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Evaluation of the Addition of Yeast Mannoprotein to *Oenococcus oeni* Starter Cultures to Improve Wine Malolactic Fermentation

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Abstract: Malolactic fermentation (MLF) in wine is driven by the lactic acid bacterium *Oenococcus oeni* in most cases. Although this bacterium is resistant to wine stress conditions, it often faces difficulties completing MLF. Previous studies indicate that yeast mannoproteins may improve *O. oeni* growth and survival in wine. However, very little is known about this topic. This study evaluated the effect of the addition of mannoprotein extracts to culture media on *O. oeni* growth and its survival to stress conditions and MLF performance. Three commercial mannoprotein extracts were characterized in terms of polysaccharide and protein richness and were used for *O. oeni* culture media supplementation. The addition of mannoprotein extracts improved the survival of the two evaluated *O. oeni* strains, PSU-1 and VP41, after acid shock (pH 3.2) in comparison to that of the control. The transcriptional response of four genes involved in mannose metabolism was different depending on the strain, indicating the complexity of sugar metabolism in *O. oeni*. PSU-1 cells grown with two of the mannoprotein extracts performed faster MLF compared with the control condition, indicating that mannoprotein addition may improve the performance of *O. oeni* starter cultures, although this effect depends on the strain.

Keywords: yeast mannoprotein; malolactic fermentation; *Oenococcus oeni*; starter culture; wine

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1. Introduction

In wine, malolactic fermentation (MLF) is driven by lactic acid bacteria (LAB). This process consists of the decarboxylation of L-malic acid into L-lactic acid. MLF is a relevant step in wine production and may improve by decreasing acidity, increasing microbial stability, and improving the sensory properties of the final product [1,2]. This fermentation is particularly interesting in red wines and high-acidity white wines.

MLF typically occurs after alcoholic fermentation (AF) and is carried out mainly by the LAB species *Oenococcus oeni*. After AF, wine has several physicochemical characteristics that could inhibit MLF and the growth of *O. oeni*. The main factors inducing stress in *O. oeni* are high ethanol concentration (up to 16% *v*/*v*), low pH (3.0–3.5), low temperatures, total sulfur dioxide (50–100 mg/L), yeast medium-chain fatty acids, and nutrient depletion due to its consumption by yeasts during AF [3,4]. However, this LAB species is the most resistant to wine-related stress [5,6]. MLF can occur spontaneously or by using starter cultures. Due to the various stress factors encountered by *O. oeni* in wine, MLF may slow or, in the worst case, stop. Using starter cultures can be a good solution to avoid this problem. Different inoculation strategies have been proposed to ensure MLF completion,

including the coinoculation of yeast and LAB to avoid wine stress conditions. Also, the preadaptation of LAB starter cultures to prepare cells for wine hash conditions can improve MLF performance [7].

O. oeni has been described as the most resistant LAB species to wine conditions due to its mechanisms of stress response that include the activation of ATPases, which regulate pH homeostasis and prevent intracellular acidification, and cell membrane modulation [8,9]. *O. oeni* also protects itself from cellular stress by synthesizing stress proteins like Hsp18, encoded by the *hsp18* gene, which acts as a chaperone and has been related to different types of stress [10–12].

The optimal pH for *O. oeni* growth is between 4.3 and 4.8 [13]. Acidification of the environment causes reversible modification of the *O. oeni* cell membrane. Nevertheless, it can grow at a pH of 3.2, making it resistant to this type of stress [3,14]. MLF occurs more slowly at pH values less than 3.4 [5]. Exposure to ethanol destabilizes cell membrane lipids, increasing membrane fluidity and permeability. This significantly reduces the viability of *O. oeni* when inoculated into wine [2,15]. *O. oeni* is considered to be a microorganism with ethanol tolerance, but at concentrations greater than 10% (*v*/*v*) ethanol in an acidic environment, membrane destabilization occurs, producing cell damage [15,16].

Mannoproteins are glycoproteins composed of 80–90% mannose polysaccharides and approximately 5–10% protein, usually amino acids [17]. They are naturally found in the cell walls of yeast species and are released at the end of AF or during aging on lees [18]. These compounds can contribute positively to the physical, chemical, organoleptic, and sensory characteristics of the final product [19,20]. As a result, they are increasingly being used in winemaking or added as purified commercial extracts. The beneficious role of mannoproteins on MLF was already described by Guilloux-Benatier et al. in 1991 [21]. This could be due to the adsorption of the medium-chain fatty acids synthesized by *Saccharomyces* [22]. These compounds have been shown to inhibit bacterial growth and, therefore, their removal improves malolactic fermentation. Moreover, malolactic bacteria is able to hydrolyze mannoproteins, thus enhancing the nutritional content of the medium and also stimulating their activity [23]. However, since then, few studies have addressed this topic [24–27]. Mannoproteins may have a positive impact on MLF development by adsorbing the medium-chain fatty acids produced by the yeasts and phenolic compounds of grape must and stimulating bacterial growth. *O. oeni* glycosidase and peptidase activities may enable the release of sugars and amino acids from mannoproteins and other macromolecules, thereby increasing the nutritional content and survival of *O. oeni* in wine [28]. Mannose, which is released from yeast mannoproteins because of mannosidase activity by *O. oeni*, may be a phosphotransferase system (PTS) substrate and thus may be involved in stimulating LAB growth in the presence of yeast mannoproteins or yeast extracts. The phosphoenolpyruvate (PEP) and phosphotransferase (PTS) systems have been described as the most relevant systems for *O. oeni* sugar metabolism [29]. Specifically, the PTS system plays a significant role because it consists of integral membrane proteins capable of translocating substrates through the membrane via a phosphorylation cascade [25]. In addition to regulating the metabolism of some of the polysaccharides, it also participates in stress responses and allows rapid sugar uptake from the environment without the need for a concentration gradient.

The PTS system in *O. Oeni* includes genes such as *ptsI*, which encodes Enzyme I (EI), and *ptsH*, which encodes a histidine-containing phosphocarrier protein (HPr), which are highly conserved in most strains of *O. oeni*. Other important genes in the PTS system are *manA*, *manB,* and *manC*, which are overexpressed, especially in the presence of glucose and mannose, particularly *manA* and *manB* [25,29]. The activation of some of these genes could be associated with improved responses of *O. oeni* to the stress conditions of wine [25].

The addition of yeast mannoprotein extract during the growth of *O. oeni* could have a protective effect against various stress conditions associated with wine. This could be useful for obtaining starter cultures that improve the development and control of MLF. Therefore, this work aimed to determine the effect of adding different commercial mannoprotein

extracts on the viability of *O. oeni* when it is subjected to various stress conditions and on the development of MLF.

2. Materials and Methods

2.1. Microorganisms and Culture Media

In this study, two *O. oeni* strains were used: PSU-1 (ATCC BAA-331) and the commercial VP41 (Lallemand, Inc., Montreal, BC, Canada). To maintain *O. oeni* strains, MRSmf plates were used as described by Balmaseda et al. [24], and plates were stored at 4 °C. To prepare the inoculum, a single colony was selected from the plates and cultured in liquid medium at 27 °C within a 10% CO_2 environment. Subsequently, 500 µL of this culture was inoculated into 50 mL of the same fresh liquid medium.

To achieve optimal growth of *O. oeni*, MRS medium (Difco™ Lactobacilli MRS Broth, Becton, Dickinson and Co., Le Part de Claix, France) was used. The growth of *O. oeni* was enhanced by the addition of 5 g/L of D-Fructose and 4 g/L D/L-malic acid (referred to as MRSmf medium). For all MRS-based preparations used in this study, the pH was adjusted to 5. For growth curves, MRS liquid medium without additional components was used, and for plate inoculation, MRSmf medium supplemented with 20 g/L of agar was used.

2.2. Characterization of Mannoprotein Extracts

Three different solid products of pure mannoprotein extract, referred to as MP1, MP2, and MP3, from *Saccharomyces cerevisiae* cell walls commercially available were evaluated. Mannoprotein extracts were selected due to their minimum sulfur dioxide content according to the manufacturers' description. The average protein percentage (%) was determined using a Y15 automatic analyzer (Biosystems SA, Barcelona, Spain) by measuring the alpha-amino nitrogen (NOPA) and ammoniacal nitrogen content. To determine the polysaccharide concentration and their molecular weight distribution from the commercial product, the soluble fractions were analyzed via high resolution size exclusion chromatography (HRSEC) using a refractive index detector (RID), after a previous extraction based on Gil et al. [30]. Briefly, a sample of 10 mL was centrifuged at 8500 rpm for 20 min. The sample was subsequently dried and concentrated with a vacuum concentrator (Univap 148 100ECH; Progen Scientific, London, UK) until 2 mL of sample was obtained. This pellet was transferred to a 50 mL tube with 10 mL of 0.3 mM acidified ethanol and left to precipitate for 24 h at 4 °C. Next, the sample was centrifuged at 8500 rpm for 15 min and the supernatant was removed. The precipitate obtained was suspended with 1 mL of double-distilled water and kept frozen at −20 °C for 24 h. The sample was subsequentially removed and lyophilized (LyoQuest, Telstar, Barcelona, Spain) for 24 h. Once this time had elapsed, the precipitate was suspended in 1 mL of 50 mM ammonium formate and filtered into HPLC vials with 0.45 µm diameter membranes. The mixture was injected into an Agilent 1200 Series system HPLC instrument (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a G1311A quaternary pump, a G1316A column oven, a G1329A autosampler (Agilent Technologies Inc.), and a refractive index detector (G1362A–RID), using two SHodex gel permeation HPLC columns (OHpak SB-186 803 HQ and SB-804 HQ, 300 mm × 8 mm I.D.; Showa Denko, Japan) and a 50 mM ammonium formate mobile phase following a flow of 0.6 mL/min or 0.5 mL/min for 70 min. The molecular weight distribution of the polysaccharide fractions was followed by calibration with pullulan and dextran (Sigma-Aldrich, Saint Louis, MO, USA) standards of different molecular weights. Polysaccharides were quantified according to the peak area for each fraction using an external standard method with commercial pectin and dextran standards (Sigma-Aldrich) in the range between 0 and 2 g/L ($R^2 > 0.99$) [31].

2.3. Growth of O. oeni with and without Mannoprotein Addition

A growth curve was established for each *O. oeni* strain in order to evaluate the effect of the addition of 2 g/L of each of the mannoprotein extract (MP1, MP2, or MP3) with respect to the control conditions without mannoprotein addition. The experiment was

conducted in triplicate in 100 mL bottles containing liquid MRS media. The initial inoculation concentration was 1×10^5 CFU/mL. In all the growth experiments, a control without inoculation was included. Monitoring was performed by measuring the optical density (O.D.) at 600 nm every 24 h, and the viable population, based on MRSf plate counts, was determined at the initial time of inoculation (t_0), at the middle of the exponential phase of growth, and when the O.D. was stable (t_f). MRSf plates for viable population counting were incubated at 27 °C with 10% CO_2 for 7 days.

2.4. Stress Shocks

When the bacteria reached the stationary growth phase, the initial volumes were divided into 10 mL tubes for each condition, replicate, and type of stress. Two types of stress shocks were applied based on previous studies [16]: (i) an acid shock lowering the pH from 5 to 3.2 adding 2 M HCL, and (ii) ethanol shock adding this pure compound to reach 14% (*v*/*v*). The bacteria was exposed to the different stress conditions for 30 min at 20 °C. The survival rate was calculated as the percentage between viability at t_f (end of growth, before the shock) and viability after the application of the stress conditions.

2.5. Procedure for Extracting Mannoproteins and Analyzing Their Consumption

The consumption of mannoproteins by *O. oeni* during growth was evaluated. Analysis of mannoprotein consumption was primarily based on Quirós et al. [32] with some modifications. As such, 5 mL samples were collected after cold precipitation (−20 °C) overnight and subsequent centrifugation (7500 rpm for 15 min). To these samples, five volumes of absolute ethanol were added, and the samples were incubated overnight at 4 °C. Subsequently, the samples were centrifuged at 4600 rpm for 10 min, and the resulting pellet was washed with two volumes of ethanol. The pellet was then resuspended in 1 mL of ethanol; after which, the sample was transferred to a 2 mL tube. These samples were subjected to further centrifugation at 8500 rpm for 5 min, and the resulting pellet was dried in a speed vacuum (Univap 148 100ECH) for 60 min. Next, the dried samples were hydrolyzed by adding 1 mL of 5 M H_2SO_4 and incubating them for one hour at 95 °C in a thermoblock (Labnet, Madrid, Spain). Once the samples reached room temperature, neutralization was carried out with 10 M NaOH for enzymatic analysis, following the methods of Balmaseda et al. [24].

2.6. Fermentation Conditions

The malolactic fermentations (MLF) were conducted in wine-like medium (WLM) [24] with 14% ethanol (*v*/*v*), 2 g/L L-malic acid, 1.25 g/L Bacto TM casamino acids (BD, Le Pont de Claix Cedex, France), and 1.25 g/L peptone (Panreac, Química SLU, Castellar del Vallès, Spain), pH 3.4 and 14% ethanol.

A population of 10^7 cells/mL of each *O. oeni* strain was inoculated into WLM in 50 mL tubes under four fermentation conditions: (i) addition of 2 g/L of mannoprotein extract (MP1, MP2, or MP3) and (ii) the control without mannoprotein addition. All fermentations were run under anaerobic and static conditions at 20 °C in triplicate. The progress of fermentation was monitored daily by measuring the consumption of L-malic acid and the growth of the bacterial population was determined at the end of MLF. MLF was deemed complete when the L-malic acid concentration decreased to less than 0.1 g/L.

2.7. Analysis of Wine Parameters

Concentrations of sugars (glucose and fructose), L-malic acid, acetic acid, D- and L-lactic acid, NH_4, alpha-amino nitrogen (NOPA), and citric acid were determined with an autoanalyzer Y15 (Biosystems SA, Barcelona, Spain). pH was determined using a Crison micro pH 2002 pH-meter (Crison, Barcelona, Spain) and alcohol contents were determined using ebulliometry (electronic ebulliometer uEBU6576, Gab Sistemática Analítica SL, Barcelona, Spain) following the methods of the Compendium of International Methods of Analysis of Must and Wines (OIV, 2009).

2.8. Analysis of Gene Expression through Real-Time Quantitative PCR (RT-qPCR)

During the growth phases and after applying stress, *O. oeni* pellets from each sample were preserved. In total, 1 mL of growth medium at t_f (end of growth) and after the shock (pH and ethanol) was taken and centrifuged at 12,000 rpm for 5 min. The supernatant was discarded, and the pellet was frozen with liquid nitrogen and stored at −80 °C for later analysis. RNA extraction and transcriptional analysis by RT-qPCR were performed following the methods of Balmaseda et al. [24] and Olguín et al. [32].

The pellets were washed twice with 1 mL of sterile 10 mM Tris-HCl buffer at pH 8 and centrifuged at 12,000 rpm for 5 min. Then, the pellet was resuspended in 200 µL of the same buffer containing 50 mg/mL lysozyme (Roche Life Science, Mannheim, Germany) and incubated in a water bath at 37 °C for 30 min. RNA was purified following the instructions of the High Pure RNA Isolation Kit Version 13 (Roche Life Science).

To remove any remaining contaminating DNA, the Invitrogen TURBO DNA-free Kit (Thermo Fisher Scientific, Madrid, Spain) was used following the manufacturer's instructions. Subsequently, reverse transcription of the purified RNA was carried out as described by Olguín et al. [32], using a thermocycler (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed to evaluate the expression of five genes: *manA*, *manB*, *ptsI*, *ptsH,* and *hsp18,* using the primers described in supplementary Table S1. The first four genes are related to mannose metabolism and the last is related to the stress response; in addition, the *gyrB* gene was used as an internal control (constitutive gene). The relative expression of each gene in the two strains was determined using the ΔΔCt method of Livak and Schmittgen [33].

2.9. Statistical Analysis

Statistical analysis of the results was performed using XLSTAT software version 2022.5.1 (Addinsoft, Paris, France). Analysis of variance was conducted via ANOVA with Tukey's HSD test to determine significant differences among the samples. A confidence interval of 95% was utilized, and the level of statistical significance was established as p-value < 0.05.

3. Results and Discussion

3.1. Mannoprotein Extracts Characterization

The mannoprotein extracts (MP1, MP2, and MP3) used in this work were characterized according to their polysaccharide and protein richness. These three extracts were chosen among the commercially available products for their purity in soluble mannoproteins according to the manufacturer's description (between 85 and 95% (*w*/*w*) of richness) and the absence of sulfur dioxide, which would have negatively affected the viability of *O. oeni*. The three products were thermally extracted from the cell walls of selected strains of the yeast *S. cerevisiae*.

According to the chromatograms obtained by HSREC, the polysaccharide content differed among the three mannoprotein extracts (Figure 1). In this sense, MP1 was the richest in polysaccharides (98.06%), followed by MP2 and then MP3; however, no statistically significant differences were observed between these last two commercial products (Table 1), which contained 74.54% and 71.29% total polysaccharides, respectively. Significant differences were observed among the mannoprotein extracts in terms of molecular weight fractions (Table 1). Despite these differences, most of the polysaccharides from the three commercial products could be included in the medium/high-molecular-weight (M/HMW) fraction defined between 3 and 440 kDa. On the one hand, the M/HMW fraction was greater for MP1 and MP2 (74.15% and 73.93%, respectively) than for MP3 (58.86%). On the other hand, regarding the low-molecular-weight fraction (LMW) comprising polysaccharides of less than 3 kDa, the content was 23.09% in MP1 and 12.04% in MP3. In contrast, this LMW polysaccharide fraction was not detected in MP2. In conclusion, all the mannoprotein extracts were significantly different in the LMW fraction and the MP1 and MP2 products significantly differed from the MP3 product in the M/HMW fraction.

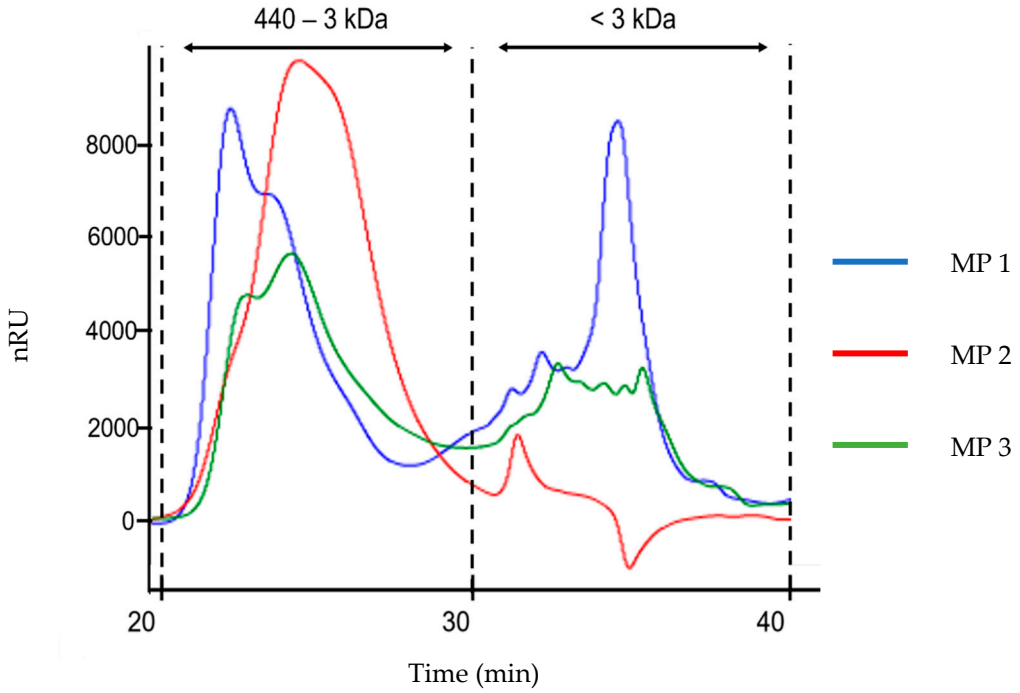

Figure 1. HRSEC-RID chromatograms of the different mannoprotein extracts, MP1, MP2, and MP3, divided in two main fractions, i.e., medium/high molecular weight (M/HMW) between 3 and 440 kDa and low molecular weight (LMW) less than 3 kDa, measured in absorbance units (nRU).

Table 1. Richness in total polysaccharide, medium/high-molecular-weight (M/HMW) polysaccharide, low-molecular-weight (LMW) polysaccharide, and total protein for the three mannoprotein extracts. Richness is expressed as the percentage (*w*/*w*) of each component referring to the total product weight. Different lowercase letters indicate the existence of significant differences among the samples (p-value < 0.05). Data are expressed as the mean arithmetic average ± standard deviation of experimental replicates (n = 3). Nd: not detected.

Mannoprotein Extract	Total Polysaccharide % (*w*/*w*)	M/HMW % (*w*/*w*)	LMW % (*w*/*w*)	Total Protein % (*w*/*w*)
MP1	98.06 ± 0.27 ^{b}	74.15 ± 1.12 ^{b}	23.91 ± 0.74 ^{b}	1.94 ± 0.01 ^{a}
MP2	74.54 ± 3.88 ^{a}	74.63 ± 5.48 ^{b}	nd	25.97 ± 0.02 ^{c}
MP3	71.29 ± 0.19 ^{a}	58.83 ± 0.78 ^{a}	12.40 ± 0.52 ^{a}	28.73 ± 0.01 ^{b}

The richness (%) in protein content was also different among the products (Table 1). Significant differences were observed among all the products, where MP3 presented the highest content, ~29%, followed by MP2, ~26%, and it is worth noting that the amount of protein in MP1 was lower (~2%).

3.2. Growth of O. oeni with Mannoprotein Addition

No significant differences were observed during the growth of the two strains of *O. oeni* VP41 and PSU-1, with the addition of mannoprotein extracts compared to the control without addition (Figure S1). Both strains entered the stationary phase after eight days. The VP41 strain exhibited a greater O.D. at 600 nm (1.67) in all conditions, whereas the PSU-1 strain exhibited an O.D. of 1.18 in the control condition and slightly higher values (approximately 1.3) in the cultures supplemented with mannoproteins. There were slight differences in terms of viability at the end of the growth in both strains, with an increase observed in CFU/mL in the cultures grown with added mannoprotein extracts compared to the control. However, these differences were not significant and the viable population was approximately 10^9 CFU/mL at the end of growth in all cases.

Significant differences in mannoprotein consumption were observed depending on the strain and the mannoprotein extract added (Table 2). The PSU-1 strain showed higher consumption with MP2 (68.10%), followed by MP1 (58.47%) and MP3 (54.75%). In the case of the VP41 strain, consumption was greater with MP1 (74.51%), followed by MP2 (55.18%) and MP3 (18.34%). These results are in accordance with previous studies that showed the consumption of mannoproteins by *O. oeni* [24]. In the two strains, MP3 was the product consumed least during growth, corresponding to the extract with a lower richness of polysaccharides (Table 1).

Table 2. Mannoprotein consumption at the end of growth (before stress shocks) and cell survival of *O. oeni* after acid shock and ethanol shock for each strain under the different growth conditions. Different lowercase letters indicate the existence of a significant difference between conditions regardless of the strain (p-value < 0.05). Different uppercase letters indicate the existence of a significant difference between strains with each mannoprotein extract (p-value < 0.05). All data are expressed as the arithmetic average ± standard deviation of biological replicates (n = 3).

Strain	Growth Condition	Mannoprotein Consumption (%)	Survival after pH Shock (%)	Survival after Ethanol Shock (%)
VP41	Control	-	67.18 ± 0.11^{Ba}	38.02 ± 0.23^{Bb}
	MP1	58.47 ± 0.05^{Ac}	74.21 ± 0.07^{Bb}	11.84 ± 0.09^{Ba}
	MP2	68.10 ± 0.25^{Ab}	70.83 ± 0.16^{Ba}	54.16 ± 0.11^{Bc}
	MP3	54.75 ± 0.62^{Aa}	91.74 ± 0.17^{Bc}	16.97 ± 0.14^{Ba}
PSU-1	Control	-	11.71 ± 0.08^{Aa}	5.14 ± 0.53^{Aa}
	MP1	74.51 ± 0.08^{Bb}	95.38 ± 0.26^{Ad}	31.23 ± 0.38^{Ab}
	MP2	55.15 ± 0.32^{Bb}	52.22 ± 0.26^{Ac}	57.33 ± 0.23^{Ac}
	MP3	18.34 ± 0.32^{Bb}	37.03 ± 0.10^{Ab}	74.07 ± 0.27^{Ad}

3.3. Effect of Mannoprotein Addition on O. oeni Survival in Response to Different Stress Shocks

Once the maximum O.D. was reached in the cultures of both *O. oeni* strains, two different types of stress shocks were applied considering the two main stress factors in wine: low pH (3.2) and high ethanol content (14% (v/v)). The viability of the cells grown with and without mannoprotein extracts was monitored before and after the shock. The survival of the PSU-1 and VP41 strains under the three stress conditions is shown in Table 2.

The PSU-1 strain demonstrated a greater survival rate after acid shock when it had been grown with added MP1 (95.39%), followed by added MP2 (52.22%) and added MP3 (37.04%). These values were statistically significant compared to those of the control, in which only 11.71% of the population survived. In the case of the VP41 strain, survival was greatest when MP3 was added to the growth media (91.74%), followed by MP1 (74.22%) and MP2 (70.83%). Significant differences were observed among these conditions and compared to the control (67.19%). Notably, VP41 exhibited a markedly greater survival percentage after stress shock in the control condition, without preculture with mannoproteins, than PSU-1. This finding indicates that VP41 is a more stress-resistant strain per se than PSU-1, regardless of the addition of mannoproteins.

The addition of 14% (v/v) ethanol had the most negative impact on both strains, with a particularly pronounced effect on VP41. For this strain, the addition of MP1 (11.85%) and MP3 (16.97%) resulted in lower survival rates than the control (38.02%). Only the addition of MP2 (54.17%) led to improved survival. There were significant differences among all these conditions. For PSU-1, the survival rates after the addition of MP3 (74.07%), MP2 (57.33%), and MP1 (31.32%) were better than that of the control (5.14%), with significant differences observed among all the studied conditions.

In conclusion, the addition of mannoprotein extracts to *O. oeni* growth media was beneficial for population survival in response to acid shock in all cases. However, this effect was found only in some cases in response to ethanol shock. Survival from ethanol shock improved with mannoprotein preculture in PSU-1 but not in VP41. In the latter case, the

resistance to ethanol improved only with the addition of MP2 to growth media. It is worth noting that survival benefits in response to all the stress shocks for the two strains were observed only with the addition of MP2.

3.4. Transcriptional Analysis of O. oeni in Response to Acid Shock

According to the previous results, mannoprotein addition would improve survival after acid shock in the two *O. oeni* strains precultured with any of the mannoprotein extracts. To evaluate changes in gene expression that could be indicative of mannose uptake, four representative genes of the PTS (phosphotransferase) system described for *O. oeni* [25,28] were analyzed via real time-qPCR: *manA* and *manB*, encoding PTS permeases; *ptsI*, which encodes Enzyme I (EI), and *ptsH*, which encodes a histidine-containing phosphocarrier protein (HPr). Additionally, the gene encoding for the heat shock protein Hsp18, which is a stress biomarker in *O. oeni* [8,34], was included in the analysis. Cultures with added MP2 were chosen for this on the basis of the previous results obtained in this work since this extract was the only one that had beneficial effects on both strains in response to all the stress conditions. In Figure 2, the results of the transcriptional analysis at the end of growth (MP2 vs. C) and after the application of the acid shock (MP2pH vs. CpH) are shown. Significant gene overexpression was considered when the relative expression (expressed as fold change, FC) exceeded 2, and inhibition of expression was considered when the relative expression was less than 0.5.

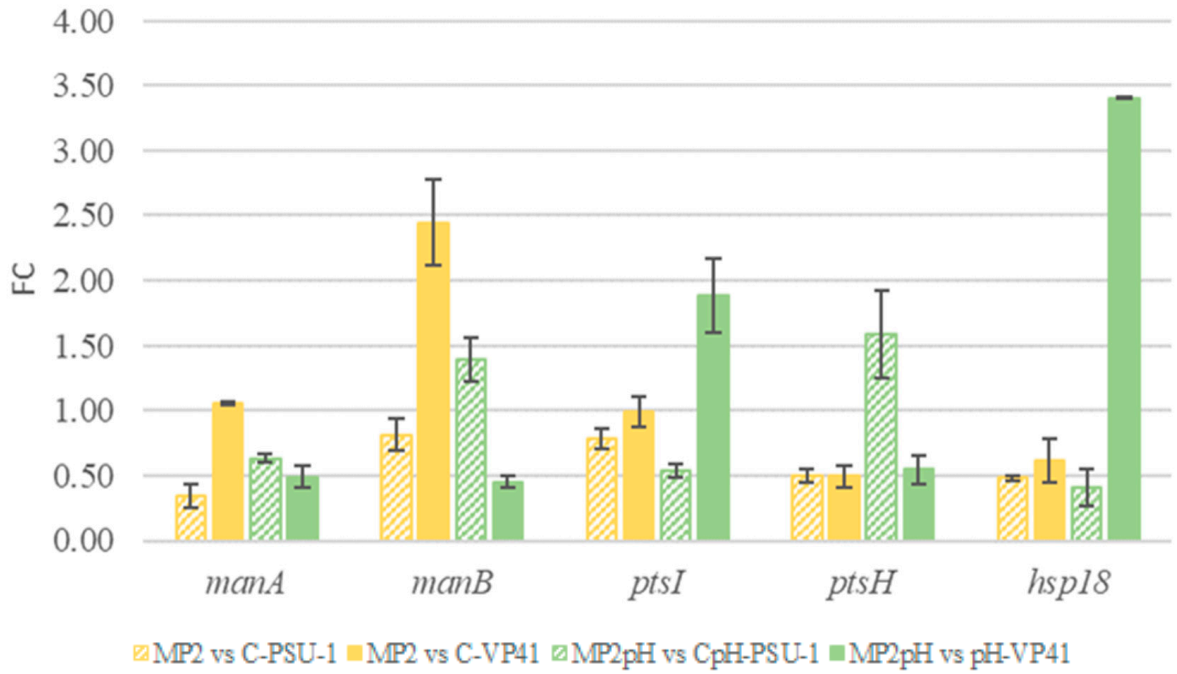

Figure 2. Relative expression (expressed in fold change, FC) of the genes *manA*, *manB*, *ptsI*, *ptsH*, *hsp18* in cells precultured with mannoprotein extract (MP2) and without mannoprotein (C) at the end of growth, and samples with and without mannoprotein added after receiving the acid shock (MP2pH and CpH, respectively) of each strain. All data are expressed as the arithmetic average of three biological replicates ± standard deviation (n = 3).

At the end of growth, an overexpression of *manB* was detected only in VP41 in cells precultured with MP2 with respect to the control. The *manB* gene encodes an active PTS permease that functions to transport sugars from the environment into the cell. It has been reported that the *manB* gene is overexpressed in *O. oeni* when it grows in media rich in glucose and fructose [26]. In fact, this permease is associated not only with mannose uptake but also with the uptake of other sugars, such as glucose. The overexpression of *ptsI* and *hsp18* genes was observed after the application of the pH shock only in VP41. The induction

of *hsp18* in response to acid stress has long been reported [28]. In the case of *ptsI*, this gene encodes for EI, one of the general PTS proteins. EI, which is a highly conserved enzyme in *O. oeni*, is used in the phosphorylation cascade for all the PTS substrates in the cell [29], therefore being associated with sugar utilization.

The strain PSU-1 did not overexpress any of the studied genes, whereas repression compared to the control of *manA* after growth with MP2 and of *hsp18* after acid shock was observed. Although significant, these changes were slight considering the standard deviation. The fact that PSU-1 showed different results compared to VP41 could be due to several reasons. On the one hand, this strain might activate stress mechanisms involving genes not included in this study since it is known that the stress response can vary among *O. oeni* strains. On the other hand, these results would be indicative of the complexity of the PTS system regulation and the variability in *O. oeni*. This is in accordance with the few studies reporting data on this topic that also showed the variability in gene regulation depending on the strain and/or the culture conditions [24,28].

3.5. Malolactic Fermentation in a Wine-like Medium

To evaluate the effect of mannoprotein addition to *O. oeni* starter culture growth on MLF development, fermentation was carried out in a model wine-like medium (WLM) inoculated with VP41 and PSU-1 strains. MLF was completed under all conditions by both strains. In the case of PSU-1, the fermentation was completed one day earlier than the control condition with the inocula precultured with MP1 and MP2, while the fermentation with the inoculum grown with MP3 was delayed by one day with respect to the control (Figure 3). VP41 showed differences only in MLF with cells grown with the addition of MP3, which was delayed by one day with respect to the control conditions, as observed for the PSU-1 strain (Figure 3). MLF activation in PSU-1 was observed for the precultures of the two mannoprotein extracts richest in the M/HMW polysaccharides, MP1 and MP2 (Table 1). Altogether, the metabolization of MP1 and MP2 during growth resulted in cells with better fermentation capacities in this strain, which is the most sensitive to acid pH and ethanol according to the results obtained in the stress shock assays (Table 2). Díez et al. [27] described the beneficial impact of medium-sized polysaccharides between 6 and 45 kDa, included here the in M/HMW fraction, on *O. oeni* growth. Therefore, polysaccharide size plays a role in the metabolization of these macromolecules by *O. oeni*, as previously suggested [27].

It is difficult to explain the delay in MLF completion observed for MP3 precultured cells of the two *O. oeni* strains since mannoprotein consumption and survival improvement in response to stress (Table 2) was also observed for this mannoprotein extract. It is worth mentioning that this was the less-consumed mannoprotein extract by the two strains. However, in addition to a lower mannoprotein consumption, some undetermined factor associated with MP3 composition and metabolization may have affected the cell state at the end of growth, resulting in a higher sensitivity to WLM conditions.

No changes in the citrate consumption or acetic acid production, which can compromise the wine sensory properties, of wine were observed. The content of nitrogen compounds was not altered by the preculture conditions (Table 3). The pH increased around 0.3 units after MLF in all cases, as expected due to L-malic acid decarboxylation. An increase in L-lactic acid was also in accordance with MLF completion. In summary, the use of mannoproteins in *O. oeni* preculturing did not alter the levels of the analyzed metabolites compared with those in the control condition.

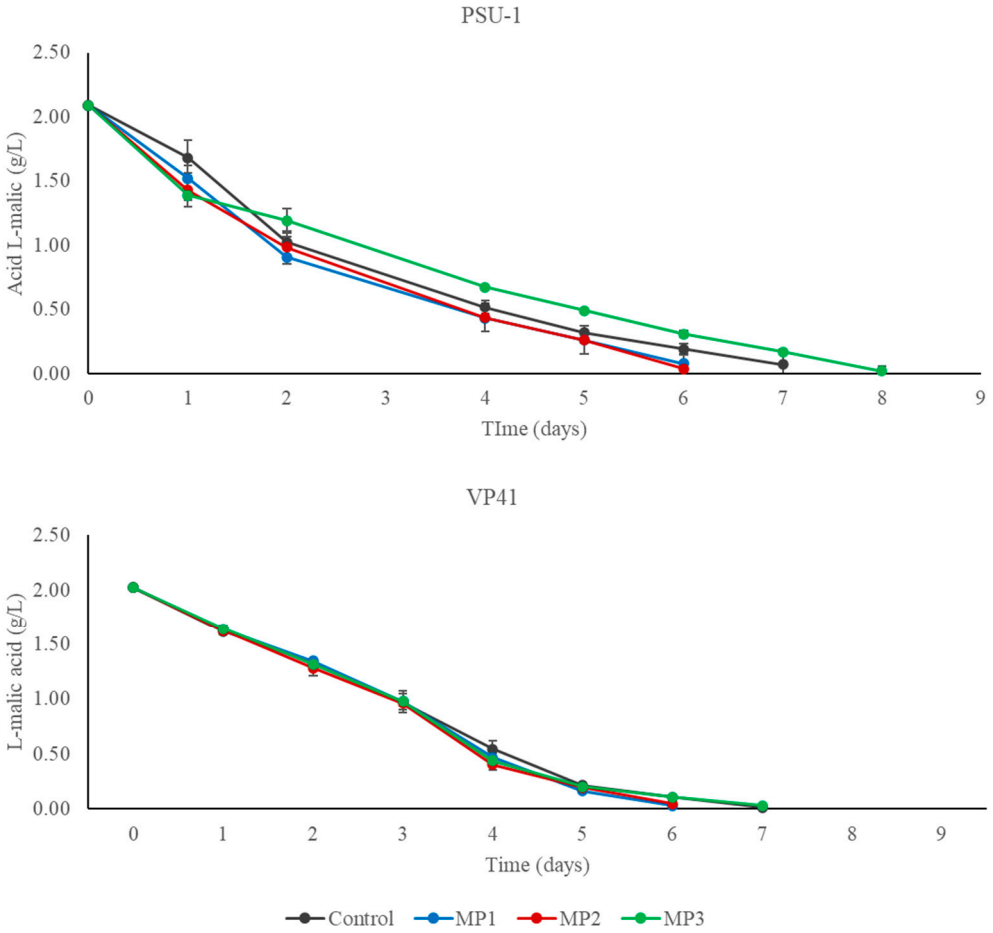

Figure 3. Evolution of malolactic fermentation kinetics fermented with *O. oeni* strain PSU-1 and VP41 in WLM. Control represents WLM inoculated with *O.oeni* precultured without added mannoproteins, while MP1, MP2, and MP3 indicate WLM inoculated with *O. oeni* precultured with MP1, MP2, and MP3 extracts, respectively. All data are expressed as the arithmetic average of three biological replicates ± standard deviation (n = 3).

Table 3. WLM analysis of wine compounds at the end of MLF. Control represents WLM inoculated with *O. oeni* precultured without added mannoproteins, while MP1, MP2, and MP3 indicate WLM inoculated with *O. oeni* precultured with mannoprotein extracts. Different lowercase letters indicate the existence of a significant difference between conditions regardless of the strain (p-value < 0.05). Different uppercase letters indicate the existence of a significant difference between strains with each mannoprotein extract (p-value < 0.05). All data are expressed as the arithmetic average ± standard deviation of biological replicates (n = 3).

Condition	Glucose/Fructose (g/L)	Citric Acid (mg/L)	L-lactic Acid (g/L)	D-lactic Acid (g/L)	Acetic Acid (g/L)	NH_4^+ (mg/L)	NOPA (mg/L)
			O. oeni VP41				
Control	0.46 ± 0.02 ^{Aa}	104.67 ± 5.57 ^{Aa}	1.44 ± 0.05 ^{Aa}	0.17 ± 0.01 ^{Aa}	0.46 ± 0.01 ^{Aa}	20.67 ± 0.58 ^{Aa}	160.33 ± 4.04 ^{Aa}
MP1	0.46 ± 0.01 ^{Aa}	106.67 ± 1.53 ^{Aa}	1.42 ± 0.05 ^{Aa}	0.17 ± 0.01 ^{Aa}	0.46 ± 0.01 ^{Aa}	18.67 ± 4.16 ^{Aa}	160.00 ± 4.00 ^{Aa}
MP2	0.44 ± 0.01 ^{Aa}	124.00 ± 6.08 ^{Aa}	1.35 ± 0.02 ^{Aa}	0.16 ± 0.01 ^{Aa}	0.48 ± 0.01 ^{Aa}	16.33 ± 1.15 ^{Aa}	154.67 ± 1.53 ^{Aa}
MP3	0.45 ± 0.01 ^{Aa}	138.33 ± 2.52 ^{Aa}	1.38 ± 0.01 ^{Aa}	0.16 ± 0.02 ^{Aa}	0.49 ± 0.02 ^{Aa}	16.33 ± 4.04 ^{Aa}	155.67 ± 1.15 ^{Aa}

Table 3. *Cont.*

Condition	Glucose/Fructose (g/L)	Citric Acid (mg/L)	L-lactic Acid (g/L)	D-lactic Acid (g/L)	Acetic Acid (g/L)	NH_4^+ (mg/L)	NOPA (mg/L)
			O. oeni PSU-1				
Control	0.49 ± 0.02 ^{Ba}	111.60 ± 7.64 ^{Ba}	1.26 ± 0.03 ^{Ba}	0.04 ± 0.01 ^{Ba}	0.41 ± 0.03 ^{Ba}	12.67 ± 3.06 ^{Ba}	139.67 ± 3.21 ^{Ba}
MP1	0.49 ± 0.02 ^{Ba}	116.67 ± 9.17 ^{Ba}	1.31 ± 0.04 ^{Ba}	0.05 ± 0.00 ^{Ba}	0.43 ± 0.01 ^{Ba}	14.67 ± 0.58 ^{Ba}	144.33 ± 5.13 ^{Ba}
MP2	0.49 ± 0.04 ^{Ba}	126.33 ± 17.06 ^{Ba}	1.3 ± 0.05 ^{Ba}	0.05 ± 0.00 ^{Ba}	0.43 ± 0.03 ^{Ba}	11.33 ± 1.15 ^{Ba}	144.00 ± 6.56 ^{Ba}
MP3	0.49 ± 0.02 ^{Ba}	138.67 ± 2.08 ^{Ba}	1.23 ± 0.06 ^{Ba}	0.03 ± 0.00 ^{Ba}	0.44 ± 0.02 ^{Ba}	11.67 ± 3.21 ^{Ba}	139.33 ± 4.93 ^{Ba}

4. Conclusions

This study showed that the addition of yeast mannoproteins could improve *O. oeni* population survival in response to acidic stress and to ethanol stress in some cases, which could be related to the consumption of mannoproteins during growth. A limited transcriptional response of the PTS system was observed due to the addition of mannoproteins, but only in the VP41 strain. This finding highlights the complex regulation of sugar metabolism and polysaccharide utilization in *O. oeni* which requires further study.

The PSU-1 strain, which is less resistant to stress conditions, benefited more from the added mannoproteins during starter culture growth in the development of MLF in WLM. The mannoprotein extracts richer in M/HMW polysaccharides were more favorable for MLF development.

The results of this study showed that the impact of yeast mannoproteins is variable and depends mainly on the *O. oeni* strain. According to these results, under less favorable conditions (i.e., more sensitive strains), the beneficial effect could be more relevant. Future research including natural wine and a wider collection of *O. oeni* strains is needed to understand the potential of yeast mannoproteins to improve the MLF performance of starter cultures.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10010052/s1, Figure S1: Growth curves of *O. oeni*; Table S1: Primers used for RT-qPCR analysis.

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