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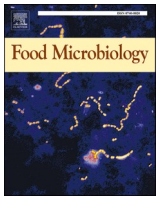

Proteomic insight into the beneficial effect of mannoproteins on *Oenococcus oeni* in wine malolactic fermentation

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ABSTRACT

Oenococcus oeni is the main species of lactic acid bacteria (LAB) responsible for malolactic fermentation (MLF) in winemaking. MLF development can present difficulties because of the harsh, stressful conditions of wine. Yeast mannoproteins have been described as possible activators of *O. oeni* and MLF. This study investigated the proteomic response of *O. oeni* PSU-1 to the presence of yeast mannoproteins in wine like-medium (WLM).

In the proteomic analysis, 956 proteins were identified, with 59 differentially expressed proteins (DEPs) when mannoproteins were added. Notably, carbohydrate metabolism and transport were activated, suggesting the use of the mannose oligosaccharides released from mannoproteins. Some of the DEP proteins identified have been associated with mannan recognition in other LAB. However, proteins associated with amino acid metabolism were relatively low in abundance in the presence of mannoproteins, indicating that the amino acid fraction of mannoproteins is not relevant to *O. oeni* metabolism under the studied conditions. Surprisingly, some stress response proteins, such as ClpP, cold-shock DNA-binding protein, and the citrate transporter MaeP, presented increased abundance. The roles of these proteins in the presence of mannoproteins require further investigation.

1. Introduction

In winemaking, the microbiological transformations carried out by the community of yeast and bacteria are key for wine quality (Moreno-Arribas and Polo, 2005). Alcoholic fermentation (AF), performed by yeasts, transforms grape must sugars into ethanol and CO_2. After AF, lactic acid bacteria (LAB) can conduct malolactic fermentation (MLF) in some wines, especially in red wine but also in some rosé and white wines, such as Chardonnay (Gambetta et al., 2014; Morata et al., 2023; Ruiz-de-Villa et al., 2023). MLF involves the decarboxylation of L-malic acid to L-lactic acid (Pilone and Kunkee, 1970). This biotransformation, mainly performed by *Oenococcus oeni*, results in a final product with lower acidity and higher pH (Liu, 2002; Lonvaud-Funel, 1999; Wibowo et al., 1985), as well as microbiological stability, by eliminating a potential carbon source for wine spoilage bacteria (Liu, 2002).

Wine lees are produced after AF and after MLF in red and white wines; their contact seems to be positive concerning the removal of most undesirable compounds from wine (Pérez-Serradilla and de Castro, 2008) and improves its organoleptic characteristics (Lubbers et al., 1994). Mannoproteins are the most oenologically interesting component of the yeast cell wall. They constitute between 30% and 50% of the cell wall (Klis et al., 2006). Chemically, mannoproteins are glycoproteins, typically with a high degree of glycosylation (80–90%), composed mainly of mannose (>90%), glucose (Guadalupe et al., 2010), and proteins (<10%) (Vidal et al., 2003). Yeast mannoproteins promote the growth of wine LAB, favouring the development of MLF (Balmaseda et al., 2024; Díez et al., 2010; Toraño et al., 2024). These molecules adsorb medium-chain fatty acids produced by yeast that are toxic for *O. oeni* (Capucho and San Romão, 1994) and thereby improve the development of MLF (Caridi, 2006; Díez et al., 2010; Guilloux-Benatier and Chassagne, 2003; Liu et al., 2017). In addition, during MLF, *O. oeni* can degrade mannoproteins, as observed by some authors (Balmaseda et al., 2021; Ganan et al., 2012).

Protease, peptidase and glycosidase activities have been described in *O. oeni* (D'Incecco et al., 2004; Grimaldi et al., 2005; Manca de Nadra et al., 1999; Michlmayr et al., 2012; Olguín et al., 2011; Ritt et al., 2009). These enzymatic activities facilitate the release of sugars and

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amino acids from mannoproteins and other macromolecules, which results in an increase in nutrients in wine, improving *O. oeni* survival (Alexandre et al., 2004). The adaptation mechanisms of *O. oeni* in wine are essential for its survival and ability to perform MLF. This bacterium possesses a highly conserved phosphotransferase system (PTS), enabling it to phosphorylate sugars such as glucose, cellobiose, trehalose, and mannose via phosphoenolpyruvate (Jamal et al., 2013).

Previous studies have linked the use of mannoproteins to the increased survival of *O. oeni* under wine conditions, especially when its preferred substrate, L-malic acid, is depleted (Balmaseda et al., 2021; Toraño et al., 2025). Yeast mannoproteins, particularly those with molecular weights between 6 and 22 kDa, have been demonstrated to be useful additives in wine fermentation, promoting bacterial growth in challenging environments, such as in the presence of high ethanol levels (Díez et al., 2010). It has been reported that *O. oeni* is capable of hydrolysing mannoproteins, thereby increasing the nutritional content of its environment and stimulating its activity (Caridi, 2006; Díez et al., 2010). Although the exact mechanisms by which mannoproteins benefit *O. oeni* are not fully understood, it has been demonstrated that mannose oligosaccharides (MOS) derived from mannoproteins promote beneficial responses in other LAB species, such as some probiotic lactobacilli (Baurhoo et al., 2007; Sharma et al., 2018; Zang et al., 2019).

To better understand the molecular mechanisms involved in the beneficial impact of mannoproteins in *O. oeni*, this work aimed to evaluate the changes in the proteome of the PSU-1 strain caused by the presence of mannoproteins.

2. Materials and methods

2.1. Oenococcus oeni strain and mannoprotein extract

The *O. oeni* PSU-1 (ATCC BAA-331) strain and the mannoprotein extract Mannostab (Laffort, Bordeaux, France) were selected because good results were obtained previously in the development of MLF when this type of mannoprotein was added.

2.2. Culture media and fermentation conditions

The *O. oeni* strain was grown in MRSmf consisting of MRS broth medium (Difco Laboratories, Detroit, MI, USA) supplemented with DL-malic acid (4 g/L) and fructose (5 g/L) at pH 5.0 at 28 °C with a 10% atmosphere of CO_2. The cells were collected at the final exponential phase ($OD_{600nm} = 1$) (Margalef-Català et al., 2017). The strains were also maintained on MRSmf solid medium that contained 20 g/L bacteriological agar (Panreac, Química SLU, Castellar del Vallès, Spain).

2.3. Fermentation conditions

MLF was carried out in wine-like medium (WLM) (Bordas et al., 2015) with 14% ethanol (v/v), 2 g/L L-malic acid, 1.25 g/L Bacto TM casamino acids (BD, France) and 1.25 g/L peptone (Panreac) adjusted to pH 3.40. The WLM was enriched with a mannoprotein extract (MP) at a concentration of 2 g/L, which, in previous studies, had a positive effect on *O. oeni* metabolism (Toraño et al., 2024). *Oenococcus oeni* was inoculated in a population of 10^7 CFU/mL in 2 L sterile bottles under static conditions at 20 °C in triplicate. *Oenococcus oeni* populations were quantified by plating on modified MRS plates, which contained 20 g/L agar (Panreac). The MLF was followed every 24 h by the measurement of L-malic acid consumption with a Y15 autoanalyzer (Biosystems S.A., Barcelona, Spain) and bacterial population evolution by counting colony-forming units (CFU) on MRSmf solid medium.

2.4. Proteomic analysis

Samples for proteomic analyses were taken on day 4 when more than half of the L-malic acid concentration had been consumed, corresponding to a L-malic acid concentration below 1 g/L.

For the proteomic analysis, 1 L of WLM was centrifuged at 4,600×*g* for 20 min at 4 °C. After centrifugation, the resulting cell pellet was washed with 10 mM Tris-HCl buffer at pH 8. The cell pellet was subsequently frozen in liquid nitrogen and stored at −80 °C until protein extraction (Margalef-Català et al., 2016).

Proteomic analysis was conducted at the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat. Initially, 30 μg of protein per sample was reduced with 4 mM 1,4-dithiothreitol (DTT) and alkylated with 8 mM iodoacetamide (IAA). Subsequently, enzymatic digestion was performed using sequencing-grade trypsin/Lys-C (Thermo Fisher Scientific, CA, USA) at an enzyme-to-protein ratio of 1:50. Following digestion, TMT (Tandem Mass Tag) 10-plex labelling (Thermo Fisher Scientific) was carried out according to the manufacturer's instructions. To ensure sample normalization, a pool of all samples labelled with the TMT-126 tag was included in each TMT batch. The TMT-labelled peptides were then fractionated using an OFFGEL fractionator (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocol. Six fractions of peptides obtained were further separated on a C18 reversed-phase (RP) nanocolumn (75 μm I.D.; 15 cm length; 3 μm particle diameter; Nikkyo Technos Co. Ltd., Japan) using an EASY-II nanoLC (Thermo Fisher). Chromatographic separation was performed with a 90-min gradient with Milli-Q water and acetonitrile (0.1% formic acid) as the mobile phase at a flow rate of 300 nL/min. Mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro (Thermo Fisher). This involved the generation of an enhanced FT-resolution MS spectrum (R = 30,000 FHMW), followed by data-dependent FT-MS/MS acquisition (R = 15,000 FHMW, 40% NCE HCD) of the ten most intense parent ions.

A charge state rejection of one and a dynamic exclusion of 0.5 min were applied. Protein identification and quantification were conducted using Proteome Discoverer software v.1.4.0.288 (Thermo Fisher Scientific), which employs multidimensional protein identification technology (MudPIT). This integrated the six raw data files (fractions) obtained for each sample. For protein identification, all MS and MS/MS spectra were analysed using the Mascot search engine (v.2.5), which was configured with the *O. oeni PSU-1* database (1682 entries) and a contaminant database (247 entries). The proteomic analysis protocol allowed for two missed cleavages during enzymatic digestion, along with tolerances of 0.02 Da for the FT−MS/MS fragmentation mass and 10.0 ppm for the FT−MS parent ion mass. The TMT-10 plex served as the quantification modification, methionine oxidation and N-terminal acetylation were dynamic modifications, and cysteine carbamidomethylation remained static. The false discovery rate (FDR) and protein probabilities were determined through Percolator analysis. Protein quantification involved calculating ratios between each TMT label and the 126-TMT label, followed by normalization based on the protein median, ensuring accurate comparative analysis.

2.5. General oenological analytical parameters

The concentrations of sugars (glucose and fructose), L-malic acid, acetic acid, D- and L-lactic acids, NH_4, primary amino nitrogen (NOPA), and citric acid were determined with a Y15 analyser (Biosystems SA), and the pH was determined using a Crison micro pH 2002 pH meter (Barcelona, Spain).

2.6. Statistical analysis

Proteomic analyses were performed using Mass Profiler Professional Software (Agilent). The raw data were normalized by log2 transformation and mean-centring for univariate analysis (Student's *t*-test), with significance set at $p < 0.05$.

For the analysis of the oenological compounds at the time of sampling, three independent biological samples were analysed. ANOVA (Tukey's honestly significant difference (HSD) test) was performed with

the XLSTAT software 2023.4 package (Addinsoft, Paris, France), with a statistical level of significance of $p < 0.05$.

3. Results and discussion

3.1. Mannoprotein addition benefits MLF with O. oeni PSU-1

The selection of *O. oeni* PSU-1 was based on previous studies in which this strain showed better performance during MLF due to its ability to metabolize mannoproteins. The strain presented high mannoprotein consumption rates, reaching 90% at 1 g/L and 80% at 2 g/L added mannoprotein extract (Toraño et al., 2024, 2025). This effective mannoprotein utilization directly contributed to improving the MLF duration and L-malic acid consumption rate. Additionally, this strain has been used as a model for molecular studies because its genome is completely annotated. The selection of the commercial mannoprotein extract (MP) was also based on previous results that showed a better effect on MLF. The analysis revealed a total polysaccharide content of 74.54% (w/w) and a total protein content of 25.97% (w/w) (Toraño et al., 2024).

In comparison to the control condition, MLF in the MP treatment group was shorter (Fig. 1), as observed in synthetic WLM media, where MLF is completed in less time (Toraño et al., 2024). The samples were collected in the second half of MLF (Day 4, when 1.4–1.6 g/L L-malic acid was consumed). The detected viabilities at that point were 2×10^7 CFU/mL in the control wine and 1.83×10^7 CFU/mL in the wine with the mannoprotein extract.

None of the oenological parameters analysed were significantly modified by the addition of MP during MLF, except for those associated with MLF development, such as the decrease in L-malic acid content and the increases in L-lactic acid content and pH (Table 1). The D-lactic acid and acetic acid concentrations slightly increased during MLF. Citric acid was consumed similarly in both the control and the MP-supplemented conditions. The consumption of both organic and inorganic nitrogen did not significantly vary, as in a previous study (data pending publication). The use of nitrogen from yeast mannoprotein extracts was evaluated, and no differences were found that could relate the protein portion of mannoproteins to the enhancement of MLF. Thus, the polysaccharide fraction of mannoproteins appeared to be metabolized primarily by *O. oeni*, while also considering the potential detoxifying role of these molecules (Guilloux-Benatier and Chassagne, 2003).

3.2. The proteome of O. oeni PSU-1 is significantly affected by mannoprotein addition

Analysis of the variability among samples with and without the addition of MP revealed significant differences in the proteome of *O. oeni* PSU-1. A total of 956 proteins were identified. An unpaired Student's *t*-test was used to compare the control condition *vs.* the addition of MP ($FC > 1$, $p < 0.05$) after log2 normalization, resulting in the identification of 59 differentially expressed proteins (DEPs) (Table 2). The functions of the DEPs were categorized based on Clusters of Orthologous Genes (COGs) classification (Fig. 2). All COG categories had at least one associated DEP. The greatest variabilities were observed in (i) carbohydrate transport and metabolism (10 DEPs, 16.95%), (ii) translation, ribosomal structure and biogenesis (8 DEPs, 13.56%), and (iii) amino acid transport and metabolism (7 DEPs, 11.86%).

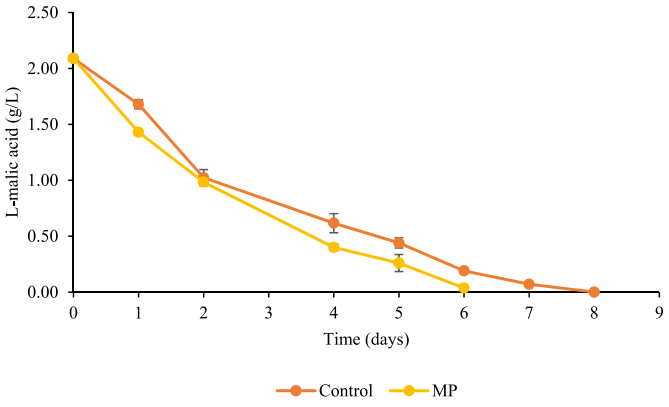

Fig. 1. Evolution of malolactic fermentation in wine-like medium (WLM) fermented with *O. oeni* PSU-1 with the addition of 2 g/L of mannoprotein extract (MP). All data are presented as the mean ± standard deviation of three biological replicates.

Table 1
Composition of the initial wine-like medium (WLM_i) and at the sampling point with mannoprotein extract addition (MP) and without it (Control). Different lowercase letters indicate a significant difference between the samples using the Tukey (HSD) test at $p < 0.05$. NOPA: Primary Amino Nitrogen. All data are expressed as the arithmetic average of three biological replicates ± standard deviation.

	WLM_i	Control	MP
L-malic acid (g/L)	1.96 ± 0.02^c	0.63 ± 0.04^b	0.40 ± 0.06^a
L-lactic acid (g/L)	0.05 ± 0.02^a	1.13 ± 0.02^b	1.12 ± 0.04^b
D-lactic acid (g/L)	0.01 ± 0.01^a	0.10 ± 0.01^b	0.12 ± 0.02^b
Citric acid (mg/L)	480 ± 3.54^b	134 ± 1.73^a	132 ± 2.13^a
Acetic acid (g/L)	0.20 ± 0.01^a	0.21 ± 0.01^a	0.24 ± 0.02^b
NOPA (mg/L)	123 ± 3.54^a	127 ± 3.79^a	124 ± 2.25^a
NH_4^+ (mg/L)	5.0 ± 0.02^a	5.0 ± 0.95^a	5.0 ± 0.73^a
pH	3.41 ± 0.01^b	3.42 ± 0.01^a	3.44 ± 0.01^a

3.2.1. Mannoproteins activate carbohydrate transport and metabolism in O. oeni

During MLF, some proteins associated with carbohydrate metabolism in *O. oeni* significantly changed with the addition of MP. Seven out of the ten most prominent DEPs significantly increased in abundance in *O. oeni* in the presence of this extract.

The abundance of malate dehydrogenase (OEOE_1325), also referred to as L-lactate dehydrogenase, increased the most in response to the addition of MP. According to Sternes et al. (2017), this enzyme (OEOE_1325) enables the conversion of L-lactate into pyruvate. Moreover, it has been hypothesized to be involved in the remetabolization of L-lactate, either to obtain ATP (through acetate formation) or to avoid the intracellular accumulation of lactate, which can be toxic to the cell (Eicher et al., 2024a), in both cases benefiting *O. oeni* development.

The results of the proteomic analysis suggest the activation of central sugar metabolism. Increases in the protein abundances of phosphoglycerate kinase (OEOE_0638) and enolase (OEOE_1650, classified in the energy production category in Table 2) were observed during fermentation with added MP. These two enzymes are involved in steps of the glycolytic pathway that convert glyceraldehyde-3-P into pyruvate and could be associated with mannose metabolism. Additionally, enolase is related to host tissue adhesion in probiotic bacteria, including *Lactiplantibacilllus plantarum*. Enolase would bind to host fibronectin, a glycoprotein containing mannose oligosaccharides (Castaldo et al., 2009). However, it has not been determined whether mannose plays any role in the binding of probiotics in this case. However, what has been reported in different studies is the recognition of yeast mannans or mannose oligosaccharides (MOS) by LAB, which is mediated by proteins localized on the LAB surface (Katakura et al., 2010). The positive effect of yeast MOS on the survival of certain LAB probiotic species has also been described (Everard et al., 2014; Tang et al., 2022). In fact, yeast MOS have been proposed as an effective prebiotic component (Baek et al., 2024; Cao et al., 2019; Faustino et al., 2021). No studies have reported MOS recognition and/or utilization by *O. oeni*. However, we can hypothesize based on the knowledge of other LAB species that *O. oeni* could recognize the MOS, previously released from yeast mannoproteins, by the β-glucanase and α-mannosidase activities described

Table 2
Differentially expressed proteins in *O. oeni* PSU-1 due to mannoprotein addition and related metabolism.

Related metabolism	Protein annotation	Gene symbol	Fold change
Cell wall/ membrane/ envelope biogenesis	Phosphoglycerol transferase-like protein	OEOE_0165	1.393
	Cell division protein FtsI/ penicillin-binding protein 2	OEOE_1149	−1.329
	Muramidase with LysM repeats	OEOE_1199	−1.456
	UDP-N-acetylmuramyl tripeptide synthase	OEOE_1599	1.499
Defense mechanism and secondary metabolites	Glucose-1-phosphate thymidylyltransferase	OEOE_1449	1.401
Translation. ribosomal structure and biogenesis	50S ribosomal protein L9	OEOE_0014	−1.386
	50S ribosomal protein L36	OEOE_0617	1.946
	Elongation factor Tu	OEOE_0792	−1.178
	Peptide deformylase	OEOE_0806	1.209
	50S ribosomal protein L27	OEOE_0958	−1.166
	Aspartyl/glutamyl-tRNA(Asn/ Gln) amidotransferase	OEOE_1693	−1.267
	Glutamyl-tRNA(Gln) amidotransferase subunit A	OEOE_1694	−1.216
	Probable DNA-directed RNA polymerase subunit delta	OEOE_1788	2.217
Transcription	Transcriptional regulator	OEOE_0916	1.214
	Transcriptional regulator MraZ	OEOE_1152	−1.497
	Cold-shock DNA-binding protein	OEOE_1376	3.531
Replication. recombination and repair	ATP-dependent helicase/ nuclease subunit A	OEOE_0309	−1.415
	DNA repair ATPase	OEOE_0903	−1.126
	DNA polymerase III. delta prime subunit	OEOE_1405	1.453
	Recombination protein RecR	OEOE_1409	1.495
	Uracil-DNA glycosylase	OEOE_1436	1.974
Energy production and conversion	Citrate permease MaeP	OEOE_0419	3.938
	Enolase	OEOE_1650	1.392
Amino acid transport and metabolism	Aminopeptidase C. Cysteine peptidase	OEOE_0461	−1.323
	ATP-dependent Clp protease proteolytic subunit	OEOE_0570	1.664
	Aspartate-semialdehyde dehydrogenase	OEOE_0776	−1.431
	Serine/threonine protein phosphatase	OEOE_0788	−1.242
	Saccharopine dehydrogenase-like protein	OEOE_1649	−1.331
	Methylmalonyl-CoA epimerase	OEOE_1705	−1.392
	Threonine dehydrogenase	OEOE_1707	−1.166
Nucleotide transport and metabolism	Inosine-uridine nucleoside N-ribohydrolase	OEOE_1673	−1.388
Carbohydrate transport and metabolism	D-Ala-D-Ala carboxypeptidase A. Serine peptidase	OEOE_0121	−1.117
	Phosphoglycerate dehydrogenase	OEOE_0413	2.714
	Phosphotransferase system. mannose/fructose-specific component IIA	OEOE_0464	1.193
	Putative gluconeogenesis factor	OEOE_0568	1.318
	Phosphoglycerate kinase	OEOE_0638	1.677
	Undecaprenyl-diphosphatase 1	OEOE_0848	3.168
	Malate dehydrogenase (NAD+)	OEOE_1325	3.364
	Galactoside O-acetyltransferase	OEOE_1501	1.228
	Acetyl-CoA carboxylase carboxyl transferase	OEOE_1583	−1.129
	Acetyl-coenzyme A carboxylase carboxyl transferase	OEOE_1584	−1.735
Coenzyme metabolism	Pyridoxal 5′-phosphate synthase	OEOE_1036	−1.684
Inorganic ion transport and metabolism	Phosphate ABC transporter substrate-binding protein	OEOE_0555	1.377
	Cation transport ATPase	OEOE_1647	1.267
General function prediction	Ribosome biogenesis GTPase A	OEOE_1018	1.663
	FMN bind domain-containing protein	OEOE_1090	1.600
	UPF0297 protein	OEOE_1163	1.919
Function unknown	Uncharacterized protein	OEOE_0428	−1.328
	Uncharacterized protein	OEOE_0822	−1.344
	Uncharacterized protein	OEOE_0857	−1.636
	Uncharacterized protein	OEOE_0898	−1.697
	Uncharacterized protein	OEOE_0942	2.260
	Uncharacterized protein	OEOE_0993	1.585
	Uncharacterized protein	OEOE_1080	1.378
	Uncharacterized protein	OEOE_1166	1.481
	Uncharacterized protein	OEOE_1239	1.158
	Uncharacterized protein	OEOE_1485	2.000
	Uncharacterized protein	OEOE_1626	2.735
	Uncharacterized protein	OEOE_1655	−1.280

in *O. oeni* (Cibrario et al., 2016; Guilloux-Benatier and Chassagne, 2003). MOS could be taken up—as described for other gram-positive bacteria—and converted into free mannose in the cytosol by intracellular mannosidases (Ladevèze et al., 2017). Mannose can be converted into fructose-6-P by *O. oeni* and further metabolized to pyruvate and then into D-lactate, CO_2, acetate or ethanol via the heterolactic pathway. In this work, the possible activation of sugar metabolism, due to the presence of the mannoprotein extract, could not be associated with significant increases in the expected metabolites from sugar catabolism, such as D-lactate or acetate (Table 1). The enzymes phosphoglycerate kinase and enolase, which were significantly more abundant in the presence of mannoproteins, are involved in pyruvate production. Pyruvate is a central metabolite that can be redirected to different end products, including C4 products such as diacetyl, acetoin and 2,3-butanediol. These C4 compounds are usually obtained primarily from pyruvate produced by citrate catabolism, but the accumulation of pyruvate in the cell can lead to the major production of these C4 compounds as a mechanism of the detoxification of excess pyruvate (Eicher al., 2024b). Further research is needed to understand the metabolism associated with mannoproteins in *O. oeni* and the biochemical pathways involved in this process.

The mannose/fructose-specific component IIA (OEOE_0464) of the phosphotransferase system (PTS), which is involved in the uptake and metabolism of sugars, presented greater abundance in *O. oeni* PSU-1 in the presence of mannoproteins. Kim et al. (2011) showed the induction of the gene OEOE_0464 by glucose and fructose. These data suggest that the phosphotransferase component is responsible for part of the transport of hexoses at acidic pH. The higher concentration of the phosphotransferase component associated with mannose/fructose transport (OEOE_0464) could be related to the uptake of free hexoses (such as mannose or glucose present in the MP extract) during MLF. Moreover, this IIA component of the PTS system has also been associated with the recognition of yeast mannan by *Lactobacillus paracasei* (Yamasaki-Yashiki et al., 2017), as it is one of the genes activated due to the presence of mannan (either associated with inactivated yeast cells or soluble mannans).

Some *O. oeni* proteins associated with cell wall biosynthesis and maintenance presented significant differences in abundance during fermentation with mannoproteins. Specifically, a very high concentration of undecaprenyl diphosphatase (OEOE_0848) was detected during fermentation with added MP, whereas D-Ala-D-Ala carboxypeptidase (OEOE_0121) was less abundant than it was in the control. Undecaprenyl diphosphatase catalyses the dephosphorylation of undecaprenyl pyrophosphate to undecaprenyl phosphate, which is an essential glycan lipid carrier in bacterial cell wall synthesis. The sugar is linked to the lipid carrier and is translocated towards the periplasm, where the glycan moiety is transferred to the growing polymer (Manat et al., 2014).

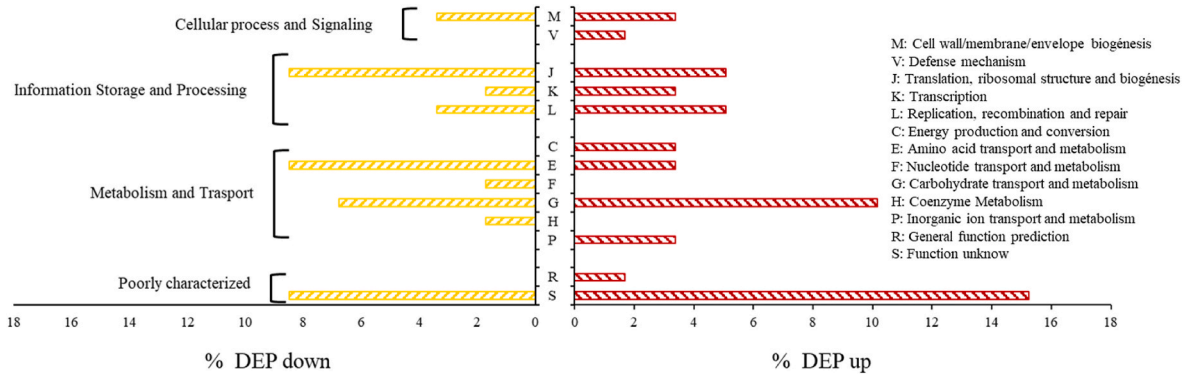

Fig. 2. Distribution of differentially expressed proteins (DEP) in *O. oeni* PSU-1 fermenting with mannoprotein extract added categorized by clusters of orthologous groups (COGs). Positive bars (right) represent the % of increased abundance of DEPs. Negative bars (left) represent the % of decreased abundance of DEPs. Absence of bars means no DEPs for that COG.

D-Ala-D-Ala carboxypeptidase A (OEOE_ 0121) is a bacterial enzyme involved in the final steps of peptidoglycan biosynthesis (Kijek et al., 2019) and is associated with maintaining the balance between bacterial cell wall synthesis and degradation (Obando and Dörr, 2023). All these changes in cell wall biosynthesis regulation could be related to the possible interaction of MOS with this structure, but this is only a hypothesis that requires further investigation. Notably, other enzymes that use sugars as substrates were detected at higher concentrations in the fermentations with added MP. This was the case for phosphoglycerate dehydrogenase (OEOE_1583), which is involved in the biosynthesis of serine from glucose (Zogg, 2014), and galactoside O-acetyltransferase (OEOE_1501), which is involved in sugar transport (Bon et al., 2009). Additionally, some proteins with decreased abundance were observed, such as the two subunits of a carboxyl transferase (OEOE_1583, OEOE_1584) that plays a critical role in fatty acid synthesis. This biotin-dependent enzyme catalyses the carboxylation of acetyl-CoA to produce malonyl-CoA through its two catalytic activities, biotin carboxylase (BC) and carboxyltransferase (Tong, 2005).

3.2.2. Mannoproteins do not activate O. oeni amino acid transport and metabolism

In general, proteins related to amino acid metabolism in the proteome of *O. oeni* decreased in abundance in the presence of the mannoprotein extract. Only one out of seven DEPs presented a greater concentration than did those without MP. Even if mannoproteins have an amino acid fraction, it seems that they do not activate amino acid transport or metabolism in *O. oeni* under the studied conditions (Table 2). The protein showing a relatively high concentration is a known stress protein, the ATP-dependent Clp protease proteolytic subunit (OEOE_0570).

3.3. Some stress proteins are increased due to mannoprotein addition

As mentioned above, the stress protein ClpP (OEOE_0570) was more abundant in the fermentation with added mannoproteins (Table 2). Another stress protein whose concentration markedly increased in the presence of mannoproteins was the cold-shock DNA-binding protein (OEOE_1376). ClpP is a protease that responds to different types of stress (Beltramo et al., 2004, 2006). The cold shock protein (OEOE_1376) is a chaperone that has been associated with adaptation to cell acclimation to wine stress conditions (Cecconi et al., 2009).

In the conditions of this study, there were no stress factors associated with the proteomic analysis. The samples compared were those with or without added mannoproteins in the same medium (WLM). Therefore, the cells faced the same concentration of ethanol and pH, the main stress factors present in the medium. The addition of mannoproteins seemed to be beneficial for *O. oeni,* according to the faster development of MLF. Therefore, these findings do not explain the increased abundance of stress proteins during fermentation with added MP.

Notably, some stress proteins have been associated with the recognition of yeast mannan by other LAB. More specifically, in *Lactococcus lactis*, the stress proteins DnaK and GroEL were detected in the purified extract of the cell wall. Curiously, glycolytic enzymes such as pyruvate kinase dehydrogenase and glyceraldehyde-3-phosphate were also detected. DnaK bound to both bacteria and mannan yeast at pH 4 and aggregated at concentrations above 0.1 mg/mL (Katakura et al., 2010). DnaK and GroEL, which have chaperone functions and are controlled by the master regulator CtsR, have also been described in *O. oeni* (Grandvalet et al., 2005). However, no changes were detected in these proteins in this study. In this work, only the soluble proteins were analysed. Therefore, those embedded in the cell wall were not included.

The possible role of stress proteins, such as ClpP, and also the enyme enolase, both associated with mannan recognition and adhesion, require further analysis to understand if they could be involved in the formation of a protective cell coating of mannose oligosaccharides that could improve the stress tolerance of *O. oeni.*

The abundance of the citrate transporter is highly increased in response to mannoproteins.

The increased abundance of the citrate permease MaeP (OEOE_0419) detected in the fermentation with added mannoproteins was surprising. No differences in citrate consumption were observed between the fermentations with and without MP. However, the concentration of MaeP was almost 4-fold greater in cells in contact with mannoproteins than in control cells (Table 2), with the highest fold change observed among all the DEPs. Citrate metabolism has been reported to have beneficial effects on *O. oeni* physiology, particularly its acid tolerance (Ramos et al., 1994). Indeed, as with malate metabolism, citrate metabolism can activate the proton motive force by citrate uptake under its anionic form and when oxaloacetate is converted to pyruvate, leading to the consumption of one scalar proton (Ramos et al., 1994). The consumption of protons during citrate catabolism also helps the cell maintain its intracellular pH (Ramos et al., 1994). Moreover, some studies have shown that *maeP* gene expression is induced by wine stress conditions (Augagneur et al., 2007; Margalef-Català et al., 2016). Overall, citrate metabolism may be helpful for *O. oeni* adaptation to wine conditions. However, we could not find a link between *O. oeni* mannoprotein utilization and citrate metabolism associated with the conditions evaluated in this work.

In *Lactococcus lactis*, the citrate permease CitP (named MaeP in *O. oeni*) catalyses the uptake of citrate in exchange for oxaloacetate and/or pyruvate when these compounds accumulate in the cytoplasm. In addition to these two intermediates, citrate can also be taken up in exchange for acetate (Pudlik and Lolkema, 2012). In this work, several proteins related to pyruvate production were in higher concentration in

the presence of mannoproteins (phosphoglycerate kinase and enolase), thus MaeP higher concentration could be associated with a cellular response to the increase in intracellular pyruvate. However, more knowledge about *O. oeni* MaeP specificity and transport mechanisms is necessary to understand whether MaeP is related to pyruvate metabolism and citrate transport.

Finally, other proteins associated with general functions in translation, replication and nucleotide metabolism were affected by mannoprotein addition (Table 2). These changes might be associated with cell processes involved in the global changes observed in the *O. oeni* PSU-1 proteome.

4. Conclusions

Proteomic analysis revealed that mannoprotein addition resulted in increased protein abundance related to carbohydrate transport and metabolism, with notable increases in enzymes such as L-lactate dehydrogenase, phosphoglycerate kinase and enolase. These changes suggest the utilization of mannose oligosaccharides released from mannoproteins. In contrast, proteins involved in amino acid transport and metabolism presented decreased abundances, indicating that the amino acid fraction of mannoproteins does not have an impact on *O. oeni* metabolism. Some of the proteins with higher abundances in the presence of mannoproteins have been associated with mannan recognition in other LAB species and could play a role in the protection of *O. oeni* cells.

This study demonstrated that mannoproteins modulate the proteomic profile of *O. oeni,* affecting its metabolism and enhancing its fermentative capacity. However, some aspects, such as the roles of some stress proteins and the citrate transporter in mannoprotein utilization by *O. oeni*, remain unclear and require further research.

CRediT authorship contribution statement

Paloma Toraño: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Aitor Balmaseda:** Writing – review & editing, Formal analysis, Data curation. **Albert Bordons:** Writing – review & editing. **Nicolas Rozès:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Conceptualization. **Cristina Reguant:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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