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RESEARCH ARTICLE

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Impact of ergosterol content on acetic and lactic acids toxicity to *Saccharomyces cerevisiae*

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Abstract

Organic acid stress often represents a major hurdle in industrial bio-based microbial processes. Organic acids can be released from lignocellulosic feedstocks pretreatment and can also be desirable products obtained by microbial fermentation with applications in different industrial sectors. Yeasts are prominent cell factories. However, the presence of organic acids can compromise yeast metabolism, impairing fermentation performances and limiting the economic feasibility of the processes. Plasma membrane remodeling is deeply involved in yeast tolerance to organic acids, but the detailed mechanisms and potentials of this phenomenon remain largely to be studied and exploited. We investigated the impact of ergosterol on *Saccharomyces cerevisiae* tolerance against organic acid stress by coupling in vitro and in vivo assays. In the in vitro assay, synthetic lipid vesicles were prepared containing different concentrations of ergosterol. We observed changes in organic acids diffusion through the membrane as a function of ergosterol content. Then, we extended our approach in vivo, engineering *S. cerevisiae* with the aim of changing the ergosterol content of cells. We focused on *ECM22*, an important transcription factor, involved in the regulation of ergosterol biosynthesis. The overexpression of *ECM22* was sufficient to increase ergosterol levels in *S. cerevisiae*, resulting in an enhanced tolerance toward lactic acid stress. In this work we propose an in vitro approach, using synthetic lipid vesicles, as a complementary method to be used when studying the impact of the plasma membrane lipid composition on the diffusion of organic acids.

KEYWORDS

ergosterol, organic acids, plasma membrane, *Saccharomyces cerevisiae*, synthetic lipid vesicles, tolerance

1 | INTRODUCTION

The yeast *Saccharomyces cerevisiae* has been one of the earliest domesticated living organisms, used since ancient times for baking and brewing (Hittinger et al., 2018). Since the era of r-DNA it also became a widely used workhorse for recombinant production processes, and it is among the most frequently used microorganisms in biotechnology with successful applications in the production of both bulk and fine chemicals from renewable biomasses, such as starch or sugar or lignocellulosic biomass (Hong & Nielsen, 2012). However, during bio-based industrial processes yeasts are subjected to different kinds of stress associated with industrial process conditions, such as non-optimal temperature, inhomogeneous mixing, unfavorable pH, high osmotic pressure. These stress factors might inhibit cellular metabolism and compromise the performance of the fermentative process (Deparis et al., 2017; Piper, 2011). Among the different stresses, organic acids represent a double edge sword in bio-based processes. Organic acids can be released from the pretreatment of biomass, inhibiting the fermentative process but, at the same time, they can be desirable products obtained by microbial fermentation as valuable building block chemicals with many different industrial applications (Sauer et al., 2008). For some of these applications, as for obtaining biopolymers, the undissociated form of organic acids is preferable, therefore it is preferable to run the fermentations at low pH (below the pKa of the acid). Low pH is also critical to avoid unwanted bacterial contamination. However, the desired undissociated form can cross the plasma membrane by simple diffusion (Mira et al., 2010). Once inside the cell, in a higher pH environment, the acid dissociates leading to the release of protons and the respective counter ion. The accumulation of both species inside the cell has detrimental effects ranging from intracellular acidification, inhibition of metabolic activities, inducing oxidative stress, interfering with lipid organization, plasma membrane permeability and integrity, leading to decreased cellular performance and eventually to cell death (Mira et al., 2010; Piper, 2011). Moreover, when organic acids are produced by yeast and high titers are reached, the interaction of the accumulating products with the plasma membrane might become an additional stress factor (Jezierska & Van Bogaert, 2017). The plasma membrane plays a key role in these processes, acting as a selective gate, controlling the flux of compounds into and out of the cell (Ferraz et al., 2021). As such, the plasma membrane has been proven to be an important target for stress adaptation when designing strategies for the development of versatile, robust, and efficient cell factories ready to tackle the harshness of industrial processes while delivering high yield, titer, and productivity (Russell et al., 1995). Lipid composition, in particular, is fundamental to determining the biophysical characteristics of the plasma membrane: changes in the lipid composition can have profound effects on cellular functions, including signal transduction, membrane elasticity, and membrane trafficking (Santos & Preta, 2018). Remarkably, the activity and stability of membrane proteins are also dependent on the lipids that surround them (Coskun & Simons, 2011). Membrane proteins require specific lipids, as cofactors for their functions or as "costructures" for their correct folding and stability. Therefore, the composition of the lipid bilayer must be optimal for obtaining the correct activity or the desired biological function of the membrane proteins (A. G. Lee, 2004). Certain plasma membrane lipid compositions can be more advantageous than others under specific stress conditions. For this reason, cells are able to adapt their plasma membrane according to the surrounding environment (Guo et al., 2018; Klose et al., 2012). Sterols are an essential component of the plasma membrane of eukaryotic cells and the major sterol present in yeast is ergosterol. Sterols contribute to membrane integrity, fluidity, and permeability as well as to the sorting of lipid rafts, which are sterols and sphingolipids-enriched clusters that house several membrane proteins, among them the proton pump H^+-ATPase Pma1 (Dufourc, 2008; Ferreira et al., 2001). Additionally, sterols increase membrane rigidity by ordering the fatty acyl chains and thus allowing a tighter lipid packing (Caron et al., 2014). The accumulation of different ratios, amounts and structures of sterols in the plasma membrane can lead to different membrane properties (Dufourc, 2008).

Take-away

- The protective effect of ergosterol varies depending on different organic acids.
- Ergosterol levels are crucial for *Saccharomyces cerevisiae* tolerance to lactic acid.
- *S. cerevisiae* strain with increased tolerance to lactic acid was obtained.

In this work, we are particularly interested in organic acid stress of yeast cells and how membrane composition, in terms of ergosterol content, relates to stress tolerance. The impact of individual lipid species on the plasma membrane physicochemical properties is difficult to predict (Ferraz et al., 2021). Nevertheless, a more rigid and less permeable membrane is likely to reduce the diffusion of acids into the cell and, therefore, likely to increase resistance toward the acids. In this work, we used complementary in vitro and in vivo approaches to study the impact of ergosterol on yeast plasma membrane tolerance against organic acid stress. Acetic acid was selected for this study, as it is the major inhibitory compound released from the pretreatment of lignocellulosic biomass (Jönsson & Martín, 2016). We also studied lactic acid—a chemical platform molecule with an ample spectrum of industrial applications, including food preservation, and use as pharmaceutical additive, also having the potential to be used for bioplastic production from a renewable source (Porro & Branduardi, 2017).

The in vitro assay, based on synthetic lipid vesicles, prepared from commercially available yeast lipids, enriched, or not, with different amounts of ergosterol, takes advantage of the use of a pH-responsive fluorescent dye, loaded into the vesicles. This assay allows to monitor pH and pH changes inside the liposomes and, indirectly, investigate the diffusion of organic acids through the liposomal membranes, independent of yeast metabolism. We observed that, at low pH, in the presence of acetic acid, higher levels of ergosterol

decrease the acid diffusion into the liposomes. Differently, in the presence of lactic acid, we observed that increasing ergosterol content is not directly proportional with decreased diffusion of the acid into the liposomes. Given the promising indications observed, we extended our approach to in vivo, taking advantage of *S. cerevisiae* cells engineered to modulate the quantity of ergosterol by overexpressing *ECM22*, encoding the transcription factor described as the main regulator of ergosterol biosynthesis (Vik & Rine, 2001). The overexpression of *ECM22* revealed to be crucial for increased yeast tolerance toward lactic acid. Moreover, coherently in the in vitro and in vivo assays, we observed that the impact of ergosterol depends on the organic acid under investigation.

2 | RESULTS AND DISCUSSION

2.1 | Impact of ergosterol content of the lipid membrane of artificial liposomes on organic acids diffusion

To study the influence of ergosterol on the diffusion of acetic and lactic acids through the plasma membrane, an indirect in vitro technique based on a liposomal fluorescent assay was designed. Liposomes incorporated 10 mM Na-HEPES buffer solution with pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt, HPTS), a ratiometric fluorophore, at pH 7.4. Pyranine is a water-soluble, membrane-impermeable fluorophore, having fluorescent excitation and emission spectra that are highly dependent on the pH. Therefore, it is used as a pH sensitive fluorescent probe to monitor pH and, indirectly, can allow evaluating the membrane diffusion to organic acids. Liposomes were prepared starting from Yeast Lipid Total extract, acquired from Avanti Polar lipids, and were enriched, or not, with different concentrations of ergosterol. Five different liposome compositions were used in this study. As a reference, in *S. cerevisiae* ergosterol concentration is about 10 mol% of the total lipids (Klose et al., 2012). The polydispersity index (PDI) (Table 1) indicates that all liposomes preparations produced are homogeneous (see Section 4).

TABLE 1 Ergosterol concentration of different liposome preparations, liposome average size (nm), and polydispersity index (PDI)

Ergosterol concentration (Mol%)	Average size (nm)	PDI
Standard	148 ± 0.3	0.141 ± 0.011
+3	143.6 ± 1.5	0.152 ± 0.013
+10	141.2 ± 3.6	0.258 ± 0.025
+20	151.7 ± 5.6	0.163 ± 0.012
+30	146.6 ± 6.1	0.134 ± 0.02

Note: Standard deviations were calculated from three independent replicates.

Liposomes were exposed for 5 min to 65 mM of acetic or lactic acid solutions at different pH values, and then the final pH values inside the different liposomes were calculated through the correlation with the measured fluorescence. The results are shown in Figure 1. We observed that both acetic and lactic acid caused pH drops in all the liposomes under all conditions tested. Upon the exposure to acetic and lactic acid at pH 7 and pH 5, no differences were observed in the final internal pH among the different liposomes (Figure 1a,b). Nonetheless, at these pH values, acetic acid caused larger pH drops than lactic acid. Our data are in accordance with the study of Gabba et al. (2020). Using a liposome mixture composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE): 1-pal- mitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) sodium-salt (POPG): 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (2:1:1 weight ratio), the authors observed that, at pH 7, liposomes permeability to acetic acid is higher than to lactic acid. However, the effect of ergosterol on organic acid diffusion is strongly dependent on lipid compositions. Frallicciardi et al. (2022) made use of pure lipid preparations of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or 1,2- dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). They reported that the enrichment of POPC liposomes with 15% ergosterol resulted in a decreased permeability to lactic acid, which was not further influenced by increasing the concentration of ergosterol to 30% and 45%. Differently, when ergosterol was added to liposomes prepared with only DPPC, the permeability to lactic acid increases. These data support our choice of using Yeast Lipid Total extract, to resemble, as much as possible, the lipid composition of *S. cerevisiae*. We then tested our system at pH 3, (Figure 1c), where differences were observed between the different liposomes. In the presence of acetic acid at pH 3, liposomes enriched with 30 mol% ergosterol were able to maintain a higher internal pH when compared with the reference composition. The pH difference between the control and the 30 mol% ergosterol enriched liposomes was about 0.41 pH units (p-value = 0.0316). Also, under this condition, we observed a trend: increasing amounts of ergosterol lead to smaller decrease in the liposomes internal pH. Differently, in the presence of lactic acid at pH 3, liposomes enriched with 10 mol% ergosterol were able to sustain a higher internal pH than the other liposomes. The pH difference between the control and the 10 mol% ergosterol liposomes was about 0.25 pH units (p-value = 0.0335). Also, under this condition, a significant drop in the internal liposomal pH was observed between liposomes enriched with 10 and 20 mol% ergosterol (p-value = 0.0027). This pH drop was unexpected and further testing will be needed to clarify this difference. Control conditions where water was added instead of organic acids can be found in the supplementary material (Supporting Information: Figure S1).

In solution, organic acids exist in a pH-dependent equilibrium between dissociated and undissociated states, as described by the Henderson–Hasselbalch equation: $pH = pK_a + \log [A^-]/[HA]$, where A^- and HA are the dissociated and undissociated species, respectively. This equation indicates that at pH values above the acid pK_a value (acetic acid $pK_a = 4.76$, lactic acid $pK_a = 3.86$), more than 50% of the

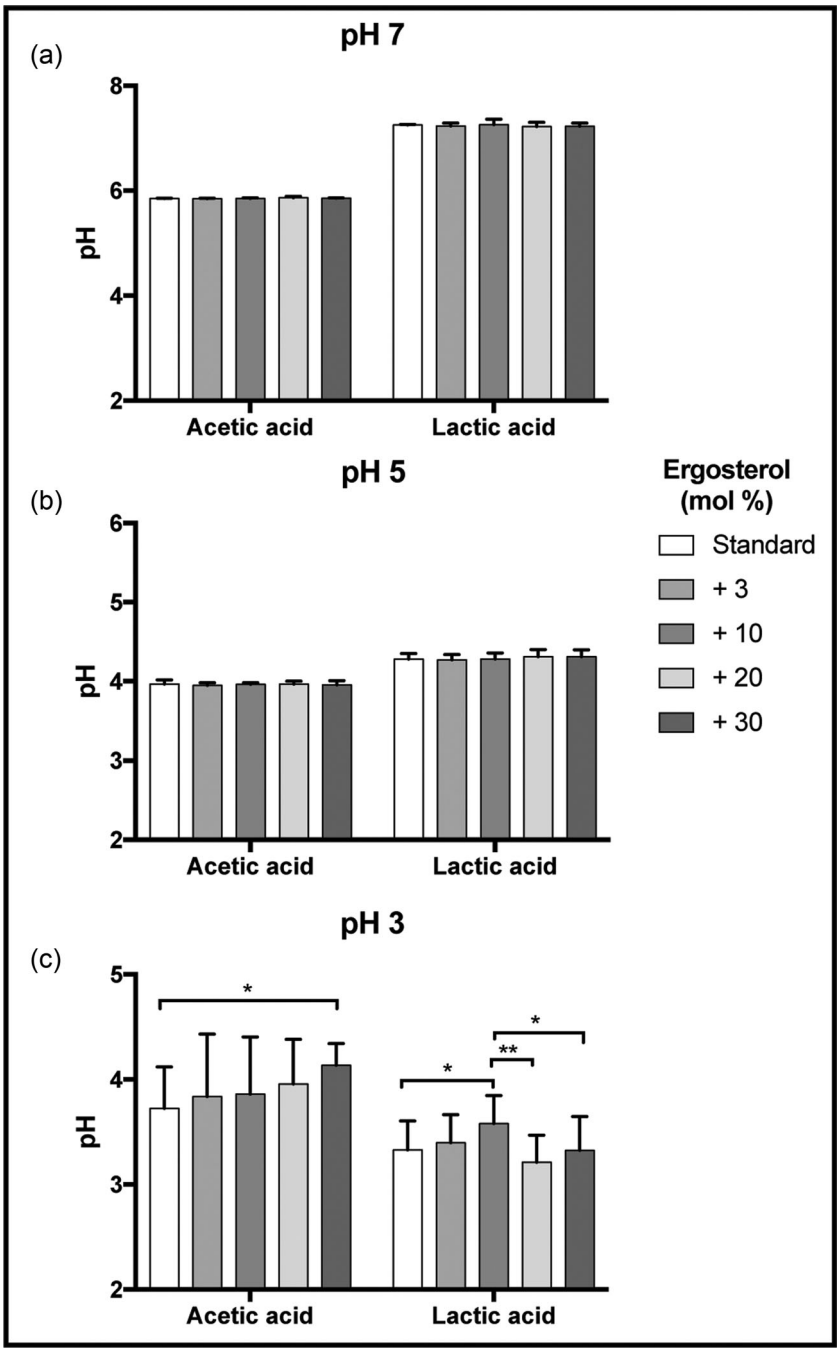

FIGURE 1 Internal pH (pH_i) of the different liposomes after exposure to 65 mM of Acetic acid or Lactic acid solutions, at different pH values (a) pH 7, (b) pH 5, and (c) pH 3. The initial pH inside the liposomes was 7.4. $^{*}p < 0.05$ and $^{**}p < 0.01$. Please note that "y" axes are different.

acid is dissociated and that the concentration of the undissociated acid increases exponentially as the pH declines below this threshold. Undissociated weak acids diffuse passively through the plasma membrane until an equilibrium is established (Figure 2). As the undissociated acid enters the liposome lumen, it dissociates at the higher internal pH (pH_i). This results in the formation of charged anions and protons, which cause a reduction of the internal pH once the buffering capacity is exceeded. The concentration of these molecules differs based on the molar concentration of the acids in the medium and the pH_e and pH_i values.

Our results showed that at $pH_e = 3$, the pH drop caused by lactic acid was higher than acetic acid, which may be related with the lower pK_a of lactic acid. Organic acids with a lower pK_a have a higher tendency to dissociate and therefore have a greater ability to donate protons and collapse the pH, when going from a medium at a pH where they are mostly undissociated to an environment with a pH higher than their pKa.

Maintaining cytoplasmic pH is essential for optimal cellular metabolism, growth, and proliferation (Casey et al., 2010). Several studies have shown that the ability to maintain pH_i is crucial for acid stress resistance (Arneborg et al., 2000; Halm et al., 2004). The pH differences obtained by the modulation of the ergosterol composition of liposomal membranes (0.41 and 0.25 pH units in the presence of acetic and lactic acid, respectively, for the best-performing setting)

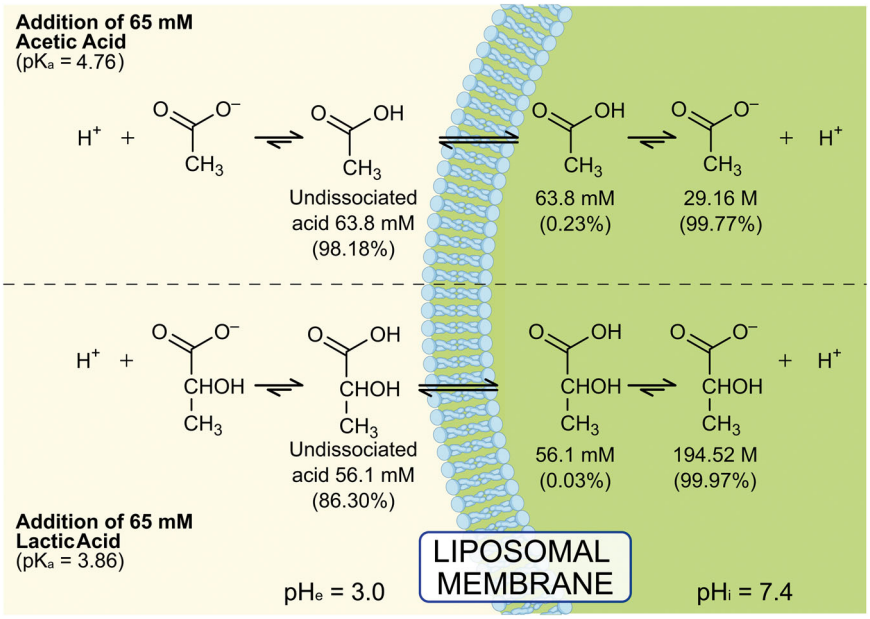

FIGURE 2 Illustration of the concentrations of anions and undissociated acids that would be present in the medium and inside the liposomes (based on the pH_e and pH_i) when 65 mM of either acetic acid or lactic acid is present. The concentrations were calculated based on the Henderson–Hasselbalch equation and assuming the initial pH values do not change. The theoretical concentration of dissociated acids (anions) would reach 29.16 M acetic acid and 194.35 M lactic acid under the stated conditions, illustrating the higher capacity of lactic acid to release protons. This figure is illustrative and is not meant to reflect the real situation as, in most situations, decreases in pH_i will occur and influence the values shown. The diffusion of H^+ through the membrane was also not taken into account, this fact does not change the concept illustrated.

have the potential to be essential for an efficacious cellular response to organic acid stress. Srivastava et al. (2007) reported that changes of pH_i within a range of 0.2–0.3 units function as a signaling mechanism to regulate a number of cellular processes such as cell cycle progression, proliferation/apoptosis, and differentiation.

Ergosterol is essential for maintaining the correct structure of the plasma membrane. The accumulation of ergosterol may protect the cell membrane against acid stress (Vanegas et al., 2012). Importantly, we observed in vitro that the effect of ergosterol changes with the organic acid in question. An enrichment with 30 mol% of ergosterol was required to maintain a higher pH_i in the presence of acetic acid, whereas 10 mol% was enough to maintain a higher pH_i in the presence of lactic acid. Interestingly, a larger quantity did not show the same effect. As there are no similar studies in vitro as comparison, it is not trivial to comment on our findings. One possibility could be that the quantity of ergosterol needed to have a protective effect against acid diffusion is higher in the case of acetic acid. This might be in agreement with an in vivo study where the authors demonstrated that at low pH the permeability of the yeast membrane to acetic acid is much higher than to lactic acid (Casal et al., 1996). Therefore, it is rational and consistent that a higher amount of ergosterol is needed to decrease the diffusion of acetic acid through the liposomal membrane than lactic acid. Nonetheless, further studies are necessary to better describe these findings, also because it is not clear why the diffusion of lactic acid is minimal with an addition of 10 mol% of ergosterol, but increases again with 30 mol%. This indicates that an increasing ergosterol abundance is not straightforwardly proportional with a decreased diffusion of organic acids in general, but also that the effect depends on the type of organic acid.

2.2 | From in vitro to in vivo: Overexpression of *ECM22* in yeast cells changes the ergosterol content

Given the significant differences, correlating with different incorporation of ergosterol in the liposomal membrane, we attempted to modulate the ergosterol composition of a *S. cerevisiae* strain to test the effects in vivo. The plasma membrane is a complex and dynamic system, whose behavior is challenging to predict due to the connections, competitions, dependencies, or other types of interactions between its components. For this reason, it is not trivial to predict which element(s) should be changed to trigger a specific rewiring of the overall system. Multiple genetic modifications are often required to unlock phenotypes of interest. Therefore, engineering a desired phenotype would be facilitated by simultaneous multiple gene modifications. In the case of ergosterol, the overexpression of a few enzymes has been revealed to be insufficient to modulate its metabolism (He et al., 2003, 2007; Polakowski et al., 1999). Given the ability of transcription factors to regulate multiple genes, they can be recruited to modulate entire metabolic pathways, such as the sterol biosynthesis in *S. cerevisiae*. We focused on the transcription factor encoded by the *ECM22* gene. Ecm22 acts as the main regulator of ergosterol biosynthesis by binding to sterol regulatory elements in the promoter of ergosterol biosynthesis

(*ERG*) genes (Vik & Rine, 2001). Hypothesizing a pivotal role of Ecm22 in the overall ergosterol composition of *S. cerevisiae*, we overexpressed *ECM22* and investigated the ergosterol levels of the two different strains: CEN.PK 102-5B WT and CEN.PK 102-5B *ECM22*.

The mRNA levels of the *ECM22* gene, measured in exponentially growing cells (Figure 3a), were almost five-fold higher in the overexpressing strain, CEN. PK 102-5B *ECM22*, compared to the wild-type (WT) strain.

The ergosterol analysis (Figure 3b) revealed differences between the wild type and CEN. PK 102-5B *ECM22* strain, the latter revealing an increased ergosterol content of almost 50% (Figure 3b). The increased levels of ergosterol caused by the overexpression of *ECM22* have also been evidenced indirectly by Wang et al. (2018).

2.3 | Effect of the overexpression of *ECM22* on organic acids tolerance

Sterols play a crucial role in the architecture of the plasma membrane, namely by ordering fatty acyl chains allowing tighter lipid packing and thus reducing membrane permeability. A less permeable plasma membrane might be able to reduce the permeation of organic acids into the cells, contributing to other counteraction mechanisms, and therefore resulting in more tolerant strains. The different ergosterol content observed between the strains opened the question of a possible effect of these changes on the cellular response to organic acid stress, and about the possibility to compare the data with these obtained with the in vitro assay. We investigated the effect of *ECM22* overexpression on the growth of cells challenged with acetic or lactic acid, at low pH, and in particular at pH 3 as this value revealed significant differences in the in vitro assay. First, strains were used for a drop test on solid minimal media with 2% w/v glucose at pH 3 in the presence or absence of organic acids (Figure 4). Acetic acid concentrations used in this study were initially based on literature findings reporting the range of acetic acid released from different pretreatment methods as well as different lignocellulosic biomass (Almeida et al., 2007; Baral & Shah, 2014). Acetic acid is more toxic to yeast cells than lactic acid (Narendranath et al., 2001). Therefore, to impair yeast growth, the concentration of lactic acid used in the following studies was increased, similar to the work of Berterame et al., 2016, as it was proven to be industrially relevant to screen for improved yeast tolerance.

In the absence of organic acids, no differences between the parental and the mutant strain were observed. Similarly, although the

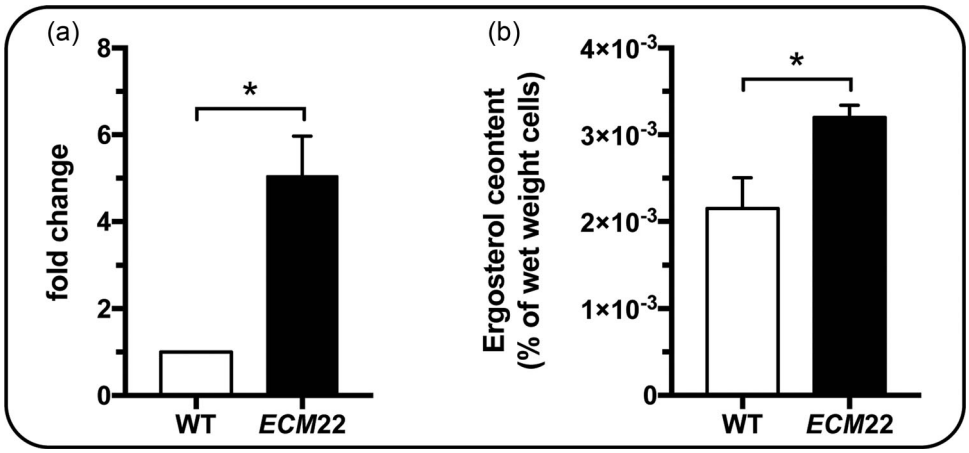

FIGURE 3 (a) Expression levels of *ECM22* in the CEN.PK 102-5B wild-type (WT) (white bar) and CEN.PK 102-5B *ECM22* (black bar) strains analysis during exponential growth phase. (b) Ergosterol content in CEN.PK 102-5B WT (white bar) and CEN.PK 102-5B *ECM22* (black bar) strains. $^*p < 0.05$.

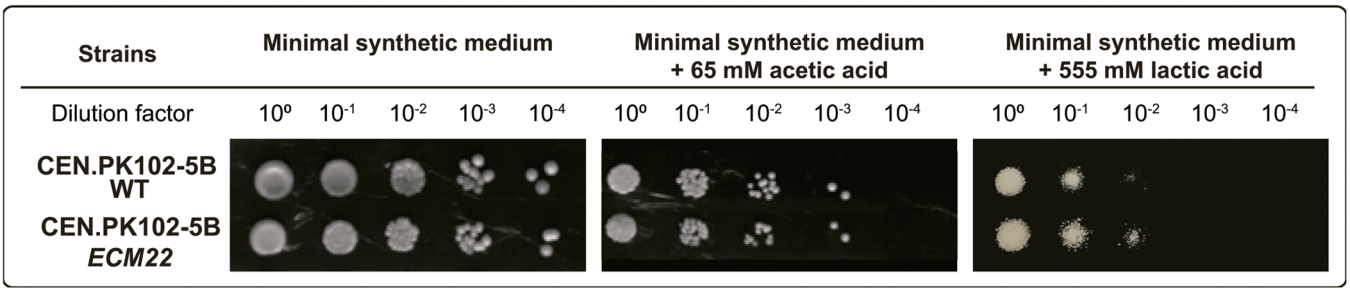

FIGURE 4 Spot assay of CEN.PK 102-5B wild-type and CEN.PK 102-5B *ECM22* cells in the absence and presence of acetic (65 mM) or lactic (555 mM) acids, pH 3 at 30°C. The images were taken after 72 h of growth.

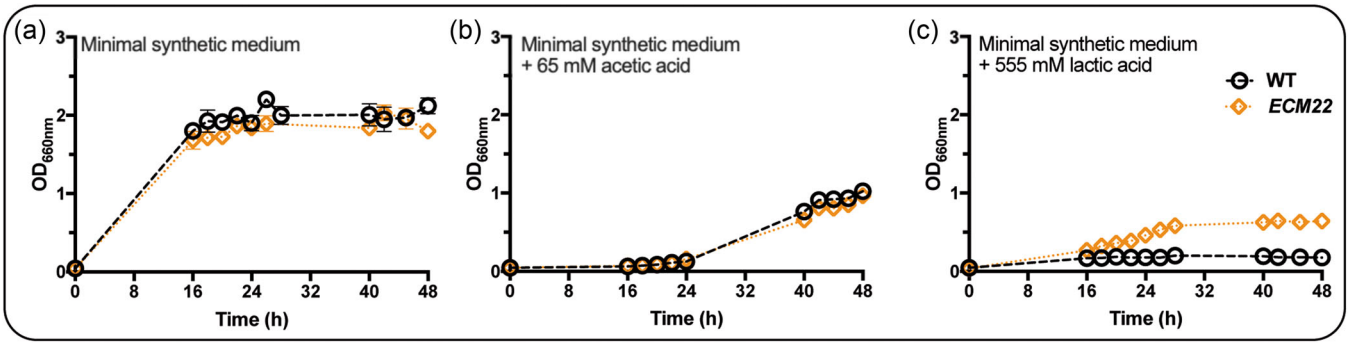

FIGURE 5 Growth of CEN.PK 102-5B WT and CEN.PK 102-5B *ECM22* cells in the absence and presence of acetic or lactic acid. Yeast cells were grown in shake flasks in minimal synthetic media with 2% w/v glucose at pH 3, without (a) or with (b) 65 mM of acetic acid or (c) 555 mM of lactic acid. Growth was assessed by measuring the OD at 660 nm. CEN.PK 102-5B WT (black open circles -o-) and CEN.PK 102-5B *ECM22* strains (orange open diamonds ...◊...). Values are the mean of three independent experiments. Standard deviations are included. For some points, the error bars are shorter than the height of the symbol, in these cases, the error bars are not visible.

addition of acetic acid had a negative effect on the growth of both strains, there are no significant differences among them. The addition of lactic acid also impaired the growth of both strains. However, under this condition, differences in the growth between the strains were observed. The strain CEN.PK 102-5B *ECM22*, with a higher content of ergosterol, demonstrated an increased growth capacity in comparison with the CEN.PK 102-5B WT strain.

To further characterize the consequences of the stress imposed by the organic acids, we performed growth kinetics experiments in shake flasks (Figure 5). In the absence of stress, no differences were found between the parental and the mutant strain, none of the strains exhibited lag phase and the maximum specific growth rate was 0.15 and 0.16 h^{-1} from the wt and the overexpressing strain, respectively (Figure 5a). Consistently with drop test results, the addition of acetic acid had a negative impact on growth, leading to an increased lag phase. However, after approximately 20 h both strains were able to resume growth in a similar way, the maximum specific growth rate was 0.04 h^{-1} for both the strains (Figure 5b). Lactic acid had a strong negative effect on the growth of both strains. The CEN.PK 102-5B WT was not able to resume growth for the entire period of observation, while the strain overexpressing *ECM22* was able to resume growth after a long lag phase (Figure 5c). These results indicate that the increased levels of ergosterol are crucial to improve *S. cerevisiae* tolerance toward lactic acid stress.

One important mechanism for yeast cells to counteract stress caused by organic acids is by modulating the plasma membrane lipid composition (Mira et al., 2010). Recently, Guo et al. (2018) performed a comparative analysis of lipids in *S. cerevisiae* grown in the presence of four different organic acids, acetic, formic, cinnamic, and levulinic acid. The authors observed that *S. cerevisiae* adapts its lipid composition differently according to the organic acid present in the media. In particular, during growth on glucose in the presence of the organic acids studied, levels of ergosterol increased to different extents according to the organic acid in question, revealing a specific response.

The importance of ergosterol has also been associated with the remarkable resilience against acetic acid stress of the yeast *Zygosaccharomyces bailii*. *Z. bailii* is a food spoilage yeast that is typically isolated as a contaminant during wine fermentation, as well as from many acidic, high-sugar, and canned foods (Kuanyshev et al., 2017). Lindberg et al. (2013) investigated the lipidomic response of *Z. bailii* to acetic acid stress. Differently from *S. cerevisiae*, this yeast is capable of maintaining its intracellular pH and plasma membrane integrity upon acetic acid stress (Fleet, 1992). Lipid analysis revealed *Z. bailii*'s ability to maintain high levels of ergosterol, upon acetic acid exposure, as one of the main reasons for its high tolerance. Our data suggest that the increased level of ergosterol obtained is enough to boost *S. cerevisiae*'s tolerance to lactic acid stress but not to acetic acid. In fact, the results obtained with the liposomal membranes (Figure 1) suggest that higher levels of ergosterol are required to decrease the diffusion of acetic acid than lactic acid. We speculate that the ergosterol levels obtained in the *ECM22* overexpressing strain were high enough to diminish the lactic acid diffusion into the cell, but had no effect on acetic acid.

It has been reported that changes in the yeast lipid composition can have profound effects on different cellular functions including signal transduction, membrane elasticity, membrane trafficking, and cell wall (Santos & Preta, 2018). The transcription factor *ECM22* has also been shown to play a role in cell wall synthesis (Abramova et al., 2001) and in other biological processes such as mating (Joshua & Höfken, 2017) or in the biosynthesis of sphingolipids (Vik & Rine, 2001). The cell wall has been shown to be involved in the adaptation of cells to weak acid stress (Ribeiro et al., 2021). This means that the increased tolerance of *S. cerevisiae* to lactic acid, which we see after *ECM22* overexpression might be a combination of effects of the increased ergosterol levels and changes in other structural constituents of the cells. The results obtained in vitro showed that ergosterol has as an individual factor an important effect on the diffusion of organic acids through the membrane. It would be desirable to have a way to increase ergosterol in vivo individually without affecting other points. However, at the time being all attempts to do so failed and we lack the genetic tools to obtain this. We therefore opted to take the compromise and look at the combined effects in vivo, which are in

line with our in vitro findings. In future, it will be interesting to see the effects of the individual targets.

2.4 | Plasma membrane response to lactic acid stress

Given the growth differences observed, we tried to understand, at the plasma membrane level, if there were differences that could explain the increased tolerance to lactic acid of the overexpressing strain. Lipid peroxidation is another of the reported effects of the organic acids counter-anions on *S. cerevisiae* cells (Mira et al., 2010). Lipid peroxidation is a sudden molecular rearrangement that starts with the attack of a radical reactive oxygen species to a double bond of a polyunsaturated fatty acid, resulting in the formation of radical polyunsaturated fatty acids. These species, due to their high reactivity, can lead to the formation of several products, including malondialdehyde (MDA), which can be used as an index of lipid peroxidation level. Here, we were interested to determine if lipid peroxidation can occur after sudden exposure to organic acids, and how wild-type and engineered strains could react. We observed that lactic acid stress correlates with a significant increase in peroxidized lipid content when compared with the control condition (Figure 6a), with no significant differences between the two strains. So far, it remains to be elucidated how membrane remodeling influences the fatty acid composition and degree of saturation and unsaturation. Literature analysis seems to indicate that the relative ratio of the degree of unsaturation of fatty acids varies widely among species, between strains of the same species, with environmental conditions and in respect to the different organic acids tested. Guo et al. (2018) reported that the exposure of *S. cerevisiae* cells to the acetic, formic, and levulinic acids leads to a continuous increase in the unsaturation index of fatty acids. Differently, authors reported that the exposure to cinnamic acid did not affect the saturation index. Berterame et al. (2016), using a different *S. cerevisiae* background, observed a reduction of lipid peroxidation after cell exposure to lactic acid. Authors speculated that unsaturated membrane lipids decreased in favor of saturated ones after acid treatment.

Plasma membrane proteins represent around 40% of the membrane composition. A total of about 1000 different proteins are estimated to be located in the yeast plasma membrane (Stewart, 2017). The abundance and activity of membrane proteins changes according to different cellular stimuli. Remarkably, the activity and stability of membrane proteins is dependent on the lipids that surround them (Coskun & Simons, 2011). Membrane proteins require specific lipids, as cofactors for their functions or as "costructures" for their correct folding and stability. Therefore, the composition of the lipid bilayer must be optimal for obtaining the correct activity or the desired biological function of the membrane proteins (A. G. Lee, 2004). In this work, we are particularly interested in understanding how the plasma membrane ergosterol composition can influence *S. cerevisiae* robustness toward acetic and lactic acids. Nonetheless, intracellular acidification is considered one of the major causes of growth inhibition for *S. cerevisiae* cells upon organic acid stress (Ullah et al., 2012). To counteract this acidification, cells strongly rely on the proper functioning of the proton pump H^+-ATPase to maintain the intracellular pH close to neutral (Ferreira et al., 2001). Pma1, the major proton pump present in the yeast plasma membrane, is localized at plasma membrane lipid rafts. Lipid rafts are heterogeneous, highly dynamic, ergosterol and sphingolipid-enriched domains that compartmentalize cellular processes and house several membrane proteins (Ferreira et al., 2001; Pike, 2006). The importance of this proton pump for yeast cells under different stress conditions has been highlighted in several studies (Y. Lee et al., 2015, 2017; Serrano et al., 1986). Eisenkolb et al. (2002) suggested that the correct positioning and activity of Pma1 into plasma membrane lipid rafts has been correlated with the presence of sterols. When we measured the effect of lactic acid stress on proton extrusion by assessing the extracellular acidification rate, we observed an increased flux of protons in both strains, with respect to the control condition (Figure 6b). Our results show that, under lactic acid stress, the proton efflux rate was higher in CEN.PK 102-5B WT than in CEN.PK 102-5B *ECM22*. According to the in vitro data, higher levels of ergosterol result in a lower acidification of the liposomes in presence of lactic acid (Figure 1). We hypothesize that the drop of the internal pH of the wild-type strain is higher than in the *ECM22* overexpressing strain. Therefore, the wild-type strain requires a higher proton efflux to maintain pH homeostasis. Maintenance of pH_i homeostasis can be energetically expensive, resulting

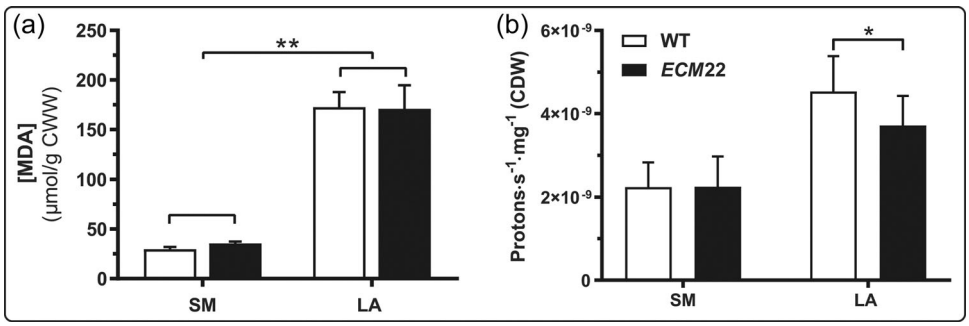

FIGURE 6 Effect of lactic acid pulse on (a) lipid peroxidation, measured as MDA levels, and (b) rates of the net proton efflux. Cells were grown in minimal synthetic media (pH 3) until the exponential phase and then were treated (LA) or not (SM) with a 30 min pulse of lactic acid 555 mM at pH 3. CEN.PK 102-5B WT (white bars), CEN.PK 102-5B *ECM22* (black bars). $^*p < 0.05$ and $^{**}p < 0.01$.

in the membrane H^+-ATPase consuming between 40% and 60% of the total cellular ATP (Holyoak et al., 1996). Therefore, the maintenance of pH_i homeostasis in the presence of organic acids may deplete cellular ATP levels. We speculate that the higher levels of proton efflux observed in the wild-type strain depletes the ATP, thereby preventing growth. These results can explain the growth of CEN.PK 102-5B *ECM22* in the presence of lactic acid (Figure 5c).

3 | CONCLUSION

In this work, using in vitro and in vivo complementary approaches, we demonstrate that a modulation of the ergosterol content is crucial for *S. cerevisiae* to gain tolerance toward lactic acid. Synthetic lipid vesicles were used to characterize the role of ergosterol on organic acid diffusion through different membrane compositions. This allowed us to further comprehend the structural role of ergosterol in the yeast plasma membrane, independently of yeast metabolism. Given the promising indications observed, we evaluated the response to acetic and lactic acid of *S. cerevisiae* mutant strains with different ergosterol compositions. Increased ergosterol levels in a *S. cerevisiae* mutant strain resulted in an increased tolerance toward lactic acid stress, but not acetic acid stress. This is in line with in vitro observation that a moderate increase of the ergosterol content decreases lactic acid diffusion through the membrane, but not acetic acid diffusion. To decrease acetic acid diffusion, a large increase of the ergosterol content was required, which was interestingly not suitable to inhibit lactic acid diffusion. Therefore, both the in vitro and in vivo *assay* indicated that the same membrane ergosterol composition led to different outcomes in the presence of different organic acids. Membrane engineering strategies must be specific for different kinds of acids.

4 | MATERIALS AND METHODS

4.1 | Liposomes preparation

Liposomes were prepared by an adapted dried reconstituted vesicle (DRV) method (Suleiman et al., 2019), initially developed by Kirby and Gregoriadis (1984). Yeast lipid total extract was purchased from Avanti Polar Lipids and ergosterol from Sigma-Aldrich. The standard amount of ergosterol present in Yeast Lipid Total extract is not reported by the company. The desired amount of yeast lipid extract and ergosterol were transferred into a rotating round-bottomed flask and the organic solvents were evaporated, at 60°C in a water bath at reduced pressure until a dried lipid film was generated. Liposomes also incorporate Biotin-X-DHPE (N-((6-(Biotinoyl)amino)hexanoyl)-1,2-dihexadecanoylsn-glycero-3-phosphoethanolamine, triethylammonium salt), to allow binding to streptavidin coated plates, used to study the diffusion of organic acids into the liposomes. The lipid film was rehydrated with 2 ml of 10 mM Na-HEPES buffer solution (pH 7.4) containing 500 µM of Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt, HPTS). Rehydration of the lipid film with buffer, as well as, liposome size reduction by extrusion was performed at 60°C. The final lipid suspension (5 mg/ml) was extruded on a LIPEX™ Extruder (Northern Lipids Inc.) equipped with Whatman® Nuclepore track-etc0hed membranes. Up to 10 extrusion cycles with 800, 400, and 200 nm membranes were performed, to obtain homogeneous suspension. Five different liposome formulations were prepared, consisting of either pure yeast lipid total extract or enriched with different concentrations of ergosterol: 3, 10, 20, or 30 mol %. The resulting suspension was stored at 4°C. The liposome mean diameter and the homogeneity were measured by DLS (dynamic light scattering) with a Zetasizer Nano-ZS with software version 7.03 (Malvern Instruments) at 25°C. Liposomal suspensions were diluted 100-fold with double distilled water prior to the measurement. Each suspension was measured three times, and each consisted of 15 repetitions. Size distribution was calculated by the intensity report. The homogeneity of the liposomes was determined by the PDI. PDI values range from 0 (for a perfectly uniform sample, with respect to the particle size) to 1 (for a highly polydisperse sample with multiple particle size populations).

4.2 | Pyranine ph calibration

A pH calibration curve (Supporting Information: Figure S2) was established for the ratiometric fluorophore pyranine. Pyranine solutions (500 µM) were prepared in 10 mM Na-HEPES buffer in the pH range from 3 to 8. The fluorescence intensity upon excitation at both 405 and 453 nm, emission ratio at 510 nm, was recorded using the Infinite M200 plate reader (Tecan). The ratio 453/405 nm was calculated for each pH. Later, equation ($y = 2 \times 10^{-5} e^{1.485\,x}$) was used to convert the measured ratio to pH values. The pH determination using pyranine is based on ratiometric measurements between two fluorescence excitations: one at 453 nm, which is pH dependent, and the other at 405 nm, which is a pH-independent isosbestic point. Therefore, the total amount of the fluorophore does not influence the pH calculation.

4.3 | Liposomes exposure to acetic and lactic acids

Streptavidin-coated 96-well plates (Thermo Scientific) were used to study the impact of organic acids on liposomes internal pH. Wells were washed with 10 mM Na-HEPES buffer (pH 7.4). After, 50 µl of biotinylated liposomes were added to the wells and incubated at room temperature for 30 min, with shaking. After the binding step, wells were washed with 10 mM Na-HEPES buffer (pH 7.4), to remove unbound liposomes and traces of pyranine. The fluorescence (emission at 510 nm) of the bound liposomes was measured in the M200 plate reader (Tecan) and the ratio between excitation 453 nm and 405 nm was converted to pH using the above equation. Next, organic acid solutions, 65 mM at different pH values, were added to the wells containing the liposomes. The pH of the organic acids solutions was adjusted to the desired pH by the addition of KOH or HCl 1 M. Incubation with organic acids was done at room temperature for 5 min, with shaking. We tested different incubation times

(10, 20, and 30 min, data not shown). It turned out that 5 min are enough for the organic acid diffusion to occur. Therefore, this was the time chosen to perform the study. Finally, M200 plate reader (Tecan) was used to monitor the fluorescence intensity changes caused by the addition of organic acids to the wells. Final pH values inside the liposomes were obtained using the equation.

4.4 | Yeast strains, transformation, and growth conditions

The *S. cerevisiae* strain CEN.PK 102-5B [*MATa his3Δ1, ura 3–52, leu2-3,112 MAL2–8c SUC2*] was transformed with the integrative plasmids pYX022 and pYX042. This strain was used for the overexpression of *ECM22*, encoding the transcription factor *ECM22* (Table 2). Yeast transformation was performed according to the LiAc/PEG/ss-DNA protocol (Gietz, 2014). For each set of transformations, at least three independent transformants were tested, showing no significant differences among them. All the strains used and created in this work are listed in Table 2. Yeast cultures were grown in minimal synthetic media (0.67% w/v YNB media with ammonium sulfate; cat. No. 919-15, Difco Laboratories) with 2% w/v D-glucose as carbon source. Uracil and the required amino acids were added to a final concentration of 50 mg/L. For the kinetics of growth, acetic and lactic acid stress was imposed by adding the desired amount of the organic acids (Sigma Aldrich) to the culture media. The final media were prepared starting from different stock solutions, 1.54 mol of acetic acid, 2.22 mol of lactic acid, and synthetic minimal media 2X. The pH of the organic acids stock solutions and of the culture media was adjusted to 3 by the addition of KOH or HCl 1 M. Cell growth was monitored by measuring the OD at 660 nm at regular time intervals and cells were inoculated at an initial OD of 0.05. All cultures were incubated in shake flasks at 30°C degrees and 160 rpm.

TABLE 2 List of strains and plasmids used in this work

Parental strain		
Strains	**Genotype**	**Source**
CEN.PK 102-5B	*MATa, ura 3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*	Entian and Kötter (1998)
Transformed strains		
Strains	**Plasmids**	**Source**
CEN.PK 102-5B WT	pYX022, pYX042, YCplac33	This work
CEN.PK 102-5B *ECM22*	pYX022, pYX042, YCplac33_*ECM22*, YLR228C::*kanMX*	This work
Plasmids	**Segregation**	**Markers**
pYX022	Integrative	*HIS3*
pYX042	Integrative	*LEU2*
YCplac33	Centromeric	*URA3*
YCplac33_*ECM22*	Centromeric	*URA3*

4.5 | Gene amplification and plasmid construction

All the primers used in this work are listed in Table 3. Endogenous *ECM22*, of the CEN.PK 102-5B strain, was deleted by the insertion of a *KanMX* cassette that integrates into the genome by homologous recombination, precisely replacing the target coding sequence. The *KanMX* resistance cassette was amplified from pUG6 (Güldener et al., 1996) with the primers Kan_Fw and Kan_rv, design with specific flanks complementary to the upstream and downstream regions of the *ECM22* gene to allow homologous recombination and used to transform the strain CEN.PK 102-5B, deleted in the endogenous *ECM22*. Transformed clones were selected in yeast extract/peptone/dextrose (YPD) agar plates supplemented with the antibiotic G418 (Merck Millipore) at the final concentration of 0.5 mg/mL. Correct integration of the cassette into the genome was confirmed by PCR analysis with the primers Del_fw and Del_rv (Table 3). After, *ECM22*

TABLE 3 List of primers used in this work

Name	**Sequence (5′-3′)**
Kan_fw	ATGACATCCGATGATGGGAATGCTGGACAAGAAAGAGAGAAGGATGCTGAGACATGGAGGCCCAG
Kan_rv	TTACATAAAAGCTGAAAAGTTTGTAGTGGTCATAGAAGGTAACCCACCTCCAGTATAGCGACCAGCATT
Del_fw	CCTCGTTCTTGTCGGAAAA
Del_rv	TTGATGCTCGATGAGTTTTTCTAA
Ecm_fw	AGATTCCCGGGATCTAACATAACATGACATC
Ecm_rv	AGGCTCTCGAGTTACATAAAAGCTGAAAAGT
RtEcm_fw	CCAGGGAATTCGCCATTGAG
RtEcm_rv	CTGCTGCAATCCATTCGTCA
RtAct_fw	CATTGCCGACAGAATGCAGA
RtAct_rv	ACGGAGTACTTACGCTCAGG

gene sequence was amplified by PCR, with primers Ecm_fw and Ecm_rv (Table 3), using genomic DNA from CEN.PK strain as template, extracted by standard methods (Maniatis et al., 1988). The obtained fragment was digested with *Sma*I and *Xho*I and then ligated to YCpLac33 (Gietz & Akio, 1988) previously digested with *Sma*I and *Xho*I and dephosphorylated. Clones were confirmed by enzymatic digestion. The obtained plasmid, in which the *ECM22* gene is under the control of the *TPI* promoter (TPIp), was named YCplac33_*ECM22*. All the restriction enzymes utilized are from NEB (New England Bio-labs, UK). The plasmid YCplac33_*ECM22* was transformed in the CEN.PK 102-5B strain, deleted for the endogenous *ECM22*, to obtain the strain CEN.PK 102-5B *ECM22*. The starting strain, CEN.PK 102-5B was transformed with an empty YCpLac33 plasmid, named CEN.PK 102-5B WT and used as control.

4.6 | Reverse transcription-quantitative PCR

Total RNA was extracted from the transformant strains CEN.PK 102-5B *ECM22* and from the wild-type strain, CEN.PK 102-5B WT, using the kit ZR Fungal/Bacterial RNA Miniprep (Zymoresearch/The epigenetics company). Strains were grown until exponential growth phase in minimal synthetic media with 2% w/v glucose. The retrotranscription to obtain cDNA was performed using the iScriptTM cDNA Synthesis (Bio-Rad Laboratories, Inc.) kit. The obtained cDNA was used to perform a reverse transcription quantitative PCR using SsoFastTM EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, Inc.) with the specific primers RtEcm_fw and RTEcm_rev (Table 3), according to the manufacturer's instructions. Expression levels of *ECM22* were normalized to the expression of the Actin housekeeping gene. Relative expression was calculated using the DDCt method.

4.7 | Ergosterol quantification in yeast cells

Total sterols were extracted as described before (Arthington-Skaggs et al., 1999) with slight modifications. Briefly, 2 ml overnight culture from a single *S. cerevisiae* colony was used to inoculate 50 ml of minimal synthetic media with 2% w/v glucose, overnight. Exponential-phase cells were collected by centrifugation at 2700 rpm for 10 min and washed twice with distilled water. Cell densities were measured based on OD_{660nm} of the cultures and equal amounts of cells were processed for sterol extraction. 5 ml of 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 ml of sterile distilled water, brought to 100 ml with 96% ethanol) was added to each pellet and vortex mixed for 1 min. Cell suspensions were transferred to glass tubes and were incubated in a 90°C water bath for 2 h. Following incubation, the tubes were allowed to cool to room temperature. Sterols were then extracted by addition of 5 ml of n-hexane followed by centrifugation at 2700 rpm for 5 min to facilitate phase separation. The hexane layer was transferred to a clean tube and scanned spectrophotometrically between 240 and 300 nm against a hexane blank with a V-770 UV-Visible/NIR spectrophotometer. The presence of ergosterol in the extracted sample resulted in a characteristic four-peak curve. The ergosterol content was determined using equations previously reported by Demuyser et al. (2019).

4.8 | Spot assay

Spot assays were performed by cultivating yeast cells in minimal synthetic media with 2% w/v glucose. Cells were cultivated until mid-exponential phase and then diluted to an OD_{660nm} of 0.5. Four 1:10 serial dilutions were made and spotted on solid minimal synthetic media containing agar (2% w/v) and 2% w/v glucose, supplemented with acetic or lactic acid. The pH of the organic acids stock solutions and of the culture media was adjusted to 3 by the addition of KOH or HCl 1 M. Plates were incubated for 72 h at 30°C.

4.9 | Evaluation of lipid peroxidation

Cells were grown until exponential phase in minimal synthetic media (pH 3) with 2% w/v glucose, collected and transferred to flasks containing lactic acid (555 mM), adjusted to pH 3. Cells were incubated at 30°C and 160 rpm for 30 min. An estimation of lipid peroxidation was based on the level of malondialdehyde formed after incubation in minimal synthetic media with or without lactic acid, as described by Fernandes et al. (2000). Briefly, after treatment with or without lactic acid, cells were collected, resuspended in 100 mM Tris pH 7.8 and broken by glass beads. After centrifugation, the supernatant was collected and 250 µl of the extract were mixed with 500 µl of the mix TBARS (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, 0.25 N hydrochloric acid). The solution was heated for 1 h in a thermomixer. The absorbance of the sample was determined at 535 nm against a blank that contained all the reagents, except the extract. Results are expressed as micromoles of malondialdehyde per gram of wet weight biomass.

4.10 | Proton movements assays

S. cerevisiae cells were grown until exponential-phase in minimal synthetic media with 2% w/v glucose, at pH 3. Cells were harvested and inoculated in minimal synthetic media with 2% w/v glucose containing 555 mM of lactic acid (pH 3) for 30 min. Cells were again harvested, washed with distilled water and resuspended in distilled water with a final concentration of 50 mg cell dry weight ml^{-1} and kept on ice. Proton movements were measured, at room temperature, by recording the pH of cell suspensions with a standard pH meter (PHM 82; Radiometer) connected to a potentiometer recorder (BBC-GOERZ METRAWATT, SE460). The pH electrode was immersed in a water-jacketed chamber of 10 ml capacity with magnetic stirring. To the chamber, 4.5 ml water and 0.5 ml yeast

suspension were added (Leão & Van Uden, 1984). For the measurement of proton efflux, the initial pH was adjusted to 5 using HCl (100 mM) and a baseline was established. The addition of 2% w/v glucose triggered H^+ efflux, leading to an acidification of the extracellular environment. When glucose is added to *S. cerevisiae* cells, protons are extruded after a delay of 10–30 s and the external pH drops, the rate constants were calculated from the maximum instant slope of the records. The rate of acidification, calibrated with 10 mM HCl, was taken as a measure of proton extrusion activity.

4.11 | Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism 7. In all the assays, means and standard deviations were determined based on three independent experiments ($n = 3$). Results were compared using *t*-test (real time quantitative PCR; evaluation of lipid peroxidation; proton movements assays) and one-way analysis of variance, with Turkey's multiple comparison statistical test (Liposomes exposure to organic acids). All tests were performed with a confidence level of 95%.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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