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Evaluating the potential of natural product combinations with sorbic acid for improving preservative action against food-spoilage yeasts

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ABSTRACT

Fungal control methods commonly involve the use of antifungals or preservatives, which can raise concerns about broader effects of these stressors on non-target organisms, spread of resistance and regulatory hurdles. Consequently, control methods enabling lower usage of such stressors are highly sought, for example chemical combinations that synergistically inhibit target-organisms. Here, we investigated how well such a principle extends to improving efficacy of an existing but tightly controlled food preservative, sorbic acid. A screen of ~200 natural products for synergistic fungal inhibition in combinations with sorbic acid, in either 2% or 0.1% (*w/v*) glucose to simulate high or reduced-sugar foods, did not reveal reproducible synergies in either of the spoilage yeast species *Saccharomyces cerevisiae* or *Zygosaccharomyces bailii*. Potentially promising screen candidates (e.g. lactone parthenolide, ethyl maltol) or a small additional panel of rationally-selected compounds (e.g. benzoic acid) all gave Fractional Inhibitory Concentration Indices (FICI) $\geq$ 0.5 in combinations with sorbic acid, corroborating absence of synergy in either glucose condition (although FICI values did differ between the glucose conditions). Synergies were not achieved either in a tripartite combination with screen candidates or in a soft-drink formulation as matrix. In previous work with other stressors synergy 'hits' have been comparatively frequent, suggesting that sorbic acid could be unusually resistant to forming synergies with other potential inhibitors and this may relate to the weak acid's known multifactorial inhibitory-actions on cells. The study highlights a challenge in developing appropriate natural product or other chemical combinations applicable to food and beverage preservation.

1. Introduction

Food spoilage by fungi, from crop disease to post-processing spoilage, is a substantial threat to food security and a strong economic burden globally. To mitigate this loss, control methods commonly involve the use of stressors such as fungicides (for crop protection) and food preservatives (Davies et al., 2021). Generally, such control methods may provoke concerns about broader stressor effects, such as on non-target organisms, including in the environment (Lebeaux et al., 2022; Schneeberger et al., 2018) and development of microbial drug/stressor resistance (Swiecilo, 2016). Consequently, tight regulatory hurdles govern the deployment of these agents and can discourage development of new agents. Because of these concerns, fungal control methods with reduced stressor (bioactives) load are highly sought, especially in the food industry where microbial spoilage accounts for as much as 25% of global food loss annually (Bondi et al., 2014).

One approach to address these concerns is the exploitation of synergistic interactions between microbial inhibitors, whereby two compounds in combination inhibit microbial growth at concentrations potentially many-fold lower than when supplied individually (Davies et al., 2021; Tyers and Wright, 2019). Such synergies may arise from targeting of a common biological process or structure by different molecular targets and can be target-organism specific (Vallieres et al., 2018), so potentially less harmful to non-target organisms than individual, broad-spectrum agents.

Demand for healthier, low sugar and "clean label" food products, from both consumers and government legislation, might lead to altered spoilage propensity of new drinks formulations, while also inviting a switch to more natural preservation methods such as from natural products (NPs). Recently, a high hit-rate of antimicrobial synergies was reported among pairwise combinations of

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agents of a natural product chemical library, revealed by high-throughput combinatorial screening against yeast (Augostine and Avery, 2022). That study used a natural product library of 200 NP compounds that have mostly been described in foodstuffs and traditional medicine, possessing relatively low toxicity (http://www.caithnessbiotechnologies.com/puretitre.html), making it an ideal library also for mining potential food preservatives. Here, we sought to test how well such a principle for reducing the level of bioactives-use may extend to improve efficacy of an existing, major food preservative, sorbic acid. Accordingly, we interrogated the Puretitre library of natural products for discovery of synergistic activities with sorbic acid, against growth of known food spoilage yeasts. Many natural products are considered to have broad (promiscuous) effects, but the gain of inhibitory action attainable with synergy is thought to add specificity, given that synergy is typically focused on a common target of the individual agents (Augostine and Avery, 2022). Regarding sorbic acid, the allowable concentration of this weak acid in foods is tightly controlled and it is typically used as a single preservative (Younes et al., 2019). The possibility of synergy with natural products could open up new, acceptable food preservation practices.

2. Materials and methods

2.1. Yeast and culture conditions

The organisms used in this study were the haploid *Saccharomyces cerevisiae* W303 strain (*MATα ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3112*) and *Zygosaccharomyces bailii* S1 (NCYC 1766). Yeast strains were maintained and grown in YEP medium [2% peptone (Oxoid), 1% yeast extract (Oxoid)] supplemented with 2% (*w/v*) D-glucose (YEPD). For experiments in a soft drink formulation, commercial samples of 'Ribena Light – Pineapple & Passion Fruit' were filtered sterilised (pore diameter, 0.22 μm) before use without further modification. For experiments, single yeast colonies were used to inoculate 10 ml of medium in 50 ml Erlenmeyer flasks and incubated overnight with orbital shaking (New Brunswick Scientific) at 120 rev min^{-1}, 24 °C. Overnight cultures were subsequently diluted to optical density (OD_{600}) 0.5 and grown for 4 h to reach exponential phase before harvesting by centrifugation at 4500*g* for 4 min. Harvested cells were washed then resuspended in fresh YEPD medium at an appropriate dilution before use for experiments.

2.2. Determining sub-inhibitory concentrations of sorbic acid and assay time points

To determine the sub-inhibitory concentration (SIC) of sorbic acid for subsequent high-throughput screening, growth assays of *S. cerevisiae* W303 were conducted by incubating exponential phase cells (adjusted as described above to OD_{600} 0.1) with a range of sorbic acid concentrations in YEP (pH 4) supplemented with either 2% or 0.1% (w/v) glucose in 100 μl volumes in 96-well microtitre plates (Greiner Bio-One). Plates were incubated statically at 24 °C for 96 h, with growth monitored by OD_{600} measurement every 30 min immediately after shaking plates for 2 min (to mix cell suspensions) using an Epoch 2 Microplate Spectrophotometer (BioTek) plate reader, which was also used for OD determination. Growth curves were produced to ensure that behaviour was similar between *S. cerevisiae* and *Z. bailii* in high and low glucose conditions, and to ensure that the growth phase of cells when sampled for subsequent assays were comparable between the glucose conditions (Supplementary Fig. 1).

2.3. High-throughput NP compound screening

The Puretitre natural compound library (http://www.caithnessbiotechnologies.com/puretitre.html) is a collection of 200 characterised natural product (NP) compounds, provided in 96-well microtitre plates at 10 mM concentrations in dimethyl sulfoxide (DMSO). To screen this library for synergistic inhibition of growth of *S. cerevisiae* W303 in combinations with sorbic acid, a dilution of the library was first prepared by adding compounds into YEP medium (pH 4) to produce a 2 mM stock of each compound. Aliquots (5 μl) of these diluted stocks together with 5 μl of exponential phase yeast culture (OD_{600} 2) in YEP, pH 4, were added to 90 μl of YEP (pH 4) containing either 2% or 0.1% (*w/v*) D-glucose, supplemented with either sorbic acid (SA) or water (for library-compound only controls). This produced preparations with a final volume of 100 μl containing 100 μM NP compound, 100 μM sorbic acid (or water in library-compound only controls), and cells at OD_{600} 0.1. Solvent matched controls at 0.35% (*v/v*) DMSO were used for control assays without any added compounds.

Subsequent growth was determined by OD_{600} measurement using a BioTek EL800 Microplate spectrophotometer after static incubation at 24 °C for 24 h or 48 h for plates at 2% or 0.1% glucose, respectively (growth being slower at 0.1% glucose). OD_{600} from growth with compounds was expressed as a percentage of growth without compounds. Screens were performed in biological triplicate.

2.4. Checkerboard assays

Checkerboard assays were conducted as described in Bellio et al. (2021), with the exception that growth medium was used at 1X concentration instead of 2X and cell suspensions were prepared in YEP with either 0.1% or 2% (*w/v*) glucose instead of water. Plates were incubated statically at 24 °C and OD_{600} measured as above after 24 or 48 h of growth (depending on whether at 2 or 0.1% glucose, respectively) using a BioTek EL800 microplate spectrophotometer. Fractional inhibitory concentration indices (FICI's) were used to assess potential synergy from the checkerboard results, calculated as [(compound 1 MIC in combination) / (compound 1 MIC alone)] + (compound 2 MIC in combination) / (compound 2 MIC alone)]. Three-way checkerboards were produced as above for the paired compounds, but with each checkerboard replicated four times with sorbic acid added to each replicate at two-fold increasing concentrations.

3. Results

3.1. Preliminary natural product library screen

To survey a wide range of potential candidate natural product (NP) compounds for synergistic activity with sorbic acid, the Puretitre NP compound library was screened alone and in combination with sorbic acid for growth inhibition of *S. cerevisiae* W303. The concentration of 100 μM NP used for the screen is as per previous work (Augostine and Avery, 2022) and, for nearly all the library NPs, was conveniently sub-inhibitory to *S. cerevisiae* (Fig. 1). Similarly, the 100 μM screen concentration used for sorbic acid was nearly sub-inhibitory to *S. cerevisiae* W303 (<5% reduction in growth yield after 48 h versus control). These pairwise combinations of agents at near sub-inhibitory concentrations maximised the likelihood of detecting potential synergies (i.e., any observed reduction in growth by combination of agents that are sub-inhibitory individually would likely be due to synergistic interaction). In both high (2%) and low (0.1%) glucose conditions, the vast majority of the combinations of sorbic acid with natural product gave ≥85% growth *versus* natural product alone (Fig. 1). One strong

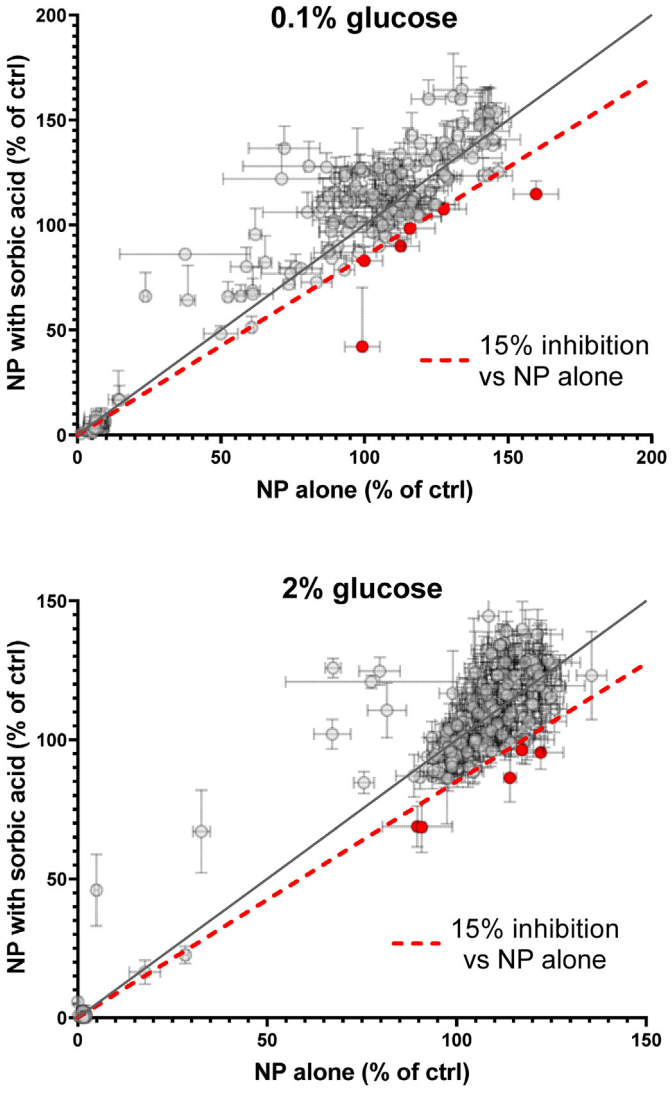

Fig. 1. Screen of NP compound library for synergy with sorbic acid against yeast growth.
NPs from the Caithness Puretitre library (see Methods) were screened at 100 μM against a sub-inhibitory concentration (100 μM) of sorbic acid for synergistic inhibition of growth of *S. cerevisiae* W303. Screens were conducted in YEP broth containing either 0.1% (top) or 2% (bottom) glucose. Each point represents growth (% OD_{600} versus minus-NP/minus-sorbic acid control condition) with a library compound alone (x axis) or in combination with sorbic acid (y axis) after 48 or 24 h for 0.1% or 2% glucose, respectively. The black line indicates y = x and the red line indicates y = 0.85x, with points in red below this line representing possible hits. Error bars represent SEM of three independent biological replicates. Underlying data for each natural product from the screen are presented in Supplementary Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

candidate (<50% growth vs control), sclareol, was identified in the 0.1% glucose assay, alongside six others weaker candidates in assays at either high or low glucose (<85% growth versus control) (Table 1; underlying data from the screens are presented in Supplementary Table 1). There was no overlap in identified candidate synergies between the 2% and 0.1% glucose screen. It was noted also that a greater number of compounds were inhibitory alone when in the 0.1% glucose condition.

3.2. Validation of NP screen hits

Following the screen, candidate NP compounds that were: (i) potentially synergistic with sorbic acid (see above), and (ii) suitable for food use (i.e., already present in foodstuffs or supplements), were subjected to synergy validation tests using checkerboard assays. *S. cerevisiae* was challenged with 2-fold serially increasing sorbic acid and NP concentrations in pairwise combinations, and the ratios of concentration differences needed to inhibit growth for each agent alone versus in combination [the fractional inhibitory concentration index (FICI)] was calculated (see Methods; FICI $\leq$ 0.5 is indicative of synergy) (Fig. 2A). Despite exhibiting combinatorial growth inhibition in the library screen, none of the candidate NP compounds showed a clear synergistic interaction with sorbic acid in the checkerboard assays, with all FICI values > 0.5 (Fig. 2B).

3.3. Testing near-synergistic hits in the spoilage yeast Z. bailii

Because baicalein, ethyl maltol, parthenolide and sclareol were near-synergistic with sorbic acid (FICI between 0.56 and 0.75), we additionally tested these combinations for synergistic inhibition of growth of the common spoilage yeast *Z. bailii*, in YEPD (Fig. 2C) and in a sorbic acid-free soft drink formulation (Fig. 2D) (*S. cerevisiae* did not grow in the soft-drink formulation). In *Z. bailii*, FICI values in YEPD were generally higher for the four NPs with sorbic acid than they had been for *S. cerevisiae* at both 0.1% and 2% glucose (Fig. 2C) and this was the case also when assayed in the soft drink formulation (Fig. 2D). The preceding screen threshold had been set low (15% growth inhibition vs control) so as not to miss potential synergies (Fig. 1). Consequently, it was not expected that all or even most candidates from the screen would satisfy the greater stringency of dedicated checkerboard tests. Nevertheless, the absence of corroborated synergies here (Fig. 2) contrasted markedly with previous screens (Augostine and Avery, 2022; Flobak et al., 2019; Pemovska et al., 2018; Tyers and Wright, 2019; Vallieres et al., 2018), potentially highlighting a particular issue for synergies that may enhance weak acid preservative action. To interrogate this further, a three-way combination was tested, including both ethyl maltol and sclareol together with sorbic acid. However, only a small additional reduction in growth was observed compared to any of the three pair-wise combinations of these agents (Supplementary Fig. 2).

3.4. Screening a select panel of food-present antimicrobials with sorbic acid

In addition to the NP library screening strategy for finding synergies with sorbic acid, we used a more targeted approach by testing combinations of sorbic acid with a select panel of natural products or preservatives already found in or used in food and beverages (Fig. 3). These selected compounds (potassium benzoate, cinnamic acid, quinine, caffeine) are known to exhibit some antifungal activity individually (AlEraky et al., 2022; Geoghegan et al., 2020; Islahudin et al., 2014), with some already reported to synergise with certain other compounds (Vallieres et al., 2018). The alternative weak-acid preservative potassium benzoate combined with sorbic acid gave an FICI value ~0.58 in 0.1% glucose, just above the threshold for synergy (Fig. 3B). The other compounds tested with sorbic acid gave FICI outcomes indicative of additivity or antagonism (Fig. 3B). As above, we also tested near synergistic combinations, in this case potassium benzoate + sorbic acid, for growth inhibition in *Z. bailii.* Similar to *S. cerevisiae*, this combination was only near-synergistic in *Z. bailii* (FICI ~0.58 in 0.1% glucose) (Fig. 3C).

In the case of potassium benzoate, it was noted that the same FICI values could be achieved with several-fold decreased concentrations of either compound, i.e., a two-fold reduction in one compound coupled with an eight-fold reduction in the other (Fig. 3A). In contrast, the other combinations from the preservative

Table 1
Candidate NP interactions with sorbic acid arising from the NP library screen.

Glucose conc. (%)	NP that was combined with sorbic acid	% Growth with NP + sorbic acid vs NP alone[a]
0.1	Sclareol	42.36 ± 28.53
0.1	Betulin	84.31 ± 2.52
0.1	Vanillylacetone	71.83 ± 3.85
2	Baicalein	76.78 ± 8.18
2	Ethyl maltol	78.09 ± 4.89
2	Parthenolide	82.03 ± 16.41

[a] Table shows NP + sorbic acid combinations giving ≤ 85% growth vs NP alone.

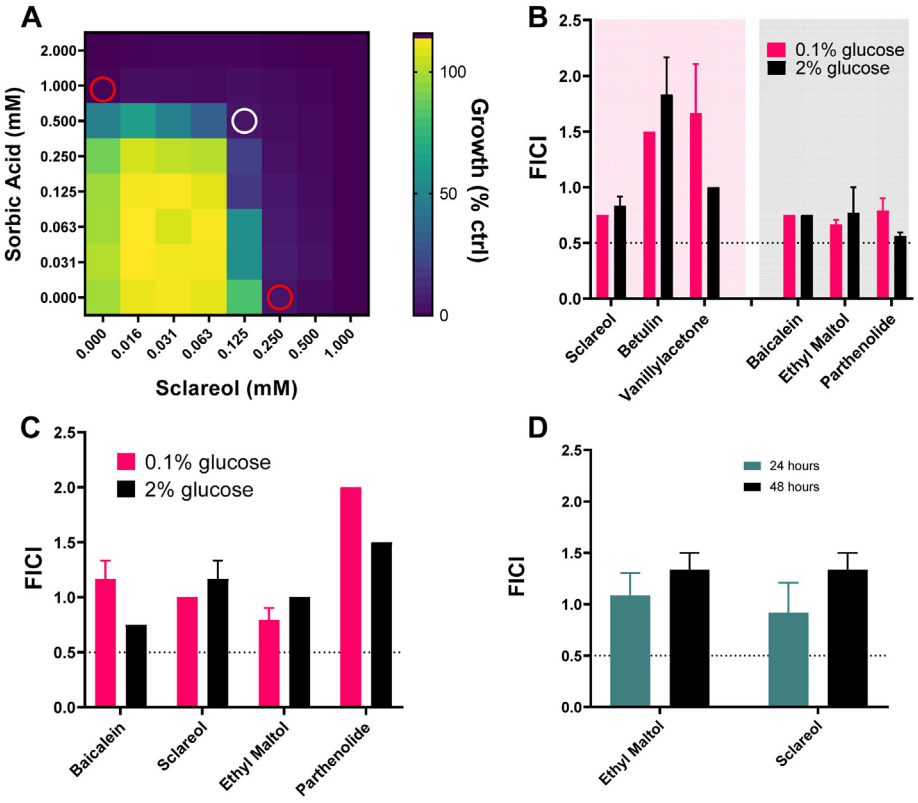

Fig. 2. Assays for synergy between candidate NP compounds in combination with sorbic acid.
(A) Example checkerboard assay of the candidate hit, sclareol + sorbic acid, in YEP 0.1% glucose, presented as the mean values from three biological replicates. Growth values (represented by the colour scale) for *S. cerevisiae* were according to % OD_{600} versus controls lacking inhibitors. Red circles indicate MIC of compounds alone and white indicates MIC in combination. (B) FICI values (fractional inhibitory concentration indices) for sclareol and other NP compounds in combination with sorbic acid, derived from checkerboard assays of yeast growth at 2% or 0.1% glucose after 24 or 48 h, respectively. Compounds are grouped according to whether the candidate NP was identified initially from screens performed at 0.1% (pink background) or 2% glucose (grey). The dotted line represents the cut-off for synergistic FICI values (≤0.5). (C) FICI values for selected combinations against *Z. bailii*. (D) FICI values for selected combinations in soft drinks formulation after 24 and 48 growth of *Z. bailii*. Values shown are means from three biological replicates, error bars represent SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

panel and earlier screen tended to reduce the MIC of the test compound more so than the MIC of sorbic acid (which only ever decreased two-fold in these experiments). This could reflect the overlap in modes of action of benzoic and sorbic acids, meaning their inhibitory effects should be at least partly interchangeable (consistent with an additive interaction), whereas different thresholds of contribution to an interaction may apply to pairs of agents with more distinct modes of action.

Collectively, the targeted and screening approaches used here indicate that previous evidence for antifungal synergies being common among pairs of different agents does not extend to natural products combined with the weak-acid food preservative sorbic acid. That conclusion from the present work with spoilage yeasts also fits with indications from tests of filamentous fungi with sorbic acid and alternative chemical libraries (discussed below).

4. Discussion

In this study, we set out to use a high-throughput screening approach to identify natural products (NPs) that may interact synergistically with the preservative sorbic acid in inhibiting growth of food spoilage fungi. Using such combinations to control microbial spoilage could help decrease the chemical additives load in foods (Tyers and Wright, 2019), allay concerns over spread of microbial (cross)stressor resistance and of harmful incidental effects on non-target organisms such as gut biota (Laudisi et al., 2019), while addressing consumer and regulatory pressures for cleaner label products.

Previous studies have reported a comparatively high frequency of antimicrobial synergies between natural products (Augostine and Avery, 2022) and repurposed drugs (Vallières et al., 2020).

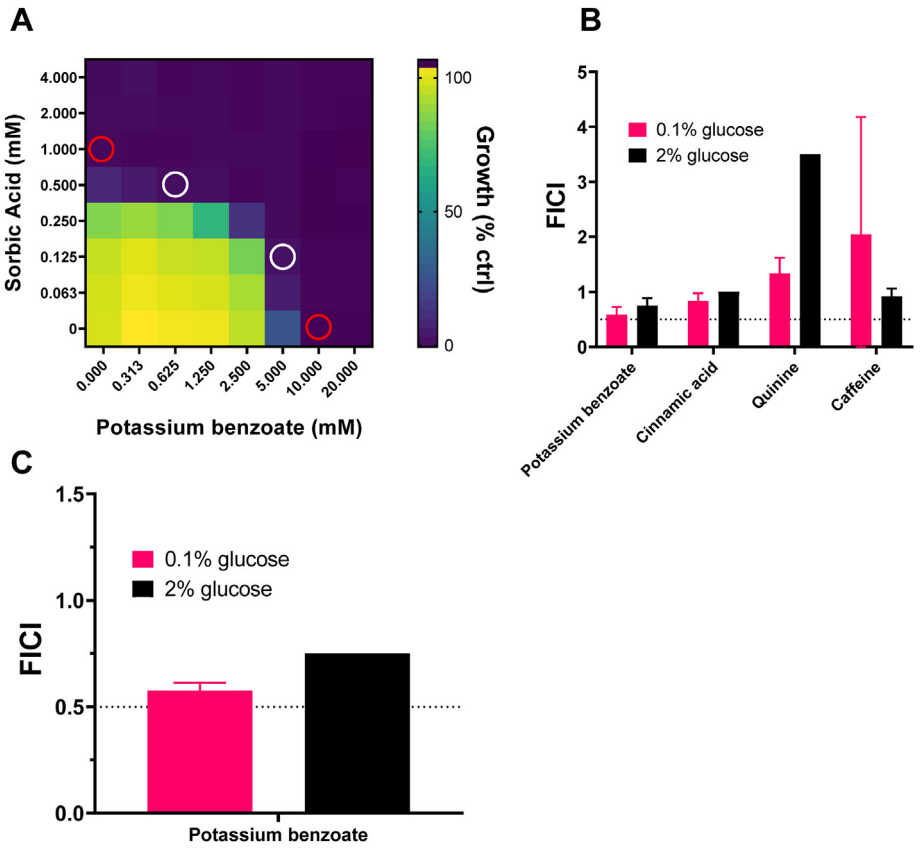

Fig. 3. Pairwise combinations of a select panel of food-relevant compounds with sorbic acid.
(A) Example checkerboard assay against *S. cerevisiae* of sorbic acid with benzoic acid in YEP 2% glucose. Data shown are means from three biological replicates. Red circles indicate MICs of the individual compounds; white circles represent combination MICs from which FICI values were calculated. (B) FICI values for other tested panel-compounds in combination with sorbic acid at both 0.1% and 2% glucose conditions after 48 and 24 h growth of *S. cerevisiae*, respectively. (C) FICI values for near-hit benzoic acid + sorbic acid against *Z. bailii*. Values shown are means from three biological replicates, error bars represent SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

However, of over 200 NPs screened here, none were clearly synergistic with sorbic acid. Only four natural products (baicalein, ethyl maltol, parthenolide, sclareol) suggested near-synergistic activity (FICI approx. 0.5–0.75), while benzoic acid was near-synergistic in a separate test of agents currently found or used in foods. Although not quite synergistic, the NP tested that could be of greatest relevance for use in foodstuffs was ethyl maltol. It is relatively inexpensive, soluble, has a fruity flavour, is already used in some baked goods and beverages as a flavourant (Durrani et al., 2021) and is generally recognised as safe for consumption (Durrani et al., 2021; Hua et al., 2019). Together, this suggests that small quantities of ethyl maltol in combination with sorbic acid may have minimal formulation impact, while somewhat reducing spoilage propensity, albeit not to the same degree as a synergistic combination. We did note that generally, NPs alone tended to be more inhibitory at 0.1% glucose compared to 2% glucose conditions. This could provide promise and further avenues for new NP preservatives in low sugar foods and beverages that may not have been as effective in higher sugar environments.

Besides the results of this study focused on yeasts and natural products, other work on combinatorial screens of sorbic acid with more-general chemical libraries (the Prestwick Chemical Library, and the University of Nottingham Managed Chemical Compound Collection (MCCC), testing ≥1000 compounds in each case), against the filamentous fungus *Aspergillus niger,* also has not revealed any clear growth-inhibitory synergies (Reis TF, Geoghegan IA, Goldman GH, Avery SV: Unpublished data). This reinforces the inference that sorbic acid may be relatively impervious to forming synergies. Factors potentially explaining this could relate to certain properties of sorbic acid including its broad modes-of-action that should minimise the potential for synergistic inhibitory actions. Synergistic growth inhibition commonly arises by mechanisms including where one drug facilitates influx of another or inhibits the breakdown or metabolism of another (Islahudin et al., 2013; Jia et al., 2009). However, sorbic acid is thought to diffuse across the cell membrane (Stratford et al., 2013), and decarboxylation of sorbic acid in *S. cerevisiae* is not a prominent resistance mechanism (Stratford et al., 2007), so reducing the potential for those routes of synergy. A further mechanism of synergy is where each inhibitor targets different parts of the same cellular pathway or process (Vallieres et al., 2018). Sorbic acid is purported to have broad, cumulative action in cells: reducing internal pH, disrupting enzyme function, uncoupling membrane potential and promoting oxidative stress with recent studies elucidating cellular respiration as a target in yeasts (van Beilen et al., 2014; Sofos et al., 1986; Stratford and Anslow, 1998; Stratford et al., 2013, 2020). Therefore, we suggest that the apparent relative sparsity (or absence) of discoverable sorbic acid synergies in the present screen and others, may in part be because it does not present a particular major target-route for synergistic interaction but instead several 'minor' targets. As such, additional targets of sorbic acid action may dampen phenotypic impacts of synergies that only affect one (or two) of its multiple cellular targets. This may also somewhat explain the near-synergy present between sorbic acid and benzoic acid; the two weak acid

preservatives have overlaps in their broad modes of action, and so offer potential scope for synergies over a larger range of these agents' targets.

Future research might consider utilising the near-synergistic combinations identified in this study in conjunction with additional agent(s) offering mode(s) of action overlapping the two, to elicit a synergistic inhibition with a defined "cocktail" of natural preservatives. Such approaches may be needed to overcome the unusual imperviousness of sorbic acid to combinatorial synergy indicated by the present study and to help address preservation challenges faced by the food industry.

Declaration of competing interest

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2023.01.004.

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