. A) Typical replicates showing the intensity of each spot versus the equivalent spot in a replicate filter. B), C) and D) Averaged spot intensities from a sample taken at $t = 0h$ (X-axis) versus ethanol-treated and control samples (Y-axis); $t = 1h$ H₂O (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, $P < 0.05$).

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	AACATGACGGCGTTGTTC	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 ^a	qPCR ^b
<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

^aMicroarray and ^bRT-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-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
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	Lysozyme 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: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
Inorganic Ion Transport and Metabolism		
OEOE 1355	Kef-type K ⁺ transport system. membrane component	-4.29
OEOE 0827	Mn ²⁺ and Fe ²⁺ 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
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^aThe gene names are taken from the NCBI database for *Oenococcus oeni* PSU-1 complete genome

^bThe 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/>)

^cThe 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 ^s .	Gene symbol	Gene name	Functional category Protein name (EC number)	Fold change*	Theoretical M _r (kDa)	Theoretical pI
Nucleotide transport and metabolism						
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
Coenzyme metabolism						
9	OEOE_1036	<i>pxs</i>	Pyridoxine biosynthesis enzyme, SOR/SNZ family	-3.1	31.44	5.58
Translation, ribosomal structure and biogenesis						
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 ^{Asn} /Glu-tRNA ^{Gln} 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
Energy production and conversion						
16	OEOE_1248	<i>eutG</i>	Iron-binding alcohol dehydrogenase / aldehyde dehydrogenase family domain (EC1.1.1.1)	-3.8	99.1	6.04
Cell envelope biogenesis						
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
Posttranslational modification, protein turnover, chaperones						
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
Carbohydrate metabolism						
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
Amino acid transport and metabolism						
29	OEOE_0845	<i>appF</i>	ABC-type oligopeptide transport system, ATPase component	-2.7	34.49	5.74
Signal transduction mechanisms						
30	OEOE_0807	<i>typA</i>	Stress response membrane GTPase	-3.1	68.22	5.24
General function prediction only						
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
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.

ACCEPTED MANUSCRIPT

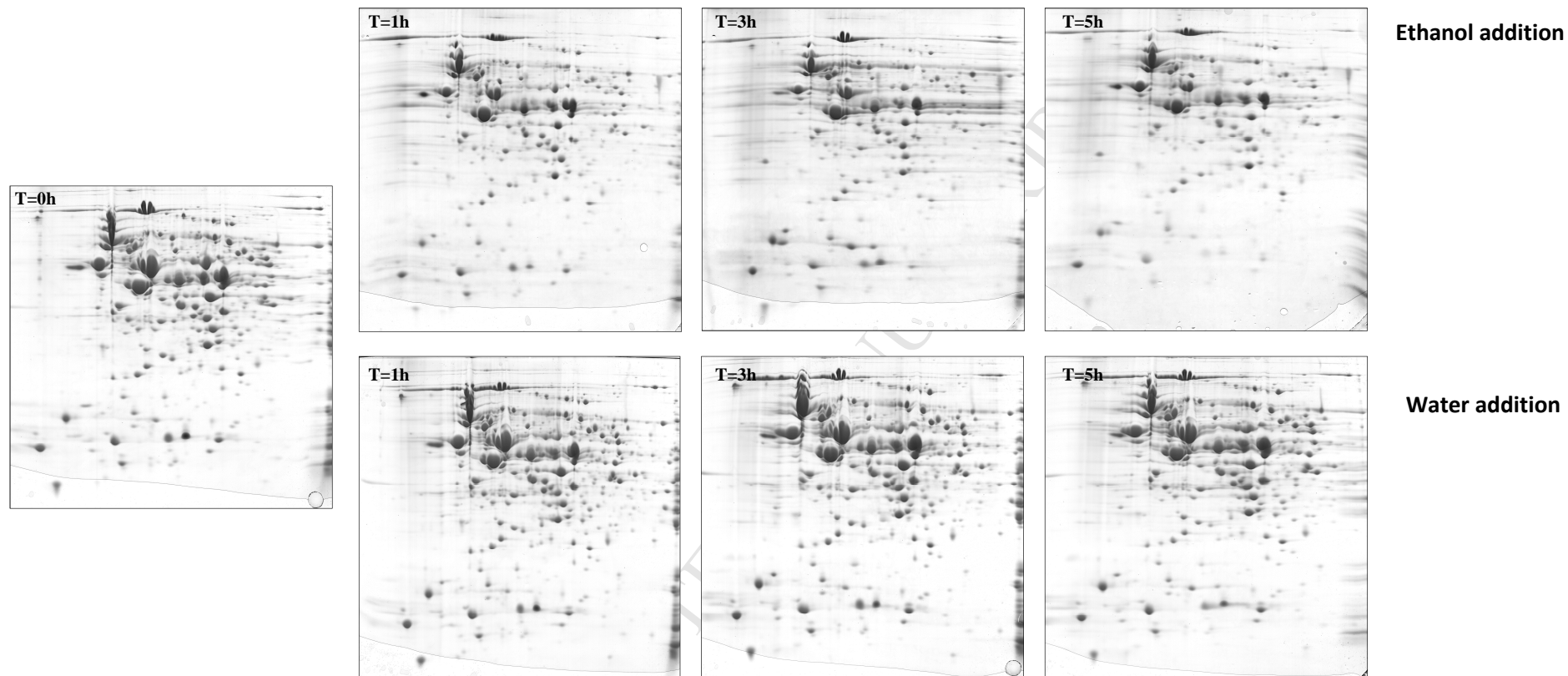


Figure S1. Pictures of 2D protein gel of the different conditions assayed: T=0h, T=1, 3 and 5 h after ethanol addition (upper side) and after water addition (lower side).

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.