A Differential Proteomic Approach to Characterize the Cell Wall Adaptive Response to CO₂ Overpressure during Sparkling Wine-Making Process

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Abstract: In this study, a first proteomic approach was carried out to characterize the adaptive response of cell wall-related proteins to endogenous CO₂ overpressure, which is typical of second fermentation conditions, in two wine Saccharomyces cerevisiae strains (P29, a conventional second fermentation strain, and G1, a flor yeast strain implicated in sherry wine making). The results showed a high number of cell wall proteins in flor yeast G1 under pressure, highlighting content at the first month of aging. The cell wall proteomic response to pressure in flor yeast G1 was characterized by an increase in both the number and content of cell wall proteins involved in glucan remodeling and mannoproteins. On the other hand, cell wall proteins responsible for glucan assembly, cell adhesion, and lipid metabolism stood out in P29. Over-represented proteins under pressure were involved in cell wall integrity (Ecm33p and Pst1p), protein folding (Ssa1p and Ssa2p), and glucan remodeling (Exg2p and Scw4p). Flocculation-related proteins were not identified under pressure conditions. The use of flor yeasts for sparkling wine elaboration and improvement is proposed. Further research based on the genetic engineering of wine yeast using those genes from protein biomarkers under pressure alongside the second fermentation in bottle is required to achieve improvements.

Keywords: sparkling wine; yeast; cell wall; flocculation; protein; CO₂ overpressure

1. Introduction

The production of sparkling wines following the traditional method (or Méthode Champenoise) implies a characteristic stage where yeast cells are subjected to a second fermentation in sealed bottle and an aging period in contact with lees. This whole stage is known as setting the foam or second fermentation in bottle, and yeast cells must be able to cope with stress mainly caused by ethanol toxicity (10–12% v/v), low temperature (9–12 °C), nutrient starvation, and CO₂ overpressure (6–7 bar). Moreover, the aging period is known to contribute considerably to the wine quality and organoleptic properties through the release of cell wall and intracellular compounds during autolysis [1–4].

The yeast cell wall is a dynamic macromolecular complex in which components (β-1,3 and β-1,6-glucans, mannoproteins, and chitin) are linked, forming a molecular network with several functions [5]. Numerous studies have reported the structural and morphological changes of the cell wall, mainly during aging [6–9]. These authors confirmed that although the cell wall structure showed folds and morphological changes, it remained unbroken in yeast cells at long-term aging. The cell wall is essential not only for maintaining the cell morphology during growth, mating,
or sporulation, but also for dealing with stress conditions that destroy the cell integrity. Under stress conditions, cell wall composition varies in response to environment, and the existence of a cell response illustrates its dynamic nature [10,11]. This response to stress, which is known as the “compensatory mechanism”, has been characterized by an increase in the bulk of cell wall proteins, chitin content increase, glucans synthesis, and cell wall components’ redistribution and remodeling [12–14].

Among the cell wall components, mannoproteins and those involved in flocculation are the most relevant from the industrial and enological point of view. Mannoproteins represent between 35% and 40% of the cell wall, and numerous studies have associated these glycoproteins with the wine quality and organoleptic properties such as aroma [15], color [16], and foam [17]. In this context, the overproduction of mannoproteins has become one of the most desirable aspects for the yeast selection [16]. On the other hand, flocculation implies a nonssexual, homotypic, reversible, multivalent, and Ca²⁺-dependent process in which yeast cells aggregate, forming flocs. The flocculent capacity of yeast cells is considered a distinctive feature allowing a fast, cost-effective, and environmentally friendly way to remove yeast cells during wine clarification [18,19]. In addition, the higher ethanol stress resistance of Saccharomyces cerevisiae in biofilms provides an efficient ethanol fuel production, in respect to free cells, during industrial cell immobilization [20]. Among the genes that regulate flocculation, FLO11 is the main flocculin in yeasts required also for cell adhesion, invasive and pseudohyphal growth, and biofilm formation [21–23]. This last process is carried out by a special type of yeasts known as flor yeasts, which are capable of forming a biofilm on the wine surface and assimilating ethanol under oxidative conditions [24].

In this study, a novel proteomic approach was developed to identify cell wall-related proteins with the aim of characterizing and comparing their response to CO₂ overpressure along the second fermentation in two industrial S. cerevisiae strains (a sparkling wine strain P29 and a flor yeast G1 implicated in sherry wine production). Understanding of the yeast behavior and cell wall proteomic response to such special conditions would provide relevant insight into the yeast cell wall and would allow improving the industrial process of sparkling wine elaboration and second fermentation yeast strains.

2. Materials and Methods

2.1. Yeast Strains and Conditions

In this work, two industrial yeast strains were used: Saccharomyces cerevisiae P29 CECT 11770, a yeast strain commonly used in sparkling wine elaboration and isolated from INCAVI (Catalan Institute of Vines and Wines, Vilafranca del Penedès, Barcelona, Spain), and a flor yeast G1 ATCC MYA-2451, which is responsible for the biological aging of sherry wines and is isolated from a wine flor velum biofilm from Montilla-Moriles region, Spain.

Previously, yeast strains were grown in Yeast Extract–Peptone–Dextrose medium (YPD, 1% yeast extract, 2% peptone, and 2% glucose) and later acclimated separately in a pasteurized must (Macabeo white grape variety with 174.9 g/L of sugar, 3.6 g/L total acidity, and pH 3.4) during 5 days at 22 °C. After reaching high cellular concentration, viability, and ethanol content (similar to base wine), “tirage” was carried out according to INCAVI in a commercial base wine (Macabeo and Chardonnay 6:4, 10.21% v/v of ethanol, 0.3 g/L of sugar, pH 3.29, 5.4 g/L of total acidity, and 0.21 g/L of volatile acidity) added with 22 g/L of sucrose and 1.5 × 10⁶ cells/mL. Each yeast strain was fermented in two conditions: PC or pressure condition, using sealed bottles with a bidule and metal overcap; and NPC or non-pressure condition, using a perforated bidule. Sampling was performed at two points along the second fermentation: T1 or the middle of the second fermentation (3 bar), and T2 or one month after the end of the second fermentation (6.5 bar). Cells in sealed bottles were collected considering the pressure levels (T1: 3 bar and T2: 6.5 bar). Samples of control bottles without pressure were taken at the same times, just taking into consideration the similar values of ethanol content under both conditions.
Culture mediums, study conditions, sampling, kinetics of second fermentation, and cell viability are described in detail by Porras-Agüera et al. (2019) [25].

2.2. Proteomic Analysis

Protein extraction and identification was developed using the methods described in Porras-Agüera et al. (2019) [25] and Ishihama et al. (2005) [26] for protein quantification. Proteins were properly separated using an OFFGEL High Resolution kit pH 3–10 (Agilent Technologies, Palo Alto, CA, USA) according to their isoelectric point. Once separated, these proteins were identified through mass spectrometry, after digestion with trypsin, using an LTQ Orbitrap XL (Thermo Fisher Scientific, San José, CA, USA) and a nano LC Ultimate 3000 system (Dionex Corporation, Sunnyvale, CA, USA). After identification, cell wall-related proteins were selected using the Gene Ontology section from the *Saccharomyces* genome database (SGD, http://www.yeastgenome.org, access date: September 2019) and Uniprot (http://www.uniprot.org, access date: September 2019) databases, which were later quantified through the protein content (mol %).

2.3. Confidence Parameters and Statistics

From the total of proteins, only those identified with a score $>2$ and observed peptides $\geq 2$ were used in the analysis. These proteins were discussed according to their ratio content PC/NPC as over-represented (ratio $\geq 2$) or under-represented (ratio $\leq 0.5$). Moreover, proteins obtained with high contents and those found specifically in both strains were considered.

Cell wall proteins were sorted by biological processes (GO, Gene Ontology, Terms) using the tool “GO Term Finder” from the SGD database. For each GO Term, $p$-values and the FDR (False Discovery Rate) were calculated, and a $p$-value $< 0.01$ was considered at the time of selecting the GO Terms. The multiple sample comparison procedure (MSC) was performed using the software Statgraphics Centurion v. XVI (StatPoint Technologies, Warrenton, VA, USA), considering a confidence level of 95.0%, according to Fisher’s least significant difference (LSD) method. Furthermore, the software STRING v. 11.0 (https://string-db.org) was used to build the protein interaction map. All data were normalized (square root) and auto scaled prior to analysis.

3. Results and Discussion

In *S. cerevisiae* P29, a total of 594 proteins were detected under PCT1, 1517 were detected under NPCT1, 419 were detected under PCT2, and 392 were detected under NPCT2; whereas in *S. cerevisiae* G1, 568 proteins were obtained under PCT1, 1000 were obtained under NPCT1, 94 were obtained under PCT2, and 218 were obtained under NPCT2. In this study, 32 proteins specifically located in the yeast cell wall were identified in each condition and sampling time, as well as those proteins related to flocculation (Table S1 (Supplementary Materials)). In the case of cell wall-related proteins in the P29 strain, 12 were found under PCT1 (2.02%, 2.7 mol%), 6 were found under PCT2 (1.43%, 2.1 mol%), 26 were found under NPCT1 (1.71%, 2.7 mol%), and 7 were found under NPCT2 (1.79%, 3.2 mol%). On the contrary, higher frequencies and protein content were observed in flor yeast G1, especially at T2: 12 proteins were found under PCT1 (2.02%, 2.7 mol%), 6 were found under PCT2 (1.43%, 2.1 mol%), 26 were found under NPCT1 (1.71%, 2.7 mol%), and 7 were found under NPCT2 (1.79%, 3.2 mol%).

The protein number in P29 decreased under both conditions along the second fermentation and the first month of aging, while the number, content, and frequency values in flor yeast at T2 considerably exceeded those in P29. In fact, in terms of content, the difference was 3.6-fold and 4.1-fold under PCT2 and NPCT2, respectively, in flor yeast G1. These results suggest a high requirement of cell wall proteins in flor yeast once second fermentation is over, which is probably for a later biofilm formation once nitrogen and fermentable carbon sources are limited [24,27]. However, the proteins associated with this process only appeared under NPCT1 in both P29 (5 proteins, 0.33%, 0.1 mol%) and G1 (3 proteins, 0.30%, 0.1 mol%), and under NPCT2 just in flor yeast G1 (1 protein, 0.46%, 0.3 mol%).
In order to find the biological processes in which these proteins are involved, a GO analysis was carried out (Tables S2 and S3 (Supplementary Materials)). In general, processes associated with cell wall organization or biogenesis and external encapsulating structure organization were highly enriched in both strains and conditions. Nevertheless, the negative regulation of cell aging was highlighted especially at T2 under both conditions and yeast strains (except under PCT2 in G1), although it was also found under PCT1 in G1. Moreover, processes related to carbohydrate and polysaccharide metabolism were found in P29 and G1 under NPCT1, and also under this condition, a fungal-type cell wall (1→3)-β-D-glucan biosynthetic process was observed in flor yeast G1. As for the proteins associated with cell adhesion and flocculation, these were obtained only under NPCT1. Besides, processes such as invasive or pseudohyphal growth, which take place under glucose and nitrogen limitation, are highlighted in flor yeast G1.

To analyze the connections between the different proteins identified, a protein interaction network map, based on the 32 cell wall and flocculation-related proteins identified in total in *S. cerevisiae* P29 and G1, was built using the STRING v. 11.0 database (Figure 1). In the map, proteins are shown as nodes, and the edges represent the interactions between nodes. A PPI (protein-protein interaction) enrichment *p*-value < $1 \times 10^{-16}$ indicates that the nodes are not random and the observed number of edges is significant. From the 32 nodes, a total of 135 edges were established. Nodes with different colors represent specific clusters obtained from an MCL (Markov Cluster Algorithm) clustering method. The strength of the connections is indicated by the edges thickness, the red nodes being those which showed the strongest interactions, and representing proteins required mainly for cell wall organization and structure. In addition, proteins required for cell separation and cytokinesis (clear green nodes) also obtained strong connections, along with the proteins involved in flocculation and response to glucose starvation (blue nodes). Green nodes represent proteins responsible for folding and response to stress, and just the protein Plb2p did not show interactions.
In general, most of the cell wall proteins were detected at T1 and especially under NPC in both yeast strains. However, although the protein number decreased in samples at T2 under both PC and NPC, and also PCT1, the content increased considerably under these conditions (Figure 2). For a better understanding, the most relevant processes in which cell wall proteins in both strains are involved are described below. Furthermore, the over and under-represented proteins under PC, as well as those found specifically in a yeast strain and proteins that obtained high content, have been discussed in detail.

![Figure 2](image-url)  
**Figure 2.** Protein content increases (mol%) observed in (A) *S. cerevisiae* P29 and (B) *S. cerevisiae* G1, under pressure conditions (PC) compared to non-pressure condition (NPC). T1 (middle of the second fermentation), T2 (one month after it).

### 3.1. Glucan Processing and Remodeling

The main core of the cell wall is formed by β-1,3-glucan chains connected to β-1,6-glucan polymers. This polysaccharides network is continuously subjected to remodeling by numerous enzymes, allowing yeast cells to grow, bud, and deal with lysis [28]. In this study, a high amount of enzymes involved in glucan processing were identified in both strains. Among them, there is Exg2p, an exo-1,3-β-glucanase with a glycosylphosphatidylinositol (GPI) anchor attachment site required for β-glucan assembly [29], and the protein Scw4p, which is highlighted in flor yeast G1 to be detected 2.4 and 3-fold under PC at T1 and T2, respectively (Table 1). Even though Scw4p has been associated with the glucanases, studies by Capellaro et al. (1998) [30] reported that it may have an involvement in mating. Furthermore, deletion of this gene has been related to abnormal morphology, affecting the β-1,3-glucan and mannoproteins network, as well as increasing chitin content [31]. As it is observed in Figure 2, the protein content of Scw4p showed a different pattern, decreasing and increasing in P29 and G1, respectively, along the second fermentation and the first month of aging. This behavior might be explained due to the increase in pressure levels (2-fold at T2) and typical stresses as high ethanol content or starvation, which could compromise the cell wall integrity. Apart from the detection of Scw4p, the protein Scw10p was found in both strains, showing decreases in content under PC, which is more remarkable in flor yeast G1 at T2 (Figure 2). While the expression of SCW4 is constitutive,
the gene SCW10 is cell-cycle regulated [32]. Deletions of these homologous proteins (sharing 63% of amino acids) have resulted in cell wall changes and demonstrated their role in the mating process [30]. Besides, these proteins have been suggested to participate in concert with other cell wall proteins to maintain cell wall integrity [33]. The high requirement of enzymes in flor yeast responsible for cell wall remodeling, especially glucans, would allow yeast cells to make the cell wall rigid, tolerate the stress conditions, and avoid cell lysis [12,34].

Other relevant hydrolases identified in this study were the two major proteins of the cell wall, the endo-β-1,3-glucanase Bgl2p and the exo-β-1,3-glucanase Exg1p [35,36]. These proteins were found with high content in both strains, especially at T2 under both conditions. However, this content was different depending on the yeast strain, since while in P29, Bgl2p was more abundant under NPCT2, in G1, it stood out under PCT2; and the same behavior was observed for Exg1p. This can be better appreciated in Figure 2, where the content of both Bgl2p and Exg1p increased lightly under PCT2 (versus NPCT2) in flor yeast G1, and on the contrary, in P29, their content under PCT2 showed a considerable drop of 0.25 and 0.24 mol%, respectively. Deletions of both genes BGL2 and EXG1 have been reported to increase the chitin and glucan levels in the cell wall, respectively [37,38]. Moreover, Bgl2p has been implied in the incorporation of GPI-anchored cell wall proteins [39] and also in the limitation of the reproductive life span during aging [40]. Cell wall degradation, which takes place during the autolysis process, is carried out by numerous hydrolytic enzymes, of which the glucanases are the most relevant. Although autolysis has not been observed until 3–6 months of aging in sparkling wine with conventional strains [1], the observed increase of glucanases under pressure conditions in flor yeast G1 might promote an earlier release of cell wall components and therefore shorten the period of aging under lees.

3.2. Glucan and Chitin Assembly

Once cell wall components are synthetized, these are assembled and cross-linked to the cell wall due to the action of different glycoproteins and enzymes. In this context, the glycoprotein Kre9p was identified exclusively in P29 under NPCT1. KRE9 encodes an O-glycoprotein reported to be involved in β-1,6-glucan synthesis and assembly [41]. These authors confirmed that the disruption of this gene results in serious growth impairment and an altered cell wall containing less than 20% of β-1,6-glucan. In addition to this protein, those belonging to the GAS family (Gas1p, Gas3p, and Gas5p) were found in both strains (under PCT1 and NPCT1 in P29, and under all conditions in G1). According to Figure 2, the only difference was observed in Gas1p, whose content decreased at both T1 and T2 in flor yeast G1, while it increased just at T1 in P29. The results obtained by Matsushita et al. (2017) [42] support that the use of strains overexpressing the gene GAS1 have several advantages for fermentation processes under stress conditions, especially for low-pH conditions. These proteins are known to be β-1,3-glucanosyltransferases required for the maintenance and formation of β-1,3-glucan [10,43,44]. Based on their expression patterns, they appear to play partially overlapping roles throughout the development: whereas GAS1 and GAS5 are induced during vegetative growth, GAS2 is expressed exclusively during sporulation and is required for normal spore wall formation [45]. On the other hand, the protein Crh1p and the chitin transglycosylase Crh1p was found in both strains (under NPCT1 in P29, and under all conditions in G1; Table S1 (Supplementary Materials)). It functions in the transfer of chitin to β-1,6 and β-1,3 glucan in the cell wall [46], and it is known to be induced under cell wall stress [47]. From an industrial point of view, the addition of chitin has been demonstrated to reduce wine haze formation and improve the clarification process [48]. Moreover, the presence of chitin attached to glucans in the cell wall has been observed under stress conditions through the action of Crh1p. This component accumulates as much as 10 times more in cells with mutations affecting the synthesis of glucans, mannoproteins, or glycoproteins, in order to compensate for the cell integrity [49]. Therefore, the activation of glucan and chitin synthesis could be induced as a response to cell wall stress via the cell wall integrity pathway [13,50,51].
Table 1. Over-represented proteins detected under pressure conditions (PC) in both yeast strains *S. cerevisiae* P29 and G1, at the middle of the second fermentation (T1) and one month after it (T2). Molecular function and fold change in brackets are shown. Only proteins with fold changes of protein content ≥ 1.8 are shown.

GPI: glycosylphosphatidylinositol.

| Yeast Strains         | *S. cerevisiae* P29 |          |          | *S. cerevisiae* G1 |          |          |
|-----------------------|---------------------|----------|----------|-------------------|----------|----------|
| Conditions            | T1                  | Funcion  | T2       | Function          | T1       | Function |
| Protein               | Ecm33p (2.1)        | GPI-anchored protein | -        | -              | Cis3p (1.8) | Mannoprotein |
|                       | Gas1p (1.8)         | β-1,3-glucanosyltransferase | -        | -              | Exg2p (2.4) | β-glucan assembly |
|                       | Pst1p (2.3)         | Cell wall protein with GPI-attachment site | -        | -              | Pst1p (1.9) | Cell wall protein with GPI-attachment site |
|                       | Ssa1p (2)           | Protein folding | -        | -              | Ssa1p (2.1) | Protein folding |
|                       | Ssa2p (1.9)         | Protein folding | -        | -              | Ssa2p (2.1) | Protein folding |

O-mannosylated heat shock protein
Cell wall protein with GPI (Glycosylphosphatidylinositol) attachment site
Cell wall protein with similarity to glucanases
3.3. Mannoproteins

Apart from glucan and chitin, mainly mannoproteins represent between 35% and 40% of the cell wall, and they are covalently joined to the β-1,3-glucan network [10]. Among them, mannoproteins belonging to the PIR family (Pir1p, Hsp150p/Pir2p, Pir3p, and Cis3p/Pir4p) were detected in both strains; however, they were more abundant in terms of content in flor yeast G1 (Table S1 (Supplementary Materials)). The authors revealed that these yeast genes are homologous, containing internal tandem repeats of amino acids, and PIR1 and PIR2 were observed to participate during heat shock tolerance [52,53]. The mannoprotein Cis3p/Pir4p, exclusively located in the bud scars of vegetative cells [54,55], experimented a marked decrease in content at T2, comparing both PC and NPC (Figure 2). As for the rest, it highlighted both the increase of Hsp150/Pir2p and the decrease of Pir1p in flor yeast under PCT2 (Figure 2). Moreover, Pir3p was found with high content in flor yeast G1 under both PCT2 and NPCT2 (Table S1 (Supplementary Materials)), although it did not show great differences in content. These PIR proteins have been associated with numerous functions and processes such as cell wall stability and synthesis, and also heat and nutrient stress [56–58]. In addition to these mannoproteins, others such as Ccw14p, Cwp1p, Dan4p, and Pst1p were found in both strains. The covalently linked cell wall mannoprotein Ccw14p [59,60] was found in both strains, although its content was more relevant in flor yeast G1 (only identified at T1 under both conditions). Furthermore, deletion of this gene confirmed their role in biofilm formation in flor yeast G1, showing a decrease of the biofilm weight and cell adhesion [61]. Cwp1p localizes to the birth scars of daughter cells [62], and Dan4p was obtained in the two yeast strains and specifically in P29, both under NPCT1, respectively. Studies by Abramova et al. (2001) [63] confirmed that CWP1 is down-regulated under anaerobic conditions, which agree with our results, since this protein was not found under PC (Table S1 (Supplementary Materials)). Lastly, the mannoprotein Pst1p, which is secreted by yeast-regenerating protoplasts [64], was found over-represented in P29 (Table 1), and it also stood out in flor yeast G1 in terms of content under PC (Figure 2). This protein has been observed to be important for cell wall integrity [65] and during the response to cell wall damage, along with Cwp1p [66].

The high amount of mannoproteins detected, some of them under pressure conditions, and more abundant in flor yeast G1 could be interesting from an industrial and enological point of view. These glycoproteins have been reported to positively affect the wine quality and organoleptic properties, improving parameters such as the wine aroma [15], color [16], and foam [17]. In this context, the overproduction of mannoproteins represents one of the most desirable aspects for the yeast strains selection. The abundance of mannoproteins observed in flor yeast may open a door to the use of this type of yeast in the sparkling wine elaboration or improvement. Besides, the accumulation of mannoproteins in the cell wall has been recently associated with enhanced stress resistance and fermentation performance [67].

3.4. Cell Separation

The enzymes required for cell separation and cytokinesis located in the cell wall (Figure 1) were detected also. The endoglucanase Egt2p stood out to be found specifically in flor yeast G1 under NPCT1. This protein has been associated with cell separation during the cell cycle after cytokinesis [68]. However, studies carried out by Pan and Heitman (2000) [69] revealed a new role of this protein in pseudohyphal growth, which could give us an insight into the yeast behavior, since in addition, this result is in accordance with the GO Terms “invasive filamentous growth” and “pseudohyphal growth” detected under NPCT1 in flor yeast (Table S3 (Supplementary Materials)). Another endoglucanase such as Dse4p, located in the cell wall and required for cell separation [70], was found under PCT1 and NPCT1 in P29, and under NPCT1 and NPCT2 in G1. The content of this protein decreased significantly in both strains (Table S1 (Supplementary Materials)), although this difference in content was higher in P29 (Figure 2). Furthermore, Sun4p, a member of the SUN family of proteins, is involved in the remodeling of the yeast cell wall and cell septation process during the various phases of yeast culture development and under various environmental conditions [71,72], and it
was identified only under NPCT1 in both yeast strains (Figure 2). Additionally, the glucosidase Scw11p also was detected in both strains, and it seems to play a role in cell separation and conjugation during mating [30]. The detection of these proteins may indicate that yeast cells are dividing, and despite the fact that nutrients are available for yeast cells at T1, some cells of the colony could exhibit invasive and pseudohyphal growth. This would require a coordinated cell wall synthesis and remodeling in order to deal with the changes in cell morphology.

3.5. Proteins Related to Flocculation, Cell Adhesion, and Biofilm Formation

The selection of wine yeasts with flocculent capacity represents a desirable factor in sparkling wine elaboration, since this process would allow a fast clarification of fermenting product, thus reducing time and production costs [73]. The proteins required for this process, along with others involved in cell adhesion and biofilm formation, were found in both yeast strains and mainly under NPCT1 (Table S1 (Supplementary Materials)). Among them, Flo11p stands out as the main flocculin responsible for cell adhesion-related phenotypes in *S. cerevisiae* and whose analysis revealed complex mechanisms of genetic regulation [21,74]. However, this protein was not relevant in terms of content under PC (Figure 2), since it only was observed under NPCT1 in both strains and NPCT2 just in flor yeast G1. Protein kinase Snf1p and the subunit beta-3 Gal83p were identified in both strains. Studies by Kuchin et al. (2002) [75] showed evidence that Snf1p kinase regulates the transcription of *FLO11* during pseudohyphal growth and biofilm formation in response to glucose limitation. Moreover, it has been observed that the interaction of Snf1p–Gal83p (Figure 1) is required for invasive growth through *FLO11* activation [76]. Apart from these proteins, others such as the heat shock protein Hsp12p and the transcriptional regulator Snf2p were found just in *S. cerevisiae* P29 under NPCT1 (Table S1 (Supplementary Materials)). While the first one is responsible for membrane organization during stress [77] and is essential for biofilm formation [78], the second one is a catalytic subunit of the SWI/SNF chromatin-remodeling complex involved in *FLO11* activation [21]. Biofilm formation is known to be induced under nutrient limitation as glucose or nitrogen and oxidative conditions, which is in agreement with the detection of these proteins under NPC. The switch of fermentative to oxidative metabolism by flor yeasts is essential to allow cells to remain at the wine surface and metabolize ethanol into acetaldehyde [24]. The presence of proteins involved in cell adhesion and flocculation under NPCT1 in both strains could indicate that cells are forming flocs under these conditions. On the other hand, the detection of Flo11p in flor yeast G1 with high content under NPCT2 suggests that this yeast strain is developing a biofilm formation phenotype.

3.6. Other Cell Wall Proteins

Apart from the cell wall proteins mentioned above, others participating in processes such as folding (Ssa1p and Ssa2p), lipid metabolism (Plb2p), and two proteins with unknown specific function (Ecm33p and Sim1p) were also detected in this study. The ATPase Ssa1p and the ATP-binding protein Ssa2p, both belonging to the HSP70 protein family [79], were relevant in both strains. These proteins were found to be over-represented in both yeast strains: Ssa1p just in P29 and the two in flor yeast G1 (Table 1). Since the main function of these proteins is to serve as molecular chaperones, binding newly translated proteins to assist in proper folding and prevent aggregation/misfolding [80], their presence under PC may be explained as a response to cell wall damage and stress. On the other hand, the lysophospholipase 2 or Plb2p, which is required for lipid metabolism [81], appeared exclusively in P29 just at T1 under both conditions (Table S1 (Supplementary Materials)). Ethanol stress has been proposed as the main factor to activate lipid membrane remodeling, in order to increase its stability and resistance [82]. Additionally, the cell surface glycosylphosphatidylinositol (GPI)-anchored protein Ecm33p, which is required for proper cell wall integrity and for the correct assembly of the mannoprotein outer layer [65,83,84], and the protein Sim1p—a member of the SUN family of proteins probably with a role in DNA replication [85]—were found: the first one over-represented in P29.
(Table 1) and the second one in both strains, although the differences in content were not relevant (Figure 2).

4. Conclusions

According to the results, pressure seems to affect the number of cell wall proteins, mainly in *S. cerevisiae* P29. The results obtained in flor yeast G1 under CO$_2$ overpressure conditions agree with those observed during a typical cell wall response to stress, and the abundance observed in mannoproteins makes this type of yeasts an interesting and innovative option for the improvement and elaboration of new sparkling wines. On the other hand, in *S. cerevisiae*, P29 stood out among the cell wall proteins responsible for glucan assembly and lipid metabolism. On the contrary, those proteins related to cell adhesion phenotypes (flocculation, cell adhesion, and biofilm formation) were not relevant under pressure, being observed exclusively in open bottles in both strains. Over-represented proteins under pressure were involved in cell wall integrity (Ecm33p and Pst1p) and folding (Ssa1p) in *S. cerevisiae* P29, and glucan remodeling (Exg2p and Scw4p) and folding (Ssa1p and Ssa2p) in flor yeast G1. The genes that codify these proteins may be interesting for exploration in the search for mechanisms involved in endogenous CO$_2$ overpressure adaptation in sparkling wine yeasts, and the other hand, these target proteins might be involved in an accelerated aging process.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/8/1188/s1.

Table S1: List of total proteins related to cell wall and flocculation identified in *S. cerevisiae* P29 and G1, under both study conditions (PC, CO$_2$ overpressure condition; and NPC, non-pressure conditions) and sampling times (T1, middle of the second fermentation; T2, one month after it). Data show protein and gene names, accession number of Uniprot, molecular function, score, peptides, and protein content (mol%) ± standard deviation. Different letters (a–f) indicate significant differences in each condition at 0.05 level according to Fisher’s least significant difference procedure. Proteins showing between 4 and 8 homogeneous groups (HG) are marked with an asterisk *. n.f.; not found. ns.; not significant. Table S2: Biological processes (GO terms) of cell wall-related proteins detected in *S. cerevisiae* P29 under PC (CO$_2$ overpressure condition) and NPC (non-pressure condition), and in each sampling time: T1 (at the middle of the second fermentation) and T2 (one month after it). GO terms were obtained considering a p-value < 0.01. Table S3: Biological processes (GO terms) of cell wall-related proteins detected in *S. cerevisiae* G1 under PC (CO$_2$ overpressure condition) and NPC (non-pressure condition), and in each sampling time: T1 (at the middle of the second fermentation) and T2 (one month after it). GO terms were obtained considering a p-value < 0.01.

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References

1. Alexandre, H.; Guilloux-Benatier, M. Yeast autolysis in sparkling wine—a review. *Aust. J. Grape Wine Res.* 2006, 12, 119–127. [CrossRef]

2. Kemp, B.; Alexandre, H.; Robillard, B.; Marchal, R. Effect of production phase on bottle-fermented sparkling wine quality. *J. Agric. Food Chem.* 2015, 63, 19–38. [CrossRef]

3. Penacho, V.; Valero, E.; Gonzalez, R. Transcription profiling of sparkling wine second fermentation. *Int. J. Food Microbiol.* 2012, 153, 176–182. [CrossRef] [PubMed]
4. Torresi, S.; Frangipane, M.T.; Anelli, G. Biotechnologies in sparkling wine production. Interesting approaches for quality improvement: A review. *Food Chem.* **2011**, *129*, 1232–1241. [CrossRef] [PubMed]

5. Kalebina, T.S.; Rekstina, V.V. Molecular organization of yeast cell envelope. *Mol. Biol.* **2019**, *53*, 850–861. [CrossRef]

6. Charpentier, C.; Van Long, T.N.; Bonaly, R.; Fuellrat, M. Alteration of cell wall structure in *Saccharomyces cerevisiae* and *Saccharomyces bayanus* during autolysis. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 405–413. [CrossRef]

7. Martínez-Rodríguez, A.J.; Polo, M.C.; Carrascosa, A.V. Structural and ultrastructural changes in yeast cells during autolysis in a model wine system and in sparkling wines. *Int. J. Food Microbiol.* **2001**, *71*, 45–51. [CrossRef]

8. Piton, F.; Charpentier, M.; Troton, D. Cell Wall and Lipid Changes in *Saccharomyces cerevisiae* during Aging of Champagne Wine. *Am. J. Enol. Vitic.* **1988**, *39*, 221–226.

9. Tudela, R.; Gallardo-Chacón, J.J.; Rius, N.; López-Tamames, E.; Buxaderas, S. Ultrastructural changes of sparkling wine lees during long-terms aging in real enological conditions. *FEMS Yeast Res.* **2012**, *12*, 466–476. [CrossRef]

10. Klis, F.M.; Mol, P.; Hellingwerf, K.; Brul, S. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEBS Microbiol. Rev.* **2002**, *26*, 239–256. [CrossRef]

11. Sanz, A.B.; García, R.; Rodríguez-Peña, J.M.; Arroyo, J. The CWI pathway: Regulation of the transcriptional adaptive response to cell wall stress in yeast. *J. Fungi* **2018**, *4*, 1. [CrossRef] [PubMed]

12. Bowman, S.M.; Free, S.J. The structure and synthesis of the fungal cell wall. *Bioessays* **2006**, *28*, 799–808. [CrossRef] [PubMed]

13. Levin, D.E. Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: The cell wall integrity signaling pathway. *Genetics* **2011**, *189*, 1145–1175. [CrossRef] [PubMed]

14. Durán, A.; Nombela, C. Fungal cell wall biogenesis: Building a dynamic interface with the environment. *Microbiology* **2004**, *150*, 3099–3103. [CrossRef] [PubMed]

15. Juega, M.; Núñez, Y.P.; Carrascosa, A.V.; Martínez-Rodríguez, A.J. Influence of yeast mannoproteins in the aroma improvement of white wines. *J. Food Sci.* **2012**, *77*, 499–504. [CrossRef]

16. Guadalupe, Z.; Martínez, L.; Ayestarán, B. Yeast Mannoproteins in Red Winemaking: Effect on Polysaccharide, Polyphenolic, and Color Composition. *Am. J. Enol. Vitic.* **2010**, *61*, 191–200.

17. Blasco, L.; Viñas, M.; Villa, T.G. Proteins influencing foam formation in wine and beer: The role of yeast. *Int. Microbiol.* **2011**, *14*, 61–71. [CrossRef] [PubMed]

18. Pozo-Bayón, M.A.; Martínez-Rodríguez, A.; Pueyo, E.; Moreno-Arribas, M.V. Chemical and biochemical features involved in sparkling wine production: From a traditional to an improved winemaking technology. *Trends Food Sci. Technol.* **2009**, *20*, 289–299. [CrossRef]

19. Soares, E.V. Flocculation in *Saccharomyces cerevisiae*: A review. *J. Appl. Microbiol.* **2010**, *110*, 1–18. [CrossRef] [PubMed]

20. Yang, L.; Zheng, C.; Chen, Y.; Ying, H. *FLO* genes family and transcription factor MIG1 regulate *Saccharomyces cerevisiae* biofilm formation during immobilized fermentation. *Front. Microbiol.* **2018**, *9*, 1860. [CrossRef]

21. Barrales, R.R.; Jimenez, J.; Ibeas, J.I. Identification of novel activation mechanisms for FLO11 regulation in *Saccharomyces cerevisiae*. *Genetics* **2008**, *178*, 145–156. [CrossRef] [PubMed]

22. Van Dyk, D.; Pretorius, I.S.; Bauer, F.F. Mss11p is a central element of the regulatory network that controls *FLO11* expression and invasive growth in *Saccharomyces cerevisiae*. *Genetics* **2005**, *169*, 91–106. [CrossRef] [PubMed]

23. Zara, G.; Zeidan, M.B.; Fancellu, F.; Sanna, M.S.; Mannazzu, I.; Budroni, M.; Zara, S. The administration of L-cysteine and L-arginine inhibits biofilm formation in wild-type biofilm-forming yeast by modulating *FLO11* gene expression. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 7675–7685. [CrossRef] [PubMed]
24. Legras, J.L.; Moreno-Garcia, J.; Zara, S.; Zara, G.; Garcia-Martinez, T.; Mauricio, J.C.; Mannazzu, I.; Coi, A.L.; Zeidan, M.B.; Dequín, S.; et al. Flor yeast: New perspectives beyond wine aging. Front. Microbiol. 2016, 7, 503. [CrossRef] [PubMed]

25. Porras-Agüera, J.A.; Moreno-Garcia, J.; Mauricio, J.C.; Moreno, J.; Garcia-Martinez, T. First Proteomic Approach to Identify Cell Death Biomarkers in Wine Yeasts during Sparkling Wine Production. Microorganisms 2019, 7, 542. [CrossRef] [PubMed]

26. Ishihama, Y.; Oda, Y.; Tabata, T.; Sato, T.; Nagasu, T.; Rappsilber, J.; Mann, M. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol. Cell. Proteom. 2005, 4, 1265–1272. [CrossRef]

27. Alexandre, H. Flor yeasts of Saccharomyces cerevisiae—Their ecology, genetics and metabolism. Int. J. Food Microbiol. 2013, 167, 269–275. [CrossRef]

28. Teparić, R.; Mrša, V. Proteins involved in building, maintaining and remodeling of yeast cell walls. Curr. Genet. 2013, 59, 171–185. [CrossRef]
43. Mazáñ, M.; Ragni, E.; Popolo, L.; Farkaš, V. Catalytic properties of the Gas family β-(1,3)-glucanoyltransferases active in fungal cell-wall biogenesis as determined by a novel fluorescent assay. *Biochem. J.* 2011, 438, 275–282. [CrossRef] [PubMed]

44. Mouyna, I.; Fontaine, T.; Vai, M.; Monod, M.; Fonzi, W.A.; Diaquin, M.; Popolo, L.; Hentrich, R.P.; Latgé, J.P. Glycosylphosphatidylinositol-anchored glucanoyltransferases play an active role in the biosynthesis of the fungal cell wall. *J. Biol. Chem.* 2000, 275, 14882–14889. [CrossRef]

45. Ragni, E.; Coluccio, A.; Rolli, E.; Rodríguez-Peña, J.M.; Colasante, G.; Arroyo, J.; Neiman, A.M.; Popolo, L. GAS2 and GAS4, a pair of developmentally regulated genes required for spore wall assembly in *Saccharomyces cerevisiae*. *Eukaryot. Cell* 2007, 6, 302–316. [CrossRef]

46. Cabib, E.; Blanco, N.; Grau, C.; Rodríguez-Peña, J.M.; Arroyo, J. Crh1p and Crh2p are required for the cross-linking of chitin to beta(1-6)glucan in the *Saccharomyces cerevisiae* cell wall. *Mol. Microbiol.* 2007, 63, 921–935. [CrossRef]

47. Bermejo, C.; Rodríguez, E.; García, R.; Rodríguez-Peña, J.M.; Rodríguez de la Concepición, M.L.; Riva, C.; Arias, P.; Nombela, C.; Posas, F.; Arroyo, J. The sequential activation of the yeast HOG and SLT2 pathways is required for cell survival to cell wall stress. *Mol. Biol. Cell* 2008, 19, 1113–1124. [CrossRef]

48. Ndlovu, T.; Divol, B.; Bauer, F.F. Yeast cell wall chitin reduces wine haze formation. *Appl. Microbiol. Biotechnol.* 2004, 66, 352–358. [CrossRef] [PubMed]

49. Moukadiri, I.; Armero, J.; Abad, A.; Sentandreu, R.; Zueco, J. Identification of a mannoprotein present in the cell wall of *Saccharomyces cerevisiae* by reducing agents. *Microbiology* 2009, 155, 199–207. [CrossRef] [PubMed]

50. Popolo, L.; Gualtieri, T.; Ragni, E. The yeast cell wall salvage pathway. *Med. Mycol.* 2001, 39, 111–121. [CrossRef]

51. Lesage, G.; Bussey, H. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 2006, 70, 317–343. [CrossRef] [PubMed]

52. Toh-e, A.; Yasunaga, S.; Nisogi, H.; Tanaka, K.; Oguchi, T.; Matsui, Y. Three yeast genes, GAS2, GAS3, and GAS4 are required for cell survival to cell wall stress. *J. Bacteriol.* 1993, 175, 481–494. [CrossRef] [PubMed]

53. Yang, N.; Yu, Z.; Jia, D.; Xie, Z.; Zhang, K.; Xia, Z.; Lei, L.; Qiao, M. The contribution of Pir protein family to yeast cell surface display. *Appl. Microbiol. Biotechnol.* 2014, 98, 2897–2905. [CrossRef] [PubMed]

54. Moukadiri, I.; Jaafar, L.; Zueco, J. Identification of two mannanproteins released from cell walls of a *Saccharomyces cerevisiae* mnn1 mnn9 double mutant by reducing agents. *J. Bacteriol.* 1999, 181, 4741–4745. [CrossRef] [PubMed]

55. Sumita, T.; Yokoo, T.; Shimma, Y.; Jigami, Y. Comparison of Cell Wall Localization among Pir Family Proteins and Functional Dissection of the Region Required for Cell Wall Binding and Bud Scar Recruitment of Pir1p. *Eukaryot. Cell* 2005, 4, 1872–1881. [CrossRef] [PubMed]

56. Kaptetyn, J.C.; Van Egmond, P.; Sievi, E.; Van Den Ende, H.; Makarow, M.; Klis, F.M. The contribution of the O-glycosylated protein Pir2p/Hsp150 to the construction of the yeast cell wall in wild-type cells and beta 1,6-glucan-deficient mutants. *Mol. Microbiol.* 1999, 31, 1835–1844. [CrossRef]

57. Mrša, V.; Tanner, W. Role of NaOH-extractable cell wall proteins Ccw5p, Ccw6p, Ccw7p and Ccw8p (members of the Pir protein family) in stability of the *Saccharomyces cerevisiae* cell wall. *Yeast* 1999, 15, 813–820. [CrossRef]

58. Russo, P.; Simonen, M.; Uimari, A.; Teesalu, T.; Makarow, M. Dual regulation by heat and nutrient stress of the yeast HSP150 gene encoding a secretory glycoprotein. *Mol. Gen. Genet.* 1993, 239, 273–280. [CrossRef]

59. Moukadiri, I.; Armero, J.; Abad, A.; Sentandreu, R.; Zueco, J. Identification of a mannanprotein present in the inner layer of the cell wall of *Saccharomyces cerevisiae*. *J. Bacteriol.* 1997, 179, 2154–2162. [CrossRef]

60. Mrša, V.; Ecker, M.; Strahl-Bolsinger, S.; Nimtz, M.; Lehle, L.; Tanner, W. Deletion of new covalently linked cell wall glycoproteins alters the electrophoretic mobility of phosphorylated wall components of *Saccharomyces cerevisiae*. *J. Bacteriol.* 1999, 181, 3076–3086. [CrossRef]

61. Moreno-Garcia, J.; Cui, A.L.; Zara, G.; Garcia-Martinez, T.; Mauricio, J.C.; Budroni, M. Study of the role of the covalently linked cell wall protein (Ccwc14p) and yeast glycoprotein (Ygp1p) within biofilm formation in a flor yeast strain. *FEMS Yeast Res.* 2018, 18. [CrossRef] [PubMed]

62. Van der Vaart, J.M.; Caro, L.H.; Chapman, J.W.; Klis, F.M.; Verrips, C.T. Identification of three mannanproteins in the cell wall of *Saccharomyces cerevisiae*. *J. Bacteriol.* 1995, 177, 3104–3110. [CrossRef] [PubMed]
63. Abramova, N.; Sertil, O.; Mehta, S.; Lowry, C.V. Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in Saccharomyces cerevisiae. *J. Bacteriol.* 2001, 183, 2881–2887. [CrossRef] [PubMed]

64. Pardo, M.; Monteoliva, L.; Pla, J.; Sánchez, M.; Gil, C.; Nombela, C. Two-dimensional analysis of proteins secreted by *Saccharomyces cerevisiae* regenerating protoplasts: A novel approach to study the cell wall. *Yeast* 1999, 15, 459–472. [CrossRef]

65. Pardo, M.; Monteoliva, L.; Vázquez, P.; Martínez, R.; Molero, G.; Nombela, C.; Gil, C. PST1 and ECM33 encode two yeast cell surface GPI proteins important for cell wall integrity. *Microbiology* 2004, 150, 4157–4170. [CrossRef]

66. Terashima, H.; Yabuki, N.; Hamada, K.; Kitada, K. Up-regulation of genes encoding glycosylphosphatidylinositol (GPI)-attached proteins in response to cell wall damage caused by disruption of FKS1 in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 2000, 264, 64–74. [CrossRef]

67. Li, X.; Wang, J.; Phornsanthia, S.; Yin, X.; Li, Q. Strengthening of Cell Wall Structure Enhances Stress Resistance and Fermentation Performance in Lager. *J. Am. Soc. Brew. Chem.* 2014, 72, 88–94. [CrossRef]

68. Kovacech, B.; Nasmyth, K.; Schuster, T. *EGT2* gene transcription is induced predominantly by Swi5 in early G1. *Mol. Cell. Biol.* 1996, 16, 3264–3274. [CrossRef]

69. Pan, X.; Heiman, J. SSK2 Regulates Yeast Pseudohyphal Differentiation via a Transcription Factor Cascade That Regulates Cell-Cell Adhesion. *Mol. Cell Biol.* 2000, 20, 8364–8372. [CrossRef]

70. Baladrón, V.; Ufano, S.; Dueñas, E.; Martín-Cuadrado, A.B.; del Rey, F.; Vázquez de Aldana, C.R. Eng1p, an endo-1,3-beta-glucanase localized at the daughter side of the septum, is involved in cell separation in *Saccharomyces cerevisiae*. *Eukaryot. Cell* 2002, 1, 774–786. [CrossRef]

71. Kuznetsov, E.; Kucerova, H.; Vachova, L.; Palkova, Z. SUN family proteins Sun4p, Uth1p and Sim1p are secreted from *Saccharomyces cerevisiae* and produced dependently on oxygen level. *PLoS ONE* 2013, 8, e73882. [CrossRef] [PubMed]

72. Mouassite, M.; Camougand, N.; Schwob, E.; Demaison, G.; Laclau, M.; Guérin, M. The ‘SUN’ family: Yeast SUN4/SCW3 is involved in cell septation. *Yeast* 2000, 16, 905–919. [CrossRef]

73. Tofalo, R.; Perpetuini, G.; Di Gianvito, P.; Arfelli, G.; Shirone, M.; Corsetti, A.; Suzzi, G. Characterization of specialized flocculent yeasts to improve sparkling wine fermentation. *J. Appl. Microbiol.* 2016, 120, 1574–1584. [CrossRef]

74. Barrales, R.R.; Korper, P.; Jimenez, J.; Ibeas, J.I. Chromatin modulation at the FLO11 promoter of *Saccharomyces cerevisiae* by HDAC and Swi/Snf complexes. *Genetics* 2012, 191, 791–803. [CrossRef]

75. Kuchin, S.; Vyas, V.K.; Carlson, M. Snf1 Protein Kinase and the Repressors Nrg1 and Nrg2 Regulate FLO11, Haploid Invasive Growth, and Diploid Pseudohyphal Differentiation. *Mol. Cell. Biol.* 2002, 22, 3994–4000. [CrossRef]

76. Vyas, V.K.; Kuchin, S.; Berkey, C.D.; Carlson, M. Snf1 Kinases with Different β-Subunit Isoforms Play Distinct Roles in Regulating Haploid Invasive Growth. *Mol. Cell. Biol.* 2003, 23, 1341–1348. [CrossRef]

77. Welker, S.; Rudolph, B.; Frenzel, E.; Hagn, F.; Liebisch, G.; Schmitz, G.; Scheuring, J.; Kerth, A.; Blume, A.; Weinkauf, S.; et al. Hsp12 is an intrinsically unstructured stress protein that folds upon membrane association and modulates membrane function. *Mol. Cell* 2010, 39, 507–520. [CrossRef]

78. Zara, S.; Antonio Farris, G.; Budroni, M.; Bakalinsky, A.T. *HSP12* is essential for biofilm formation by a Sardinian wine strain of *S. cerevisiae*. *Yeast* 2002, 19, 269–276. [CrossRef]

79. Lopez-Ribot, J.L.; Chaffin, W.L. Members of the Hsp70 family of proteins in the cell wall of *Saccharomyces cerevisiae*. *J. Bacteriol.* 1996, 178, 4724–4726. [CrossRef] [PubMed]

80. Bukau, B.; Horwich, A.L. The Hsp70 and Hsp60 chaperone machines. *Cell* 1998, 92, 351–366. [CrossRef]

81. Merkel, O.; Fido, M.; Mayr, J.A.; Prüger, H.; Raab, F.; Zandonella, G.; Kohlwein, S.D.; Palfau, F. Characterization and function in vivo of two novel phospholipases B/lysophospholipases from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 1999, 274, 28121–28127. [CrossRef] [PubMed]

82. Aguilera, F.; Peinado, R.A.; Millán, C.; Ortega, J.M.; Mauricio, J.C. Relationship between ethanol tolerance, H+ -ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. *Int. J. Food Microbiol.* 2006, 110, 34–42. [CrossRef] [PubMed]
83. Terashima, H.; Hamada, K.; Kitada, K. The localization change of Ybr078w/Ecm33, a yeast GPI-associated protein, from the plasma membrane to the cell wall, affecting the cellular function. *FEMS Microbiol. Lett.* 2003, 218, 175–180. [CrossRef] [PubMed]

84. Zhang, J.; Astorga, M.A.; Gardner, J.M.; Walker, M.E.; Grbin, P.R.; Jiranek, V. Disruption of the cell wall integrity gene *ECM33* results in improved fermentation by wine yeast. *Metab. Eng.* 2018, 45, 255–264. [CrossRef] [PubMed]

85. Mouassite, M.; Guérin, M.G.; Camougrand, N.M. The SUN family of *Saccharomyces cerevisiae*: The double knock-out of *UTH1* and *SIM1* promotes defects in nucleus migration and increased drug sensitivity. *FEMS Microbiol. Lett.* 2000, 182, 137–141. [CrossRef] [PubMed]

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