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Efficacy of antimicrobial combinations to reduce the use of sodium hypochlorite in the control of planktonic and sessile *Escherichia coli*

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ABSTRACT

Foodborne illness outbreaks linked to fresh produce are becoming more frequent and widespread. The types and properties of the chemical agents used for washing, cleaning and disinfection procedures, particularly their toxicity are the key indicators of environmental performance of a minimally processed vegetables (MPV) industry. The main aims of this work were focused on the evaluation of selected disinfectants (chlorine dioxide, peracetic acid, hydrogen peroxide, copper sulphate, vanillin and sodium bicarbonate) alone and combined with sodium hypochlorite on the control of *Escherichia coli* planktonic and sessile cells. The most effective disinfectants tested in planktonic cells were peracetic acid (6 mM) and chlorine dioxide (3 mM) and the best combination with sodium hypochlorite (3 mM) was obtained with peracetic acid (2 mM). In sessile cells, hydrogen peroxide and vanillin had antagonistic effects in combination with sodium hypochlorite whereas sodium bicarbonate efficiency was enhanced when combined with sodium hypochlorite.

Keywords: Biocidal combination, biofilms, control, immobilized cells, microbial growth, sodium hypochlorite

LIST OF ABBREVIATIONS

CD	Chlorine dioxide
CFU	Colony forming units
CS	Copper sulphate
HP	Hydrogen peroxide
LR	Log CFU reduction index
LR_c	Log CFU reduction of the compound in the combination
LR_i	Log CFU reduction of the compound used individually
MBC	Minimum bactericidal concentration
MHB	Mueller–Hinton broth
MIC	Minimum inhibitory concentration

MIC_c	MIC of the compound in the combination
MIC_i	MIC of the compound used individually
PA	Peracetic acid
PCA	Plate count agar
PS	Polystyrene
SB	Sodium bicarbonate
SH	Sodium hypochlorite
SS	Stainless steel
VN	Vanillin

1. INTRODUCTION

Nowadays, consumers are more conscious of the importance of a healthy life and with what they eat [1]. Since fresh produce is a good and natural source of vitamins and nutrients, its consumption has increased. In parallel, foodborne illness outbreaks are becoming more frequent due to an increasing availability of fresh produce [2-4]. Current decontamination techniques show limited efficiency in reducing pathogen levels [1, 5, 6]. The chemicals used for washing, cleaning and disinfection procedures, as well as the toxicity of these chemical agents are the key indicators of environmental performance of a minimally processed vegetables (MPV) industry [7]. Sodium hypochlorite (SH) is widely used for MPV, but it produces unhealthy by-products and its efficiency in disinfection is largely reduced by the presence of organic matter [8, 9]. Moreover, the possible formation of carcinogenic chlorinated compounds in water raised concerns on the use of SH in food processing [10]. In fact, SH is included in the indicative list of the Directive on Industrial Emissions (IPCC, 2007/0286 (COD)) [11] as a major pollutant for water emissions and on the formation of carcinogenic and mutagenic products in the presence of organic matter [12, 13]. Alternative disinfection methods have been recently proposed: physical methods, such as pulsed light and ultrasound [7, 14]; and chemical methods, such as ozone [12], phytochemicals [15], hydrogen peroxide (HP) [12], copper sulphate (CS) [16], peracetic acid (PA) [17, 18], sodium bicarbonate (SB) [19] and chlorine dioxide (CD) [20]. These methods can help to reduce the use of SH in cleaning and disinfection steps, especially if used in combination. This can result in a synergistic

effect, i.e., the combination of disinfectants can lead to a reduction of the disinfectants concentration, compared to when they are applied individually [21].

HP can have a bactericidal (death) or a bacteriostatic (inhibitory) effect on the microorganisms [12, 22]. It can be applied on food surface material [23] and its main advantage is the rapid decomposition into water and oxygen by catalase [12]. Despite the fact of having the Generally Recognized As Safe (GRAS) status [24], HP is not allowed by the USA Food and Drug Administration (FDA) [12].

CS is extensively used as a fungicide [25]. The application of copper combined with lactic acid was previously reported [26, 27]. The growth of *Salmonella* spp. and *Escherichia coli* O157:H7 were inhibited when both lactic acid and copper were applied. The authors concluded that this combination produced a synergistic inhibition of microbial growth [26]. Gyawali et al. (2011) observed a significant growth inhibition of *E. coli* O157:H7, after a 8 h incubation at 37 °C with a combination of copper (40 ppm) and lactic acid (0.2%) [27].

PA is used as a disinfectant in water [28], in the food and biomedical sectors because of its effectiveness against a broad range of microorganisms (bacteria, fungi, and viruses) [29, 30]. Due to its high oxidizing potential, this acid is an ideal antimicrobial agent [17, 18]. This disinfectant has advantages over SH: (i) it does not react with proteins to produce toxic or carcinogenic compounds; (ii) it has low environmental impact; (iii) and it has been reported to be more active against biofilms [31, 32]. Furthermore, the by-products originated by this acid (water, acetic acid and oxygen) are environmentally friendly [33]. The main drawback associated with PA disinfection is the increase of organic content in the effluent due to acetic acid [34].

CD has attracted interest for the fresh cut industry [20]. It has a higher oxidation capacity than SH and does not react with nitrogen or ammonia to form dangerous products [23], as the major compound formed (chlorite) is classified as non-carcinogenic [20]. This disinfectant is accepted by the FDA for washing vegetables [35, 36], but it is not allowed by the EU Food legislation [23]. Furthermore, it has lower reactivity with organic matter and is less corrosive than SH and ozone [12]. The main drawbacks are: (i) its maximum allowed concentration (3 ppm) [35] which is a relatively low value since studies demonstrated that higher concentrations are required to promote a reduction in microbiological content; (ii) its instability, since it is explosive (it has to be generated on

site); (iii) its efficiency is pH dependent and the pH values have to be between 6.5 and 7.5 [12]; (iv) and its decomposition when exposed to sunlight [20].

Vanillin (VN) is a phytochemical that is used as flavouring agent in food. This phytochemical has GRAS status and antioxidant properties and is also used as food preservative due to its antimicrobial activity against Gram-positive and Gram-negative bacteria [37-39]. The main drawbacks are the unawareness of the VN mode of action and also its low solubility in water (1% w/w) [38, 40].

Sodium bicarbonate (SB) is usually used as a food additive and has GRAS status. It has been used to control green and blue molds in citrus [41, 42]. It is commonly applied due to its wide acceptance in the food industry, low cost, non-toxic properties and because it does not damage the fruits [42]. It can also be applied to eliminate microorganisms from food contact surfaces, such as stainless steel (SS) [19].

The aim of the present study was to test alternative chemical compounds and to explore the combination with sodium hypochlorite, for water and surfaces' disinfection. Therefore, the main objectives were to determine the antimicrobial activity of selected disinfectants (CD, PA, SH, HP, CS, VN and SB) against planktonic cells of *E. coli*. Additionally, combinations between disinfectants were studied to promote the reduction of chlorine in the microbial control process. Also, the biofilm and dispersedly adhered cells removal from AISI 316 SS and polystyrene (PS) surfaces was assayed for both individual and disinfectant combinations.

2. MATERIALS AND METHODS

2.1. MICROORGANISM AND CULTURE CONDITIONS

E. coli CECT 434 was the microorganism studied since it is recommended by the European standard EN 1276 for the development of disinfection strategies [43]. The bacterium was obtained from overnight cultures grown in 250 mL flasks with 100 mL of Mueller–Hinton broth (MHB) (Merck, Germany), incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany).

2.2. DISINFECTANTS

SH 13% (w/w) was obtained from Acros Organics (Belgium) and VN was acquired from Sigma-Aldrich (Switzerland). PA 38-40% (w/v), HP 30% (w/v), SB and CS pentahydrate were obtained from Merck (Germany). CD 2 g/L was provided by Loehrke (Deutschland).

2.3. MIC AND MBC DETERMINATION

The minimum inhibitory concentration (MIC) was determined in 96-well flat-bottomed PS tissue culture plates with a lid (Orange Scientific, USA) using a total volume of 200 µL. In each microtiter plate well, 180 µL of bacterial inoculum (containing 4×10^7 CFU/mL) was added to 20 µL of increasing concentrations of disinfectants. After 24 hours exposure to the disinfectants, the OD_{600} was measured in a SynergyTM HT 96-well microplate reader (Biotek Instruments, Inc., USA) and the MIC was determined as the lowest concentration of the antimicrobial that inhibits the growth of the microorganism [44, 45]. To determine the MBC, the motion drop method [46] was used on Plate Count Agar (PCA) plates (Merck, Germany). The plates were incubated overnight at 30 ºC after a neutralisation step, by diluting the chemicals to sub-inhibitory levels [47]. MBC was determined as the lowest concentration of an antimicrobial that kills a microorganism [44, 45] after a 24 hours incubation period. At least three independent experiments were performed for each condition tested.

2.4. COMBINATION OF SODIUM HYPOCHLORITE WITH OTHER DISINFECTANTS

Disinfectant combinations were assayed using the previously described method by applying 50% (v/v) of each disinfectant in a total volume of 20 µL. SH concentration used in combination with the other disinfectants was half of the individual MIC (3.0 mM). In the case of SH combined with HP and CS the disinfectants were applied at the following levels 50% (v/v), 40% (v/v) and 10% (v/v), respectively [48]. At least three independent experiments were performed for each condition tested.

The presence or absence of synergism was determined by the calculation of the MIC ratio (Table 1). Where MIC_c is the MIC of the compound in the combination and MIC_i is the MIC of the compound used individually. If the MIC ratio is lower than 0.5 potentiation is occurring; if it is lower than 1 but higher than 0.5 it is considered as a modest

enhancement in antimicrobial activity; and if it equal or higher than 1 it means that the combination is antagonistic.

2.5. BACTERIAL ADHESION AND BIOFILM FORMATION

The efficacy of disinfectants was assessed on dispersedly adhered bacteria and biofilms. AISI 316 SS and PS coupons (dimensions of 1.0 × 0.9 cm) were used. The coupons were placed in 48-wells flat-bottomed PS tissue culture plates (Thermo Fisher Scientific, Korea) using a total volume of 1000 µL with an initial number of cells of 4×10^7 CFU/mL. The plates were incubated for 2 hours and 24 hours for dispersed cell adhesion and biofilms formation, respectively (Fig. 1), at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany).

2.6. DISINFECTION OF DISPERSEDLY ADHERED CELLS AND BIOFILMS

After the incubation period, the medium was removed and replaced by the disinfectant solution (MIC, 5× MIC and MIC for the combination with SH 3 mM) for 20 min, at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). After the disinfectant exposure the coupons were placed in 5 mL saline solution (0.85% (w/v) NaCl), the cells were removed by vigorously vortex and the neutralisation step was performed by dilution to sub-inhibitory concentrations [47]. The necessary dilutions were prepared to determine the number of colony forming units (CFU) using the motion drop method [46]. At least three independent experiments were performed for each condition tested.

In this case, the presence or absence of synergism was determined by the calculation of the log CFU reduction index (LR) (Table 2). Where LRc is the log CFU reduction of the compound in the combination and LRi is the log CFU reduction of the compound used individually. If the LRc is higher than the LRi it represents an enhancement of the disinfectant activity; if it is equal to LRi, a neutral effect is considered; and if it is lower than the LRi antagonism is present.

2.6. STATISTICAL ANALYSIS

The results were analysed using paired samples *t*-test from the statistical software SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistical calculations were based on a confidence level of ≥ 95% ($P < 0.05$ was considered statistically significant).

3. RESULTS AND DISCUSSION

Antimicrobial products, particularly chlorine, have been used for disinfection in industrial systems [5, 49]. Although disinfection with chlorine is widespread, there is a global concern on developing alternative disinfection strategies and on minimizing its environmental and public health impacts [50]. In fact, chlorine can react with organic compounds to produce disinfection by-products that have potential carcinogenic effects [51]. Therefore, optimization on its use for disinfection is required. No chlorine is probably an unreachable target, based on the recent work of Kim et al. and considering that this agent is efficient in the control of anaerobic bacteria and biofilms [52]. Therefore, the present study aimed at developing strategies to reduce the use of chlorine in the control of bacterial planktonic and sessile growth.

SH and other selected disinfectants were tested and the individual MIC and MBC were determined (Table 3). The inhibitory and bactericidal effect for which a lower concentration of the disinfectants was necessary was, in an ascending order of concentrations: CD < PA = SH < HP < CS.

For CD, the minimum concentration necessary to inhibit *E. coli* was 3.0 mM. This disinfectant was already used by Maillard [53] to test its sporicidal efficacy against *Bacillus cereus*. The author obtained a MBC of 2.96 mM for spores of *B. cereus*, which is similar to the MBC and MIC value obtained for *E. coli* in the present study. The main drawback on the industrial use of this biocide is the maximum FDA [35] allowed concentration (40 µM). This is a very low value and not suitable to promote significant reduction in the microbiological load of vegetables [12]. Moreover, this biocide can also induce alterations in the organoleptic properties of fresh food products as it was demonstrated by Mahmoud and Linton [54] on lettuce leaves when CD was applied in the gas form at 7 µM. PA is another oxidizing agent that had a MIC of 6.0 mM. Bridier et al. [55] determined a MBC of PA of 0.097 mM for *E. coli* PHL 628 which is much lower than the value obtained in the present study. On the other hand Penna et al. [56] obtained a MIC obtained of 30 mM which is 5 times higher than the value obtained in this study. These results clearly reinforce that antimicrobial susceptibility is dependent on the microbial species/strains and on the methods used [55]. The mode of action of PA is not known but it is suggested that this acid disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane or causes rupture of cell walls [34].

The MIC of SH was equal to that of PA. When comparing with previous published studies, the MIC of SH (6.0 mM) was higher than the value obtained by Cerioni et al. [16] (4.03 mM) although in the same order of magnitude. This is arguably due to the different microorganisms tested [22]. In fact, Cerioni et al. [16] used a filamentous fungi (*Penicillium digitatum*). Also, the methods used to determine the MIC were different [16]. Abadias et al. [57] used 1.3 mM SH and only achieved 1 log CFU/mL reduction of *E. coli*. Penna et al. [56] obtained inhibitory concentrations in the range of 2-15 mM for *E. coli*. Heling et al. [58] needed 24 mM and 383 mM to inhibit and have a bactericidal effect against *Enterococcus faecalis*, respectively. In a more recent work, Cerioni et al. [48] determined MIC of SH and HP for *Penicillium expansum* of 50 and 400 mM, respectively. Cerioni et al. [16] also determined the MIC of HP which is higher (300 mM) than the value obtained in the present study (15 mM). Miyasaki et al. [59] described that the MBC of HP varies from 0.75 to 10 mM for *Haemophilus aphrophilus*, *Eikenella corrodens* and *Capnocytophaga gingivalis*. Pericone et al. [60] could not determine the MIC of HP, but obtained a MBC of 15 mM against *E. coli* RS218 which is close to the value obtained in the present study (16 mM). The bactericidal or bacteriostatic effect of HP on the microorganisms can be justified by the fact that this disinfectant is an oxidizer and can form toxic species that are responsible for its antimicrobial properties [12, 61]. In the case of CS, Cerioni et al. [16, 48] did not determined the MIC but stipulated that it was 6.0 mM, the concentration generally used for plant fumigation [16, 62]. In this study a MIC of 27 mM was obtained.

The MIC and MBC of VN were not determined, since the maximum concentration that could be tested (4.5 mM, due to solubility limitations) was not sufficient to inhibit or kill *E. coli*. VN has low water solubility and this is a significant disadvantage for its application [40]. However, there are studies demonstrating the antimicrobial activity of VN. Fitzgerald et al. [38] found that the MIC of VN was 15 mM for *E. coli*, when this phytochemical was prepared in ethanol. Abadias et al. [57] prepared the solution in acetic acid and achieved 1 log CFU/mL reduction of *E. coli* with VN at 79 mM. Likewise, the MIC and MBC were not determined for SB. In fact, the solubility of SB in water is also a problem for food related disinfection. Miyasaki et al. [59] already demonstrated that a very high concentration of SB was necessary to achieve an inhibitory (23-182 mM, depending on the microorganism) or bactericidal effect (182-728 mM). Additionally, Abadias et al. [57] concluded that 1.19 M caused no *E. coli* reduction.

Overall, PA and CD were the most promising alternative disinfectants to SH ($P < 0.05$) to inhibit and to eliminate *E. coli* in suspension. When comparing the MIC and the MBC values, only those for CS, HP and PA were different, although the differences were not statistically significant ($P > 0.05$). The significant action of CD was probably due to its low pH value (0.96). Also, PA had a pH of 4.00. The other disinfectants had pH values near neutrality (7.07, 7.00, 5.50, 7.00 and 6.50 for SH, HP, CS, SB and VN, respectively). These pH values do not reflect the mode of action of the disinfectants.

After the individual MIC and MBC determinations, the disinfectants were combined with 3.0 mM of SH in order to ascertain their putative antimicrobial potentiation (Table 4). The inhibitory and bactericidal effect for which a lower concentration of disinfectants was necessary was, in an ascending order: CD < PA < HP + CS < CS < HP.

The combination of SH with CD promoted modest enhancement on antimicrobial activity. Nevertheless, the difference between the MIC assayed individually or in combination with SH was not statistically significant ($P > 0.05$). The results obtained for PA when combined with SH seem very promising. Indeed, of all the combinations tested this was the one with the most promising results ($P < 0.05$), as the concentration of PA in the combination, necessary to have a bactericidal effect, was reduced 3-fold when compared to the individual tests.

The combination of SH with HP and CS potentiated its antimicrobial activity. The concentration of HP was greatly reduced ($P < 0.05$), apparently due to the presence of copper. Cerioni et al. [16] demonstrated that copper acts as a mediator of hydrogen peroxide inducing damage in *E. coli*. This process is irreversible and affects the respiratory chain, with the consequent loss of bacterial viability [16]. Furthermore, when HP is combined with metal ions like copper, the Fenton and Haber-Weiss reactions occur and HP is converted to the strongly reactive hydroxyl radical [63]. However, the application of copper has limitations since concentrations ranging from 50 to 202 mM have been reported as 96 h LC50 median lethal concentration values for juvenile *Penaeus monodon* [64]. In the present study the CS MIC used was lower, being greatly reduced ($P < 0.05$) in the combination with SH. The results demonstrate that the CS + SH + HP combination can be advantageous in the control of planktonic *E. coli*. The enhancement of the inhibitory or bactericidal action of SH can be due to the fact that the disinfectants target the cell wall, causing structural changes or penetrate the cell and attack intracellular targets [65]. According to Denyer [65], copper ions act in the

cytoplasmatic membrane and HP and PA act in the cytoplasm, inhibiting catabolic and anabolic processes.

In the food industry, the complete biofilm eradication is not always the objective but rather a logarithmic reduction [66]. Moreover, the microbial contaminants are not only in the planktonic phase, but also as cells dispersedly adhered to the surfaces [6] and as biofilm structures. Therefore, the selected disinfectants were tested for their ability to remove dispersedly adhered cells (Fig. 2) and biofilms (Fig. 3) from SS and PS surfaces. When comparing the materials tested, it is possible to observe that PS surface had a higher dispersedly cell adhesion (4×10^6 CFU/cm^2 in PS *vs* 1×10^6 CFU/cm^2 in SS, $P > 0.05$) and biofilm development (1×10^7 CFU/cm^2 in PS *vs* to 3×10^6 CFU/cm^2 in SS, $P < 0.05$). According to Simões et al. [67] adhesion is higher when both cell and substratum surfaces are hydrophobic. In this study, *E. coli* [68], SS 316 [67] and PS [69, 70] surfaces are hydrophobic. Therefore, adhesion was favored by the thermodynamic interactions established between cell and substratum surfaces. However, the hydrophobicity of SS (-55.1 mJ/m^2) [67] and PS (-55.2 mJ/m^2) [69] was not, apparently, the main aspect causing the different cell densities.

The use of the selected disinfectants demonstrated that their disinfecting potential was higher in adhered cells than in biofilms. In fact, *E. coli* already demonstrated increased antimicrobial resistance in biofilms [71]. Observing the results for dispersedly adhered cells (Fig. 2), the removal from PS surfaces is less efficient than on SS. On SS surfaces (Fig. 2a), all the disinfectants were effective except: VN at 4.5 and 22.5 mM, that promoted reductions of 1.2 and 2.2 log CFU/cm^2 ($P < 0.05$), respectively; and SB 90 mM that promoted 0.26 log CFU/cm^2 reduction ($P > 0.05$). On the PS surfaces (Fig. 2b) SH, PA and CD completely removed the cells. As for HP, CS, VN and SB they were not significantly effective ($P > 0.05$). Only when the concentrations were increased from the MIC to 5 × MIC a significant reduction was obtained: HP 75 mM caused 4.58 log CFU/cm^2 reduction ($P < 0.05$), CS completely eliminated *E. coli* ($P < 0.05$) and SB 450 mM reduced 2.67 log CFU/cm^2 ($P < 0.05$). VN at 22.5 mM had no significantly different ($P > 0.05$) result from VN 4.5 mM.

Concerning biofilm control (Fig. 3), HP, VN and SB were not significantly efficient ($P > 0.05$) in controlling biofilms on the SS surfaces (Fig. 3a). When the concentration of HP was increased to 5 × MIC a complete log CFU/cm^2 reduction was achieved ($P < 0.05$). For the PS surface (Fig. 3b), VN at 4.5 mM and SB were also not efficient ($P > 0.05$) in

biofilm control. Only by increasing the concentration of VN to 5 × MIC a 1.17 log CFU/cm^2 reduction was achieved ($P < 0.05$). HP at 15 and 75 mM caused 1.68 and 2.55 log CFU/cm^2 reduction, respectively ($P < 0.05$). As for CS at 27 mM, a 1.27 log CFU/cm^2 reduction was achieved ($P < 0.05$), and 135 mM of CS were required to completely eradicate *E. coli* biofilm ($P < 0.05$). However, the application of copper has limitations for concentrations between 50 and 202 mM [64].

The findings on biofilm control with PA are similar to those of Martín-Espada et al. [17], when they achieved total eradication using PA at 35 mM PA against *P. aeruginosa* biofilms formed on PS. Abadias et al. (2011) achieved 4 log CFU/mL *E. coli* reduction with PA 1 mM on apples. In fact, the surface disinfection properties of PA were already proposed by Carpentier and Cerf [72].

In general, SH, PA and CD were the best disinfectants while HP, SB and CS were less efficient in the removal of both dispersedly adhered cells and biofilms from both surfaces. The removal of dispersedly adhered cells with SB was only possible on SS surfaces. This is an interesting compound as it is a non-toxic food additive [19].

The results obtained for the combination of disinfectants against dispersedly adhered cells and biofilms are shown in Figures 4 and 5, respectively. For both surfaces tested and type of tests performed (dispersedly adhered cells and biofilms) the results were similar, *i.e.* when the disinfectants were combined with SH at 3 mM the removal was efficient for all the tested conditions ($P < 0.05$) ($LR_c = LR_i$), except for HP and VN. These two combinations were antagonistic ($LR_c < LR_i$), since when SH at 3 mM was applied alone it reduced 3 log CFU/cm^2 ($P<0.05$) and when SH was combined with the other disinfectants (HP and VN) the log CFU/cm^2 reduction was lower. It is important to note that SB combined with SH was effective for complete control of dispersedly adhered cells and biofilms from both PS and SS surfaces ($LR_c > LR_i$). This is an expected result, based on the performance of SB when used alone and when combined with SH against planktonic cells. In biofilms, the microbial growth rate is reduced and there are less nutrients available, which can explain why SB was more efficient against the sessile cells [73]. In practice SH and SB demonstrated to be the most promising combination to be used in the disinfection of food surfaces. In fact, the concentration of SH can be reduced and SB has a GRAS status and is already applied in the disinfection of food surfaces [19, 42].

4. CONCLUSIONS

In conclusion, PA and CD, alone and combined with SH, were the most effective biocides to control planktonic and sessile *E. coli*. Interestingly, SB was potentiated by SH in the control of sessile *E. coli*. Taking into account that SH is considered a major risk for the formation of carcinogenic and mutagenic products [11], the overall results demonstrate that the reduction of SH concentration in disinfection is possible using alternative biocide combinations.

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Figure captions

Figure 1 - Microscopy visualization of adhered cells (2 h) on PS (a) and SS (b), and biofilm (24 h) on PS (c) and SS (d). Magnification ×1000 and scale bar 10 µm. Adhered cells and biofilms were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, Portugal). Each slide was stained with 20 µL of DAPI at a concentration of 0.5 µg/mL. After 10 min of incubation in the dark, the slides were mounted with non-fluorescent immersion oil on glass microscope slides. The slides were examined using an epifluorescence microscope (LEICA DMLB2) with a filter with the following characteristics: excitation filter 340 – 380 nm, dichromatic mirror of 400 nm and suppression filter LP 425. It is possible to observe that biofilms cells are embedded within the extracellular polymeric matrix.

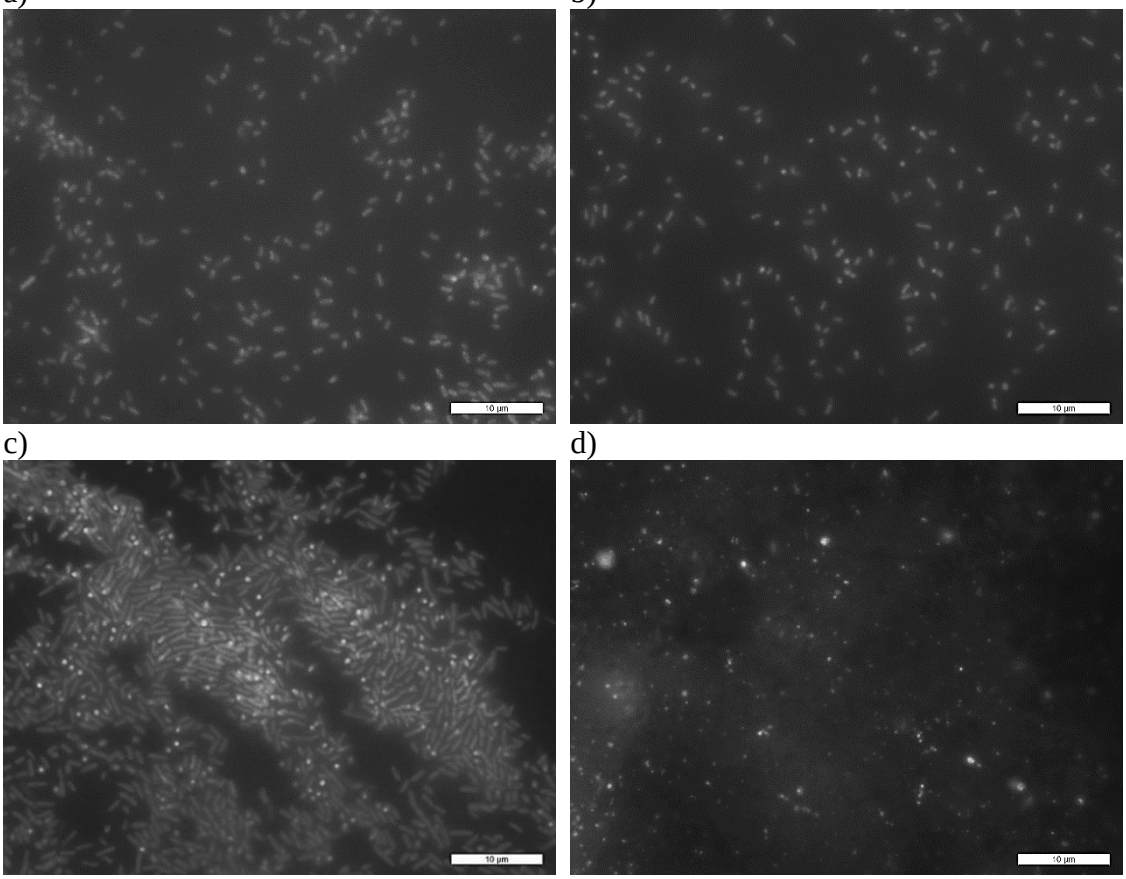
Figure 2 - Log CFU/cm^2 reduction achieved after the application of the individual disinfectants at their MIC and 5 × MIC against dispersedly adhered cells on SS (a) and on PS (b). The line indicates the method detection limit (2.06 log CFU/cm^2). The symbol * represents that no CFU was detected. Different letters represent statistically different values ($P<0.05$). (SH – sodium hypochlorite, HP – hydrogen peroxide, CS – copper sulphate, PA – peracetic acid, CD – chlorine dioxide, VN – vanillin, SB – sodium bicarbonate).

Figure 3 - Log CFU/cm^2 reduction achieved after the application of the individual disinfectants at their MIC and 5 × MIC against biofilms formed on SS (a) and on PS (b). The line indicates the method detection limit (2.06 log CFU/cm^2). The symbol * represents that no CFU was detected. Different letters represent statistically different values ($P<0.05$). (SH – sodium hypochlorite, HP – hydrogen peroxide, CS – copper sulphate, PA – peracetic acid, CD – chlorine dioxide, VN – vanillin, SB – sodium bicarbonate).

Figure 4 - Log CFU/cm^2 reduction achieved after the application of the combined disinfectants against dispersedly adhered cell on SS (a) and on PS (b). The line indicates the method detection limit (2.06 log CFU/cm^2). The symbol * represents that no CFU was detected. Different letters represent statistically different values ($P<0.05$). (SH – sodium hypochlorite, HP – hydrogen peroxide, CS – copper sulphate, PA – peracetic acid, CD – chlorine dioxide, VN – vanillin, SB – sodium bicarbonate).

Figure 5 - Log CFU/cm^2 reduction achieved after the application of the combined disinfectants against biofilms formed on SS (a) and on PS (b). The line indicates the method detection limit (2.06 log CFU/cm^2). The symbol * represents that no CFU was detected. Different letters represent statistically different values ($P<0.05$). (SH – sodium hypochlorite, HP – hydrogen peroxide, CS – copper sulphate, PA – peracetic acid, CD – chlorine dioxide, VN – vanillin, SB – sodium bicarbonate).

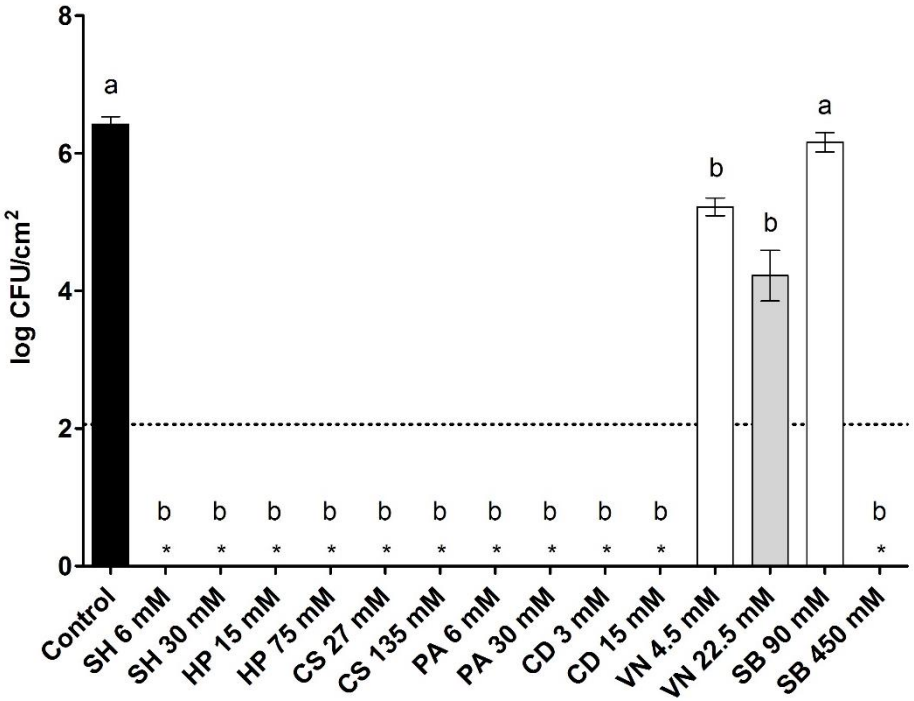
a) b)



c) d)

Figure 1

a)


log CFU/cm²
0
2
4
6
8
a
b
b
b
b
b
b
b
b
b
b
b
b
a
b
*
Control
SH 6 mM
SH 30 mM
HP 15 mM
HP 75 mM
CS 27 mM
CS 135 mM
PA 6 mM
PA 30 mM
CD 3 mM
CD 15 mM
VN 4.5 mM
VN 22.5 mM
SB 90 mM
SB 450 mM

b)

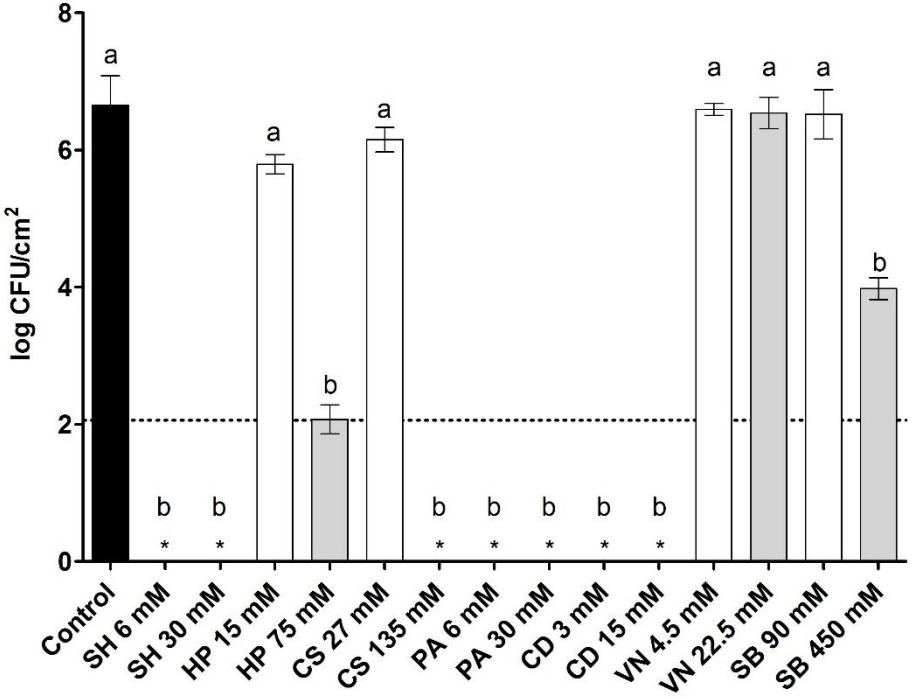
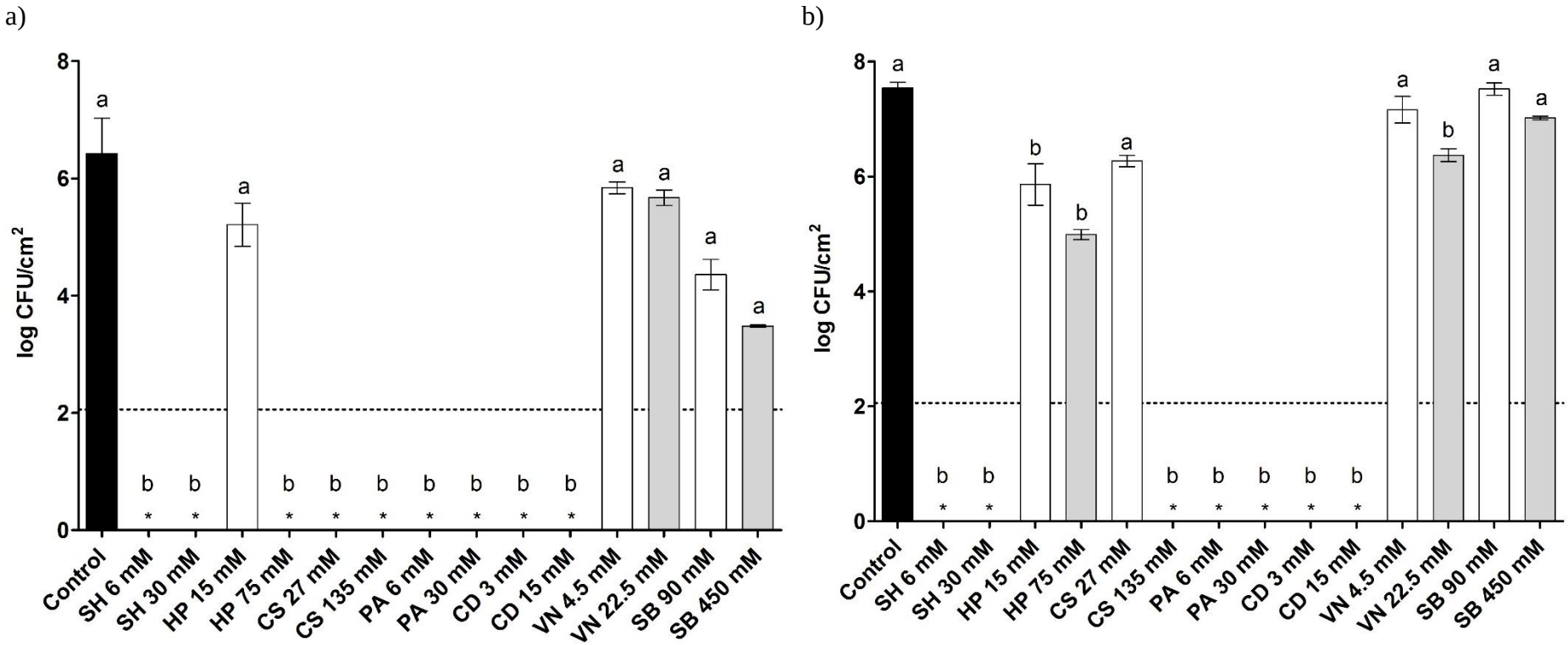

log CFU/cm²
0
2
4
6
8
a
b
b
a
b
a
b
b
b
b
b
a
a
a
b
*
Control
SH 6 mM
SH 30 mM
HP 15 mM
HP 75 mM
CS 27 mM
CS 135 mM
PA 6 mM
PA 30 mM
CD 3 mM
CD 15 mM
VN 4.5 mM
VN 22.5 mM
SB 90 mM
SB 450 mM

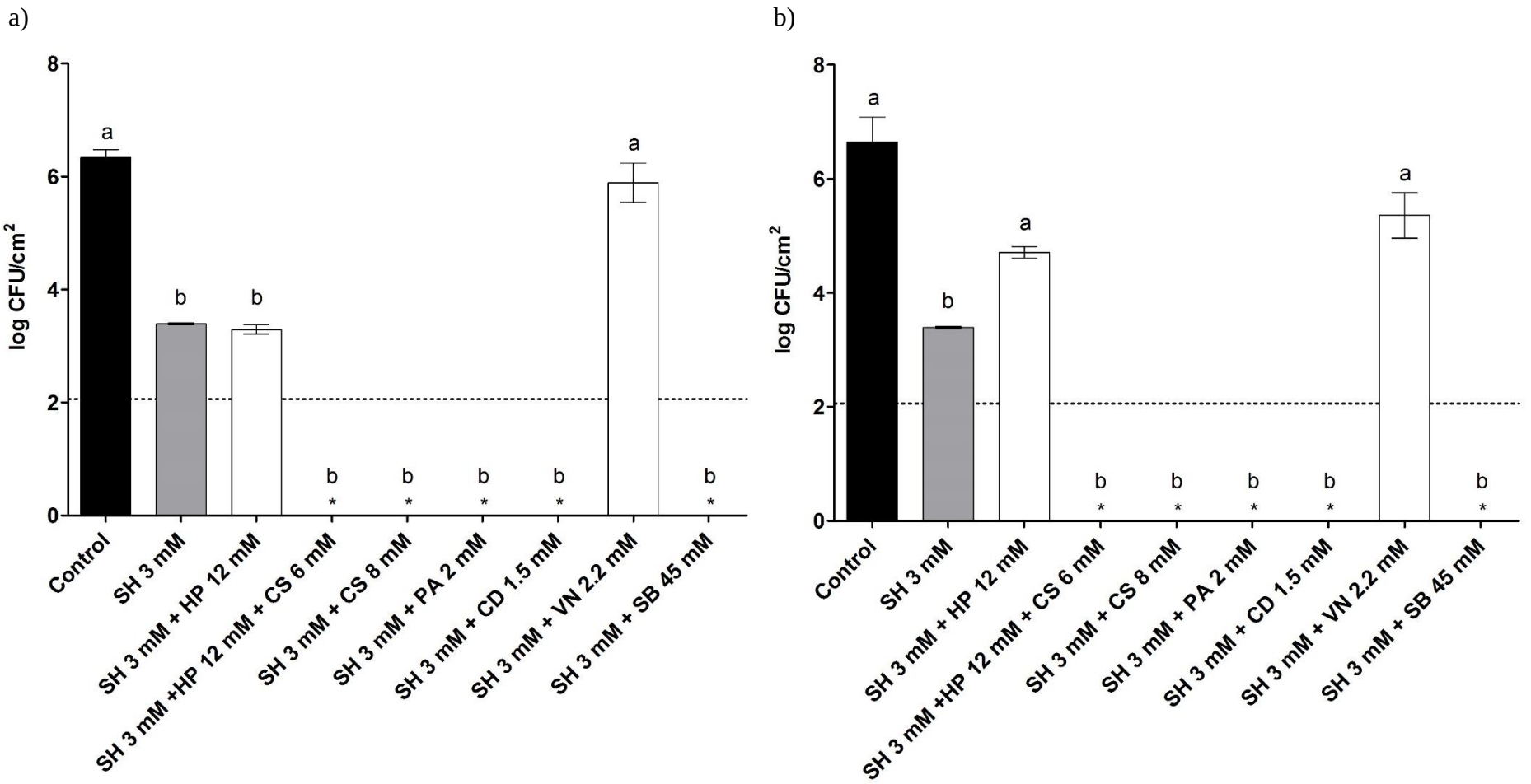
Figure 2

1


a)
b)
log CFU/cm²
0
2
4
6
8
Control
SH 6 mM
SH 30 mM
HP 15 mM
HP 75 mM
CS 27 mM
CS 135 mM
PA 6 mM
PA 30 mM
CD 3 mM
CD 15 mM
VN 4.5 mM
VN 22.5 mM
SB 90 mM
SB 450 mM

2 Figure 3

3

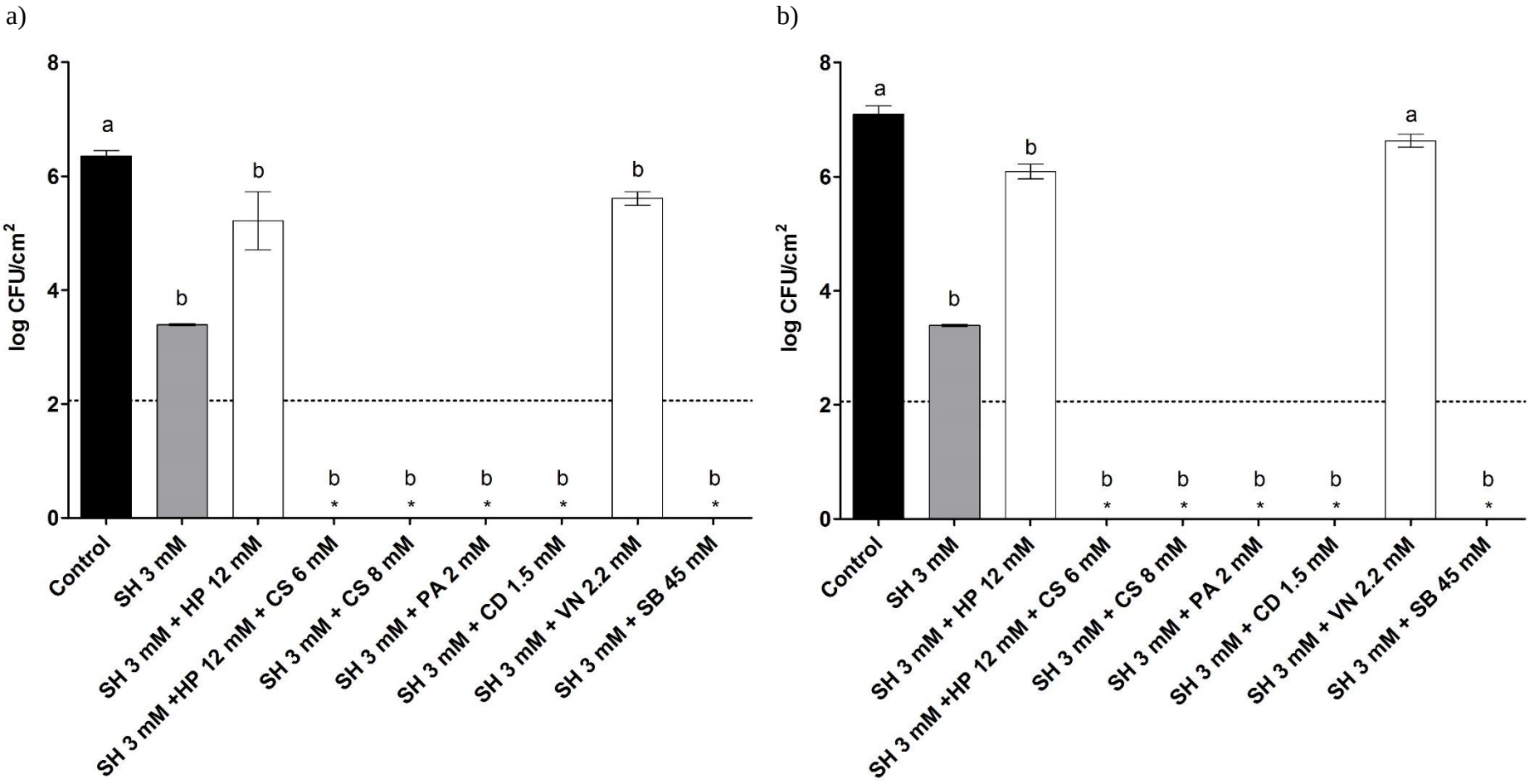

a)
b)
log CFU/cm²
0
2
4
6
8
a
b
*
Control
SH 3 mM
SH 3 mM + HP 12 mM
SH 3 mM +HP 12 mM + CS 6 mM
SH 3 mM + CS 8 mM
SH 3 mM + PA 2 mM
SH 3 mM + CD 1.5 mM
SH 3 mM + VN 2.2 mM
SH 3 mM + SB 45 mM

1 Figure 4

2

3

1


a)
b)
log CFU/cm2
8
6
4
2
0
a
b
b
b
b
b
b
b
b
b
b
b
*
Control
SH 3 mM
SH 3 mM + HP 12 mM
SH 3 mM +HP 12 mM + CS 6 mM
SH 3 mM + CS 6 mM
SH 3 mM + CS 8 mM
SH 3 mM + PA 2 mM
SH 3 mM + CD 1.5 mM
SH 3 mM + VN 2.2 mM
SH 3 mM + SB 45 mM

2 Figure 5

3

Table 1 – Calculation and significance of the MIC ratio

MIC ratio	Value	Result
	$0 < \frac{MIC_c}{MIC_i} < 0.5$	Potentiation
	$0.5 \leq \frac{MIC_c}{MIC_i} < 1$	Modest enhancement
	$\frac{MIC_c}{MIC_i} \geq 1$	Antagonism

MIC_c is the MIC of the compound in the combination and MIC_i is the MIC of the compound when used individually

Table 2 – Calculation and significance of the LR value

LR value	**Result**
$LR_c > LR_i$	Enhancement
$LR_c = LR_i$	Neutral
$LR_c < LR_i$	Antagonism

LR_c is the log CFU reduction of the compound in the combination and
LR_i is the log CFU reduction of the compound when used individually

1 Table 3 – MIC and MBC obtained for the individual disinfectants

Disinfectant	**MIC (mM)**	**MBC (mM)**
Chlorine dioxide	3.0 ± 0.0 [a]	3.0 ± 0.0 [a]
Peracetic acid	6.0 ± 0.0 [b]	7.0 ± 1.2 [b]
Sodium hypochlorite	6.0 ± 1.0 [b]	6.0 ± 1.0 [c]
Hydrogen peroxide	15 ± 1.2 [c]	16 ± 0.0 [d]
Copper sulphate pentahydrate	27 ± 2.3 [d]	28 ± 0.0 [e]
Vanillin	>4.5 [e]	>4.5 [f]
Sodium bicarbonate	>90 [f]	>90 [g]

2 Values are presented as the mean±standard deviation of three independent
3 experiments. Different letters within the same column represent statistically different
4 values ($P < 0.05$).

5

1 Table 4 – MIC and MBC obtained for different compounds when combined with SH 3
2 mM

Disinfectants	**MIC (mM)**	**MBC (mM)**	**MIC ratio**	**Result**
Chlorine dioxide	1.5 ± 0.0 [a]	1.5 ± 0.0 [a]	0.50	Modest enhancement
Peracetic acid	2.0 ± 0.0 [b]	2.7 ± 1.2 [b]	0.33	Potentiation
Hydrogen peroxide (with copper sulphate pentahydrate)	4.0 ± 0.0 [c]	6.7 ± 0.7 [c]	0.27	Potentiation
Copper sulphate pentahydrate	8.0 ± 2.0 [d]	10 ± 0.0 [d]	0.30	Potentiation
Hydrogen peroxide	12 ± 0.0 [e]	12 ± 0.0 [e]	0.80	Modest enhancement
Vanillin	>2.2 [f]	>2.2 [f]	-	-
Sodium bicarbonate	>45 [g]	>45 [g]	-	-

3 Values are presented as the mean±standard deviation of three independent experiments. Different
4 letters within the same column represent statistically different values ($P < 0.05$).
5