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RESEARCH ARTICLE

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Sodium and lithium exert differential effects on the central carbon metabolism of *Debaryomyces hansenii* through the glyoxylate shunt regulation

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Abstract

Debaryomyces hansenii is a halotolerant/halophilic yeast usually found in salty environments. The yeast accumulated sodium at high concentrations, which improved growth in salty media. In contrast, lithium was toxic even at low concentrations and its presence prevented cell proliferation. To analyse the responses to both cations, metabolite levels, enzymatic activities and gene expression were determined, showing that NaCl and LiCl trigger different cellular responses. At high concentrations of NaCl (0.5 or 1.5 M) cells accumulated higher amounts of the intermediate metabolites glyoxylate and malate and, at the same time, the levels of intracellular oxoglutarate decreased. Additionally, 0.5 M NaCl increased the activity of the enzymes isocitrate lyase and malate synthase involved in the synthesis of glyoxylate and malate respectively and decreased the activity of isocitrate dehydrogenase. Moreover, transcription of the genes coding for isocitrate lyase and malate synthase was activated by NaCl. Also, cells accumulated phosphate upon NaCl exposure. None of these effects was provoked when LiCl (0.1 or 0.3 M) was used instead of NaCl. Lithium induced accumulation of higher amounts of oxoglutarate and decreased the concentrations of glyoxylate and malate to non-detectable levels. Cells incubated with lithium also showed higher activity of the isocitrate dehydrogenase and neither increased isocitrate lyase and malate synthase activities nor the transcription of the corresponding genes. In summary, we show that sodium, but not lithium, up regulates the shunt of the glyoxylic acid in *D. hansenii* and we propose that this is an important metabolic adaptation to thrive in salty environments.

KEYWORDS
glyoxylate shunt, lithium, salt tolerance, sodium, stress, yeast

1 | INTRODUCTION

The excess of salt in the environment is one of the main abiotic stresses for most of living organisms. For example, the importance of soil salinity for agricultural yields is enormous and it has been well defined that all salts affect the behaviour of the plant although not all inhibit growth (Tester, 2003). Sodium is the most abundant cation in living environments and sodium salts impose both a sodium-specific and an osmotic stress on living cells (Munns, 2002). Yeast is used as model organisms when studying eukaryotic physiological processes and, in the case of salt-induced stress, the growth of many species, including the well-studied *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* is inhibited at relatively low amounts of sodium (Calero et al., 2000; Yenush, 2016). However, one of the most fascinating properties of the nonconventional *Debaryomyces hansenii* is its 'salt-loving' character which we are far to fully understand. *D. hansenii* is an ascomycetous yeast that was first defined as a marine occurring yeast (Norkrans, 1966, 1968; Norkrans & Kylin, 1969). It is abundant in salty food, sausages, and dry meat products where it plays an important role during the ripening process (Ramos-Moreno et al., 2019a, 2021).

Research in *D. hansenii* has traditionally been limited by some physiological and molecular characteristics. It is a very heterogeneous species, and it belongs to the CTG-clade yeasts in which the CUG codon can be ambiguously translated to serine or to the standard leucine (Santos et al., 2011). In addition, the lack of robust molecular tools to engineer this yeast has hampered the process until very recently, when two studies reporting the successful adaptation of the CRISPR/Cas9 methodology for *D. hansenii* (Spasskaya et al., 2021; Strucko et al., 2021) have been published.

As aforementioned, the specific mechanisms responsible for the halotolerant/halophilic character of *D. hansenii* remain uncertain. However, some hints of this behaviour that may help to solve this puzzle seem to be related to the existence/activity of ion transporters (González-Hernández et al., 2004; Martínez et al., 2011; Prista et al., 1997, 2005), the structural changes and composition of the plasma membrane in response to salt (Turk et al., 2007) and/or in the activity of some specific enzymes (Aggarwal et al., 2005; Chawla et al., 2017; Garcia-Neto et al., 2017). Additionally, a differential effect triggered by the exposure to either KCl or NaCl salts has been reported in batch cultivations using controlled lab-scale bioreactors (Navarrete et al., 2021a), as well as a positive effect in the cell performance when low environmental pH is combined with a high sodium concentration in the media (Almagro et al., 2000; Navarrete et al., 2021a).

Nevertheless, information about the effects on cell performance and metabolism exerted by the presence of lithium salts is extremely scarce. Lithium is usually considered a toxic sodium analogue and there is only one report describing an inhibitory effect of LiCl on *D. hansenii* (Prista et al., 1997). Concerning the targets of sodium and lithium toxic effects, it has been reported that *HAL2*, which encodes a nucleotidase that dephosphorylates 3′-phosphoadenosine 5′-phosphate (PAP) and 3′-phosphoadenosine 5′-phosphosulfate, is inhibited by Na^+ and Li^+ (Albert et al., 2000; Murguía et al., 1996).

Take-away

- Sodium is a beneficial element for *D. hansenii*. By contrast, lithium is toxic.
- Sodium, but not lithium, up regulates the glyoxylic acid shunt in *D. hansenii*.
- Activation of glyoxylic acid cycle may be an important adaptation to salt stress.

In addition, phosphoglucomutase activity is inhibited by lithium in both yeast and humans, making it an important in vivo lithium target (Csutora et al., 2005).

In relation to the effects of high salt concentrations on the metabolism, the group of Prof Peña published a series of works hypothesizing that high salinity affects energy pathways and growth in *D. hansenii*. A first study concluded that in the presence of 1 M NaCl, *D. hansenii* increases its fermentative capacity (Sánchez et al., 2008), while on a follow up study the conclusion was that the presence of salt increased its respiratory metabolism instead (Calahorra et al., 2009). Finally, a third study indicated an activation of the glyoxylate shunt as a consequence of the salt stress. In addition, a microarray-based gene expression analysis of cells incubated under saline conditions and high pH revealed a down-regulation in the expression of genes related to energy-producing pathways and in some genes involved in the cell cycle and DNA transcription (Sánchez et al., 2018).

Very recently, a multiomics study performed in chemostat cultivations, has highlighted the complexity of the subject. Sodium and potassium triggered different responses at both expression and regulation of key enzyme activity, showing that the metabolic response to sodium is highly coordinated and even more regulated than the response to high potassium in the medium. The same work also revealed cellular events never linked to halotolerance before such as protein trafficking, endocytosis, or the stimulation of the biosynthetic pathways of long fatty acids (Navarrete et al., 2021b).

To get further details on the effect of salt on the performance and the energy metabolism of *D. hansenii*, we have accomplished a series of experiments in which growth, intracellular ion content, key metabolic precursors in the TCA cycle, enzymatic activities, and gene expression were determined in cells exposed to different NaCl or LiCl concentrations. Our results confirm a difference among the physiological and metabolic effects of both salts, which can be related to their beneficial or toxic effect in *D. hansenii*'s performance and fitness.

2 | MATERIALS AND METHODS

2.1 | Yeast strain, media, and culture conditions

The wild-type strain *Debaryomyces hansenii* CBS767 (PYCC2968) (Prista et al., 1997) was used in this study. Yeasts were maintained in

solid YPD medium (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and for selective purposes synthetic complete YNB medium (0.17% YNB, 2% glucose) adjusted at pH 5.8 with NH_4OH was used. Cells were routinely cultured at 26°C, in liquid media, 180 rpm, for 20–48 h.

2.2 | Growth and viability assays

The effect of salts on growth was determined in solid and liquid media. Drop tests were performed with cells resuspended in sterile water and adjusted to the same 1.0 initial A_{600}. Ten-fold serial dilutions were prepared, and 5 µL aliquots of each dilution were spotted on the appropriate plates supplemented with a variety of KCl, NaCl or LiCl amounts. Plates were then incubated during 48 h at 26°C.

Growth curves were performed in flasks with 50 mL of YNB medium supplemented with the indicated concentrations of NaCl or LiCl. Cultures were inoculated at A_{600} 0.06, and growth was followed for 48 h using a Spectronic 20 (Bausch and Lomb) (Ruiz-Castilla et al., 2021).

Cell viability in the presence of salt was determined in liquid YNB supplemented with the indicated NaCl or LiCl amounts. Cultures were inoculated at A_{600} 0.06, and cells were grown overnight. When A_{600} reached values of 0.2 the required concentration of salt was added. At time zero and after 24 h, samples were taken, inoculated on the surface of YNB plates and the number of colony-forming units (cfu) was quantified after 48 h incubation at 26°C.

2.3 | Intracellular cation content

To estimate intracellular sodium or lithium content, cells were inoculated (A_{600} 0.06) in YNB liquid media supplemented with several concentrations of NaCl or LiCl. When the cultures reached early exponential phase (A_{600} 0.2–0.3), samples of cells were withdrawn from growth media, cells were collected on Millipore filters (0.8 µm pore size), then they were rapidly washed with a 20 mM $MgCl_2$ solution and acid extracted (0.2 M HCl, 10 mM $MgCl_2$) for 3 h. Finally, they were analysed by atomic emission spectrophotometry as described previously (Ramos et al., 1990). All intracellular cation values are expressed in nmols of cation per mg dry weight of cells (Navarrete et al., 2010).

2.4 | Cell-free extracts preparation

Extracts used for determination of metabolites were obtained from YNB cells grown in the presence of different salt concentrations up to A_{600} 0.5–0.7. On the other hand, extracts used for the study of enzymatic activities and transcriptional regulation were obtained from YNB-grown cells (A_{600} 0.5–0.7) then exposed (0, 2, and 6 h) to salt.

In all cases, cells were harvested by centrifugation (5000*g*, 4°C, 10 min), and washed once with sterile water. Extracts were then prepared resuspending yeasts in 10 mM Tris–HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted by vigorous shaking for 1 min with sea sand. The process was repeated with at least a 1-min interval on ice until breakage, which was checked microscopically. Cell extracts were separated from cell debris and glass beads by centrifugation (20,000*g*, 4°C, 15 min).

2.5 | Determination of metabolites

The concentration of ethanol was measured in cell-free supernatant, while the concentration of metabolites relative to tricarboxylic and glyoxylic acid cycle was measured in cell extracts. As mentioned above, cells were grown at different concentrations of NaCl and LiCl.

The concentration of metabolites was determined by HPLC (model 1100–1200 Series HPLC System, Agilent Technologies). The injection volume was 20 µL, the eluent 5 mM H_2SO_4 and the flow rate was set at 0.6 mL/h. The temperature of a Bio-Rad Aminex HPX-87H column was kept at 60°C (Navarrete et al., 2021a).

To measure phosphate levels, cells were grown in YNB supplemented with the corresponding salt concentration (A_{600} 0.5–0.7). Phosphate was extracted with 10% trichloroacetic acid in cold. After centrifugation, phosphate levels were determined by the method of Fiske and Subbarow (1925) modified by Sanui (1974). This is a colorimetric method consisting in the quantification of phosphomolybdic acid formed by the reaction of phosphate extracted with molybdic acid (A_{310}). Results were expressed in µmol Pi/mg cell protein.

2.6 | Enzymatic activities

These determinations were assayed spectrophotometrically using a DU®650 Spectrophotometer (Beckman Coulter).

Isocitrate dehydrogenase (IDH) activity was measured by the appearance of NADH formed by the reaction of D,L-isocitrate and NAD^+ carried out by IDH. The process was monitored spectrophotometrically at 324 nm. The reaction mixture contained 40 mM MOPS-HCl pH 7.0 buffer, 1.5 mM $MgCl_2$, 0.7 mM NAD^+ and 10 mM D,L-isocitrate (Bernt & Bergmeyer, 1974).

Isocitrate lyase (IL) activity was followed by the appearance of a glyoxylate-phenylhydrazine coloured compound that can be measured at 324 nm. The reaction mixture contained 30 mM imidazole pH 8.0, 5 mM $MgCl_2$, 4 mM phenylhydrazine, 1 mM D,L-isocitrate and 4 mM EDTA (Chell et al., 1978).

Malate synthase (MS) activity was determined by the appearance of a CoA-DTNB coloured compound measured at 412 nm. The reaction mixture contained 30 mM imidazole pH 8.0, 10 mM $MgCl_2$, 0.25 mM acetyl-CoA, 1 mM glyoxylate and 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Chell et al., 1978; Silverstein, 1975).

Protein content of the extracts was determined by using Bradford quantification kit reagent (Bio-Rad) following the manufacturer's instructions and using bovine serum albumin as a standard (Kruger, 1994).

2.7 | RNA isolation and reverse transcription

D. hansenii cells were incubated in liquid YNB plus NaCl or LiCl. At different times (0, 2 and 6 h after addition of salt), cells were collected, washed with sterile cold water and resuspended in 1 mL of TRI-REAGENT (Sigma-Aldrich) plus approximately 200 µL of sea sand (Washed, thin grain QP, Panreac). For disruption, yeasts were vortexed 10 times for 1 min with intervals of at least 1 min on ice, incubated 5 min at 70°C, and followed by other 10 times 1 min vortexing with cooling intervals. Afterwards, the standard TRI-REAGENT protocol for RNA isolation was followed. Isolated RNA samples were treated using DNase I (New England Biolabs) to remove contaminating DNA until no polymerase chain reaction (PCR) amplification was observed without prior complementary DNA (cDNA) synthesis. RNA sample quality and quantification were performed spectrophotometrically (NanoDrop 2000). At least two RNA preparations were isolated for each experimental condition. One microgram from each RNA sample was retrotranscribed with Kit iScript™ cDNA Synthesis Kit (Bio-Rad) on three separate reactions that were pooled together before PCR amplification.

2.8 | Real-time PCR

D. hansenii gene sequences for primer design were obtained from the National Center for Biotechnology Information Search database (NCBI, https://www.ncbi.nlm.nih.gov/). Primers were designed with OLIGO 7.60 (Molecular Biology Insights) (Supporting Information: Table S1). All primer pairs specifically amplified the desired target sequence, no primer dimers were detected. All amplification efficiencies were close to 100%.

The PCR amplification was carried out in a mixture (20 µL final volume) with IQTM SYBR® Green Supermix (Bio-Rad), 1 µL of cDNA, plus 0.1 µM of the specific primers. PCR reactions were performed at least in triplicate (three biological replicates and three technical replicates each). Real-time PCR conditions were an initial denaturation step, 95°C 3 min, followed by 40 PCR cycles consisting of 15 s of denaturation at 95°C, and 30 s of annealing plus elongation at 70°C. Finally, melting curves were determined.

2.9 | Statistics

At least, three biological replicates for each experiment were performed. Data were analysed in Microsoft Excel software 2019. Statistical significance was evaluated using analysis of variance, followed by post hoc multiple comparison according to Dunnet for parametric analysis using GraphPad Prism 9 (Dotmatics). Significant differences are indicated with asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3 | RESULTS

3.1 | LiCl has a toxic effect for *D. hansenii*

While yeast cells require potassium for growth, sodium and lithium usually behave as toxic elements when accumulated at certain amounts (Gómez, 1996; Marquina et al., 2012). However, it has been reported that, in *D. hansenii*, the presence of relatively high concentrations of sodium improves the performance of the cells not only under control conditions but also in the presence of some additional stress factors such as extreme pH or temperatures (Almagro et al., 2000; Navarrete et al., 2021a). We first wondered whether lithium may have a similar effect in this yeast. Figure 1a shows the proliferation of cells spotted on YNB plates supplemented with either KCl, NaCl or LiCl. While potassium or sodium at medium-high concentrations had a beneficial effect, LiCl hampered cell proliferation at very low concentrations and after 48 h cultivation. To get further insights we used liquid media supplemented with the same concentrations of NaCl or LiCl and followed growth (Figure 1b). In the case of sodium, the presence of 0.5 M increased the growth rate compared to the control, and only 1.5 M importantly affected growth by increasing the lag phase. However, the addition of lithium, even at much lower concentrations than those used in the case of sodium, had a detrimental effect in the growth rate and even at relatively low LiCl (0.1 M) growth was already significantly slowed in comparison. On the other hand, the results on cell viability showed that the number of cfu was, after 24 h, much higher in cells growing in 0.5 M NaCl than in the control and that the toxic cation lithium did not immediately kill the cells. For example, even after 24 h in the presence of 0.5 M LiCl, although cells did not proliferate, a number of cfu similar to the one obtained immediately after inoculation (time 0) was observed (Figure 2). The noxious effect of internal lithium was also evident when intracellular amounts of lithium and sodium were determined since the cell population was already intoxicated when lithium reached values around 30 nmol/mg cell (Table 1). Altogether, results in Figure 1 and Table 1 show that intracellular lithium was at least one order of magnitude lower than sodium under any condition studied and that these low intracellular amounts were already producing a detrimental effect on cell growth. In summary, these results show that, on contrary to the situation with sodium, lithium triggers a toxic effect in *D. hansenii* significantly affecting its cell performance.

3.2 | Effect of NaCl and LiCl on ethanol production and phosphate accumulation

We also measured the presence of two important compounds previously reported to increase in the presence of salt: ethanol extrusion and phosphate accumulation (Calahorra et al., 2009; Sánchez et al., 2008). It has been demonstrated that *D. hansenii* has a high respiratory activity at

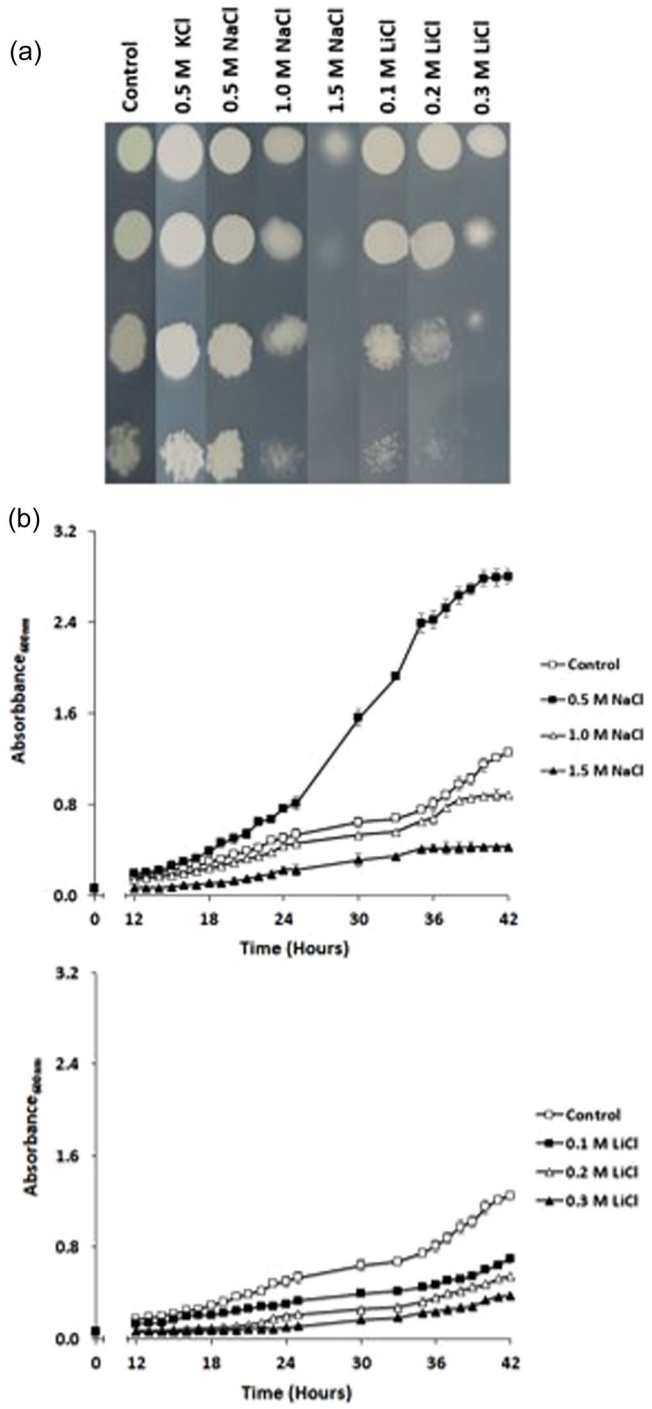

FIGURE 1 Effect of different salts on growth of *D. hansenii*. (a) Tenfold serial dilutions of cells were spotted on YNB plates supplemented with KCl, NaCl or LiCl. Plates were incubated at 26°C, and images were taken after 48 h. (b) Growth at several concentrations of NaCl or LiCl in liquid media. Cultures were inoculated at A_{600} 0.06, and growth was monitored for 42 h. Mean values ± standard deviation obtained in three independent experiments are plotted.

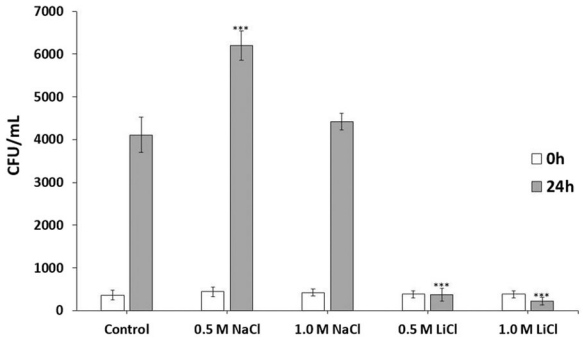

FIGURE 2 Effect of NaCl and LiCl on the viability of *D. hansenii*. Cells were incubated up to 24 h in liquid media supplemented with the required salt. Samples were taken and colony-forming-units were counted and compared with the control in the absence of added salt. Mean values ± standard deviation obtained in three independent experiments. Statistically significant data respect to control were expressed: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

TABLE 1 Intracellular sodium and lithium content.

	External cation			
	NaCl		LiCl	
	0.5 M	1.5 M	0.1 M	0.3 M
Internal cation (nmol/mg cell)	400 ± 12**	413 ± 32*	27 ± 1***	30 ± 3***

Note: Cells were grown in YNB supplemented with NaCl or LiCl. Mean values ± standard deviation obtained in three independent experiments. Statistically significant data were expressed:
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

moderate to high concentration of sodium, however, the fermentative capacity of this yeast has been controversial, and it may depend on strains or conditions. On the one hand, it has been reported that cells grown with salt increased fermentation capacity and ethanol production using glucose as single carbon source (Calahorra et al., 2009), however, several other authors did not observe any ethanol production even in the presence of high salt and the amounts of this compound were negligible under all conditions reported (Garcia-Neto et al., 2017; Navarrete et al., 2021a). These results prompted us to measure the possible effect of LiCl or NaCl on ethanol production. In our experimental conditions, *D. hansenii* cells did not produce ethanol (neither in control nor in treated cells), therefore confirming a fully respiratory metabolism on glucose in the presence of either salt.

Another reported effect on the presence of high salt in the medium is the increase in phosphate levels of *D. hansenii* (Sánchez et al., 2008). Results in Figure 3 show that cells grown in 0.5 M NaCl increased intracellular phosphate but that neither supraoptimal concentrations of sodium (1.5 M) nor lithium produced that effect. In fact, accumulated phosphate levels were lower under exposure to either 1.5 M NaCl or LiCl (0.1 or 0.3 M) than those measured under control conditions.

3.3 | Changes in TCA intermediate's levels and enzymatic activities

It has been proposed that *D. hansenii* cells grown in either 1 M NaCl or KCl activate the glyoxylate shunt (Sánchez et al., 2008). To get

further insight about that hypothesis and extend the study to the case of lithium salts, we grew yeast in liquid cultures with several concentrations of either NaCl (0.5 or 1.5 M) or LiCl (0.1 or 0.3 M) and measured the concentration of several key TCA intermediates against control conditions (no salt added). We observed important differences in response to the presence of lithium or sodium. Table 2 shows the levels of the intermediates measured in cells grown in NaCl (0.5 or 1.5 M) or in LiCl (0.1 or 0.3 M). The first important finding was that the oxoglutarate levels strongly decrease in sodium-grown cells while significantly increased in lithium. Moreover, the levels of glyoxylate and malate followed a similar pattern, increasing when cells were cultured in sodium (especially in the case of 0.5 M NaCl) and decreasing in lithium-grown cells until nondetectable levels. The presence of other intermediaries such as succinate or fumarate was never detected in our conditions.

Due to the important quantitative differences in the level of these intermediates of the TCA cycle induced by sodium or lithium, we decided to measure the activity of three key enzymes that would confirm the hypothesis that sodium but not lithium activates the glyoxylate shunt in *D. hansenii*. Cells were grown in the absence of salt and then resuspended in NaCl (0.5 and 1.5 M) or LiCl (0.1 and 0.3 M). After 2- and 6-h of incubation, samples were taken and the activity of the enzymes isocitrate dehydrogenase (IDH), isocitrate lyase (ICL) and malate synthase (MS) was determined. Results in Figure 4a,b show that when NaCl was present, the activity of IDH clearly decreased (0.5 M NaCl) or it was close (1.5 M NaCl) to the control cells incubated in the absence of added salt. On the contrary, the addition of LiCl significantly activated IDH under any of the conditions analysed (Figure 4c,d). Moreover, the activities of ICL and MS, the two key enzymes responsible for the glyoxylate shunt, followed the same pattern and their activities increased during incubation in 0.5 M NaCl (Figure 4a), a concentration of sodium that is not only nontoxic but, as we have shown in Figure 1, improves cell performance. Additionally, 1.5 M NaCl did not significantly affect the activity of both enzymes neither after 2 h nor after a longer period of exposure (6 h) (Figure 4b). Once again, the effect of LiCl was very different from that produced by NaCl, since activation of these enzymes was never observed and, in fact, enzymatic activity inhibition was measured under most of our working conditions (Figure 4c,d). It is relevant that after 6 h incubation in lithium, the activity of both, ICL or MS was significantly decreased by either 0.1 or 0.3 M LiCl.

Taken together these results clearly point to very different metabolic responses induced by sodium or lithium.

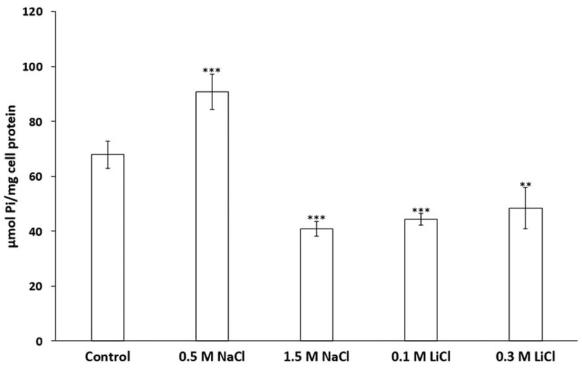

FIGURE 3 Levels of phosphate in cells grown in YNB supplemented with NaCl or LiCl. Values are expressed in µmol Pi/mg of cell protein. Mean values ± standard deviation obtained in three independent experiments. Statistically significant data respect to control were expressed: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.4 | Transcriptional regulation of IDH, ICL and MS genes

The results shown above point to the fact that sodium, but not lithium, activates the glyoxylate shunt in *D. hansenii*. To try to gain additional support to our hypothesis, the expression levels of the genes coding for IDH (*DEHA2C10758g* and *DEHA2G05786g* coding for the two known IDH subunits required for the functioning of the enzyme), ICL (*DEHA2D12936g*) and MS (*DEHA2E13530g*) were studied by real-time PCR (Figure 5). The conditions of the experiment were the same than those used to measure enzymatic activities, that is, cells incubated in NaCl (0.5 M or 1.5 M) or LiCl (0.1 M or 0.3 M) during 2 or 6 h. The presence of NaCl did not induce changes in the transcription of the genes coding for IDH. Moreover, NaCl increased ICL and MS transcription levels, which resulted especially relevant

TABLE 2 The concentration of different metabolites relative to tricarboxylic and glyoxylic acid cycles.

	External cation				
		NaCl		LiCl	
Metabolites	Control	0.5 M	1.5 M	0.1 M	0.3 M
Oxoglutarate	190.62 ± 5.75	90.00 ± 7.12**	78.00 ± 5.23**	369.09 ± 10.03**	277.65 ± 16.52**
Glyoxylate	0.85 ± 0.15	4.48 ± 0.29***	1.48 ± 0.11**	N.D.	N.D.
Malate	2.10 ± 0.07	9.00 ± 0.15**	5.12 ± 0.12*	N.D.	N.D.

Note: Cells were grown in YNB supplemented with different concentrations of NaCl or LiCl. When A_{600} reached values of 0.5–0.7, metabolites were extracted and measured by high-performance liquid chromatography. Results are expressed in µg/mg of cell protein. N.D.: Nondetectable. Mean values ± standard deviation obtained in three independent experiments. Statistically significant data were expressed:
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

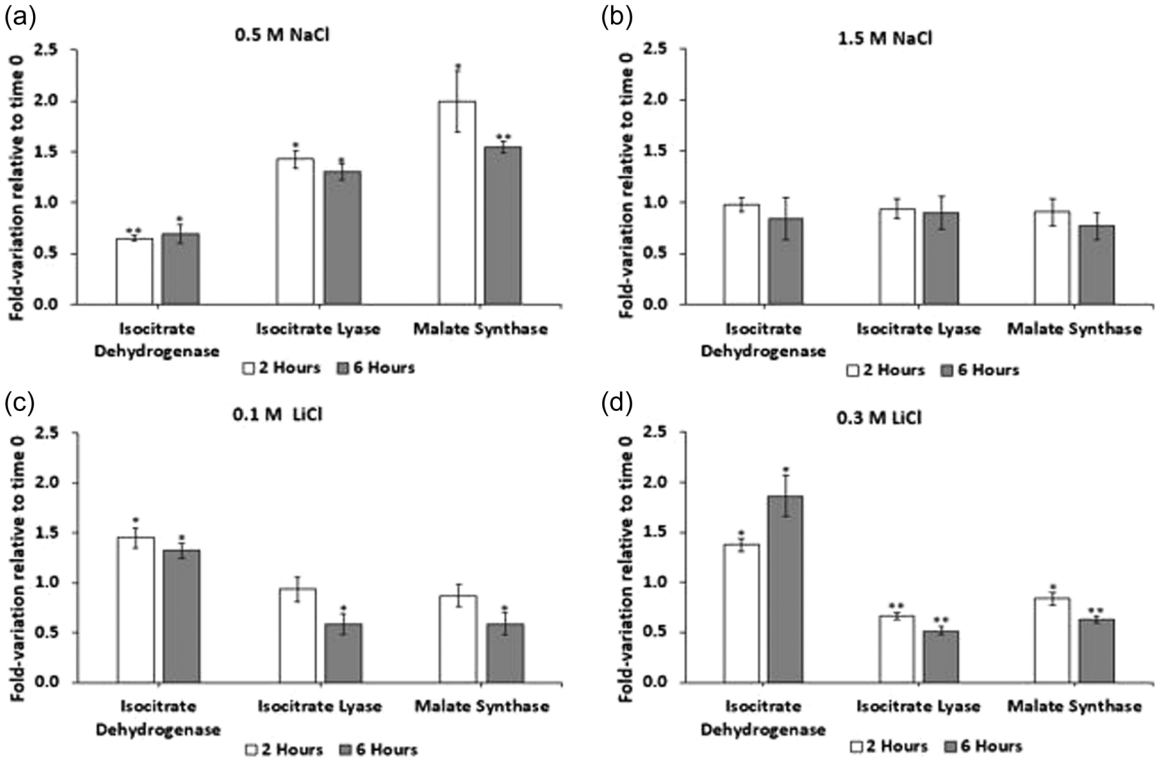

FIGURE 4 Enzymatic activities of isocitrate dehydrogenase, isocitrate lyase and malate synthase at different times after exposure to (a, b) NaCl or (c, d) LiCl. Values (U/mg) were expressed as Fold-variation relative to time 0 ratio. Mean values ± standard deviation obtained in three independent experiments. Statically significant data were expressed: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

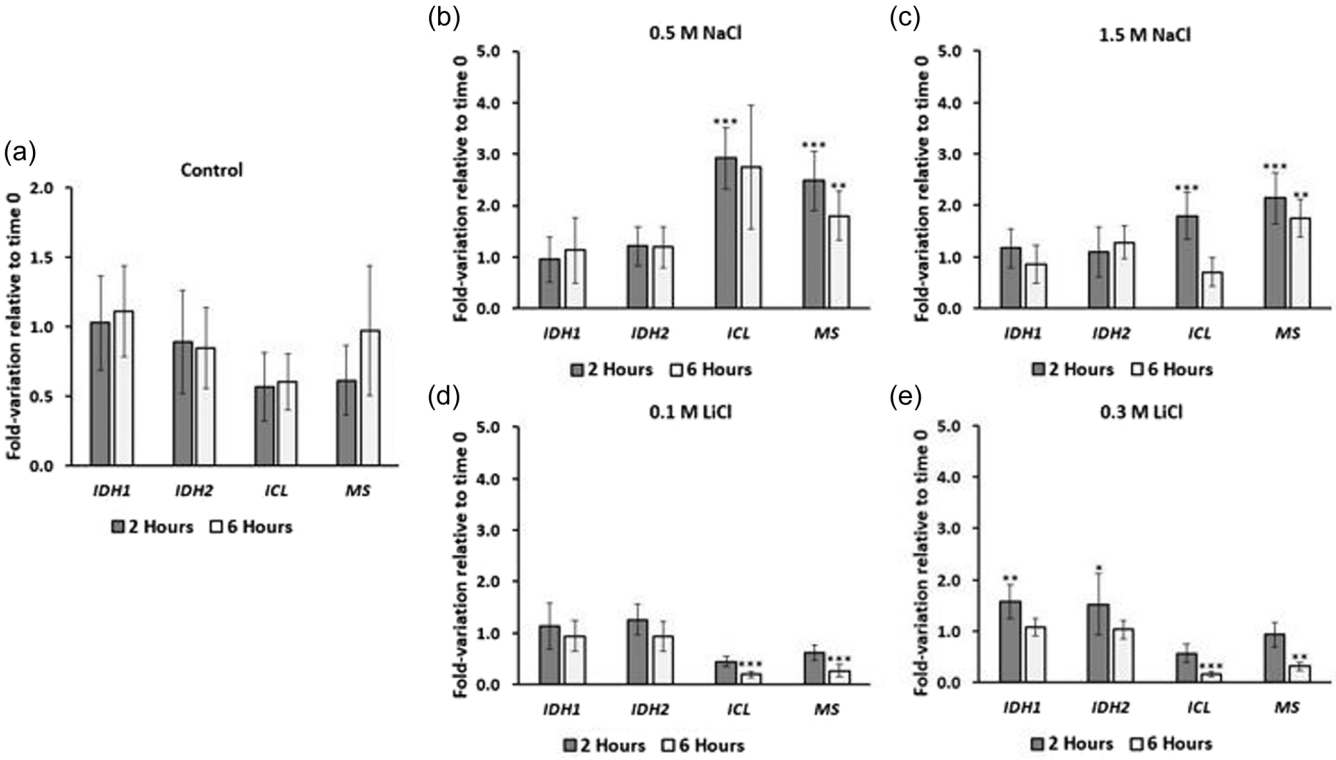

FIGURE 5 Changes in expression levels of DEHA2D12936g (ICL), DEHA2E13530g (MS), DEHA2C10758 (IDH_1) and DEHA2G05784g (IDH_2). (a) Control cells were incubated in the absence of added salt and treated cells were exposed to different concentrations of (b,c) NaCl or (d,e) LiCl. Samples were taken at different times. All transcript levels are referred to those for time 0, 2, or 6 h incubation in the absence of added salt. Mean values ± standard deviation obtained in three independent experiments. Statistically significant data respect to control were expressed: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

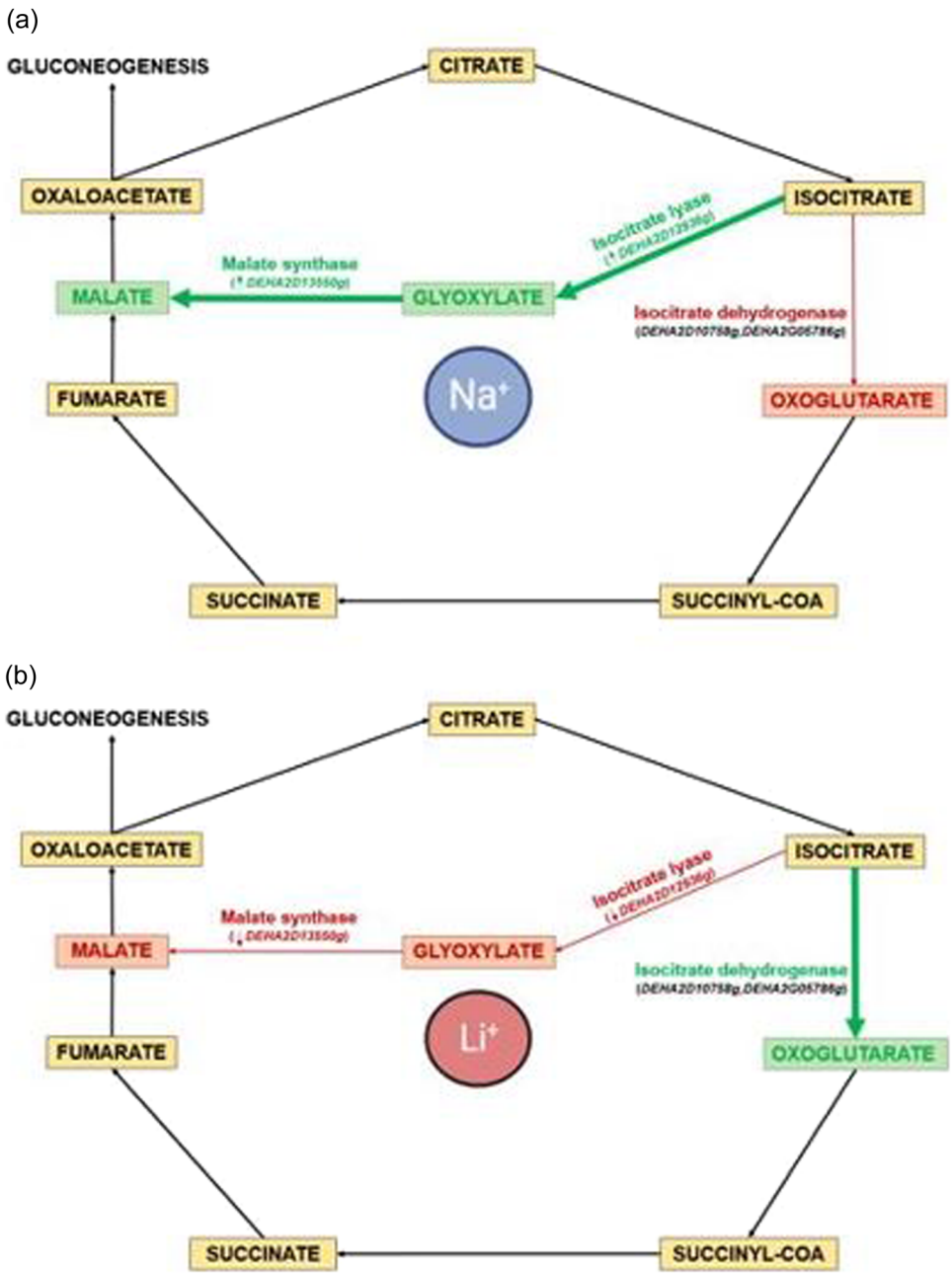

FIGURE 6 Schematic drawing of the proposed effects of (a) NaCl or (b) LiCl on the glyoxylic and tricarboxylic acid cycles. Globally, sodium increases the amounts of glyoxylate and malate, the activity of isocitrate lyase and malate synthase enzymes and the transcription of the genes coding for those enzymes. On the contrary, lithium causes an increase in oxoglutarate, in the activity of isocitrate dehydrogenase and in the transcription of the two genes coding for the subunits of the enzyme.

after 2 h incubation in 0.5 M NaCl (Figure 5b,c). On the other hand, when the effect of lithium was studied, we observed that IDH expression changed after 2 h incubation at 0.3 M LiCl. Under this condition, the two genes coding for IDH subunits were slightly activated. More relevant, and in contrast to our observation with sodium, lithium negatively affected the transcription level of the genes coding for ICL and MS in most of the conditions studied and, in any case, it never increased the transcription in comparison with the control conditions (Figure 5d,e).

4 | DISCUSSION

During decades, many different salt tolerance determinants have been identified in *D. hansenii*, including ion transporters, changes in plasma membrane composition or intracellular cation distribution (Prista et al., 2016).

We show in this work that while sodium is not harmful for *D. hansenii* cells, lithium is toxic even at low intracellular concentrations and this is accompanied with very different physiological and

metabolic responses to the presence of these cations in the external milieu.

It has been previously reported that salts activate fermentation, and consequently ethanol production, in *D. hansenii* (Calahorra et al., 2009). However, these results were not confirmed by other authors (Navarrete et al., 2021a, 2021b). In the present work, the levels of ethanol were undetectable under any of the different conditions used, confirming *D. hansenii* as a crabtree-negative yeast.

Phosphate is accumulated in *D. hansenii* in response to high salt (Sánchez et al., 2008) and this seems to agree with the fact that expression of the phosphate transporters coded by *PHO84* and *PHO89* is stimulated under these conditions (Navarrete et al., 2021a). At this respect, it is worth to mention that, similarly to the situation in *S. cerevisiae*, in *D. hansenii*, PHO84 and PHO89 work as phosphate/H^+ and phosphate/Na^+ symporters, respectively (Navarrete et al., 2021a). We confirmed that under optimal sodium concentrations (0.5 M NaCl) phosphate accumulation occurs and under any other stressful condition (excess of sodium or the presence of lithium) there is a decrease in the intracellular phosphate content.

In 2008 it was proposed that 1.0 M KCl or NaCl induce a change in the metabolic pattern of *D. hansenii* in favour of the glyoxylate cycle although important differences between the effects triggered by sodium or potassium were detected (Sánchez et al., 2008). Since then, no further information has been provided. In addition to allow the utilization of two carbon compounds (C2) to regenerate glucose (C6) via replenishment of the oxaloacetate pool to undergo gluconeogenesis instead of following the TCA path, the glyoxylate shunt has also been implicated in a variety of processes related to stress responses. For example, it is required for fungal virulence (Lorenz & Fink, 2001), and it is upregulated under conditions of oxidative stress (Ahn et al., 2016). An increase in the respiratory metabolism of *D. hansenii* has been observed in the presence of high salt using different approaches (Navarrete et al., 2021a; Sánchez et al., 2008), overlapping responses between salt and oxidative stress in *D. hansenii* have been reported (Ramos-Moreno et al., 2019b) and, additionally, proteins related to the cellular responses to oxidative stress have been found to be more abundant in the presence of salt (Navarrete et al., 2021b). Therefore, our results suggest the existence of a link in *D. hansenii* between the glyoxylate shunt, oxidative stress responses and the presence of NaCl.

Figure 6 is a schematic drawing summarizing our main findings. The metabolite analysis, enzyme activities and gene expression experiments show that under optimal cultivation conditions (0.5 M NaCl) the glyoxylate cycle is activated in *D. hansenii*. The decrease in oxoglutarate, the increase in glyoxylate and malate pools together with the decrease in the activity of IDH and the activation of ICL and MS support that hypothesis, which is reinforced by the increased expression of the genes coding for these two enzymes. The situation in the presence of 1.5 M NaCl is more complex and difficult to discuss since cells are dealing with an excessive amount of salt (hence additional osmotic stress) as shown in our growth experiments (Figure 1). However, our results also point to an activation of the cycle under those conditions as indicated by the changes in ketoglutarate, glyoxylate and malate as well as the activated transcription of the genes coding for ICL and MS. On the other hand, lithium which behaved as a toxic cation under all conditions tested, did not activate the bypass.

The glyoxylate shunt is an alternative to the TCA cycle and it is essential for the utilization of acetate and short chain organic acids as carbon sources (C2 and C3). It still remains uncertain to us the ultimate reason to explain the activation of the bypass by sodium, but it is reasonable to think that the utilization of alternative carbon sources in the presence of salt can be an advantage to thrive under increasing salt concentrations in natural environments against other microbial competitors. In fact, it has been recently proposed that in *D. hansenii*, rapid metabolization of available carbon sources when salt increases and other nutrients and pH decrease is a successful survival strategy for growing in extreme environments (Navarrete et al., 2021a, 2022).

Living in salty environments is a complex and multifactorial process. Metabolic adaptations of *D. hansenii* to salt have been poorly explored. In this sense, the activation of the glyoxylate shunt by NaCl deserves to be studied in more detail. It would be important to define the situation in salt-sensitive yeasts. At this respect, future research should establish the relevance of that variant of the tricarboxylic acid cycle on the whole adjustment to halotolerance/halophilism.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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