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Prenylated (iso)flavonoids as antifungal agents against the food spoiler *Zygosaccharomyces parabailii*

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ABSTRACT

Species belonging to the *Zygosaccharomyces bailii* sensu lato clade are some of the most troublesome spoilage yeasts of acidic food products, primarily due to their resistance towards synthetic food preservatives, like weak organic acids. Therefore alternative, powerful and more natural antifungal agents are needed. Herein, prenylated isoflavonoids, present mainly in the Leguminosae family, were investigated for their antifungal properties (including potency and mode of action) against a *Z. bailii* food isolate, its interspecies hybrid, *Z. parabailii*. The mono-prenylated isoflavonoids, wighteone and glabridin were the most active, with minimum fungicidal concentrations (MFC) of 12.5 μg/mL (37 μM) and 25 μg/mL (77 μM), respectively, at pH 4.0, rendering them over 20 times (on weight basis) more potent than sorbic acid (MFC 550 μg/mL, 4.5 mM). The two active mono-prenylated isoflavonoids induced killing within 15 min, suggesting potential membrane activity. Membrane permeabilization by these molecules was confirmed by propidium iodide uptake and TEM imaging. Membrane permeabilization was accompanied with leakage of intracellular material. Molecular properties such as the electrostatic energy potential and the hydrophobic integy moment were correlated to activity through a binary classification quantitative structure-activity relationship (QSAR) model. Altogether, mono-prenylated isoflavonoids can serve as highly potent, novel and natural antifungal against *Z. parabailii* acting by severely compromising the membrane integrity.

1. Introduction

Spoilage yeasts constitute one of the most important challenges in food and drink preservation. The *Zygosaccharomyces bailii* sensu lato clade is probably the most troublesome yeast species complex (Group 1) (Davenport, 1996). This species complex comprises *Zygosaccharomyces bailii* together with two recently revealed, phylogenetically related species (hybrids), namely *Z. parabailii* and *Z. pseudobailii* (Suh et al., 2013). *Z. bailii* is strongly associated with spoilage of foods with a high sugar content and/or acidic foods e.g. fruit juices and sauces (pH 3.0–4.0) (Beuchat, 1982; Stumbo, 1973), and it is also known for its resistance towards cleaning agents and disinfectants (high concentration of ethanol (>15% v/v)) (Davenport, 1996), impeding the cleaning of food-processing equipment (Deak et al., 1994; Fleet, 1992).

Weak organic acids such as sorbic, acetic and lactic acid are typically used to control the growth of *Z. bailii* (Beuchat, 1982; Martorell et al., 2007). They are known to inhibit microbial growth primarily via cytoplasmic acidification (Fujita et al., 2002, 2005; Krebs et al., 1983; Neal et al., 1965) but other fundamentally different actions have also been proposed (Piper et al., 1998; Stratford et al., 1998, 2009, 2020). *Z. bailii* shows extreme resistance towards them. In particular, *Z. bailii* is approximately 3 times more resistant to acetic and lactic acid than the model yeast, *Saccharomyces cerevisiae* (Guerreiro et al., 2012; Mira et al., 2014; Stratford et al., 2013b). This enhanced resistance has been hypothesized to develop due to (i) cell heterogeneity (preservative heteroresistance), i.e. the ability of a few hyper-resistant cells to cause spoilage (Steels et al., 2000; Stratford et al., 2013b; van Esch, 1987), (ii) degradation and metabolism of weak organic acids by the yeast, especially in the presence of sugars (Sousa et al., 1998) and (iii) the proposed efflux of weak organic acids due to the presence of a sorbate pump (Warth, 1977). Therefore, high amounts of weak organic acids are needed to ensure food safety, but this is prohibited by law and unacceptable to consumers due to the safety complications being associated with them (Piper, 2011). For example, the limit concentrations

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approved for use of sorbic acid range from 0.5 to 2 g/L (4.5–18 mM), depending on the food product (European Commission, 2011), but over four times higher concentrations are required to inhibit the growth of *Z. parabailii* (Stratford et al., 2013b). In addition, high concentrations of sorbic acid are associated with strong and undesirable fatty acid odor (Fujita et al., 2008).

Taken together, this indicates that alternative, effective food preservatives which are also well-perceived by consumers, are needed. Some natural compounds such as fatty acids, sesquiterpenes, terpenoid alcohols and phenols have already been tested against *Z. parabailii* (Fujita et al., 2008; Ribes et al., 2019; Rivera-Carriles et al., 2005). The most promising antifungal against *Z. parabailii* is the sesquiterpene polygodial, isolated from *Polygonum hydropiper*, shown to have a minimum fungicidal concentration (MFC) of 50 μg/mL (213 μM) (Fujita et al., 2005). Yet, polygodial cannot be classified as a good antimicrobial (MIC $\leq$ 25 μg/mL) based on Gibbon's classification system on natural compounds (Gibbons, 2004). To enhance the antimicrobial potency, synergistic combinations of (natural) antimicrobials are increasingly explored on the basis that synergy occurs when the combined agents act in different, yet complementary fashions (Eliopoulos et al., 1982; Fujita et al., 2005; Rivera-Carriles et al., 2005).

Prenylated flavonoids and isoflavonoids (i.e. (iso)flavonoids) are phenolic compounds found in the Leguminosae and are acknowledged for their enhanced antimicrobial potency. (Iso)flavonoids, encompassing 21 different subclasses in total, can be decorated with C5-isoprenoid moieties (prenyl-groups), a feature known to increase the potency of these compounds against bacteria (Araya-Cloutier et al., 2018b; Mitscher et al., 1983), molds (Ammar et al., 2013; Kim et al., 2010) and yeasts (Han et al., 2007; Lima et al., 2018). The mono-prenylated isoflavonoids, glabridin and wighteone, for example, have shown high antimicrobial potency against Gram-positive and Gram-negative bacteria with minimum inhibitory concentrations (MICs) between 10 and 20 μg/mL (30–62 μM) (Araya-Cloutier et al., 2018b; Fukai et al., 2002; Kalli et al., 2021). Glabridin has shown antimicrobial activity against *Candida* spp. (MIC of 6–64 μg/mL, 8–197 μM) (Liu et al., 2014; Messier et al., 2011), whereas wighteone was active against *S. cerevisiae* with a MIC of 4 μg/mL (12 μM) (Yin et al., 2006). Prenylation-based hydrophobicity seems to (partly) explain the high antimicrobial potency of prenylated (iso)flavonoids due to their increased affinity to membranes and proteins (Botta et al., 2009; Kalli et al., 2021). Glabridin and wighteone were shown to exert their antimicrobial action through permeabilization of the microbial membrane (Araya-Cloutier et al., 2018b; Liu et al., 2014), although other mechanisms have also been proposed (Moazeni et al., 2017; Peralta et al., 2015; Singh et al., 2015).

To the best of our knowledge, the antimicrobial properties of prenylated (iso)flavonoids against *Z. parabailii* have not been systematically evaluated. In this study, we explored the antifungal potency of these molecules and the mode of action of the two most active ones against a food isolate. Lastly, we analyzed the most important molecular properties that influence the antifungal activity of prenylated (iso)flavonoids using a QSAR approach.

2. Materials and methods

2.1. Materials and reagents

Prenylated (iso)flavonoids (glabrene, 3′-hydroxy-4′-*O*-methyl-glabridin, 4′-*O*-methyl-glabridin, hispaglabridin B, glyceollidin II, dehydroglyceollidin II, dehydroglyceollin I, dehydroglyceollin II, dehydroglyceollin, III, dehydroglyceollin IV and glabrol) were previously purified and chemically characterized (van de Schans et al., 2015, 2016). Glabridin was supplied by FUJIFILM Wako Pure Chemical Corporation (Neuss, Germany). Propidium iodide (PI), hydrochloric acid (36.5–38.0% v/v) and sorbic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetic acid (glacial) and glutaraldehyde 25% w/v in water) were supplied by VWR International B.V. (Amsterdam, the Netherlands). Isowighteone and neobavaisoflavone were purchased from ChemFaces (Wuhan, Hubei, China). Wighteone, lupiwighteone, luteone, and 6,8-diprenygenistein were purchased from Plantech UK (Reading, UK). 6′-Prenylpiscidone was supplied by Carbosynth Ltd. (Berkshire, UK). Yeast extract peptone dextrose broth (YPD) was purchased from Brunschwig Chemie B.V. (Amsterdam, the Netherlands) and bacteriological agar from Oxoid Ltd (Basingstoke, UK), and peptone physiological salt solution (PPS) from Tritium Microbiologie (Eindhoven, the Netherlands). DMSO was purchased from Duchefa Biochemie B.V. (Haarlem, the Netherlands). For transmission electron microscopy glutaraldehyde, paraformaldeyde, OsO_4, UranyLess staining, Spurr's resin and lead citrate were purchased from EMS (Hatfield, USA). Gelatin (type B, powder) and the ethanol (100% v/v) were supplied by Merck/Sigma-Aldrich and the 96% ethanol from TechniSolv.

2.2. Microorganisms

The food isolate *Z. parabailii* UL 3699 was kindly provided by Unilever Nederland B.V. (Wageningen, the Netherlands). *Z. parabailii* ATCC 60483 was purchased from the American Type Culture Collection (Wessel, Germany).

2.3. Methods

2.3.1. Screening of potency of prenylated (iso)flavonoids

Nineteen prenylated (iso)flavonoids were screened for their potency against the food isolate *Z. parabailii*. Yeasts cells were streaked from a −80 °C glycerol stock onto a YPD agar plate and incubated 48 h at 30 °C. One colony was transferred to 10 mL YPD broth and further incubated for 16 h at 30 °C. The overnight cultures were diluted 50 times with YPD (final inoculum concentration 4.4 ± 0.3 $\log_{10}$ CFU/mL). Stock solutions of the different prenylated (iso)flavonoids in DMSO (wighteone, glabridin, luteone, isowighteone and 6′-prenyl piscidone) or 70% ethanol (the rest of the compounds) were subsequently diluted with YPD (1–50 μg/mL). No solubility issues were associated with prenylated (iso)flavonoids in hydrophilic growth media (YPD) at any of the concentrations tested. Equal volumes (100 μL) of yeast inoculum and prenylated (iso) flavonoid solutions in YPD were mixed in a sterile 96-well plate (with a maximum solvent percentage of 2.0%). The 96-well plate was incubated in a Spectramax ID3 (Molecular Devices, Sunnyvale, CA, USA) at 30 °C for 48 h with double orbital, high intensity shaking before each measurement for 5 s. The optical density (OD) was measured at 600 nm every 10 min.

Growth was determined by measuring the time to detection (TTD), i.e. the time to reach a change in OD of 0.05 units (Aryani et al., 2015). In addition, negative control (YPD yeast cell suspension with maximum solvent percentage) and blanks were used for optical comparison and sterility control.

When the change in optical density (ΔOD) was less than 0.05, i.e. flat lines after 48 h, viable cell count was performed. In brief, 100 μL from each well was decimally diluted and spread onto an YPD agar plate. Plates were incubated at 30 °C for 48 h after which colonies were counted. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of compound that resulted in a cell count equal or lower than that of the initial inoculum. The minimum fungicidal concentration (MFC) was determined as the lowest concentration of compound that resulted in > 99% yeast inactivation from the initial inoculum. Prenylated (iso)flavonoids were screened in three biological replicates, each performed in duplicate.

2.3.2. Antifungal activity of most active prenylated (iso)flavonoids at acidic pH

The most potent prenylated isoflavonoids found upon screening were tested against *Z. parabailii* UL 3699 and *Z. parabailii* ATCC 60483 at pH 4.0 (or pH 3.0). Overnight cultures were prepared and assayed for 72 h (or 96 h for pH 3.0) as described above (final inoculum concentration

4.3 ± 0.3 $\log_{10}$ CFU/mL for UL 3699 and 4.5 ± 0.2 $\log_{10}$ CFU/mL for ATCC 60483 at pH 4.0, and 4.5 ± 0.3 $\log_{10}$ CFU/mL for UL 3699 at pH 3.0). The assays were performed in three biological replicates, each performed in duplicate.

2.3.3. QSAR binary model

The binary classification QSAR model was chosen (over for e.g. a continuous one) as it can accommodate molecules with non-established MICs (i.e. ≫ 25 μg/mL). Incorporation of molecules with unestablished MICs is crucial when small datasets are employed to increase the chemical space used for model development. Chemical structures were extracted from literature and inputted to the modeling software (Molecular Operating Environment, MOE, v.2019.08, Chemical Computing Group) using the canonical SMILES codes from PubChem. If not available, the chemical structure was drawn manually using the PubChem sketcher to obtain the SMILES code before importing to MOE. A conformational search (LowModeMD, RSM gradient 0.1 kcal/mol/Å, other settings default) was performed for all compounds in the database. The conformation with the lowest energy was further refined using MOPAC force field (RSM gradient 0.01 kcal/mol/Å).

Optimized chemical structures were used to calculate different molecular descriptors available in MOE. Descriptors were calculated for fully undissociated molecular species. After eliminating descriptors that were identical for all molecules or redundant descriptors (inter-correlation $R_{pearson} > 0.99$), a total number of 120 descriptors was finally incorporated into the database.

A binary QSAR model was constructed to predict activity of mono-prenylated isoflavonoids, using the QuaSAR-model application of MOE. Genetic algorithm was used to explore which variables best describe the data (Shahlaei, 2013). The binary model was constructed with the two descriptors that were most frequently indicated by the GA as the best predictors of the antifungal potency. Given the number of compounds used (15), only 2 descriptors (Shen et al., 2004) were selected following the principle of "Occam's Razor". This principle supports that a reasonable QSAR model with the smallest number of descriptors should be sought to avoid interpretation complexity (Cherkasov et al., 2014). According to Karelson et al. (2009), the number of descriptors should be maximum 5 times more than the number of data-points.

In this study, an activity threshold of pMIC of 4.1 (MIC ≤ 25 μg/mL) was set to discriminate between actives and inactives. When a MIC range was determined for a molecule, the worst case scenario (highest MIC value of the range) was considered. For the four mono-prenylated isoflavonoids, which showed no change in TTD at the maximum concentration tested (i.e. MIC ≫ 25 μg/mL), a MIC value of 100 μg/mL was inputted for the analysis. The quality of the binary model was first assessed by the total accuracy of the QSAR model, which is the fraction of observations correctly predicted, as well as the accuracy on actives (sensitivity) and accuracy on inactives (specificity), which are the fractions of correctly predicted sample actives and inactives, respectively. The descriptor importance was estimated to show the degree to which each descriptor is useful in distinguishing actives from inactives. The model was validated by the leave-one-out cross validation scheme where the cross-validated accuracies (total, active and inactive) were also considered to select the final binary model. Since the size of our experimental set did not allow splitting of the compounds into a training and a test set (Tropsha, 2010), we selected the best model by predicting the activity of an external set of prenylated isoflavonoids tested against other yeasts (*S. cerevisiae* and *Candida albicans*). The external set comprised molecules from the same subclasses as the ones used for model construction, which were within the applicability domain (AD) of the model. The (AD) of the models was calculated by means of the William's plot.

2.3.4. Combination studies

The two-dimensional checkerboard microdilution assay (Norden et al., 1979) was used to assess the types of interactions between prenylated isoflavonoids (glabridin and wighteone) and between prenylated isoflavonoids and sorbic acid at pH 4.0 against *Z. parabailii* UL 3699 (inoculum size 4.7 ± 0.1 $\log_{10}$ CFU/mL). Combination experiments with the two prenylated isoflavonoids were performed in 3 biological replicates, each performed in duplicate. Assays between prenylated isoflavonoids and sorbic acid were performed in two biological replicates per combination each performed in duplicate.

Two-fold dilutions of one compound (horizontally) were tested in combination with two-fold dilutions of the other compounds (vertically), in a concentration range based on MIC and MFC determination of the individual compound. The fractional inhibitory fungicidal concentration indices (FICI or FFCI) were calculated based on the checkerboard data obtained after a 72 h-incubation at 30 °C. The ΣFIC of the combinations refer to ($MIC_{compound1\ in\ combination}/MIC_{compound1\ alone}$) + ($MIC_{compound2\ in\ combination}/MIC_{compound2\ alone}$). The following criteria were used to determine the type of interaction between the two agents: ΣFIC ≤ 0.5 synergy; 0.5 < ΣFIC ≤ 1.0 addition; 1.0 < ΣFIC ≤ 4 indifference; ΣFIC > 4 antagonism (Bassolé et al., 2012; Mulyaningsih et al., 2010; van Vuuren et al., 2011). The same equation applies for ΣFFCIs determination.

2.3.5. Killing kinetics

An overnight yeast culture was diluted to 4.8 ± 0.3 $\log_{10}$ CFU/mL with acidified YPD (pH 4.0) and mixed with the most active prenylated isoflavonoids, glabridin and wighteone, at their MFC. Samples were incubated in duplicate at 30 °C/300 rpm. At different time points (0, 15 min, 30 min, 45 min, 60 min, 24 h and 48 h), 100 μL of culture medium were taken and decimally diluted in PPS. Dilutions were spread on YPD agar plates and incubated for 48 h at 30 °C, after which, colonies were counted. Sorbic acid at 1.65 mg/mL (=3 × MFC) was used as control antifungal agent. Samples of the sorbic acid-yeast mixture were taken at (0–48 h). Experiments were performed in three biological replicates against both yeast strains.

2.3.6. Membrane permeabilization

Quantification of membrane permeabilization was performed by measuring the fluorescence signal of propidium iodide (PI). PI is a fluorescence probe that intercalates to DNA through van der Waal's stacking (Banerjee et al., 2014). *Z. parabailii* (UL 3699) cell cultures grown for 30 h at 30 °C in YPD were centrifuged at 4900 rcf, 4 °C for 15 min. The pellet was washed twice with 9 mL PPS. Cells were re-suspended in acidified (pH 4.0) PPS (cell density 9.2 ± 0.2 $\log_{10}$ CFU/mL). One hundred μL of the cell suspension was incubated at room temperature (RT) with 90 μL of the most active compounds (glabridin or wighteone) at 6.25–50 μg/mL in a black well plate with clear bottoms. To limit the interaction of PI with prenylated isoflavonoids (Fig. A1), yeast cells were pre-incubated with prenylated isoflavonoids for 1 h, prior the addition of PI. Next, 10 μL of propidium iodide (PI) (4.5 μM final concentration) were added and the fluorescence signal was measured in a Spectramax ID3. Emission of fluorescence was measured every 2 min at 620 nm (bottom read mode) for 2 h, while exciting the samples at 520 nm. Photomultiplier tube detector gain was set to high. As a positive control, cells heated at 99 °C in a thermomixer for 10 min were used. Background fluorescence was accounted for by the use of controls and blanks (live cells with PI, without compounds; prenylated isoflavonoids alone; PI alone; and PPS alone). Viable cell counting was performed after 24 h. Experiments were performed in three biological replicates.

2.3.7. Fluorescence microscopy

Z. parabailii (UL 3699) cell suspensions (200 μL) were prepared as described in **2.3.6** and mixed with 180 μL stock compounds (final concentration 25 μg/mL). Different eppendorf tubes were used for the different time-points (from 5 to 180 min). Twenty μL PI (4.5 μM final concentration) was added to each mixture just before visualization of

the cells. Cells were visualized with an Olympus BX41 microscope (Olympus, Tokyo, Japan) and a red fluorescent protein (RFP) filter as described elsewhere (Araya-Cloutier et al., 2018a). Viable cell counts were performed at the different time-points. The experiment was performed in two biological replicates.

2.3.8. Transmission electron microscopy (TEM)

For TEM analysis, 10^9 CFU/mL *Z. parabailii* (UL 3699) cells grown in acidified YPD (pH 4.0) were treated with glabridin and wighteone at their MFC at these conditions (50 and 25 μg/mL, respectively). After specific time of exposure (from 5 to 180 min), (un)treated cells were centrifuged for 5 min at 4900 rcf at RT. The pellet was fixed with 2.5% (w/v) glutaraldehyde 0.1 M phosphate/citrate buffer, pH 7.2). Fixed cells were collected by centrifugation (4900 rcf, 5 min) and resuspended in 3% (w/v) gelatin in phosphate buffer (pH 7.2) and allowed to cool until solidified cubes formed. The cubes were manually sliced with a sharp razor blade into pieces of approximately 0.5 × 0.5 × 0.5 mm. The specimens were post-fixed in 1% OsO_4 (in 0.1 M phosphate/citrate buffer, pH 7.2) for 60 min at RT. Subsequently, the cells were dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 96%, 2 × 100% [v/v]). The dehydrated cells were then infiltrated in mixtures of 1:2, 1:1 and 1:2 resin:ethanol (30 min with each ratio). Then, the mixture was replaced with resin for 60 min and the cells were resuspended in fresh resin for overnight storage. The resin was polymerized for 8 h at 70 °C. Sections (50 nm coupes) were cut using a Leica EM UC7 microtome (Leica Microsystems B·V., The Netherlands) and post-stained with UranyLess staining and lead citrate. The ultrathin sections were observed with a JEOL JEM-1400 plus electron microscope (JEOL USA Inc., USA) at an acceleration voltage of 120 kV. Images correspond to different cells per sample.

2.3.9. Statistical analysis

Statistical analysis was performed using the SPSS statistics software package (Version 25, IBM, Armonk, NY, USA). Significant decrease in the viable counts during killing kinetic experiments was determined by independent samples t-tests between samples at t = 0 and samples from different time-points of exposure to any antimicrobial. The significance level used was 0.05. The standardized residuals and leverage values of the compounds needed for the William's plot in **2.3.3** were also calculated through SPSS.

3. Results

3.1. Antifungal potency of prenylated isoflavonoids against Z. parabailii

Fifteen mono-prenylated and four di-prenylated (iso)flavonoids were screened for their antifungal potency against *Z. parabailii* (UL 3699) (Table 1 and Fig. A2). Screening was performed at the native pH of the yeast growth medium, 6.5, to ensure that the influence of only one hurdle (i.e. prenylated (iso)flavonoids) on yeast growth was investigated.

Eight mono-prenylated isoflavonoids had a MIC value ≤ 25 μg/mL, which is associated with a good antimicrobial activity (Gibbons, 2004) (Table 1). The mono-prenylated isoflavones, wighteone (**9**) and luteone (**7**) and the mono-prenylated isoflavan glabridin (**3**) showed the highest antifungal activity with MICs of 3.13–6.25 μg/mL (9–18 μM), 12.5 μg/mL (35 μM) and 6.25–12.5 μg/mL (19–39 μM μg/mL), respectively. It should be noted that the primary focus of this study is the membrane-activity of prenylated (iso)flavonoids and thus only the undissociated species are considered. However, the highly active isoflavones have low pKas and are therefore largely dissociated at pH 6.5 (Table 1), implying that they might also act elsewhere than the membrane.

The four di-prenylated (iso)flavonoids tested in this study were not active against *Z. parabailii*, as they did not induce any significant change in the time to detection (TTD) at the maximum concentration tested (C_{max} 25 μg/mL) (Fig. A3). Lack of activity of di-prenylated molecules was independent of the subclass to which they belong (flavanone (**16**), isoflavan (**17**) and isoflavones (**18** and **19**)) or the prenylation patterns they bear (Fig. A2).

Table 1
Antifungal potency (in μg/mL) of mono- and di-prenylated (iso)flavonoids against *Z. parabailii* (UL 3699) at pH 6.5. When the MIC is depicted in between two tested values, then it means that no inhibition was observed in the lower value but killing was already observed in the higher one. The percentage of undissociated species of prenylated isoflavonoids at pH 6.5 is given in parentheses (only if this is less than 90%) (calculated using MarvinSketch 20.3). (–) means not tested.

No.	Subclass	Compound	MIC	MFC
Mono-prenylated				
1	Isoflavan	3′–OH–4′-*O*-methyl glabridin	≫25	–
2		4′-*O*-methyl glabridin	25	–
3		Glabridin	6.25 < MIC < 12.5	12.5
4	Isoflavene	Glabrene	25	50
5	Isoflavone	Isowighteone (69%)	12.5 < MIC < 25	25
6		Lupiwighteone (60%)	≫25	–
7		Luteone (58%)	12.5	25
8		Neobavaisoflavone (65%)	50	–
9		Wighteone (59%)	3.13 < MIC < 6.25	6.25
10	Pterocarpan	Glyceollidin II	≫25	–
11		Dehydrolyceollidin II	25 < MIC < 50	50
12	Pterocarpene	Dehydroglyceollin I	12.5 < MIC < 25	25
13		Dehydroglyceollin II	25	–
14		Dehydroglyceollin III	≫25	–
15		Dehydroglyceollin IV	≫25	–
Di-prenylated				
16	Flavanone	Glabrol	≫25	–
17	Isoflavan	Hispaglabridin B	≫25	–
18	Isoflavone	6,8-diprenylgenistein (49%)	≫25	–
19		6′-prenyl piscidone (68%)	≫25	–

* Measurements were performed in three biological replicates; the standard deviation between the triplicates for established MIC/MFC was 0.0.

3.2. (Quantitative) structure-activity relationships of mono-prenylated isoflavonoids against Z. parabailii

3.2.1. Development of binary classification QSAR

To obtain an insight in the overall molecular properties underlying antifungal activity, a QSAR model was developed using the activity data (MICs) of the 15 mono-prenylated isoflavonoids tested (Table 1). Mono-prenylated isoflavonoids had a range of activities and a variety of structural features (5 different isoflavonoid subclasses and different configuration and position of the prenyl group), aspects important during QSAR modeling. In contrast, the di-prenylated (iso)flavonoids were excluded for the QSAR analysis as they were unanimously inactive, irrespective of their structural features other than prenylation. A binary QSAR model was chosen (over for e.g. a continuous one) as it can accommodate the molecules for which no MIC was established. It should be noted that descriptors were calculated for the undissociated species of prenylated isoflavonoids, since these are expected to interact with the membrane. Genetic algorithm (GA) was used to select the two variables that best describe the dataset. *E_ele* and *vsurf_ID* descriptors were the most frequently selected variables by the GA (36% and 57% frequencies, respectively). *E_ele* and *vsurf_ID7* were used to construct a binary QSAR model which resulted in a 93% total accuracy, discriminating active and inactive compounds with high (cross-validated) accuracies, i.e. ≥ 86% (Table A1). The choice of the best QSAR model was also based on the quality of prediction of an external set of prenylated isoflavonoids tested against other yeasts (*S. cerevisiae* and *C. albicans*) (Table A2 and Fig. A4), which fell into the applicability domain of the developed model (Fig. A5). The proposed model predicted correctly the activity of 67% of the molecules tested against other yeast species (Table A2).

E_ele refers to the electrostatic component of potential energy and

vsurf_ID7 represents the hydrophobic integy moment at −1.4 kcal/mol, a measure of unbalance between the centre of mass of a molecule and the barycentre of the hydrophobic regions (Sharifi et al., 2014). Both descriptors were positively correlated to the antifungal activity. The two descriptors had a low correlation coefficient (Pearson coefficient < 0.4) and their importance in the binary model was equally high (35% for *E_ele* and 30% for *vsurf_ID7*).

3.2.2. Effect of electrostatic potential energy and hydrophobic integy moment on the activity of mono-prenylated isoflavonoids

Fig. 1 demonstrates the effect of location, presence and type of substituents on the electrostatic potential energy and hydrophobic integy moment and the activity of prenylated isoflavonoids per subclass against *Z. parabailii*.

In isoflavones (Fig. 1A), luteone (**7**), the only studied molecule with 4 free hydroxyl groups had the highest absolute *E_ele* value (Table A3). The *E_ele* values and the antifungal activity decreased as the number of hydroxyl groups decreased (for example, from (**7**) to (**9**) and from (**5**) to (**8**)) (Fig. 1A). Furthermore, wighteone (**9**) and luteone (**7**), both having A-prenylation, had higher *vsurf_ID7* values and higher antifungal activity than isowighteone (**5**) (B-ring prenylated) (Fig. 1A). Yet, *vsurf_ID7* does not distinguish prenylation at different locations of the A-ring since *C*6-prenylated molecules (as in (**7**) and (**9**)) had the same descriptor value as the *C*8-prenylated isoflavone (**6**), while the latter showed no activity (Table A3).

For pterocarpenes (Fig. 1B), methylation of a free –OH group at the *C*3 (as in dehydroglyceollin IV (**15**) from dehydroglyceollidin II (**11**)) negatively affected *E_ele* and abolished the antifungal activity (Fig. 1B). A furan-over a pyran-prenyl configuration decreased both the *E_ele* and the *vsurf_ID7*, cancelling the antifungal activity of dehydroglyceollin III (**14**) as compared to dehydroglyceollin II (**13**) (Fig. 1B).

In contrast, methylation of isoflavans (Fig. 1C) did not significantly influence *E_ele*, even though it decreased the antifungal activity more than 2-fold (4′-*O*-methyl glabridin (**2**) compared to glabridin (**3**)) (Fig. 1C). Strikingly, an additional –OH on the B-ring even decreased the value of the energy descriptor, abolishing the activity, despite the increase in *vsurf_ID7*.

Overall, *E_ele* seems to partially relate to the number of free –OH groups present in the molecules, whereas *vsurf_ID7* seems to correlate more with the location of the prenyl-group. However, isoflavans do not seem to follow the trends observed for isoflavones and pterocarpenes. Possibly, the absence of a double bond at the C-ring and the subsequent discontinued electron distribution might be associated with this deviating behaviour.

It should be noted that the di-prenylated isoflavones and isoflavans tested in this study had electrostatic potential energies (*E_ele*) ranging from −33 to −52 kcal/mol and hydrophobic integy moments (*vsurf_ID7*) ranging from 0.6 to 2.0 Å (Table A3). These values do not deviate from those of the mono-prenylated (iso)flavonoids, thus, do not explain the absence of activity based on the developed QSAR model.

3.3. Influence of low pH on the antifungal activity of prenylated isoflavonoids

Z. parabailii is thought to develop resistance mechanisms at low pH, such as activation of efflux pumps and alteration of membrane permeability (Lindahl et al., 2016; Stratford et al., 2013b, 2020). Thus, it was of high importance to assess the potency of prenylated isoflavonoids at low pH. For this, the molecules which demonstrated potency (MIC ≤ 50 μg/mL) at pH 6.5 were tested at the most relevant pH for products commonly contaminated by *Z. parabailii* (pH 4.0) and at an extreme pH where *Z. parabailii* has been previously shown to survive (pH 3.0) (Berry, 1979; Pitt et al, 1973, 1998) (Table 2).

Most prenylated isoflavonoids showed slightly higher MICs/MFCs towards lower pH. Prenylated isoflavonoids which were highly potent at pH 6.5 (Table 1), i.e. glabridin (**3**), luteone (**7**) and wighteone (**9**) retained their potency also at acidic pH, both at pH 4.0 and 3.0 (Table 2). These three molecules showed 2–44 times higher activity (MFCs) than the traditional food preservative, sorbic acid, at acidic pH (Table 2). The MICs of sorbic acid at the two acidic pH were in line with the MICs

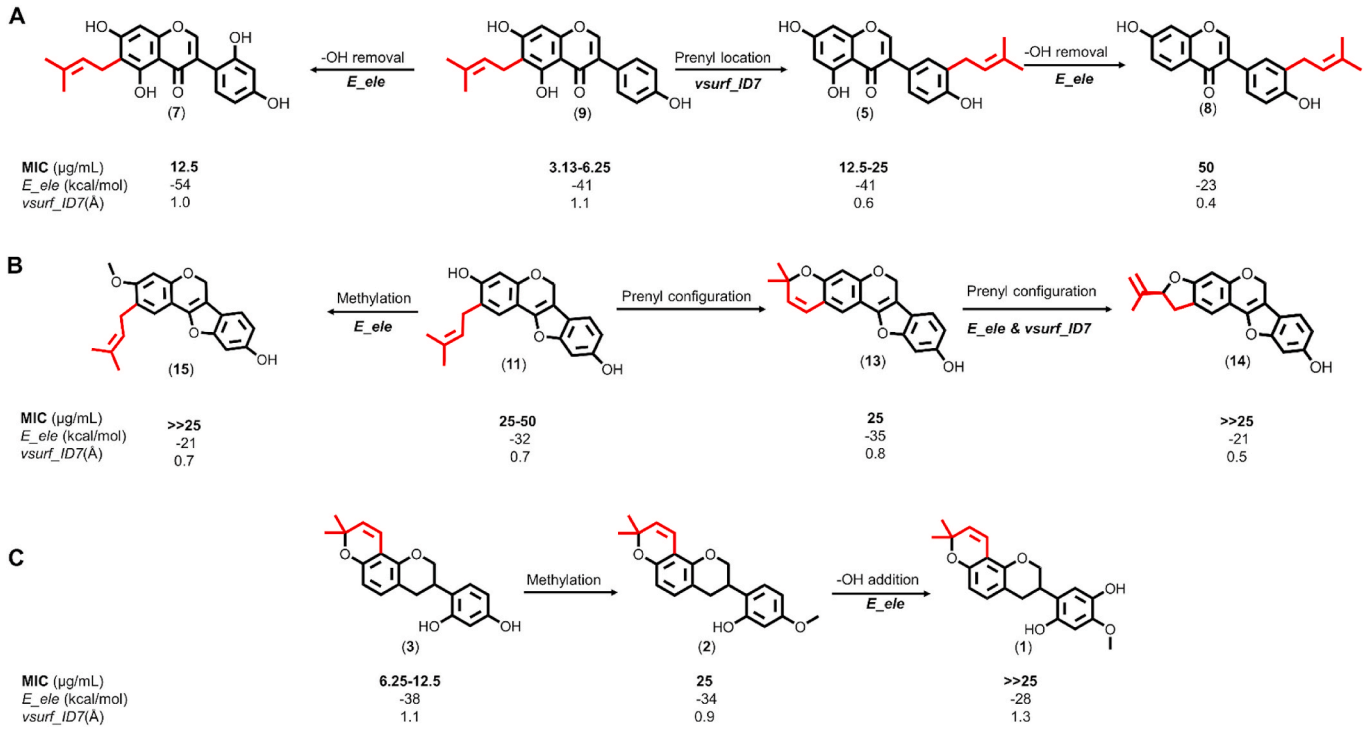

Fig. 1. Effect of type, location and configuration of substituents on the main QSAR-derived molecular properties (electrostatic potential, *E_ele* and hydrophobic integy moment, *vsurf_ID7*) and their subsequent effect on the activity of prenylated isoflavonoids of three subclasses; isoflavones (**A**), pterocarpenes (**B**) and isoflavans (**C**). The structural difference and the affected molecular property are illustrated above and below the arrows, respectively.

Table 2
Antifungal potency (in μg/mL) of mono-prenylated isoflavonoids against *Z. parabailii* (UL 3699) at pH 4.0 and 3.0. (−) means not tested. When the MIC is depicted in between two tested values, then it means that no inhibition was observed in the lower value but killing was already observed in the higher one. In parentheses, the percentage of undissociated sorbic at each pH is given (calculated using MarvinSketch 20.3). Prenylated isoflavonoids are fully protonated at these pH values.

			pH 4.0		pH 3.0	
No.	**Subclass**	**Compound**	**MIC**	**MFC**	**MIC**	**MFC**
3	Isoflavan	Glabridin	12.5	25	12.5 < MIC ≤ 25	33.3 [±11.8]
4	Isoflavene	Glabrene	50 < MIC < 75	75	125	150
5	Isoflavone	Isowighteone	50 < MIC < 75	75	50 < MIC < 75	75
7	Isoflavone	Luteone	25 < MIC < 37.5	37.5	25 < MIC < 37.5	37.5
8	Isoflavone	Neobavaisoflavone	>150	–	–	–
9	Isoflavone	Wighteone	6.25 < MIC < 12.5	12.5	12.5	25
12	Pterocarpene	Dehydroglyceollin I	>100	–	–	–
13	Pterocarpene	Dehydroglyceollin II	>100	–	–	–
15	Preservative	Sorbic acid	275 (86%)	550 (86%)	138 (98%)	275 (98%)

* 4′-*O*-methyl glabridin (**2**) and dehydroglyceollidin II (**11**) were not tested at low pH due to limited purified amounts.
** Measurements were performed in three biological replicates; the standard deviation between the triplicates for established MIC/MFC was 0.0. If not, then the mean concentration is shown together with its standard deviation in brackets.

reported by Fujita et al. (2005). Neobavaisoflavone (**8**) and the pterocarpenes (dehydroglyceollin I and II, **12** and **13**) became completely inactive (MICs >100 μg/mL) at pH 4.0 and thus were not tested at pH 3.0 (Table 2).The potency of prenylated isoflavonoids was also confirmed against the reference strain (ATCC 60483) (Table A4) at its most relevant pH conditions (pH 4.0).

3.4. Killing kinetics

The inactivation rate of *Z. parabailii* at pH 4.0 by the two most active prenylated (iso)flavonoids, glabridin (**3**) and wighteone (**9**) at their corresponding MFCs at pH 4.0 (25 and 12.5 μg/mL), respectively, is shown in Fig. 2.

Within the first 15 min, both prenylated isoflavonoids significantly (p-value < 0.001) reduced the viable counts by 99.99% of UL 3699. This fast killing was confirmed with the ATCC 60483 strain (Fig. A6), although for glabridin a statistically significant reduction in the valuable counts was only observed within 30 min (p-value 0.018). In contrast, sorbic acid showed slower killing rates for the two isolates. Three hours were needed for a significant (p-value 0.048) 99% inactivation of the UL 3699 isolate by sorbic acid, whereas 24 h were needed for 97% inactivation of the ATCC 60483 strain (p-value 0.002) at a sorbic acid concentration of 1650 μg/mL (Fig. A6). The latter finding is in line with the slow killing activity of sorbic acid (1600 μg/mL) against *Z. parabailii* (ATCC 60483), which was observed within 12 h (Fujita et al., 2005) (no intermediate time-point was obtained between 6 and 24 h in this study).

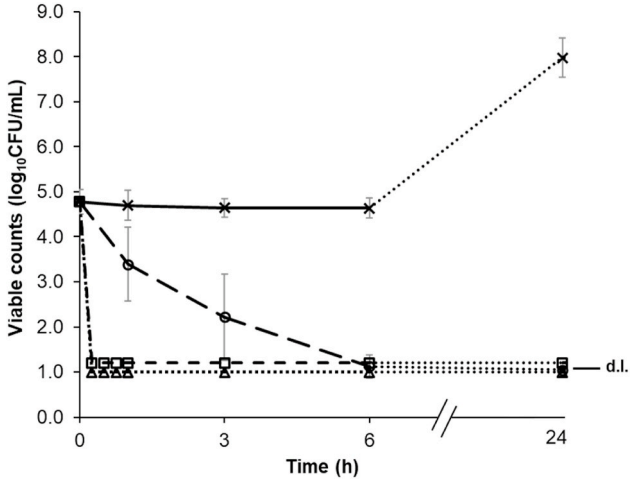

Fig. 2. Inactivation kinetics of *Z. parabailii* (UL 3699) cells (10^5 CFU/mL) at pH 4.0 in the presence of 12.5 μg/mL wighteone (dashed line with triangle markers), 25 μg/mL glabridin (squared dotted line with square markers) and sorbic acid at a concentration of 1650 μg/mL (long dashed line with circle markers). Control cells (solid line) are also depicted. Error bars indicate the standard deviation of three biological replicates (when error bars are not visible, these are smaller than the symbol size). d.l. refers to the detection limit.

3.5. Combination experiments

Given that prenylated isoflavonoids are thought to act in a different fashion to sorbic acid (putative membrane activity versus cytoplasmic acidification and generation of oxidative stress, respectively) (Araya-Cloutier et al., 2018b; Stratford et al., 2013a, 2020), we explored the synergistic potential of their mixture. However, when the two prenylated (iso)flavonoids were (separately) combined with sorbic acid, more than half of the corresponding MFC of the agents was needed to induce complete lethality, suggesting an indifferent effect between the two agents (Fig. A7).

3.6. Membrane permeabilization

We assessed the membrane permeabilization capacity of the two most active prenylated isoflavonoids against *Z. parabailii* in a qualitative and a quantitative way. In both cases, the fluorescent probe, propidium iodide (PI) was used to stain damaged cells. PI staining, and therefore *Z. parabailii* membrane permeabilization, started within 15 min of exposure to the two prenylated isoflavonoids at pH 4.0 (Fig. 3A) and increased with time of exposure. For glabridin-treated cells, intracellular material (most probably including PI) leaked out of the cells within 1 h (Fig. 3A), accompanied with 99.99% decrease in the viable counts (Fig. 3C). Leakage of intracellular material is more pronounced in wighteone-treated cells, as no PI stain was visible after 30 min, accompanied with 99% decrease in cell viability. In contrast, heated cells maintained their integrity (although metabolically inactive as determined by plate counting), retaining the PI taken up intracellularly (Fig. 3A). This is in line with the higher accumulation of PI in cells exposed to glabridin in time compared to those exposed to wighteone, even though these were still lower than in heated cells (Fig. 3B). Similar (quantitative and qualitative) observations were obtained at pH 7.2 (Fig. A8 and A9). Sorbic acid did not cause any PI uptake at pH 4.0 at a concentration sufficient to kill (data not shown). These findings agree with the generally accepted mechanism that weak organic acids act through acidification of the cytoplasm by diffusing easily through the membrane.

3.7. Transmission electron microscopy

Transmission electron microscopic (TEM) images were obtained after 15 min and 180 min of exposure to the two most active prenylated isoflavonoids. Prenylated isoflavonoids, at their MFC, induced

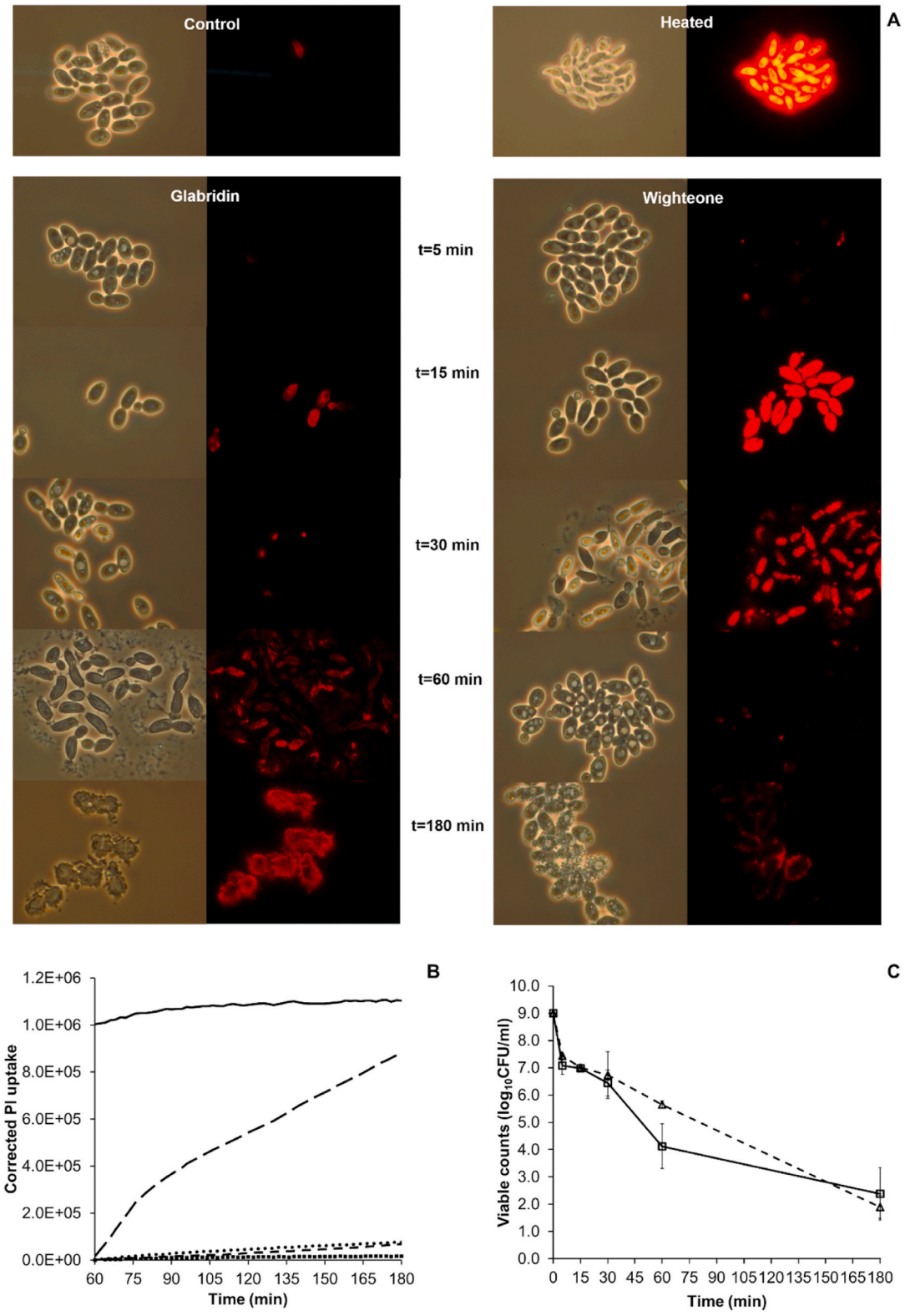

Fig. 3. Light and fluorescence microscopy images of *Z. parabailii* (10^9 CFU/mL) at different time points after exposure to 25 μg/mL of glabridin and wighteone. Control and heated cells are shown after 180 min (**A**). Propidium iodide uptake in time after incubating the yeast cells for 1 h at pH 4.0 with different concentrations of glabridin (dashed lines) and wighteone ((squared) dotted lines); small dashes and squared dotted lines correspond to 25 μg/mL prenylated isoflavonoid whereas long and dotted lines correspond to 50 μg/mL prenylated isoflavonoid. Heated cells (solid line) served as the positive control and the fluorescence signal of PI from untreated cells was subtracted from the signal of the treated cells. Data are means of three biological replicates (**B**). Viable cell counting after exposure to 25 μg/mL of glabridin and wighteone over time (solid line represents glabridin and dashed line represents wighteone). Error bars indicate the standard deviation of two biological replicates (when error bars are not visible, these are smaller than the symbol size) (**C**).

disruption of the membrane integrity compared to the control cells (exemplified in Fig. 4). Glabridin distorted the order in the membrane within the first 15 min (Fig. 4A and A′) and induced large discontinuities (pores (Aleksić et al., 2014)) after 180 min (Fig. 4B and B′). Wighteone-treated cells showed membrane elongations engulfing a cluster (Fig. 4C) and some more vaguely visible protrusions together with membrane discontinuities (Fig. 4C'). After 180 min, cells treated with wighteone completely lost their cell membrane (Fig. 4D and D'). More images can be found in Fig. A10.

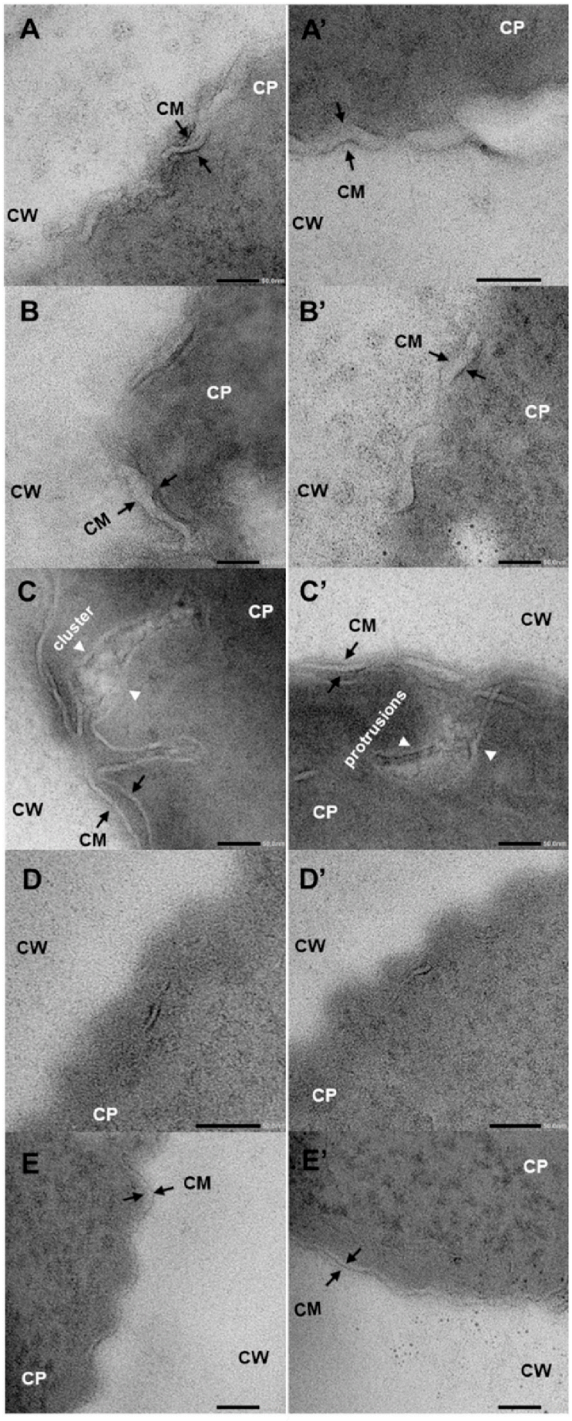

Fig. 4. Representative TEM images (black scale bars at 50 nm) of cells (10^9 CFU/mL) exposed to 50 μg/mL glabridin (**A**, **B**) and 25 μg/mL wighteone (**C**, **D**) for 15 (**A**, **C**) and 180 min (**B**, **D**), compared to untreated cells at 180 min (**E**). Letters with a prime refer to duplicates. Arrows point to the cell membrane (CM), CP stands for the cytoplasm and CW designates the cell wall. More images can be found in Fig. A10.

4. Discussion

4.1. Mono-prenylated isoflavonoids have superior anti-Z. parabailii activity to di-prenylated isoflavonoids

Ten mono-prenylated isoflavonoids from four different subclasses showed moderate to good antifungal activity against *Z. parabailii* (MIC ≤ 50 μg/mL) at pH 6.5. The A-ring prenylated isoflavone, wighteone (**9**) and the prenylated isoflavan, glabridin (**3**), in particular, showed the lowest MICs (MIC < 12.5 μg/mL), classifying them as very active antimicrobials (Gibbons, 2004). The fungicidal activity of wighteone and glabridin was 4–8 times higher than that of polygodial (MIC 50 μg/mL, 213 μM) at pH 6.5, the most potent natural agent against *Z. parabailii* reported so far (Fujita et al., 2005).

In contrast, the di-prenylated analogue of wighteone, 6,8-diprenyl genistein (**18**) and of glabridin, hispaglabridin B (**17**) (along with two other di-prenylated isoflavonoids, (**16**) and (**19**)) did not exert any antifungal activity at Cmax, MIC ≫ 25 μg/mL. The differential activity of mono- and di-prenylated (iso)flavonoids has not been systematically studied in yeasts, and in *Z. parabailii* in particular. Most di-prenylated (iso)flavonoids found in literature were also inactive against other yeast species (for an overview see Table A5). The absence of activity of isoflavones (**18**) and (**19**) is in line with the absence of activity (up to 60 μg/mL) of the structural analogue flavones, morusin and kuwanon C against *C. albicans* (Sohn et al., 2004) (Fig. A11). Similarly, the lack of activity of the flavanone, glabrol (**16**) found in this study, is corroborated by the lack of activity of the flavanones, euchrestaflavanone A and lonchocarpol A (MIC > 200 μg, determined by the TLC method) against *Cladosporium herbarum* reported in literature (Tahara et al., 1994) (Fig. A11).

Active mono-prenylated isoflavonoids tested in this study have a *logD* value of ≤ 4.5. Possibly, very hydrophobic molecules (*logD* > 5.0) are not able to cross the hydrophilic, complex cell wall of yeasts. Yet, the three di-prenylated (iso)flavonoids reported to inhibit the growth of *S. cerevisiae* and/or *C. albicans* (**D3**, **D7** and **D8**, Table A5) had *logD* values ranging from 5.0 to 6.1, implying that there might be additional factors that influence the activity of di-prenylated (iso)flavonoids against yeasts.

4.2. The interplay between hydrogen bonding and hydrophobic integy moment dictates the potency of mono-prenylated isoflavonoids

In Fig. 5, the relationship between the two descriptors derived from the QSAR model (*E_ele* and *vsurf_ID7*) for prenylated isoflavonoids with promising activity (MIC ≤ 50 μg/mL at pH 6.5) is displayed. Furthermore, the extent of decrease in activity (log reduction in MFC, ΔMFC) of the compounds at lower pH is depicted with different colors or patterns.

Electrostatic interactions (*E_ele*) are expressed through hydrogen-bonding in (iso)flavonoids, given that only the undissociated species are expected to interact with the membrane (Cramariuc et al., 2012). Hydrogen bonding is generally known to stabilize the interacting molecules with the polar head groups of the membrane (Li et al., 2018). Besides, intermolecular hydrogen-bonding between prenylated phenolics has been proposed to shield polarity of the free hydroxyl groups inside the membrane (Li et al., 2018). The location of hydrophobic moieties (shown to be associated with *vsurf_ID7,* in Fig. 1) might be important for effective hydrophobic interactions with the interior (lipid core) of the membrane. These molecular properties are frequently related to antimicrobial activity of prenylated (iso)flavonoids (Araya-Cloutier et al., 2018b; Sadgrove et al., 2020). Balance between these two properties is considered optimal for effective interactions with the membrane (Araya-Cloutier et al., 2018b; Fujita et al., 2008; Sadgrove et al., 2020).

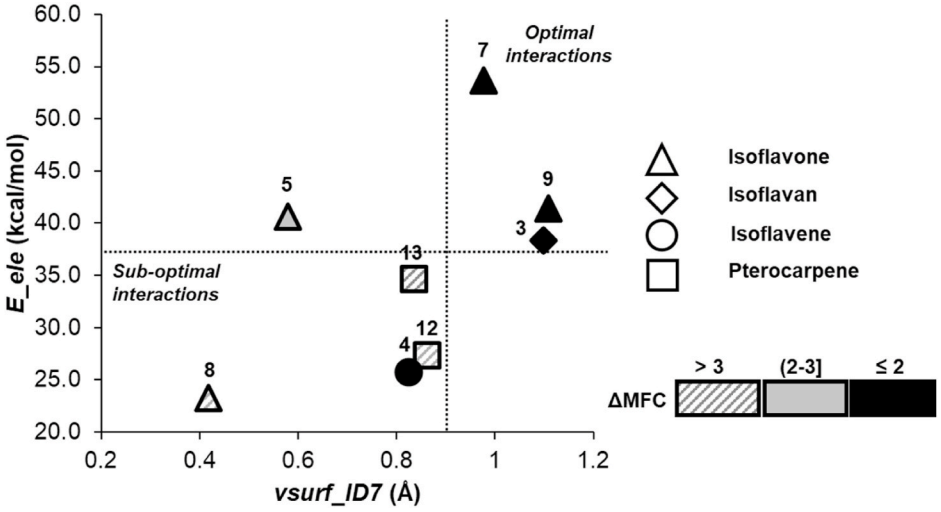

Fig. 5. Relationship between the two descriptors derived from the QSAR model (*E_ele* and *vsurf_ID7*) and the decrease in the antifungal activity (from pH 6.5 to pH 4.0) of prenylated isoflavonoids with promising potency (MIC $\leq$ 50 μg/mL) at pH 6.5. The extent of activity decrease upon lowering the pH (log reduction in MFC, ΔMFC) is shown with different fill colors or patterns; black color denotes a ΔMFC $\leq$ 2, grey color a 2 < ΔMFC $\leq$ 3 and patterned fill is used to represent a ΔMFC > 3. Numbers on top of the symbols refer to compounds and structures in Fig. A2 and symbols represent the isoflavonoid subclasses. Dashed lines suggest the descriptor thresholds for optimal and sub-optimal interactions of prenylated isoflavonoids with the membrane.

Based on the current set of prenylated isoflavonoids, highly active molecules, such as luteone (**7**), wighteone (**9**) and glabridin (**3**), had *E_ele* values of $\geq$ 38 kcal/mol and *vsurf_ID7* values of > 0.9 Å (Fig. 5 and Table A3). These molecules are expected to interact well with the membrane via hydrogen-bonding and hydrophobic interactions. Based on Li et al. (2018) and the number of free hydroxyl groups in the molecules, molecules (**7**) and (**9**) are expected to form clusters inside the membrane, whereas molecule (**3**) might also do it but to a lesser extent. The B-ring prenylated isoflavone, isowighteone (**5**), scored equally high in *E_ele* as (**7**) and (**9**), but moderately in *vsurf_ID7*, suggesting effective hydrogen-bonding, but without having optimal orientation of the prenyl group, which may affect its interaction with the interior of the membrane. Molecules, such as the A-ring prenylated pterocarpenes (**12** and **13**) and the A-ring prenylated isoflavene (**4**) scored moderately, whereas the B-ring prenylated isoflavone, neobavaisoflavone (**8**), scored low on both descriptors. These molecules are expected to make moderate to poor interactions with the membrane, respectively.

The mono-prenylated (iso)flavonoids with promising activity (MIC $\leq$ 50 μg/mL) at pH 6.5 showed a lower, although to different extents, antifungal activity at pH 4.0 (shown with different colors or patterns in Fig. 5) irrespective of the subclass. Given the different pKa of the molecules from different subclasses (only isoflavones are charged at pH 6.5), it is unlikely that pH-dependent changes in the compounds are responsible for the decrease in activity. Instead, the cell envelop of *Z. parabailii* is known to undergo remodelling, as a resistant mechanism to cope with acidic stress (Kuanyshev et al., 2016; Lindahl et al., 2016). At low pH, the membrane has been reported to become enriched in saturated (highly ordered (Simons et al., 2011)) glycerophospholipids and complex sphingolipids, which can result in reduced porosity and diffusion of molecules (Lindberg et al., 2013). Upon re-ordering of the membrane, interactions with prenylated isoflavonoids might be hindered.

Compounds with high scores on both descriptors (*E_ele* > 38 kcal/mol and *vsurf_ID7* > 0.9 Å) and thus better proposed interactions with the membrane, showed the least change in activity at lower pH (ΔMFC $\leq$ 2, black filled symbols in Fig. 5). Molecules scoring sub-optimally on one descriptor (such as molecule (**5**)) or on both descriptors (such as molecules (**8**), (**12**) and (**13**)) showed moderate (2 < ΔMFC $\leq$ 3, grey filled symbols in Fig. 5) and detrimental activity change (ΔMFC > 3, patterned symbols in Fig. 5), respectively. The only exception to the above is molecule (**4**). Performing this analysis with more representatives from the different subclasses would help to establish more generalized trends.

4.3. The most active prenylated isoflavonoids induce different membrane deformations in the cell membrane of *Z. parabailii*

The most potent prenylated isoflavonoids which maintained their high activity also at low pH (MIC $\leq$ 25 μg/mL) induced rapid killing of the yeast (within 15 min). Membrane permeabilization experiments confirmed fast permeabilization (within 1 h) of the membrane of *Z. parabailii* by glabridin (**3**) and wighteone (**9**), which was accompanied by complete lethality of Z. *parabailii* cells. Fast membrane distortion (within 15 min) after exposure to a killing concentration of the two compounds, observed through TEM imaging, corroborated the killing kinetics and the membrane permeabilization. Disruption of the membrane integrity as the primary mode of antimicrobial action of prenylated (iso)flavonoids against *Z. parabailii* confirms what was shown against bacteria and *C. albicans* (Araya-Cloutier et al., 2018a; Liu et al., 2014; Mun et al., 2014; Pang et al., 2019). Nevertheless, differences in the yeast cell membrane were observed through TEM imaging when cells were treated with wighteone or glabridin. The membrane elongations and the cluster observed in wighteone-treated yeast cells is reminiscent to endocytosis-dependent changes in the membrane (Buser et al., 2013). Endocytosis has been associated with the antifungal action of some antimicrobial peptides (Rossignol et al., 2011). The possibility of endocytosis-dependent antifungal activity of wighteone and the reason behind the differences induced by the two prenylated isoflavonoids require further investigation.

5. Conclusion

This is the first report on the antifungal properties of prenylated (iso) flavonoids against the common food spoilage yeast, *Z. parabailii.* Wighteone and glabridin were the most active prenylated isoflavonoids (MFC 12.5–25 μg/mL, 37–77 μM, respectively at pH 4.0) and their activities are superior to any other natural antifungal reported so far and over 20 times (on weight basis) higher than sorbic acid against *Z. parabailii*. The antimicrobial properties of prenylated isoflavonoids were correlated with electrostatic potential energy and hydrophobic integy moment. Wighteone and glabridin scored high on those properties which seem to be related to membrane-activity. The effect of the two molecules on the membrane showed some differences, as visualized through TEM imaging. Prenylated isoflavonoids are promising antimicrobial agents with a distinct mode of action compared to traditional food preservatives. Study of the interaction of prenylated isoflavonoids with other components in food matrices and their potency towards a wider range of *Z. parabailii* strains is necessary to understand their full potential as novel food preservatives.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Sylvia Kalli: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft. **Carla Araya-Cloutier:** Conceptualization, Methodology, Writing – review & editing. **John Chapman:** Conceptualization, Supervision. **Jan-Willem Sanders:** Conceptualization, Supervision. **Jean-Paul Vincken:** Writing – review & editing, Supervision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.108434.

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