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Adaptation to sorbic acid in low sugar promotes resistance of yeast to the preservative

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ABSTRACT

The weak acid sorbic acid is a common preservative used in soft drink beverages to control microbial spoilage. Consumers and industry are increasingly transitioning to low-sugar food formulations, but potential impacts of reduced sugar on sorbic acid efficacy are barely characterised. In this study, we report enhanced sorbic acid resistance of yeast in low-glucose conditions. We had anticipated that low glucose would induce respiratory metabolism, which was shown previously to be targeted by sorbic acid. However, a shift from respiratory to fermentative metabolism upon sorbic acid exposure of *Saccharomyces cerevisiae* was correlated with relative resistance to sorbic acid in low glucose. Fermentation-negative yeast species did not show the low-glucose resistance phenotype. Phenotypes observed for certain yeast deletion strains suggested roles for glucose signalling and repression pathways in the sorbic acid resistance at low glucose. This low-glucose induced sorbic acid resistance was reversed by supplementing yeast cultures with succinic acid, a metabolic intermediate of respiratory metabolism (and a food-safe additive) that promoted respiration. The results indicate that metabolic adaptation of yeast can promote sorbic acid resistance at low glucose, a consideration for the preservation of foodstuffs as both food producers and consumers move towards a reduced sugar landscape.

1. Introduction

Annual soft drink consumption by adults in Europe is reportedly ~40 L per capita [1]. Traditionally, soft drink beverages tend to contain relatively high sugar concentrations (up to 14 % w/v), have a pH range of 2.4–5.0, and are commonly stored at ambient temperatures [2]. These conditions are conducive for fungal growth and, therefore, soft drinks are prone to spoilage by filamentous fungi or yeasts such as *Zygosaccharomyces bailii, Brettanomyces bruxellensis* and *Saccharomyces cerevisiae* [3,4]. To mitigate spoilage, the weak acid (WA) sorbic acid is a common preservative used in soft drink beverages at concentrations in the parts per million range (e.g. the UK legal limit is 300 mg/L, or 2.7 mM), having been widely shown to possess strong antifungal properties [5–7]. In more recent years, consumer concerns over high-sugar foods and implementation of sugar tax levies in countries such as the UK, has led many beverage companies to transition towards low-sugar drink formulations [8]. However, potential impacts of such reformulation on preservative resistance and spoilage propensity have yet to be fully explored. Considering that nutrient (e.g. sugar) availability is well known to affect microbial physiology, e.g., nutrient assimilation rates [9], mitochondrial morphology [10], and general stress response

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[11–13], there is strong rationale for anticipating impacts of altering food formulation in this way on the propensity for spoilage.

The concern raised above may be especially pertinent to spoilage by yeasts, as many yeasts are known to switch from fermentative to respiratory metabolism as extracellular glucose levels are decreased [14] and sorbic acid has been shown to exert greater inhibition of cells growing on respiratory substrates (e.g. glycerol) than fermentative substrates (high glucose) [3]. The latter was related to observations in the same study that the weak acid selectively targets respiration. This evidence suggested that a bias to respiratory metabolism by yeasts in low-sugar beverage conditions would sensitize them to inhibition by sorbic acid.

In this study, we tested this hypothesis by investigating effects of sorbic acid in low glucose conditions, giving new insights to the relationship between carbon metabolism and preservative resistance.

2. Methods

2.1. Yeasts and culture conditions

The yeast species and strains used in this study were: *Saccharomyces cerevisiae* W303 (*MATα ura3-1 ade 2-1 trp 1-1 his3-11,15 leu2-3112*); *S. cerevisiae* BY4743 (*MAT**a**/MATα his3Δ 1/his3Δ 1 leu2Δ 0/leu2Δ 0 met15Δ 0/MET15 LYS2/lys2Δ 0 ura3Δ 0/ura3Δ 0*) and its isogenic deletion strains (purchased from EUROSCARF, Frankfurt); *Zygosaccharomyces bailii* NCYC 1766 [3]; *Brettanomyces bruxellensis* (an isolate from a food factory in the UK); and fermentation negative strains *Rhodotorula mucilaginosa* CMCC2663, *Rhodotorula glutinis* NCYC59, *Rhodotorula glutinis* (an isolate from a food factory in Israel), *Cryptococcus laurentii* (an isolate from a food factory in Rio de Janeiro, Brazil) and *Cryptococcus magnus* (an isolate from a food factory in Samara, Russia). Yeasts were maintained and grown in YPD medium [2 % w/v peptone (Oxoid), 1 % w/v yeast extract (Oxoid), 2 % w/v D-glucose]. Where required, medium was solidified with 2 % (w/v) agar. For starter cultures, single colonies were used to inoculate 10 ml of medium in 50 ml Erlenmeyer flasks and incubated with orbital shaking (New Brunswick Scientific) at 120 rev min^{-1} at 24 °C. Overnight cultures were then used to inoculate fresh medium to an OD_{600} ~0.5 before incubating for ~4 h as described above to ensure all were actively growing in the fresh medium.

2.2. Growth assays

Growth assays were conducted in 96-well microtiter plates (Greiner), with each well containing 100 μl YP [2 % peptone (Oxoid), 1 % yeast extract (Oxoid)] pH 4.0 (adjusted with HCl)] supplemented with either 2 % or 0.1 % (v/w) D-glucose. For addition of weak acids, aliquots from stock solutions were added to each well to achieve the desired final acid concentration. Stock solutions of sorbic acid, propionic acid and decanoic acid were dissolved in water. Acetic acid was dissolved in YP to prevent the substantial dilution of culture medium by the large volumes of acetic acid needed. Typically, final weak acid concentrations were ~55–60 % of the MIC. Wells were inoculated with exponential-phase cells to a final optical density (OD_{600}) of 0.1. Growth was monitored during incubation at 24 °C in a BioTek Powerwave XS microplate spectrophotometer, shaking for 2 min before OD_{600} measurements every 30 min.

For experiments in anaerobic conditions, growth assays were conducted as above, with the exception that plates were incubated in an anaerobic chamber [Whitley DG250 anaerobic workstation; Don Whitley Scientific (10 % CO2, 10 % H2, 80 % N2)] for 5 h to remove oxygen from culture media before inoculation of cells, as above, within the chamber to a final OD_{600} 0.1. Growth yield was assessed by OD_{600} readings after 30 h incubation in the anaerobic chamber at 24 °C.

2.3. Intracellular pH measurements

Prior to intracellular pH measurements, exponential phase cells were incubated for 4 h with shaking at 120 rev min^{-1}, 24 °C in 10 ml of medium in 50 ml Erlenmeyer flasks, containing the weak acids and carbon sources as described in Fig. S2. Intracellular pH was measured with a method adapted from Ref. [15]. Cells were harvested from 1 ml of culture at OD_{600} ~0.5 in each experimental condition by centrifugation at 3000*g*, 4 min and resuspended in 1 ml phosphate buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 11.9 mM phosphate buffer) containing the pH sensitive intracellular stain 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) at a final concentration of 5 mg/L. Cells were incubated in the CFDA-SE solution for 20 min at 24 °C, before centrifugation at 4000*g*, 3 min and aspiration of the staining solution. Stained cells were then resuspended in the spent medium that they had been harvested from (medium was filter sterilised beforehand using a 0.22 μm filter) and incubated for 1 h before examination by flow cytometry, described below.

A standard curve for intracellular pH was produced by staining exponential phase cells as above, followed by 1 h incubation in permeabilization solution (PBS with 100 μm nigericin) that had been pH-adjusted between 4.0 and 7.5 in pH 0.5 increments, using HCL or NaOH. This procedure yields cells with defined intracellular pH [16]. These were analysed by flow cytometry as described below. The resultant standard curve was used to convert ratiometric fluorescence values from experimental cultures to median intracellular-pH values.

CFDA-SE ratiometric fluorescence was determined for 10^6 cells per sample by flow cytometry, with a FACSCanto A (BD Biosciences) instrument. Laser excitation was at 488 nm and emission was collected through a 530/30 nm filter for pH-dependent emission and a 585/42 nm filter for pH-independent emission from CFDA-SE. Events (cells) were gated by forward scatter and side scatter to exclude doublets and debris. Median fluorescence of gated cells was then calculated using Kaluza Analysis V2.1 software.

2.4. Fermentation measurements

To measure pressure resulting from gas evolution during fermentation, McCartney bottles containing 5 ml YP supplemented with glucose as specified in the Results were inoculated with exponential phase cells to a final OD_{600} 0.2. Bottles were then sealed with hole-punched metal caps containing intact rubber seals to prevent gas leakage. After static incubation for either 1 or 7 days at 24 °C, pressure accumulated from culture gas evolution was measured using a 2000P Differential Manometer (Digitron), alongside culture OD_{600} measurements, as above. Pressure readings were subsequently normalised to OD_{600} readings.

2.5. Oxygen depletion measurements

To measure oxygen depletion from culture media as a result of respiration, McCartney bottles were each fitted with a single Planar Oxygen-Sensitive Spot (PreSens, Regensburg). These spots are coated with an oxygen-quenching fluorescent material, which can both be non-invasively excited, and emission (oxygen-dependent) read, using a FitBox 4 (PreSens, Regensburg). YP medium (5 ml), supplemented with glucose as described in Results, was added to the McCartney bottles and inoculated with exponential phase cells to a final OD_{600} 0.5. Bottles were then sealed before baseline oxygen measurements were taken, followed by static incubation for 2 h at 24 °C. After incubation, endpoint readings were taken to determine oxygen depletion from the medium as a result of respiration. Although oxygen depletion occurred over a 2 h period (Fig. 1A), 1 h incubation was sufficient to measure oxygen depletion from the medium before substantial culture growth occurred and 1 h was used as the endpoint for subsequent assays.

3. Results

As recent work has shown that the food preservative sorbic acid inhibits respiration more strongly than fermentation of yeasts [3], we tested the hypothesis that the greater relative respiratory (versus fermentative) metabolism of yeasts at low glucose may sensitize them to sorbic acid. *Saccharomyces cerevisiae* was cultured with either 2 % w/v glucose (~111 mM, a standard concentration in media) or 0.1 % w/v glucose (~5.55 mM, the minimum concentration found to permit reproducible batch growth) in the absence or presence of sorbic acid. As expected, cells in 0.1 % glucose had the greater respiratory activity, consuming oxygen more rapidly than cells in 2 % glucose, as assessed by oxygen depletion from culture medium in sealed gas-tight bottles (Fig. 1A).

Yeast growth was compared in these two glucose conditions in the absence and presence of sorbic acid. Culture plates were not gas-tight, unlike the above respiration assays, to allow continued oxygen availability during growth. Contrary to the starting hypothesis, cells in 0.1 % glucose were not more sensitive to sorbic acid than at 2 % glucose (Fig. 1B). In fact, sorbic acid was less inhibitory to these yeasts at low glucose, an effect that could be visualised more clearly after normalising the OD values with sorbic acid to the corresponding control ODs, for each glucose condition (Fig. 1B, right panel). Regardless of normalisation to the respective controls, between ~20 and ~50 h there was a small, absolute increase in cell density with sorbic acid at low-compared with high-glucose. This followed a shortened lag phase with sorbic acid when at low glucose (Fig. 1B, left). Despite an extended lag phase, maximum growth rate was eventually higher in the high glucose with sorbic acid treatment compared to the low glucose sorbic acid treatment. After ~60 h cells in high glucose began to outgrow those in low glucose, possibly due to excess remaining glucose.

Other weak acids were tested for possible similar effects. In addition to sorbic acid, there was some relative resistance at low glucose to decanoic acid (Fig. 2A and B), tested here because longer-chain acids have been shown to selectively inhibit respiring vs fermenting cells [3]. In contrast, a resistance phenotype at low glucose was not evident in cells cultured with acetic or propionic acids (Fig. 2). These are shorter-chain acids that show little selective inhibition of cells growing by respiration [3]. The resistance phenotype was not dependent on the weak acid concentration as the effect was reproducible across a range of sorbic acid concentrations, even when only weakly inhibitory (Fig. S1).

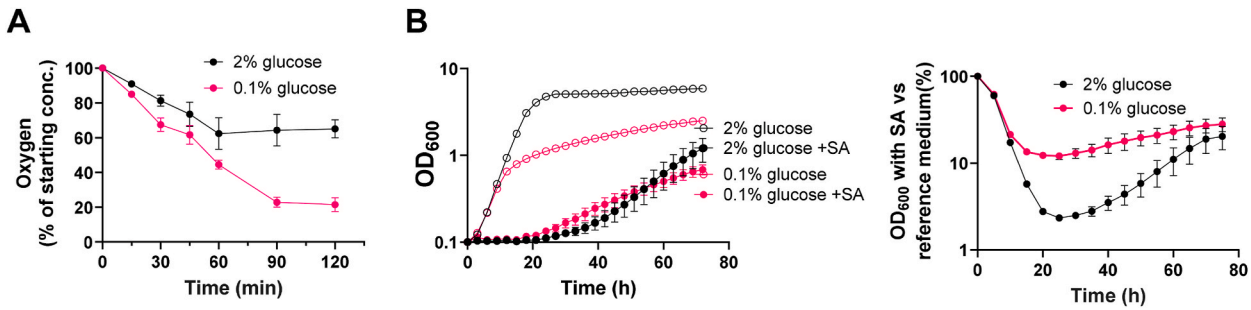


Fig. 1. Yeast shows relative resistance to sorbic acid (SA) in low- (respiratory) versus high- (fermentative) glucose concentrations. (**A**) Time courses of oxygen depletion reflecting relative respiratory activity of *S. cerevisiae* W303 at either 2 % (black) or 0.1 % (pink) glucose. Assays performed in YP medium with yeast at OD_{600} 0.5 in sealed McCartney bottles. (**B**) Growth of *S. cerevisiae* is presented as raw OD_{600} values (left panel) or OD_{600} percentage versus no-sorbic acid controls (right) in 2 % (black) or 0.1 % (pink) glucose, either without (open symbols) or with (filled) 1 mM sorbic acid. Assays performed in microtiter plates with lids but unsealed. Points represent means from three biological replicates; error bars (shown where they are larger than the dimensions of the symbols) represent SEM.

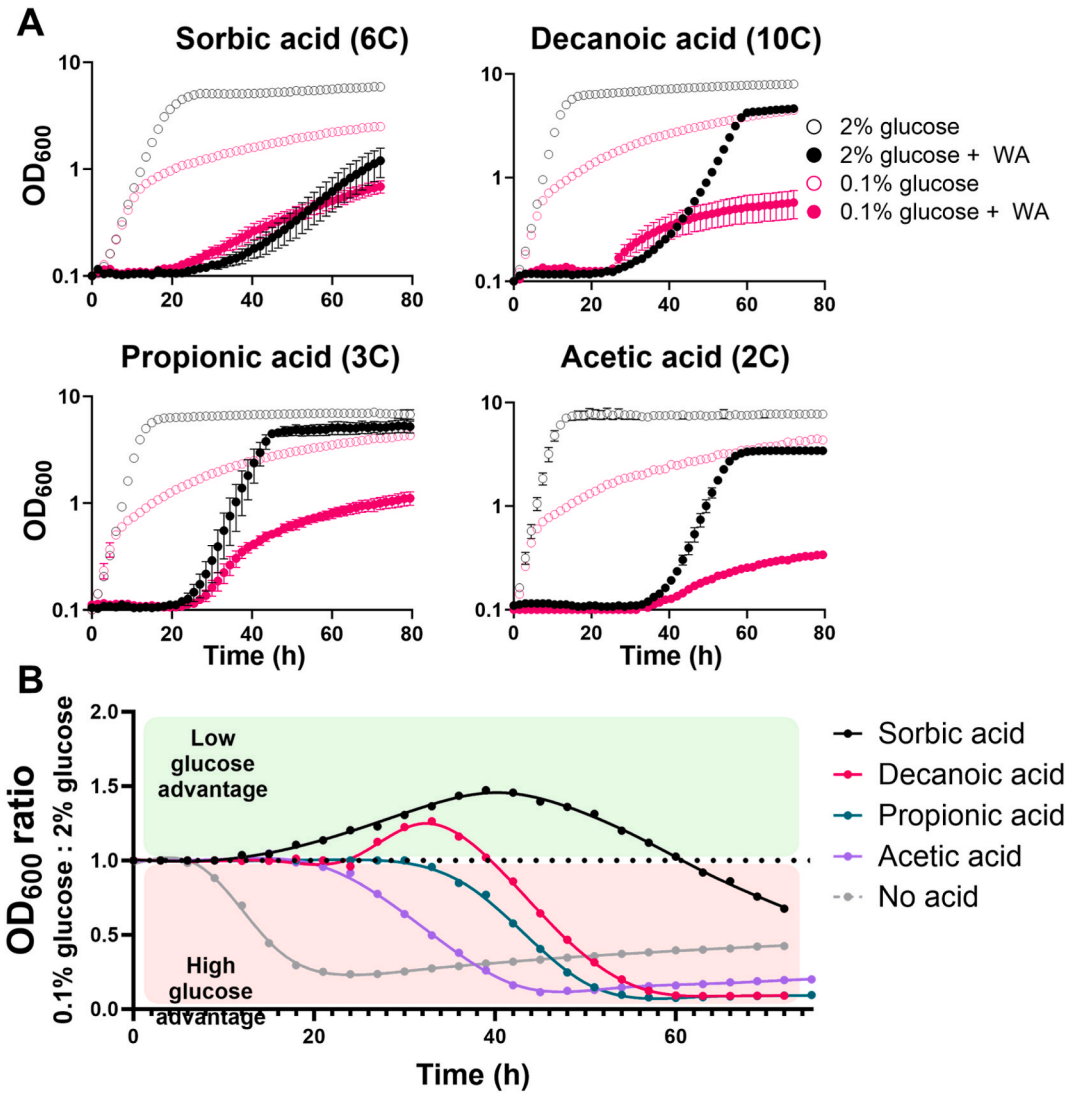


Fig. 2. Growth in low glucose supports relative-resistance to weak acids that selectively inhibit respiring cells **A.** Growth of *S. cerevisiae* W303 in the presence of different weak acids (WA), with either 2 % (black) or 0.1 % (pink) glucose in the absence (open symbols) or presence (filled) of WA. Points represent means from three biological replicates. Carbon chain lengths are indicated after the relevant WA name. **B.** Growth data from A presented as the ratio of optical density in 0.1 % versus 2 % glucose cultures with each WA, where a ratio greater than 1.0 (above the dotted line) represents higher OD_{600} with the WA when in low glucose. Error bars (shown where larger than the dimensions of the symbols) represent SEM from three biological replicates.

Since sorbic acid is known to inhibit respiration, we also widened out the panel of yeast isolates to encompass yeasts reported to be fermentation negative [3]. This was in order to determine the prevalence of the resistance phenotype when respiration was the predominant energy generating mechanism. We found that fermentation negative yeasts typically did not show relatively enhanced resistance at low glucose, with the phenotype present in only one (one of two *Rhodoturula glutinis* isolates) out of five fermentation negative isolates tested (Fig S3). Alongside this panel, yeasts that are fermentation positive were assayed (besides *S. cerevisiae,* already described above). Similar to *S. cerevisiae* (Fig. 1), these additional fermentation positive yeasts (*Z. bailii and B. bruxellensis)* showed enhanced relative-resistance to sorbic acid when at low glucose (Fig. S3). While differences and similarities between the fermentation-positive and -negative species are of course not limited to fermentative capacity, the apparent separation of the present phenotype between them was consistent with a possible requirement for fermentative metabolism in eliciting sorbic acid resistance at low glucose.

To further probe whether fermentation was involved in sorbic acid resistance at low glucose, sorbic acid challenge experiments were repeated but this time in anaerobic conditions, to restrict cells to fermentative growth only. Measurements of OD_{600} were taken at timepoints where the greatest difference in resistance between high and low glucose had been observed under aerobic conditions. In both of the glucose conditions, sorbic acid resistance was greater in the anaerobic environment (inhibiting respiration but not

fermentation) than in the aerobic environment (Fig. 3). Moreover, the difference between aerobic and anaerobic growth with sorbic acid was more than 50 % greater at low glucose compared to high glucose. This indicated that sorbic acid resistance conferred by anaerobicity (i.e. forced fermentative growth) was greater for the cells in low glucose (i.e. the condition that supports the greatest respiration when aerobic; Fig. 1A).

To further characterise the above findings, fermentation rate was measured according to pressure generated by gas evolution [3] of cells in 2 % or 0.1 % glucose in the presence or absence of sorbic acid. Because yeast cells produce excess CO_2 as a product of fermentation, whereas the net change in CO_2 during respiration is balanced by O_2 consumption, any increased gas pressure during yeast culture is predominantly the result of fermentation (apart from negligible amounts of other volatile molecules) [3]. Endpoint pressures determined after 24 h of growth in sealed bottles revealed that cells produced more gas (i.e., fermented more) with than without sorbic acid in both low and high glucose (Fig. 4). This suggested that the presence of sorbic acid stimulates fermentative metabolism. A negative control of cells cultured with glycerol as carbon source, an obligatory respiratory substrate, showed no detectable fermentative activity. Notably, cells in low glucose exhibited little fermentation in the absence of sorbic acid, but more than a doubling in fermentative activity with the inclusion of sorbic acid. This compared with a <45 % increase for the equivalent comparison in 2 % glucose (Fig. 4). This greater relative shift to fermentation at low glucose mirrored the greater relative resistance to added sorbic acid seen at low glucose (Figs. 1–3) and the known resistance of fermentative cells to sorbic acid [3]. Consistent with this, growth on galactose (which can be fermented or respired, without the repression of respiration that occurs with glucose) was not associated with increased fermentation in the presence of sorbic acid and, likewise, was not associated with sorbic acid resistance at 0.1 % versus 2 % carbon source (Fig. S4). This evidence also allayed concerns that other impacts of anaerobicity, such as limiting the biosynthetic capacity of oxygen requiring reactions, were solely responsible for this phenotype.

We additionally considered previous evidence that implicates intracellular pH as a factor in WA resistance or sensitivity [7,15,17], while culturing with low glucose has been reported to acidify the cytosol of yeast cells [18]. To examine whether differences in internal pH could be related to WA resistance at low glucose, the intracellular pH of exponential-phase *S. cerevisiae* cells was measured with the pH dependent stain CFDA-SE [15] and analysis by ratiometric flow cytometry, after 4 h of growth in either 2 % or 0.1 % glucose with acetic, sorbic, or decanoic acids (Fig. S2). Whereas intracellular pH was higher in low versus high glucose for cells incubated with decanoic acid, there was no significant effect of the glucose concentration with acetic acid, sorbic acid or without WA (Fig. S2). This suggested that the resistance phenotype at low glucose was not related to differences in intracellular pH. In addition, culture medium at the end of growth experiments remained unchanged at pH 4.0.

After examining intracellular pH, attention was refocused on the apparent association between increased fermentation and sorbic acid resistance at 0.1 % glucose. We sought to narrow down carbon sensing and metabolism pathways that may be integral to this phenotype. *S. cerevisiae* deletion strains lacking genes of the glucose sensing and repression pathways were assayed for retention of the relative sorbic acid resistance at 0.1 % glucose seen in the wildtype. In total, 11 deletion strains were tested, as summarised in Fig. 5 (see Fig. S5 for underlying data, see Fig. 5A and B for example of data transformation). Deletion of most of these genes had deleterious effects on sorbic acid resistance, both at high and low glucose. This was especially apparent with the deletion of *RGT2* (encoding a sensor of extracellular glucose), *YCK1* (kinase regulator of Rgt2), *MIG1* (transcriptional repressor of respiratory genes) and *REG1* (regulator of *MIG1*) (Fig. 5C and D). Specific to the present report of sorbic acid resistance in low glucose, deletion of *YCK1, MIG1,* or *RGT1* was more impactful in reducing sorbic acid resistance in low glucose conditions than in high glucose, implying that the relevant gene functions are indeed involved in sorbic acid resistance at low glucose.

There were also exceptions to the trend of decreased resistance in deletion strains. Deletion of *SNF3* (a high affinity glucose sensor) or of downstream *MTH1* enhanced sorbic acid resistance in low glucose. Interestingly, Snf3 is a key sensor of low extracellular glucose

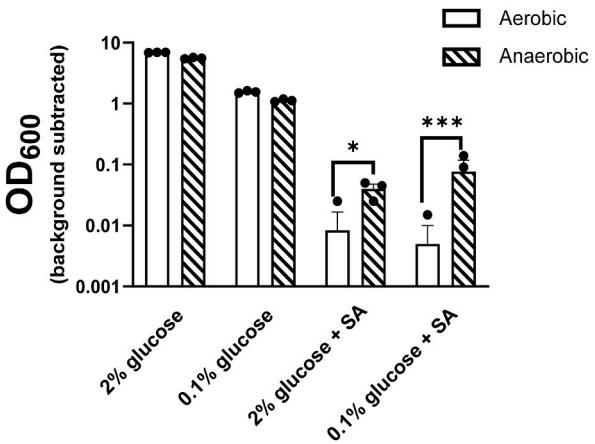


Fig. 3. Growth in anaerobic conditions increases sorbic acid resistance, particularly at low glucose.
Growth yields (OD_{600}) of *S. cerevisiae* W303 were compared after 40 h static incubation at 24 °C in the different conditions with or without 1 mM sorbic acid (SA). Bars are means from three biological replicates (points). Where not all points are visible, the absent points are OD_{600} value of zero and cannot be displayed on a logarithmic axis. Error bars represent SEM. * = $p < 0.05$, *** = $p < 0.001$, according to one-way ANOVA.

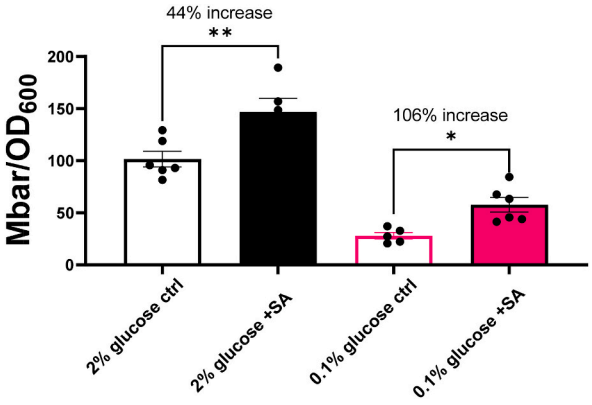


Fig. 4. Sorbic acid increases fermentation by cells, particularly at low glucose.
Pressure generated by *S. cerevisiae* W303 grown from starting inocula of OD_{600} 0.2 in the absence or presence of 1 mM sorbic acid in either 2 % (black) or 0.1 % (pink) glucose after 24 h static incubation at 24 °C, normalised by final OD_{600} readings. Error bars represent SEM from at least five biological replicates (points).

concentrations, so this result suggests that normal (Snf3-dependent) sensing of low glucose in the wild type would mediate sorbic acid sensitivity, rather than resistance of the wild type to sorbic acid at low glucose as described in this paper. However, there are many alternative reasons why loss of a key regulatory sensor could lead to resistance to sorbic acid. Deletion of the transcription factor encoded by *SIP4*, a positive regulator of gluconeogenesis, also yielded sorbic acid hyper-resistance at low glucose but hyper-sensitivity in high glucose. However, deletion of *PCK1* encoding phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis, yielded only mild, negative impacts on sorbic acid resistance at both glucose concentrations.

The collective results, coupled with the evidence above that galactose (which does not directly interact with these signalling pathways) at low concentrations minimally induces sorbic acid resistance (Fig. S4), indicate that elements of upstream glucose sensing and glucose repression can be required for the relative sorbic acid resistance of cells at low glucose.

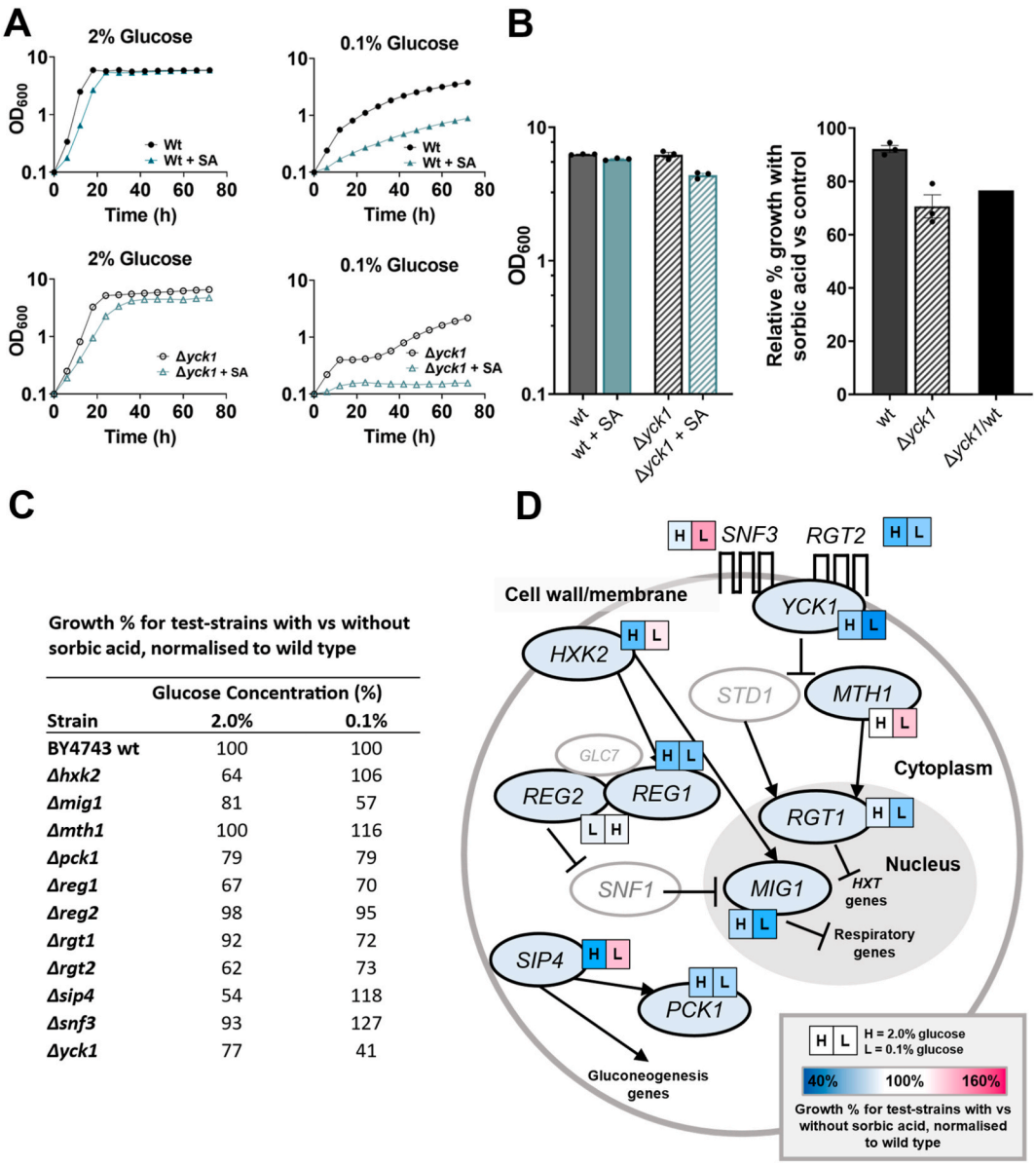
As an increase in fermentation in response to sorbic acid at low glucose appeared to be associated with sorbic acid resistance in this condition, we hypothesised that encouraging respiratory metabolism could restore inhibition by sorbic acid. To achieve this, we supplemented cultures with succinic or malic acids, intermediate compounds of the tricarboxylic acid (TCA) cycle, during culture with sorbic acid. Supplementation of low glucose medium with succinic acid (a substrate of mitochondrial complex II; succinate dehydrogenase) both stimulated cellular respiration (Fig. 6A), as also reported by Ref. [19], and markedly sensitized cells to sorbic acid (Fig. 6B). Increased respiration in response to succinic acid supplementation was absent at 2 % glucose (Fig. 6A). Similar relative effects on growth were apparent also with malic acid supplementation at 0.1 % glucose (Fig. 6B), but these were weaker than with succinic acid; possibly because not all malic acid is used for succinate production via the TCA cycle and can instead feed into the malate-aspartate NADH shuttle [20].

While succinic acid did increase respiration in line with previous literature, there could be other explanations for its efficacy in sensitizing cells to sorbic acid. In *S. cerevisiae*, succinate is transported into the mitochondria via the succinate-fumarate carrier (Sfc1) antiporter, and deletion of the *SFC1* gene prevents growth on respiratory substrates [21]. To help corroborate that succinic acid confers sorbic acid sensitivity at least in part via increased respiration, we conducted growth assays with the *S. cerevisiae Δsfc1* deletant. Results are presented as % growth versus the control condition without sorbic or succinic acid for easier visual comparison of the wild type and mutant. Sensitization to sorbic acid in the presence of succinic acid at 0.1 % glucose became apparent only after around 40 h in the BY4743 wildtype background that is isogenic with the *Δsfc1* deletant (Fig. 6C). Nevertheless, this succinic acid-dependent hypersensitivity was alleviated in the mutant lacking Sfc1 function. While not excluding other possible explanations, this result is consistent with respiration being involved in the wild-type phenotype.

Taken together, the present findings support a hypothesis that a metabolic shift from respiration (Fig. 1A) to fermentation (Fig. 4) enables yeast adaptation and resistance to sorbic acid resistance at low glucose, and they offer a potential means of re-sensitizing yeasts to sorbic acid at low glucose.

4. Discussion

Many food producers have been transitioning to reduced-sugar foods and beverages, in response to government legislation (in some countries) or consumer preferences [8]. However, impacts of these changes on microbial preservative resistance and spoilage are barely characterised. The present results indicate that yeast in low glucose can be more resilient to inhibition by added sorbic-acid preservative than in high glucose. This resilience was characterised partly by a reduced lag phase. There was also an effect on maximum growth rate, which itself could affect stress resistance [22]. The overall finding contrasted with our starting hypothesis, which had been based on prior knowledge that: (i) sorbic acid targets yeast respiratory metabolism, evidenced during growth on a carbon source such as glycerol that can only be respired [3]; (ii) glucose scarcity favours respiratory over fermentative metabolism in many yeasts [14]. The present findings suggest an explanation for this apparent contradiction, as they indicated that cells in low



Growth % for test-strains with vs without sorbic acid, normalised to wild type

	Glucose Concentration (%)	
Strain	**2.0%**	**0.1%**
BY4743 wt	100	100
Δhxk2	64	106
Δmig1	81	57
Δmth1	100	116
Δpck1	79	79
Δreg1	67	70
Δreg2	98	95
Δrgt1	92	72
Δrgt2	62	73
Δsip4	54	118
Δsnf3	93	127
Δyck1	77	41

Fig. 5. Deletion of key glucose sensing and repression genes alters sorbic acid (SA) sensitivity **A.** Example growth assays for *S. cerevisiae* BY4743 (wt) and an isogenic deletion strain (example shown is Δ*yck1*), demonstrating sorbic acid hypersensitivity of the mutant at 0.1 % glucose. Results from similar assays for other test-deletants are presented in Fig. S5. **B.** Example of data processing from growth assays in (A) to % inhibition values show in (C). Left panel: Wild type (solid) and *Δyck1* (hashed) OD_{600} at 60 h from the growth assays in 2 % glucose either without (black) or with (green) 0.75 mM sorbic acid. Right panel: Using values from the left panel, growth of each strain with sorbic acid as a percentage of control growth (without sorbic acid). The final bar normalises the relative growth of the mutant with sorbic acid to that of the wild type and is the value used in panel (C). **C.** Growth of all tested deletants with versus without sorbic acid, expressed relative to the corresponding effect measured for the wild type, where values less than or greater than 100 % represent relative sensitivity or resistance, respectively of the deletant to sorbic acid. **D.** Simplified scheme of the glucose sensing and signalling cascades examined in *S. cerevisiae* and phenotypes of corresponding deletion mutants. The results of (C) are presented here next to each gene as a colour scale for 2 % (H) and 0.1 % (L) glucose from blue to pink, with blue representing a sensitization to sorbic acid and pink representing resistance, of the corresponding deletant relative to the wild type. Greyed-out components represent untested mutants.

glucose switch to fermentation in response to sorbic acid addition, therefore alleviating any respiration-associated sensitivity. Other tested parameters like intracellular pH changes, which can be effected both by altered extracellular glucose levels [23,24] and weak acid exposure [7,25], were not correlated with sorbic acid resistance in the present conditions. However, organelle-specific or localised pH within different parts of the cell were not measured and may warrant attention in future work. Nevertheless, relative resistance to sorbic acid at low glucose was suppressed in certain deletants lacking key genes of the glucose signalling and repression pathway. That

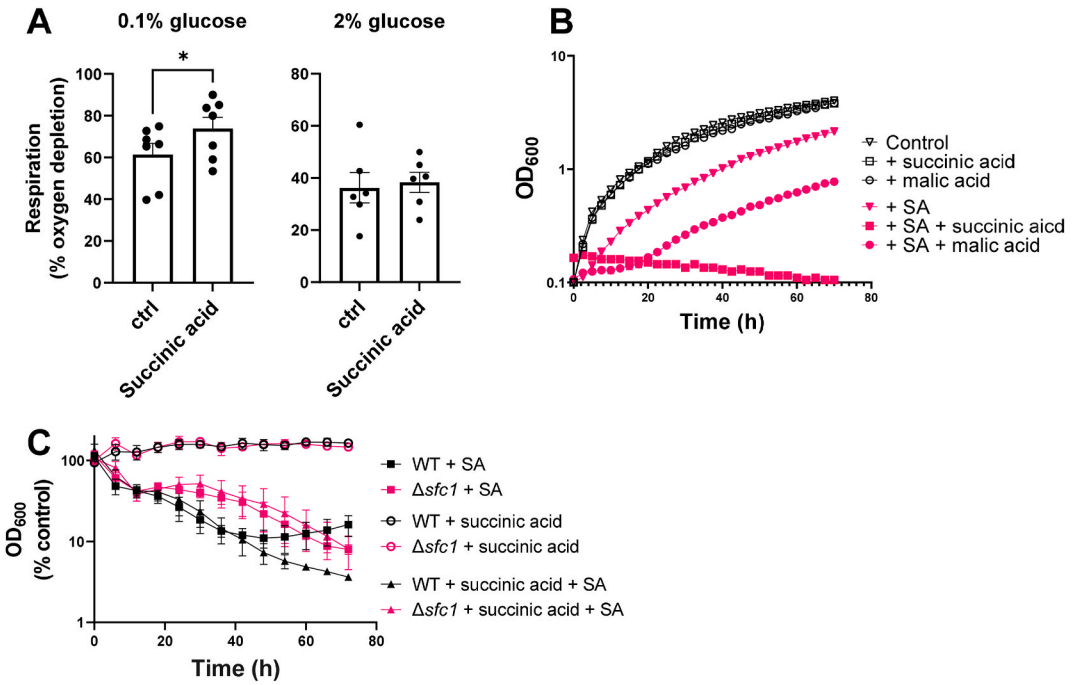


Fig. 6. Supplementing yeast with succinic acid promotes both respiration and sorbic acid hyper-sensitivity at low glucose **A.** Measurements of respiration by *S. cerevisiae* W303 grown in the presence or absence of 111 mM succinic acid in YP supplemented with 0.1 % glucose are presented as percentage oxygen depleted from culture medium after 1 h incubation at OD_{600} 0.5 (for time course of oxygen depletion, see Fig. 1A). **B.** Growth curves with or without 1 mM sorbic acid and 111 mM of either succinic or malic acid are presented in 0.1 % glucose. Growth curves are the average of three biological replicates; error bars (shown where they are larger than the dimensions of the symbols) represent SEM. (C) Growth of *S. cerevisiae* BY4743 (wt) and the isogenic *Δscf1* deletant in the presence of 1 mM sorbic acid, 111 mM succinic acid, or both, with OD_{600} presented as a percentage of values obtained at the corresponding timepoints for growth in medium without sorbic acid or succinic acid. Error bars represent SEM from three biological replicates.

is consistent with this pathway playing a role in the increased fermentation and resistance response.

We propose that an ability to shift to a more fermentative metabolism in response to sorbic acid at low glucose promotes resistance because it mitigates the usual need for (sorbic acid-susceptible) respiration [3]. Generally, cells must expend energy to mount an adaptive stress response, which requires the generation of ATP. In response to industrially relevant stressors (e.g. ethanol, salt, temperature), it has been demonstrated that respiration can be insufficient to meet these energy demands, and yeast cells can switch to respiro-fermentative metabolism to mitigate the ATP deficit at the cost of biomass accumulation [26]. However, unlike in Ref. [26], where increased fermentation was coincident with decreased biomass accumulation, in the present study increased fermentation at low glucose with sorbic acid was correlated with increased biomass relative to the effect of sorbic acid at high glucose. This could reflect the additional respiration-targeting effect of sorbic acid compared to certain other stressors (i.e. decreased respiration alone helps protect against sorbic acid) or some other sorbic-acid specific element of the response. One effect of decreased respiratory activity would be decreased production of reactive oxygen species (ROS) [27]. This is relevant here as it should limit ROS production by sorbic acid itself, an effect thought to lead to the depletion of functional respiratory complexes (i.e. respiratory inhibition) through petite-cell formation and FeS cluster targeting [3].

One question is how sorbic acid specifically might trigger such a switch in carbon metabolism. In the bacterium *Bacillus subtilis*, sorbic acid has been shown to promote responses like those seen during nutrient limitation, including upregulation of TCA-cycle associated genes [28]. However, results from a transcriptomic and proteomic study of sorbic acid exposure in *S. cerevisiae* indicated little change in carbon metabolic or fermentation-associated gene expression that might also impact sorbic acid resistance [29]. For example, whereas expression of the glycolytic gene *TDH1* in that study was upregulated in response to sorbic acid, deletion of the gene did not elicit any measurable change in sorbic acid resistance. However, it is important to note that this previous study was at 2 % glucose and the importance of these or other genes up/down-regulated by sorbic acid at, say, 0.1 % glucose may have greater impacts for sorbic resistance in the lower glucose condition.

As any alleviation of preservative sensitivity in spoilage yeasts in reduced sugar foods could be a significant concern for the industry and consumers, we considered ways to mitigate the effect that could be feasible in real-world foodstuffs. Succinic acid is a metabolic intermediate of respiratory metabolism and a food-safe additive [30]. Stimulation of yeast respiration in the presence of succinic acid (at low glucose) in the present study was consistent with previous findings [19]. Moreover, the bias to respiration that arose from this addition was coincident with loss of sorbic acid resistance at low glucose. This suggested a feasible approach for restoring yeast sensitivity to preservative in low sugar formulations. Of course, any additions to foods (e.g., succinic acid) would require careful

consideration also of potential flavour, texture or regulatory consequences.

As producers and consumers transition towards reduced-sugar food and drink formulations, efforts should be made to understand how these alterations change the spoilage-propensity landscape. The results of this study indicate that low sugar environments can facilitate preservative resistance, relative to high sugar environments, and an adaptation of carbon metabolism appears to play a role in this. However, reduced sugar formulations often substitute sugar with low-calorie sweeteners, such as aspartame, acesulfame K, sucralose, and stevia [31]. Although these 'artificial sweeteners' have little to no calorific value to humans, many can be metabolised by microorganisms [31], potentially providing an alternative energy source for spoilage organisms. Furthermore, additional formulation changes such as reducing acidity to accommodate reduced sugar or replacing the glucose in energy drinks with caffeine or B vitamins, may also constitute changes to the susceptibility to spoilage that warrant investigation.

Data availability statement

All data are available either in the present Results section, in the Supplemental material or on request.

CRediT authorship contribution statement

Harry J. Harvey: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Alex C. Hendry:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Marcella Chirico:** Data curation, Investigation. **David B. Archer:** Investigation, Writing – review & editing. **Simon V. Avery:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22057.

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