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Antimicrobial and antibiofilm potentiation by a triple combination of dual biocides and a phytochemical with complementary activity

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ABSTRACT

The failure of current sanitation practices requires the development of effective solutions for microbial control. Although combinations using antibiotics have been extensively studied to look for additive/synergistic effects, biocide combinations are still underexplored. This study aims to evaluate the antimicrobial effectiveness of dual biocide and triple biocide/phytochemical combinations, where phytochemicals are used as quorum sensing (QS) inhibitors. The biocides selected were benzalkonium chloride (BAC) and peracetic acid (PAA) – as commonly used biocides, and glycolic acid (GA) and glyoxal (GO) – as alternative and sustainable biocides. Curcumin (CUR) and 10-undecenoic acid (UA) were the phytochemicals selected, based on their QS inhibition properties. A checkerboard assay was used for the screening of chemical interactions based on the cell growth inhibitory effects against *Bacillus cereus* and *Pseudomonas fluorescens*. It was observed that dual biocide combinations resulted in indifference, except the PAA + GA combination, which had a potential additive effect. PAA + GA + CUR and PAA + GA + UA combinations also triggered additive effects. The antimicrobial effects of the combinations were further evaluated on the inactivation of planktonic and biofilm cells after 30 min of exposure. These experiments corroborated the checkerboard results, in which PAA + GA was the most effective combination against planktonic cells (additive/synergistic effects). The antimicrobial effects of triple combinations were species- and biocide-specific. While CUR only potentiate the antimicrobial activity of GA against *B. cereus*, GA + UA and PAA + GA + UA combinations promoted additional antimicrobial effects against both bacteria. Biofilms were found to be highly tolerant, with modest antimicrobial effects being observed for all the combinations tested. However, this study demonstrated that low doses of biocides can be effective in bacterial control when combining biocides with a QS inhibitor, in particular, the combination of the phytochemical UA (as a QS inhibitor) with GA and PAA.

1. Introduction

In industrial settings, microbial safe levels are ensured by the implementation of Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Points (HACCP) plans, which settle on biofilm prevention by the identification, assessment, and control of hazards (Blom, 2015; Sharma & Anand, 2002). The selection of sanitation practices to prevent or readily control the established biofilms depends on the process conditions, such as surface material, type of biocide used, contact time, and microbial contaminants (Parish et al., 2003). Sanitation comprises cleaning (*i.e.* application of alkaline- or acid-based cleaning formulations) and disinfection steps (*i.e.* application of physical methods, like UV light and ultrasounds, or antimicrobial compounds/biocides) that trigger the removal of organic and mineral residues, and microbial damage or eradication (Iniguez-Moreno et al., 2021). However, even after regular sanitation, bacteria can persist on surfaces, within biofilms (Stoller et al., 2019).

The increased antimicrobial tolerance of biofilm cells has been related to several mechanisms, including the process conditions that affect biofilm development and the intrinsic properties of the biofilm resident cells (Simões et al., 2010). For example, Iniguez-Moreno et al. (2021) demonstrated that biofilm removal (by enzymes and biocides) was influenced by the type of surface material (stainless steel and polypropylene) and nutrient load (presence of food debris). The intrinsic properties comprise low cell accessibility and chemical interaction of biocides with biofilm constituents, microenvironment heterogeneity,

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nutrient limitation and slow growth rate, production of degradative enzymes, cell-to-cell communication by quorum sensing (QS) mechanisms, and presence of persister cells (Bridier et al., 2011).

Nowadays, the possibility of cross-resistance between biocides and antibiotics or other biocides is a well-recognized phenomenon (Pereira et al., 2020). Capita et al. (2019) demonstrated that the continuous exposure to sub-lethal concentrations of disinfectants [*i.e.* sodium hypochlorite, peracetic acid (PAA), and benzalkonium chloride (BAC)] promoted acquired tolerance to other biocides and the resistance to antibiotics by *Cronobacter sakazakii* and *Yersinia enterocolitica*. Furthermore, several operating and geometric characteristics result in the use of sub-lethal concentrations, particularly the use of low biocide doses, inappropriate storage with loss of antimicrobial effectiveness, presence of high organic load as interfering substances, and a gradient distribution around corners and difficult-to-reach of specific parts of the system, particularly the so-called dead zone (Capita et al., 2019).

Given the systematic failure of current sanitation practices against biofilm cells and the rising biocide tolerance and antibiotic cross-resistance, the demand for new antimicrobial solutions has emerged. The development of antimicrobial formulations based on already approved active compounds is an attractive alternative strategy for the development and registration of novel disinfecting products (European Union, 2012). Combination-based approaches have been explored to obtain improved antimicrobial effects, particularly for antibiotic combinations (with specific cellular targets) (Gonzales et al., 2015; Mei et al., 2022; Stein et al., 2015). The combination of antibiotics with QS inhibitors has also revealed improved antimicrobial effects (Brackman et al., 2011; 2016; Hawas et al., 2022). QS inhibition can mitigate microbial pathogenesis and virulence (by attenuating the expression of virulence factors and preventing biofilm formation) and increase antimicrobial susceptibility (Borges et al., 2017; Li et al., 2022; Mayer et al., 2020; Wei et al., 2020). However, the outcomes from the combinations with biocides (with multiple cellular targets) remain underexplored since it is expected that additional molecules would have little room for a suitable activity that would enable synergism (Noel et al., 2021).

The present study aims to evaluate the potential of dual biocide and triple biocide/QS inhibitor combinations on the antimicrobial action against planktonic and biofilm cells. *Bacillus cereus* and *Pseudomonas fluorescens* were selected as model food spoilage microorganisms that are typically found in food industry settings (Scales et al., 2014; Soni et al., 2016). Firstly, the potential of glycolic acid (GA) and glyoxal (GO) (Fernandes et al., 2020; 2022a) in enhancing the antimicrobial effects of commonly used biocides (BAC and PAA) was assessed (dual biocide combinations). Triple combinations were further tested by combining these biocides with selected QS inhibitors [phytochemicals with low toxicity – curcumin (CUR) and 10-undecenoic acid (UA) (Fernandes, Borges, Gomes, Sousa, & Simões, 2023)]. Two- and three-dimensional checkerboard assays and fractional inhibitory concentration index (FICI) calculations were performed to evaluate the role of the chemical interactions on cell growth inhibition (during 24 h of exposure). The antimicrobial effects of dual biocide combinations (at distinct concentrations) were evaluated in the inactivation of planktonic and biofilm cells, after 30 min of exposure. The most effective dual biocide combinations, based on the triple FICI values and cell inactivation, were selected for assessing the antimicrobial and antibiofilm activity of triple combinations (dual biocide/QS inhibitor).

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacillus cereus strain isolated from a disinfection solution and identified by 16S rRNA gene sequencing (Simões et al., 2007) and *Pseudomonas fluorescens* ATCC 13525 ^{T} were selected as representative Gram-positive endospore-forming and Gram-negative bacterial models, respectively. Selected strains have been used as target bacteria for antimicrobial tests against planktonic and biofilm cells (*i.e.* antimicrobial activity and mode of action) (Fernandes, Gomes, & Simões, 2020, 2022; Fernandes, Gomes, Sousa, & Simões, 2022). Both bacteria were grown overnight using a sterile synthetic nutrient medium (5 g/L of glucose, 2.5 g/L of peptone and 1.25 g/L of yeast extract in 0.2 M phosphate buffer, pH 7, all from Merck, Germany) at 30 °C with agitation (120 rpm). Overnight grown cultures were centrifuged (3772 × g, 10 min) and washed once with phosphate buffer saline [PBS, 8 g/L of NaCl (VWR, Belgium), 0.2 g/ L of KCl (VWR, Belgium), 1.44 g/L of Na_2HPO_4 (Chem-Lab NV, Belgium) and 0.24 g/L of KH_2PO (VWR, Belgium), pH 7.4]. The cell pellets were then resuspended in tryptic soy broth (TSB) or PBS and adjusted to a cellular density according to the requirements of each assay.

2.2. Biocides and quorum sensing inhibitors/phytochemicals

The selection of biocides for dual combinations was performed based on previous results about their antimicrobial effects on planktonic and biofilm cells (Fernandes, Gomes, & Simões, 2020, 2022). Glycolic acid 99% (*w/w*) (GA; Sigma-Aldrich, USA) and glyoxal 40% (*w/v*) (GO; Sigma-Aldrich, Denmark) were selected as sustainable biocide alternatives to improve the antimicrobial effects of benzalkonium chloride (BAC; Sigma-Aldrich, Denmark) and peracetic acid 38–40% (*w/v*) (PAA; Merck, Germany) – two commonly used biocides (Simões et al., 2010). Biocide solutions were aseptically and freshly prepared in sterile TSB for checkerboard assays or sterile distilled water for antimicrobial inactivation tests against planktonic and biofilm cells. For triple biocide/QS inhibitor combinations, the selected QS inhibitors/phytochemicals were curcumin (CUR) 95% (total curcuminoid content), from *Turmeric rhizome*, and 10-undecenoic acid (UA) 99%, both from Alfa Aesar (Germany). These were previously characterized as QS inhibitors (Fernandes, Borges, Gomes, Sousa, & Simões, 2023). Stock phytochemical solutions were freshly prepared in 100% DMSO, adequate dilutions were performed to ensure a final concentration of 6% (*v/v*) of DMSO.

2.3. Combination screening – Checkerboard assay

Dual biocide combinations evaluated the potential of GA/GO to improve the antimicrobial activity of BAC/PAA. For that, a two-dimensional checkerboard assay was used to determine the role of chemical interactions on the effects of cell growth inhibition after 24 h of exposure (Buchmann et al., 2022). Bacterial pellets were resuspended in TSB and adjusted to 10^8 colony forming units per mL (CFU/mL). In total, 20 μL of bacterial suspension was added to 80 μL of TSB and 100 μL of biocidal solution (50 μL/50 μL of BAC/GA, BAC/GO, PAA/GA, or PAA/GO). Each column contained increasing concentrations of PAA (12.5, 25, 50, 100, 160, 200, 320, 400, 800, 1000, and 1600 μg/mL) or BAC (0.5, 1, 2, 3, 4, 5, 10, 15, 20, 40, and 80 μg/mL) and each row contained increasing concentrations of GA (200, 400, 800, 1000, 1250, 1500, 2000, 2500, and 5000 μg/mL) or GO (12.5, 25, 50, 100, 250, 300, and 500 μg/mL). The selected concentrations were based on the minimum inhibitory concentration (MIC) of each biocide (see supplementary file).

The effects of triple combinations (dual biocide/QS inhibitor) were assessed through a three-dimensional checkerboard assay (Stein et al., 2015). For that, each well was filled with 20 μL of bacterial suspension, 68 μL of TSB, 100 μL of biocidal solution (50 μL/50 μL of BAC/GO, PAA/GA, or PAA/GO), and 12 μL of the QS inhibitor (CUR or UA). Two-dimensional checkerboard plates were prepared as previously described by adding BAC or PAA and GA or GO. Then, each plate was filled with different concentrations of QS inhibitor (third compound) – CUR at 9.375, 18.75, and 37.5 μg/mL for *B. cereus* and at 18.75, 37.5, and 75 μg/mL for *P. fluorescens*; UA at 50, 100, and 200 μg/mL for *B. cereus* and at 125, 250, and 500 μg/mL for *P. fluorescens* (concentrations below the MIC, see Table S1). Two- and three-dimensional checkerboard plates were incubated for 24 h at 30 °C with agitation (160 rpm). Absorbance at 610 nm was measured before (0 h) and after incubation

(24 h) using a microtiter plate reader (SPECTROstarNano; BMG Labtech, Germany).

For all wells with no detectable growth, the Fractional Inhibitory Concentration (FIC) was calculated according to Equation (1), where MIC_X corresponds to the lowest concentration of the biocide or QS inhibitor tested (X) which inhibited cell growth. The Fractional Inhibitory Concentration Index (FICI) was estimated as the sum of each FIC ($FIC_A + FIC_B$ for dual biocide combinations and $FIC_A + FIC_B + FIC_C$ for triple combinations).

$$FIC = \frac{MIC_X \text{ in combination}}{MIC_X \text{ alone}} \quad (1)$$

The antimicrobial effects of the combination were defined as synergistic for FICI ≤ 0.5, additive for $0.5 <$ FICI ≤ 1, indifferent for $1 <$ FICI < 4, and antagonist for FICI ≥ 4 (Ju et al., 2022). The FICI represents the mean of three independent experiments, whereas the lowest FICI was selected (corresponding to the best combination).

Additionally, experimental data of dual biocide combinations were modelled using Combenefit software (version 2.021, available at https://sourceforge.net/projects/combenefit/, accessed on June 2022), which allowed the visualization of antimicrobial effects of combinations according to the Loewe model as a function of concentration (Di Veroli et al., 2016) – see Figures S1-S4. Furthermore, two-dimensional checkerboard plates were analysed for biofilm prevention. For that, after incubation during 24 h of bacterial suspension with dual biocide combinations, biofilm formation was quantified by crystal violet staining as described by Fernandes et al. (2022) and experimental data, as a percentage of biofilm inhibition, was modelled using Combenefit software (Figures S5-S8).

2.4. Potential for cell inactivation by dual biocide and triple combinations

The antimicrobial activity of dual biocide combinations (BAC/GO, PAA/GA, and PAA/GO) against planktonic and biofilm cells was assessed based on both FICI values and cell inactivation after 30 min of exposure, where the most promising triple combinations (dual biocide/QS inhibitor) were picked for assessing the inactivation of planktonic/biofilm cells.

2.4.1. Inactivation of planktonic cells

The antimicrobial activity as the inactivation of planktonic cells was performed according to the European Standard EN 1276 (2009) with some modifications. Bacterial pellets were resuspended in PBS and adjusted to 10^8 CFU/mL. A volume of 1 mL of cell suspension was added to 1 mL of sterile distilled water and maintained in contact for 2 min. For dual biocide combinations, 8 mL of biocidal solution (4 mL/4 mL of BAC/GO, PAA/GO, or PAA/GA) was added to reach the desired concentrations (BAC: 4, 10, and 40 μg/mL; GA: 100, 200, 400, and 1000 μg/mL; GO: 12.5, 25, 125, and 5000 μg/mL; PAA: 1.25, 5, 25, 50, and 100 μg/mL). Triple combinations were performed by adding 3.7 mL/3.7 mL of PAA/GA and 0.6 mL of QS inhibitor to reach the desired concentrations: GA at 100 μg/mL; PAA at 1.25 and 10 μg/mL; CUR at 150 μg/mL; and UA at 100 μg/mL. Biocide concentrations were selected based on data modelled using Combenefit software, corresponding to synergy/indifference (Figures S1-S4). Furthermore, to enable the quantification of synergistic/additive effects of tested combinations, concentrations were selected to ensure that substantial inactivation of planktonic cells was not achieved for individual compounds, *i.e.* the sum of cell inactivation of each compound tested alone should not correspond to the total inactivation. QS inhibitor concentrations were selected based on the MIC (Table S1), ensuring that it provided negligible inactivation during the exposure time, when tested alone. Control samples comprised single biocide effects (replacing a biocide by sterile distilled water and QS inhibitor with DMSO), single QS inhibitor effects (replacing both biocides with sterile distilled water), and dual biocide/QS inhibitor combination effects (replacing a biocide by sterile distilled water). Positive controls were performed by adding sterile distilled water/DMSO instead of biocides and QS inhibitors. After 30 min of exposure at room temperature, biocide neutralization using a universal neutralizer [30 g/L of polysorbate 80 (VWR Chemicals, France), 30 g/L of saponin (VWR Chemicals, Belgium), 1 g/L of L-histidine (Merck, Japan), 3 g/L of lecithin (Alfa Aesar, Germany), 5 g/L of sodium thiosulphate (Labkem, Spain) in 0.0025 M phosphate buffer (EN 1276, 2009) was performed according to Fernandes et al. (2020). Then, surviving cells were quantified by CFU counting onto tryptic soy agar (TSA, TSB with 1.5% *w/v* agar) after appropriate serial dilution in sterile saline solution (0.85% *w/v* NaCl) and incubation at 30 °C for 24 h. For each condition, at least three independent experiments were performed with two replicates. The limit of detection of the method is 2.7-Log CFU/mL. The inactivation of planktonic cells was evaluated by logarithmic reduction (Log CFU/mL reduction) as $Log(X_0/X)$, where X_0 and X are the counts of CFU/mL for unexposed (positive control) and exposed bacteria, respectively.

Additionally, for different concentrations of PAA + GO combinations, the measurement of pH (phenomenal pH 1100 L, VWR, Germany) was performed over time (during 30 min) to assess the chemical decomposition of PAA (Figure S9).

2.4.2. Inactivation of biofilm cells

The 48 h-old biofilms (mature biofilms) were grown in 96-well polystyrene microtiter plates as previously described (Fernandes et al., 2022). For dual biocide combinations, biofilms were exposed to 200 μL of biocide solution (100 μL/100 μL of BAC/GO, PAA/GO, or PAA/GA) at the desired concentrations (BAC: 40 and 100 μg/mL; GA: 100, 400, 1000, 2000, and 10000 μg/mL; GO: 100, 125, 250, 300, and 5000 μg/mL; PAA: 100, 125, 200, 400, and 500 μg/mL). Triple combinations were performed by adding 94 μL/94 μL of PAA/GA and 12 μL of QS inhibitor to reach desired concentrations: GA at 1000 μg/mL; PAA at 200 μg/mL; CUR at 150 μg/mL; and UA at 1000 μg/mL. Biocide concentrations were selected based on data simulation using Combenefit software for the prevention of 24 h-old biofilm formation, corresponding to synergy/indifference effects (Figures S5-S8). Furthermore, to enable the evaluation of synergistic/additive effects of the combinations tested, concentrations were selected to ensure that substantial inactivation of biofilm cells was not achieved for individual compounds, *i.e.* the sum of inactivation of each compound tested alone should not correspond to the total inactivation. QS inhibitory concentrations were selected based on MIC (Table S1), ensuring that it provided negligible inactivation during the exposure time when tested alone. Control samples were performed by replacing a biocide with sterile distilled water and QS inhibitor by DMSO (single-biocide effect), both biocides by sterile distilled water (single QS inhibitor effect), and a biocide with sterile distilled water (dual biocide/QS inhibitor combination effect). Positive controls were performed by adding sterile distilled water/DMSO instead of biocides and QS inhibitors. After exposure for 30 min, antimicrobial solutions were discarded and neutralized for 15 min (Fernandes et al., 2022). Then, surviving cells were quantified in terms of cell culturability (CFU/cm^2). For each condition, at least three independent experiments were performed with two replicates. The limit of detection of the method is 1.5-Log CFU/cm^2. The inactivation of biofilm cells was evaluated by logarithmic reduction (Log CFU/cm^2 reduction) as $Log (X_0/X)$, where X_0 and X are the count of CFU/cm^2 for unexposed (positive control) and exposed biofilms, respectively.

2.5. Classification of antimicrobial effect of combinations

The antimicrobial effect of dual and triple combinations was classified according to the definitions of “Synergism”, “Antagonism”, “Indifference”, and “Additive effects” from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2000). Synergism occurred when the combination caused cell inactivation statistically significantly higher than the sum of inactivation from biocide/QS inhibitor alone ($P < 0.05$). Antagonism was observed when the

combination caused cell inactivation statistically significantly lower than the most active compound ($P < 0.05$). Indifference occurred when no statistically significant differences were observed for the cell inactivation promoted by the combination compared to the most active compound ($P > 0.05$). Finally, the additive effect occurred when no statistically significant differences were found between the cell inactivation caused by the combination and the sum of cell inactivation from biocide/QS inhibitor alone ($P > 0.05$) and the cell inactivation from the combination was statistically significantly higher than the most active compound ($P < 0.05$).

2.6. Statistical analysis

Experimental data were analysed through the calculation of the mean and standard deviation (SD) for all conditions. The antimicrobial effects of dual biocide, dual biocide/QS inhibitor, and triple combinations were classified based on statistical differences between samples using unpaired *t*-test with Welch's correction from the statistical program GraphPad Prism 6.0 for Windows (GraphPad software, La Jolla California, USA). Statistical differences were established for a probability level of 95% ($P < 0.05$).

3. Results and discussion

Combination-based approaches are gaining increasing interest to exploit synergistic/additive effects using already approved compounds to help control bacterial tolerance/resistance worldwide and to provide solutions for the lack of development of new effective antimicrobial compounds (Buchmann et al., 2022; Ju et al., 2022; Oliveira et al., 2022; Pietsch et al., 2020). These approaches have been extensively addressed for antibiotics, to increase the therapeutic activity/decrease antimicrobial resistance (Tabcheh et al., 2023). Synergistic effects were achieved for different dual antibiotic and antibiotic-biocide combinations (Ju et al., 2022; Pietsch et al., 2020). The antimicrobial effects were found as specific to the antibiotic-biocide combination. For example, chemical interactions from gentamicin and meropenem caused synergistic and antagonistic effects, respectively (Pietsch et al., 2020). Phytochemicals (compounds from the secondary metabolism of plants) were also used in combination-based approaches. Very few authors have demonstrated synergistic effects from phytochemicals with recognized anti-QS activity combined with antimicrobial compounds (Brackman et al., 2016; Malheiro et al., 2019; Monte et al., 2014; Ning et al., 2021). These molecules were found to improve the antimicrobial activity of antibiotics by the downregulation of resistance mechanisms of bacteria and inhibition of virulence factors (Buchmann et al., 2022; Oliveira et al., 2022).

Studies concerning biocide combinations are required to increase the antimicrobial susceptibility of planktonic and biofilm cells to disinfection processes. Looking for more effective antimicrobial strategies against planktonic/biofilm cells than the current antimicrobial solutions, the present study focused on dual biocide combinations (PAA and BAC combined with GA or GO), followed by triple combinations using phytochemicals with recognized anti-QS activity (CUR and UA). In a first attempt, chemical interactions of dual biocide and triple combinations on cell growth inhibition were evaluated by checkerboard assay, after 24 h of exposure. Generally, synergistic effects from antibiotic combinations are validated by time-kill assays (during 24 h) (Buchmann et al., 2022; Ju et al., 2022). However, the exposure time of biocides is generally quite short (5 min). Thus, synergistic/additive effects were confirmed by the quantification of the inactivation of planktonic and biofilm cells of *B. cereus* and *P. fluorescens* after 30 min of exposure. The checkerboard assay was a good screening approach, even considering a high contact time, which allowed not only the evaluation and selection of potential synergistic/additive combinations but also the definition of concentrations to be validated in the following antimicrobial inactivation tests against planktonic/biofilm cells using typical exposure time of a disinfection process (Chino et al., 2017).

3.1. Chemical interactions of dual biocide and triple combinations – Checkerboard assay

The antimicrobial effects of dual biocide combinations against *B. cereus* and *P. fluorescens* according to the checkerboard assay are summarized in Table 1. All dual biocide combinations resulted in indifferent effects ($1 \leq FICI < 4$) against both bacteria, except PAA + GA which resulted in additive effects ($0.5 < FICI < 1$). There was a 2-fold and 4-fold reduction of MIC for PAA when measured alone *versus* in combination with GA against *B. cereus* and *P. fluorescens*, respectively. Additionally, Combenefit analysis demonstrated that synergy/antagonism can occur across a wide range of concentrations for all combinations, but not for all the concentrations (Fig. 1 and Figures S1-S4). For example, against *B. cereus*, the PAA + GA combination resulted in synergy for a specific range of concentrations (12.5–200 μg/mL of PAA and 200–400 μg/mL of GA), while another range triggered antagonistic effects (12.5–200 μg/mL of PAA and 1000–1500 μg/mL of GA). Distinct antimicrobial effects from synergy to antagonism on combination patterns were also demonstrated by Kashif et al. (2017) for different concentrations of clinically relevant anticancer drugs. In addition, the BAC + GA combination resulted in a wide-range antagonism zone (zone coloured from yellow to red in Fig. 1). BAC causes the greatest antimicrobial activity under neutral to slightly alkaline conditions and its activity decreases under acidic conditions (Frozza et al., 2021). Thus, the combination of BAC with a weak acid (GA) may potentiate the reduction of antimicrobial activity and consequently, BAC + GA did not proceed for the triple combinations and the inactivation of planktonic/biofilm cells was not evaluated.

The potential chemical interactions of triple combinations were also screened by the checkerboard assay. CUR and UA (at sub-inhibitory concentrations) were combined with dual biocide combinations (BAC + GO, PAA + GA, and PAA + GO) against *B. cereus* and *P. fluorescens*. According to the FICI results (Table 2), additive effects ($0.5 < FICI < 1$) were observed for all triple combinations, except for the BAC + GO + CUR against *B. cereus*. The low FICI values for triple combinations compared to dual biocide combinations are indicators of high antimicrobial effects when QS inhibitors were added. In general, the best triple combinations occurred when UA was used in comparison to CUR, and for PAA + GA or PAA + GO combinations in comparison to BAC + GO. The antimicrobial effects of PAA were improved, being reflected in the reduction of MIC against both bacteria, ranging from 4- to 32-fold reduction. On the other hand, MIC for BAC only reduced 1.25- to 15-fold in triple combinations *versus* BAC alone.

Table 1
Results of checkerboard assay for dual biocide combinations against *B. cereus* and *P. fluorescens*. MIC values were determined for biocides alone and in combination. Values are the means of three independent assays.

	MIC (μg/mL)				FICI	Effect [1]
	Alone		Combination			
	A	B	A	B		
BAC (A) + GA (B)						
B. cereus	5	2500	5	200	1.08	Indifference
P. fluorescens	15	2500	1	2500	1.07	Indifference
BAC (A) + GO (B)						
B. cereus	5	300	5	12.5	1.04	Indifference
P. fluorescens	15	300	15	12.5	1.04	Indifference
PAA (A) + GA (B)						
B. cereus	800	2500	400	800	0.82	Additivity
P. fluorescens	400	2500	100	1500	0.85	Additivity
PAA (A) + GO (B)						
B. cereus	800	300	25	300	1.03	Indifference
P. fluorescens	400	300	12.5	300	1.03	Indifference

[1] Synergism: $FICI \leq 0.5$; additivity: $0.5 < FICI < 1$; indifference: $1 \leq FICI < 4$; and antagonism: $FICI \geq 4$.

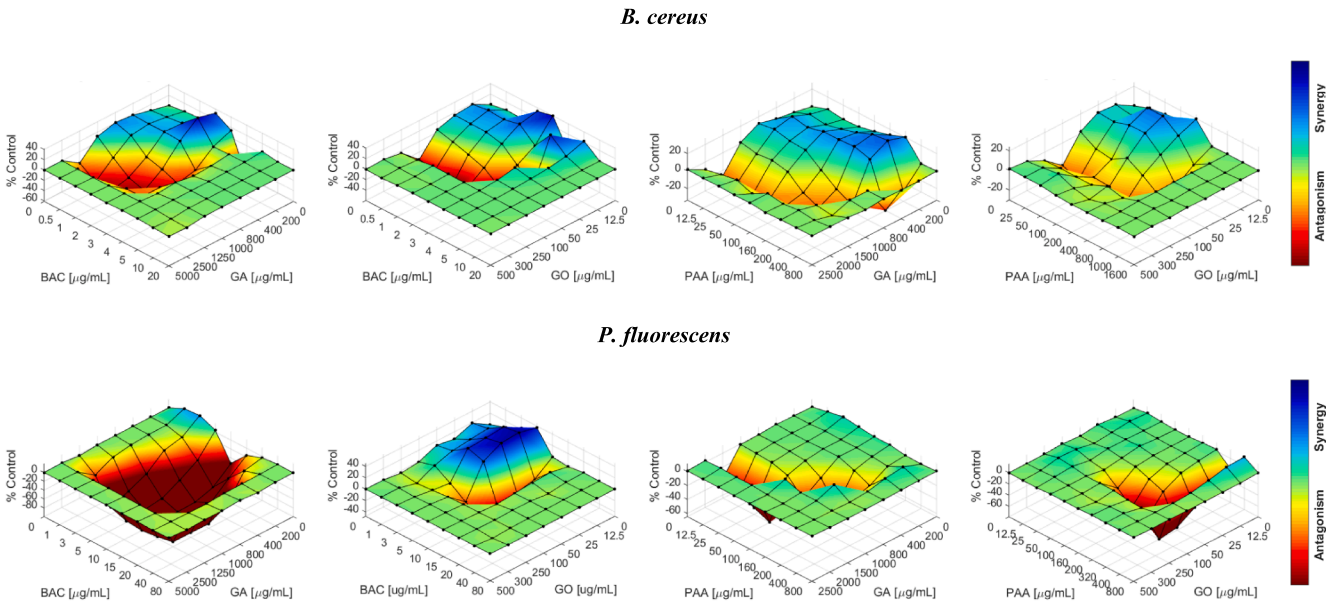

Fig. 1. Loewe dose–response graph showing the effects of dual biocide combination with increasing BAC/PAA and GA/GO concentrations on *B. cereus* and *P. fluorescens* cell growth inhibition.

Table 2
Fractional inhibitory concentration index (FICI) for each combination in cell growth inhibition of curcumin and 10-undecenoic acid (C) with BAC + GO, PAA + GA, and PAA + GO against *B. cereus* and *P. fluorescens*. MIC values were determined for biocides/phytochemicals alone and in combination. Values correspond to the mean of three independent assays.

	MIC (μg/mL)						FICI	Effect [1]
	Alone			Combination				
	A	B	C	A	B	C		
Curcumin (C)								
BAC (A) + GO (B)								
B. cereus	5	300	75	1	100	37.5	**1.03**	Indifference
P. fluorescens	15	300	150	1	150	18.75	**0.69**	Additivity
PAA (A) + GA (B)								
B. cereus	800	2500	75	100	200	37.5	**0.71**	Additivity
P. fluorescens	400	2500	150	100	1000	37.5	**0.90**	Additivity
PAA (A) + GO (B)								
B. cereus	800	300	75	100	50	37.5	**0.79**	Additivity
P. fluorescens	400	300	150	12.5	150	18.75	**0.66**	Additivity
10 – Undecenoic acid (C)								
BAC (A) + GO (B)								
B. cereus	5	300	400	4	12.5	50	**0.97**	Additivity
P. fluorescens	15	300	>1000	10	12.5	125	**<0.83**	Additivity
PAA (A) + GA (B)								
B. cereus	800	2500	400	50	200	200	**0.64**	Additivity
P. fluorescens	400	2500	>1000	100	1000	125	**<0.78**	Additivity
PAA (A) + GO (B)								
B. cereus	800	300	400	100	12.5	200	**0.67**	Additivity
P. fluorescens	400	300	>1000	12.5	150	125	**<0.66**	Additivity

[1] Synergism: FICI ≤ 0.5; additivity: 0.5 < FICI < 1; indifference: 1 ≤ FICI < 4; and antagonism: FICI ≥ 4.

3.2. Potential for cell inactivation by dual biocide combinations

For dual biocide combinations (BAC + GO, PAA + GA, and PAA + GO), the antimicrobial activity as the inactivation of planktonic and biofilm cells of *B. cereus* and *P. fluorescens* was evaluated after 30 min of exposure (Figs. 2-4).

In general, BAC + GO combinations resulted in indifferent effects against planktonic/biofilm cells of both bacteria (Fig. 2, $P > 0.05$). Except for the BAC + GO combination (100 μg/mL + 5000 μg/mL) that resulted in antagonistic effects against *B. cereus* biofilms ($P < 0.05$). The specific cellular phenotype (persister cells/endospores) and/or the interference with the extracellular polymeric substances from the biofilm matrix (Fernandes, Gomes, & Simões, 2022; Fernandes, Gomes, Sousa, & Simões, 2022) may help to explain the antagonism observed for *B. cereus* biofilms with a high concentration of GO.

PAA + GO combinations caused indifferent and antagonistic effects against both bacteria in planktonic and biofilm states (Fig. 3). The combination of PAA with organic compounds (including aldehydes) caused its decomposition to deprotonated species (PAA^-) that are agents with low oxidative power (Kim and Huang, 2021). According to the Baeyer-Villiger mechanism, PAA will react with GO resulting in the decomposition of PAA and increasing H^+ concentration (Kim and Huang, 2021). The continuous decrease of pH evidenced the chemical decomposition of PAA (Figure S9). Increasing GO/PAA concentration triggered a high reaction rate between the compounds (measured by the increase of H^+), and consequently, a high PAA decomposition rate is

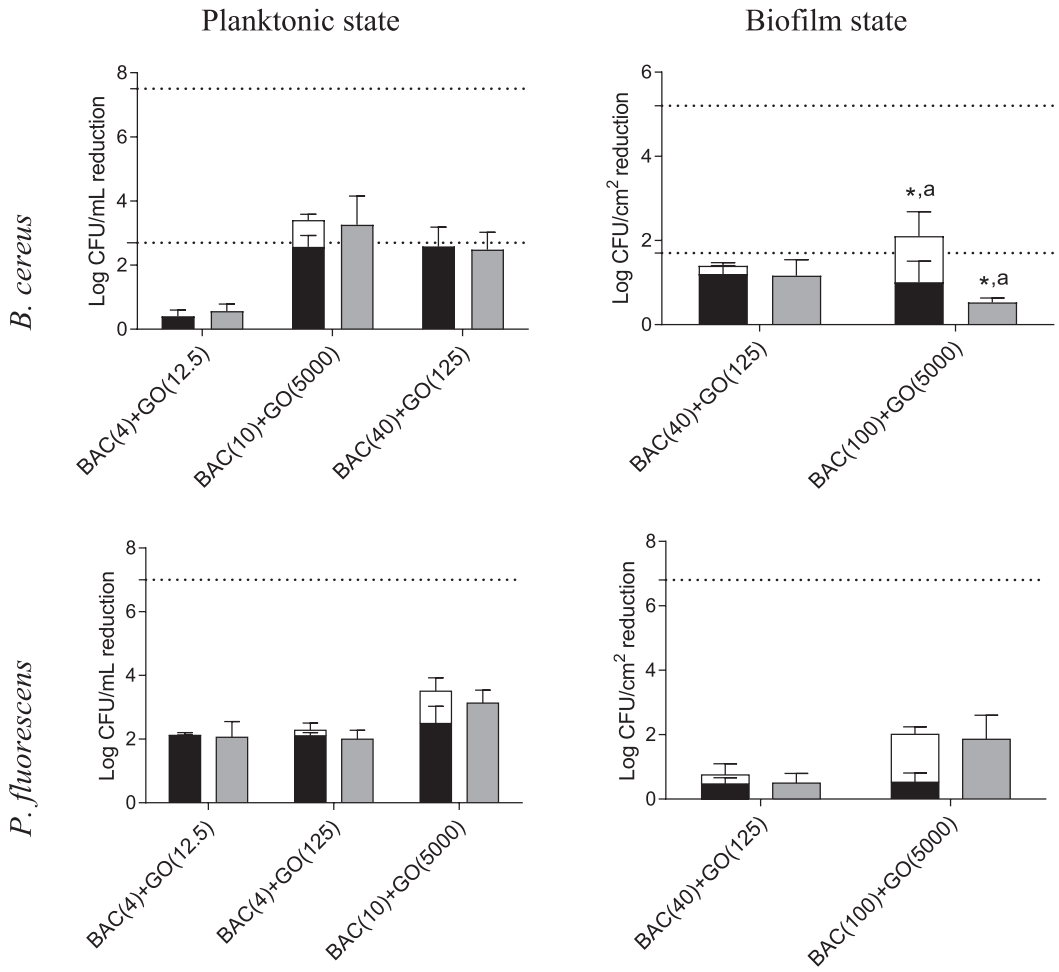

Fig. 2. Antimicrobial effects of BAC + GO combination against *B. cereus* and *P. fluorescens* in planktonic and biofilm states. Distinct biocide concentrations (at μg/mL) were tested. Black and white bars represent the cell inactivation caused by BAC and GO alone in a stacked position, respectively. Grey bars represent the cell inactivation promoted by dual biocide combinations. Dashed lines correspond to Log CFU/mL or Log CFU/cm^2 of control cultures and *B. cereus* non-endospores cells after exposure time. Values are means ± SDs of at least three independent assays. * – Cell inactivation caused by dual biocide combination was statistically different from the sum of inactivation caused by single biocides (unpaired *t*-test with Welch's correction, $P < 0.05$). a – Cell inactivation caused by dual biocide combination was statistically different from the most active biocide (unpaired *t*-test with Welch's correction, $P < 0.05$).

achieved, which may be related to the loss of antimicrobial activity observed. For example, increasing PAA from 50 to 100 μg/mL (planktonic *B. cereus*) or 1.25 to 5 μg/mL (planktonic *P. fluorescens*) combined with GO at 25 μg/mL resulted in indifferent to antagonistic effects. A similar pattern occurred when increasing GO from 300 to 5000 μg/mL (*B. cereus* biofilm) and 125 to 250 μg/mL (*P. fluorescens* biofilm) combined with PAA at 200 μg/mL. Ocampo et al. (2014) also found that 61% of the 204 antibiotic combinations tested were antagonistic, demonstrating the occurrence of antagonistic effects associated with the combination of bacteriostatic and bactericidal compounds. The authors hypothesized that the highest antimicrobial effects of the bactericidal compound against active cells were compromised by bacteriostatic action that induced cell stasis/inhibited cell growth (Ocampo et al., 2014).

PAA + GA combinations caused distinct antimicrobial effects against *B. cereus* and *P. fluorescens* (Fig. 4). Indifferent effects were verified against *B. cereus*, for all combinations tested against planktonic and biofilm cells ($P > 0.05$), except for the PAA + GA combination at 10 μg/mL + 100 μg/mL against planktonic cells (additive effect) and 200 μg/mL + 1000 μg/mL against biofilm cells (antagonistic effect). These antimicrobial effects may be explained by the presence of endospores that remained culturable after biocidal exposure (Fernandes et al., 2022). The available PAA concentration (5–400 μg/mL) (sporicidal agent) was not sufficient to cause total eradication of planktonic [> 500 μg/mL (Fernandes et al., 2020)] and biofilm cells [> 10000 μg/mL (Fernandes et al., 2022)]. On the other hand, PAA + GA combinations caused indifferent and synergistic effects against planktonic *P. fluorescens*. Specifically, for PAA at 1.25 μg/mL, a minimum quantity of GA was required for synergism (GA ≥ 200 μg/mL). The minimum bactericidal concentration of PAA and GA alone that caused complete eradication of planktonic *P. fluorescens* after 30 min of exposure were 100 and 5000 μg/mL, respectively (Fernandes et al., 2020). Thus, the PAA + GA combination allowed the reduction of effective concentrations to 1.25 and 200 μg/mL of PAA and GA, respectively. However, indifferent effects were observed for all the combinations tested against *P. fluorescens* biofilms ($P > 0.05$). Noel et al. (2021) also pbserved that the synergistic/additive effects were not ubiquitous across all bacterial species, being species-specific. From dual combinations between membrane-active agents [BAC, didecyldimethylammonium chloride (DDAC), polyhexamethylene biguanide (PHMB), chlorocresol] and a reactive oxygen species (ROS) generation agent (bronopol), BAC + chlorocresol showed synergistic effects against both *Staphylococcus aureus* and *Enterococcus faecalis*; PHMB + chlorocresol combination caused synergism against *E. faecalis*; while no effect was obtained against *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Noel et al., 2021).

Overall, the antimicrobial effects of dual biocide combinations based on the inactivation of planktonic cells corroborated the checkerboard results. However, very few combinations (*i.e.* biocides and concentrations) resulted in distinct outcomes, such as antagonism from PAA + GO

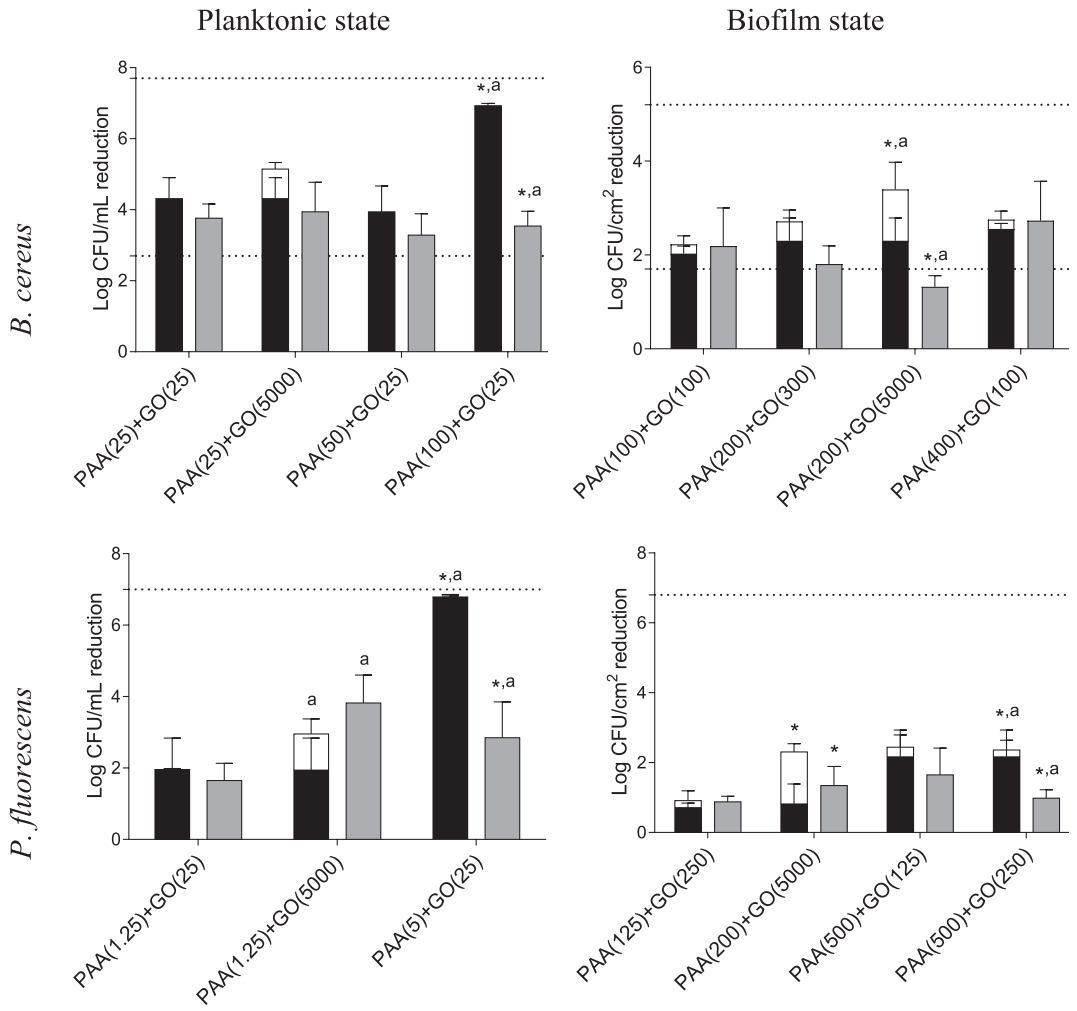

Fig. 3. Antimicrobial effects of PAA + GO combination against *B. cereus* and *P. fluorescens* in planktonic and biofilm states. Distinct biocide concentrations (at μg/mL) were tested. Black and white bars represent the cell inactivation caused by PAA and GO alone in a stacked position, respectively. Grey bars represent the cell inactivation caused by dual biocide combinations. Dashed lines correspond to the Log CFU/mL or Log CFU/cm^2 of control cultures and *B. cereus* non-endospores cells after exposure time. Values are means ± SDs of at least three independent assays. * – Cell inactivation caused by dual biocide combination was statistically different from the sum of cell inactivation caused by single biocides (unpaired *t*-test with Welch's correction, $P < 0.05$). a – Cell inactivation promoted by dual biocide combination was statistically different from the most active biocide (unpaired *t*-test with Welch's correction, $P < 0.05$).

and indifference/synergy from PAA + GA combination, which can be explained by the ability of dual biocide combinations to inhibit cell growth, which is different from the ability to inactivate cells after 30 min of exposure. In general, dual biocide combinations were found to cause indifferent or additive effects, since the non-specific antimicrobial action (multiple cellular targets) of biocides may not provide an open space for additional effects beyond the sum of their parts (Noel et al., 2021). The synergistic effects of the PAA + GA combination against planktonic *P. fluorescens* can be explained by cell membrane destabilization from GA exposure, facilitating PAA diffusion into cells, and causing higher antimicrobial effects. Other authors also reported a synergistic effect between colistin (membrane-active compound) and rifampicin, in which the high antimicrobial activity of rifampicin was promoted by cell permeability changes caused by colistin (Zhou et al., 2020). Against biofilm cells, diffusion–reaction mechanisms could be involved in the reduction of the active concentration of biocides, and synergistic effects were not observed. Regardless of the absence of sporicidal activity, the PAA + GA combination showed great potential for improving the antimicrobial activity against planktonic cells. Even without additional antimicrobial effects against biofilm cells, the PAA + GA combination may be effective in the control of dispersed biofilm cells, preventing their dissemination, cross-contamination, and the reseed of a new biofilm. Thus, the PAA + GA combination was selected as the most promising for use in triple combinations.

3.3. Potential for cell inactivation by dual biocide/QS inhibitor and triple combinations

The antimicrobial activity of dual biocide/QS inhibitor combinations (PAA + CUR, GA + CUR, PAA + UA, and GA + UA) and triple combinations (PAA + GA + CUR and PAA + GA + UA) was assessed based on the inactivation of planktonic and biofilm cells of *B. cereus* and *P. fluorescens* after 30 min of exposure (Figs. 5 and 6). According to the pH (close to 3) measured and pKa values [for CUR, $pKa_1 = 7.8$, $pKa_2 = 8.5$, and $pKa_3 = 9.0$ (Zebib et al., 2010); for UA, pKa = 5.02, predicted from ChemAxon (https://chemaxon.com/, accessed on November 2022)], both QS inhibitors were mainly present in neutral forms, which enable the cross of the cell membrane. Checkerboard results for triple combinations were not so accurate as for dual biocide combinations, since predicted additive effects for triple combinations resulted in antagonism for PAA + GA + CUR against both bacteria, while the PAA + GA + UA combination triggered indifference against *B. cereus* and synergism against *P. fluorescens*. As previously mentioned, these differences can be explained by the distinct exposure times (from 30 min to 24 h) that

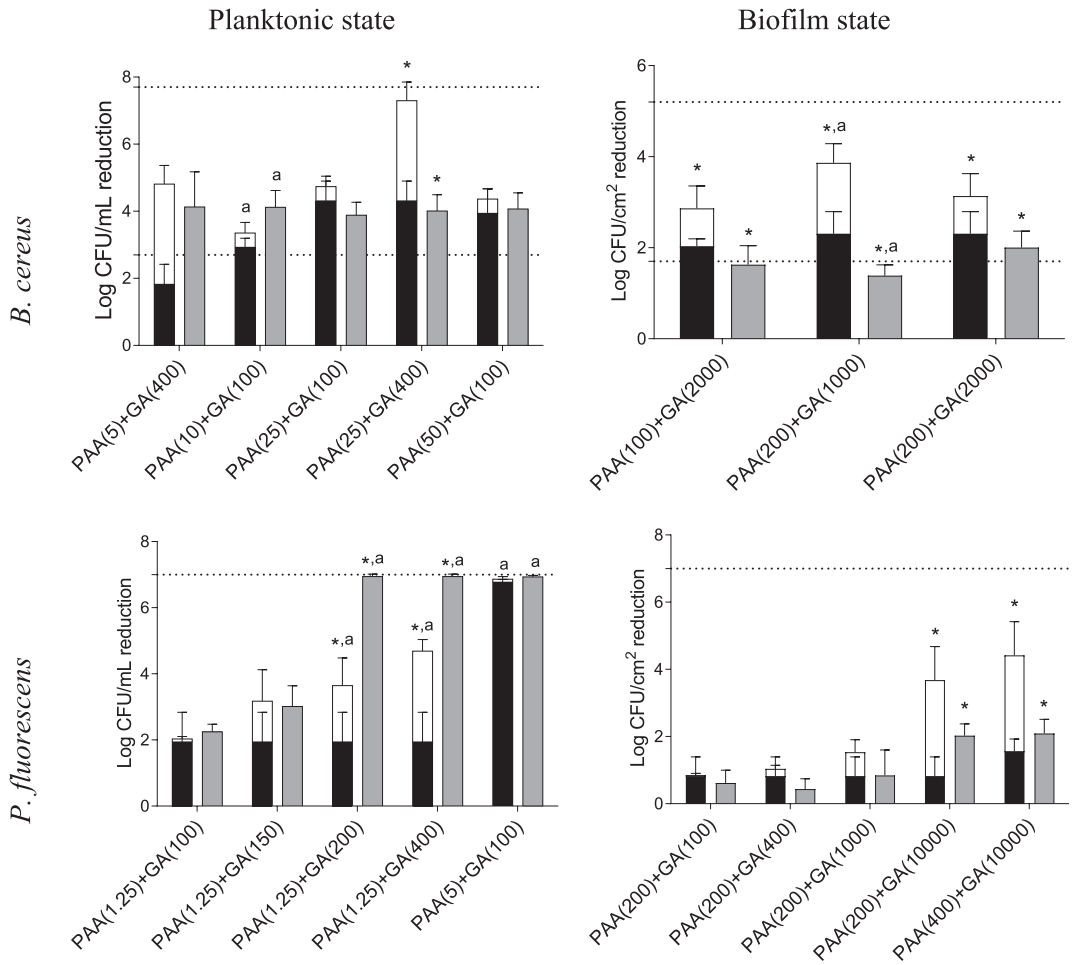

Fig. 4. Antimicrobial effects of PAA + GA combination against *B. cereus* and *P. fluorescens* in planktonic and biofilm states. Distinct biocide concentrations (at μg/mL) were tested. Black and white bars represent the cell inactivation caused by PAA and GA alone in a stacked position, respectively. Grey bars represent the cell inactivation promoted by dual biocide combination. Dashed lines correspond to the Log CFU/mL or Log CFU/cm^2 of control cultures and *B. cereus* non-endospores after exposure time. Values are means ± SDs of at least three independent assays. * – Cell inactivation caused by dual biocide combination was statistically different from the sum of cell inactivation caused by single biocides (unpaired *t*-test with Welch's correction, $P < 0.05$). a – Cell inactivation promoted by dual biocide combination was statistically different from the most active biocide (unpaired *t*-test with Welch's correction, $P < 0.05$).

caused different antimicrobial effects.

CUR has a broad-spectrum antimicrobial activity against bacteria, fungi, viruses, protozoa, and parasites (Adamczak et al., 2020). Its mode of action is related to cell growth inhibition by the generation of ROS, and changes in cell membrane permeability (Dai et al., 2022; Hussain et al., 2022). Against planktonic cells, CUR at 150 μg/mL caused negligible antimicrobial effects (approximately 0.2-Log CFU/mL reduction). Whilst the antimicrobial effects of PAA + CUR, GA + CUR, and PAA + GA + CUR combinations were species-specific (Fig. 5). The antimicrobial effects of PAA and GA against planktonic *B. cereus* increased (additive and synergistic effects, respectively) by the presence of CUR ($P < 0.05$), while indifference was observed against planktonic *P. fluorescens* ($P > 0.05$). The highest antimicrobial effects (additive/synergy) of PAA + CUR and GA + CUR against *B. cereus* can be associated to the potentiation of sporicidal effects by CUR as it already demonstrated to be effective in the inactivation of fungal spores and spore-forming bacteria (Dong et al., 2022; Huang et al., 2021). The differences in cell membrane/cell wall components between *B. cereus* (Gram-positive bacteria) and *P. fluorescens* (Gram-negative bacteria) can be related to the different levels of susceptibility to biocide combinations with CUR. Adamczak et al. (2020) verified that Gram-negative bacteria were less susceptible to CUR than these Gram-positive. Furthermore, the PAA + GA + CUR combination triggered antagonistic effects against both bacteria in the planktonic state ($P < 0.05$), proposing potential chemical interactions between the biocides combined and CUR, and a consequent loss of antimicrobial activity. The bacteriostatic action of CUR improved the antimicrobial activity of several antibiotics (synergism), as reviewed by Hussain et al. (2022) and Teow et al. (2016). However, as previously mentioned, the use of bacteriostatic compounds usually causes antagonistic effects when combined with these bactericidal (Ocampo et al., 2014).

In terms of biofilm control, it is known that CUR interferes with cell adhesion and consequent biofilm formation through QS inhibition (Abdulrahman et al., 2020; Raorane et al., 2019). Regardless of the high antimicrobial effects of CUR against planktonic cells, its action against mature biofilms was less pronounced, causing partial biofilm removal and cell inactivation (Tan et al., 2019). Gobin et al. (2022) found CUR as a promising antimicrobial compound, among different phytochemicals, against planktonic *S. aureus* and *Pseudomonas aeruginosa*, but weak antibiofilm effects were obtained by 500 μg/mL of CUR. Likewise, in this study, it was observed that CUR at 150 μg/mL caused negligible inactivation of biofilm cells of both bacteria (approximately 0.2-Log CFU/cm^2 reduction) (Fig. 5). In general, PAA, GA, and PAA + GA triggered indifferent effects against biofilm cells when combined with CUR ($P > 0.05$). To improve the antibiofilm activity of CUR, several strategies have been implemented to increase its solubility and delivery into the

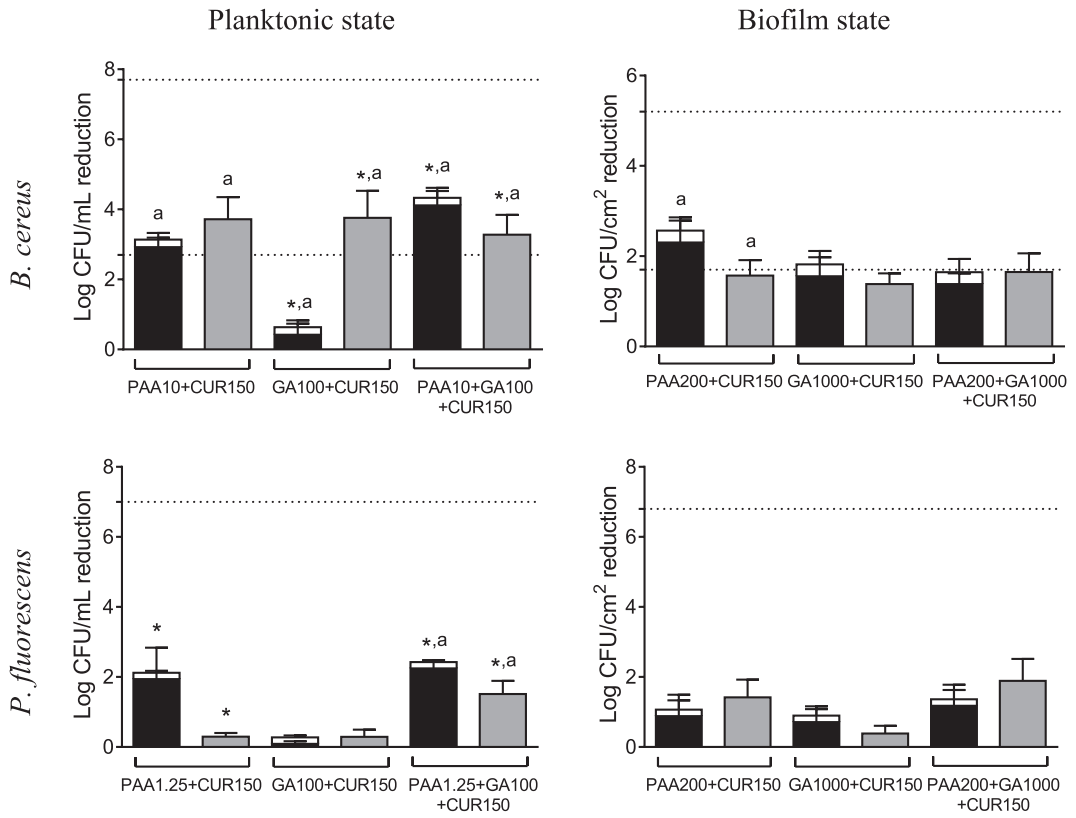

Fig. 5. Antimicrobial effects of PAA + CUR, GA + CUR, and PAA + GA + CUR combinations against *B. cereus* and *P. fluorescens* in planktonic and biofilm states. Distinct biocide/curcumin concentrations (at μg/mL) were tested. The cell inactivation caused by single or dual biocide (black bar) and curcumin (white bar) were represented in a stacked bar. Grey bars corresponded to the cell inactivation promoted by tested combinations. Dashed lines correspond to the total Log CFU/mL or Log CFU/cm^2 of control cultures and Log CFU/mL or Log CFU/cm^2 of total *B. cereus* non-endospores cells after exposure time. Values are means ± SDs of at least three independent assays. * – Cell inactivation caused by dual/triple combination was statistically different from the sum of cell inactivation caused by each compound alone (unpaired *t*-test with Welch's correction, $P < 0.05$). a – Cell inactivation promoted by the triple combination was statistically different from the highest cell inactivation of a single compound (unpaired *t*-test with Welch's correction, $P < 0.05$).

mature biofilm structure (Dai et al., 2022; Urosevic et al., 2022). Barros et al. (2020) demonstrated the enhancement of the antibiofilm activity of CUR by functionalization of silica nanoparticles. CUR-nanoparticles (at 5000 μg/mL with 1% *w/w* of curcumin) caused 54% biomass reduction (CV staining) and 33% cell viability reduction (MTT assay) against *Pseudomonas putida* biofilms, after 24 h of exposure, while free curcumin (50 μg/mL) did not cause substantial antibiofilm effects (Barros et al., 2020).

Regarding the antimicrobial activity of UA (Fig. 6), applying a sub-inhibitory concentration (100 μg/mL) caused negligible inactivation of planktonic cells (0.2 and 0.3-Log CFU/mL reduction of *P. fluorescens* and *B. cereus*, respectively). Combinations (including UA) caused the inactivation of planktonic cells in a biocide-dependent manner. PAA + UA caused indifferent antimicrobial effects against both bacteria ($P > 0.05$) and the GA + UA triggered synergistic effects ($P < 0.05$). The triple combination PAA + GA + UA caused synergistic effects against *P. fluorescens* ($P < 0.05$), while indifference was obtained against *B. cereus* ($P > 0.05$). These differences in the performance of the PAA + GA + UA combination can be related to the endospore-forming ability of *B. cereus* (Fernandes et al., 2022).

GA is an oxidative and membrane-active agent (Fernandes et al., 2020), while UA causes changes in cell membrane permeabilization, inhibition of fatty acid synthesis, and intracellular pH reduction (Leclercq et al., 2021). Leclercq et al. (2021) also demonstrated antimicrobial potentiation by cumulative damages on *S. aureus* and *Candida albicans* when combining UA (as an enzyme inhibitor) with carvacrol (as a membrane fluidizer). Diverse biocide formulations exist combining fatty acids (including UA) with organic acids (including GA) for surface cleaning and disinfection purposes (Kany et al., 2005; Richter et al., 2003; Trauten et al., 2002; Wang, 1982). In these combinations, the organic acid has been used to solubilize and stabilize the fatty acid, improving antimicrobial activity and reducing foam formation (Richter et al., 2003). UA at the sub-inhibitory concentration tested can act as a QS inhibitor, being an additional mechanism that can allow the synergistic activity of the GA + UA combination. Considering the antimicrobial activity of UA against biofilms, inhibition of *Candida albicans* biofilm formation was already reported (Kumar et al., 2020; Shi et al., 2016). Indeed, UA is already used clinically to treat topical and nail fungal infections (Van der Steen and Stevens, 2009). To the best of the authors' knowledge, the present study was the first one demonstrating the antibiofilm activity of UA (alone and in combination) against Gram-positive and Gram-negative bacteria. UA at 1000 μg/mL caused distinct inactivation of *B. cereus* (0.9-Log CFU/cm^2 reduction) and *P. fluorescens* biofilms (0.3-Log CFU/cm^2 reduction). Yuyama et al. (2020) also demonstrated that Gram-positive bacteria were more susceptible to fatty acids than these Gram-negative. According to the MIC values (Table S1), the concentrations of UA applied may cause distinct antimicrobial effects against both bacteria. UA mainly behaved as a QS inhibitor against *P. fluorescens*, since the concentration applied is below the MIC (> 1000 μg/mL) (Kumar et al., 2020). On the other hand, *B. cereus* was exposed to UA at a concentration above MIC (400 μg/mL), which may have promoted antimicrobial effects. Other authors found that different unsaturated fatty acids (*i.e.* palmitic acid, palmitelaidic acid, palmitoleic acid, oleic acid, linoleic acid, γ-linolenic acid, arachidonic acid, and 7(Z),10 (Z)-hexadecadienic acid) did not cause any effect on mature biofilms, but inhibited biofilm formation (Yuyama et al., 2020). Regardless of the

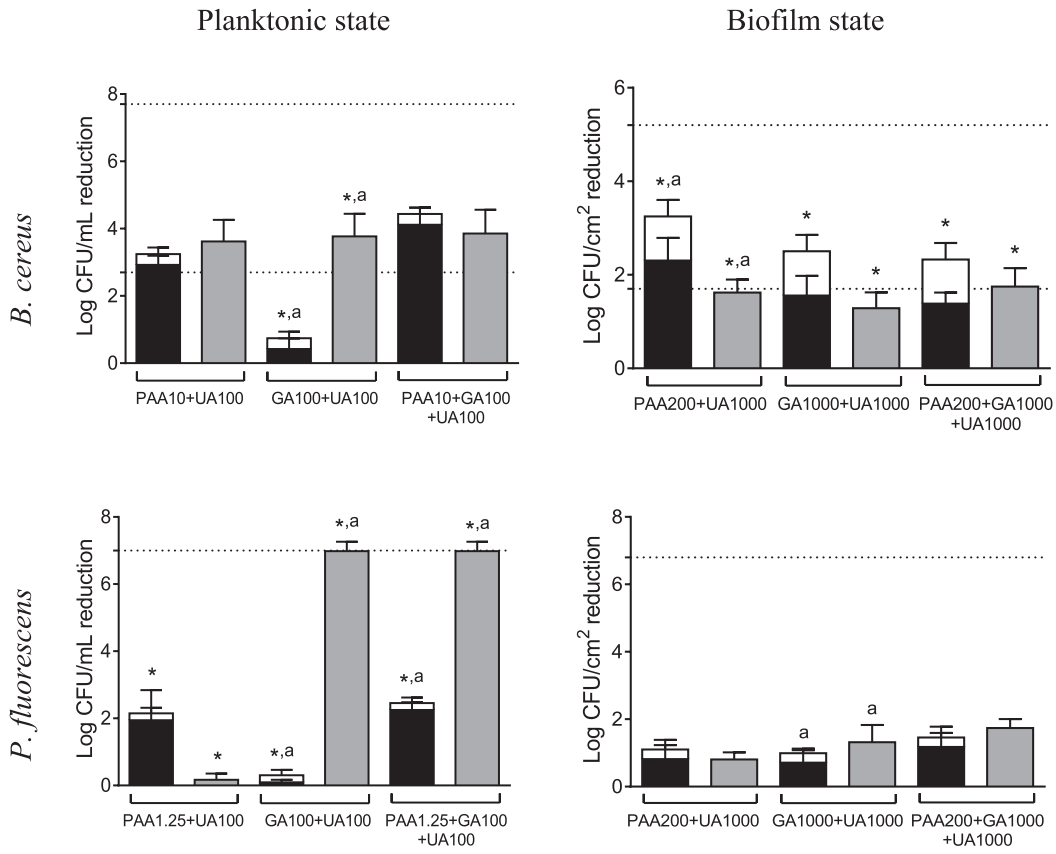

Fig. 6. Antimicrobial effects of PAA + UA, GA + UA, and PAA + GA + UA combination against *B. cereus* and *P. fluorescens* in planktonic and biofilm states. Distinct biocide/UA concentrations (at μg/mL) were tested. The cell inactivation caused by single or dual biocide (black bar) and UA (white bar) were represented in a stacked bar. Grey bars corresponded to the cell inactivation promoted by tested combinations. Dashed lines correspond to the total Log CFU/mL or Log CFU/cm^2 of control cultures and Log CFU/mL or Log CFU/cm^2 of total *B. cereus* non-endospores cells after exposure time. Values are means ± SDs of at least three independent assays. * – Cell inactivation caused by dual/triple combination was statistically different from the sum of cell inactivation caused by each compound alone (unpaired *t*-test with Welch's correction, $P < 0.05$). a – Cell inactivation promoted by the triple combination was statistically different from the highest cell inactivation of a single compound (unpaired *t*-test with Welch's correction, $P < 0.05$).

synergistic effects against planktonic cells, indifference was generally attained by the combinations with UA against biofilm cells. Sepehr et al. (2014) demonstrated increasing biofilm dispersion/removal by the combination of another unsaturated fatty acid (*cis*-2-decenoic acid) with commercial disinfectants (based on hydrogen peroxide and PAA) and antibiotics (*i.e.* ciprofloxacin, vancomycin, and ampicillin). For example, the combination of a commercial disinfectant containing 70 μg/mLof PAA and *cis*-2-decenoic acid resulted in a 5-fold decrease in CFU counts, while only a 2-fold decrease was obtained using PAA alone. The distinct nature of the fatty acids tested (*i.e.* carbon saturation, chain length, molecular configuration, hydrogenation, and hydroxylation) was responsible for the different antimicrobial effects (Kumar et al., 2020). In this study, UA at 1000 μg/mL caused no additional antimicrobial effect against biofilm cells when combined with the biocides. Regardless of the absence of additional antimicrobial effects against the biofilm cells, the potentiation found for GA + UA and PAA + GA + UA combinations against planktonic cells can result in the use of lower concentrations of acidic compounds to achieve a similar level of disinfection.

4. Conclusions

Dual biocide combinations developed in this study were classified as indifferent, according to the predicted chemical interactions based on cell growth inhibition (checkerboard assay), except the PAA + GA combination which was classified as additive. This behaviour was corroborated by the results from the inactivation of planktonic *B. cereus* and *P. fluorescens* after 30 min of exposure. A synergistic effect was found for PAA + GA, antagonistic for PAA + GO, and indifferent for BAC + GO. The antimicrobial effects from the addition of a QS inhibitor to biocide combinations (PAA + GA) against planktonic cells were dependent on the phytochemical molecule and the bacteria. The combinations that contained CUR resulted in indifferent or antagonistic effects, while the GA + UA combination triggered synergism and the PAA + GA + UA combination caused indifferent and synergistic effects against *B. cereus* and *P. fluorescens*, respectively. Overall, this study suggests PAA + GA, GA + UA, and PAA + GA + UA combinations as potential effective formulations for sanitation, which required low GA and PAA doses to achieve a similar disinfection degree. Synergism from biocide/phytochemical combinations can be related to chemical interactions between the compounds, but also the anti-QS activity of phytochemicals at sub-inhibitory concentrations. In general, biofilm cells remained resilient (indifferent effects) to all the combinations tested, proposing that the industry should implement effective strategies to prevent the formation of strongly adhered biofilms. Nevertheless, the promising combinations against planktonic cells can potentiate biofilm control through the effective reduction of dispersed biofilm cells, preventing their dissemination, cross-contamination, and the reseed of a new biofilm. It is important to consider that all antimicrobial effects from the combinations tested were related to selected concentrations of each biocide/QS inhibitor, *i.e.* the effects of other combinations (distinct concentrations of each compound) can cause distinct results.

CRediT authorship contribution statement

Susana Fernandes: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Funding acquisition. **Inês B. Gomes:** Investigation, Writing – review & editing. **Manuel Simões:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112680.

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