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The effects of ferulic and salicylic acids on *Bacillus cereus* and *Pseudomonas fluorescens* single - and dual - species biofilms

Madalena Lemos, Anabela Borges, Joana Teodósio, Paula Araújo, Filipe Mergulhão, Luís Melo, Manuel Simões*
LEPAE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal

Abstract

Biofilms are a problem to food industries, causing equipment damage, increased energy costs, and food spoilage, and they are a potential harbour of pathogenic microorganisms. Their extreme anti- microbial resistance means that novel control strategies are necessary. Plant secondary metabolites (phyto-chemicals) have demonstrated promising antimicrobial properties when applied against planktonic cells and biofilms. The aim of this study was to test the effectiveness of two phenolic acids: ferulic (FA) and salicylic (SA), alone and in combination (FSA) on the prevention and control of *Bacillus cereus* and *Pseudomonas fluorescens* biofilms. Additional tests were performed to ascertain the effects of FA and SA on bacterial motility, surface properties (physicochemical properties and surface charge), and quorum sensing inhibition (QSI). The effects of a concentration two times the minimum inhibitory concentration (500 $\mu g\ mL^{-1}$) were assessed on single- and dual-species biofilms. The results demonstrated that only swimming was affected by FA and SA and no clear relationship was obtained between the effects of phenolic acids on motility and biofilm prevention. The bacterial physico- chemical surface properties and charge were affected by the phenolic acids. Salicylic acid demonstrated capacity for QSI. However, both bacteria were able to form single- and dual-species biofilms in the presence of the phenolic acids. The application of FA and SA (single and combined) to biofilms caused low to moderate inactivation and removal. However, dual-species biofilms formed in the presence of phenolic acids were highly susceptible to a second exposure to the chemicals. The continuous exposure of dual-species biofilms to the phenolic acids decreased their resilience and resistance to inactivation and removal. This study clarifies the role of FA and SA in the prevention and control of biofilms formed by two important food spoilage bacteria.

1. Introduction

Microbial adhesion to surfaces and consequent biofilm formation is a survival strategy that has been studied and documented in recent decades (Watnick and Kolter, 2000). Biofilms can be defined as a complex structure composed of microbial cells adhered to a surface and enclosed in a matrix of hydrated extracellular polymeric substances (EPS) (Sauer et al., 2007), constituting a major problem for the biomedical, food, and environmental areas (Ludensky, 2003; Meyer, 2003). In the food industry, biofilms are usually indicative of microbial contamination (food spoilage and outbreaks of diseases) that can cause corrosion of surfaces, decrease heat transfer and pressure drop increase in conduits (Cloete et al., 1998; Beech, 2004). Studies have been carried out to collect information about biofilms in food-processing environments, which are important to understand the interactions among abiotic entities and microorganisms, and to assess the relationship between the proliferation of food-borne pathogens and the efficiency of cleaning and disinfection processes (Kumar and Anand, 1998; Simões et al., 2010c). In this context, the research focus should include disinfection procedures against mixed-culture biofilms, as in food industries the microbial diversity is very different from the pure cultures found in controlled environments (Hood and Zottola, 1995). The evolution of microbial resistance mechanisms is also related to the biofilm phenotype, and emergent strategies are required for their control. In fact, biofilm cells are more resistant to antimicrobial products than their planktonic counterparts (Simões et al., 2010a,c). Therefore, new antimicrobial products need to be identified and their antimicrobial action against biofilms must be assessed. The lack of new antibacterial products for biofilm control has been recognized as a major unmet industrial and biomedical need. Plant secondary metabolites (phytochemicals) can provide interesting solutions for biofilm control (Furiga, et al., 2008; Jagani et al., 2009; Simões et al., 2009a). Natural antimicrobial products can be attractive to the food industry in that they control natural spoilage microorganisms (Tajkarimi et al., 2010). Previous studies (Oussalah et al., 2007; Sandasi et al., 2008) have demonstrated that essential oils from selected plants inhibited the growth of four pathogenic bacteria (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Staphylococcus aureus*) and reduced the metabolic activity of *L. monocytogenes* biofilms.

Phenolic substances, including simple phenols and phenolic acids, are a major class of phytochemicals that have already demonstrated significant antimicrobial properties (Cushnie and Lamb, 2005; Vaquero et al., 2007; Simões et al., 2009a). Their mechanism of action may include enzyme inhibition by the oxidized products and consequent disruption of energy production (Simões et al., 2009a; Saavedra et al., 2010; Borges et al., 2012). Phenolic acids have a carboxylic acid functionality and in the realm of plant metabolites they constitute a distinct group of organic acids, divided into two characteristic constitutive carbon frame-works: the hydroxycinnamic and hydroxybenzoic structures (Robbins, 2003).

Chemically, their basic structure is similar, but the position and number of the hydroxyl groups on the aromatic ring defines the difference between the molecules. Phenolic acids are commonly used as preservatives in food applications, due to their antioxidant and antimicrobial activities (Robbins, 2003). However, diverse phenolic acids, including ferulic and salicylic acids, are promising candidates for cleaning and disinfection due to their antimicrobial properties and low cutaneous toxicity (Teichberg et al., 1993; Phan et al., 2001; Lin et al., 2005; Ergün et al., 2011; Borges et al., 2012).

The main purpose of this work was to study the action of two selected phenolic acidsdferulic acid, a hydroxycinnamic acid; and salicylic acid, a hydroxybenzoic acidd against *Bacillus cereus* and *Pseudomonas fluorescens* single- and dual-species biofilms. These bacteria are commonly found in biofilms formed in industrial systems (Simões et al., 2010b). The effects of phenolic acids were assessed on biofilm formation/prevention and control. Additional tests on bacterial motility, quorum sensing inhibition (QSI), and cell surface physicochemical properties and charge were performed in order to assess potential mechanisms of action of the selected products.

2. Materials and methods

2.1. Bacteria and culture conditions

The bacteria used in this work were *P. fluorescens* ATCC 13525^{T} and a *B. cereus* strain isolated from a disinfectant solution and identified by 16S rRNA gene sequencing, according to Simões et al. (2009b). Bacterial growth conditions were 27±2 ^{o}C and pH 7, with glucose as the main carbon source. Both bacteria were allowed to grow in a sterile synthetic nutrient medium consisting of 5 g l^{-1} glucose, 2.5 g l^{-1} peptone, and 1.25 g l^{-1} yeast extract, in 0.2 M phosphate buffer (PB) at pH 7 (Simões et al., 2006). *Chromobacterium violaceum* CV12472 was also used to determine QSI. This strain was kindly provided by Professor Robert McLean, of Texas State University. *C. violaceum* was routinely cultured aerobically in Luria-Bertani broth (LB; Liofilchen, Italy) at 30 ^{o}C with 150 rpm agitation in an orbital incubator (AGITORB 200, Aralab, Portugal), prior to the experiments. LB agar was used to test the activity of phytochemicals on QSI.

2.2. Chemicals tested

Two phenolic acids were tested, ferulic acid (FA) and salicylic acid (SA) (Sigma-Aldrich, Portugal) (Fig. 1). Due to their low solubility in water, FA and SA were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The tests were performed in the presence of FA and SA in a ratio of 1:1 (FSA). Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce

inhibitory concentrations in the range of 100 to 1000 μg ml^{-1} (Simões et al., 2009a). In this work, the concentration of FA, SA, or FSA tested on biofilms was 1000 μg ml^{-1}. Negative controls were performed with DMSO.

2.3. *Growth-inhibitory activity - minimum inhibitory concentration*

To determine whether the presence of the FA and SA had some effect on the bacterial growth in liquid medium, the minimum inhibitory concentration, or MIC (considered the lowest concentration of an antimicrobial that will inhibit the growth of a micro- organism after incubation) was determined using a microtiter plate-based assay method (Casey et al., 2004). Several concentrations of the phenolic acids were prepared by diluting the stock solutions in the appropriated DMSO. Overnight grown cultures were diluted with fresh sterile grow medium, in order to set the optical density, at 610 nm (OD610), to 0.4±0.02 for *P. fluorescens* and 0.8±0.02 for *B. cereus*, corresponding to approximately $1x10^8$ cells ml^{-1}. A sterile 96-well microtiter plate, (Orange Scientific), was inoculated with fresh sterile growth medium, the bacterial suspension, and the phenolic acids at different concen- trations. After incubation in an orbital shaker (24 h, 120 rpm, 27 °C), the OD610 in the microtiter plate was measured using an absorbance microplatereader (Spectramax M2e). The lowest concentration of phenolic acid where no growth was detected was considered the MIC of the product.

2.4. *Motility assays*

Swimming and swarming motilities were assessed in the presence of FA and SA. The plate medium for this assay was composed of 1% (w/w) Tryptone, and 0.25% (w/w) NaCl and agar at different concentrations (w/w), 0.3% for swimming and 0.7% for swarming assays (Sperandio et al., 2002; Borges et al., 2012). The use of different concentrations of agar (with the medium porosity directly related to the concentration of agar, so various levels of bacterial diffusion can be selected) enables the characterization of different types of bacterial motility (Borges et al., 2012). All the components were obtained from Merck (VWR, Portugal). The FA and SA at 100 μg ml^{-1} were incorporated into the growth medium (tempered at 45 °C). A sub-inhibitory concentration was used to ensure that the effects on motility inhibition were not due to antimicrobial activity. Overnight grown cultures were washed three times (3777 g, 5 min) with PB and resuspended in PB to a final concentration of $1x10^8$ cells ml^{-1}. Fifteen microliters of these cultures were applied in the center of the plates, producing an 8-mm halo (defined as the baseline). The motility halos were measured at 12, 24, and 48 h. Three plates were used to evaluate the motility of each bacterium and three independent experiments were performed.

2.5. *Physicochemical characterization of the bacterial surfaces*

The physicochemical properties of the bacterial surface were determined by the sessile drop contact angle measurement on bacterial layers, prepared as described by Busscher et al. (1984). The PS surfaces were prepared for characterisation by immersion in a solution of commercial detergent (SonasolPril, Henkel Ibérica S. A.) in ultrapure water for 30 min. After rising with ultrapure water, the surfaces were dried at 65 ^{o}C for 3 h.

The contact angles of the bacteria (non-exposed and exposed to FA and SA at different concentrations for 1 h) and the polystyrene (PS) were determined by sessile drop contact angle measurements. The measurements were performed at room temperature (25±2 ^{o}C), automatically, using a model OCA 15 Plus (DATA- PHYSICS, Germany) that allowed image acquisition and data analysis. The surface tension components of bacteria and PS were obtained by measuring the contact angles with three pure liquids: water, formamide, and α-bromonaphthalene (Sigma, Portugal). The surface tension components of the reference liquids were taken from the literature (Janczuk et al., 1993). Contact angle data were obtained from at least 25 determinations for each liquid and for each experiment. Afterwards, the hydrophobicity of bacteria and the PS surfaces were evaluated from contact angle measurements by the method of van Oss et al. (1987, 1988, 1989). With this method, the degree of hydrophobicity of a given material (i) is expressed as the free energy of interaction between two entities of that material immersed in water (w) - (ΔG_{iwi}-mJ m^{-2}). A ΔG_{iwi} value was calculated from the surface tension components of the interacting entities, according to the equation:

$$\Delta G_{iwi} = -2\left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_i^+\gamma_w^-} + \sqrt{\gamma_i^-\gamma_w^+} - \sqrt{\gamma_i^+\gamma_i^-} - \sqrt{\gamma_w^+\gamma_w^-}\right); \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. The surface tension components were estimated by the simultaneous resolution of three equations of the type:

$$(1 + \cos\theta)\gamma_i^{Tot} = 2\left(\sqrt{\gamma_s^{LW}\gamma_i^{LW}} + \sqrt{\gamma_s^+\gamma_i^-} + \sqrt{\gamma_s^-\gamma_i^+}\right); \quad (2)$$

where θ is the contact angle and $\gamma^{Tot} = \gamma^{LW} + \gamma^{AB}$.

2.6. *Free energy of adhesion*

The free energy of adhesion between the bacterial cells and PS surfaces was assessed according to Simões et al. (2008). When studying the interaction (free energy of adhesion - Δ_{iwl}^{Tot} between surface i (bacterium) and I (PS) that immersed or dissolved in water, the total interaction energy, Δ_{iwl}^{Tot}, can be expressed as:

$$\Delta G_{iwI}^{Tot} = \gamma_{iI}^{LW} - \gamma_{iw}^{LW} - \gamma_{Iw}^{LW} + 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_i^-} + \sqrt{\gamma_I^-} - \sqrt{\gamma_w^-}\right) + \sqrt{\gamma_w^-}\left(\sqrt{\gamma_i^+} + \sqrt{\gamma_I^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\gamma_i^+\gamma_I^-} - \sqrt{\gamma_i^-\gamma_I^+}\right] \quad (3)$$

Thermodynamically, if $\Delta_{iwl}^{Tot} < 0$, adhesion is favoured, whereas adhesion is not expected to occur if $\Delta_{iwl}^{Tot} > 0$ mJ m^{-2}.

2.7. *Bacterial surface charge* e *zeta potential*

The zeta potential of bacterial suspensions, before and after contact (1 h) with different concentrations of FA and SA (100, 500, and 1000 mg ml^{-1}) was determined using a Nano Zetasizer (Malvern Instruments, UK). Cell suspensions in ultrapure water (pH 6) without phytochemical were used as controls. The zeta potential was measured by applying an electric field across the bacterial suspensions. The experiments were repeated at least three times.

2.8. *Bioassay for detection of QSI*

A standard disc diffusion assay (Bauer et al., 1966) was per- formed with biosensor strain *C. violaceum* CV12472 to detect QSI activity of the selected phytochemicals, according to McLean et al. (2004). Briefly, LB agar plates were inoculated with 100 ml ($1.4x10^8$ CFU ml^{-1}) of overnight culture of *C. violaceum* CV12472. Afterwards, sterile paper disks (6 mm in diameter) (Oxoid, Spain) were placed over the plates and were loaded with 15 µl of different concentrations of each phytochemical. DMSO and LB broth were used as controls. Plates were incubated for 24 h at 30 °C to check the inhibition of pigment production around the disc (a ring of colourless but viable cells). Antimicrobial activity was indicated by the lack of microbial growth. Bacterial growth inhibition by the phytochemical was measured as diameter (d1) in millimeters while phytochemicals showing both growth and pigment inhibition were measured as diameter (d2) in millimeters. Quorum sensing inhibition, QSI, assessed by pigment inhibition, was determined by subtracting bacterial growth inhibition diameter (d1) from the total diameter (d2): QSI = (d2 – d1), according to Zahin et al. (2010).

2.9. *Biofilm formation*

Biofilms were developed according to a modified microtiter plate test proposed previously (Stepanović et al., 2000). Single- and dual-species 24-h-aged biofilms were grown in sterile 96-well flat- bottomed polystyrene tissue culture microtiter plates (Orange Scientific, USA). The microtiter plates were inoculated with fresh sterile growth medium and a bacterial suspension with a cell density of $1x10^8$ cells ml^{-1}. Dual-species biofilms were formed with a mixture of equal volumes of *P. fluorescens* and *B. cereus*, with the same cell density as described for single-species biofilms (Simões et al., 2010a). The microtiter plates were incubated for 24 h in an orbital shaker (120 rpm, 27 ± 2 °C) and the biofilms formed were used to assess the effects of phenolic acids on biofilm control (inactivation and biofilm reduction). Negative controls were obtained by incubating the wells only with growth medium without adding any bacteria. All experiments were performed in triplicate with three repeats.

2.10. *Biofilm cellular density*

Single- and dual- species biofilms were grown in 96-well microtiter plates as described in the previous section. After 24 h of incubation, the content of each well was discarded and the biofilm was gently washed with sterile PB to remove loosely attached micro-organisms. A volume of 200 µl of PB was added to the wells and the biofilm was scraped with a stainless steel scraper as described by Simões et al. (2010b). The resuspended cells were used to assess total counts. After dilution, a volume (up to 3 mL as a function of the bacterial concentration) was filtered through a 25-mm black Nucleopore™ polycarbonate membrane with a pore size of 0.2 µm (Whatman, UK). After filtration, cells on the membrane were stained with 400 ml of 4,6-diamino-2-phenylindole (DAPI) (Sigma) at 0.5 µg ml^{-1} and left in the dark for 5 min (Simões et al.,2007). Cells were visualized under an epifluorescence microscope (Leica DMLB2 with a mercury lamp HBO/100W/3) incorporating a CCD camera to acquire images using IM50 software (Leica), using a x100 oil immersion fluorescence objective, and a filter sensitive to DAPI fluorescence (359-nm excitation filter in combination with a 461-nm emission filter).

A total of 20 fields were counted and at least three independent membranes were used to calculate total cells per square centimeter of biofilm.

2.11. *Biofilm prevention and effects of a second dose of phenolic acids*

Single- and dual-species biofilms were grown in the presence of the phenolic acids. Microtiter plates where inoculated with a bacterial suspension with a cell density of $1x10^8$ cells ml^{-1}, fresh sterile growth medium, and FA, SA, or FSA at

1000 μg ml^{-1}. After 24 h of incubation in an orbital shaker (120 rpm, 27±2 °C), the biofilms were used to assess the preventive effects of FA, SA, or FSA on biofilm formation. With this purpose, the biomass and activity of biofilms formed in the presence of phenolic acids was compared with those formed only with growth medium (the presence of DMSO caused no effects on biofilm formation e results not shown). Final results are presented as a percentage of biofilm reduction and inactivation.

Those biofilms formed in the presence of phenolic acids were also used to assess their presumptive adaptation to the chemicals by exposing the biofilms to a second dose of the product. Before the second exposure to phenolic acids, new growth medium was inserted in the microtiter plates and the remaining biofilms and the plates were incubated for 24 h. After 1 h exposure to the second treatment with phenolic acids, the biofilms were analysed in terms of biomass and activity and the final results are presented as percentages of biofilm reduction and inactivation.

2.12. *Biofilm control*

To determine whether the phenolic acids had an effect on biofilm control, microtiter plates with single- and dual-species biofilms were exposed to 1000 μg ml^{-1} of FA, SA, or FSA, according to Simões et al. (2010b). One hour after exposure, the biofilms were analysed in terms of biomass and metabolic activity and the results are presented as percentages of biofilm reduction and inactivation.

2.13. *Biomass quantification*

The biofilm mass was quantified using crystal violet (Merck, Portugal) staining according to Borges et al. (2012). The absorbance was measured at 570 nm using an absorbance microplate reader (Spectramax M2e, Molecular Devices, Inc.). The percentage of reduction was assessed based on the following equation:

$$\text{Percentage of reduction} = \frac{[C - T]}{C} \times 100\% \qquad (4)$$

where C denotes the absorbance for control wells (absence of phenolic acids), and T is the absorbance for biofilms exposed to FA, SA, or FSA.

2.14. *Biofilm activity*

To assess bacterial viability, a modified alamar blue microtiter plate assay was used as described in Sandberg et al. (2009) and Borges et al. (2012). Fluorescence was

measured at an emission wavelength of 570 nm and an excitation wavelength of 590 nm using an absorbance microplater eader (Spectramax M2e, Molec- ular Devices, Inc.).

The percentage of inactivation was calculated from the fluo- rescence of the control samples and the biofilms treated with phenolic acids:

$$\text{Percentage of inactivation} = \frac{[C - T]}{C} \times 100\% \qquad (5)$$

The C value is the fluorescence for the biofilm control, and T denotes the fluorescence for the biofilm exposed to the phenolic acids.

2.15. *Biofilm control activity classification*

The biofilm control effects of the phenolic acids were classified according to the following scheme, where I refers to inactivation and R to removal: Low efficacy - I or $R < 25\%$; moderate efficacy - $25\% \leq I$ or $R < 60\%$; high efficacy - $60\% \leq I$ or $R < 90\%$; and excellent efficacy - $90\% \leq I$ or $R \leq 100\%$.

2.16. *Statistical analysis*

The experimental data were analysed using the statistical pro- gram SPSS version 17.0 (Statistical Package for the Social Sciences). The mean and standard deviations within samples were calculated for all cases. At least three independent experiments were performed for each condition tested. All data were analysed by the application of the non-parametric Kruskal-Wallis tests (confidence level ≥95%).

3. Results

3.1. *Minimum inhibitory concentration*

Both FA and SA had inhibitory effects on the growth of the tested bacteria. The MIC of the phenolic acids was 500 μg ml^{-1} for both bacteria.

3.2. *Motility*

Swimming and swarming motilities increased over time in the absence of phenolic acids, for both bacteria (Table 1). However, this increase was only significant for swimming motility ($P < 0.05$). In the presence of FA and SA, swimming motility of both bacteria was significantly reduced when compared with the control experiments ($P < 0.05$). No significant changes over time were detected for the swimming motility of both bacteria when they were exposed to FA or SA ($P > 0.05$).

The swarming motility of *P. fluorescens* and *B. cereus* was not significantly affected by FA or SA ($P > 0.05$). There are no statistically significant differences ($P > 0.05$) when comparing the action of the two phenolic acids on swimming and swarming motilities of *B. cereus* and *P. fluorescens*.

3.3. *Effect of phenolic acids on physicochemical surface properties*

B. cereus and *P. fluorescens* untreated and phenolic acid-treated cells were analysed in order to determine their surface tension parameters and hydrophobicity (Table 2). Both *B. cereus* and *P. fluorescens* cells are naturally hydrophilic, as their $\Delta G_{iwi} > 0$ mJ m^{-2}, with this characteristic more pronounced for *B. cereus* ($P < 0.05$). The exposure to the phenolic acids caused a significant increase on ΔG_{iwi} ($P < 0.05$) for the *B. cereus* cells when they were exposed to SA at 100 mg ml^{-1}. For *P. fluorescens,* the value of this component increased significantly ($P < 0.05$) after exposure to FA at 500 and 1000 μg ml^{-1} and to SA at 500 and 1000 μg ml^{-1}.

The Lifshitz-van der Waals component γ^{LW} and the Lewis acid-base component γ^{AB} indicate the a polar or polar surface properties of the bacterial cell, respectively. Regarding the *B. cereus* cells, the exposure to the phenolic acids had no significant effect on the γ^{LW} ($P > 0.05$), with the exception of SA at 100 μg ml^{-1}. The γ^{LW} was significantly increased by both phenolic acids ($P < 0.05$) for *P. fluorescens*, with the smaller difference caused by FA at 100 μg ml^{-1}; the most significant increase was observed with SA at 100 μg ml^{-1}. Moreover, in the case of *B. cereus*, the γ^{AB} component was significantly affected by both phenolic acids ($P < 0.05$), except for FA at 1000 μg ml^{-1} and SA at 500 μg ml^{-1}. For *P. fluorescens* the magnitude of this parameter decreased significantly ($P < 0.05$) for all the conditions tested, with the minimum value being observed with FA at 1000 μg ml^{-1} and the maximum with FA at 100 μg ml^{-1}. The $\gamma+$ and $\gamma-$ values indicate if the cells have more ability to accept or donate electrons, respectively. The electron donor component of *cereus* increased significantly after the treatments with SA at 100 μg ml^{-1} ($P < 0.05$). The electron acceptor ability of *B. cereus* cell surfaces increased after treatment with FA at 500 μg ml^{-1} and SA at 100 μg ml^{-1} ($P > 0.05$). The $\gamma-$ of *P. fluorescens* cell surfaces increased significantly ($P < 0.05$) with exposure to FA at 1000 μg ml^{-1} and SA at 1000 μg ml^{-1}. The γ^{+} component of *P. fluorescens* cell surfaces decreased significantly with exposure to the phenolic acids and with an increase of concentration ($P < 0.05$).

3.4. *Free energy of adhesion*

The surface tension values allowed the assessment of the free energy of adhesion between the bacterial surfaces and PS (Table 3). *B. cereus* untreated cells had a ΔG^{Tot} of -1.80 mJ m^{-2}. After exposure to the phenolic acids there was an increase of the free energy of adhesion, for all the conditions. The maximum value of ΔG^{Tot} 12.2 mJ m^{-2} was observed for the treatment with SA at 100 μg ml^{-1}. In the case of *P.* fl*uorescens*, the untreated cells had a ΔG^{Tot}=13:5 mJ m^{-2}. The exposure to phenolic acids caused a decrease in the free energy of adhesion, for all the conditions. The minimum value of ΔG^{Tot} = -5.74 mJ m^{-2} was after exposure to SA at 500 μg ml^{-1}.

3.5. *Effect of phenolic acids on bacterial surface charge*

The bacteria tested have negative cell surface charge: -19.1 mV (*B. cereus*) and -9.07 mV (*P.* fl*uorescens*). The zeta potential values (Fig. 2a and b) of both bacteria after exposure to the phenolic acids were significantly different from the control ($P < 0.05$). The surface charge of *P.* fl*uorescens* became less negative with increasing phenolic acid concentration, for all the compounds ($P < 0.05$). Also, the surface charge changes were concentration-dependent ($P < 0.05$). Exposure of *B. cereus* to SA and FA increased the sur- face charge ($P < 0.05$). This change in surface charge was not concentration-dependent ($P > 0.05$).

3.6. *Screening for quorum sensing inhibition of phenolic acids*

A disc diffusion assay was performed for QSI screening using *violaceum* CV12472. Diverse concentrations of the phenolic acids were tested, in order to ascertain if the halos produced around the biosensor strain were due to bacterial growth inhibition and/or QSI (Table 4). Hence, loss of purple pigment by *C. violaceum* CV12472 was indicative of QSI by phytochemicals. No pigment inhibition was observed with FA at the concentrations tested. However, growth inhibition halos between 6.5 and 9.6 mm were detected. Inhibition of pigment production was observed for SA at 500 and 1000 μg ml^{-1}. Salicylic acid caused QSI halos of 4.9 (500 μg ml^{-1}) and 7.9 mm (1000 μg ml^{-1}). For these cases, the white zones of inhibition were opaque and not transparent, indicating that the halo around the disks was caused by inhibition of violacein production, and not due to the inhibition of cell growth. Moreover, antimicrobial activity, in addition to QSI, was also observed for SA.

3.7. Biofilm cellular density

The single- and dual-species biofilms were characterized in terms of total cells. *B. cereus* biofilms had total cell counts of 6.49±0.16 log cells cm^{-2}, whereas *P. fluorescens* had 6.64±0.13 log cells cm^{-2}. Dual-species biofilms presented higher values of total cells (6.77±0.23 log cells cm^{-2}), although this result was not statistically different from the cell densities of single-species bio- films ($P > 0.05$).

3.8. Biofilm prevention with phenolic acids

The preventive action of FA, SA, and SFAon biofilm formation was assessed by developing *B. cereus* and *P. fluorescens* single- and dual-species biofilms in the presence of these chemicals. The concentration used (1000 μg ml^{-1}) was twice the minimum inhibitory concentration for both bacteria. In terms of inactivation, due to the presence of the phenolic acids (Fig. 3A), dual-species biofilms were equally affected in terms of reduction of biofilm activity by FSA and SA ($P > 0.05$), whereas a significantly lower percentage was found for FA ($P < 0.05$). *P. fluorescens* biofilms had the highest percentage of inactivation with FSA, compared with FA and SA. However, the inactivation percentages are statistically similar ($P > 0.05$). The SA had low effects on *B. cereus* biofilm inactivation. The effects of FSA were more pronounced than those of FA on *B. cereus* biofilms ($P < 0.05$).

The reduction on *P. fluorescens* biofilm formation (Fig. 3B) was similar ($P > 0.05$) for FSA and FA, and showed a moderate decrease ($P < 0.05$) for SA. In the case of *B. cereus* biofilms, FA had the most significant preventive action ($P < 0.05$) when it was compared with FSA and SA ($P > 0.05$). The highest preventive effects were observed with FA and FSA ($P < 0.05$) for the dual- species biofilms.

3.9. Biofilm control with phenolic acids

The phenolic acids were applied to 24-h-aged biofilms and their effects were assessed on inactivation (Fig. 4A) and biomass removal (Fig. 4B). The phenolic acids had low effects on the inactivation (<25%) of single- and dual-species biofilms of *B. cereus* and *P. fluorescens*. Despite the lower percentage (<20%), FSA promoted the most significant inactivation for *P. fluorescens* and *B. cereus* single- and dual-species biofilms ($P < 0.05$). In the case of *P. fluorescens* biofilms, SA was more effective than FA ($P < 0.05$), while the opposite occurred for *B. cereus* biofilms ($P < 0.05$). The FA and SA had similar ($P > 0.05$) effects on the inactivation of dual- species biofilms.

In terms of biofilm removal, a moderate action (removal > 25%) was found for *P. fluorescens* biofilms treated with FSA and FA and for *B. cereus* biofilms exposed to FA. The remaining treatments had low effects in terms of biofilm removal. The biomass reduction of *P. fluorescens* and *B. cereus* single biofilms (Fig. 4B) was similar ($P > 0.05$) for FA and SA. For the dual-species biofilm, the biomass reduction was low and statistically similar for the diverse treatments ($P > 0.05$).

3.10. *Control of biofilms formed in the presence of phenolic acids*

The effects of a second exposure to phytochemicals were assessed on single- and dual-species biofilms formed in the presence of phenolic acids (Fig. 5A and B). The exposure of presumptively adapted biofilms to the phytochemicals promoted a high inactivation (>80%) of dual-species biofilms, with FSA, FA, and SA showing similar effects ($P > 0.05$). The metabolic activity of single-species biofilms was affected to a low extent and the inactivation promoted by the phenolic acids was similar when comparing both *B. cereus* and *P. fluorescens* single-species biofilms ($P > 0.05$).

The action of phenolic acids on the removal of presumptively adapted biofilms was moderate (removal > 25%) for all conditions analysed. The dual-species biofilms were the most significantly affected by the chemicals, with FA and SA promoting the higher removal percentages ($P < 0.05$). The application of FA, SA, and FSA caused similar removal of *B. cereus* and *P. fluorescens* single-species biofilms ($P > 0.05$).

4. Discussion

Interest in the use of phytochemicals as an alternative to con- ventional antimicrobials has been increasing significantly in recent years (Cowan, 1999; Simões et al., 2009a; Saavedra et al., 2010). These molecules can act on multiple biochemical targets of the cell, preventing the emergence of antimicrobial resistance events (Simões et al., 2009a; Abreu et al., 2012). There are studies on the inhibition of planktonic cell growth with phenolic products (Fernández et al., 1996; Puupponen-Pimiä et al., 2001; Cueva et al., 2010; Saavedra et al., 2010). However, studies on their biofilm control potential are scarce (Furiga et al., 2008; Jagani et al., 2009; Ergün et al., 2011; Borges et al., 2012). Furiga et al. (2008) tested compounds from red wine, grape marc, and pine bark, and demonstrated interesting anti-plaque activity in vitro against *Streptococcus mutans*. Jagani et al. (2009) verified that phenol and natural phenolic compounds showed a significant reduction in biofilm formation by *Pseudomonas aeruginosa*. Ergün et al. (2011) evaluated the antimicrobial properties of aromatic ester derivatives of ferulic acid, which showed significant biofilm eradication potential, comparable to their MIC for planktonic cultures against Gram-positive bacteria and fungi. It seems fundamental to understand the role of

phytochemicals as a source of new anti- microbial products for biofilm prevention and control. In the present work, the in vitro activity of two phenolic acids was assessed, as single compounds e FA and SA, and in combination e FSA, against single- and dual-species biofilms formed by *B. cereus* and *P. fluorescens*. Those bacteria are ubiquitous in industrial systems, and are particularly problematic in food-processing plants (Simões et al., 2010c). The phenolic acids had MICs of 500 μg ml^{-1} against both bacteria.

The phenolic acids (single and in combination) at twice the MIC had low effects on the inactivation of single- and dual-species biofilms of *B. cereus* and *P. fluorescens*. The 24-h-aged biofilms used in this study had statistically comparable cell densities and they were similar to those of biofilms formed by other bacteria on 96-well polystyrene microtiter plates (Simões et al., 2010b). It is conceivable that the small differences in cell density of *P. fluorescens* and *B. cereus* single- and dual-species biofilms are not the reason for the distinct susceptibility and behaviour to phytochemical exposure. In terms of biofilm removal, moderate efficacy was only detected for FSA (*P. fluorescens* single biofilms) and FA (*B. cereus* and *P. fluorescens* single biofilms). These results are in accordance with the findings of Jagani et al. (2009). These authors compared the effects of various natural phenolic compounds on *P. aeruginosa* biofilm removal, showing that SA caused a moderate reduction (45%) when compared to other molecules. Saavedra et al. (2010), in studies with planktonic bacteria (*E. coli*, *P. aeruginosa*, *L. monocytogenes*, and *S. aureus*) found low levels of inhibitory effects of hydroxycinnamic acids (ferulic and caffeic acids) and a hydroxybenzoic acid (gallic acid), when compared to selected antibiotics (gentamicin, ciprofloxacin, and streptomycin). It is assumed that hydroxycinnamic and hydroxybenzoic acids have similar antibacterial action mechanisms, promoting enzyme inhibition, possibly through reaction with sulfhydryl groups or through nonspecific interactions such as the reversible or irreversible re- action of quinonic compounds with amino acids or with peptides (Cowan, 1999; Bittner, 2006).

Comparing the effects of FA or SA on biofilm control (inactiva- tion and removal), no significant differences were obtained. Also, their combination (FSA) was not clearly more effective than the individual chemicals. The comparison of the results from this study with previous reports clearly demonstrates that different bacterial species display distinct antimicrobial susceptibilities. A study with hydroxycinnamic and hydroxybenzoic acids demonstrated that those hydroxycinnamics cause higher inhibition of a strain of *Oenococcus oeni* in planktonic state (Campos et al., 2003). Borges et al. (2012) found that both gallic and ferulic acids had strong potential to inactivate and remove biofilms of *E. coli*, *L. monocytogenes*, *P. aeruginosa*, and *S. aureus*. Prithiviraj et al. (2005), using the *Arabidopsis thaliana-P. aeruginosa* pathosystem, gathered evidence that suggests that SA, besides triggering defense responses, could also act on *P. aeruginosa* by disruption of biofilm formation on biotic and abiotic surfaces and by repression of

a number of virulence factors. Other authors (Rosenberg et al., 2008; Nowatzki et al., 2012) found that salicylic acid-based and realising polymers significantly reduced biofilm formation by *Salmonella enterica* serovar Typhimurium and *E. coli*. Ergün et al. (2011) demonstrated that simple aromatic esters of FA inhibited biofilm formation by *S. aureus* at a concentration <8 μg ml^{-1}.

The action of phenolic acids (single and in combination) on presumptively adapted biofilms (development in the presence of phenolic acids) was significant only on those formed by dual species. This is a surprising result as in previous studies (Simões et al., 2009b, 2011) it was found that *B. cereus*-*P. fluorescens* association increased antimicrobial resistance to antimicrobial products (an aldehyde-based biocide and a quaternary ammonium compound) comparatively to their single-species biofilms. This study demonstrates that dual-species biofilm formation in the presence of FA, SA, or FSA will increase their susceptibility to a second exposure to the chemicals. This is a new finding and indicates that the interactions established by *B. cereus* and *P. fluorescens* in the formation of dual-species biofilms seem to be affected by the presence of FA, SA, or FSA. In fact, single-species biofilms were almost un-affected by the second exposure to the phenolic acids. Houry et al. (2012) demonstrated that the infiltration of *Bacillus thuringiensis* into a *S. aureus* biofilm promotes the penetration of macro-molecules macromolecules, such as antimicrobials, into the biofilm. The authors suggested that these tunneling bacteria can be used to in- crease the efficacy of biocides in both industrial abiotic surface disinfection and human infections. This is one of the probable aspects that might be involved in the increased susceptibility of the dual-species biofilms to the phytochemicals tested.

In order to ascertain putative aspects involved in the behaviour of *B. cereus* and *P. fluorescens* single- and dual-species biofilms, studies on the effects of FA and SA on bacterial motility, surface physicochemical properties, and charge and QSI activity were performed. Bacterial motility and biofilm formation are manifestations of functional responses to surface colonization. It is believed that motility may be implicated in the stabilization of cell surface and cell-to-cell interactions (Harshey, 2003). In this study, the effects of phenolic acids were tested on swimming and swarming motilities of *B. cereus* and *P. fluorescens*. Swimming and swarming motilities are flagella-mediated. However, swimming is considered to be individual motility in liquid medium, while swarming is a social movement of a group of bacteria, and permits rapid migration over a surface (Shapiro, 1998). It is accepted that biofilm formation is enhanced by cell motility, particularly when it is mediated by flagella. This occurs because bacteria overcome the long-range repulsive forces that facilitate close approach to the surfaces, enabling accelerated surface adhesion (Karatan and Watnick, 2009). Houry et al. (2010) reported the importance of motility in *B. cereus* biofilm formation in microtiter plates. The application of phenolic acids affected the swimming motility of *B. cereus* and *P. fluorescens*. In a previous study, Muller et al. (1998) found that the inclusion of 5 mM of SA in medium inhibited both growth

and biofilm production by *Staphylococcus epidermidis* by up to 55%. Farber and Wolff (1993) found that SA prevented the adherence of bacteria (*P. aeruginosa*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*) and yeast (*Candida albicans*) to silastic catheters. Borges et al. (2012) found that gallic and ferulic acids had potential to inhibit motility and to prevent biofilms of four important human pathogenic bacteria (*E. coli*, *L. monocytogenes*, *P. aeruginosa,* and *S. aureus*). Those results clearly demonstrate that the action of FA and SA in motility and further biofilm prevention is strongly dependent on the bacterial species used. In this work, the assays of biofilm prevention show low to moderate effects of FA, SA, and FSA on the inhibition of single- and dual-species biofilm formation, indicating that the effects of phytochemicals on bacterial motility were not significant on biofilm prevention. Therefore, it was important to understand other factors involved in biofilm formation and development, particularly the aspects involved in the initial adhesion processes.

Adhesion is a complex process that is affected by many aspects, such as the physicochemical characteristics of bacteria, the material surface properties, and the environmental factors (An and Friedman, 1998; Machado et al., 2011). Moreover, it is known that cell surface properties (e.g., hydrophobicity and surface charge) are relevant to the process of bacterial adhesion and biofilm formation (Simões et al., 2008, 2010a). When there is contact with the sub- stratum, bacterial adhesion can be mediated by hydrophobic inter- actions. It is commonly observed that cell surface hydrophobicity can affect bacterial adhesion to different types of substrata, including hydrophobic surfaces, such as PS (Pang et al., 2005). Comparing the thermodynamic capability for adhesion between the bacteria and PS, the results obtained shown that the exposure to the phenolic acids works against the adhesion of *B. cereus* to PS but favours *P. fluorescens*. These results based on thermodynamic analysis indicate that FA and SA may prevent *B. cereus* biofilm formation. The effects of phytochemicals on the prevention of biofilm formation by *E.*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* was also demonstrated by Borges et al. (2013) using microtiter plate tests. For *P. fluorescens* biofilms, no correlation was verified be- tween the thermodynamic adhesion and biofilm prevention tests. This result indicates that the surface physicochemical properties are not the main factor regulating the initial adhesion process. However, even if distinct information on the physicochemical surface properties and free energy of adhesion was obtained for *B. cereus* and *P. fluorescens*, biofilm prevention was not achieved. This indicates that initial adhesion did not predict the ability of the tested bacteria to form a biofilm, suggesting that other events such as phenotypic and genetic switching during biofilm development and the production of extracellular polymeric substances, may play a significant role in biofilm formation and differentiation (Simões et al., 2010a).

The surface charge of the cell was also assessed to provide in- formation on the potential electrostatic repulsive/attractive events between the bacterial cell surfaces and PS. It was observed that under physiologically conditions the bacteria have

negative surface charges. When exposed to phenolic acids the surface charge of the bacteria increased significantly, particularly for *B. cereus*. This effect seems to increase the interaction between the bacterial surfaces and PS and may help in understanding the failure of FA and SA (single and combined) to prevent biofilm formation. In fact, PS has a zeta potential of 32 mV (Simões et al., 2010a).

Quorum sensing (QS), a form of cell-to-cell communication in bacteria, is an important regulatory mechanism in biofilm formation and differentiation. Interference with QS can affect the biofilm development and make the bacteria more susceptible to antimicrobials (Landini et al., 2010). Data provided by some studies showed that mutants with lack of QS form biofilms that are more unstructured, and susceptible to chemical agents compared to wild types (Davies et al., 1998; Bjarnsholt et al., 2005). The results obtained in this study demonstrated that SA had potential for QSI, despite the small size of halos produced. Moreover, SA had antimicrobial activity in addition to QSI. This effect of SA on QSI can help in understanding the results obtained with biofilms, particularly the behaviour of dual-species biofilms after a second exposure to phytochemicals. However, the QSI activity verified with this phenolic acid needs to be further characterized, particularly in relation to the mechanisms involved in the inhibition process. In fact, it is probable that FA may have QSI activity, not detectable by the method used (McLean et al., 2004).

In conclusion, FA and SA can significantly reduce bacterial swimming motility, increase the surface charge value, and promote changes in the physicochemical surface properties of both *B. cereus* and *P. fluorescens*. Salicylic acid demonstrated potential for QSI. Their distinct chemical structure does not seem to cause differences in the biofilm control activity, when the molecules were applied individually (FA and SA) and in combination (FSA). This result reinforces the inadequacy of planktonic tests to the design of disinfection procedures to be applied to control biofilms. The development of biofilms in the presence of phenolic acids increased the susceptibility of dual-species biofilms to a second exposure to the chemicals, arguably due to an interference with the interactions involved in *B. cereus*-*P. fluorescens* biofilm formation and structure. To our knowledge this is the first study on the evaluation of FA, SA, and their combination on *B. cereus* and *P. fluorescens* biofilm prevention and control. Additional studies are in progress in order to ascertain the potential of FA, SA, and other "green chemicals" to act as synergistic agents with conventional biocides for biofilm control; the aim would be to reduce the strong dependency of disinfection practices on high doses of conventional chemicals.

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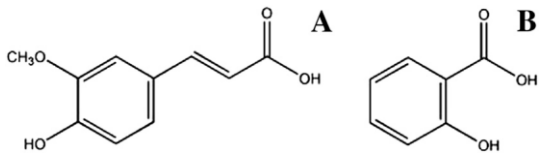

Fig. 1. Chemical structures of ferulic (A) and salicylic acids (B).

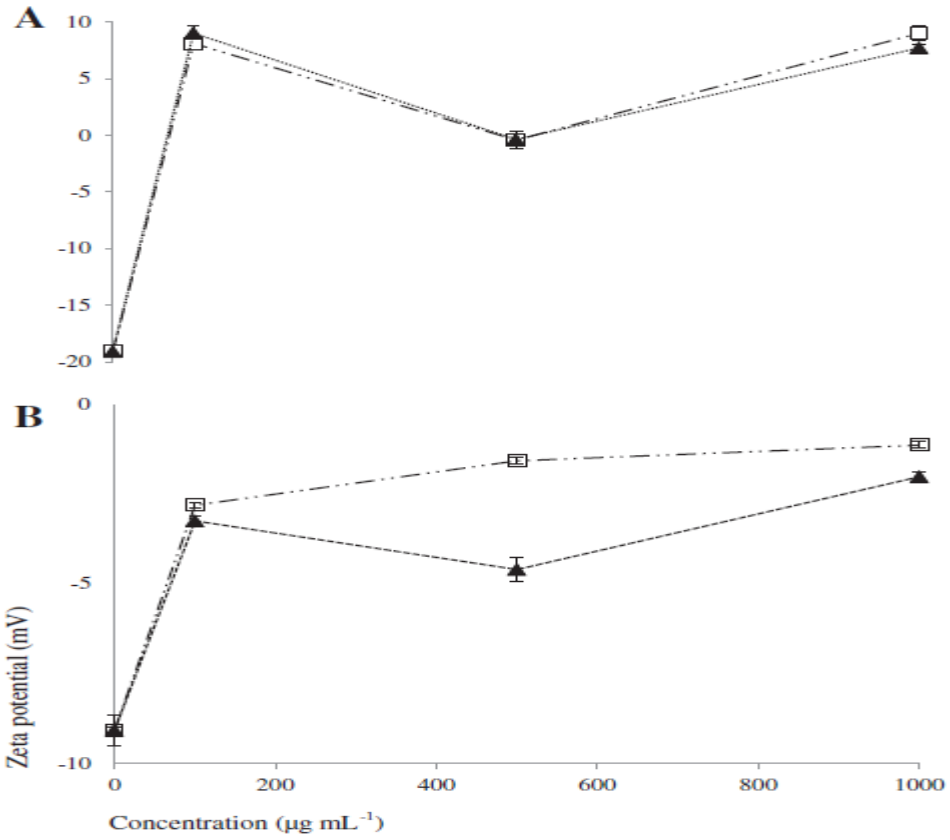

Fig. 2. Zeta potential values (mV) of suspensions of *B. cereus* (a) and *P. fluorescens* (b) when exposed to different concentrations (0, 100, 500, and 1000 µg mL^{-1}) of SA (‚) and FA (:) for 1 h. The means ± SD for at least three replicates are illustrated.

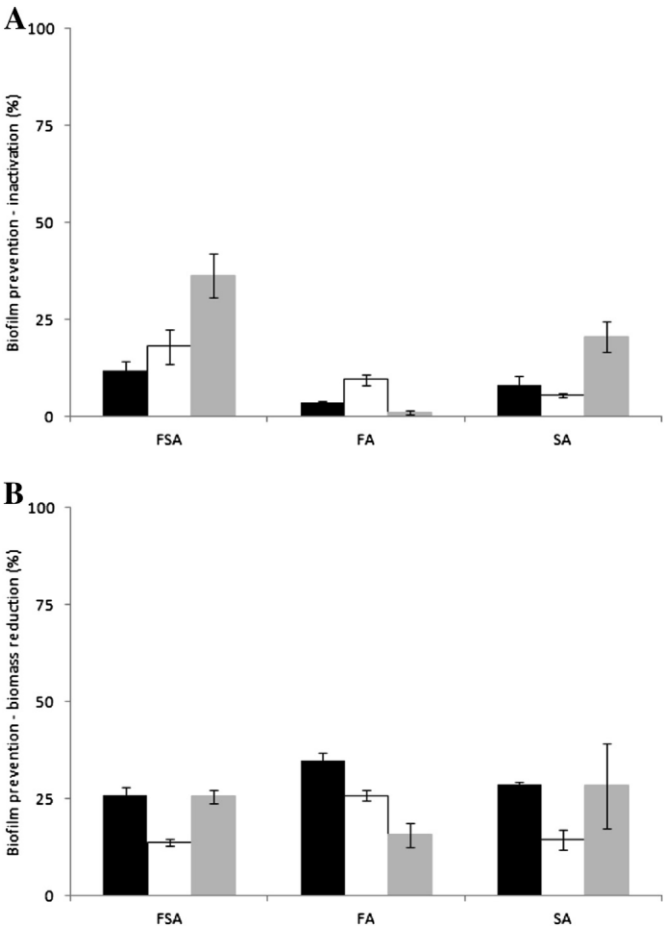

Fig. 3. Preventive action (24-h-aged biofilms formed in the presence of phenolic acids) of FA, SA, and FSA on the activity (A) and biomass formation (B) of *P. fluorescens* (—) and *B. cereus* (▪) single- and dual- (—) species biofilms. Mean values ± standard deviation for at least three replicates are illustrated.

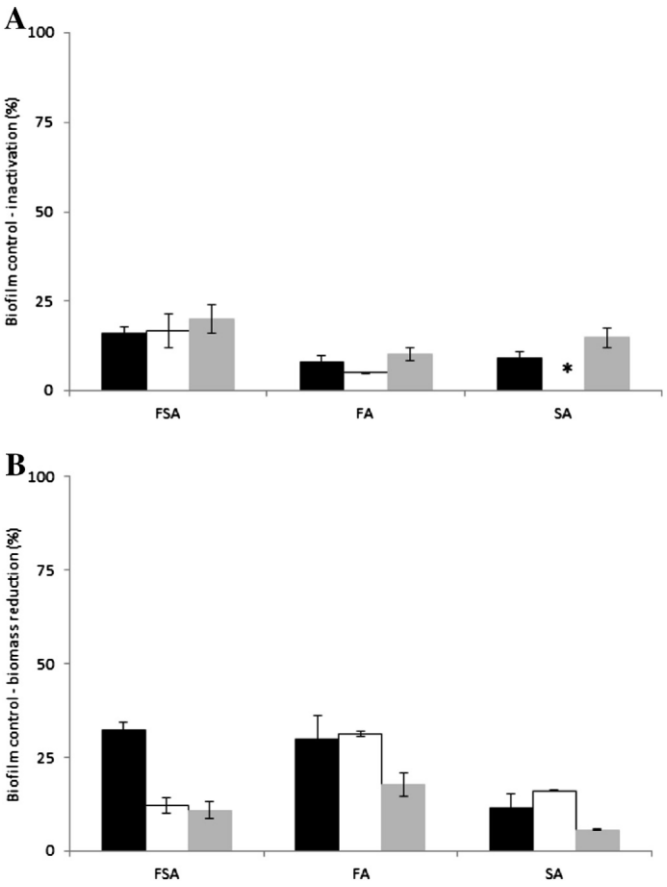

Fig. 4. Percentage of inactivation (A) and reduction (B) of *P. fluorescens* (—) and *B. cereus* (▪) single- and dual- (—) species biofilms treated with FA, SA, and FSA for 1 h. * No inactivation was found. Mean values ± standard deviation for at least three replicates are illustrated.

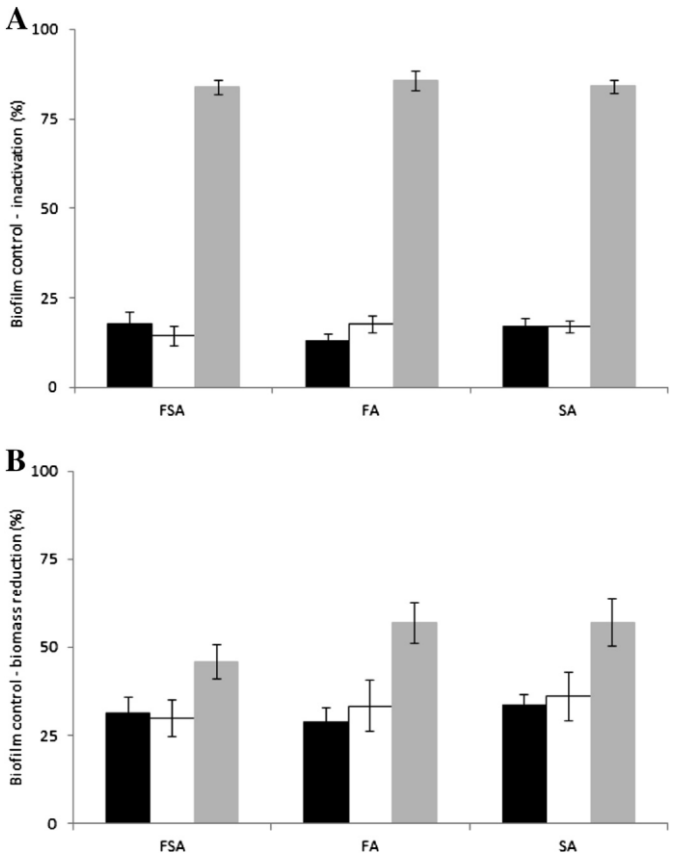

Fig. 5. Percentage of inactivation (A) and reduction (B) of *P.* fl*uorescens* (—) and *B. cereus* ( ) single- and dual- (—) species biofilms formed in the presence of FA, SA, and FSA for 24 h and subsequently exposed to the phenolic acids for 1 h. Mean values ± standard deviation for at least three replicates are illustrated.

Table 1 - Swimming and swarming motilities (mm) of *B. cereus* and *P. fluorescens* in the absence (control) and presence of FA and SA. Results are shown as mean ± standard deviation of at least three independent experiments. The 15 μL of bacterial culture produced an 8-mm (baseline) spot on the agar.

	B. cereus			*P. fluorescens*		
	24 h	48 h	72 h	24 h	48 h	72 h
Swimming						
Control	3.67 ± 0.6	7.33 ± 0.5	12.3 ± 1.5	13.3 ± 2.3	19.3 ± 2.5	24.7 ± 3.1
FA	1.00 ± 0.2	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.5
SA	1.00 ± 0.0	1.00 ± 0.3	1.00 ± 0.0	1.67 ± 1.1	2.00 ± 0.4	2.00 ± 0.0
Swarming						
Control	4.00 ± 0.0	5.00 ± 0.0	6.67 ± 0.6	3.00 ± 1.0	3.67 ± 1.2	4.67 ± 0.6
FA	3.00 ± 0.0	3.00 ± 0.8	3.00 ± 1.9	2.33 ± 0.5	3.33 ± 0.6	3.33 ± 1.1
SA	3.33 ± 0.6	3.33 ± 0.6	3.33 ± 1.6	3.33 ± 0.6	3.67 ± 0.8	3.33 ± 0.8

Table 2 - Surface tension parameters and hydrophobicity (DGiwi) of the untreated (control) and phenolic acids-treated cells. The means ± SD for at least three replicates are given.

	Phenolic acid	Concentrations (μg mL^{-1})	Surface tension parameters (mJ/m^2)				ΔG_{iwi} (mJ m^{-2})
			γ^{LW}	γ^{AB}	γ^+	γ^-	
B. cereus	Control		37.9 ± 2.5	15.9 ± 3.5	1.2 ± 0.4	47.4 ± 7.5	29.5 ± 6.0
	FA	100	39.0 ± 0.3	11.8 ± 1.0	0.7 ± 0.1	52.4 ± 1.7	32.2 ± 2.3
		500	39.1 ± 1.2	9.4 ± 2.2	0.4 ± 0.1	51.0 ± 6.0	34.1 ± 6.5
		1000	38.9 ± 0.9	12.6 ± 1.6	0.8 ± 0.2	50.5 ± 2.3	29.4 ± 3.2
	SA	100	41.2 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	75.8 ± 17.9	69.2 ± 16
		500	37.8 ± 0.3	11.9 ± 1.1	0.7 ± 0.2	56.2 ± 3.9	38.2 ± 6.6
		1000	38.7 ± 1.2	12.2 ± 2.0	0.8 ± 0.2	53.5 ± 4.5	33.3 ± 6.4
P. fluorescens	Control		18.0 ± 0.9	9.6 ± 1.0	9.6 ± 1.0	48.9 ± 2.3	14.8 ± 2.2
	FA	100	30.3 ± 0.6	15.5 ± 2.6	1.5 ± 0.4	44.0 ± 6.2	19.6 ± 3.6
		500	35.7 ± 1.6	15.1 ± 3.7	0.3 ± 0.3	54.6 ± 8.6	39.0 ± 14
		1000	35.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	68.0 ± 8.4	60.7 ± 10.7
	SA	100	37.0 ± 0.5	10.1 ± 3.0	0.7 ± 0.4	47.9 ± 8.0	22.3 ± 5.3
		500	35.9 ± 0.4	9.4 ± 3.2	0.0 ± 0.0	46.2 ± 8.5	41.2 ± 5.4
		1000	33.9 ± 2.0	3.0 ± 0.7	0.0 ± 0.0	64.2 ± 7.9	54.1 ± 11

Table 3 - Free energy of adhesion (ΔG^{Tot} mJ m^{-2}) between *B. cereus* and *P. fluorescens* untreated (control) and phenolic acids-treated cells to PS.

Phenolic acid	Concentration (μg mL^{-1})	*B. cereus*	*P. fluorescens*
Control		−1.80	13.51
FA	100	1.04	−1.25
	500	−0.69	2.42
	1000	0.08	8.91
SA	100	12.18	−1.70
	500	3.90	−5.74
	1000	2.13	7.21

Table 4 - Screening for the QSI effects and antimicrobial activity (AM) of FA and SA against C. violaceum CV12472. The halos are present as the total final halo minus the diameter of the paper disk (6 mm).

Phenolic acid	Concentration (μg mL $^{-1}$)	Pigment production[a]	AM[b] (mm)	QSI[c] (mm)
FA	100	+	Y (6.5)	N
	500	+	Y (8.5)	N
	1000	+	Y (9.6)	N
SA	100	+	Y (6.9)	N
	500	+/−	Y (9.9)	Y (4.9)
	1000	+/−	Y (13.3)	Y (7.9)
DMSO		+	N	N
LB		+	N	N

[a] (+) Indicates visualization of pigment; (−) indicates absence of purple pigment; (+/−) indicates partial visualization of purple pigment.

[b] Y − antimicrobial activity observed as a clear halo; N − absence of antimicrobial activity. Diameter of zone of inhibition in millimetre in parentheses.

[c] Y − QSI observed as a colorless halo of viable cells; N − absence of QSI. Diameter of QSI in millimetre in parentheses.