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The production of preconditioned freeze-dried *Oenococcus oeni* primes its metabolism to withstand environmental stresses encountered upon inoculation into wine

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Abstract

Oenococcus oeni is the most resistant lactic acid bacteria species to the environmental stresses encountered in wine, particularly the acidity, presence of ethanol and phenolic compounds. Indigenous strains develop spontaneously following the yeast-driven alcoholic fermentation and may perform the malolactic fermentation whereby improving taste, aroma, and the microbial stability of wine. However, spontaneous fermentation is sometimes delayed, prolonged or incomplete. In order to better control its timing and quality, *O. oeni* strains are selected and developed to be used as malolactic starters. They are prepared under proprietary manufacturing processes to survive direct inoculation and are predominantly provided as freeze-dried preparations. In this study, we have investigated the physiological and molecular alterations occurring in *O. oeni* cells prepared by an industrial process that consists of preconditioning protocols and freeze-drying, and compared them to the same strain grown in a grape juice medium. We found that compared to cultured cells, the industrial production process improved survival under extreme conditions, i. e. at low pH or high tannin concentrations. In contrast, cultured cells resumed active growth more quickly and strongly than freeze-dried preparations in standard pH wines. A proteomic analysis showed that during the industrial production most non-essential metabolic processes are shut down and components of the general and the stringent stress response are upregulated. The presence of major components of the stress response facilitates protein homeostasis and physiological changes that further ensure the integrity of cells.

Keywords: lactic acid bacteria, malolactic fermentation, stress response, comparative proteomics, freeze-drying

Abbreviations: AF, alcoholic fermentation; C-cells, cultured cells; F-cells, freeze-dried cells; F/C ratio, ratio of protein expression of freeze-dried vs. cultured cells; LAB, lactic acid bacteria; MLF, malolactic fermentation; MRM, multiple reaction monitoring.

1. Introduction

The biotechnological significance of the lactic acid (LAB) bacterium *Oenococcus oeni* lies not only in its ability to perform the malolactic fermentation (MLF) in a number of alcoholic beverages of economic importance – including wine and cider – but also in the fact that it can survive and thrive in an environment that is inhospitable to most microorganisms. During MLF L-malic acid is decarboxylated by the malolactic enzyme into L-lactic acid and carbon dioxide (Lonvaud-Funel, 1999). In the wine industry this property is used to deacidify most red wines and some white wines, particularly those produced in colder climates. Although the reduction in acidity is still the main aim, other microbial metabolic processes modulate the flavour and quality of wines (Bartowsky, 2005; Bartowsky and Henschke, 2004; Liu, 2002). These include the production and/or degradation of aromatic compounds, and the degradation of other organic acids and remaining sugars after alcoholic fermentation, compounds which could facilitate the growth of unwanted microorganisms (Davis et al., 1985). MLF hence ameliorates the stability of wines through the depletion of nutrients.

Following alcoholic fermentation, wine is characterised by a low pH, a high level of ethanol, phenolic compounds at more or less important concentrations, and it may contain significant amounts of sulphur dioxide. *O. oeni* adapts best to these conditions. It develops faster than all other wine-resident LAB and becomes the predominant species immediately after AF (Bartowsky, 2005; Bech-Terkilsen et al., 2020). *O. oeni* is also associated with the production of other acidic, low alcohol-containing beverages such as cider and kombucha (Lorentzen and Lucas, 2019). In the last decade a large number of strains have been isolated and sequenced, and phylogenetic studies showed that they can be divided into four main groups (Bilhère et al., 2009; Campbell-Sills et al., 2015; Lorentzen et al., 2019): group A strains stem from wines undergoing spontaneous MLF (high alcohol), group B and C strains were mainly isolated from grape must and cider (low alcohol), and the most phylogenetically distinct group D consists of kombucha strains. Previous studies showed that within the "wine-group" A, phylogenetic clades exist consisting of strains isolated from the same type of wine (Campbell-Sills et al., 2017). The adaptation to

these specific environments is achieved through genetic predisposition of certain strains providing superior resistance to lower pH or higher concentrations of phenolic compounds (Breniaux et al., 2018).

Many studies have investigated the resistance of *O. oeni* to wine stressors. A number of stress-related genes and proteins have been identified which include F0F1-H^+ ATPases, members of the Clp protease family, the molecular chaperon Lo18 and thioredoxin (Bech-Terkilsen et al., 2020; Mills et al., 2005). F0F1-H^+ ATPases are directly coupled to MLF and are induced at low pH (Fortier et al., 2003; Tourdot-Maréchal et al., 1999). They are involved in the maintenance of the intracellular pH by exporting protons from the cytoplasm to the extracellular space. The Clp proteolytic complexes are involved in the protein quality control of the bacterial cell (Frees et al., 2007; Truscott et al., 2011). They exert functions as chaperons and in the unfolded protein response under both normal and stress conditions. Clp proteases are for example involved in the degradation of the CtsR repressor during heat and other stresses (Darsonval et al., 2018). Once CtsR is removed, the expression of numerous stress-related genes is induced, including the small heat shock protein Lo18. This latter is a molecular chaperon that prevents protein aggregation and contributes to the stabilisation of the cell membrane in response to ethanol, acid and heat stress (Delmas et al., 2001; Guzzo et al., 1997; Weidmann et al., 2017). Thioredoxins are small proteins known to be involved in the maintenance of the redox balance of the cell (Holmgren, 1989). They function as antioxidants by using a disulphide exchange mechanism and are particularly important during times of oxidative stress. The genome of the strain *O. oeni* PSU-1, for example, encodes three thioredoxin genes which are all overexpressed after inoculation of the bacterium into a wine-like medium (Margalef-Català et al., 2017).

In order to overcome problems associated with spontaneous MLF such as slow progression or stuck fermentations, as well as the production of undesired compounds, malolactic starter strains have been developed since the late 1970s (Lonvaud-Funel, 1999; Torriani et al., 2011). Resistance to the stresses encountered in wine is another one of the major selection criteria. Traditionally, strains are selected based on phenotypic tests. Nowadays, genetic tests are used for the analysis of certain characteristics, such as the production of biogenic amines. Most of malolactic starters are produced industrially in freeze-dried

99 form. After inoculation into wine, either directly or after an acclimation step (e.g. cultivation for 24h in
100 half-strength wine), growth is resumed following a few hours to several days of latency (Maicas et al.,
101 2000; Nielsen et al., 1996). Possible reasons for this delay are structural damage to the cells during the
102 dehydration process as well as a metabolic state that is unfavourable to growth in the extreme
103 environmental conditions of the wine (Bouix and Ghorbal, 2015; Bravo-Ferrada et al., 2018; Lievense et
104 al., 1994). However, the use of cryoprotective agents (e.g. certain sugars or amino acids) as well as
105 preconditioning by acid or ethanol treatments of cells prior to freeze-drying facilitates survival and faster
106 re-growth following the inoculation into wine (Hua et al., 2009; Martos et al., 2007, Yang et al., 2021,
107 2020; Zhao and Zhang, 2009).

108 In this study we have investigated the physiological and molecular changes occurring in *O. oeni*
109 strains prepared by a proprietary industrial process including a preconditioning step and freeze-drying.
110 This was achieved by comparing rehydrated, freeze-dried cells (F-cells) to cells cultivated (C-cells) in a
111 grape juice medium. First, we carried out phenotypic tests by analysing the survival and growth of F-cells
112 and C-cells of five different strains in wines adjusted to different pH values or tannin concentrations.
113 Then we used a proteomics approach to identify the molecular changes between the two types of cell
114 preparations. The results revealed important physiological changes and the involvement of stress response
115 components, some of which have never been associated with the survival of *O. oeni* in wine.
116

117 **2. Materials and methods**

118 *2.1. Bacterial strains and growth conditions*

119 The five *O. oeni* strains used in this study are selected malolactic starters produced by Lallemand
120 SAS. The pure cultures used here were designated LAB6, LAA1, LAD1, LAL01 and LAB2013. F-cells
121 provided by Lallemand SAS were produced using a proprietary industrial process. The cells were kept at
122 -20 °C and rehydrated before use in chlorine-free water following the manufacturer's recommendations.
123 Cell populations were fixed to 2.10^8 cells/mL. C-cells were prepared by inoculation into liquid grape
124 juice medium containing 25 % (v/v) commercial red grape juice, 5 g/L yeast extract, 0.1 % (v/v) Tween

125 80, and 0.1 g/L pimaricin (DSM, NL), pH 4.0 (KOH). Cultures were incubated at 25 °C until late
126 exponential phase.
127 Bacterial growth was monitored by using three principal methods: (1) The enumeration of cultivable cells
128 by plating appropriate serial dilutions on grape juice agar plates (liquid grape juice medium, 20 g/L agar).
129 Plates were incubated under aerobic conditions at 25 °C. The number of colony-forming units (CFU) was
130 determined; (2) The direct detection of viable cells by epifluorescence microscopy, whose mechanism is
131 based on the degradation of 5-carboxyfluorescein diacetate, a cell-permeant esterase substrate and the
132 release of a green-fluorescent dye (Laforgue and Lonvaud-Funel, 2012); (3) By measuring the optical
133 density of cultures at 600 nm.
134
135 *2.2. Phenotypic tests*
136 Bacterial strains were tested for their resistance to acidity and phenolic compounds as described by
137 Breniaux and colleagues (Breniaux et al., 2018) with some modifications. The resistance to polyphenolic
138 compounds was determined in a Pinot Noir red wine (12.6 % ethanol, 3.05 g/L total acidity, 0.3 g/L
139 volatile acidity, 1.5 g/L malic acid, 1.37 g/L tannins) adjusted to pH 3.6 and supplemented with 1, 2.5 or
140 5 g/L of a grape seed tannin extract, 24 hours before addition of the bacteria. The extract was previously
141 described and designated "T3" fraction (Breniaux et al., 2018): mean degree of polymerisation = 4.5,
142 1000.0 ± 64.3 mg/g total proanthocyanidins, 32.4 ± 1.0 mg/g total tannins including 7.6 ± 0.2 mg/g (+)-
143 catechin, 12.9 ± 0.3 mg/g (-)-epicatechin, and 11.9 ± 0.3 mg/g procyanidin dimers B1-B4. The resistance
144 to acidity was tested in a Gamay red wine (12.6 % ethanol, 4.57 g/L total acidity, 0.3 g/L volatile acidity,
145 2.4 g/L malic acid, 1.53 g/L tannins) adjusted to pH 2.8, 3.0, 3.3, 3.6 and 4.0, respectively, using
146 orthophosphoric acid or potassium hydroxide.
147 For all phenotypic tests, *O. oeni* strains in freeze-dried and cultured form were inoculated at
148 2.10^6 cells/mL. The development of preparations of cultured *O. oeni* strains prior to the inoculation into
149 wine was followed by measuring the optical density (OD) at 600 nm. Then, the number of viable cells
150 was determined by direct detection using epifluorescence microscopy (Laforgue and Lonvaud-Funel,

2012) to estimate the volume of culture necessary to reach about 2.10^6 cells/mL. Cells were pelleted by centrifugation and transferred into wine. Following inoculation, samples were taken immediately to determine the number of cultivable cells at time point zero (T0) by plating them onto grape juice agar plates. Thereafter, cell numbers were determined two, seven and 14 days after inoculation (Fig. S1, S2). Plates were incubated at 25 °C under aerobic conditions.

The Log10 of the CFU per mL was determined for each time point and condition and was then normalised against T0. Box and Whisker Plots, and Scatter Plots were created using the boxplot and scatterchart function in RStudio. Pairwise comparisons between freeze-dried and cultured cells under different conditions were carried out using the Wilcoxon-Mann-Whitney rank-sum test. A nonparametric test was chosen, first, because data of the majority of datasets followed a non-normal distribution (Shapiro-Wilk) and second, because the power of the parametric Student's t-test is low when analysing small datasets (ten data points each).

2.3. Preparation of samples for quantitative proteomics

Freeze-dried LAB6 cells were rehydrated, pelleted and frozen at -80 °C. C-cells were prepared by inoculating freeze-dried *O. oeni* LAB6 into grape juice medium at 10^6 CFU/mL. Cells were cultured at 20 °C for 7 days and then at 25 °C until they reached the end of the exponential growth phase. Cell pellets were washed with 0.9 % NaCl solution and frozen at -80 °C. For each condition five replicates were prepared. Cell pellets were resuspended in 120 µL lysis buffer (50 mM sodium bicarbonate, 1 U/µL mutanolysin per 4.10^7 cells) and incubated for 1 h at 37 °C. A final concentration of 5 % sodium deoxycholate (DOC) and 20 mM dithiothreitol (DTT) were added to each sample, followed by sonication on ice using a Bioruptor system (Diagenode) with 30 s on/ off cycles (15 times) at high level, to complete cell lysis. After centrifugation at 16,000 x*g* for 5 min to remove cell debris, the total protein concentration was determined in the supernatants using the Bradford protein assay.

2.4. Whole proteome analysis by label-free relative quantification using nano LC-MS/MS

177 *2.4.1. Sample preparation*

178 Before proteolytic digestion, the DOC concentration was adjusted to 1 %. For each sample, 10 µg
179 protein was denaturated for 5 min at 95 °C, reduced with 0.2 mM DTT for 30 min at room temperature
180 and alkylated with 0.7 mM iodoacetamide for 30 min at room temperature and protected from light.
181 Proteins were then digested by the addition of trypsin (Promega) at a 1:50 (enzyme:protein) ratio.

182 After the addition of 50 % formic acid (pH 2) to stop the reaction and to precipitate the DOC, the
183 samples were centrifuged at 16,000 x*g* for 5 min. The peptides collected in the supernatants were purified
184 using StageTips (Rappsilber et al., 2007) and vacuum-dried. Peptides were resuspended at a concentration
185 of 0.2 µg/µL in loading buffer (2 % acetonitrile (ACN), 0.05 % trifluoroacetic acid (TFA)).
186 5 µL of each sample (1 µg of peptides) were analysed by nano LC-MS/MS using an UltiMate 3000
187 RSLCnano liquid chromatography system coupled to an Orbitrap Fusion Tribrid Mass Spectrometer
188 (ThermoFisher Scientific). The peptides were trapped at 20 µL/min in loading buffer on a C18
189 PepMap100 pre-column (300 µm ID x 5 mm) for 5 min. Then, the pre-column was switched online with
190 an analytical C18 PepMap100 column (75 µm ID x 500 mm length, 3 µm, 100 Å,) equilibrated in 95 %
191 buffer A (0.1 % FA) and 5 % buffer B (80 % ACN/0.1 % FA) at a flow rate of 300 nL/min. The peptides
192 were eluted with a linear gradient of 5-40 % buffer B during 90 min. Mass spectra were acquired using a
193 data-dependent acquisition mode and the Xcalibur software v.4.1.50 (ThermoFisher Scientific). Survey
194 scans were acquired in the Orbitrap at a resolution of 120,000 setting the automatic gain control (AGC)
195 target to 4x 10^5 and the maximum ion injection time to 50 ms. Each MS scan was followed by the
196 acquisition of MS/MS spectra of the most intense precursor ions for a total cycle time of 3 s (TopSpeed
197 mode). The selected ions were isolated using the quadrupole analyser in a window of 1.6 m/z and
198 fragmented by higher energy collisional dissociation (HCD) with 35 % normalised collision energy
199 (NCE). The resulting fragmented ions were detected by the linear ion trap at rapid scan rate with an AGC
200 target of 1e4 and a maximum injection time of 50 ms. A dynamic exclusion of previously fragmented
201 precursors was set for a duration of 20 s with a tolerance of 10 ppm. Internal lock mass calibration was
202 used on the 445.12003 m/z siloxane ion.

203

204 *2.4.2. Database search and label-free quantification*

205 Spectra were searched against the complete reference proteome of *O. oeni* AWRIB429 database
206 (ID: 655225, proteome ID: UP000003075, protein count: 2159) using the Andromeda module of the
207 MaxQuant software v.1.6.6.0 (Tyanova et al., 2015). The following parameters were selected: Trpysin/P
208 with two possible missed cleavages; fixed modification: cysteine carbamidomethylation; variable
209 modifications: methionine oxidation, asparagine and glutamine deamidation; mass search tolerances:
210 5 ppm (MS) and 0.5 Da (MS/MS). For validation, a maximum false discovery rate (FDR) of 1 % at
211 peptide and protein level was used based on a target/decoy strategy. MaxQuant was also used for label-
212 free quantification with a minimum ratio count of 1. The 'match between runs' algorithm was used. The
213 alignment time window was set to 10 min and the match time window to 3 min. Only unique razor
214 peptides were used for quantification. All other parameters were left at default values.

215

216 *2.4.3. Data treatment and statistical analysis*

217 The peptide.txt file generated by MaxQuant was imported into the R software (Cox and Mann,
218 2008; The R Core Team, 2017). Intensity values were first normalised using the median of all the
219 intensity values in each sample. Only peptides with a quantification value in four samples over the five
220 replicates in at least one of the two groups were considered as quantifiable. The remaining missing values
221 were attributed using a noise value calculated as the first percentile of all the intensity values per sample.
222 The intensities of all the quantifiable peptides were then summed by their *leading razor protein* accession
223 number in order to obtain quantification at the protein level. Only proteins with at least two quantified
224 peptides were kept for further analysis. For differential expression analysis, a protein ratio between
225 freeze-dried/cultured (F/C ratio) conditions was calculated using the average of protein intensities in the
226 five samples of each group. The ratios were then converted into z-scores to centre the data. A *p*-value was
227 also calculated between the two groups using the Limma Bioconductor package and was adjusted with
228 the Benjamini-Hochberg method for multiple testing (*q*-value) (Benjamini and Hochberg, 1995).

Proteins with a F/C ratio >2 were considered upregulated, while a ratio <0.5 indicated down-regulation. Candidate proteins with a |z-score| >1.96 and a *q*-value <0.05 were considered as significantly regulated between the two conditions.

2.5. Validation of label-free experiments by targeted proteomics using Multiple Reaction Monitoring (MRM)

2.5.1. Sample preparation

18 proteins of interest were selected from the label-free experiment to be validated by targeted proteomics using MRM analysis. For that, five biological replicates of each group (freeze-dried and cultured) were prepared following the same procedure as described in section 2.4.1.

2.5.2. Stable-isotope-labelled standard (SIS) peptides

For each protein, one or two peptides were selected to perform quantification. For each of them, crude synthetic peptides (PEPotec) containing [^{13}C6]-Lys and [^{13}C6]-Arg (ThermoFisher Scientific) were synthesised to be used as internal standards. Peptides were diluted and pooled to a final concentration ranging from 0.1 to 100 pmol/µL. 1 µL of peptide standard solution was added to 6 µL of reconstituted protein sample prior to nano LC-MRM.

2.5.3. LC-MRM analysis

1 µg of peptides (in 6 µL) were analysed on an UltiMate 3000 RSLCnano (ThermoFisher Scientific) liquid chromatography system coupled via a NanoSpray ionisation source to a ABSciex 6500QTRAP^{TM} hybrid triple quadrupole/linear ion trap mass spectrometer controlled by the Analyst 1.6. software. Chromatographic conditions were kept identical as those used in the label-free experiments, except the duration of the elution gradient was reduced from 90 to 30 min. MS analysis was conducted in positive ion mode with an ion spray voltage of 2500 V. Nebulizer gas was set to 12 (Gas1), curtain gas to

25 and interface heater to 150 °C. Four transitions of each peptide were scheduled during 5 min along the gradient, with a fixed cycle time of 1.5 s.

2.5.4. MRM data analysis

The quantification was done with Skyline v.19.1. and was based on the relative areas of the SIS and endogenous peptides (MacLean et al., 2010). The area of the most intense transition per peptide was first normalised by applying a normalisation factor calculated on the median signal across the samples of the heavy peptide. The sum of the two peptides was calculated in order to obtain quantification at the protein level. Proteins with a F/C ratio >2 were considered upregulated, while a ratio <0.5 indicated down-regulation. Candidate proteins with Welch *p*-value <0.05 were considered as significantly regulated between the two conditions.

3. Results

3.1. Resistance of F- and C-cells of five O. oeni strains to different stressors in wine

The effect of the industrial process of preconditioned freeze-drying was evaluated in terms of the resistance of different *O. oeni* strains to increasing acidity and tannin concentrations in wine. For this, five different strains prepared according to the industrial process by Lallemand SAS were used: LAB6, LAD1, LAA1, LAL01 and LAB2013. Cells were either inoculated directly into the wine after rehydration of the F-cells or first cultured in grape juice medium and harvested at late exponential phase for inoculation (C-cells). The number of cultivable cells on grape juice agar plates was determined at inoculation (day 0) and 2, 7 or 14 days after inoculation.

Generally, the differences between C-cell and F-cell preparations and the effect on cell viability are less apparent in regard to pH as compared to increasing tannin concentrations (Fig. 1). Cells of both preparations resisted well a decrease in pH (Fig. 1A, Suppl. Fig. S1). At day 2, little difference is observed, albeit C-cells survive significantly better at higher pH (3.6 and 4.0). At day 7, it becomes clear that there are large strain-depended variations at the lowest pH, though the variability is less among F-cell

280 strains. At day 14, there is a tendency, particularly for F-cells, to exhibit better viability with increasing
281 pH. As the resistance to particularly challenging environmental conditions is linked to the genotype, the
282 largest difference between strains was seen at the lowest pH (pH 2.8). The observed inter-strain variations
283 are, however, smaller for F-cells compared to C-cells. Furthermore, although one would expect to see
284 differences between strains originally isolated from red wine (LAB6, LAL01) and white wine (LAD1,
285 LAA1, LAB2013), none were identified under the experimental conditions used (Suppl. Fig. S1).

286 The differences between the two cell preparations are more striking in the case of increasing
287 concentrations of tannins (Fig. 1B, Suppl. Fig. S2). F-cells are hardly affected and tolerate high
288 concentrations of tannins remarkably well. At all time points and all tannin concentrations there is only
289 little loss in cell viability. A large variability between strains is, however, observed in the case of C-cell
290 preparations as well as a significant decrease in viable cells with increasing tannins concentrations. The
291 red wine strains LAB6 and LAL01 exhibit a better resistance to tannins compared to the white wine
292 strains (Suppl. Fig. S2).

293

294 *3.2. Comparative proteomics analysis*

295 In order to identify metabolic differences between F- and C-cells of *O. oeni*, a mass spectrometry-
296 based proteomics approach was taken. The LAB6 strain was chosen as it exhibits good survival and
297 growth capacities for both F- and C-cell preparations under standard wine conditions. These are
298 considered to be at a pH above 3.3 and a polyphenol content of around 2.5 g/L (Herderich and Smith,
299 2005). Furthermore, it is one of the most widely used strains for wine production (communication:
300 Lallemand SAS) and hence of interest to the wine industry. The genome sequence of *O. oeni* AWRIB429
301 (Borneman et al., 2012) was used as reference as it is closely related to the genome sequence of LAB6,
302 which is not currently available (manuscript in preparation). An overview of the workflow is given in
303 Fig. 2. Briefly, proteins from five replicates of each condition (F-cells and C-cells) were extracted and
304 trypsin digested. Label-free relative protein quantification was performed to identify proteins of interest
305 which are differentially expressed between the two different sample sets. From these initial data, 18

306 proteins were selected for the targeted approach and analysed by MRM mass spectrometry. The selection
307 of the proteins was based on the results of the non-targeted approach and the potential relevance of
308 proteins in the context of stress adaptation.
309
310 *3.2.1. Non-targeted approach/ whole proteome analysis*
311 The non-targeted approach was used to obtain an overview of pathways that are differentially
312 regulated in F-cells relative to C-cells.
313 In preparation of the experiments, freeze-dried LAB6 cells were rehydrated, and two sample sets
314 were generated: Set 1 was immediately frozen after rehydration (F-cells); cells of set 2 were cultured in
315 "low stress" grape juice medium until reaching late exponential growth phase and then frozen (C-cells).
316 Subsequently, both sets were analysed by LC-MS/MS using label-free quantification of relative protein
317 expression levels (Bantscheff et al., 2012).
318 Among a total of 297 proteins identified with this approach, 125 proteins were identified with at
319 least two peptides and used for relative quantification (Tab. S1). The relative amounts of these 125
320 proteins were determined in F-cells relative to C-cells as shown (Fig. 3, Tab. S1). An unusually high
321 proportion of the proteins were either present or absent in one or the other condition, suggesting that the
322 industrial preparation process results in some important changes. To obtain a better understanding of
323 altered functions and metabolic pathways in F-cells, identified proteins were sorted into Cluster
324 Orthologues Groups (COG) categories. Using this approach, we saw a strong division in the processes
325 which were either up or downregulated. Most remarkably, 83 proteins, and hence the majority of COG-
326 identified processes, were downregulated. These included the transport and metabolism of amino acids,
327 lipids, nucleotides and secondary metabolites, as well as the biogenesis of cell envelope components,
328 signal transduction processes and transcription. Of the 42 upregulated proteins, most participated in two
329 COG processes which fall into the main functional group of "Information Storage and Processing".
330 Upregulated proteins included several ribosomal proteins, elongation factor G and peptide chain release
331 factor 3 (COG category "Translation, ribosomal structure and biogenesis") along with stress response

factors such as the chaperonins GroEL, Hsp/Lo18 and trigger factor (COG category "Posttranslational modification, protein turnover, chaperons"). A mixture of up as well as downregulated enzymes were found in the categories of carbohydrate transport and metabolism, as well as energy production and conservation. The expression of six proteins was significantly regulated in F-cells relative to C-cells (see asterisks in Fig. 3), based on z-score and *q*-value criteria (see Methods section). Statistically significant downregulation was observed for a putative oligopeptide ABC transporter DppE (OEOE_v1_10138), a putative metal-dependent hydrolase VtkL (OEOE_v1_60039) and chromate reductase ChrR (OEOE_v1_30035), while significant upregulation was observed for the known stress-response factors Lo18 (OEOE_v1_90017) and the large subunit of the chaperonin GroE (OEOE_v1_10025), as well as for polyphosphate kinase Ppk (OEOE_v1_10285).

3.2.2. Targeted approach

From the non-targeted approach, 18 proteins were selected for validation by a targeted MRM mass spectrometry approach. Target proteins were selected (Tab. 1) either based on a marked difference in expression detected in the non-targeted approach and/or because they are known to be involved in the cellular stress response and resistance. One or two peptides for each protein were selected for detection. For each of them a synthetic, labelled peptide was synthesised to be used as internal standard for quantification and added to the samples before analysis (Tab. S2). The same samples as in 3.2.1. were used for analysis.

Six out of 18 proteins were identified with a F/C ratio ≥2 suggesting their upregulation in F-cells relative to C-cells (Tab. 1). Those six proteins were already detected as up-regulated proteins in the label-free approach. This is interesting due to their importance in the context of stress resistance. Specifically, these were the small heat shock protein Lo18 (Hsp), the polyphosphate kinase (Ppk), the large subunit of the chaperonin GroE (GroEL), the 50S ribosomal protein L11 (RplK), the elongation factor G (FusA) and the molecular chaperon DnaK/Hsp70.

Six out of 18 proteins were determined to be significantly downregulated in F-cells with a F/C ratio ≤0.5 (Tab. 1), including the organic pyrophosphatase Ppc, glutathione reductase (GshR), glyceraldehyde-3-phosphate dehydrogenase (Gap), the substrate binding subunit of an ABC-type amino acid transporter (Aat), the substrate binding subunit of an oligopeptide-binding protein (DppE) and the trigger factor (Tig). The protein enolase (Eno) was not downregulated with statistical significance, neither in the targeted nor the non-targeted approach.

Five proteins turned out to be not regulated with an F/C ratio between 0.5 and 2. They were initially chosen for the targeted analysis because they either showed upregulation in the non-targeted approach and/or they are known to be involved in stress resistance and adaptation. Among which were two ClpC protease subunits (Grandvalet et al., 2005), as well as the F0 and the F1 subunits of a F0F1-type ATP synthetase (Fortier et al., 2003).

4. Discussion

The survival of *O. oeni* after inoculation into wine is one of the most critical steps when malolactic starters are used to promote MLF. This is because the rate of cell survival is directly linked to the onset and duration of the MLF (Lonvaud-Funel, 2002). Among the currently available products of malolactic starter strains, freeze-dried preparations are the most conventional ones. This process removes water from a frozen sample by sublimation, with the advantage of providing high bacterial viability (Papadimitriou et al., 2016). However, the process of freeze-drying exposes cells to several stresses including low temperature, the reduced availability of nutrients as well as osmotic and oxidative stress (Schott et al., 2017). This can have negative effects for example on membrane integrity, protein folding, the redox status, and the overall viability of cells. To mellow the negative effects of freeze-drying, cells can be preconditioned to certain stresses (Yang et al., 2019, 2020, 2021), suggesting that cross stress-response reactions may occur. The conditions must, however, be carefully chosen depending on the microorganism and the specific strain as well as its application (Gaucher et al., 2019). In this study, the process used for preparing the freeze-dried strains is called MBR (membrane re-enforced). It is

intellectual property of the Lallemand company, and the protocol is hence not publicly available. The physiological state and the composition of the membrane are affected by culture conditions including the temperature (Chu-Ky et al., 2005; Maitre et al., 2014). Thus, stabilising or re-enforcing the membrane certainly impacts its composition by activating enzymes that allow cyclisation of fatty acids or their saturation. Indeed, it has been known for some time that *O. oeni* is capable of altering its membrane lipid composition to adjust bilayer fluidity in response to alcohol or low pH stress (Grandvalet et al., 2008; Ingram, 1976; Maitre et al., 2014; Teixeira et al., 2002). Furthermore, it has been shown that modifications in the production of exopolysaccharides (EPS) improve the survival of *O. oeni* in wine and wine-like media, especially when cells have been preconditioned in the presence of ethanol and/or low pH before freeze-drying (Dimopoulou et al., 2018; Yang et al., 2019, 2020, 2021). Our data confirmed that preconditioned freeze-dried cell preparations show less overall variation in cell viability between strains as compared to cultured cells. Under certain conditions, particularly in red wine at low pH and high concentrations of tannins, F-cells showed superior survival. This suggests the activation of stress adaptation processes during the industrial production of these malolactic starter strains. Bravo-Ferrada and colleagues studied the survival of cells after inoculation into wine-like medium comparing two different freeze-drying protocols to cultured cells (Bravo-Ferrada et al., 2018). In agreement with our data, they found that F-cells exhibit a higher survival rate after direct inoculation, and they performed better at MLF compared to cultured cells. This again suggests a superior ability of F-cells to acclimate to typical wine conditions by developing an appropriate stress response and adapting their general metabolism during the freeze-drying process through cross stress-response reactions.

This improved survival of F-cells after inoculation into wine may be related to a pool of chaperones present in the bacteria that can protect cells. Indeed, it is well known that under stressful conditions a number of different heat shock proteins are produced (chaperons and proteases). They ward off the potentially deleterious effects of aggregating denatured proteins by either refolding and repair or proteolytic degradation and removal (Nakamoto and Vígh, 2007; Narberhaus, 2002). The production of heat shock proteins under various stress conditions that assist with refolding of damaged proteins and/or

act at the membrane level is well documented in *O. oeni* (Coucheney et al., 2005; Darsonval et al., 2016; Guzzo et al., 2002; Li et al., 2018; Maitre et al., 2014). Our study shows that chaperons including GroEL, Lo18 (small heat shock protein, sHsp) and DnaK were upregulated in F-cells. The expression of these three chaperones is controlled in *O. oeni* by the negative master regulator of the stress response, CtsR (class three stress-response regulator) (Grandvalet et al., 2005). The chaperones GroES/L and DnaK/J are also expressed under normal conditions in order to maintain general protein homeostasis. However, they are upregulated when exposed to stress factors (Fukuda et al., 2002; Grandvalet et al., 2005; Rosenzweig et al., 2019). The sHsp Lo18 is specifically expressed in response to stress and fulfils a dual role as chaperonin and as stabiliser of the cell membrane to ensure cellular integrity (Maitre et al., 2014). Other genes that are under the control of CtsR include a number of proteases such as ClpC, ClpP and ClpE (Beltramo et al., 2006; Darsonval et al., 2018). Both ClpC proteins were detected in slightly higher quantities in the non-targeted approach but were found not to be regulated in the targeted approach (Fig. 3, Tab. 1). No other Clp protein was detected in either of the approaches. Clp proteases are part of the general stress response systems and are responsible for the degradation of unfolded or damaged proteins. ClpC/L1 has also been identified to be responsible for the recruitment of CtsR to the ClpP-ClpE/L2 proteasome (Darsonval et al., 2018). The fact that none of the Clp protease components have been detected to be upregulated could suggest on the one hand that protein damage was low before and during freeze-drying and as a consequence the expression of the proteases was not induced. On the other hand, it is possible that these components were not yet induced as they represent a second "escalation level" of the cellular stress response which is the case when pure chaperoning is not enough anymore to restore or maintain protein homeostasis.

Among the upregulated proteins, results also indicate the production of proteins related to the stringent response such as Ppk (polyphosphate kinase) and the L11 ribosomal protein. The stringent response usually develops under nutritional deprivation, however, it has been shown that the ability of bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Lacticaseibacillus casei* (formerly *Lactobacillus casei)* and others, to adapt to various stresses is greatly diminished in the absence of Ppk

(Gray and Jakob, 2015; Rao and Kornberg, 1999; Rashid et al., 2000). Ppk is an enzyme involved in the production of polyphosphate (poly-P) from ATP. Work on *L. casei* and *Limosilactobacillus fermentum (*formerly *Lactobacillus fermentum)* strains isolated from mozzarella cheese whey has shown that the appearance of poly-P granules may represent a defence mechanism to counteract the low pH found in this particular environment (Aprea et al., 2005). Moreover, an inactive mutant *ppk* in *L. casei* BL23 impaired growth under high-salt or low-pH conditions and increased sensitivity to oxidative stress compared to a wildtype *ppk* containing strain (Alcántara et al., 2014). Interestingly, (p)ppGpp, a molecule characteristic of the stringent response, is involved in the stress response in some LAB (Rallu et al. 2000), where it plays a role in modulating poly-P levels by inhibiting Ppx (exopolyphosphatase) activity (Rallu et al., 2002; Rao and Kornberg, 1999). Furthermore, the ribosomal protein L11, a regulator of the stringent response by interaction with the ribosome-associated (p)ppGpp synthetase RelA, is upregulated under various stress conditions (Agrawal et al., 2018), and also increased in our study. Finally, the downregulation of the enzyme inorganic pyrophosphatase/ exopolyphosphatase, which is involved in the degradation of polyphosphate is consistent with these data.

The adaptation to the modification of environmental conditions is accompanied by metabolic remodelling occurring in *O. oeni* during inoculation into wine with and without acclimation (Cecconi et al., 2009; Margalef-Català et al., 2016; Yang et al., 2019), and which is crucial for the survival and the malolactic metabolism of the bacteria. Interestingly, the non-targeted approach revealed downregulation of processes involved in lipid metabolism and cell envelop biogenesis. We presume that LAB6 F-cells used in this study were preconditioned to wine-like conditions or certain physicochemical parameters which would induce a modification of the lipid composition of the cell membrane, thanks to Fab or CFA synthase and the production of EPS (Dimopoulou et al., 2018; Maitre et al., 2014; Yang et al., 2019, 2020, 2021). This would explain why enzymes of the lipid metabolism are negatively regulated: the adaptation has already been completed during preconditioning, and the enzymatic components involved will have already been degraded or would be in the process of degradation. Furthermore, C-cells which were harvested at the end of the exponential growth phase, showed high concentrations in enzymes

involved in cell envelop biosynthesis, compared to F-cells, that were most likely not actively growing before freeze-drying.

In the same context another observation was, that most processes involved in protein synthesis were downregulated in respect to cultured cells, whereby many proteins of the "Translation, ribosomal structure and biogenesis" COG category were detected with F/C ratios suggesting upregulation. This is a bit puzzling as one would expect to have a larger number of ribosomes or translation associated proteins present in actively growing cells. However, we do not actually know the state of the ribosomes and associated proteins, as mass spectrometry only detects peptides and does not tell us whether a protein is properly folded or active. It is possible that following a lush growth phase these ribosomal and accessory proteins were actually damaged during preconditioning. In order to avoid further cytotoxic effects, the cell would stop synthesis of all new proteins (Albert et al., 2019; Rene and Alix, 2011). A glutathione reductase which can signal oxidative stress in cells was found to be strongly downregulated in F-cells in the targeted approach (Tab. 1). In fact, this enzyme may not be a glutathione-specific reductase as in most *O. oeni* strains and related LAB it is annotated as a more general NAD(P)/FAD-dependent oxidoreductase. Among proteins linked to transport functions, a particularly interesting one is the dipeptide ABC transporter substrate binding subunit DppE which was significantly downregulated in both approaches in F-cells. Its gene is only present in the genomes of about 40 % of all sequenced *O. oeni* (data not shown). DppE is highly expressed in actively growing cells (Fig. 3, Tab. 1) and might provide additional advantages to certain strains in accessing nitrogen sources. An orthologous gene (OEOE_v1_260001, Tab. S1) can be found as part of the *opp*-operon which encodes subunits of an oligopeptide ABC transporter.

Under conditions of environmental stress, LAB alter metabolic and energy fluxes by adapting their carbon source metabolism. This was shown for example for the growth of *L. lactis* at two different pHs, where significant alteration in protein abundance and phosphorylation status were observed (Koponen et al., 2012). Therefore, the ability of LAB to efficiently transport and metabolise carbohydrates and other

carbon sources, such as malate or citrate, under conditions of environmental stress is crucial for growth and persistence.

5. Conclusion

It is important to understand the direct consequences of preconditioning industrial freeze-dried cell preparations on the stress resistance of *O. oeni* in wine as they strongly influence the MLF performance of strains. Furthermore, due to modifications of the enzyme pool, and in particular of metabolic enzymes, other parameters of the wine could be affected, such as the productions of aromatic compounds. By comparing F-cells and C-cells this work revealed the superior survival of F-cells in harsh wine conditions, whichever the strain. It also revealed the shut-down of non-essential metabolic processes and the up-regulation of the general and stringent stress response in these F-cells. In agreement with previous studies, this work disclosed molecular components (e.g. Lo18, GroEL, DnaK) which were already known to contribute to the stress response in *O. oeni.* Interestingly, it also uncovered new components (the polyphosphate kinase Ppk, the ribosomal protein L11), which had never been associated with the stress response in this bacterium and deserve future investigations.

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Author's contributions

SM, MB, OC, CG and SB contributed to the study design, performed experiments, data analyses and manuscript revision. MDB and SK provided biological material. SW and JR performed data analyses and prepared the manuscript. PL contributed to the study design, performed data analyses and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Figure legends

Fig. 1. Comparison of freeze-dried (F) and cultured (C) cell preparations and their survival in wine. Boxplots showing plate cell counts of colony-forming units (CFU) are shown for F-cells (empty boxes) and C-cells (grey boxes) preparations of *O. oeni* strains LAB6, LAD1, LAA1, LAL01 and LAB2013 in red wine. A) Cells were inoculated into red wine adjusted to pH 2.8, 3.0, 3.3, 3.6 and 4.0. The same data are depicted in form of a scatterplot (insets): square, pH 2.8, circle, pH 3.0, triangle, pH 3.3, cross, pH 3.6, diamond, pH 4.0. B) Cells were inoculated into red wine adjusted to pH 3.6 supplemented with 0, 1, 2.5 and 5 g/L of grape seed tannin extract. Experiments were carried out in duplicate. The number of cultivable cells was determined 2, 7 and 14 days after inoculation. The same data are depicted in form of a scatterplot (insets): square, 0 g/L, circle, 1 g/L, triangle, 2.5 g/L, diamond, 5 g/L. Cell numbers were normalised against T0 (inoculation time) and pairwise comparisons between the different conditions were conducted using the nonparametric Wilcoxon-Mann-Whitney rank-sum test. F- and C-cell dataset pairs, that were significantly different, are indicated by a square bracket and the *p*-values are reported as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Fig. 2. Workflow of the proteomics experiments. The expression levels of proteins were measured in freeze-dried, rehydrated *O. oeni* LAB6 (F-cells) or cells grown in grape juice medium (C-cells) as controls. In the non-targeted (1) or label-free mass spectrometry experiments, the relative expression levels were determined for detected peptides of the whole proteome. 18 proteins were then selected for validation in a targeted approach (2) using multiple reaction monitoring (MRM) mass spectrometry. Expression levels were calculated against internal controls of synthesised protein-specific peptides. Five biological replicates were prepared and analysed for each condition.

Fig. 3. Relative protein expression levels determined by non-targeted mass spectrometry. 125 proteins were quantified and sorted into clusters of orthologous groups (COG) belonging to the following

categories: black, Cellular Processes and Signalling; red, Information Storage and Processing; blue, Metabolism; grey, Poorly Characterised. The ratio (F/C) of protein expression was determined in freeze-dried *O. oeni* LAB6 cells (F) vs. cultured cells (C). Upregulated expression is shown in green and downregulated expression is indicated with yellow horizontal columns. Statistically significant (|z-score| >1.96 and *q*-value <0.05) samples are indicated with an asterisk.

Table 1. Results of the non-targeted vs. targeted mass spectrometry approaches.

protein name based on annotation	COG Cat.	Locus tag (LAB6)	Gene	NON-TARGETED APPROACH				TARGETED APPROACH		
				ratio F/C	*p*-value	*q*-value	signif *	ratio F/C	*p*-value	signif **
molecular chaperon, small heat shock protein (Lo18)	▲O	90017	***hsp***	**2954**	3.23	9.35×10^{-8}	up	**188.1**	7.04×10^{-2}	-
polyphosphate kinase	■F	10285	***ppk***	**92.6**	1.96	1.46×10^{-4}	up	**28.7**	2.20×10^{-1}	-
chaperonin GroE large subunit	▲O	10025	***groEL***	**125.3**	2.07	6.33×10^{-5}	up	**25.0**	1.28×10^{-1}	-
50S ribosomal protein L11	★J	10048	***rplK***	**73.4**	1.88	1.36×10^{-4}	-	**8.1**	1.21×10^{-1}	-
elongation factor G	★J	60010	***fusA***	**27.4**	1.52	4.95×10^{-7}	-	**2.2**	5.20×10^{-2}	-
molecular chaperone DnaK (HSP70)	▲O	60015	*dnaK*	1.2	0.38	6.02×10^{-1}	-	**2.2**	9.89×10^{-3}	up
F0F1-type ATP synthase, subunit α (component F1)	■C	110007	*atpA*	**45.2**	1.70	1.34×10^{-7}	-	0.9	7.14×10^{-1}	-
Clp protease, ATP-binding subunit	▲O	160009	*clpC*	**4.4**	0.85	5.29×10^{-3}	-	0.9	4.46×10^{-1}	-
F0F1-type ATP synthase, subunit c (component F0)	■C	110004	*atpE*	**2.9**	0.70	1.19×10^{-2}	-	0.8	3.77×10^{-1}	-
Penicillin V acylase or related amidase	▲M	30198	*yxeI*	**2.6**	0.67	3.83×10^{-3}	-	0.8	4.59×10^{-1}	-
Clp protease (putative), ATP-binding subunit	▲O	50083	*clpC*	**3.3**	0.75	1.81×10^{-1}	-	0.7	1.14×10^{-1}	-
FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor)	▲O	10390	*tig*	**4.8**	0.88	1.56×10^{-1}	-	**0.5**	4.57×10^{-2}	down
inorganic pyrophosphatase/ exopolyphosphatase (Mn2+-dependent)	■C	30188	***ppaC***	**0.12**	-0.46	3.27×10^{-5}	-	**0.5**	1.06×10^{-3}	down
enolase	■G	10305	*eno*	**4.0**	0.82	2.32×10^{-1}	-	**0.3**	9.69×10^{-2}	-
glutathione reductase	■C	80078	*gshR*	**11.2**	1.19	4.49×10^{-4}	-	**0.03**	2.60×10^{-2}	down
glyceraldehyde-3-phosphate dehydrogenase	■G	40064	***gap***	**0.07**	-0.66	9.34×10^{-8}	-	**0.005**	2.65×10^{-5}	down
ABC-type amino acid transport/signal transduction system (substrate binding)	■E	10321	***aat***	**0.01**	-1.52	8.21×10^{-9}	-	**0.003**	1.04×10^{-5}	down
putative oligopeptide ABC transporter, periplasmic oligopeptide-binding protein	■E	10138	***dppE***	**0.002**	-1.98	1.08×10^{-9}	down	**0.003**	3.29×10^{-5}	down

▲ cellular processes and signaling; ■ metabolism; ★ information storage and processing

Bold, regulation (up or down) is the same in both approaches; green, F/C ratio ≥ 2 (up-regulation); red, F/C ratio ≤ 0.5 (down-regulation). F, value for freeze-dried cells; C, value for cultured cells; signif., significant regulation; * z-score > |1.96| and q-value (Limma) < 0.05; ** ratio ≥ 2 (up) or ≤ 0.5 (down) and p-value (Welch) < 0.05.

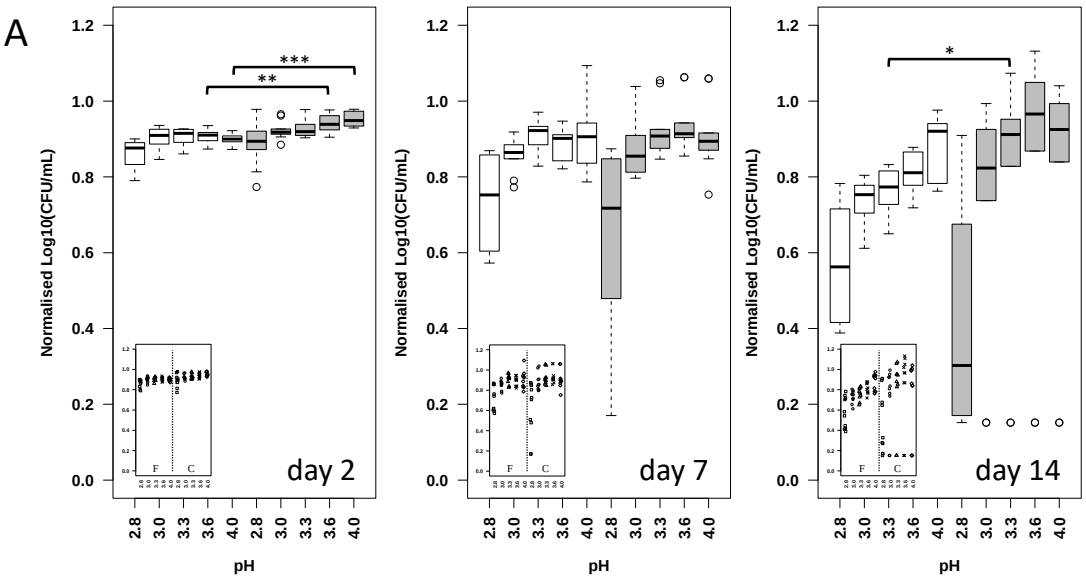
Supplementary material

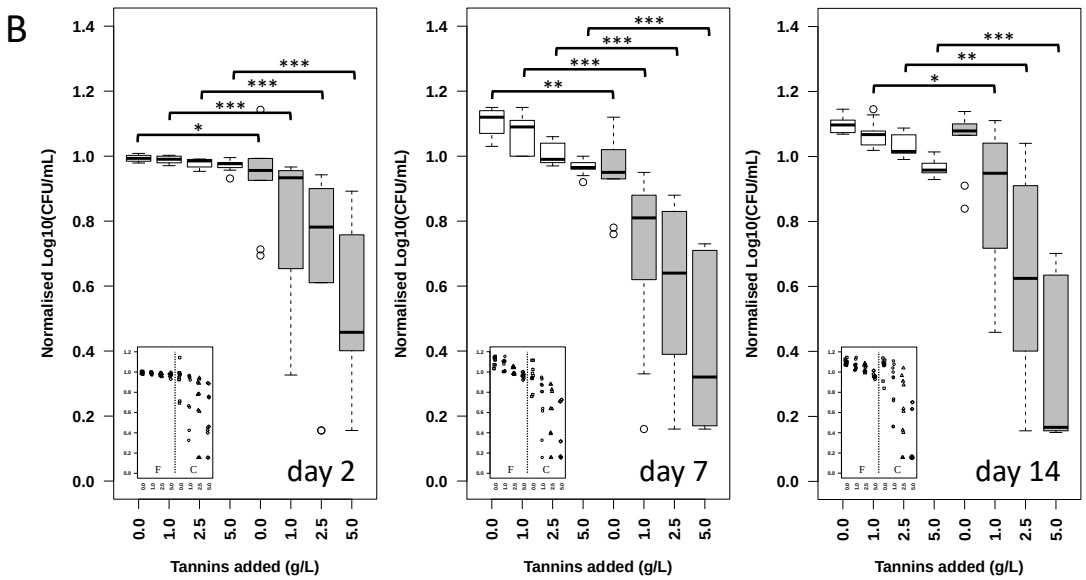
Suppl. Fig. 1. Comparison of freeze-dried (F) and cultured (C) cells and their survival in wine adjusted to different pH values. A) Cell numbers of F-cells (blue boxes) and C-cells (orange boxes) preparations of *O. oeni* strains LAB6, LAD1, LAA1, LAL01 and LAB2013. Cells were inoculated into red wine adjusted to pH 2.8, 3.0, 3.3, 3.6 and 4.0. The number of colony forming units (CFU) for cultivable cells was determined 2, 7 and 14 days (D2, D7, D14) after inoculation. Experiments were carried out in duplicate. B) Data were normalised to day 0 (D0 = 1) for each condition to facilitate a comparison of between F- and C-cells preparations as shown in the bar graph on the left.

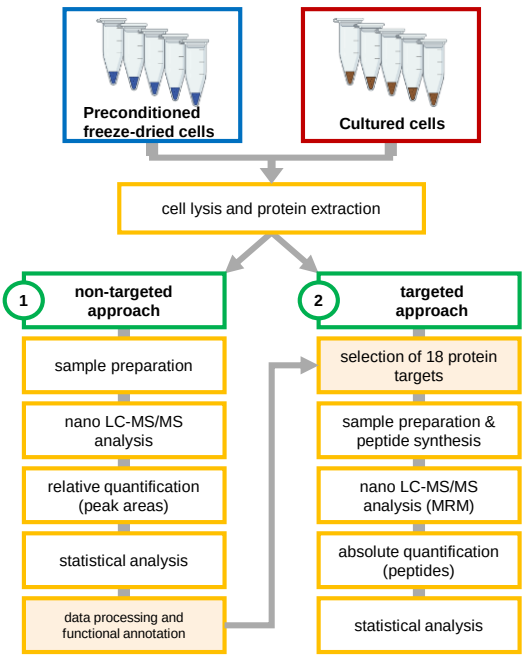
Suppl. Fig. 2. Comparison of freeze-dried (F) and cultured (C) cells and their survival in wine supplemented with different concentrations of grape seed tannins. A) Cell numbers of F-cells (blue boxes) and C-cells (pink boxes) preparations of *O. oeni* strains LAB6, LAD1, LAA1, LAL01 and LAB2013. Cells were inoculated into red wine adjusted to pH 3.6 and supplemented with 0, 1, 2.5 and 5 g/L of grape seed tannin extract. The number of colony forming units (CFU) for cultivable cells was determined 2, 7 and 14 days (D2, D7, D14) after inoculation. Experiments were carried out in duplicate. B) Data were normalised to day 0 (D0 = 1) for each condition to facilitate a comparison between F- and C-cells preparations as shown in the bar graph on the left.

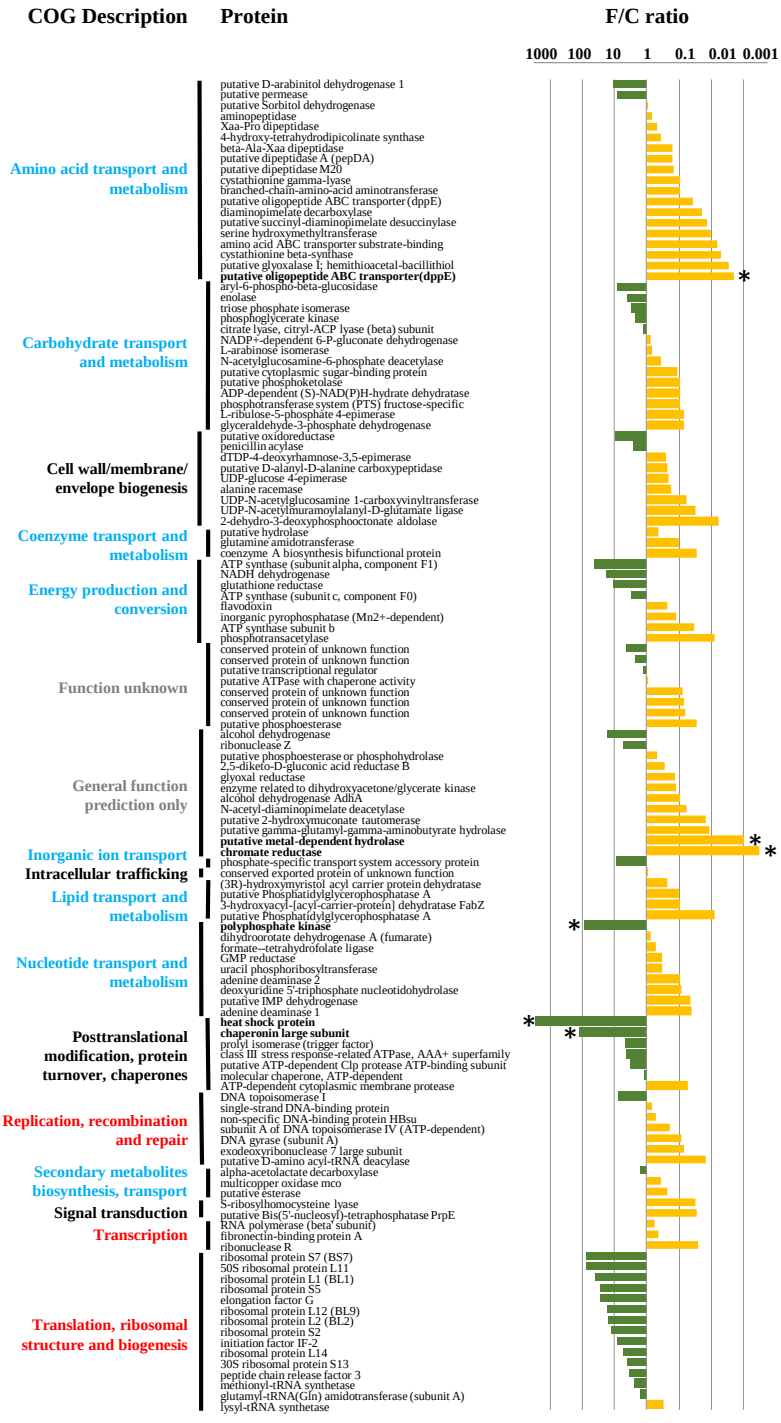
Suppl. Table 1. Results of the non-targeted quantitative proteomics approach

Suppl. Table 2. Results of the targeted quantitative proteomics approach


A
Normalised Log10(CFU/mL)
pH
day 2
day 7
day 14
F
C


B
Normalised Log10(CFU/mL)
Tannins added (g/L)
day 2
day 7
day 14
F
C


Preconditioned freeze-dried cells
Cultured cells
cell lysis and protein extraction
1
non-targeted approach
sample preparation
nano LC-MS/MS analysis
relative quantification (peak areas)
statistical analysis
data processing and functional annotation
2
targeted approach
selection of 18 protein targets
sample preparation & peptide synthesis
nano LC-MS/MS analysis (MRM)
absolute quantification (peptides)
statistical analysis


COG Description
Protein
F/C ratio
1000 100 10 1 0.1 0.01 0.001
Amino acid transport and metabolism
putative D-arabinitol dehydrogenase 1
putative permease
putative Sorbitol dehydrogenase
aminopeptidase
Xaa-Pro dipeptidase
4-hydroxy-tetrahydrodipicolinate synthase
beta-Ala-Xaa dipeptidase
putative dipeptidase A (pepDA)
putative dipeptidase M20
cystathionine gamma-lyase
branched-chain-amino-acid aminotransferase
putative oligopeptide ABC transporter (dppE)
diaminopimelate decarboxylase
putative succinyl-diaminopimelate desuccinylase
serine hydroxymethyltransferase
amino acid ABC transporter substrate-binding
cystathionine beta-synthase
putative glyoxalase I; hemithioacetal-bacillithiol
putative oligopeptide ABC transporter(dppE)
Carbohydrate transport and metabolism
aryl-6-phospho-beta-glucosidase
enolase
triose phosphate isomerase
phosphoglycerate kinase
citrate lyase, citryl-ACP lyase (beta) subunit
NADP+-dependent 6-P-gluconate dehydrogenase
L-arabinose isomerase
N-acetylglucosamine-6-phosphate deacetylase
putative cytoplasmic sugar-binding protein
putative phosphoketolase
ADP-dependent (S)-NAD(P)H-hydrate dehydratase
phosphotransferase system (PTS) fructose-specific
L-ribulose-5-phosphate 4-epimerase
glyceraldehyde-3-phosphate dehydrogenase
Cell wall/membrane/ envelope biogenesis
putative oxidoreductase
penicillin acylase
dTDP-4-deoxyrhamnose-3,5-epimerase
putative D-alanyl-D-alanine carboxypeptidase
UDP-glucose 4-epimerase
alanine racemase
UDP-N-acetylglucosamine 1-carboxyvinyltransferase
UDP-N-acetylmuramoylalanyl-D-glutamate ligase
2-dehydro-3-deoxyphosphooctonate aldolase
Coenzyme transport and metabolism
putative hydrolase
glutamine amidotransferase
coenzyme A biosynthesis bifunctional protein
Energy production and conversion
ATP synthase (subunit alpha, component F1)
NADH dehydrogenase
glutathione reductase
ATP synthase (subunit c, component F0)
flavodoxin
inorganic pyrophosphatase (Mn2+-dependent)
ATP synthase subunit b
phosphotransacetylase
Function unknown
conserved protein of unknown function
conserved protein of unknown function
putative transcriptional regulator
putative ATPase with chaperone activity
conserved protein of unknown function
conserved protein of unknown function
conserved protein of unknown function
putative phosphoesterase
General function prediction only
alcohol dehydrogenase
ribonuclease Z
putative phosphoesterase or phosphohydrolase
2,5-diketo-D-gluconic acid reductase B
glyoxal reductase
enzyme related to dihydroxyacetone/glycerate kinase
alcohol dehydrogenase AdhA
N-acetyl-diaminopimelate deacetylase
putative 2-hydroxymuconate tautomerase
putative gamma-glutamyl-gamma-aminobutyrate hydrolase
putative metal-dependent hydrolase
Inorganic ion transport
chromate reductase
Intracellular trafficking
phosphate-specific transport system accessory protein
conserved exported protein of unknown function
Lipid transport and metabolism
(3R)-hydroxymyristol acyl carrier protein dehydratase
putative Phosphatidylglycerophosphatase A
3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ
putative Phosphatidylglycerophosphatase A
Nucleotide transport and metabolism
polyphosphate kinase
dihydroorotate dehydrogenase A (fumarate)
formate--tetrahydrofolate ligase
GMP reductase
uracil phosphoribosyltransferase
adenine deaminase 2
deoxyuridine 5'-triphosphate nucleotidohydrolase
putative IMP dehydrogenase
adenine deaminase 1
Posttranslational modification, protein turnover, chaperones
heat shock protein
chaperonin large subunit
prolyl isomerase (trigger factor)
class III stress response-related ATPase, AAA+ superfamily
putative ATP-dependent Clp protease ATP-binding subunit
molecular chaperone, ATP-dependent
ATP-dependent cytoplasmic membrane protease
Replication, recombination and repair
DNA topoisomerase I
single-strand DNA-binding protein
non-specific DNA-binding protein HBsu
subunit A of DNA topoisomerase IV (ATP-dependent)
DNA gyrase (subunit A)
exodeoxyribonuclease 7 large subunit
putative D-amino acyl-tRNA deacylase
Secondary metabolites biosynthesis, transport
alpha-acetolactate decarboxylase
multicopper oxidase mco
Signal transduction
putative esterase
S-ribosylhomocysteine lyase
Transcription
putative Bis(5'-nucleosyl)-tetraphosphatase PrpE
RNA polymerase (beta' subunit)
fibronectin-binding protein A
ribonuclease R
Translation, ribosomal structure and biogenesis
ribosomal protein S7 (BS7)
50S ribosomal protein L11
ribosomal protein L1 (BL1)
ribosomal protein S5
elongation factor G
ribosomal protein L12 (BL9)
ribosomal protein L2 (BL2)
ribosomal protein S2
initiation factor IF-2
ribosomal protein L14
30S ribosomal protein S13
peptide chain release factor 3
methionyl-tRNA synthetase
glutamyl-tRNA(Gln) amidotransferase (subunit A)
lysyl-tRNA synthetase