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Effect of Mitophagy-Related Gene Regulation on Antioxidant Activity of Lager Yeast

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Abstract: Lager yeast, which is mainly used for lager beer brewing, withstands significant oxidative stress during brewing process, leading to its rapid aging in serial beer brewing. Mitophagy plays a critical role in the antioxidant stress response of yeast. However, the relationship between mitophagy and the antioxidant capacity of yeast is still unclear. Previous studies indicated that *ATG* gene family in mitophagy significantly affects the antioxidant capacity of yeast cells in beer brewing. Herein, the expression of *ATG8*, *ATG11*, *ATG32*, *DNM1*, and *MMM1* genes was regulated. The results showed that the overexpression of *ATG8* and *ATG11* significantly reduced the intracellular ROS contents to 52.05% and 22.57% of the initial state, respectively, and helped to maintain a high mitochondrial vitality during serial fermentation. Disruption of *ATG8*, *ATG11* and *ATG32* resulted in significant decrease in cell vitality when exposed to H_2O_2 stimulation. Meanwhile, the disruptions of these genes were detrimental to the balance of intracellular ROS. Excess *DNM1* activity could affect the cellular energy balance and ATP depletion under prolonged stress conditions. The repression of *MMM1* led to lower ATP levels during serial beer fermentation. The *ATG8*, *ATG11*, and *ATG32* genes might be potential targets for regulating the antioxidant capacity of yeast. The current work provides new insights into improving the antioxidant capacity of yeast through mitophagy regulation.

Keywords: mitophagy; *ATG* family genes; lager yeast; serial beer fermentation; antioxidant response

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1. Introduction

In eukaryotes, mitochondria provide energy to cells through oxidative phosphorylation. As the center of intracellular catabolism, mitochondria play an important role in cellular senescence in many organisms [1]. Mitochondria are the major source of reactive oxygen species (ROS). The accumulation of ROS during aerobic respiration causes mitochondria to be injured [2,3]. The injured mitochondria subsequently produce increased ROS levels, disrupting the mitochondrial network and damaging the intracellular redox homeostasis, further resulting in a vicious cycle. In order to maintain homeostasis and prevent mitochondrial dysfunction, cells selectively remove damaged or aged mitochondria through a process known as mitophagy [4].

Evidently, the balance of ROS production and elimination is one of the key factors that affects the yeast's response to a variety of stressors during the fermentation process. Studies have shown that targeted mitochondrial photosensitizers inducing a rapid rise in ROS in mitochondria led to the loss of mitochondrial membrane potential (MMP) and the activation of mitophagy [5]. Conversely, an overexpression of the antioxidant protein

superoxide dismutase inhibited photosensitizer-induced mitophagy. This supports the findings that ROS are an important upstream activator of mitophagy [6].

Atg32p, an indispensable receptor protein for mitophagy in yeast, localizes to the outer mitochondrial membrane [7]. After the induction of mitophagy, Atg32p interacts with Atg11p [7], a key protein involved in cellular autophagy, to recruit mitochondria to the pre-autophagosomal structure/phagophore assembly site (PAS) where autophagosomes are generated. Autophagosomes carrying mitochondria eventually fuse with vesicles for mitochondrial degradation [8,9]. In parallel, Dnm1p, Mdv1p, and Fis1p are also required to target mitochondria for the mitophagy pathway. Mao et al. [10] reported that Atg11p interacts with the mitochondrial fission factor Dnm1p to induce mitochondrial fission and achieve effective mitophagy, while Fis1p mediates ethanol-induced apoptosis and mitochondrial breakage [11,12]. Atg8p is involved in the expansion of the autophagosome membrane and is crucial for the autophagy pathway, making it an important target for examining mitophagy and the oxidative stress response of yeast. Atg32p interacts with ATg8p, facilitating the extension of the isolation membrane along the mitochondrial surface [13]. The endoplasmic reticulum (ER)–mitochondrial encounter structural complex (ERMES) that mediates ER–mitochondrial contact sites is another factor involved in mitophagy. Mmm1p is one of the complex subunits of ERMES, and it is needed to have normal levels of autophagy, and the deletion of the *MMM1* was responsible for inhibiting mitophagy in yeast [14].

Lager yeast (*Saccharomyces pastorianus*) suffers from multiple stresses (including high osmotic pressure, high alcohol content, and nutritional deficiencies) in fermentation [15,16]. These stresses lead to the disruption of ROS homeostasis and gradually result in a decline in fermentation performance during serial beer fermentation. Previous studies showed that fluctuations in energy metabolism cause fluctuations in ROS levels. Excessive ROS could cause intracellular injuries to the cells, resulting in yeast aging, autolysis, and an irreversible decrease in cell vitality [17].

Previous studies on lager yeast showed that mitophagy-related genes might have functions in regulating the antioxidative stress response [18]. Herein the mitophagy-related genes *ATG8*, *ATG11*, *ATG32*, *DNM1,* and *MMM1* were mutated to study the intrinsic interactions between mitophagy and antioxidant response in lager yeast. The results revealed important functions of *ATG* family genes in regulating the ROS balance and mitochondrial and cell vitality under oxidative stress.

2. Materials and Methods

2.1. Strains and Medium

Pilsner lager yeast isolated from a Chinese brewery (*S. pastorianus*, triploid, group II) [19] was used as the host strain, and YEp352 was used as a yeast expression vector. *Escherichia coli* BL21 and plasmid pUG6 were used for sub-cloning the genes used in this study. G418 was used as a selection marker for gene modifications. All the strains and plasmids used are listed in Table 1.

Table 1. The strains and plasmids used in this study.

Plasmids	Description	Resource
YEp352	A multicopy episomal plasmid	Stored in author's lab [20]
pUG6	Cloning vector	Stored in author's lab [21]
YEp352-*DNM1*	Native *DNM1* with PGK1p and ADH1t expressed in pYEP352	This study
YEp352-*ATG8*	Native *ATG8* with PGK1p and ADH1t expressed in pYEP352	This study
YEp352-*ATG11*	Native *ATG11* with PGK1p and ADH1t expressed in pYEP352	This study

Table 1. *Cont.*

Plasmids	Description	Resource
YEp352-*ATG32*	Native *ATG32* with PGK1p and ADH1t expressed in pYEP352	This study
YEp352-*MMM1*	Native *MMM1* with PGK1p and ADH1t expressed in pYEP352	This study
Strains		
Pilsner	Lager yeast	Chinese brewery [17]
P-O-DNM1	Pilsner transformed with pYEP352-DNM1	This study
P-O-ATG8	Pilsner transformed with pYEP352-ATG8	This study
P-O-ATG11	Pilsner transformed with pYEP352-ATG11	This study
P-O-ATG32	Pilsner transformed with pYEP352-ATG32	This study
P-O-MMM1	Pilsner transformed with pYEP352-MMM1	This study
Pil-dnm1Δ	Pilsner with *DNM1* disrupted	This study
Pil-atg8Δ	Pilsner with *ATG8* disrupted	This study
Pil-atg11Δ	Pilsner with *ATG11* disrupted	This study
Pil-atg32Δ	Pilsner with *ATG32* disrupted	This study
Pil-mmm1Δ	Pilsner with *MMM1* disrupted	This study

YPD medium (2% glucose, 2% peptone, 1% yeast extract) and MEB medium (17% malt extract, 3% peptone) were used for the yeast culture, LB medium (1% sodium chloride, 1% peptone, 0.5% yeast extract) was used for the *E. coli* culture, and 2% agar and an appropriate amount of G418 were added to solid media when necessary.

2.2. Construction of Recombinant Strains

The recombinant yeast strains derived from Pilsner are listed in Table 1. The homologies of *DNM1*, *ATG8*, *ATG32*, and *MMM1* between *Saccharomyces eubayanus* and *S. cerevisiae* were 85.73%, 87.29%, 77.85, and 81.31%, respectively. The *ATG11* gene has no reported homolog gene in *S. eubayanus*. Therefore, the primers were designed according to the genome of *S. cerevisiae* S288C.

2.2.1. Overexpression Experiments

Plasmid YEp352 was used to overexpress the target genes including *DNM1*, *ATG8*, *ATG11*, *ATG 32*, and *MMM1*. The target fragments with *Nhe* I and *Xho* I restriction sites were amplified (Table S1). The target gene was separately inserted between the promoter PGK1 and the terminator ADH1 at the *Nhe* I and *Xho* I sites and then ligated into the YEp352 expression vector using G418 as a selective marker [22].

2.2.2. Knockdown Experiments

Knockdown strains were constructed through one-step fragment homologous recombination. The constructed knockout cassette contained the upstream and downstream fragments of the target gene and a G418 selection marker. Knockdowns were verified via qRT-PCR using validation primers (Table S1).

2.3. Quantitative Real-Time PCR

The relative expression levels of target genes were determined via quantitative real-time PCR (qRT-PCR) analysis. Yeast cells were cultured in YPD liquid medium to OD_{600} of 1.0 and yeast cells were collected and treated with 4 mmol/L H_2O_2 for 3 h. Total RNA was extracted using the spin column yeast total RNA purification kit (Sangon, Shanghai, China). Subsequently, RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent kit with gDNA eraser (Takara, Dalian, China). The qRT-PCR was performed with TB Green Premix Ex Taq™ II kit (Takara, Dalian, China) in ABI StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with the specific primers (Table S1). The relative expression level of each target gene was determined by the $2^{-\Delta\Delta CT}$ method using *ACT1* as a reference [23]. All the experiments were performed in triplicate.

2.4. Measurements

2.4.1. Growth Analysis

A 1 mL sample of 1×10^7 CFU/mL cells was inoculated onto YPD medium supplemented with 0 mmol/L, 2 mmol/L, 4 mmol/L, and 6 mmol/L H_2O_2 and cultured at 28 °C, 180 r/min, respectively. Samples were taken until the strains reached the stationary phase. The absorbance at 600 nm was measured in a Synergy™ HT fluorescence-mode microplate reader (Bio Tek, Marshall Scientific, Hampton, NH, USA).

2.4.2. Intracellular ROS Assessment

Yeast was incubated in YPD medium at 28 °C until it reached the stationary phase. Cells were washed and suspended in phosphate-buffered saline (PBS). A 1 mL sample of 1×10^7 CFU/mL cell suspension was mixed with 1 mL of 2′,7′-dichlorofluorescin diacetate (DCDH-DA) (10 μmol/L) and left in the dark for 30 min at 28 °C [24]. The fluorescence intensity of the solution was monitored in a Synergy™ HT fluorescence mode microplate reader with λ_{ex} = 488 nm and λ_{em} = 525 nm.

2.4.3. Mitochondrial Membrane Potential Analysis

Yeast samples were collected in the stationary phase and a 0.5 mL sample of 5×10^6 CFU/mL cell suspension was mixed with 0.5 mL of JC-1 staining solution. Subsequently, cells were incubated at 37 °C for 20 min in the dark. After washing 2 times with JC-1 buffer, the samples were resuspended with 0.5 mL JC-1 buffer and then their fluorescence intensities were monitored in a Synergy™ HT fluorescence mode microplate reader with λ_{ex} = 485 nm and λ_{em} = 590 nm.

2.4.4. Intracellular ATP Determination

Intracellular ATP was measured using the BactTiter-Glo™ Microbial Cell Vitality Assay Kit (Promega, Shanghai, China). Yeast cells cultured to stationary phase were collected and diluted to 1×10^7 CFU/mL with PBS buffer. A 1 mL sample was disrupted with glass beads (BioSpec Products, Bartesville, OK, USA) of 0.5 mm in diameter [25]. A 100 μL sample and 100 μL detection reagent were mixed and the luminescence light signal was detected using a LumiStation 1800 Chemiluminescence Microplate Reader (Flash, Shanghai, China). The luminescence signal was proportional to the content of ATP [17].

2.4.5. Confocal Microscopy and Image Analysis of Mitochondria

The yeast was incubated in MEB medium at 28 °C for 24 h. The samples were resuspended in PBS buffer and stained with TMRE dye for 15 min. Cells were observed using a confocal fluorescence microscope from Andor xD revolution on an Olympus IX81 platform (Oxford instruments, Abingdon, UK) at 100× objective magnification. An analysis of the images was carried out using the Mitochondria-Analyzer plugin for the Fiji1 platform [26].

2.4.6. Intracellular Antioxidant Capacity Assay

Intracellular antioxidant capacity was measured using the ORAC assay [27]. The yeast was incubated in MEB medium at 28 °C to stationary phase. Cells were resuspended in phosphate-buffered saline (PBS) to produce 1 mL samples at a concertation of 20 mg/mL and the samples were disrupted with a One Shot cell disruptor (Constant Systems, Northamptonshire, UK). Samples were mixed with dipyridamole in a 96-well plate. Then, AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride) was added to each sample to initiate an oxidative reaction. Antioxidant capacity was measured in a Synergy^{TM} HT fluorescence-mode microplate reader with λ_{ex} = 415 nm and λ_{em} = 480 nm.

2.5. Serial Beer Fermentation Test

Serial beer fermentation tests were carried as described in previous study using strain Pilsner and recombinant strains [17]. Fermentation was carried out at 11 °C until the daily weight loss was less than 0.1 g/100 mL. Yeast cells collected from the first batch of fermentation were labeled as the first-generation (G1) yeast. Yeasts from five generations (G1-G5) were individually collected from serial beer fermentation. The fermentation parameters for each generation, including the degree of alcohol and real attenuation were measured by an automatic beer analyzer (Alcolyzer Beer Analyzing System, Anton Paar, Graz, Austria).

2.6. Statistical Analysis

Data were presented as means ± standard deviations (SDs) and the means were from at least three independent assays. To analyze the differential significance of the data between groups, one-way ANOVA tests were carried out using Statistical Product and Service Solutions 22.0 (SPSS, International Business Machines Corporation, Armonk, NY, USA). $p < 0.05$ indicated statistically significant differences.

3. Results

In yeast, mitochondrial degradation depends on autophagy-related genes, with oxidative stress being a key factor in inducing mitophagy. Investigating the regulation of mitophagy-related genes and their relationship with oxidative stress can enhance our understanding of yeast stress response mechanisms. Ten recombinant strains were constructed. The relative expression levels of the *ATG8*, *ATG11*, *ATG32*, *DNM1,* and *MMM1* genes in 10 recombinant strains were measured by qRT-PCR using specific primers (Table S1). The result showed that the target genes were efficiently overexpressed and repressed, respectively (Table S2). Compared with the parental strain, Pilsner, the expression levels of knockdown strains were reduced to lower levels of 3–37%, indicating that most of the target genes were repressed.

3.1. Modifications to Mitophagy-Related Genes Affect the Resistance of Yeast Cells to H_2O_2

H_2O_2 is one of the most commonly used oxidants in the field of biochemistry. When cells are stimulated by H_2O_2, the antioxidant system adjusts the intracellular redox state to tolerate a certain concentration of H_2O_2. Therefore, the ability of yeast to grow under stress conditions with or without H_2O_2 can be used to evaluate the antioxidant capacity of strains. Assessing growth performance in liquid media with varying H_2O_2 concentrations can offer a more accurate reflection of the yeast's resistance to stress. As shown in Figure 1, the recombinant strains showed similar growth trends to the parental strain in the absence of H_2O_2, except for Pil-mmm1Δ, which exhibited a notably slower growth. Under H_2O_2 stress (6 mmol/L), strains with disrupted *DNM1*, *ATG11*, *ATG32*, and *MMM1* displayed longer lag phases, with Pil-mmm1Δ being the most affected, entering the logarithmic phase only after 30 h. None of the strains grew within the first 36 h under 8 mmol/L H_2O_2. The disruption of the *ATG8*, *ATG32*, and *MMM1* genes reduced cell viability under oxidative stress, with Pil-atg8Δ achieving a lower biomass (OD600 = 3.14 at 4 mmol/L H_2O_2). In contrast, overexpression of *ATG8*, *ATG11*, and *ATG32* enhanced growth under 6 mmol/L

H_2O_2, with P-O-ATG32 reaching a higher biomass (OD600 = 3.80) than the parental strain (OD600 = 3.48) at 4 mmol/L H_2O_2. Meanwhile, a colony formation analysis was performed on solid medium and the results obtained in the solid medium are consistent with those from the liquid medium, reinforcing this approach (Figure S1). These results suggested that *ATG8*, *ATG32*, and *MMM1* played crucial roles in maintaining yeast growth and viability under oxidative stress.

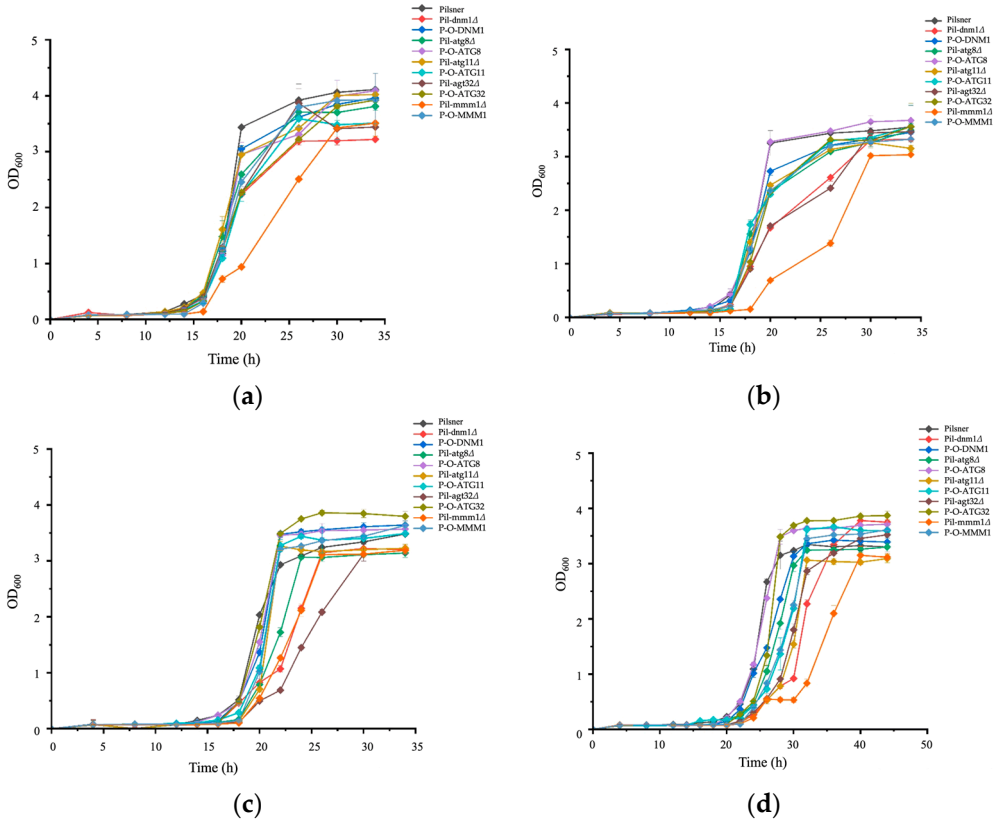


Figure 1. The growth of recombinant strains and the parental strain under different concentrations of H_2O_2. (**a**) Control, 0 mmol/L H_2O_2; (**b**) 2 mmol/L H_2O_2; (**c**) 4 mmol/L H_2O_2; (**d**) 6 mmol/LH_2O_2.

3.2. Modulation of Mitochondrial Homeostasis upon Oxidative Stress

3.2.1. Oxidative Stress Affected Mitophagy-Related Genes Expression

It is well established that mitophagy is induced by ethanol stress [28]. In order to confirm whether mitophagy is induced by oxidative stress in *S. pastorianus* Pilsner, the expression of mitophagy-related genes was measured by qRT-PCR after treatment with 4 mmol/L H_2O_2 for 3 h. As shown in Figure 2, under oxidative stress, the expression levels of the *ATG8*, *ATG11,* and *ATG32* genes in the Pilsner strain were significantly increased by 2.07, 2.02, and 1.79-fold, respectively, compared to the levels of cells without H_2O_2 treatment. In addition, the expression level of *MMM1* in Pilsner significantly decreased under H_2O_2 stimulation. The expression levels of *ATG8*, *ATG11,* and *ATG32* increased by 1.90, 0.19, and 1.59 times in P-O-ATG8, 2.18, 4.86, and 6.86 times in P-O-ATG11, and 2.87, 5.03, and 4.03 times in P-O-ATG32, respectively. The results indicated that an overexpression of the *ATG8*, *ATG11,* and *ATG32* genes enhances the whole mitophagy pathway. On the other hand, in the knockouts, the disruption of one gene could have an impact on the regulation of other genes. For example, in the Pil-atg8Δ strain, the expression level of *ATG11* increased by 2.10 times, and the expression level of ATG32 decreased by 0.16 times. In the Pil-atg32Δ strain, the expression level of *ATG11* increased by 0.12 times, and the expression level of *ATG8* decreased by 0.37 times. In summary, the results demonstrate that H_2O_2 stimulation significantly upregulated key mitophagy-related genes (*ATG8*, *ATG11*, and *ATG32*) in Pilsner. The overexpression of these genes further enhanced related pathways, which likely

support a stronger mitophagic response. While oxidative stress triggers significant changes in the expression of autophagy-related genes, the knockout or overexpression of single gene modulates this response to compensate for the lack/abundance of a certain protein.

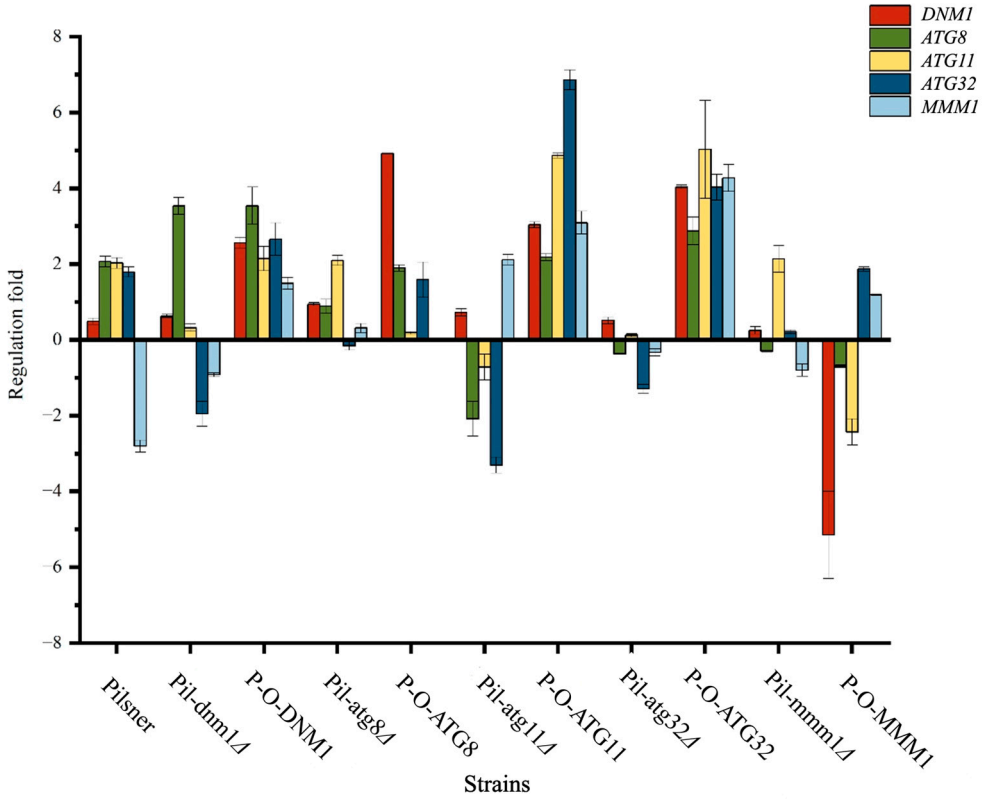


Figure 2. Expression levels of target genes in Pilsner after H_2O_2 treatment (4 mmol/L) for 3 h. Values indicate mean ± standard deviation (n = 3). The relative expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. The fold change above the bars in the chart indicates the upregulation of the gene relative to the control group. The fold change below the bars represents the downregulation of the gene.

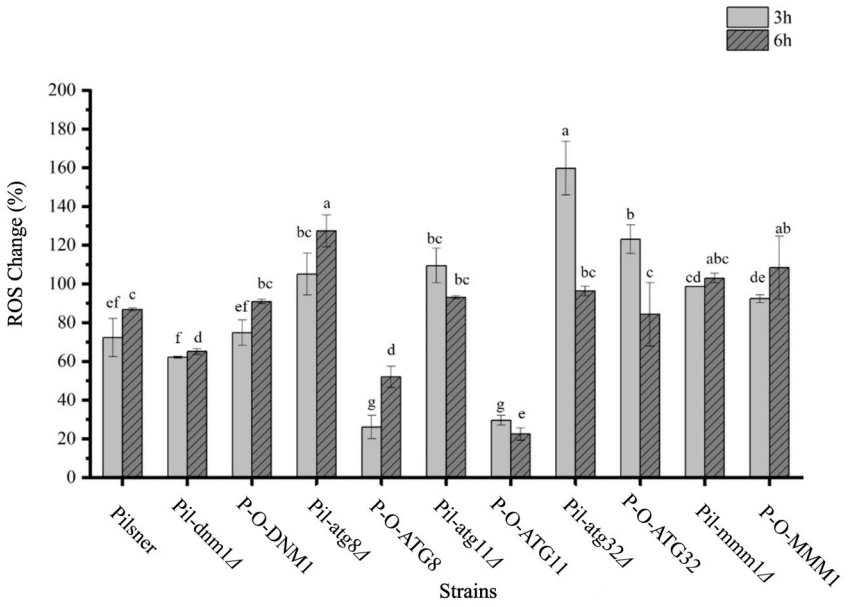
3.2.2. Regulation of Mitophagy-Related Genes Decreases ROS Content and Maintains Mitochondrial and Cell Vitality in Yeast Exposed to Oxidative Stress

A certain concentration of intracellular ROS is important to maintain redox homeostasis and cell signaling. The ability to regulate ROS to a relatively low level reflects the antioxidant capacity of cells. Using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as a probe, intracellular ROS levels were measured after 4 mmol/L H_2O_2 exposure for 3 h and 6 h. As shown in Figure 3a, after a 6 h treatment with H_2O_2, the ROS level in the Pilsner strain reduced to 84% of its initial state. The overexpression of the *ATG8* and *ATG11* genes caused a severe reduction in the ROS levels, with the ROS levels being at 52% and 23% of their initial states, respectively. In contrast, *ATG8* knockdown caused an increased ROS level to 127%, while *ATG11* knockdown did not cause much fluctuation in the ROS level. The ROS level of Pil-dnm1Δ showed a 65% reduction compared with Pilsner. However, neither the overexpression nor disruption of *DNM1* affected ROS fluctuations during the 6 h treatment. Most fluctuations were seen during *ATG32* regulation. Upregulating *ATG8* and *ATG11* expression helps maintain relatively lower ROS levels in yeast cells during oxidative stress.

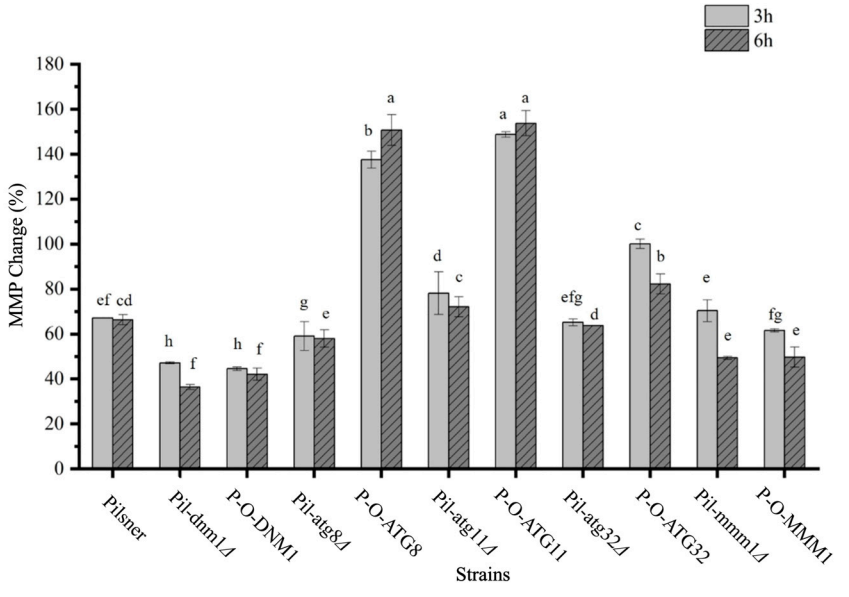
MMP is crucial for mitochondrial function, with higher levels indicating a stronger vitality. As shown in Figure 3b, after 6 h treatment with 4 mmol/L H_2O_2, the MMP value of Pilsner reduced to 69%, while the MMP levels of the overexpressed *ATG8* and *ATG11* strains increased to 150% and 153% of their initial states, respectively. The MMP level of P-O-ATG32 reduced to 82% after 6 h of treatment, which was still higher than that of the

Pilsner strain. The disruption of *ATG8*, *ATG11*, and *ATG32* caused a similar MMP reduction to the Pilsner. Meanwhile, *DNM1* and *MMM1* modifications caused a greater decline in MMP levels than the Pilsner. The results indicated that the overexpression of *ATG8*, *ATG11*, and *ATG32* would help to maintain a high mitochondrial vitality under oxidative stress.

Cell vitality was measured based on the changes in intracellular ATP content upon treatment with H_2O_2. As shown in Figure 3c, after 6 h of treatment, the ATP value of the Pilsner reduced to 82% of its initial state. *ATG8* overexpression initially boosted the ATP to 137% after 3 h but could not sustain it. *ATG11* and *ATG32* overexpression helped to maintain stable ATP levels, while the disruption of *ATG11* and *ATG32* caused drops in the ATP. Interestingly, *DNM1* disruption led to an enhancement of the ATP content (185% after 6 h exposure), whereas *MMM1* regulation exhibited the opposite effect. The above results show that the repression of *ATG8*, *ATG11*, *ATG32*, and *MMM1* negatively impacted cell viability under oxidative stress.

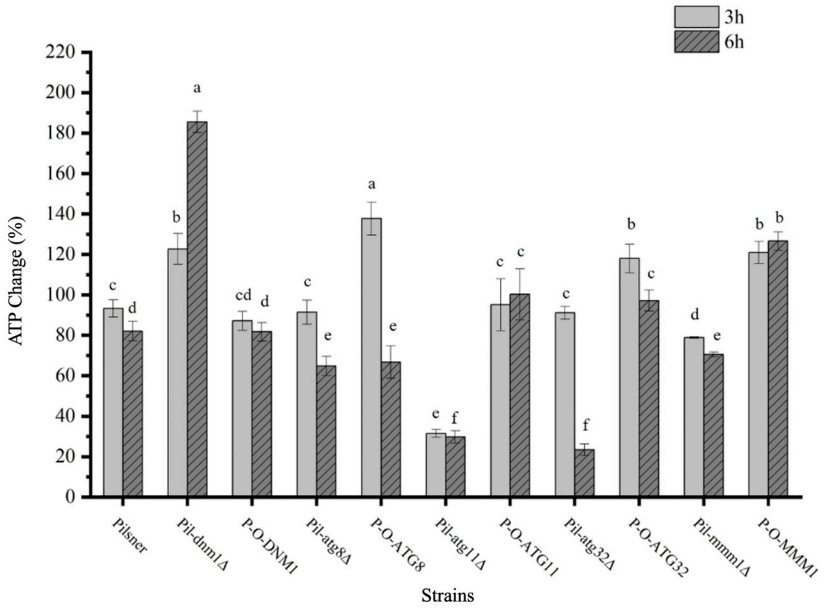


(**a**)



(**b**)

Figure 3. *Cont.*



(**c**)

Figure 3. Measurements of ROS (**a**), MMP (**b**), and ATP (**c**) values of recombinant strains and the parental strain after 4 mmol/L H_2O_2 stimulations for 3 h and 6 h. Values were calculated using the initial state as a control. Values indicate mean ± standard deviation (n = 3). Different letters indicate significant differences ($p < 0.05$) based on one-way ANOVA. Values in the same column with different superscript letters are significantly different ($p < 0.05$). Bars labeled with different letters for 3 h and 6 h indicated a significant difference between these two time points. At each time point, bars with different letters across different strains indicated a significant difference in change rate.

3.2.3. Modifications of Mitophagy-Related Genes Regulate the Amount of Mitochondria

To understand how mitophagy-related gene modifications affect the mitochondrial abundance and distribution, yeasts cultured for 24 h in MEB medium were stained with tetramethylrhodamine; which is a probe accumulating in mitochondria as a function of membrane potential. The mitochondria were then inspected under a spinning-disk confocal microscope. The changes in the number of mitochondria in the mutant yeast cells were quantified as a count and the area of mitochondria in the strains compared to those in the parental strain using confocal microscopy (Table 2, Figure S2). The results indicated that neither the disruption nor overexpression of genes *ATG8*, *ATG11*, and *DNM1* had any significant effect on the number of mitochondria in cells. Interestingly, the number of mitochondria in the knockdown strains Pil-atg32Δ and Pil-mmm1Δ significantly increased compared to parental strain ($p < 0.05$), while the overexpression of their respective genes seemed to have no effect on the number of mitochondria. Similarly, Pil-atg32Δ and Pil-mmm1Δ were the only strains with an increased area of mitochondria per cell compared to the parental strain ($p < 0.05$). Occasionally, Pil-mmm1Δ cells were found without mitochondria-related signals, which may be due to the presence of dysfunctional mitochondria lacking a sufficient mitochondrial membrane potential. Therefore, while the overexpression of autophagy-related genes protects bioenergetics under oxidative stress, it has no effect on mitochondrial mass under normal conditions. On the contrary, the deletion of crucial genes may apparently slow down mitochondrial turnover leading to higher mitochondrial mass even under normal conditions.

Table 2. Number and area of mitochondria per cell.

Strain	Mitochondria Number	Mitochondria Area
Pil-dnm1Δ	1.06 ± 0.06	1.13 ± 0.23
P-O-DNM1	1.11 ± 0.08	1.01 ± 0.13
Pil-atg8Δ	1.06 ± 0.04	1.00 ± 0.27
P-O-ATG8	1.09 ± 0.07	1.00 ± 0.28
Pil-atg11Δ	1.09 ± 0.09	1.12 ± 0.27
P-O-ATG11	1.00 ± 0.12	1.06 ± 0.26
Pil-atg32Δ	1.42 ± 0.18 *	1.41 ± 0.14 *
P-O-ATG32	1.09 ± 0.08	1.21 ± 0.17
Pil-mmm1Δ	1.13 ± 0.04 *	1.31 ± 0.04 *
P-O-MMM1	1.13 ± 0.05	1.04 ± 0.21

* $p < 0.05$. The numbers and area of mitochondria are given relative to Pilsner.

3.2.4. Modifications of Mitophagy-Related Genes Affect the Overall Antioxidant Capacity of Yeast Cells

To evaluate the impact of mitophagy-related gene modifications on the antioxidant capacity of yeast cells, the antioxidant capacity assay was performed using the modified ORAC method (Figure 4). After culturing the yeast cells for 24 h, AAPH was used to induce intracellular oxidative stress to evaluate the antioxidant capacity of cells or samples. A lower ability to scavenge radicals was found in most of the recombinant strains compared with the parental strain. The only exceptions were strains overexpressing *ATG8* and *ATG11*, which were shown to have an insignificant impact on the antioxidant capacity of yeasts. The most significant decrease was observed in the Pil-dnm1Δ and Pil-mmm1Δ strains, suggesting the crucial role of *DNM1* and *MMM1* genes in the maintenance of the proficient antioxidant response. The roles of *DNM1* and *MMM1* may be more focused on maintaining mitochondrial function, cell membrane stability, and cellular energy metabolism, which indirectly contribute to antioxidant capacity but do not necessarily significantly reduce ROS levels. Increases or decreases in localized ROS may not show a noticeable change in whole-cell ROS measurements but can impact overall antioxidant capacity. These results demonstrate the importance of fine-tuning the mitophagy machinery for the overall redox homeostasis of the yeast cell.

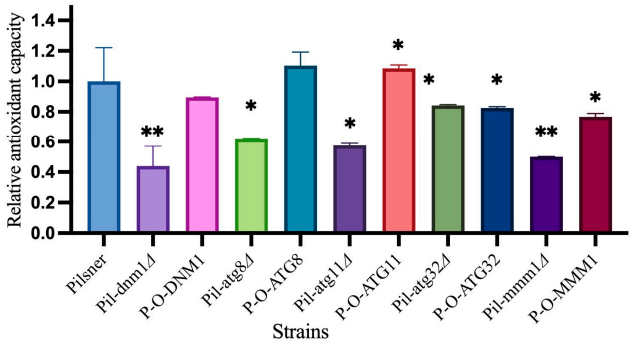


Figure 4. Antioxidant capacity as the value of the breaking point time during measurement. Values are given as mean ± standard deviation ($n = 4$), relative to Pilsner strain. One-way ANOVA showed significant differences ($p < 0.05$) between strains. Differences in antioxidant capacity of recombinant strains compared to Pilsner strain were confirmed by post hoc analysis (two-tail *t*-test) (* $p < 0.05$; ** $p < 0.01$).

3.3. Regulation of Mitophagy-Related Genes Affects the Antioxidant Abilities During Serial Beer Fermentation

In beer fermentation, yeast cells are typically collected after fermentation and re-pitched several times before discarding, leading to a gradual increase in oxidative stress. During the serial fermentation process, ROS, MMP, and the ATP levels of yeast were

measured at the end of each generation, using the parameters at the start of that generation as a reference to determine the rate of change throughout fermentation. Changes in these values were measured across five generations of serial fermentation, and each generation's rate of change was compared to that of the first generation to evaluate physiological changes in yeast, particularly in antioxidant capacity, over successive fermentations. As shown in Table 3, fermentation attenuation initially peaked at G3 and then declined. Yeasts adapt to wort after 1–2 re-pitchings, with most strains completing fermentation in 7 days by G3 [29]. However, *ATG32* and *MMM1* disruption delayed the fermentation process. The main fermentation process of beer fermented with Pil-atg32Δ and Pil-mmm1Δ finished 1–2 days later compared to other strains. Moreover, Pil-mmm1Δ had a significantly lower attenuation (56% W/W) and alcohol content (3.58% V/V) in G1 fermentation compared to the parental strain. The results indicated that the disruption of *ATG32* and *MMM1* caused a decline in cell vitality during serial fermentation.

Table 3. Beer fermentation parameters of different strains.

Strain	Generation	Fermentation Time (Day)	Alcohol Content %V/V	Real Attenuation %W/W
Pilsner	G1	9	4.11 ± 0.13	56.96 ± 0.26
	G2	9	3.97 ± 0.09	57.23 ± 0.14
	G3	7	4.16 ± 0.04	57.76 ± 0.24
	G4	6	3.91 ± 0.11	57.13 ± 0.23
	G5	7	4.02 ± 0.20	55.06 ± 0.51
Pil-dnm1Δ	G1	9	3.97 ± 0.10	57.38 ± 0.12 **
	G2	9	3.86 ± 0.07	56.94 ± 0.18
	G3	7	4.36 ± 0.11 *	57.92 ± 0.21
	G4	7	3.93 ± 0.06	56.93 ± 0.19
	G5	7	3.96 ± 0.17	55.02 ± 0.15
P-O-DNM1	G1	9	4.19 ± 0.06	57.1 ± 0.14
	G2	9	3.91 ± 0.13	57.12 ± 0.31
	G3	7	4.14 ± 0.09	57.81 ± 0.19
	G4	7	4.01 ± 0.12	57.25 ± 0.13
	G5	7	4.07 ± 0.18	55.12 ± 0.09
Pil-atg8Δ	G1	9	4.09 ± 0.05	57.06 ± 0.12
	G2	9	3.98 ± 0.04	56.85 ± 0.23 *
	G3	7	4.26 ± 0.06	58.03 ± 0.31
	G4	7	4.09 ± 0.04 *	57.31 ± 0.05
	G5	7	3.94 ± 0.21	55.12 ± 0.16
P-O-ATG8	G1	9	4.17 ± 0.14	57.45 ± 0.21 **
	G2	9	4.04 ± 0.07	56.87 ± 0.31 *
	G3	7	4.33 ± 0.14 *	57.9 ± 0.27
	G4	6	3.95 ± 0.09	57.14 ± 0.14
	G5	7	3.77 ± 0.22	54.89 ± 0.17
Pil-atg11Δ	G1	9	4.05 ± 0.09	57.02 ± 0.23
	G2	9	4.03 ± 0.12	57.00 ± 0.15
	G3	7	4.05 ± 0.13	57.4 ± 0.15
	G4	7	4.09 ± 0.04	57.07 ± 0.17
	G5	7	4.00 ± 0.21	55.24 ± 0.36
P-O-ATG11	G1	9	4.17 ± 0.12	57.3 ± 0.08 *
	G2	8	4.07 ± 0.13	56.96 ± 0.13
	G3	7	4.28 ± 0.02	58.06 ± 0.17
	G4	7	3.93 ± 0.15	57.01 ± 0.09
	G5	7	4.32 ± 0.16 *	55.25 ± 0.33

Table 3. *Cont.*

Strain	Generation	Fermentation Time (Day)	Alcohol Content %V/V	Real Attenuation %W/W
Pil-atg32Δ	G1	10	4.24 ± 0.06	56.88 ± 0.22
	G2	11	4.00 ± 0.04	57.12 ± 0.22
	G3	10	4.22 ± 0.07	57.42 ± 0.14
	G4	9	3.91 ± 0.06	57.46 ± 0.32
	G5	8	3.82 ± 0.06	54.87 ± 0.41
P-O-ATG32	G1	9	4.09 ± 0.03	56.97 ± 0.17
	G2	9	4.02 ± 0.11	56.93 ± 0.09
	G3	7	4.11 ± 0.05	57.96 ± 0.23
	G4	7	4.03 ± 0.17	57.2 ± 0.36
	G5	7	4.21 ± 0.07	54.93 ± 0.29
Pil-mmm1Δ	G1	9	3.58 ± 0.13 ***	56.14 ± 0.22 ***
	G2	11	3.94 ± 0.04	56.82 ± 0.08 *
	G3	8	4.12 ± 0.07	57.79 ± 0.11
	G4	9	3.97 ± 0.14	57.69 ± 0.19 *
	G5	9	4.29 ± 0.19	55.18 ± 0.07
P-O-MMM1	G1	8	4.20 ± 0.08	57.26 ± 0.12
	G2	9	4.21 ± 0.01	57.29 ± 0.14 *
	G3	7	4.16 ± 0.12	57.08 ± 0.31
	G4	7	4.01 ± 0.03	56.8 ± 0.37
	G5	7	4.29 ± 0.13	55.06 ± 0.15

The main fermentation was considered as finished when the daily weight loss of the fermentation system was less than 0.1 g/100 mL, and the alcohol content and the real attenuation were determined. Results are shown as the mean ± standard deviation of three parallel cultures from independent experiments (n = 3). * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

Increased oxidative damage in yeast results from both higher ROS production and reduced turnover of macromolecules and mitochondria. The intracellular ROS, MMP, and ATP contents of yeast during serial fermentations (G1-G5) were measured, with G1 as the reference (Figure 5). Yeast strains showed the best performance (lowest ROS, highest MMP, and ATP) during G3, consistent with earlier fermentation data [17]. Differences between strains became significant at G5.

The downregulation of *ATG8*, *ATG11*, and *ATG32* expression led to increased ROS and reduced MMP. In particular, Pil-atg32Δ showed a 503% ROS increase and a 30% MMP reduction at G5 compared to G1. The overexpression of *ATG32* helped maintain the intracellular redox balance, with the relative ROS value being 99% at G5 compared to G1. However, the upregulation of *ATG32* did not significantly influence the intracellular ATP fluctuation. Similarly, Pil-atg8Δ had a 207% ROS increase and a 23% MMP decrease at G5, while the overexpression of *ATG8* kept ROS and MMP levels more stable. The result was consistent with that of the Pilsner strain. The ATP content of Pil-atg8Δ significantly reduced to 80% at G5 compared to G1. *ATG11* disruption caused a 321% ROS increase and a 22% MMP decrease at G5. P-O-ATG11 overexpression slightly improved MMP and ATP levels during fermentation.

In addition, *MMM1* disruption accelerated the ROS imbalance during the late period of serial fermentation, with the levels rising to 310% at G5 compared to G1. On the contrary, the overexpression of *MMM1* did not affect the ROS balance, and the ROS level of P-O-MMM1 was similar to that of Pilsner at G5. Meanwhile, relatively lower MMP and ATP values were measured in Pil-mmm1Δ, and higher MMP and ATP values were measured in P-O-MMM1. *DNM1* regulation did not cause severe ROS fluctuation as shown in Figure 5, though *DNM1* was speculated to interfere with other genes, resulting in the decline of cell vitality. Both *MMM1* and *DNM1* seem to have a subtle effect on the yeast's antioxidant capacity.

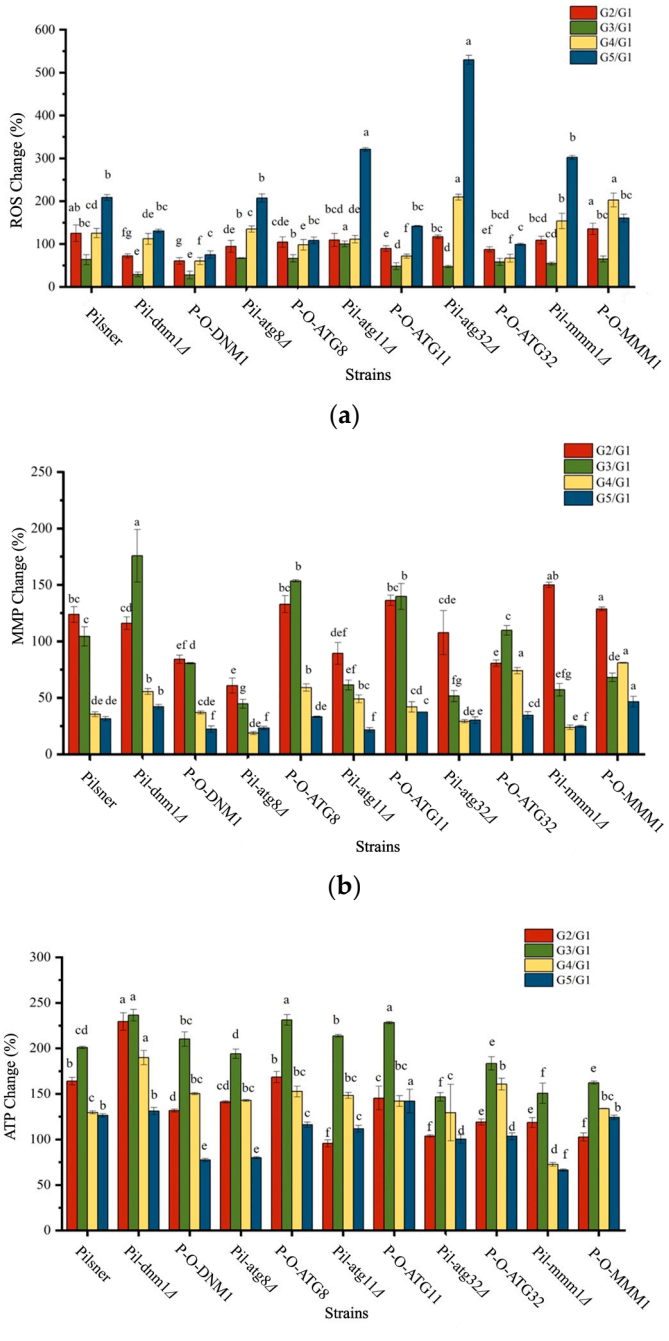


Figure 5. Measurement of ROS (**a**), MMP (**b**), and ATP (**c**) change rates for recombinant strains and parental strains during serial beer fermentation. Values indicate mean ± standard deviation ($n = 6$). Different letters indicate significant differences ($p < 0.05$) based on one-way ANOVA. Values in the same column with different superscript letters are significantly different ($p < 0.05$).

4. Discussion

Mitochondria are highly dynamic organelles continuously undergoing fission and fusion to maintain the efficient energy production of cells. The disturbance of the mitochondrial dynamic has been shown to contribute to aging and a variety of human age-related diseases, including Alzheimer's, Parkinson's, and Huntington's disease, and amyotrophic lateral sclerosis [30]. Studies have shown that oxidative stress causes mitochondria fragmentation and finally leads to an imbalance in metabolism [31,32]. Oxidative stress, defined as elevated ROS levels, can damage mitochondrial components, leading to the depolarization

of MMP and triggering mitophagy [5,33,34]. Mitophagy is an evolutionarily conserved process to remove dysfunctional or redundant mitochondria, thereby fine-tuning mitochondrial mass and maintaining the energy metabolism [34].

In budding yeast, mitochondria degradation depends on a set of autophagy-related genes. It has been proved that autophagic genes including *ATG1*, *ATG5*, *ATG9*, *ATG12*, and *ATG13* are essential for mitophagy. In addition to the above genes, *ATG32*, *ATG8*, and *ATG11* are essential for this process in yeast [35,36]. It was reported that the expression of the *ATG8*, *ATG11,* and *ATG32* genes is required for mitophagy by inducing the formation of autophagosomes [37]. Atg32p, an outer mitochondrial membrane protein, is required to initiate mitophagy by functioning as a selective receptor. It binds to Atg11p and marks mitochondria for degradation [10]. Atg32p also recruits Atg8p and is involved in the expansion of phagophore during autophagosome formation [38]. Mitophagy in yeast can be induced in several ways, including rapamycin induction, ethanol induction, the induction of nitrogen starvation [28,39,40]. Jing et al. reported that the expression levels of *ATG11* and *ATG32* varied under ethanol stress [28]. Previous studies reported that the atg32Δ strain exhibited greater MMP defects and accumulated more ROS under ethanol stress [28], and that the absence of Atg32p caused a mitophagy defect in yeast, while the overexpression of Atg32p stimulated mitophagy [7]. Herein, the disruption of *ATG32* in lager yeast caused a severe increase in intracellular ROS and a decrease in MMP. The recombinant strain showed a reduced tolerance towards H_2O_2 stress. Therefore, the downregulation of *ATG32* expression levels probably reduced the mitophagy efficiency and further affected the antioxidant activities of lager yeast. The overproduction of ROS could cause mitochondrial injury and result in MMP disruption, membrane rupture, mitochondrial matrix swelling, and mPTP opening [41]. Mitochondrial dysfunction could cause an imbalance between energy metabolism and cell apoptosis. It has been demonstrated that a lower MMP is accompanied by a simultaneous increase in ROS production [5]. Studies reported that *ATG32* was essential for yeast to maintain cell respiratory growth and cell vitality under the heat stress, and *ATG32*-mediated mitophagy promoted spermidine production [42]. During the brewing process, delayed beer fermentation cycles were found in the Pil-atg32Δ mutant, which might be caused by the interference of mitophagy. On the other hand, the use of a sake yeast strain with *ATG32* disruption was found to increase ethanol production during sake fermentation [9]. However, the ethanol content of beers brewed using Pil-atg32Δ was similar to that of beers produced using the Pilsner strain. Due to the genetic differences between lager yeast and sake yeast and the complexity of the fermentation environment, there might be some differences regarding fermentation performances [9]. The expression of *ATG32* is evidently regulated by multiple factors, and its post-translational modifications seem to control Atg32-mediated mitophagy, such as phosphorylation, ubiquitination [37]. From the published data, Atg32p phosphorylation is presumably regulated by the balance of protein kinase CK2 and protein phosphatase Ppg1. Under mitophagy-inducing conditions, CK2 efficiently phosphorylated Atg32p [43]. Conversely, the Ppg1/Far complex dephosphorylated Atg32p under non-inducing mitophagy conditions [44]. Although Atg32p, Atg8p, andAtg11p are all key proteins in mitophagy, only the amount of Atg32p could be regulated by the proteasome during mitophagy [37]. This might explain the reason for there being a lower ROS decline due to *ATG32* overexpression than due to the overexpression of *ATG8* and *ATG11*. Additional experiments are needed to further investigate how cells control the level and activity of Atg32p.

Atg32p-Atg11p and Atg32p-Atg8p interactions are crucial steps in mitophagy. Wild-type yeast cells degrade excess mitochondria through mitophagy and inhibit the excess ROS accumulation by mitochondria [45]. The disruption of *ATG8* and *ATG11* resulted in an imbalance in ROS under ethanol stress [28,46] and strains with *ATG11* and *ATG32* deletion cannot degrade excess mitochondria under nitrogen starvation [45], which was confirmed in the current study. The upregulation of *ATG8* and *ATG11* expression was beneficial for maintaining the balance of ROS fluctuation and mitochondrial vitality under oxidative

stress. Similar results were also found in plant cells as atg11Δ null plants would undergo senesce prematurely [47], indicating that *ATG11* plays a crucial role in the aging of cells.

In addition, the dynamics and functions of mitochondria depend on the interactions among organelles. The ERMES complex is related to the interaction between mitochondria and ER and Mmm1p is one of the complex subunits of ERMES. The *MMM1*Δ mutant exhibited an altered mitochondrial morphology, slow growth, and a marked loss of its mitochondrial structure [48]. Similarly, cells disrupted in the *MMM1* were inviable when grown on non-fermentable carbon sources and showed an abnormal mitochondrial morphology at all temperatures [49]. The disruption of *MMM1* inhibited the growth of lager yeast and caused a poor tolerance of H_2O_2 stress. These findings suggest that the damage of mitochondrial transcription and mtDNA shown in Pil-atg32Δ and Pil-mmm1Δ might be the main cause of the low cell viability in the presence of H_2O_2. Meanwhile, *MMM1* plays a role in lipid exchange between the ER and mitochondria. The overexpression of *MMM1* could support lipid availability for autophagosome formation during mitophagy. However, excessive levels may disrupt mitochondrial dynamics or ER function [50]. It can be speculated that the regulation of *MMM1* might affect the expression of ERMES subunit-encoding genes such as *MDM10*, *MDM12*, and *MDM34* [51], and can influence the antioxidant capacity of yeast.

Dnm1p is a dynamin-related GTPase required for mitochondrial fission. It was reported that *DNM1* was involved in the perception of oxygen and mitochondrial morphology maintenance [52,53]. The disruption of *DNM1* significantly affected the H_2O_2 tolerance of yeast. However, the vitality of the Pil-dnm1Δ strain remained unaffected. In the *DNM1* knockdown strain, higher ATP levels were observed despite a low MMP, which could be the result of a compensatory metabolic shift. The *DNM1* gene regulates mitochondrial fission, and the disruption of the *DNM1* gene would cause hyperfused mitochondria and lead to glycolysis or other non-mitochondrial ATP generation pathways. This could help maintain ATP levels even if the MMP levels were low. Moreover, a lower MMP may lead to lower energy demands for mitochondrial maintenance, preserving ATP levels in cells. It can be speculated that downregulating of *DNM1* expression might perturb the expression of other mitophagy genes such as *FIS1*, *AUP1* and *RTG3* and affect the oxidative balance of yeast cells [54–56]. Therefore, the reason for ROS and MMP fluctuations in the Pil-dnm1Δ strain may not be the impairment of mitophagy but changes in the mitochondrial ultrastructure. On the other hand, excess DNM1 activity could lead to increased mitochondrial fragmentation, which could affect the cellular energy balance and ATP depletion under prolonged stress conditions [57].

Acute ROS production also could lead to the loss of MMP, ultimately activating mitophagy in mammals [6]. In our study, we assessed mitophagy in yeast through the expression levels of key mitophagy-related genes and functional cellular outcomes, including intracellular ROS levels, MMP, and ATP. Further experiments, such as tracking labeled mitochondria delivered to the vacuole, would be necessary for direct evidence of mitophagy induction. Mitophagy is a selective autophagy mechanism and is highly conserved. Mitochondrial-targeted therapy for disease treatment has become a new strategy. For example, using targeted mitochondrial antioxidants for intervention treatment specifically inhibited mitochondrial ROS and protected mitochondria from oxidative stress damage [58]. Yeast Atg32p is a functional homolog of mammalian BCL2L13 (BCL2-like 13 [apoptosis facilitator]) [59] and Atg8p is homologous to LC3 in mammalian cells [59,60]. Studies have shown that BCL2L13 might recruit LC3 to the mitochondrial surface, leading to autophagosome formation [61]. Research on mitophagy in yeast would help to further our understanding of the relationship between mitophagy function and disease development in other cells.

5. Conclusions

Mitophagy contributes to the regulation of antioxidant capacity of yeast cells. The mitophagy-related genes including *ATG8*, *ATG11*, *ATG32*, *DNM1*, and *MMM1* were manip-

ulated in the Pilsner lager yeast to investigate the functions of these genes in the antioxidant properties of yeast. The results showed that the repression of *ATG8*, *ATG11*, *ATG32*, and *MMM1* caused a decline in yeast viability and antioxidant capacity under oxidative stress. The *ATG32* and *MMM1* knockdown strains showed delayed fermentation during serial beer fermentation. The repression of the *ATG8*, *ATG11*, and *ATG32* genes affected the intracellular MMP levels. *ATG8* and *MMM1* repression caused decreased ATP levels. Excess *DNM1* activity could affect cellular energy balance and ATP depletion under prolonged stress conditions. The current study increased our understanding of the relationship between mitophagy and oxidative response of yeast, providing a new strategy for improving the antioxidant capacity of yeast.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages10040112/s1, Figure S1: Colony forming tests of recombinant strains and the parental strain under H_2O_2 stress.; Figure S2: Visualization of mitochondria in parental and recombinant strains by confocal fluorescence microscopy using TMRE staining. Table S1: Primers for constructing overexpression and disruption plasmids; Table S2: Relative expression levels of mitophagy-related genes in recombinant strains; Table S3: The fluorescence values of ROS, MMP, and ATP of recombinant strains and the parental strain from G1 fermentation to G5 fermentation.

Author Contributions: For Conceptualization, J.W. and W.C.; writing—original draft preparation, J.W.; writing—review and editing, J.W., V.S., M.K. and J.Z.; visualization, Q.L., C.N., F.Z., T.R. and C.L. All authors have read and agreed to the published version of the manuscript.

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