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Unravelling the potential of natural chelating agents in the control of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms

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A R T I C L E I N F O

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A B S T R A C T

Iron is essential for the formation, maturation and dispersal of bacterial biofilms, playing a crucial role in the physiological and metabolic functions of bacteria as well as in the regulation of virulence. Limited availability of iron can impair the formation of robust biofilms by altering cellular motility, hydrophobicity and protein composition of the bacterial surface. In this study, the antibiofilm activity of two natural iron chelating agents, kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) and maltol (3-hydroxy-2-methyl-4-pyrone), were investigated against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In addition, the ability of these 2-hydroxy-4-pyrone derivatives in preventing and eradicating *S. aureus* and *P. aeruginosa* biofilms through the enhancement of the efficacy of two antibiotics (tobramycin and ciprofloxacin) was explored. The iron binding capacity of the kojic acid and maltol was confirmed by their affinity for iron (III) which was found to be about 90 %, comparable to the regular chelating agent ethylenediaminetetraacetic acid (EDTA, 89 %). The antibiofilm efficacy of 2-hydroxy-4-pyrone derivatives, alone and in combination with antibiotics, was evaluated by measuring the total biomass, metabolic activity, and culturability of biofilm cells. Furthermore, their impact on the membrane integrity of *S. aureus* biofilm cells was investigated using flow cytometry and epifluorescence microscopy with propidium iodide staining. It was also examined the ability of 2-hydroxy-4-pyrone derivatives and 2-hydroxy-4-pyrone derivate-antibiotic dual-combinations in inhibiting the production of virulence factors (total proteases, lipases, gelatinases and siderophores) by *S. aureus*. Regarding biofilm formation, the results showed that 2-hydroxy-4-pyrone derivatives alone reduced the metabolic activity of *S. aureus* biofilm cells by over 40 %. When combined with tobramycin, a 2-log (CFU cm^{-2}) reduction in *S. aureus* biofilm cells was observed. Moreover, the combination of maltol and kojic acid with ciprofloxacin prevented *P. aeruginosa* biomass production by 60 %, compared to 36 % with ciprofloxacin alone. In pre-established *S. aureus* and *P. aeruginosa* biofilms, selected compounds reduced the metabolic activity by over 75 %, and a 3-log (CFU cm^{-2}) reduction in the culturability of biofilm cells was noted when kojic acid and maltol were combined with antibiotics. Moreover, 2-hydroxy-4-pyrone derivatives alone and in combination with tobramycin, damaged the cell membranes of pre-established biofilms and completely inhibited total proteases production. Despite the increasing of reactive oxygen species production caused by the cellular treatment of maltol, both 2-hydroxy-4-pyrone derivatives showed good safe profile when tested in human hepatocarcinoma (HepG2) cells. The pre-treatment of HepG2 cells with both compounds was crucial to prevent the cellular damage caused by iron (III). This study demonstrates for the first time that the selected 2-hydroxy-4-pyrone derivatives significantly enhance the antibiofilm activity of tested antibiotics against *S. aureus* and *P. aeruginosa*, highlighting their potential as antibiotic adjuvants in preventing and eradicating biofilm-related infections.

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1. Introduction

Bacterial infections associated with biofilms represent a major challenge for modern medicine. About 80 % of bacterial infections are estimated to be associated with biofilms [1]. Bacterial biofilms are structured communities of bacteria that adhere to surfaces (biotic and abiotic) or form as aggregates in fluid suspension, surrounded by a protective extracellular matrix produced by themselves [2,3]. In the sessile state, the bacterial cells develop a high resistance and tolerance (*i.e.* recalcitrance) to host defences and various antibacterial agents [4]. Therefore, higher doses, combinations of antibiotics and longer treatment durations are often required to achieve the bactericidal concentrations necessary to eradicate biofilms. However, due to the toxicity and side effects of antibiotics as well as impaired kidney and liver function, this is difficult to achieve with existing therapeutic options [5].

Iron plays a crucial role in the formation and maturation of bacterial biofilms [6]. This element is essential for several bacterial physiological functions, including energy metabolism, oxygen transport, regulation of gene expression and stabilisation of the polysaccharide matrix of biofilms [7]. Furthermore, iron can promote the motility of bacterial cells and prevent the formation of durable biofilms in places where iron is not present [8]. This limitation of biofilm formation under iron starvation is also associated with the reduction of the hydrophobicity of the microbial surface by altering the protein composition [9].

Iron is one of the most difficult nutrients for bacteria to obtain, as it is only bioavailable to a limited extent, mainly due to the formation of insoluble ferric hydroxides under aerobic conditions [10]. In the host, the uptake of iron becomes even more difficult as the available iron is bound to the proteins transferrin, haemoglobin and lactoferrin, which prevent direct use by the bacteria [6]. To overcome this obstacle, bacteria have developed sophisticated mechanisms such as the production of siderophores. These molecules with a high affinity for iron have the main function of chelating this ion from the surrounding environment and transporting it back into the bacterial cells [11]. In addition, iron plays a decisive role in the bacteria virulence. The availability of iron can modulate the expression of genes involved in the production of virulence factors such as toxins and enzymes that facilitate colonisation and evade the host immune system's defences [12].

The signalling role of iron in biofilm formation is well documented in several bacterial species, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* [13]. Iron is crucial in the formation of biofilms by *S. aureus*, especially for strain SA113, where it is necessary for the primary fixation and production of polysaccharide intercellular adhesin (PIA), a fundamental component of the *S. aureus* biofilm matrix [13]. However, the availability of iron affects other strains in different ways. In the Newman *S. aureus* strain, for example, the presence of iron prevents biofilm formation. Under conditions of iron deficiency, this bacterium expresses two virulence factors, *Eap* and *Emp*, which are essential for biofilm formation [14]. Moreover, *S. aureus* uses two different pathways for iron acquisition: the production of siderophores (staphyloferrin A and staphyloferrin B) and the Isd system [14]. It is important to emphasise that the inactivation of any of these pathways restricts the colonisation of *S. aureus*, highlighting its significance in the pathogenicity and virulence of this bacterium [15].

On the other hand, in *P. aeruginosa*, the increase of iron in the airways in cystic fibrosis may be a crucial factor facilitating the initial establishment and chronicity of the infection [16]. Specifically, increased iron concentration in human cystic fibrosis airway epithelial cells has been shown to promote robust biofilm formation by *P. aeruginosa*. Just as iron limitation can impair biofilm formation in *P. aeruginosa*, excess iron can also have an inhibitory effect [16]. *P. aeruginosa* had two important siderophores for iron acquisition, pyoverdine and pyochelin. In addition to their function as iron ion chelators, these molecules play an important role in the virulence and pathogenicity of *P. aeruginosa* [17]. In this sense, pyoverdine can act as a signalling molecule for the production of other virulence factors such as the endoproteinase PrpL and exotoxin A. In turn, the production of pyochelin can contribute to the persistent inflammatory response that causes tissue damage in chronic infections, such as in patients with cystic fibrosis [18]. Furthermore, these siderophores can also chelate metals other than iron (III). For example, pyoverdine can chelate Zn2+, Cu2+ and Mn2+ ions from the extracellular environment, but with lower affinity compared to iron ions [19]. In addition to its well-documented role, extracellular DNA (eDNA) also acts as a chelating agent for cations in the biofilms of *P. aeruginosa* and enhances resistance to antimicrobial peptides and antibiotics [20].

Iron chelating agents, which limit the availability of iron to bacteria by forming stable complexes, offer a new therapeutic way by interfering with biofilm formation and production of virulence factors [6]. Furthermore, chelating agents make bacteria more susceptible to the effects of antibiotics by acting as adjuvants for these antibacterial agents and disrupting the favourable environment of biofilms [7]. Chelators can be categorised according to their binding properties to iron, with each contributing uniquely to their antibacterial efficacy [21]. This includes several synthetic iron chelators such as deferiprone, deferasirox and deferoxamine, which are currently used in clinical practice for the treatment of iron overload [21,22]. In addition, these agents have moderate antimicrobial activity, making them potential candidates for the treatment of iron-dependent bacterial biofilms. Deferiprone and deferasirox cannot bind to all six coordination sites of the iron molecule, as they are bidentate and tridentate molecules, respectively. In contrast, deferoxamine is a hexadentate chelating agent that can bind to all six coordination sites [22]. Despite their clinical use, these agents can be toxic and may cause adverse effects, particularly with prolonged use [23]. A notable example was seen in patients who transitioned from first-line therapies to deferiprone, with studies revealing inadequate hepatic iron reduction, new-onset diabetes, and hepatic dysfunction [23].

In this context, natural chelating agents are proving to be a more promising strategy. They are generally less toxic and more biodegradable, which reduces their impact on the environment [24]. They are also generally more biocompatible, which minimises the risk of side effects on the body [25]. 2-hydroxy-4-pyrone derivatives are secondary metabolites of plants or fungi that contain an unsaturated six-membered ring structure with an oxygen atom and a ketone group (Fig. 1) [26,27]. Among the 2-hydroxy-4-pyrone derivatives, maltol and kojic acid are widely used in the food industry, cosmetics and pharmaceuticals [24,27,28]. Maltol, obtained from larch bark, and kojic acid, a secondary metabolite produced by fungi of the genus *Aspergillus*, are particularly characterised by their ability to suppress bacterial virulence factors and chelate iron ions [24,27]. Despite its known properties, there are remarkably few studies investigating its potential as an antibacterial and antibiofilm agents.

Therefore, this study investigated the antibiofilm effect of two natural and structurally analogous 2-hydroxy-4-pyrone derivatives, maltol and kojic acid, against the bacteria *S. aureus* and *P. aeruginosa* was investigated. Affinity studies for the iron (III) were also performed to confirm the chelating activity of maltol and kojic acid. In addition, the ability of the selected compounds to enhance the efficacy of the antibiotics tobramycin and ciprofloxacin in preventing and eradicating biofilms of the selected bacteria was explored. Membrane integrity studies and evaluation of virulence factors produced by *S. aureus* were performed for the 2-hydroxy-4-pyrone derivatives individually and in combination with tobramycin. The cytotoxicity and the chelating capacity against iron (III) were also evaluated using an *in vitro* hepatic cell model.

2. Materials and methods

2.1. Bacteria and culture conditions

S. aureus from the Spanish Type Culture Collection (CECT 976) and

P. aeruginosa from the American Type Culture Collection (ATCC 10145) were used. These bacterial strains had already been used as model microorganisms for antibacterial tests with phytochemicals and antibiotics [17,29–31]. The strains were preserved at - 80 °C in Mueller-Hinton Broth (MHB, Merck, Darmstadt, Germany) with 30 % (v/v) glycerol (VWR, Belgium) and subcultured in MH agar (MHA, Merck, Darmstadt, Germany) before the experiments. The working bacterial suspensions were obtained from overnight growth (16–18 h) in MHB at 37 °C with agitation (150 rpm, AGITORB 200, Aralab, Rio de Mouro, Portugal).

2.2. Preparation of chelating agents and antibiotics solutions

Two natural products from the pyrone class, maltol and kojic acid, were selected for the biofilm prevention and eradication experiments. Maltol was purchased from Sigma-Aldrich (USA) and kojic acid from Tokyo Chemical Industry Co, Ltd (TCl, Tokyo, Japan). The stock solutions of the 2-hydroxy-4-pyrone derivatives were prepared in sterile distilled water or dimethyl sulphoxide (DMSO, Avantor, VWR, Radnor, PA, USA) immediately before use. The proportion of DMSO never exceeded 9 % (v/v) of the final volume of the bacterial suspensions, which was necessary to ensure the adequate dissolution of the selected compounds at 10,000 μg mL^{-1} used in the biofilm eradication assays. The antibiotics ciprofloxacin and tobramycin were purchased from Sigma-Aldrich (USA) and TCl (Tokyo, Japan), respectively. The antibiotics stock solutions were prepared in sterile distilled water according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and stored at - 20 °C until use [32]. Ethylenediaminetetraacetic acid (EDTA) was purchased from Panreac (Spain) and prepared in sterile distilled water at a concentration of 71 μg mL^{-1} [33]. This chelating agent was used as a positive control for assessing iron chelating activity.

2.3. Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the selected 2-hydroxy-4-pyrone derivatives and antibiotics was determined using the broth microdilution method according to Borges *et al.* [29]. In brief, after overnight bacterial growth at 37 °C in MHB, the optical density (OD, λ = 600 nm) was adjusted to 0.132 ± 0.02 (10^8 and 10^6 CFU mL^{-1} for *S. aureus* and *P. aeruginosa*, respectively) using fresh MHB. Sterile 96-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) were then filled with 180 μL of bacterial suspension and 20 μL of selected phytochemicals/antibiotics (10 % v/v of the well volume). Different concentrations of kojic acid and maltol (6.25–1000 μg mL^{-1}) and antibiotics (0.0625–1024 μg mL^{-1}) were used for each bacterium. Cell suspensions without phytochemicals/antibiotics were used as negative controls. Absorbance (λ = 600 nm) was measured before (t = 0 h) and after 24 h of incubation at 37 °C on an orbital shaker (150 rpm) using a microplate reader (Synergy HT, Biotek, Winooski, VT, USA). The MIC corresponds to the lowest concentration that inhibits bacterial growth (when the final DO is equal to or lower than the initial DO).

2.4. Determination of the minimum bactericidal concentration

To determine the minimum bactericidal concentration (MBC) of each compound, 10 μL of the contents of the wells corresponding to concentrations of 2-hydroxy-4-pyrone derivatives/antibiotics equal to or greater than the MIC were removed and plated on plate counter agar (PCA, Avantor, VWR, Radnor, PA, USA) and incubated at 37 °C for 24 h. The MBC of 2-hydroxy-4-pyrone derivatives/antibiotics is the lowest concentration tested at which complete inhibition of bacterial growth is observed [34]. After the determination of the MIC and MBC, these concentrations were used as the basis for further experiments.

2.5. Determination of the iron binding capacity of 2-hydroxy-4-pyrone derivatives

The ability of kojic acid and maltol to mobilise complexed iron was evaluated by adapting the test of Schwyn and Neilands [35] to the microplate system. Sterile microtiter plates were filled with 180 μL of selected compounds at different concentrations (6.25–1000 μg mL^{-1}) and with 20 μL of chrome azurol sulphonate (CAS, Fluka, Switzerland) solution. To prepare the CAS solution, 60.5 mg CAS was dissolved in 50 mL water and mixed with 10 mL iron (III) solution (1 mM $FeCl_3 \cdot 6H_2O$, (VWR, Germany), 10 mM hydrochloric acid (HCl)). This solution was added to 72.9 mg hexadecyltrimethylammonium bromide (HDTMA, Merck, Germany) dissolved in 40 mL water under stirring. The microtiter plates were then incubated for 30 min at 37 °C and the absorbance (λ = 630 nm) was measured after the incubation period using a microplate reader. Wells containing only CAS solution with EDTA (71 μg mL^{-1}) were used as positive control and wells with CAS solution without hydroxy-4-pyrone derivatives or EDTA were used as negative control [33]. The iron binding capacity was evaluated as the ability of the compounds to mobilise complexed iron, calculated on the basis of the absorption values of the negative control compared to the absorption of the tested compound.

2.6. Evaluation of the effect of dual 2-hydroxy-4-pyrone derivatives-antibiotic combinations by disc diffusion test

The antibacterial activity of 2-hydroxy-4-pyrone derivatives in combination with antibiotics was evaluated according to Oliveira *et al.* [36]. Antibiotic discs were used according to the CLSI guidelines (ciprofloxacin and methicillin (Thermo Fisher Scientific, Waltham, MA,

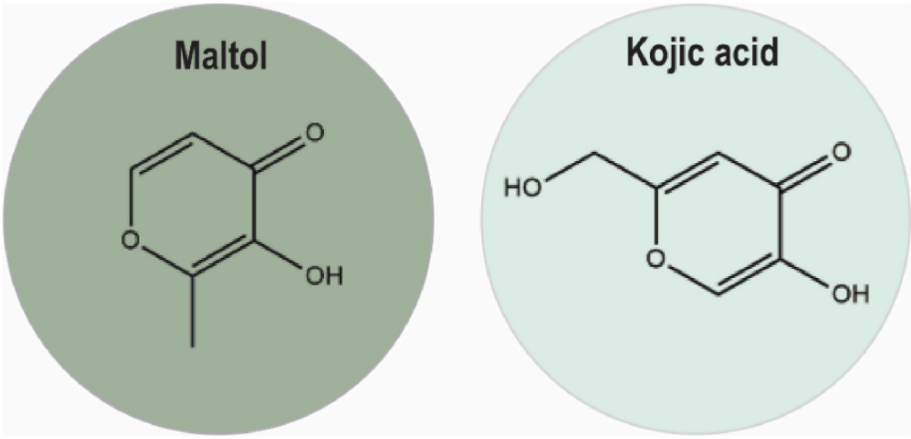

Fig. 1. Chemical structures of maltol and kojic acid.

USA): 5 μg/disc; gentamicin (AppliChem, GmbH, Darmstadt, Germany), fusidic acid (Sigma-Aldrich, St. Louis, MO, USA) and tobramycin: 10 μg/disc; and tetracycline (Sigma-Aldrich, St. Louis, MO, USA): 30 μg/disc) [37]. The kojic acid and maltol were then incorporated into MHA at the maximum tested concentration (MTC) for *S. aureus* or 0.1 × MTC for *P. aeruginosa* after autoclaving and cooling. After overnight bacterial growth on MHA, the isolated colonies were harvested from the agar surface and diluted in sodium chloride solution (NaCl, 8.5 g L^{-1}). The OD was adjusted to 0.132 ± 0.02 and the suspension was spread evenly onto 90 mm Petri dishes containing 20 mL of solidified MHA. Sterile paper discs (Enzymatic, Portugal, 6 mm diameter) impregnated with 15 μL of each antibiotic were placed on the agar plates. Negative control discs containing antibiotics were also placed on the MHA plates without 2-hydroxy-4-pyrone derivatives incorporated. The plates were then incubated at 37 °C for 24 h. After incubation, the diameter (mm) of all inhibition zones were measured and analysed according to CLSI guidelines. The effects of the dual antibiotic and 2-hydroxy-4-pyrone derivatives combinations were categorised based on the following criteria.

- Potentiation (+++): ($IZD_{antibiotic+2\text{-hydroxy-4-pyrone derivate}}$ –$IZD_{antibiotic}$) ≥ 6 mm;
- Additive (++): 6 mm > ($IZD_{antibiotic+2\text{-hydroxy-4-pyrone derivate}}$ − $IZD_{antibiotic}$) ≥ 4 mm;
- Indifferent (+): 4 mm > ($IZD_{antibiotic+2\text{-hydroxy-4-pyrone derivate}}$ − $IZD_{antibiotic}$) > - 6 mm;
- Negative (−): ($IZD_{antibiotic+2\text{-hydroxy-4-pyrone derivate}}$ − $IZD_{antibiotic}$) ≤ - 6 mm.

Where $IZD_{antibiotic}$: inhibition zone diameter of antibiotic; $IZD_{antibiotic+2\text{-hydroxy-4-pyrone derivate}}$: inhibition zone diameter of antibiotic with 2-hydroxy-4-pyrone derivatives.

2.7. Evaluation of the synergistic effects of 2-hydroxy-4-pyrone derivatives in combination with tobramycin or ciprofloxacin by checkerboard assay

The checkerboard assay was performed according to the method described by Gonçalves *et al.* [38] to evaluate the antimicrobial activity of the combination of 2-hydroxy-4-pyrone derivatives with tobramycin or ciprofloxacin. The tested concentration range was selected based on the MIC values of each compound, covering 2 × MIC to 1/64 × MIC. In brief, 10 μL of a serial dilution of 2-hydroxy-4-pyrone derivatives was added to the lines, while an equivalent volume of the antibiotic was added to the columns of the 96-well plates. The antimicrobial solutions accounted for 10 % (v/v) of the final volume of bacterial suspensions. Subsequently, 180 μL of the bacterial suspension (OD_{600nm} = 0.132 ± 0.002) was added to each well. Cell suspensions without 2-hydroxy-4--pyrone derivatives/antibiotics combinations and the respective compounds alone were used as negative controls. The absorbance (λ = 600 nm) was measured spectrophotometrically before (t = 0 h) and after 24 h of incubation at 37 °C and 150 rpm.

In addition, the MBC of the combinations was determined according to the section "*Determination of the minimum bactericidal concentration*". The interactions between the combined compounds were calculated using the fractional inhibitory concentration index (FICI), according to equations (1) and (2):

$$FICI\ (MIC) = FICA + FICB = \frac{MIC_{AP}}{MIC_A} + \frac{MIC_{PA}}{MIC_P} \quad (1)$$

$$FICI\ (MBC) = FICA + FICB = \frac{MBC_{AP}}{MBC_A} + \frac{MBC_{PA}}{MBC_P} \quad (2)$$

where MIC_A/MBC_A and MIC_P/MBC_P are the MICs and MBCs of the antibiotics and 2-hydroxy-4-pyrone derivatives when they act alone respectively, and MIC_{AP}/MBC_{AP} and MIC_{PA}/MBC_{PA} are the MICs and MBCs of the antibiotics and 2-hydroxy-4-pyrone derivatives when they are combined, respectively. The interactions were classified as follows:

- Synergy: FICI ≤0.5
- Indifferent effect: 0.5 < FICI ≤4
- Antagonism: FICI >4 [39]

The results of the checkerboard assay were also analysed using Combenefit software (version 2.021, available at https://sourceforge.net/projects/combenefit/, accessed in October 2024). This software facilitates the visualisation and analysis of the antimicrobial combination effects as a function of concentration, according to the Bliss model.

2.8. Biofilm prevention assays

The prevention of biofilm formation of *S. aureus* CECT 976 and *P. aeruginosa* ATCC 10145 by kojic acid and maltol was evaluated according to Borges *et al.* >[40]. In brief, the OD (λ = 620 nm) of the bacterial cells from overnight culture (37 °C and 150 rpm in MHB) was set to 0.04 ± 0.002 (10^8 and 10^7 CFU mL^{-1} for *S. aureus* and *P. aeruginosa* respectively). Subsequently, 180 μL of the bacterial suspension was distributed in microtiter plates together with 20 μL of selected compounds at subinhibitory concentrations (1/2 × MTC and MTC). The microtiter plates were then incubated at 37 °C for 24 h with shaking (150 rpm). Cell suspensions without 2-hydroxy-4-pyrone derivatives were used as negative controls.

2.9. Biofilm eradication assays

The effect of selected compounds on already established biofilms (24 h old) was performed according to the procedure described by Leitão *et al.* >[17]. In brief, 96-well microtiter plates were filled with 200 μL/well of a bacterial suspension, with the OD (λ = 620 nm) previously adjusted to 0.04 ± 0.002. The microtiter plates were then incubated at 37 °C and 150 rpm to allow biofilm formation (24 h aged biofilms). Subsequently, the culture medium in each microtiter plate was then replaced with 180 μL of fresh MHB and 20 μL of each 2-hydroxy-4-pyrone derivatives (MTC and 10 × MTC). The microtiter plates were then incubated for 24 h at 37 °C with shaking (150 rpm). Cell suspensions with DMSO and without selected compounds were used as negative controls.

2.10. Evaluation of the antibiofilm activity of 2-hydroxy-4-pyrone derivatives in combination with tobramycin or ciprofloxacin

The outcomes from the combination of the selected compounds and the antibiotics ciprofloxacin or tobramycin was investigated according to Baptista *et al.* [41]. The 2-hydroxy-4-pyrone derivate-tobramycin combinations were tested for *S. aureus*, while the combinations with ciprofloxacin were tested for *P. aeruginosa*. For prevention assays, 10 μL of each selected compound (maltol and kojic acid at 1/2 × MTC), 10 μL of ciprofloxacin (at 1/16 × MIC)/tobramycin (at 1/8 × MIC, 1/4 × MIC, and 1/2 × MIC) and 180 μL of bacterial suspension were used. For the biofilm eradication tests, 10 μL of each 2-hydroxy-4-pyrone derivatives (at 10 × MTC), 10 μL of ciprofloxacin (at 1/8 × MIC)/tobramycin (at 1/4 × MIC, 1/2 × MIC, and MIC) and 180 μL of fresh MHB were applied to already formed biofilms (24 h old). Cell suspensions with DMSO and without 2-hydroxy-4-pyrone derivatives/antibiotics were used as negative controls.

2.11. Preparation of biofilm samples for subsequent assays

Following the incubation period, the contents of each well were removed, and a NaCl solution (0.85 %) was used to wash away non-adherent or weakly adherent bacterial cells. In addition, each well was

treated with a universal neutralising solution (consisting of 30 g L^{-1} polysorbate 80, 30 g L^{-1} saponin, 1 g L^{-1} L-histidine, 3 g L^{-1} lecithin and 5 g L^{-1} sodium thiosulfate in 0.0025 M phosphate buffer) for 15 min [17]. The microtiter plates were then subjected to total biomass analysis using crystal violet (CV, Merck) staining, assessment of the metabolic activity of the biofilm cells using resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide, Sigma-Aldrich) staining and determination of the culturable biofilm cells by colony forming unit (CFU) for two bacterial strains used in this study. In addition, the evaluation of membrane integrity using Live/Dead BacLight™ Viability Kit (Invitrogen/Molecular Probes) and the inhibition of virulence factors production (total proteases, lipases, gelatinases and siderophores) by plate agar method was investigated for the bacterium where the combinations of chelating agents and antibiotics demonstrated the most effective *anti*-biofilm activity.

2.12. Biofilm mass determination

The biofilm mass was quantified using CV staining according to the method described by Borges *et al.* >[30]. After washing, the adherent biofilm cells were fixed with 250 μL of 99 % ethanol (Diprolar, Odivelas, Portugal) for 15 min. The contents of the microtiter plates were then discarded, and the plates were air-dried at room temperature until the ethanol evaporated. The fixed bacteria were then stained with 200 μL of 1 % (v/v) CV solution. Excess staining was carefully removed, and the dye-bound biomass was dissolved in 200 μL of 33 % (v/v) glacial acetic acid solution (Chem-Lab, Zedelgem, Belgium). The absorbance was finally measured at 570 nm using a microtiter plate reader. The results are expressed as a percentage of biomass production reduction (%BPR) for the prevention tests or as a percentage of biofilm mass removal (% BR) for the eradication assays upon exposure to the compounds and calculated according to equation (3):

$$\%BPR\ or\ \%BR = \frac{ODc - ODw}{ODc} \times 100 \quad (3)$$

Where OD_c is the OD value (λ = 570 nm) of untreated biofilms and OD_w is the OD value (λ = 570 nm) for cells or biofilm exposed to the selected compounds.

2.13. Biofilm metabolic activity determination

The metabolic activity of the biofilm cells was assessed according to the methodology described by Borges *et al.* >[31]. For this purpose, resazurin staining was used, in which resazurin (blue, not fluorescent) is converted to resorufin (pink, fluorescent) in the presence of metabolically active cells [17]. In brief, each well of the microtiter plate was filled with 190 μL of fresh MHB medium and 10 μL of resazurin indicator solution (0.4 mM) prepared in sterile distilled water. The plates were then incubated for 4 h in the dark at 37 °C. Fluorescence measurements with excitation at λ = 570 nm and emission at λ = 590 nm were then performed using a microtiter plate reader. The percentage of metabolic activity reduction (%MAR, for prevention assays) or the percentage of biofilm metabolic activity reduction (%BMAR, for eradication assays) was calculated according to equation (4):

$$\%MAR\ or\ \%BMAR = \frac{FL_C - FL_W}{FL_C} \times 100 \quad (4)$$

where FL_C corresponds to the fluorescence intensity for the biofilm cells not exposed to the compounds and FL_W corresponds to the fluorescence intensity for the cells or biofilms when exposed to the selected compounds.

2.14. Biofilm culturable cells

The culturability of biofilm cells was determined following a previously described method [40]. Briefly, biofilm cells were obtained by scraping (1 min) the microtiter plate wells and resuspending in 200 μL of sterile NaCl solution (0.85 %). The suspension from each well was then transferred to individual sterile microcentrifuge tubes (this procedure was repeated 3 times). Subsequently, 10-fold serial dilutions were prepared with NaCl. Finally, 10 μL of all dilutions were placed on PCA plates. After incubation at 37 °C for 24 h, the number of CFU was visually counted (10 < CFU <200) and expressed *per* square centimetre of the microtiter plate well (CFU cm^{-2}), according to the following equations (5) and (6):

$$\frac{CFU}{mL} = \frac{N}{SV \times Dilution} \quad (5)$$

where N is the number of CFU on the PCA plates and SV is the sample volume in mL.

$$\frac{CFU}{cm^2} = \frac{\frac{CFU}{mL} \times WV}{Wa} \quad (6)$$

where WV is the working volume of the well (0.2 mL) and Wa is the area of the well in cm^2 (1.53).

2.15. Membrane integrity of biofilm cells by propidium iodide uptake

To assess membrane integrity, the Live/Dead BacLight™ Viability Kit was used, following the method described by Gonçalves *et al.* [38]. This kit consists of two dyes (SYTO^9™ and propidium iodide-PI) that bind to nucleic acids and determine the integrity of the membrane by selective exclusion of the dyes. While SYTO9™ penetrates the bacterial membranes, resulting in a green colouration of the cells, PI only penetrates cells with damaged membranes, where it binds double-stranded nucleic acids and produces a red colouration. This approach was used in conjunction with flow cytometry and epifluorescence microscopy to assess the integrity of the membranes of *S. aureus* biofilm cells. For this purpose, the biofilm cells were collected (after 24 h of compound exposition at 37 °C) by scraping the wells of the microtiter plate and suspending them in 200 μL of sterile NaCl. The contents of each well were then transferred to sterile microcentrifuge tubes for further analysis (this process was repeated until a final volume of 1 mL was reached). For flow cytometry experiments, after collecting the biofilm cells, a volume of 950 μL was stained with 50 μL of PI (0.074 mM) and incubated for 7 min at room temperature (~ 25 °C; protected from light). Cells were then analysed on a CytoFLEX flow cytometer, model V0–B3-R1 (Beckman Coulter, Brea, CA, USA), using CytExpert software (version 2.4.0.28, Beckman Coulter, Brea, CA, USA) and the PC5.5 filter. The integrity of the cell membrane was assessed by reducing PI fluorescence. For epifluorescence microscopy analysis, 700 μL of biofilm cells were stained with 250 μL of SYTO9™ (0.0123 mM) and 50 μL of PI (0.074 mM) [29]. The dyes were allowed to react for 7 min at room temperature (~25 °C) in the dark. The mixture was then filtered using a polycarbonate membrane with 0.22 μm pores (Whatman International Ltd., Maidstone, UK). The membranes were then fixed to a microscope slide and the samples were analysed under a LEICA DMLB2 epifluorescence microscope (LEICA Microsystems Ltd., Weltzlar, Germany). The double optimal optical filter for viewing stained assemblies consisted of a 480–500 nm excitation filter in combination with a 485 nm emission filter (Chroma 61,000-V2 DAPI/FITC/TRITC). A digital colour camera with IM50 software (LEICA) and a 100 × oil immersion fluorescence objective was used to obtain epifluorescence images.

2.16. Inhibitory activity on the production of virulence factors

The effect of 2-hydroxy-4-pyrone derivatives and 2-hydroxy-4-pyrone derivatives-antibiotic combinations on the inhibition of virulence factors produced by *S. aureus* was evaluated for biofilm cells (from both prevention and eradication assays) by measuring the diameter of

the produced halo (in mm) for total proteases, lipases, gelatinases and siderophores [42]. For this purpose, biofilm cells were collected into microcentrifuge tubes using scraping processes. Then 10 μL of each condition were added to Petri dishes containing specific media for each virulence factor studied: Total proteases: PCA with 10 g L^{-1} (1 %) of skim milk powder (Merck, Darmstadt, Germany); Lipases: 2 g L^{-1} (0.20 %) $CaCl_2$ (Merck, Darmstadt, Germany), 15 g L^{-1} (1.50 %) agar (VWR, Belgium), 20 g L^{-1} (2 %) Luria-Bertani broth (LBB, Sigma-Aldrich, St. Louis, MO, USA) and 9.44 mL L^{-1} Tween 80 (VWR, Belgium); Gelatinases: gelatine agar (5 g L^{-1} peptone (0.50 %) (Merck, Darmstadt, Germany), 3 g L^{-1} (0.30 %) yeast extract (Oxoid, United Kingdom), 30 g L^{-1} (3 %) gelatine (Oxoid, United Kingdom), 15 g L^{-1} (1.50 %) agar; Siderophores: MHA with 100 mL L^{-1} of CAS.

Finally, the characteristic inhibition zones for each virulence factor were measured after 48 h (lipases, gelatinases, siderophores) and 72 h (total proteases). The presence of light zones indicated the production of total proteases, while the presence of light-yellow halos around the colonies indicated the production of lipases. Gelatinases production was identified by the presence of transparent halos around the colonies. The production of siderophores was observed by an orange-coloured halo.

2.17. Evaluation of the cytotoxicity and chelating capacity against iron (III) of 2-hydroxy-4-pyrone derivatives using an in vitro hepatic cell model

2.17.1. Reagents

Phosphate-buffered saline solution (PBS 10 ×), heat-inactivated bovine serum (FBS) and antibiotic (10,000 U mL^{-1} penicillin, 10,000 μg mL^{-1} streptomycin) were purchased from PanBiotech (Aidenbach, Germany). Trypsin (0.25 %) was acquired from Merck (Darmstadt, Germany). Minimum essential medium (MEM 0643) with 1.0 g L^{-1} glucose, SrB sodium salt, and sodium bicarbonate were acquired from Sigma-Aldrich Quimíca SA (Sintra, Portugal). Resazurin sodium salt was purchased from TCI Chemicals (Zwijndrecht Belgium). The water used was ultrapurely filtered (Millipore, Burlington, MA, USA).

2.17.2. Cells and culture conditions

HepG2 cells from the ATCC (ATCC HB-8065, Virginia, USA) were cultured in 25 cm^2 cell flasks in MEM with 5 mM glucose, supplemented with 2.2 g L^{-1} sodium bicarbonate, 10 % FBS and 1 % Penicillin-Streptomycin. Cells were maintained in culture at 37 °C-5 % CO_2 a humidified atmosphere, with the cell culture medium replaced every 3 days. Cultures were passed weekly using 0.25 % trypsin. For all biological assays, HepG2 cells were taken between the 12th and 21st passage, plated at a density of 2.5 × 10^5 cells mL^{-1} in 96 well-plates, and subsequently incubated during 24 h before the treatment with the compounds.

The cytotoxicity of 2-hydroxy-4-pyrone derivatives was evaluated by measurement of the metabolic activity and cell mass of human hepatocarcinoma (HepG2) cells after 24 h of treatment, employing the resazurin (reduction into a resorufin fluorescent product) and Sulforhodamine-B (SRB, protein binding under mild-acidic conditions) assays, respectively [43].

2.17.3. Metabolic activity and cell mass measurement

After the cellular treatment with 2-hydroxy-4-pyrone derivatives in a range of concentrations (~ 90–1000 μg mL^{-1}) for 24 h, the culture medium was replaced by 100 μL of 10 μg mL^{-1} resazurin solution, and the cells were incubated in a humidified 5 % CO_2−95 % air atmosphere at 37 °C for 45 min. After the incubation time, the fluorescence was measured on a multiplate reader (Synergy HT Multimode Reader; BioTek, Winooski, VT), using an excitation wavelength of 540 nm and emission wavelength of 590 nm. In the same plate, the cell culture medium was removed, and the cells were washed with PBS (1 ×). Subsequently, the cells were fixed with a methanolic solution of acetic acid (1 %) and left overnight at - 20 °C. On the next day, the fixing medium was removed and replaced with a 0.05 % SRB solution (prepared in 1 % acetic acid) and incubated, at 37 °C, for 1 h, after which the SRB solution was removed, and cells were washed with 1 % acetic acid (v/v) to remove the unbound dye, and the plates were allowed to air-dry at room temperature. The bound SRB was subsequently extracted with a Tris base solution (10 mM, pH 10.5), and the absorbance was measured, at 540 nm, in a multiplate reader. The results are expressed as metabolic activity percentage and cell density for resazurin reduction and SRB assays, respectively, using untreated cells as control cells (100 % of cell viability).

2.17.4. Detection of intracellular reactive oxygen species (ROS) levels

Intracellular oxidative stress was assessed using the dichlorodihydrofluorescein diacetate (DCFH-DA) probe, which is oxidized by intracellular reactive oxygen species (ROS) to form the highly fluorescent DCF [44]. On the day after cell seeding, HepG2 cells were preincubated with 20 μM DCFH-DA for 90 min at 37 °C and 5 % CO_2 air conditions in the dark. Next, the cells were washed with PBS 1X and the maltol or kojic acid at concentrations (~ 90–1000 μg mL^{-1}) were added. After 24 h of incubation, the fluorescence (excitation/emission = 485/530 nm) was measured on a Synergy™ Mx Microplate Reader (BioTek, Winooski, VT). The cells treated only with DFCH-DA were used as control cells.

2.17.5. Studies with FeNTA oxidative stressor

After the pretreatment with maltol and kojic acid (250, 375, 500, 750 μg mL^{-1}) for 1 h at 37 °C in a humidified 5 % CO_2/95 % air atmosphere, HepG2 cells were exposed with a FeNTA solution (3 mM) for 24 h. Nitrilotriacetate (NTA) solution was prepared as described previously [45]. Then, $FeCl_3$ (Fe/NTA molar ratio = 2.5/1) was added to NTA solution to obtain a final mixture with 100 mM iron concentration. After, FeNTA was diluted in culture medium to achieve the iron final concentration of 50 mM. The resulting solution was incubated for 15 min to ensure the compound was entirely in the iron (III) form. Then, the FeNTA solution was diluted in culture medium at a final concentration of 3 mM and applied in the cells. After 24 h of exposure with iron (III), HepG2 cell mass was accessed by SRB method as described above.

2.18. Statistical analysis

The statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA and multiple comparisons based on 95 % confidence levels were used to test significance ($p < 0.05$, statistically significant). Each experiment was conducted in duplicate with a minimum of three independent replicates. For the cell studies, Two-way analysis of variance was used for statistical analysis, followed by a post hoc test (Dunnett's test) for the multiple-comparison test between groups. Differences were considered statistically significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, or $****p < 0.0001$. In cellular studies, we stablished the percentage of 85 % as a threshold to determine the cell viability of treated cells.

3. Results

3.1. Inhibitory and bactericidal activities of the selected 2-hydroxy-4-pyrone derivatives and antibiotics

The results of the inhibitory and bactericidal activity of the kojic acid and maltol against *S. aureus* and *P. aeruginosa* cells are summarised in Table 1. No MIC or MBC values were found for maltol or kojic acid at the concentrations tested (6.25–1000 μg mL^{-1}). In addition, the MIC and MBC of antibiotics (ciprofloxacin and tobramycin) were also analysed to evaluate dual combinations. The antibiotics showed antibacterial effects against the tested bacteria. Tobramycin was most effective against *P. aeruginosa* with MIC and MBC values of 8 μg mL^{-1} and 16 μg mL^{-1}, respectively, while ciprofloxacin had an MIC and MBC value of 32 μg mL^{-1}. For *S. aureus*, ciprofloxacin had the lowest MIC (0.125 μg mL^{-1}),

Table 1
MIC and MBC values of the selected 2-hydroxy-4-pyrone derivatives and antibiotics for *P. aeruginosa* ATCC 10145 and *S. aureus* CECT 976 strains.

	P. aeruginosa		*S. aureus*	
Compounds	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})
Maltol	>1000	>1000	>1000	>1000
Kojic acid	>1000	>1000	>1000	>1000
Ciprofloxacin	32	32	0.125	8
Tobramycin	8	16	4	4

while tobramycin had the lowest MBC (4 μg mL^{-1}).

3.2. Iron binding capacity of the selected 2-hydroxy-4-pyrone derivatives

The ability of the kojic acid and maltol to retain iron was determined spectrophotometrically based on the formation of iron complexes with CAS solution. Both maltol and kojic acid were able to chelate iron at different concentrations, showing a dose-dependent effect (Fig. 2). Specifically, at a concentration of 1000 μg mL^{-1}, maltol led to a binding capacity of 90 %, while kojic acid resulted in an 85 %. Furthermore, it was observed that the 2-hydroxy-4-pyrone derivatives showed a significant change in colour intensity and absorption, comparable to the effect of EDTA, a known chelating agent (Fig. 2). Notably, EDTA exhibited a binding capacity of 89 % at a concentration of 71 μg mL^{-1}. In addition, analysis of the absorption spectra revealed that selected compounds exhibited a peak around 260 nm, suggesting that the observed effects were due to binding capacity rather than methodological influences (λ = 630 nm, Figs. S1 and S2).

3.3. Effect of the selected 2-hydroxy-4-pyrone derivatives on the activity of the antibiotics

To evaluate the effect of natural chelating agents in combination with antibiotics, an initial screening was carried out with four specific antibiotics for *P. aeruginosa* and six for *S. aureus*. Methicillin and fusidic acid were selected exclusively for *S. aureus*, as they are narrow-spectrum antibiotics that mainly act against Gram-positive bacteria [46,47]. Therefore, the disc diffusion method was used to evaluate possible synergies between the tested antibiotics and 2-hydroxy-4-pyrone derivatives. According to the results obtained (Table 2), combinations of kojic acid and maltol with fusidic acid, methicillin and tetracycline showed an indifferent effect on the bacteria tested. Although an additive effect between maltol and gentamicin was observed for *S. aureus*, combinations of 2-hydroxy-4-pyrone derivatives and gentamicin did not enhance the antibiotic activity against *P. aeruginosa* (indifferent effect). On the other hand, combinations between 2-hydroxy-4-pyrone derivatives and ciprofloxacin as well as tobramycin showed a potentiating effect for both *S. aureus* and *P. aeruginosa*. In particular, is worthy to note the potentiate effect for kojic acid-ciprofloxacin (in *P. aeruginosa*) and maltol-tobramycin (in *S. aureus*). In this way, the combinations that obtained a potentiation effect (2-hydroxy-4-pyrone derivatives-ciprofloxacin for *P. aeruginosa* and 2-hydroxy-4-pyrone derivatives-tobramycin for *S. aureus*) were selected for biofilm studies.

3.4. Synergistic effect of the selected 2-hydroxy-4-pyrone derivatives with ciprofloxacin or tobramycin

The checkerboard assay was performed to further corroborate the results of the disc diffusion test and quantitatively evaluate the combined antimicrobial action of the 2-hydroxy-4-pyrone derivatives with ciprofloxacin against *P. aeruginosa* and tobramycin against *S. aureus*. A FICI of 0.5 was observed for the combination with ciprofloxacin and 0.3 for the combination with tobramycin, indicating a synergistic effect. The analysis with the Combenefit software confirmed this synergy between 2-hydroxy-4-pyrone derivatives and ciprofloxacin/tobramycin, as shown in Fig. 3.

In addition, a significant reduction in both MIC and MBC values was observed for all compounds tested (Table S1). For *P. aeruginosa*, the MIC values of ciprofloxacin were reduced from 32 to 16 μg mL^{-1} when combined with maltol and kojic acid (at 15.63 μg mL^{-1}). Similarly, a fourfold reduction in the MIC and MBC of tobramycin was observed in *S. aureus*, decreasing from 4 to 1 μg mL^{-1} when combined with the selected 2-hydroxy-4-pyrone derivatives (at 15.63 μg mL^{-1}). However, a negligible effect on the MBC of ciprofloxacin (at 32 μg mL^{-1}) when combined with 2-hydroxy-4-pyrone derivatives (at 125 and 15.6 μg mL^{-1} for kojic and maltol, respectively) was observed.

3.5. Preventive effect of selected 2-hydroxy-4-pyrone derivatives and 2-hydroxy-4-pyrone derivate-antibiotic combinations on biofilms formation

The preventive effect of kojic acid and maltol alone and in combination with ciprofloxacin on *P. aeruginosa* is shown in Fig. S3. At a concentration of 1000 μg mL^{-1}, maltol showed moderate efficacy in preventing biofilm formation by reducing biomass production by 40 % and metabolic activity by 30 % ($p < 0.05$). However, at lower concentration (500 μg mL^{-1}), its effect was less pronounced (< 15 %). Kojic

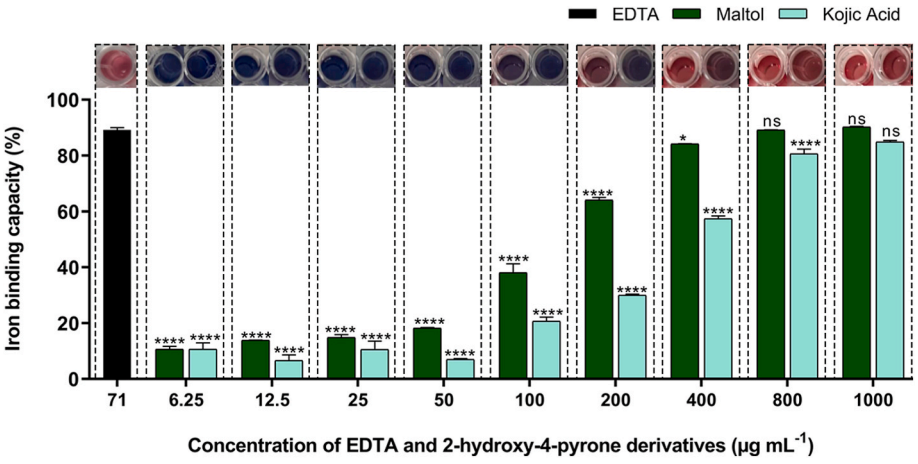

Fig. 2. Effect of increasing concentrations of maltol and kojic acid (6.25–1000 μg mL^{-1}) on iron binding capacity. EDTA (71 μg mL^{-1}) was used as a positive control. The upper panels show representative pictures of the 96-well microtiter plate assay wells containing CAS solution with 2-hydroxy-4-pyrone derivatives or EDTA. Mean values ± standard deviations for at least three replicates are illustrated. Bars with '*' are statistically different from EDTA alone for a confidence level greater than 95 % ($p < 0.05$), ns = non-significant.

Table 2
IZD values (mm) and corresponding classification of the combined application of 2-hydroxy-4-pyrone derivatives and the different antibiotics against *P. aeruginosa* and *S. aureus*.

Antibiotic	Phytochemical/concentration (100/1000 μg mL^{-1})	Antibiotic concentration (μg/disc)	*P. aeruginosa* ATCC 10145			*S. aureus* CECT 976		
			IZDa (mm)	IZDa + pyr (mm)	C	IZDa (mm)	IZDa + pyr (mm)	C
Tetracycline	Maltol	30	2.00 ±	3.50 ± 0.58	+	24.00 ±	25.75 ± 1.71	+
	Kojic acid		0.00	5.00 ± 0.00	+	1.41	27.25 ± 0.96	+
Ciprofloxacin	Maltol	5	24.25 ±	27.25 ± 0.50	+	29.25 ±	33.00 ± 0.00	++
	Kojic acid		0.50	30.25 ± 0.00	+++	0.96	30.75 ± 0.50	+
Methicilin	Maltol	5	–	–	–	19.50 ±	15.50 ± 1.29	+
	Kojic acid					1.29	16.00 ± 1.83	+
Gentamicin	Maltol	10	8.25 ±	11.75 ± 0.50	+	12.00 ±	17.50 ± 1.29	++
	Kojic acid		0.50	12.00 ± 0.00	+	1.63	9.50 ± 0.58	+
Tobramycin	Maltol	10	13.25 ±	16.25 ± 0.50	+	12.50 ±	19.00 ± 0,82	+++
	Kojic acid		0.50	17.25 ± 0.82	++	1.29	13.25 ± 0.96	+
Fusidic acid	Maltol	10	–	–	–	27.75 ±	28, 25 ± 0,96	+
	Kojic acid					0.50	26,75 ± 1,71	+

IZD: inhibition zone diameters; IZD antibiotic: inhibition zone diameter of antibiotic; IZD antibiotic + 2-hydroxy-4-pyrone derivative: inhibition zone diameter of antibiotic + 2-hydroxy-4-pyrone derivate. **Potentiation** (+++): (IZDantibiotic+2-hydroxy-4-pyrone derivate–IZDantibiotic) ≥ 6 mm; **Additive** (++): 6 mm > (IZDantibiotic+2-hydroxy-4-pyrone derivate – IZDantibiotic) ≥ 4 mm; **Indifferent** (+): 4 mm > (IZDantibiotic+2-hydroxy-4-pyrone derivate – IZDantibiotic) > - 6 mm; **Negative** (−): (IZDantibiotic+2-hydroxy-4-pyrone derivate– IZDantibiotic) ≤ - 6 mm.

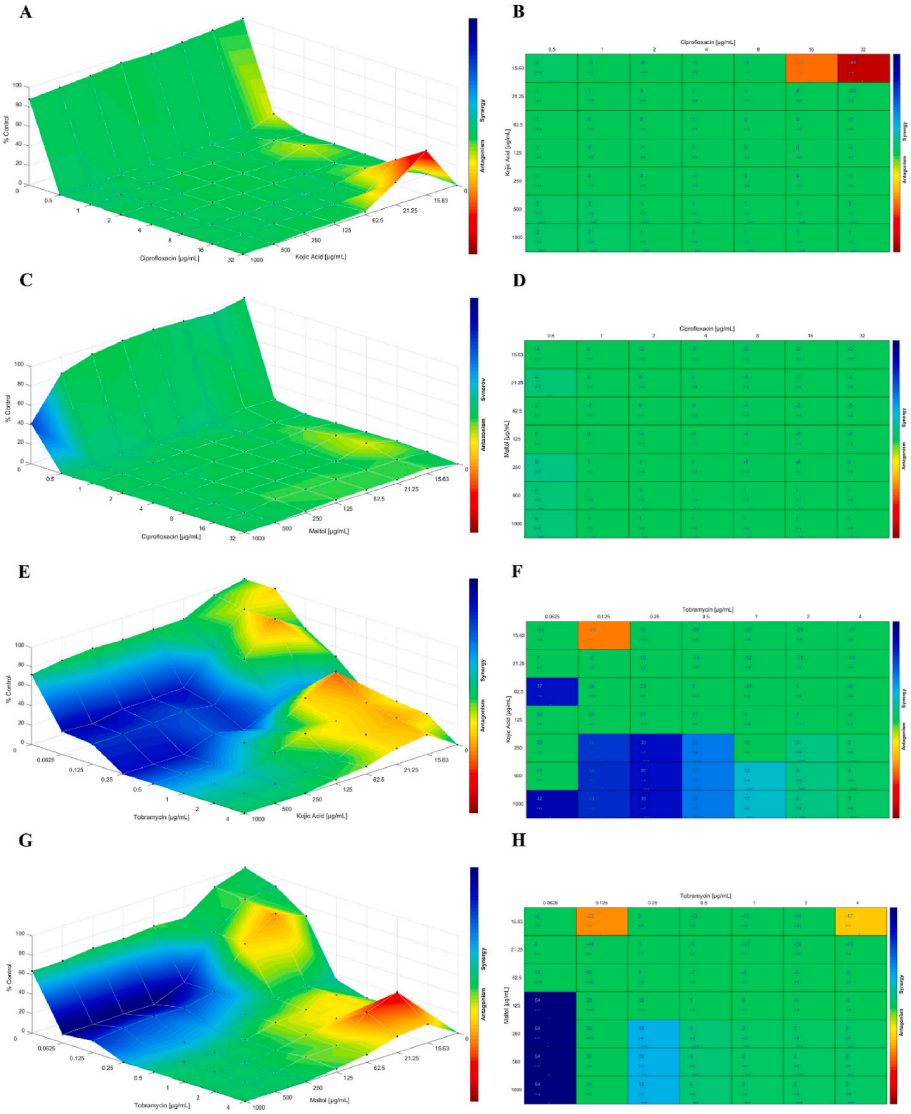

Fig. 3. Bliss synergy mapped to dose-response (A, C, E, and G) and matrix synergy plot (B, D,F, and H) for maltol- and kojic acid-ciprofloxacin combinations against *P. aeruginosa* (A and B) and for maltol- and kojic acid-tobramycin combinations against *S. aureus* (C and D).

acid did not show a significant effect in preventing biofilm formation of *P. aeruginosa* in terms of biomass production, and metabolic activity and CFU reduction at any of the concentrations tested ($p > 0.05$).

However, when maltol and kojic acid (500 μg mL^{-1}) were combined with ciprofloxacin (1/16 × MIC), there was a reduction in biofilm mass production of about 60 % ($p < 0.05$). It is noteworthy that ciprofloxacin alone only achieved a reduction of 36 % (Fig. S3A). In terms of metabolic activity and culturable cells, the use of 2-hydroxy-4-pyrone derivate-ciprofloxacin combinations did not show an improved effect, as ciprofloxacin alone showed equivalent or even better efficacy (Figs. S3B and S3C).

Similar results to those observed with *P. aeruginosa* were obtained when evaluating the inhibition of *S. aureus* biofilm formation by maltol and kojic acid (Fig. 4). Both 2-hydroxy-4-pyrone derivatives showed a reduction in biomass production of 26 % (Fig. 4A). At the same time, both 2-hydroxy-4-pyrone derivatives led to a reduction in the metabolic activity of *S. aureus* by over 40 % ($p < 0.05$, Fig. 4B). However, their effects on CFU levels were negligible ($p > 0.05$).

A slight increase in the inhibition of biomass production was observed when the 2-hydroxy-4-pyrone derivatives were used in combination with tobramycin. In particular, kojic acid in combination with 1/4 × MIC tobramycin showed a 2.6-fold stronger effect than tobramycin alone at the same concentration ($p < 0.05$, Fig. 4A). Regarding metabolic activity, the effect of the combination of 2-hydroxy-4-pyrone derivatives and tobramycin was primarily due to the effect of tobramycin (Fig. 4B). Conversely, the combination of natural chelating agents with tobramycin led to a significant reduction in culturable cells ($p < 0.05$). In particular, the combination of kojic acid with 1/8 × MIC and 1/2 × MIC of tobramycin achieved a reduction of ca. 2 log (CFU cm^{-2}) higher than the antibiotic alone, at the same concentration (Fig. 4C).

3.6. Effect of the selected 2-hydroxy-4-pyrone derivatives and 2-hydroxy-4-pyrone derivate-antibiotic combinations on the eradication of pre-established biofilms

Individual application of the selected compounds showed moderate effects on biomass removal, with maltol and kojic acid (at MTC and 10xMTC) achieving a ca. 15–20 % reduction in biomass for both bacteria studied (Figs. 5A and S4A). On the other hand, the application of kojic acid and maltol at 10xMTC led to a significant reduction in the metabolic activity of *P. aeruginosa* and *S. aureus* by 70 and 80 %, respectively ($p < 0.05$). Meanwhile, no effects on metabolic activity were observed in either bacterium tested when the 2-hydroxy-4-pyrone derivatives were used at MTC ($p > 0.05$, Figs. 5B and S4B). Similar to the metabolic activity results, only the higher concentration (10xMTC) led to a decrease in culturability. Specifically, maltol (10xMTC) achieved a decrease of 3.1 log (CFU cm^{-2}) in *P. aeruginosa* and kojic acid (10xMTC) achieved a decrease of 1.9 log (CFU cm^{-2}) in *S. aureus* ($p < 0.05$, Figs. 5C and S4C).

In pre-established *S. aureus* biofilms, all combinations of 2-hydroxy-4-pyrone derivatives and tobramycin showed increased efficacy in biomass removal, as the antibiotic alone had negligible effect at the concentrations tested (Fig. 5A). Of note, maltol-tobramycin (1/4 × MIC) achieved a 31 % reduction ($p < 0.05$). However, kojic acid-tobramycin combinations showed no improved effect, as the kojic acid alone showed a similar effect at the same concentration (10 × MTC). In terms of metabolic activity, all combinations tested showed an enhancing effect, with the combinations of 2-hydroxy-4-pyrone derivatives and tobramycin achieving a reduction of approximately 80 % compared to tobramycin alone, which only achieved a reduction of 37 % when applied at 1/4 × MIC (Fig. 5B). Conversely, the combinations of maltol and tobramycin showed the most significant improvement in culturability. When maltol was combined with 1/4 × MIC and MIC of tobramycin, this resulted in a 2-log (CFU cm^{-2}) reduction compared to tobramycin alone at the same concentration (Fig. 5C). No improved effect was observed with the 2-hydroxy-4-pyrone derivatives-ciprofloxacin combinations in terms of biomass removal, metabolic activity or CFU in *P. aeruginosa* ($p > 0.05$, Fig. S4).

3.7. Effect of the selected 2-hydroxy-4-pyrone derivatives and 2-hydroxy-4-pyrone derivate-antibiotic combinations on membrane integrity of *S. aureus* biofilm cells

In view of the promising results from studies on the control of *S. aureus* biofilms, it was decided to specifically evaluate the capacity of natural chelating agents and their combinations with tobramycin to compromise membrane integrity in this bacterium. Therefore, permeability studies using flow cytometry and epifluorescence microscopy were extended to *S. aureus* biofilms (Figs. S5 and 6).

The use of the selected compounds showed no significant effect ($p > 0.05$) on membrane integrity during *S. aureus* biofilm formation (Fig. S5). Furthermore, tobramycin at the highest concentration tested (MIC) increased PI uptake by only 20 %. However, a drastic reduction in total cell count was observed with tobramycin treatment (Figs. S5A and S5B). Furthermore, no greater effect on membrane integrity was observed with the combinations of 2-hydroxy-4-pyrone derivatives and tobramycin compared to the antibiotic alone (Figs. S5A and S5B).

In the eradication studies, it was observed that 2-hydroxy-4-pyrone derivatives at the highest concentration (10 × MTC) remarkably impaired the cells membrane integrity (90–95 %, $p < 0.05$) of pre-established *S. aureus* biofilms. In addition, combinations of selected compounds with tobramycin resulted in a 50 % of PI uptake, whereas tobramycin alone at the same concentration had no effect on membrane integrity in formed biofilms (Fig. 6A). On the other hand, similar to the biofilm prevention studies, a lower number of cells was observed in epifluorescence microscopy images when tobramycin was used alone or in combination. It is worth mentioning that the number of cells did not decrease when maltol and kojic acid were used individually, regardless of the concentration (Fig. 6A and 6B).

3.8. Effect of the selected 2-hydroxy-4-pyrone derivatives and 2-hydroxy-4-pyrone derivate-antibiotic combinations on the production of virulence factors

The efficacy of the selected compounds and their combinations with tobramycin in inhibiting the production of virulence factors during formation and in already formed biofilms is presented in Tables S2 and 3.

The results showed that *S. aureus* biofilm cells was unable to produce gelatinases. However, neither the prevention nor the eradication experiments showed that a single compound or a combination of 2-hydroxy-4-pyrone derivatives-tobramycin affected the total halo (which includes bacterial growth and the production of virulence factors, $p > 0.05$).

As for lipases, both maltol (MTC) and kojic acid (1/2 × MTC) exhibited a reduction in their production during biofilm formation ($p < 0.05$, Table S2). This effect was even more pronounced when they were combined with tobramycin, especially kojic acid (1/2 × MTC) with 1/4 × MIC of antibiotic. It is noteworthy that tobramycin did not display a similarly pronounced effect at the same concentrations. Conversely, in pre-formed biofilms, any combination with the 2-hydroxy-4-pyrone derivatives resulted in a significant reduction in lipase production, regardless of the concentration used ($p > 0.05$). Tobramycin, on the other hand, had no effect on lipase production in preformed biofilms at 1/8 × MIC and 1/4 × MIC.

The production of total proteases during biofilm formation was significantly reduced by tobramycin, regardless of the concentration used ($p < 0.05$). This effect was not observed in already established biofilms. Conversely, the 2-hydroxy-4-pyrone derivatives led to a complete inhibition of protease production when applied either individually or in combination (at all concentrations tested), both during formation and in already formed biofilms ($p < 0.05$).

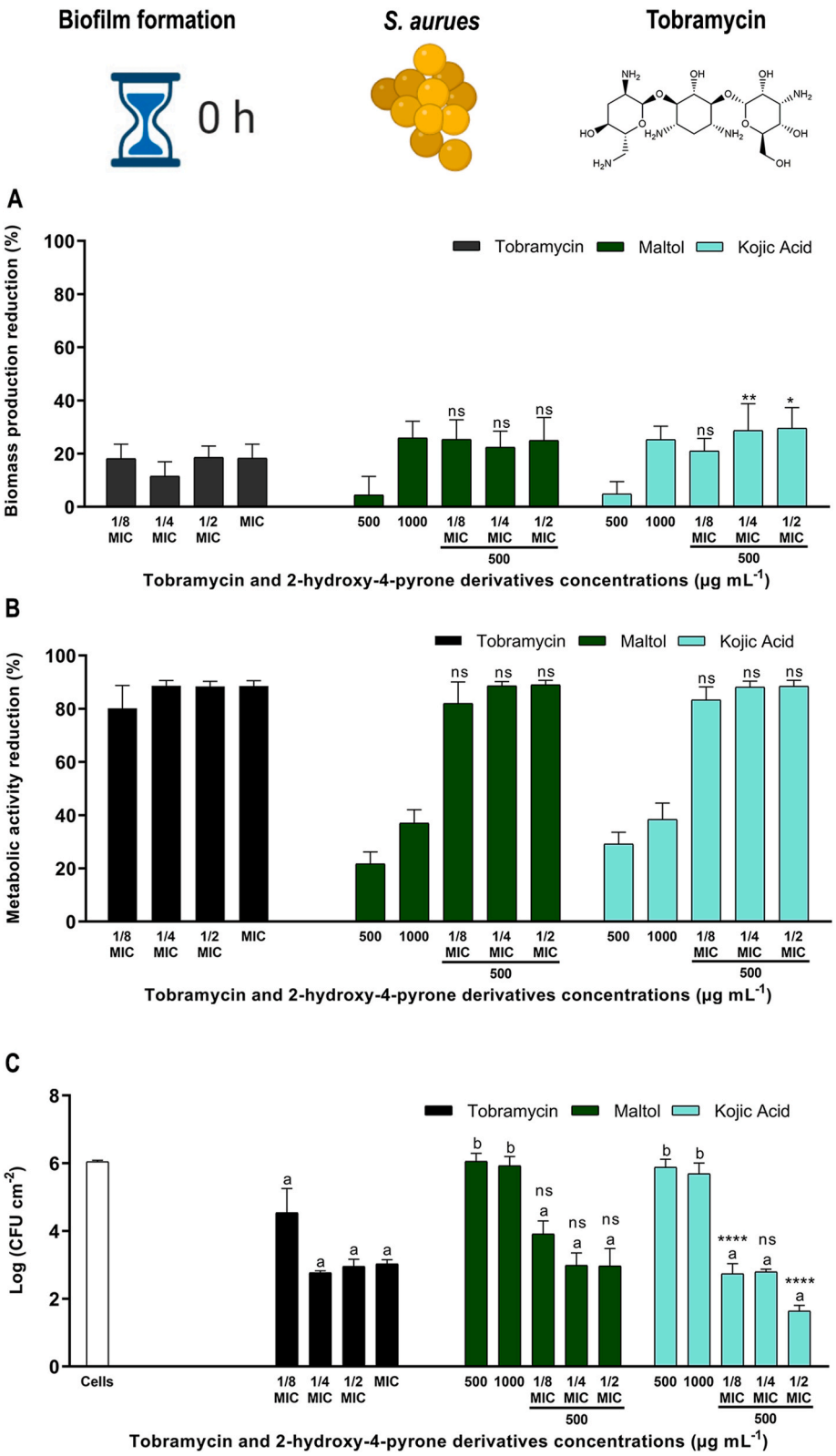

Fig. 4. Preventive effect of tobramycin, 2-hydroxy-4-pyrone derivatives and their combination (tobramycin + 2-hydroxy-4-pyrone derivatives) against *S. aureus* biofilms in terms of biomass production **(A)**, metabolic activity **(B)**, and culturable cells [log (CFU cm^{-2})] **(C)**. Mean values ± standard deviations for at least three replicates are illustrated. Bars with '*' are statistically different from tobramycin alone for a confidence level greater than 95 % ($p < 0.05$), ns = non-significant. Bars with lowercase letters are statistically different from the control (biofilms without treatment) for a confidence level greater than 95 % ($p < 0.05$, where a = **** and b = ns).

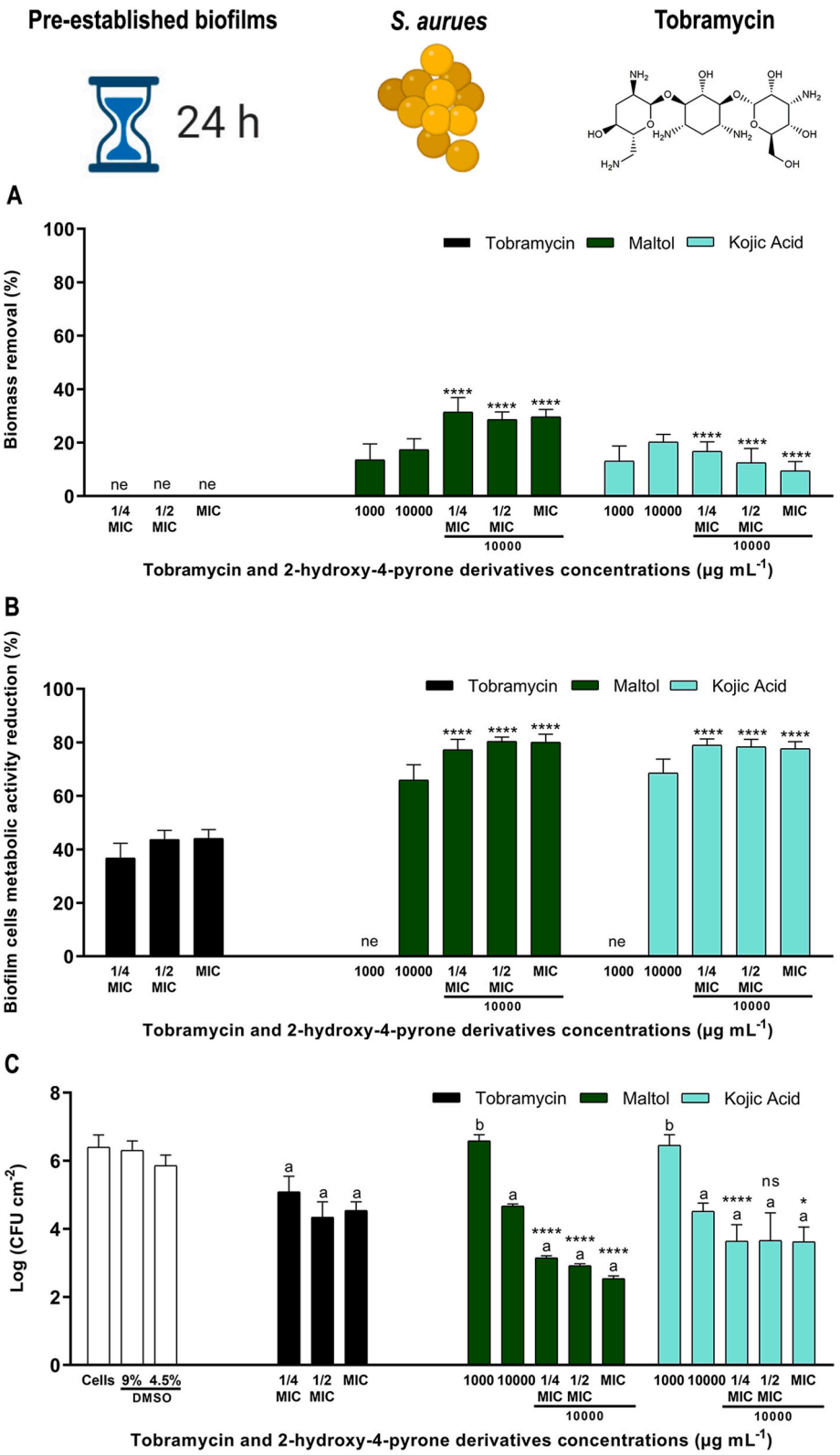

Fig. 5. Effect of tobramycin, 2-hydroxy-4-pyrone derivatives and their combination (tobramycin + 2-hydroxy-4-pyrone derivatives) against pre-established 24 h old *S. aureus* biofilms in terms of biomass removal **(A)**, metabolic activity of biofilm cells **(B),** and culturable cells [log (CFU cm^{-2})] **(C).** Mean values ± standard deviations for at least three replicates are illustrated. Bars with '*' are statistically different from tobramycin alone for a confidence level greater than 95 % ($p < 0.05$), ne = no effect, ns = non-significant. Bars with lowercase letters are statistically different from the control (biofilms without treatment and exposed to DMSO at 9 % or 4.5 %) for a confidence level greater than 95 % ($p < 0.05$, where a = **** and b = ns).

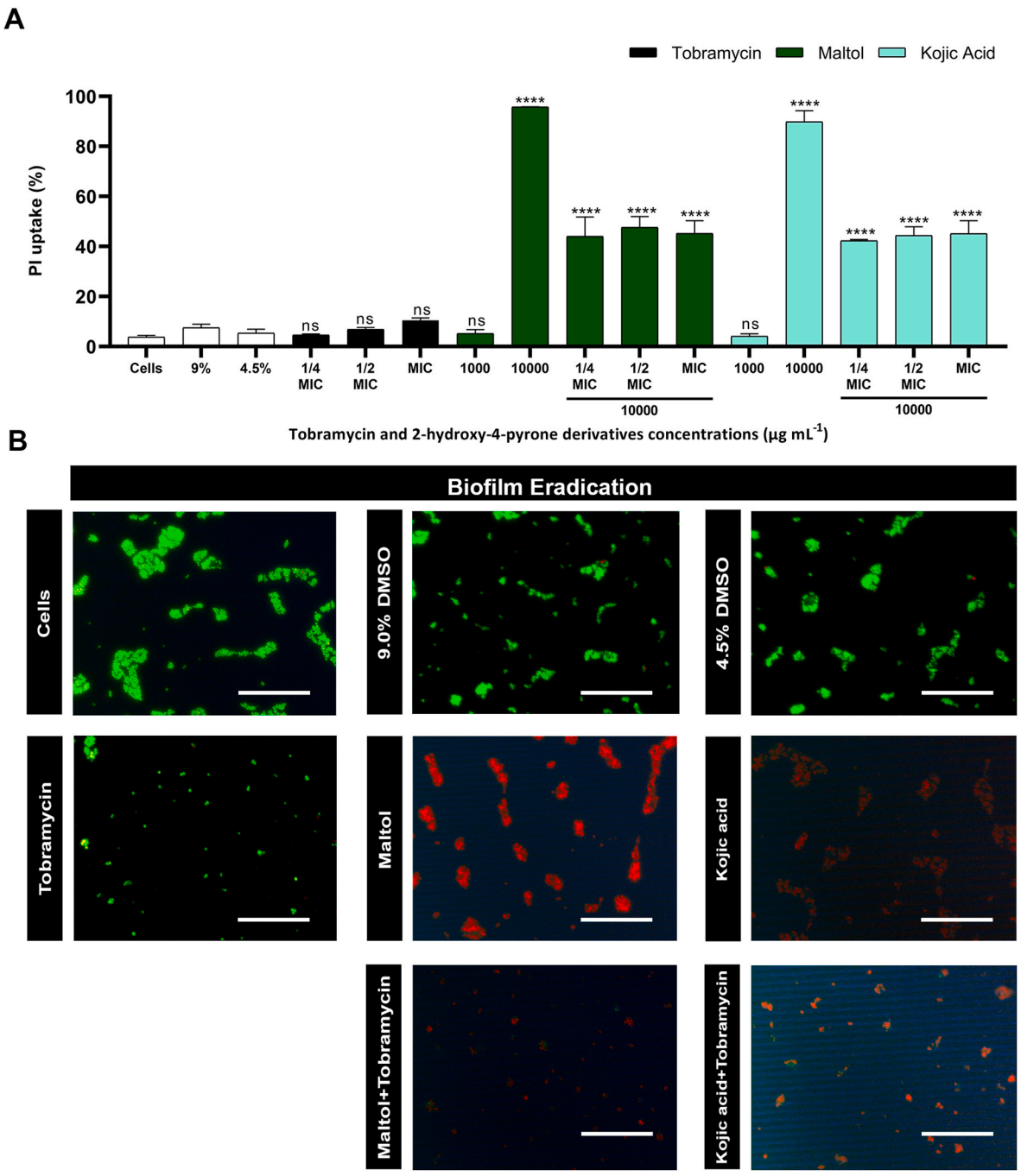

Fig. 6. Effect of tobramycin, 2-hydroxy-4-pyrone derivatives, and their combination (tobramycin + 2-hydroxy-4-pyrone derivatives) against pre-established 24 h old *S. aureus* biofilms in terms of membrane integrity evaluated by flow cytometry (**A**) and epifluorescence microscopy (**B**) using PI and live/death kit, respectively. In Fig. 6B the concentration of maltol and kojic acid was 10 × MTC, and tobramycin was set at MIC (the scale bar corresponds to 10 μm). Mean values ± standard deviations for at least three replicates are shown. Bars marked with '*' indicate statistical significance compared to the negative control (biofilms without treatment and exposed to DMSO at 9 % or 4.5 %) at a confidence level greater than 95 % ($p < 0.05$); ns = non-significant.

Regarding siderophores, the natural chelating agents also caused a significant reduction in their production during biofilm formation and in established biofilms ($p < 0.05$). When combinations were applied during biofilm formation, only kojic acid (1/2 × MTC) combined with tobramycin (1/8 × MIC) showed a superior effect compared to the individual application of the antibiotic at the same concentration ($p < 0.05$). In established biofilms, combinations of natural chelating agents with tobramycin (1/4 × MIC and 1/2 × MIC were more effective than tobramycin alone at the same concentrations ($p < 0.05$).

3.9. Cytotoxicity and iron (III) chelating capacity of 2-hydroxy-4-pyrone derivatives

The evaluation of the cytotoxic profile of both maltol and kojic acid was accomplished using human HepG2 cells, being frequently used in the safety assessment of drugs [48]. The cells were treated for 24 h with a range of concentration of both 2-hydroxy-4-pyrone derivatives, being the metabolic activity, cell mass and production of reactive species evaluated through the resazurin, SRB and DCFH-DA assays [45]. The obtained results is presented in Fig. 7.

Both compounds caused a slight depletion of metabolic activity of

Table 3
Effect of tobramycin, selected 2-hydroxy-4-pyrone derivatives and their combinations (tobramycin + 2-hydroxy-4-pyrone derivatives) in the production of virulence factors (lipases, gelatinases, and proteases) (mm) by *S. aureus* cells from pre-established 24 h old *S.* biofilms. Results are presented as mean ± standard deviations for two independent experiments with at least three replicates. Table sections with '*' are statistically different from tobramycin alone at the same concentration for a confidence level greater than 95 % ($p < 0.05$), ns = non-significant. Table sections with lowercase letters are statistically different from the control (biofilms without treatment and exposed to DMSO at 9.0 % or 4.5 %) for a confidence level greater than 95 % ($p < 0.05$, where a = ns; b = *; c = **; d = ***; and e = ****). The 10 μL of bacterial culture produced a 7.50 mm (baseline) spot on the agar plates.

Biofilm Eradication							
Conditions	Gelatinases		Lipases		Proteases		Siderophores Halo
	Cells Halo	Gelatinases Halo	Cells Halo	Lipases Halo	Cells Halo	Proteases Halo	
Cells	10.5 ± 0.6	0.0 ± 0.0	13.3 ± 0.5	4.0 ± 0.0	13.5 ± 0.6	3.8 ± 0.5	11.5 ± 0.6
DMSO 4.5 %	11.0 ± 0.0	0.0 ± 0.0	12.5 ± 0.6	4.0 ± 0.0	13.3 ± 0.5	3.8 ± 0.5	11,8 ± 0.5
DMSO 9 %	11.3 ± 0.5	0.0 ± 0.0	13.0 ± 0.0	4.0 ± 0.0	13.3 ± 0.5	3.8 ± 0.5	11.8 ± 0.5
Tobramycin (1/4 × MIC)	10.5 ± 0.6 a	0.0 ± 0.0	12.5 ± 0.6 a	4.0 ± 0.0 a	13.3 ± 0.5 a	3.8 ± 0.5 a	11.5 ± 0.6 a
Tobramycin (1/2 × MIC)	9.8 ± 0.5 a	0.0 ± 0.0	12.3 ± 0.5 a	3.8 ± 0.5 a	12.8 ± 0.5 a	3.5 ± 0.6 a	11.8 ± 0.5 a
Tobramycin (MIC)	10.8 ± 0.5 a	0.0 ± 0.0	12.8 ± 0.5 a	3.5 ± 0.6 a	12.5 ± 0.6 a	3.3 ± 0.5 a	11.3 ± 0.5 a
Maltol (MTC)	10.0 ± 0.8 a	0.0 ± 0.0	11.5 ± 0.6 b	2.8 ± 0.5 c	12.3 ± 0.5 a	3.5 ± 0.6 a	10.0 ± 0.8 d
Maltol (10 × MTC)	11.0 ± 0.0 a	0.0 ± 0.0	12.0 ± 0.0 a	2.5 ± 0.6 d	13.3 ± 0.5 a	0.0 ± 0.0 e	10.0 ± 0.8 d
Maltol (10 × MTC) + Tobramycin (1/4 × MIC)	11.3 ± 0.5 ns/a	0.0 ± 0.0	11.5 ± 0.6 ns/a	2.3 ± 0.5 ****/e	12.8 ± 0.5 ns/a	0.0 ± 0.0 ****/e	10.3 ± 0.5 ns/c
Maltol (10 × MTC) + Tobramycin (1/2 × MIC)	9.5 ± 0.6 ns/b	0.0 ± 0.0	11.5 ± 0.6 ns/a	2.3 ± 0.5 ***/e	12.3 ± 0.5 ns/a	0.0 ± 0.0 ****/e	10.0 ± 0.0 **/d
Maltol (10 × MTC) + Tobramycin (MIC)	10.0 ± 0.0 ns/a	0.0 ± 0.0	12.3 ± 0.5 ns/a	2.3 ± 0.5 **/e	12.0 ± 0.0 ns/a	0.0 ± 0.0 ****/e	10.3 ± 0.5 **/c
Kojic acid (MTC)	10.0 ± 0.0 a	0.0 ± 0.0	11.8 ± 0.5 a	2.3 ± 0.5 e	13.3 ± 0.6 a	3.5 ± 0.6 a	10.0 ± 0.0 d
Kojic acid (10 × MTC)	10.8 ± 0.5 a	0.0 ± 0.0	11.8 ± 0.5 a	2.5 ± 0.6 d	12.5 ± 0.6 a	0.0 ± 0.0 e	9.3 ± 0.5 e
Kojic acid (10 × MTC) + Tobramycin (1/4 × MIC)	10.0 ± 0.0 ns/a	0.0 ± 0.0	11.5 ± 0.6 ns/a	2.5 ± 0.6 ***/d	12.5 ± 0.6 ns/a	0.0 ± 0.0 ****/e	10.3 ± 0.5 ns/c
Kojic acid (10 × MTC) + Tobramycin (1/2 × MIC)	9.3 ± 0.9 ns/c	0.0 ± 0.0	12.3 ± 0.9 ns/a	2.0 ± 0.0 ****/e	11.8 ± 0.5 ns/b	0.0 ± 0.0 ****/e	10.0 ± 0.0 **/d
Kojic acid (10 × MTC) + Tobramycin (MIC)	9.5 ± 0.6 ns/b	0.0 ± 0.0	11.8 ± 0.5 ns/a	2.0 ± 0.0 ***/e	11.5 ± 0.6 ns/c	0.0 ± 0.0 ****/e	10.0 ± 0.0 **/d

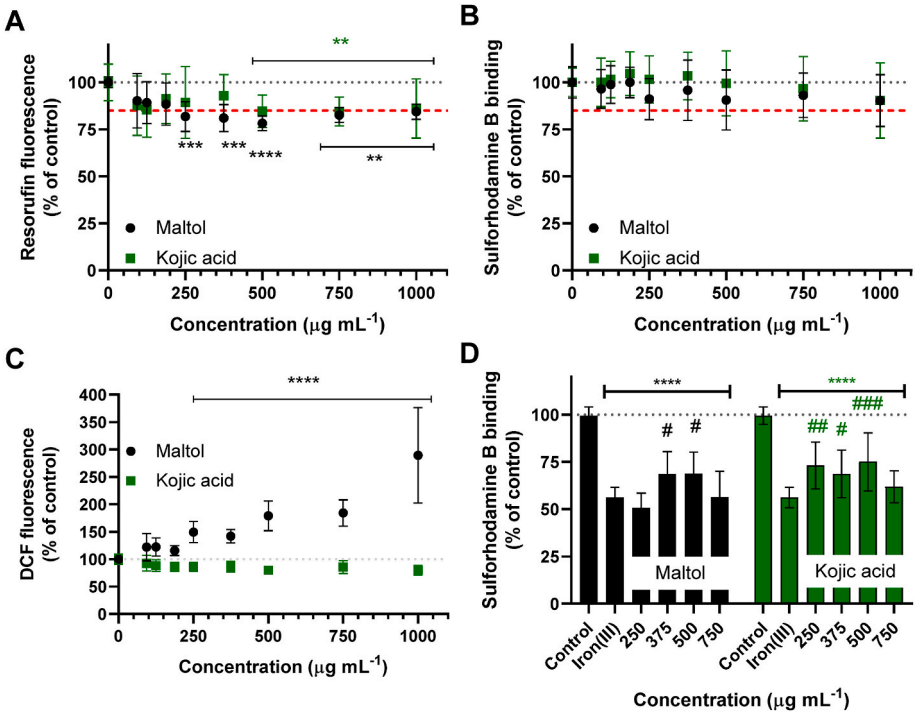

Fig. 7. Cytotoxic effects of maltol and kojic acid in HepG2 cells (93.5–1000 μg mL^{-1}) after 24 h of exposure, by measuring the metabolic activity **(A)** and cell mass **(B)** by resazurin reduction method and SRB assay, respectively. Intracellular ROS levels after exposure of HepG2 cells **(C)** were also measured after 24 h of exposure with 2-hydroxy-4-pyrone derivatives. The cell mass of HepG2 was measured after treatment with iron (III) (3 mM) in the presence and absence of 2-hydroxy-4-pyrone derivatives **(D)**. The data are expressed as the means of at least four independent experiments together with the standard deviation (Mean ± SD). Statistical comparisons were made using two-way ANOVA. In all cases, p values lower than 0.05 were considered significant (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ vs the control data; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs the cells treated only with iron (III). The grey dot line represents the mean of control cells (100 %) and the dashed orange line represents the percentage at 85 %. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the cells (resazurin reduction >80 %, Fig. 7A). Despite that, non-significant difference with control cells was observed for the measurement of HepG2 cell mass (SRB binding >90 %, Fig. 7B), which suggested the good safety profile of the 2-hydroxy-4-pyrone derivatives under study. Furthermore, it was possible to verify that the treatment with maltol led to an increase of ROS production when cells were exposed to all tested concentrations (DCF fluorescence ~ 116 %–290 %, Fig. 7C). Otherwise, kojic acid treatment caused a slight decrease of DCF fluorescence when compared with control cells for all concentrations tested (DCF fluorescence ~ 80 %–92 %, Fig. 7C). The difference between the two compounds in terms of cellular ROS production can be justified by structural features of each molecule, since maltol has the hydroxyl group adjacent to the ethyl group, which could increase the electron delocalization. This characteristic can facilitate the deprotonation of hydroxyl group, leading ultimately to the formation of radical species. Despite the overproduction of ROS caused by maltol, no cytotoxicity was observed (Fig. 7A and 7B), which could indicate that the ROS are stable and are not involved in neurotoxic cellular events.

The iron chelating capacity of both compounds were evaluated using the FeNTA method after 24 h insult of the HepG2 cells with iron (III) (Fig. 7D) as described previously by our group [45]. The treatment of the cells with iron (III) in absence of compounds caused a decreasing of cell mass (SRB absorbance = 56.2 ± 5.4 %). The chelating capacity was proved for both compounds since it was detected an increase of cell mass in comparison with cells treated only with iron (III). When Maltol was used at 375 and 500 μg mL^{-1}, the cell mass was around 68 % for both concentrations, while kojic acid was able to protect the cells from iron (III) insult at all concentrations tested (SRB absorbance >61.8 %). This data corroborates the results obtained previously (Fig. 2) and highlights the iron chelating capacity of both 2-hydroxy-4-pyrone derivatives not only in cell-free models but also in a cellular level.

4. Discussion

The increase in bacterial resistance to antibiotics, resulting from their inappropriate use and the adaptability of microorganisms, poses a major challenge in the clinical field [1]. This concern is particularly worrying in the context of infections associated with bacterial biofilms, where antibiotic recalcitrance is exacerbated. Antibiotic concentrations up to 1000 times higher are required to be effective against bacterial cells in the sessile state compared to planktonic cells [4]. Furthermore, the formation of bacterial biofilms impairs the effectiveness of the host immune response, favouring infection persistence and chronicity [4,17]. In this context, the implementation of strategies aimed at disrupting the formation and stability of biofilms can play a crucial role in reducing selective pressure and increasing the efficacy of antibiotics [41].

One promising strategy is the use of natural chelating agents, which have shown effectiveness against biofilms. These agents are particularly valuable because they can bind essential metals such as iron, which are crucial for the formation and maintenance of the structural integrity of the biofilm matrix [7]. Citric acid, for example, is widely recognised for its strong chelating properties. It forms stable complexes with metal ions such as iron, copper, calcium and magnesium through interactions with its carboxyl and hydroxyl groups [49]. Natural polyphenols, such as tannic acid, also support this strategy by chelating iron from the surrounding environment, thereby making it unavailable to bacteria. The *o*-dihydroxyphenyl groups in the tannin molecule play a key role in the chelation of iron (III) ions, forming a lattice structure that precipitates the iron along with the tannin [50,51]. In addition, quercetin, a flavonoid, demonstrated ability to inhibit Fe^{2+}-induced lipid peroxidation in adult male C57BL/6 J mice with alcoholic liver disease (at 100 mg/kg body weight). Its chelating effect against Fe^{2+} alleviates iron overload, oxidative stress and liver damage in mice [52].

Building on this foundation, the present study investigates, for the first time, two natural iron chelating agents, maltol and kojic acid, for their effect in preventing and eradicating *P. aeruginosa* and *S. aureus* biofilms when used individually and in combination with selected antibiotics. Maltol and kojic acid were selected for their diverse biological activities, such as antioxidant, antimicrobial and anti-inflammatory properties [53]. The pyrone derivatives in particular have attracted great interest as they are non-toxic and approved for use as food flavourings [54]. In fact, under this work, we evaluated the cytotoxicity of maltol and kojic acid in HepG2 cells, showing that despite a slight decrease of metabolic activity, the increasing concentrations did not lead to an increase of cell death. This data suggests the safety of these two 2-hydroxy-4-pyrone derivatives. Furthermore, their potential to form metal ion complexes supports their use in the development of innovative therapeutic strategies [24,27].

In a first phase, it was found that both 2-hydroxy-4-pyrone derivatives did not exert any antibacterial activity against *S. aureus* and *P. aeruginosa* at the concentrations tested (up to 1000 μg mL^{-1}). These results are consistent with the work of Wu *et al.* [55] who demonstrated that kojic acid has an MIC of 10 mM (approximately 1400 μg mL^{-1}) for *S. aureus* ATCC 6538. In addition, the authors found that this compound appeared to have greater activity against Gram-negative than Gram-positive bacteria. However, the MIC of kojic acid against *Escherichia coli* BNCC133721 remained above 1000 μg mL^{-1}. In another study, Li *et al.* [56] also showed the weak antibacterial activity of kojic acid for Gram-negative bacteria, with the natural chelating agent exhibiting an MIC of 24 mM (about 3410 μg mL^{-1}) for *Acinetobacter baumannii*. With regard to maltol, Ziklo and colleagues [57] observed a MIC of 4000 μg mL^{-1} for both *S. aureus* and *P. aeruginosa*.

Despite the lack of antibacterial activity at lower concentrations, the efficacy of 2-hydroxy-4-pyrone derivatives as natural chelating agents was demonstrated in this study. Their high affinity for Fe^{3+} can be attributed to the ability of pyrones to form σ- and π-bonds with metal ions and to the availability of donor groups at physiological pH [58]. In fact, kojic acid and maltol have been shown to be as effective as EDTA at concentrations of 800 μg mL^{-1} or higher, reaching levels of 80 % binding capacity. It should be noted that although EDTA was used as a positive control, its chelation mechanism differs from that of the 2-hydroxy-4-pyrone derivatives. EDTA is a hexadentate ligand, which allows it to bind to metal ions at multiple sites and results in a higher binding efficiency. In contrast, both kojic acid and maltol are bidentate ligands [59]. This difference in denticity explains the observed differences in binding capacity, with EDTA demonstrated a similar percentage of binding at significantly lower concentrations [60]. These results align with the study by Bai *et al.* >[61], which investigated the chelating effect of various natural products, including citric acid, tartaric acid and oxalic acid. The study assessed these agents alongside EDTA to evaluate their effects on the degradation capacity of the MgO_2/Fe(III) system in relation to organic contaminants. The authors reported a significant increase in the degradation efficiency of the dye rhodamine B, which increased from 6.7 % (without a chelating agent) to 42.3 % with citric acid, 98.5 % with tartaric acid, 48.9 % with oxalic acid and 25.8 % with EDTA within just 30 min. This remarkable increase was primarily attributed to the chelating interaction between the natural active agents and Fe(III). The iron chelating capacity of both maltol and kojic acid was further demonstrated using FeNTA reagent as insult for HepG2 cells. Both compounds, at concentrations lower than 750 μg mL^{-1}, were able to prevent the metal damage for the cells, causing an increasing of cell mass when compared with cells treated only with iron (III).

Preventing biofilm formation is an attractive therapeutic approach, as eradicating an established biofilm is much more difficult [5]. One of the effective strategies to prevent biofilm formation is to inhibit the initial stage of cell attachment and thus prevent the onset of infection [62]. It has been shown that high-affinity metal chelators such as EDTA and citrate are able to inhibit bacterial growth, interrupt the adhesion of microorganisms by inhibiting fibrin formation and thus prevent the formation of biofilms [63–65]. Other chelating agents of natural origin such as catechol derivatives, baicalein [66], and alizarin [67] can be used to prevent biofilm formation. In particular, Lin *et al.* >[14] showed

that the phytochemical 1,2,3,4,6-penta-*O*-gallium-β-D-glucopyranose can bind to iron ions, inhibiting the initial cell attachment and biofilm formation of *S. aureus* SA113. In addition, the authors observed the expression of genes and proteins associated with iron deficiency states and decreased production of the intercellular polysaccharide adhesin (PIA). It is noteworthy that many of the chelating agents used to prevent biofilm formation in *S. aureus* inhibit the production of staphylococcal PIA [14,68].

In this study, kojic acid was not able to prevent the formation of *P. aeruginosa* biofilms, as it had no effect on biomass, metabolic activity and CFU. This result can be explained by the ability of *P. aeruginosa* to produce siderophores. Therefore, the efficiency of the selected natural chelators depends on their ability to compete with *P. aeruginosa* for iron [69]. The results of this study are in agreement with those of Çevik and Ulusoy [70], where it was observed that kojic acid caused only a 5 % reduction in the mass of *P. aeruginosa* PAK01 biofilms. On the other hand, the authors found that kojic acid inhibited the biomass of the *P. aeruginosa* PAO1 strain by 27 %. These results show that chelating agents have different effects against different strains of the same species. This could be related to the varying ability of different strains to produce siderophores. Furthermore, O'May *et al.* >[69], show that the use of synthetic chelators (deferoxamine mesylate (DM) and ethylenediamine-*N*,*N*′-diacetic acid (EDDA)) had no significant effect on the biofilms of *P. aeruginosa* PAO1 at 2500 μM. The authors showed that the weak activity of the synthetic chelators was due to their chemical structure and low iron binding coefficients.

Regarding *S. aureus*, kojic acid showed a similar reduction in biomass as observed in the study of Wu *et al.* [55]. The authors found that kojic acid led to a 40 % reduction in biomass when used at 700 μg mL^{-1}. The authors demonstrated that the main action mechanisms of kojic acid were associated with membrane changes, leading to losses at the cytosol level and a significant interaction with DNA.

Maltol inhibited the biomass production and metabolic activity of the bacteria studied during biofilm formation by 30–40 %. These results may be related to the ability of maltol to interfere with bacterial communication mechanism, quorum sensing. In this context, Liu *et al.* >[71] demonstrated that ethyl maltol (0.1 mg mL^{-1}) can reduce the ability of *Chromobacterium violaceum* CV026 to produce violacein as well as the production of the autoinducer *N*-dodecanoyl-L-Homoserine lactone (C12-HSL) by *Aeromonas salmonicida*. In addition, the antibiofilm effect of maltol can also be explained by its ability to produce reactive oxygen species (ROS). In particular, Murakami *et al.* [72] verified that maltol can act as a pro-oxidant by generating ROS. In this context, the pro-oxidant properties are attributed to the production of the superoxide radical by the maltol-iron complex. These radicals dismutate into hydrogen peroxide, which subsequently forms the hydroxyl radical through the Fenton reaction. The hydroxyl radical is a very strong oxidising agent associated with damage to DNA and bacterial cells [38].

Well-established biofilms show even higher resistance to antibiotic therapies [73]. These mature biofilms, characterised by a dense extracellular matrix, not only shield the bacterial cells from antimicrobial agents but also enhance their survival under unfavourable conditions [4]. In this study, the 2-hydroxy-4-pyrone derivatives demonstrated a very significant effect in terms of metabolic activity and culturability reduction when tested at 10xMTC. In contrast, when the selected compounds were applied at MTC, only a slight reduction in biomass was observed in pre-established biofilms of *S. aureus* and *P. aeruginosa*. As far as we know, this study was the first to investigate the antibiofilm activity of selected natural chelating agents on mature biofilms. However, despite the high concentrations of 2-hydroxy-4-pyrone derivatives used, complete eradication was not achieved. In the study by Banin *et al.* [65], the application of EDTA in concentrations of 50 and 100 mM (approx. 14,500 and 29,000 μg mL^{-1}) in phosphate-buffered saline solution (PBS) only led to a reduction of approx. 2.5 log (CFU cm^{-2}) in established *P. aeruginosa* PAO1 biofilms.

In view of the increasing importance of chelating agents in improving the efficacy of antibiotics, the combination of 2-hydroxy-4-pyrone derivatives with ciprofloxacin and tobramycin was further investigated in biofilm tests. The synergy observed in planktonic assays emphasizes their potential in combating biofilm-associated infections, where bacterial resistance is often more pronounced [2]. In the treatment of pre-established *P. aeruginosa* biofilms, combinations with 2-hydroxy-4-pyrone derivatives showed no synergy, mainly due to the strong activity of ciprofloxacin, whose main mechanism of action is the inhibition of the enzymes topoisomerase II and IV [17]. Despite the lower concentrations of the selected compounds used in these combinations, the potent effect of ciprofloxacin may have overshadowed the contributions of the chelating agents. Tobramycin, on the other hand, showed a more modest effect when applied individually at sub-inhibitory concentrations. The main mechanism of action of this aminoglycoside antibiotic is the inhibition of protein synthesis through interactions with polyanionic 16 S rRNA regions on the 30 S ribosome [74]. In this way, it was verified that combinations with tobramycin, in particular with maltol, have a significant effect on the eradication of mature *S. aureus* biofilms. These results are consistent with the outcomes of the disc diffusion test and checkerboard assay, in which maltol achieved an improved effect with tobramycin. The combination of maltol-tobramycin improved the effect of the antibiotic alone in terms of biomass, metabolic activity and CFU for all concentrations of tobramycin tested. Furthermore, to our knowledge, this is a pioneer study investigating combinations between 2-hydroxy-4-pyrone derivatives and antibiotics for the control of biofilms. The combination of chelating agents with antibiotics has already been investigated for the treatment of biofilms. In the study conducted by Banin *et al.* >[65], the application of gentamicin at a concentration of 50 μg mL^{-1} together with EDTA (at a concentration of 50 mM) only resulted in a reduction of the already established *P. aeruginosa* PAO1 biofilms cells by approximately 3.5 log (CFU cm^{-2}). However, it should be noted that EDTA alone (at a concentration of 50 mM) caused a reduction of 2.5 log (CFU cm^{-2}). In another study, Liu *et al.* [75] showed that the combination of EDTA with ampicillin led to a reduction in biofilm cells by 2–3 log (CFU mL^{-1}) when incubated with 6-day-old *P. aeruginosa* biofilms. Again, high concentrations of EDTA (30 mg mL^{-1}) and ampicillin (320 μg mL^{-1}) were used. The effect of combinations between kojic acid and EDTA was also investigated by Ali and colleagues [76] against 23 clinical isolates. The authors demonstrated that kojic acid (6.25 mg mL^{-1}) caused a 51 % decrease in the biofilm mass when used alone, while EDTA (8 mg mL^{-1}) caused almost 80 % inhibition. The combined application of these chelating agents did not lead to a synergistic effect, as EDTA alone showed better results than in combination with kojic acid at the same concentrations.

Given the higher efficacy of 2-hydroxy-4-pyrone derivatives, particularly in combination with tobramycin, in controlling *S. aureus* biofilms, the study further investigated the effect of natural chelators, alone and in combination with tobramycin, on the disruption of biofilm cell membranes. The application of kojic acid and maltol at the MTC did not affect the integrity of the cell membranes during biofilm formation. These results are consistent with the study of Wu *et al.* >[55], which verified that the application of kojic acid (1400 μg mL^{-1}) also had no significant effect on the membrane integrity of *S. aureus* ATCC 6538 cells in the planktonic state. Similar results were observed for maltol, where Ziklo and collaborators [57] reported that treatment with maltol (5000 μg mL^{-1}) led to only a 7.7 % loss of intracellular content in *S. aureus*.

As far as tobramycin is concerned, its application does not appear to affect the membrane integrity of visualized *S. aureus* cells. However, the total number of cells was significantly reduced. This can be explained by the bactericidal activity against *S. aureus* described for this antibiotic [77]. In a study conducted by Jacob *et al.* [78] it was also observed that the antibiotic dalbavancin, a bactericidal lipoglycopeptide, reduced the number of cells without affecting the cell membrane when applied to *S. aureus* biofilms at concentrations of 4–32 mg L^{-1}. These examples and

the results obtained illustrate how bactericidal antibiotics can effectively reduce the total number of bacterial cells. In addition, the reduced number of cells in the biofilm could also be due to the effect of tobramycin on cell adhesion. Tobramycin may impair the ability of bacterial cells to adhere or cause them to adhere weakly, resulting in their removal during washing procedures. As a result, the remaining cells make up a smaller proportion of the biofilm [79]. On the other hand, the use of a 10 × MTC of natural chelating agents in already established biofilms led to an almost complete rupture of the bacterial cell membranes. However, when these agents were combined with tobramycin, a significant reduction in combined efficacy was observed, dropping to 50 %. This can be due to a possible reduction in the total number of cells by the action of tobramycin, which impairs the binding of PI to double-stranded nucleic acids and may lead to an underestimation of the proportion of bacterial cells with damaged membranes.

Following the assessment of membrane integrity, the potential of 2-hydroxy-4-pyrone derivatives to attenuate key virulence factors of *S. aureus* was further investigated. In contrast to many other bacterial pathogens, which often rely on one or a few toxins to cause disease, *S. aureus* produces a wide variety of virulence factors [80]. In this way, it can cause a broad spectrum of infections in different parts of the body of various vertebrates [81]. Its remarkable virulence is attributed to several factors that enable the bacterium to adhere to surfaces, invade the host, evade the immune system and damage the host's tissues [82]. Among the virulence factors, toxins, exoenzymes, adhesins and siderophores stand out. This study focused only on total siderophores and specific exoenzymes, particularly proteases. Like all exoenzymes, whose main goal is nutrient acquisition through the degradation of host proteins, proteases also play an important role in the modulation of the immune response and bacterial proliferation. In particular, the proteases are able to cleave immunoglobulins, plasma proteinases and elastin inhibitors, thereby inactivating various immunological defence mechanisms that are important for leukocyte chemotaxis [81]. Among the proteases, three main families stand out: metalloproteases (*e.g.* Aur), cysteine proteases (*e.g.* SspB and Scp) and serine proteases (*e.g.* SspA) [83]. In addition, other enzymes can also be produced, such as gelatinases, which target the degradation of collagen. By breaking down these structural barriers, gelatinases facilitate the spread of bacteria in the tissue and thus contribute significantly to the progression of the infection [84]. On the other hand, lipases catalyse the hydrolysis of lipids and enable *S. aureus* to use lipids as a nutrient source. In addition, the lipases of *S. aureus* are able to inactivate the host germicidal lipids, which allows the bacteria to survive and impairs the function of the host granulocytes [85].

In this study, it was observed that the kojic acid and maltol led to complete inhibition of total proteases by *S. aureus* cells during the biofilm formation and in already established biofilms. Given the importance of proteases in the processes of adhesion, proliferation and detachment, which are fundamental for the formation and maturation of biofilms, this could explain the antibiofilm activity of 2-hydroxy-4-pyrone derivatives. Furthermore, these results are consistent with the effect of EDTA, which also strongly inhibits exoenzymes, especially metalloproteases, emphasising the role of chelating agents as inhibitors of *S. aureus* virulence factors [86]. On the other hand, the application of natural chelating agents reduced the production of siderophores both in the formation and in pre-established biofilms of *S. aureus*. This suggests that these molecules have a greater affinity for iron than bacterial siderophores, limiting siderophores action [69]. The decrease in siderophore production compromises the ability of *S. aureus* to acquire the iron necessary for its metabolism and growth. In addition to affecting the formation of biofilms, this limitation weakens the bacterial defensive response against the host immune system. Consequently, reduced siderophore production can significantly impact the virulence of S. *aureus*, making the bacterium less capable of causing persistent and chronic infections [6].

The chelating properties of maltol and kojic acid play an important role in various applications, especially in the clinical field for the treatment of biofilm-associated infections. In clinical settings, these compounds can enhance the efficacy of antimicrobial agents, serving as effective adjuvants against both *S. aureus* and *P. aeruginosa* infections. This strategy has the potential to improve the activity of antibiotics that would otherwise be less effective against biofilm-forming pathogens, significantly reducing the bactericidal concentrations required for treatment. Such enhancements could significantly influence treatment protocols, particularly for patients with chronic infections, where the presence of biofilm is a major barrier to successful therapy [38]. In addition, their ability to chelate metals can be used for the removal of toxic metals and thus contribute to detoxification in medical treatments [49]. Furthermore, like citric acid, maltol and kojic acid can help prevent oxidation and discolouration in food preservation by binding metal ions, improving the shelf life and quality of food. In the environmental and industrial sectors, these natural chelators offer potential for soil remediation and water treatment by binding toxic metals and correcting chemical imbalances [49]. These diverse applications underscore the significant potential of maltol and kojic acid to promote human health and environmental safety.

Looking ahead, the structural optimization of 2-hydroxy-4-pyrone derivatives is a promising avenue for future research. By modifying their chemical structures, it may be possible to improve their iron-binding capacity, stability, or ability to penetrate biofilms. An exciting direction would be the development of antibiotic hybrids, in which one pharmacophore is designed for antibiotic activity and the other for chelating properties using natural active constituents such as kojic acid or maltol [87]. This approach could maximize therapeutic potential by simultaneously disrupting the structure of the biofilm through iron deprivation and promoting bacterial eradication through the antibiotic effect.

5. Conclusion

The natural chelating agents kojic acid and maltol exhibit significant antibiofilm activity against *S. aureus* and *P. aeruginosa*. These compounds showed an affinity for iron (III) comparable to the commercial chelating agent EDTA, demonstrating their efficacy in forming stable complexes with iron. This data was crucial to prevent the cellular damage of HepG2 after treatment with FeNTA reagent. The application of 2-hydroxy-4-pyrone derivatives resulted in a significant reduction in *S. aureus* and *P. aeruginosa* biomass, metabolic activity and culturability, both during formation and in pre-established biofilms. In combination with tobramycin and ciprofloxacin, kojic acid and maltol considerably enhanced the efficacy of these antibiotics. Furthermore, the concentration of antibiotics could be reduced without impairing their activity. This effect was even more pronounced with the 2-hydroxy-4-pyrone derivatives-tobramycin combinations in the control of *S. aureus* biofilms, as they damaged the cell membranes of the biofilm and reduced the production of virulence factors, especially the proteases. Therefore, kojic acid and maltol can be considered as promising adjuvants in the treatment of bacterial infections associated with biofilms. They extend the efficacy of conventional antibiotics and offer a new approach to the efficient control of biofilms.

CRediT authorship contribution statement

Miguel M. Leitão: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Ariana S.C. Gonçalves:** Validation, Methodology. **Joana Moreira:** Methodology, Validation. **Carlos Fernandes:** Investigation, Validation, Writing – original draft. **Fernanda Borges:** Writing – review & editing, Supervision. **Manuel Simões:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Anabela Borges:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Ethical statement

The authors confirm that all the research meets the ethical guidelines.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2024.117163.

Data availability

Data will be made available on request.

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