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Two cinnamic acid derivatives as inhibitors of *Pseudomonas aeruginosa las* and *pqs* quorum-sensing systems: Impact on biofilm formation and virulence factors

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ABSTRACT

Introduction: Quorum sensing (QS) is a bacterial communication mechanism that regulates gene expression, playing a crucial role in various physiological processes. Interfering with this signalling pathway is a promising strategy to control bacterial pathogenicity and virulence.
Objectives: This study evaluated the potential of two cinnamic acid derivatives, ferulic and sinapic acids, to inhibit the *las* and *pqs* systems in *Pseudomonas aeruginosa*. Their effects on biofilm architecture, virulence factor production and bacterial motility were also investigated.
Methods: Bioreporter strains and bioluminescence-based assays were used to evaluate the modulation of QS-activity by cinnamic acid-type phenolic acids. In addition, *in silico* docking analysis was performed to validate the binding interactions of the cinnamic acid derivatives with QS-receptors. The biofilm architecture was analysed by optical coherence tomography, and virulence factors production (pyoverdine, pyocyanin, total proteases, lipases, gelatinases and siderophores) and motility were measured by absorbance measurement and plate agar method.
Results: Ferulic and sinapic acids at 1000 μg mL^{-1} inhibited the *las* and *pqs* systems by 90 % and 80 %, respectively. The N-3-oxododecanoyl-homoserine lactone production was reduced by 70 % (6.25 μg mL^{-1}). *In silico* analysis demonstrated that cinnamic acid derivatives exhibited comparable interactions and higher docking scores than reference ligands and inhibitors. Biofilm thickness decreased from 96 μm to 11 μm, and virulence factors and swarming motility were significantly impaired. The comparable anti-QS activity of cinnamic acid derivatives suggests that the additional methoxy group in sinapic acid does not directly contribute to its anti-QS effect.
Conclusion: Ferulic and sinapic acids compromised the biofilm architecture and virulence of *P. aeruginosa* through QS inhibition.

1. Introduction

Quorum sensing (QS) is a cell-cell communication mechanism that enables bacteria to coordinate gene expression in response to population density [1]. This cross-talk allows bacteria to better adapt and survive in hostile environments or under external stress, such as exposure to antimicrobial agents [2]. Furthermore, QS helps bacteria to optimise the exploitation of resources and occupy specific niches in the environment,

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strengthening the collective defence of the bacterial population [3].

In QS systems of most Gram-negative bacteria, communication is mainly mediated by the production, release and recognition of signalling molecules known as *N*-acyl homoserine lactones (AHLs). As the bacterial density rises, the concentration of AHLs in the extracellular medium also increases [1]. When this concentration reaches a critical threshold, the AHLs bind to specific Lux-type receptors, which are cytoplasmic proteins of bacterial cells. The binding of AHLs to lux receptors triggers a signalling cascade that alters gene expression [4]. This process regulates various physiological activities, including bioluminescence, production of pigments and virulence factors, motility, biofilm formation, and plasmid transfer [3,5]. Thus, QS enables bacteria to adapt their survival mechanisms in response to unfavourable conditions [6].

Pseudomonas aeruginosa is a Gram-negative pathogenic bacterium commonly associated with chronic infections in immunocompromised patients (*e.g.*, patients with cystic fibrosis and burns) and hospital-acquired infections [7]. The ability of *P. aeruginosa* to form biofilms and exhibit high levels of antibiotic resistance makes this pathogen a challenge in the clinical setting. In particular, some strains of *P. aeruginosa* are resistant to carbapenems and third-generation cephalosporins, last-line antibiotics commonly used to treat multidrug-resistant (MDR) bacterial infections [8]. QS is fundamental for regulating *P. aeruginosa* pathogenicity and virulence. *P. aeruginosa* make use of several QS systems, including the *las* and *rhl* systems and the quinolone-based *pqs* system which use AHLs and 2-heptyl-4-quinolone (PQS) as autoinducers, respectively [9]. In addition to these systems, a fourth system has been identified, the *iqs* system, which uses the signalling molecule 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) [10].

The *las* system, which uses the autoinducer *N*-3-oxododecanoyl homoserine lactone (3-Oxo-C12-HSL), mainly controls the expression of genes related to elastase production and exotoxin synthesis. The *las* system is also essential for the regulation of genes involved in biofilm formation and differentiation [11]. On the other hand, the *pqs* system regulates the production of pyocyanin and is involved in the release of extracellular DNA (eDNA), an important component in biofilm formation [11,12]. The *pqs* system is also associated with iron acquisition, outer membrane vesicle biogenesis, and host immune response [13]. *P. aeruginosa* QS systems are organised hierarchically, with the *las* system at the top of the hierarchy, influencing the activity of the other systems [9]. However, studies have shown that inhibition of the *las* system alone may not be sufficient to stop the production of virulence factors, indicating that QS regulation should be understood as a circular interconnected model between the *las*, *rhl* and *pqs* systems [14,15].

QS plays a significant role in the formation, maintenance and spread of biofilms. Indeed, these structured bacterial communities are surrounded by an extracellular matrix that protects them from environmental stressors, including antimicrobial agents [16,17]. Biofilm-associated infections are notoriously difficult to treat, often leading to chronic disease and contributing significantly to morbidity and mortality [17]. By interrupting QS pathways, it is possible to intervene in essential bacterial processes, reduce biofilm formation and increase the efficacy of antibiotics [18]. In contrast to conventional antibiotics, which often exert selection pressure and contribute to the development of resistance, antipathogenic and antivirulence strategies aim to block bacterial communication without inhibiting growth or killing the bacteria [4].

QS inhibitors (QSI) can achieve this goal by preventing the production of autoinducers, degrading or inactivating these signalling molecules or preventing their recognition by bacterial receptors [13]. Since synthetic QSI, such as furanones, have low bioavailability and high toxicity, which impede their clinical application, natural QSIs have attracted increasing interest [18]. Cinnamic acid and its derivatives are natural compounds with diverse biological and pharmacological activities, including antimicrobial, antitumour and antioxidant properties [19]. In particular, compounds structurally related to cinnamic acid have been shown to inhibit N-decanoyl-homoserine lactone (C10-HSL) levels and reduce the production of key virulence factors in *Chromobacterium violaceum*, which harbours homologues of the LuxI/Luxor system. This emphasises the potential of cinnamic acid derivatives to disrupt bacterial communication in Gram-negative bacteria and fight bacterial infections [19,20]. Ferulic acid (4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid) and sinapic acid (3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid) are examples of phytochemicals that belong to the phenolic acid class, more specifically to the hydroxycinnamic acids, and occur naturally in plants, fruits, vegetables and cereals [21, 22]. These cinnamic acid-type phenolic acids have low toxicity and excellent biological and therapeutic properties [23].

In this study, ferulic and sinapic acids were investigated for the first time as potential inhibitors of both the *las* and *pqs* systems in *P. aeruginosa*. The anti-QS activity of these cinnamic acid derivatives was evaluated *in vitro* and supported by *in silico* molecular docking simulations. For experimental validation, (*Z*)-4-bromo-5-(bromomethylene)-2 (5 H)-furanone (FC30, 2.5 μg mL^{-1}) and furvina (12.5 μg mL^{-1}) were used as positive controls for the *las* system, while vanillin at 1000 μg mL^{-1} served as a positive control for the *pqs* system [13]. Their direct effects on the biofilm cells and architecture were investigated by analyse of the culturability and structure, using plate count agar method and optical coherence tomography (OCT), respectively. In addition, the impact of cinnamic acid derivatives on virulence factors production such as pyocyanin, pyoverdine, siderophores, proteases, gelatinases and lipases were studied. The interference of these compounds with bacterial motility was also investigated to acquire a comprehensive understanding of their role in QS-regulated processes.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *P. aeruginosa* strains used to study inhibitory activity in the *las* and *pqs* systems are shown in Fig. 1. For the *las* system, the wild-type *P. aeruginosa* PA14 (PA14-WT, burn wound isolate [24]) and the

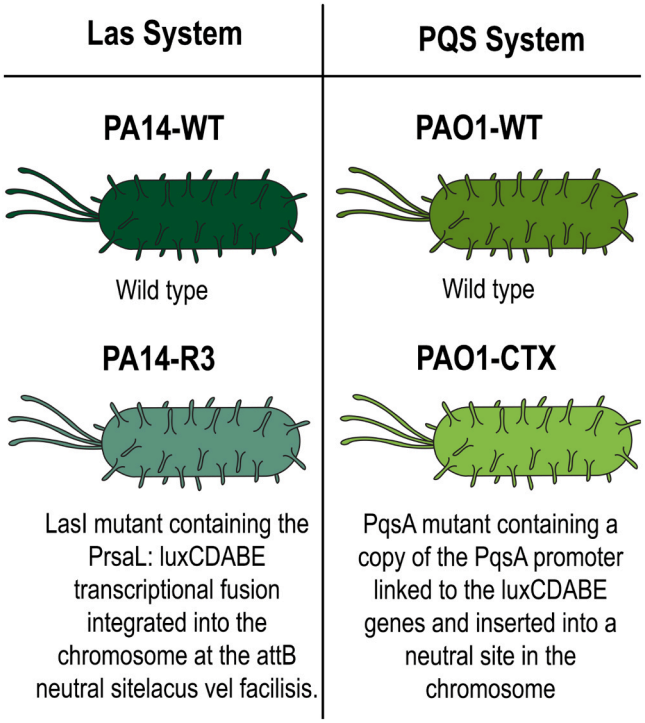


Fig. 1. Characterization of wild-type and genetically modified *P. aeruginosa* strains used in this study [25–27].

reporter strain PA14-R3 developed by Massai *et al.* [25] were used. To assess inhibition in the *pqs* system, the wild-type *P. aeruginosa* PAO1 (PAO1-WT, laboratory reference strain, originally isolated from a wound [24]) was used together with the biosensor strain *P. aeruginosa* PAO1 pqsA CTXluxHpqsA (PAO1-CTX) [26]. Professor Paul Williams kindly provided these strains from the Centre for Biomolecular Sciences at the University of Nottingham, UK. The genetically modified strains used for QS activity detection contained a *luxCDABE* operon. When this operon is activated by the interaction of autoinducers with the receptors, it triggers the production of luciferase (generated by the LuxAB proteins). This enzyme catalyses the oxidation of reduced flavin mononucleotide and a long-chain aldehyde, produced by the LuxCDE proteins, resulting in the emission of light. The light emission enables the assessment of QSI in these systems [13].

Bacterial strains were maintained at - 80 °C in cryovials containing 30 % (v.v^{-1}) glycerol (VWR, Belgium) and cultured in Luria-Bertani broth (LBB; Liofilchem, Roseto degli Abruzzi, Italy). For PAO1-CTX, the LB was supplemented with 20 μg mL^{-1} of gentamicin. Subcultures were performed on LB agar (LBA) and LBA with 20 μg mL^{-1} of gentamicin followed by incubation for 24 h at 37 °C. For the preparation of working cultures, the cells from the precultures were transferred to the LBB medium. The cultures were then incubated at 37 °C at 150 rpm (AGITORB 200, Aralab, Rio de Mouro, Portugal).

2.2. Cinnamic acid derivatives and solutions preparation

Ferulic acid and sinapic acid were obtained from Sigma-Aldrich (USA) and Tokyo Chemical Industry Co., Ltd (TCI, Tokyo, Japan), respectively. Fresh stock solutions of cinnamic acid derivatives were prepared in distilled water before use. When the minimum inhibitory concentration (MIC) was not determined, 1000 μg mL^{-1} was used as the maximum tested concentration (MTC). Vanillin, FC30, and furvina were used as positive controls in the various assays of this study [13]. Vanillin was prepared in 1 % dimethyl sulfoxide (DMSO), while FC30 and Furvina were prepared in distilled water.

2.3. Screening the antibacterial activity of selected cinnamic acid derivatives

The MIC of ferulic acid and sinapic acid were determined according to Borges *et at.* [28] and, following the Clinical and Laboratory Standards Institute (CLSI) guidelines [29]. After preparing working cultures of *P. aeruginosa,* the bacterial suspension was set to an optical density (OD) of 0.132 ± 0.02 at 600 nm (equivalent to approximately 10^8 CFU mL^{-1}).

Sterile 96-well polystyrene (PS) microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) were prepared with 180 μL of the bacterial cells and 20 μL of the ferulic and sinapic acids (ranging from 6.25 to 1000 μg mL^{-1}, 10 % v.v^{-1} of the well volume). The plates were incubated at 37 °C on an orbital shaker at 150 rpm for 24 h. Negative controls were performed with wells containing only the bacterial suspension without cinnamic acid derivatives. The absorbance at 600 nm was determined using a microplate reader (Synergy HT, Biotek, Winooski, VT, USA) before and after 24 h incubation. The MIC was identified as the lowest concentration of cinnamic acid derivatives that showed no difference in the OD values measured at two different time points (0 h and 24 h), indicating inhibition of cell growth. To determine the minimum bactericidal concentration (MBC), 10 μL of the samples from the wells with concentrations equal to or greater than the MIC were plated on LBA and incubated at 37 °C for 24 h. MBC was defined as the lowest concentration that resulted in complete inhibition of bacterial growth.

2.4. Evaluation of the las system in P. aeruginosa

To evaluate the effects of cinnamic acid derivatives on the *las* QS-system, a coculture assay was conducted with PA14-R3 and PA14-WT [25]. Following overnight bacterial growth on LBA, the colonies of both strains were collected and placed in LBB. The OD_{600} of the PA14-R3 and PA14-WT suspensions was set to 0.045 and 0.015 (equivalent to 10^7 CFU mL^{-1}), respectively. Subsequently, 96-well microtiter plates were prepared, one with a black opaque bottom (Thermo Fisher Scientific, USA) and the other with a clear bottom. Each well contained 180 μL of the coculture and 20 μL of selected cinnamic acid derivatives at concentrations ranging from 6.25 to 1000 μg mL^{-1}. A bacterial culture without cinnamic acid derivatives was used as a negative control. For the positive control, a cell suspension was treated with FC30 (2.5 μg mL^{-1}) and furvina (12.5 μg mL^{-1}, QSI previously investigated by our research group) [13,18,30]. After incubation (4 h, 37 °C, 150 rpm), LCPS and absorbance at 600 nm were recorded using a microplate reader. To normalise the luminescence values, the LCPS was divided by the absorbance at 600 nm. A detailed analysis of QS production and detection was performed, with the specific methods described in the Supplementary Information.

2.5. Evaluation of the pqs system in P. aeruginosa

The capability of cinnamic acid derivatives to disrupt the *pqs* system was evaluated as described by Leitão *et al.* [13]. After overnight growth in LBB, the OD_{600} was adjusted to 0.01, corresponding to 10^6 CFU mL^{-1}. Subsequently, 50 mL centrifuge tubes were filled with 9 mL of the previously prepared cell suspension together with 1 mL of the selected cinnamic acid derivatives (6.25–1000 μg mL^{-1}). Untreated cell suspension was used as a negative control, while vanillin (1000 μg mL^{-1}) was used as a positive control [13]. The centrifuge tubes were incubated (16–18 h) at 37 °C with constant agitation (150 rpm). The lid was kept partially open to facilitate air circulation, and the liquid volume was maintained at no more than 75 % of the total volume of the tube. To evaluate bacterial growth, 200 μL of the bacterial culture was transferred to a 96-well clear-bottom PS microtiter plate and the absorbance was measured at 600 nm. The remaining cell suspension was centrifuged at 3772 ×*g* at 4 °C for 15 min. The supernatant was collected and filtered through a filter with a pore size of 0.22 μm. Then, 100 μL of the supernatant and 100 μL of the PAO1-CTX cell suspension (adjusted to an OD_{600}= 0.01, corresponding to 10^6 CFU mL) were transferred to black and 96-well clear-bottom PS microtiter plates. After incubation (4 h, 37 °C, 150 rpm), the LCPS and absorbance at 600 nm were measured using a microplate reader. To normalise the luminescence values, the LCPS was divided by the absorbance at 600 nm.

2.6. Corroboration of the QS inhibition activity by in silico analysis

The Protein Databank (PDB) [31] and the Biofilms Structural Database (BSD) [32] were analysed to find protein structure information on the *las* and *pqs* systems of *P. aeruginosa*. In this study, four protein structures obtained by X-ray crystallography were analysed, focusing on the catalytic domains of the LasR, PqsA, PqsE and PqsR proteins (as shown in Fig. 2). The reference ligands used for these analyses were 3-Oxo-C12-HSL, 3UK (5′-O-[(S)-(2-aminobenzoyl)oxyphosphoryl]adenosine), KYX (N-{4-[(2,2-dimethylpropyl)carbamamido]phenyl}-1-H-indazole-7-carboxamide) and CZG (2-[(5-nitro-1H-benzimidazol-2-yl)sulfanyl]-N-(4-phenoxyphenyl)acetamide) (Fig. 2). These reference ligands were selected because they are known inhibitors of specific QS-related proteins, binding to the active sites that are crucial for the anti-QS activity.

Initial analysis of the PDB structures of the proteins was performed using PyMOL 2.3.0, with crystallographic waters removed and crystallographic ligands extracted and saved in separate files. Structures were aligned to guarantee consistency in coordinates and docking conditions. The Gasteiger charges and polar hydrogens were then assigned to the proteins using Gold (version 2022.2) [33]. A library with the ligands ferulic and sinapic acids was also created. All ligands were prepared for docking with OpenBabel and converted to mol2 format [34]. The Gold

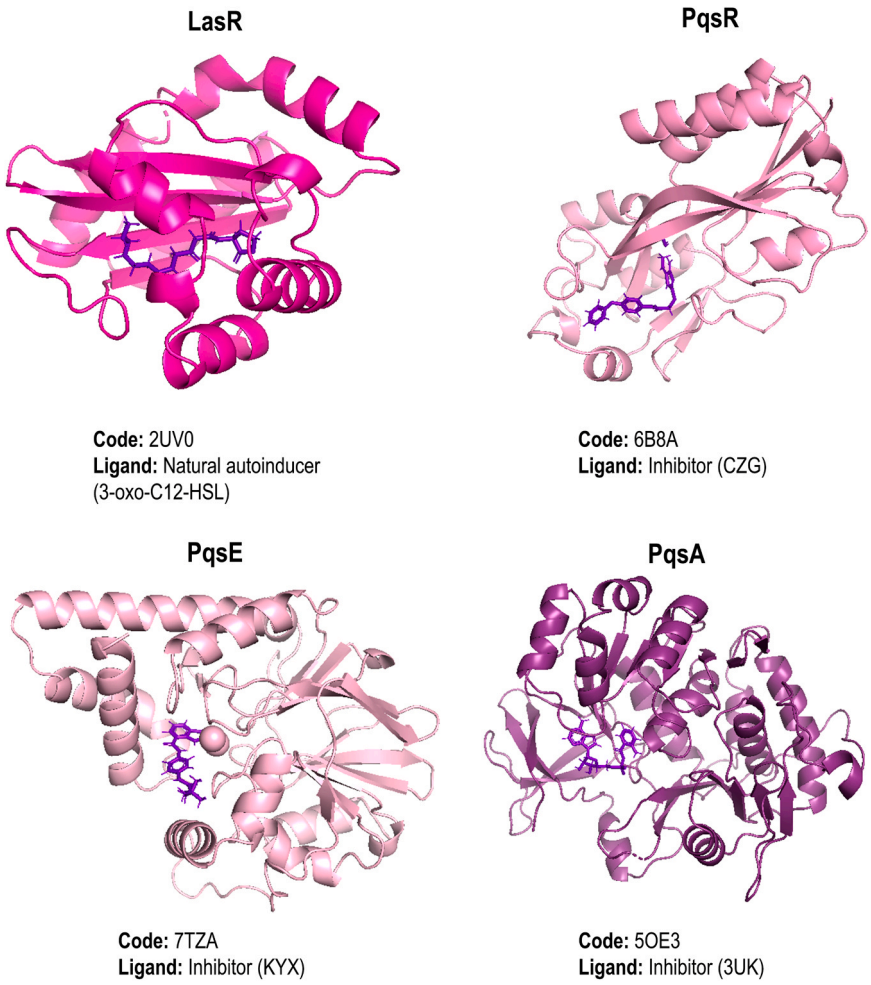


Fig. 2. X-ray structures of LasR, PqsR, PqsE, and PqsA and their ligands available in the PDB and BSD. The LasR structure, which was determined with a resolution of 1.80 Å, is complexed with the 3-Oxo-C12-HSL [37]. The PqsR structure, which was resolved at 2.65 Å, contains the ligand CZG [38]. The PqsE structure, with a resolution of 2.10 Å, shows the ligand KYX [39]. The PqsA structure, which was determined with a resolution of 1.43 Å, is complexed with the ligand 3UK [40]. All figures were created using PyMOL 2.3.0.

software was then used for molecular docking of the LasR, PqsA, PqsE and PqsR proteins using the Goldscore scoring function [35]. This function evaluates the potential binding poses of a ligand to a protein by predicting the binding affinity, with higher scores indicating more favourable interactions. GoldScore takes into account factors such as hydrogen bonds, van der Waals energy, metal interactions and torsional deformations to optimise the prediction of ligand binding sites [13].

To ensure the accuracy of the protocol (protocol validation), a redocking procedure was performed in which the crystallographic ligands were removed and then redocked. This process allows the evaluation of the ability of the docking software and protocol to reproduce the geometry and orientation of the crystallographic pose [33]. After fine-tuning the docking parameters, a library of cinnamic acid derivatives was docked into the binding sites of the LasR, PqsA, PqsE and PqsR proteins. Furthermore, interaction maps were generated using the Protein-Ligand Interaction Profiler (PLIP) to identify the key residues involved in the interaction between ferulic and sinapic acids and the target proteins [36].

2.7. Evaluation of the activity in the prevention biofilm set-up

2.7.1. Biofilm formation

Biofilm formation of PAO1-WT and PA14-WT was evaluated on white PS coupons (1 × 1 cm) using the method labelled by Pereira *et al.* [41]. In brief, the OD_{620} of overnight bacterial cultures (grown at 37 °C with shaking at 150 rpm in MHB) was adjusted to 0.04 ± 0.002. Then 900 μL of the bacterial culture was added to each well of a 24-well plate containing 100 μL of cinnamic acid derivatives at concentrations of 1000, 400, and 100 μg mL^{-1}. The 24-well plate was incubated for 24 h at 37 °C with shaking at 150 rpm. Cell suspension without cinnamic acid derivatives was used as a negative control. The substrate materials were cleaned and sterilised before being added to the wells for biofilm formation, according to the procedure described by Gomes *et al.* [42].

2.7.2. Thickness and roughness

Biofilms were characterised by thickness (μm) and roughness using optical coherence tomography (OCT) as labelled by Pereira *et al.* [43]. After incubation, the LB medium was removed, and each well was washed with a sterile NaCl solution. Then, 1 mL of NaCl solution (0.85 %) was added to each well containing a coupon before images were captured. The images were acquired with a Thorlabs Ganymede spectral domain OCT system (Thorlabs GmbH, Dachau, Germany) with a central wavelength of 930 nm and a field of view of 3.66 × 2.98 mm^3 in the X–Z plane (1024 × 1024 pixels). Each coupon was imaged in 2D over at least five fields of view to ensure accuracy and reproducibility. For automatic 2D-OCT image processing, the Biofilm Imaging and Structure Classification Automatic Processor (BISCAP) tool was used as described by Narciso *et al.* [44]. At least six 2D OCT images per condition were acquired for each independent experiment.

2.8. Evaluation of the inhibitory effect on the production of virulence factors of PA14-WT and PAO1-WT

The impact of cinnamic acid derivatives on the production of virulence factors was investigated using two sample types: culture supernatants from QS assays for the quantification of pyocyanin and pyoverdine, and biofilm cells for the quantification of virulence factors production according to the size of the produced halos. The culture supernatant was obtained following a similar protocol to the 3-Oxo-C12-HSL production experiments. PA14-WT or PAO1-WT was grown in the presence of selected cinnamic acid derivatives (ranging from 6.25 to 1000 μg mL^{-1}). After incubation (16 h, 37 °C, 150 rpm), the cells were harvested by centrifugation (15 min at 3772 ×*g*) and the resulting supernatant was saved. On the other hand, the biofilm cells from the biofilm formation assays, formed in the presence of selected cinnamic acid derivatives at 1000 μg mL^{-1}, were collected by scraping and suspended in microcentrifuge tubes. Aliquots of 10 μL of each condition were then plated on Petri dishes containing media specifically formulated to evaluate the production of virulence factors, including total proteases, lipases, gelatinases, and siderophores [45].

2.8.1. Pyocyanin

Pyocyanin was extracted from culture supernatants of PA14-WT and PAO1-WT in accordance with the method labelled by Borges *et al.* [18]. For that, 3 mL of chloroform was mixed with 5 mL of the culture supernatant. The lower blue layer was removed to a new tube and subjected to a second extraction with 1 mL of 0.2 M hydrochloric acid (HCl; Fisher Chemical, Merelbeke, Belgium). The resulting top pink layer was transferred to a microtiter plate and measured at 520 nm with a microplate reader. The pyocyanin concentration was calculated by multiplying the absorbance 520 nm values by 17.072.

2.8.2. Pyoverdine

Pyoverdine production was calculated according to the method designated by Borges *et al.* [30]. From the culture supernatant, 1 mL of each condition was collected. The concentration of pyoverdine in the culture supernatant of PA14-WT and PAO1-WT collected after exposure to cinnamic acid derivatives was determined by measuring the absorbance at 400 nm. To determine the relative pyoverdine production, the absorbance values at 400 nm were normalised by dividing them by the absorbance values at 600 nm for each sample.

2.8.3. Siderophore

Siderophore production was assessed using Mueller-Hinton agar (MHA) supplemented with 100 mL L^{-1} of Chrome Azurol S (CAS). After 48 h of incubation, the production of siderophores was indicated by the formation of orange-coloured halos around the colonies. The diameters of the zones of all virulence factors were measured in millimetres [46].

2.8.4. Extracellular enzymes

The production of total proteases was evaluated using PCA supplemented with 10 g L^{-1} (1 %) of skim milk powder (Merck, Darmstadt, Germany). After 72 h of incubation, the presence of light zones around the colonies indicated the production of total proteases [45].

The production of lipases was analysed on plates containing 2 g L^{-1} (0.20 %) $CaCl_2$ (Merck, Darmstadt, Germany), 15 g L^{-1} (1.50 %) agar (VWR, Belgium), 20 g L^{-1} (2 %) LBB, and 9.44 mL L^{-1} Tween 80 (VWR, Belgium). The production of lipases was determined after 48 h of incubation, recognisable by the formation of light-yellow halos around the colonies [18].

The production of gelatinases was determined using gelatine agar composed of 5 g L^{-1} (0.50 %) peptone (Merck, Darmstadt, Germany), 3 g L^{-1} (0.30 %) yeast extract (Oxoid, United Kingdom), 30 g L^{-1} (3 %) gelatin (Oxoid, United Kingdom), and 15 g L^{-1} (1.50 %) agar. Transparent halos around the colonies observed after 48 h of incubation indicated the production of gelatinases [46].

2.9. Motility

The effect of selected cinnamic acid derivatives on the inhibition of the motility of *P. aeruginosa* PAO1-WT and PA14-WT was investigated using biofilm cells from biofilm formation tests [47]. The biofilm cells were collected by scraping into microcentrifuge tubes and then prepared for the motility test. For each condition, 15 μL of the cell suspension was added to Petri dishes containing agar media formulated for the specific type of motility being assessed: swimming motility: 1 % (w/v) tryptone, 0.25 % (w/v) NaCl and 0.3 % (w/v) agar; swarming motility: 1 % (w/v) tryptone, 0.25 % (w/v) NaCl and 0.7 % (w/v) agar; twitching motility: 1 % (w/v) tryptone, 0.25 % (w/v) NaCl and 1.5 % (w/v) agar.

The plates were incubated at 37 ± 2 °C and the diameters of the motility halos were measured in millimetres. Halo measurements were performed after 24 h for swimming motility and after 72 h for swarming and twitching motility.

2.10. Statistical analysis

The statistical analysis was conducted with GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA). Significance was assessed using a one-way ANOVA for comparisons and multiple comparison tests with a confidence level of ≥ 95 % ($p < 0.05$ is considered statistically significant). All experiments were performed in duplicate, with a minimum of three replicates for each condition tested.

3. Results

3.1. Antibacterial activity

The antibacterial activity of ferulic and sinapic acids was evaluated for their ability to inhibit QS-*P. aeruginosa*. To determine the appropriate concentrations of the phenolic compounds in the QS assays, the MIC and MBC were measured against the different strains of *P. aeruginosa* used in this study. As shown in Fig. 3, the cinnamic acid derivatives exhibited MIC and MBC values higher than 1000 μg mL^{-1}. In the subsequent experiments, all concentrations up to the MTC were used to evaluate the possible effects of sub-inhibitory concentrations on the *las* and *pqs* QS systems of *P. aeruginosa*.

3.2. Impact of cinnamic acid derivatives on P. aeruginosa las system

Ferulic and sinapic acids showed the ability to inhibit the *las* QS system of *P. aeruginosa* in a dose-dependent manner (Fig. 4). At

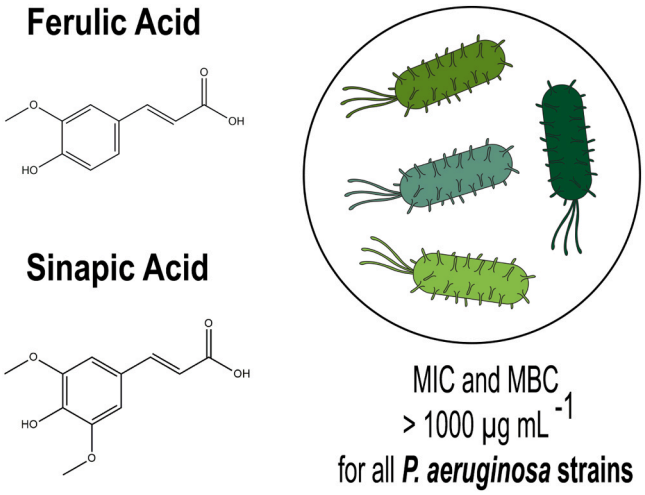


Fig. 3. Chemical structures of ferulic and sinapic acids and their MIC and MBC values for PA14-WT, PA14-R3, PAO1-WT, and PAO1-CTX strains.

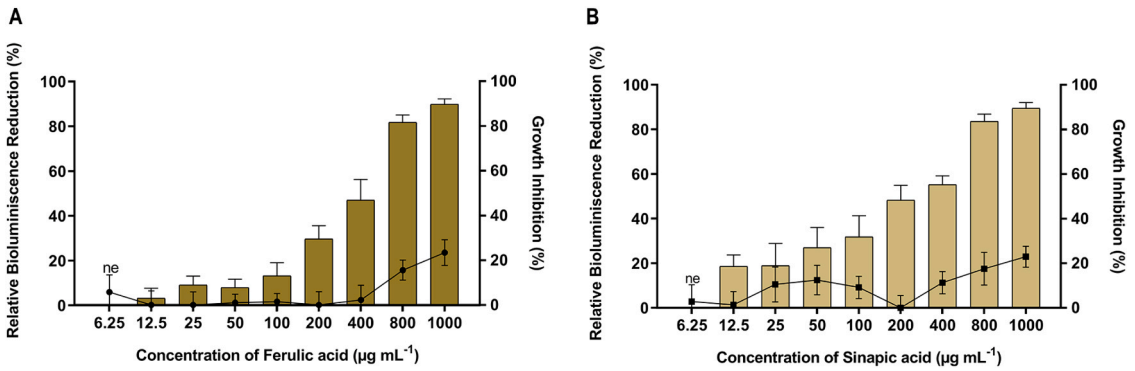


Fig. 4. Impact of ferulic acid (A) and sinapic acid (B) (6.25–1000 µg mL^{-1}) on the *las* system (shown by the bars) and inhibition of bacterial growth ($OD_{600\ nm}$, shown by the line), where "ne" means no effect. The relative bioluminescence was normalised to the cell density of the bacterial culture and expressed as a percentage compared to cells without cinnamic acid derivatives. Mean values ± standard deviations of three independent experiments are shown.

1000 µg mL^{-1}, both compounds led to a 90 % reduction in bioluminescence without significantly affecting bacterial growth (< 20 %, Fig. 4). Even at lower concentrations (400 µg mL^{-1}), ferulic and sinapic acids were able to reduce the bioluminescence by 50 % (Fig. 4A) and 55 % (Fig. 4B), respectively. Although furvina resulted in a 99 % reduction in bioluminescence at 12.5 µg mL^{-1} (Fig. S1), considerable bacterial growth inhibition was observed (57 %). In contrast, FC30, led to a 28 % reduction in bioluminescence at 2.5 µg mL^{-1}, with less impact on bacterial growth (Fig. S1).

3.3. Impact of the cinnamic acid derivatives on the production and response of autoinducers

The cinnamic acid derivatives were then screened to evaluate their capability to inhibit the production of 3-Oxo-C12-HSL *in P. aeruginosa* (Fig. 5). A significant inhibition at all concentrations tested (6.25–1000 µg mL^{-1}), resulting in a 62–72 % bioluminescence reduction, was detected for both cinnamic acid derivatives (Fig. 5A and B), without affecting cell growth. These phytochemicals also completely blocked the production of the autoinducer (Fig. 5C and D). When

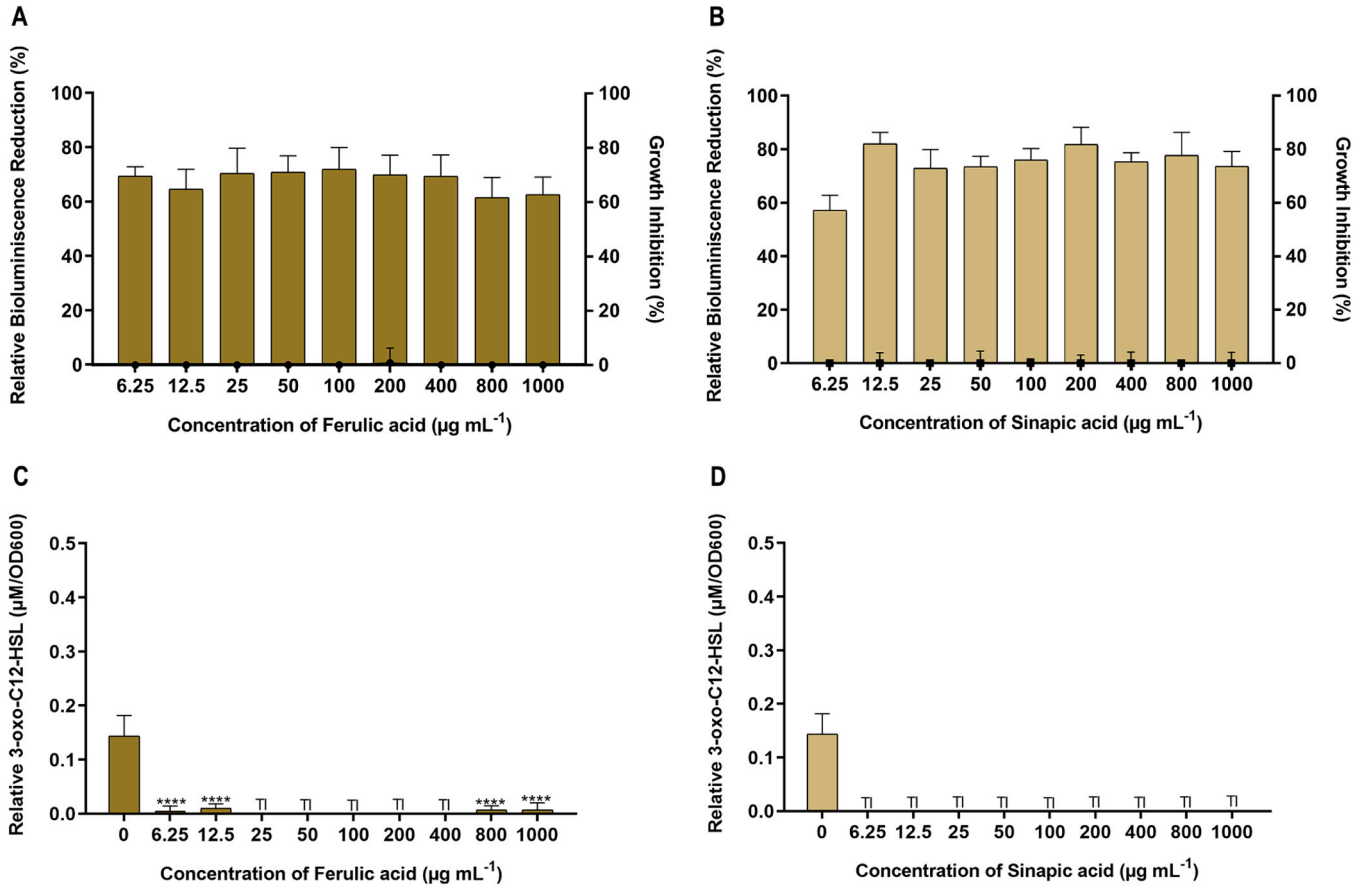


Fig. 5. Impact of ferulic acid (A and C) and sinapic acid (B and D) (6.25–1000 µg mL^{-1}) on 3-Oxo-C12-HSL production (shown by the bars) and growth inhibition ($OD_{600\ nm}$, shown by the line). Relative bioluminescence and 3-Oxo-C12-HSL levels were normalised to the cell density of the bacterial culture and expressed as a percentage compared to cells without cinnamic acid derivatives. Bars with '* ' are statistically different from the cells without cinnamic acid derivatives at a confidence level of more than 95 % ($p < 0.05$). Mean values ± standard deviations of three independent experiments are shown, where "TI" means total inhibition.

incubated with the PA14-WT strain, the cinnamic acid derivatives inhibited the production of 3-Oxo-C12-HSL at all concentrations tested. Even at the lowest concentration (6.25 μg mL^{-1}), a significant reduction in bioluminescence and a complete inhibition of autoinducer production was observed.

In contrast, furvina and FC30 led to an inhibition of bioluminescence of 59 % and 63 %, respectively (Fig. S2). Although the initial screen of the global effect on the QS system showed that furvina strongly impaired cell growth, this effect was not observed in this assay. Furvina showed a growth inhibition of less than 20 % (Fig. S2). On the other hand, the positive control compounds did not achieve complete inhibition of 3-Oxo-C12-HSL production when incubated with the PA14-WT strain, resulting in a reduction of approximately 48 % (Fig. S2).

3.4. Impact of cinnamic acid derivatives on the response of autoinducers

The selected cinnamic acid derivatives were found to inhibit bioluminescence emission by the PA14-R3 strain in the presence of exogenously added 3-Oxo-C12-HSL (interfering with autoinducer detection). Ferulic acid showed the highest inhibition of bioluminescence when tested at 400 μg mL^{-1} (31 %), while sinapic acid attained the maximum bioluminescence inhibition at 100 μg mL^{-1} (25 %) (Fig. 6). Despite the slight reduction, the cinnamic acid derivatives showed a better inhibitory effect than the positive controls, in which furvina and FC30 achieved a reduction of 19 % and 24 %, respectively (Fig. S3).

3.5. Impact of cinnamic acid derivatives on the pqs system

The cinnamic acid derivatives were also assessed for their ability to inhibit the *pqs* system. These compounds showed the ability to modify the *pqs* system in a dose-dependent manner (Fig. 7). Ferulic acid reached a maximum reduction of 81 % when applied at MTC. A significant reduction was observed when concentrations of 200 μg mL^{-1} or more were used (Fig. 7A). Similarly, sinapic acid leads to an 83 % reduction in bioluminescence when applied at MTC. Only the concentrations of 800 μg mL^{-1} and 1000 μg mL^{-1} of sinapic acid showed a reduction of above 50 % in bioluminescence (Fig. 7B). In comparison, vanillin at 1000 μg mL^{-1} achieved an 80 % bioluminescence reduction (Fig. S4). Remarkably, none of the cinnamic acid derivatives affected the growth of the PAO1-WT strain.

3.6. Corroboration of the QS inhibition activity of cinnamic acid derivatives by in silico analysis

Computer simulations were conducted to further validate the inhibitory effect of cinnamic acid derivatives on QS systems. The Goldscore values (obtained by molecular docking) for the interactions between the cinnamic acid derivatives and the proteins LasR, PqsR, PqsE and PqsA were calculated and normalised *per* heavy atom (Table 1). The results showed that, compared to the reference ligands (3UK, KYX, and CZG), the cinnamic acid derivatives achieved better docking score for each PQS protein studied. In particular, ferulic and sinapic acids showed strong interactions with PqsE, reaching normalized Goldscore values of 5.66 and 4.63, respectively. In contrast, the reference inhibitor KYX reached a value of 3.57. Similar effects were observed for the PqsA and PqsR proteins (Table 1). As for the LasR protein, the natural autoinducer (3-Oxo-C12-HSL) showed a normalized docking score of 4.49, which was similar to that of ferulic acid (4.46) and slightly higher than that of sinapic acid (3.27). Thus, the cinnamic acid derivatives exhibited docking scores comparable to those of the natural autoinducer 3-Oxo-C12-HSL and superior to the reference inhibitors, confirming their strong affinity for these proteins.

The interaction of cinnamic acid derivatives within the protein pockets was further analysed with Pymol 2.3.0. It was found that ferulic and sinapic acids tend to bind to the same pocket and occupy a similar pose as the reference inhibitors and the natural autoinducer (Fig. 8, S5, S6, and S7). In the case of the PqsR protein, the presence of two subunits and the small size of the cinnamic acid derivatives prevent them from occupying both subunits of the pocket, unlike the reference inhibitor 3UK (Fig. S6). A similar effect was observed with protein PqsA, which has a U-shaped pocket instead of two subunits (Fig. S7). Nevertheless, the cinnamic acid derivatives were found within the pocket and interacted with the same region as the reference ligands.

To assess these interactions in more detail, the PLIP tool was used to identify potential interactions of cinnamic acid derivatives and reference ligands with the key residues of each protein (Fig. 8, S5, S6, and S7). Cinnamic acid derivatives were found to bind to the same residues as the inhibitor KYX, forming two π-π stacking interactions with residues TYR72 and HIS71 (Fig. 8). In addition, KYX forms two hydrogen bonds, one with each of the above-mentioned residues. In the case of cinnamic acid derivatives, only sinapic acid forms a hydrogen bond with residue HIS71. It has also been shown that both cinnamic acid derivatives and KYX show direct coordination with the Fe ions (Fig. 8). A special feature of cinnamic acid derivatives is their ability to form salt bridges with the protein PqsE. Specifically, ferulic acid forms four salt bridges with the residues HIS71, HIS74, HIS159 and HIS221. Sinapic acid, on the other hand, forms three salt bridges with residues HIS74, HIS159 and HIS221 (Fig. 8). The formation of these salt bridges could explain the better Goldscore values obtained for the cinnamic acid derivatives compared to the reference inhibitor.

As far as the LasR protein is concerned, the natural autoinducer forms hydrogen bonds with the residues TRP60, TYR64, ASP73 and THR75. Ferulic acid also forms hydrogen bonds with TRP60 and TYR64 but also interacts with TYR93. Sinapic acid forms hydrogen bonds with TYR64, THR115 and LEU125 (Fig. S5). Like its interactions with the PqsE protein, ferulic acid also forms π-π stacking interactions with

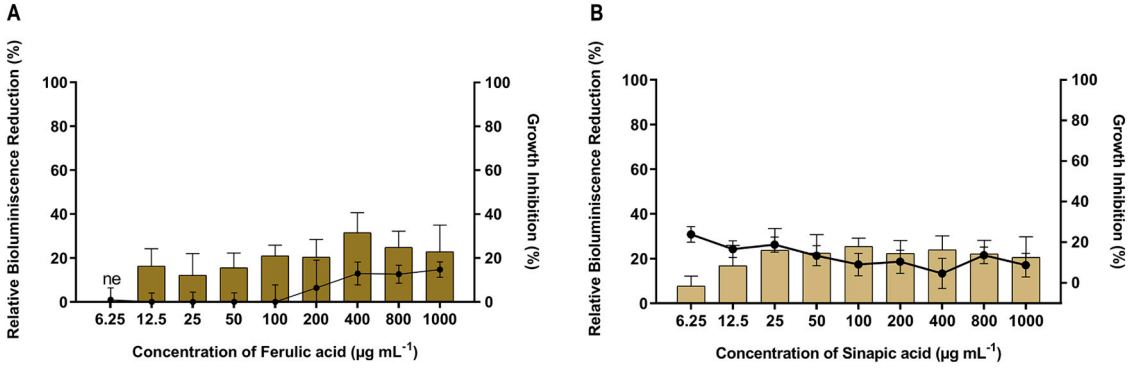


Fig. 6. Impact of ferulic acid (A) and sinapic acid (B) (6.25–1000 μg mL^{-1}) on the detection of 3-Oxo-C12-HSL (bars) and growth inhibition ($OD_{600\,nm}$, line) of *P. aeruginosa* PA14-R3. Relative bioluminescence was normalised to the cell density of the bacterial culture and expressed as a percentage compared to cells without cinnamic acid derivatives, where "ne" means no effect. Mean values ± standard deviations of three independent experiments are shown.

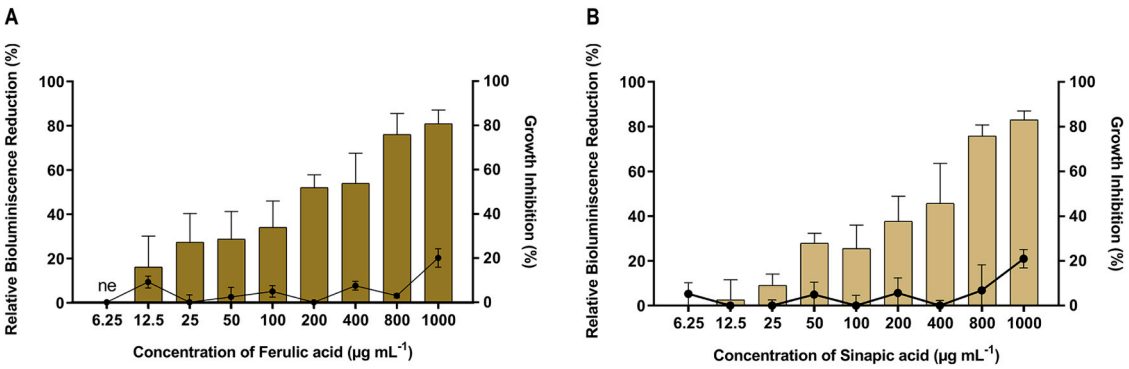


Fig. 7. Impact of ferulic acid (A) and sinapic acid (B) (6.25–1000 µg mL^{-1}) on the *pqs* system of *P. aeruginosa* PAO1 (bars) and growth inhibition ($OD_{600\ nm}$, line), where "ne" means no effect. Relative bioluminescence was normalised to the cell density of the bacterial culture and expressed as a percentage compared to cells without cinnamic acid derivatives. Mean values ± standard deviations of three independent experiments are shown.

residues TRP88 and PHE101 as well as a salt bridge with ARG61. Sinapic acid, on the other hand, does not form salt bridges or π-π stacking interactions, similar to the natural autoinducer (Fig. S5).

Only hydrogen bonds were observed for the PqsR protein in the compounds analysed (Fig. S6). Sinapic acid forms the most hydrogen bonds interacting with residues GLN194, LEU197 and LEU208. Ferulic acid forms a single hydrogen bond with residue LEU197. In contrast, the reference inhibitor forms hydrogen bonds with different residues, in particular ARG209 and TYR258 (Fig. S6).

Finally, and as for the PqsA protein, the reference inhibitor (3UK) is able to form multiple hydrogen bonds with residues GLY279, ASP299, ASP382 (x2) and ARG397 (Fig. S7). It also forms a π-π stacking interaction with residue HIS308. While the cinnamic acid derivatives do not exhibit π-π stacking, they form a salt bridge with HIS394. Common hydrogen bonds are observed between 3UK and the selected cinnamic acid derivatives with residues ASP299, GLY279 and ASP382. In addition, new hydrogen bonds are formed by sinapic acid with the residue THR323 (Fig. S7).

3.7. Impact of the cinnamic acid derivatives in the biofilm formation

The effects of ferulic and sinapic acids on *P. aeruginosa* biofilm formation were investigated using PA14-WT and PAO1-WT strains. The cinnamic acid derivatives under study had no effect on the culturability of biofilm cells in either *P. aeruginosa* strains, as no significant reduction in the log (CFU/cm^2) was observed compared to the untreated control (Fig. S8). However, clear structural changes were observed in both strains. The 2D-OCT biofilm images depicted in Fig. 9 and S9 show the presence of biofilm morphologic changes on the polystyrene coupon surfaces of PA14-WT and PAO1-WT strains.

OCT analysis also revealed significant changes in biofilm thickness and roughness for both *P. aeruginosa* strains (Fig. 10). The untreated biofilms of PAO1-WT had an average thickness of approximately 96 µm, while the biofilms formed in the presence of ferulic and sinapic acids showed a significant reduction, measuring 12 µm and 11 µm, respectively – resulting in a nine-fold decrease ($p < 0.05$). A similar effect was observed with PA14-WT biofilms: the untreated biofilms had a thickness of 25 µm, which was reduced to 9 µm with ferulic acid and 14 µm with sinapic acid ($p < 0.05$). The cinnamic acid derivatives also caused significant changes in terms of roughness. Biofilms treated with ferulic acid exhibited roughness values of 4 for PA14-WT and 6 for PAO1-WT, while biofilms treated with sinapic acid exhibited roughness values of 12 and 7, respectively ($p < 0.05$). Untreated biofilms of PA14-WT and PAO1-WT showed higher roughness values of 22 and 28, respectively ($p < 0.05$).

3.8. Impact of the cinnamic acid derivatives on the virulence factors production

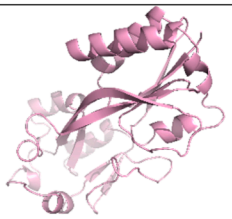
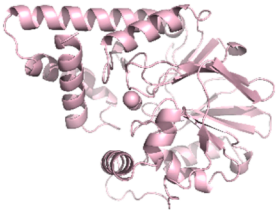
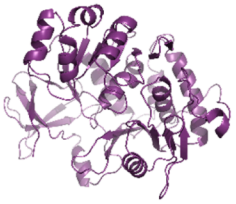
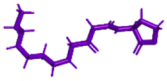
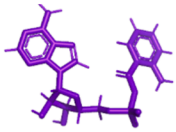
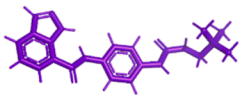
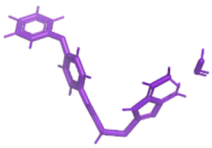
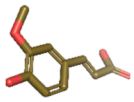
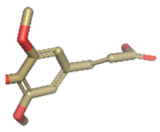
Cinnamic acid derivatives cause a slight reduction in pyocyanin production in the PA14-WT and PAO1-WT strains. In particular, in PA14-WT, the selected cinnamic acid derivatives at 1000 µg mL^{-1} led to a reduction of the production between 25 % and 32 % (Fig. 11A and B). As for the positive controls, furvina showed a similar inhibition and caused a reduction of 29 %. FC-30 achieved the most significant reduction in pyocyanin production by PA14-WT with a decrease of 40 % (Fig. S10). None of the tested compounds affected bacterial growth. In strain PAO1-WT, sinapic acid achieved the strongest reduction, inhibiting pyocyanin production by 50 % (at 1000 µg mL^{-1}). Ferulic acid had a more modest effect and led to a 35 % reduction in pyocyanin production at the same concentration (Fig. 11C and D).

The results regarding the interference of the cinnamic acid derivatives in the production of pyoverdine by PA14-WT and PAO1-WT cells are shown in Fig. 12. Only ferulic acid was able to reduce pyoverdine production by 23 % and 26 % when the higher concentrations (800 and 1000 µg mL^{-1}, respectively) were used. Sinapic acid (Fig. 12), furvina and FC30 (Fig. S11) did not affect pyoverdine production. Also, in strain PAO1-WT, none of the cinnamic acid derivatives were able to reduce pyoverdine production.

The effect of ferulic and sinapic acids on gelatinases, lipases, total proteases, and siderophores production was also evaluated for PA14-WT and PAO1-WT strains. In PA14-WT (Fig. 13), the treatment with ferulic and sinapic acids resulted in a decrease in gelatinase production, with halos decreasing from 39 mm in untreated cells to 36 mm and 37 mm, respectively ($p < 0.05$). A similar decrease was observed in lipase production, with halos decreasing from 20 mm to 18 mm after treatment with selected cinnamic acid derivatives ($p < 0.05$). Protease production also reduced significantly, with halos decreasing from 46 mm to 41 mm for ferulic acid and 42 mm for sinapic acid ($p < 0.05$). The production of siderophores was similarly reduced, with halos falling from 43 mm to 40 mm for ferulic acid and sinapic acid ($p < 0.05$). On the other hand, PAO1-WT showed a different response (Fig. S12). Treatment with ferulic and sinapic acids led to an increase in gelatinase production, with halos increasing from 33 mm in untreated cells to 37 and 38 mm for ferulic and sinapic acids, respectively ($p < 0.05$). In contrast, lipase production was significantly decreased in PAO1-WT, with halos decreasing from 22 mm to 18 mm for both cinnamic acid derivatives treatment ($p < 0.05$). No significant change was observed in protease production ($p > 0.05$), with halos remaining at approximately 42 mm. However, when PAO1-WT cells were treated with sinapic acid, a reduction to 37 mm was achieved. On the other hand, siderophore production was significantly reduced for PAO1-WT, with halos decreasing from 39 mm to 36 mm with ferulic acid and 33 mm with sinapic acid ($p < 0.05$).

Importantly, no significant differences in bacterial growth halos

Table 1
Cinnamic acid derivatives docking scores for LasR, PqsR, PqsE and PqsA proteins and representations of the different proteins and reference ligands used in this study. The result was normalised by heavy atoms.

	LasR	PqsR	PqsE	PqsA
				
3-Oxo-C12-HSL 	4.49	-	-	-
3UK 	-	2.84	-	-
KYX 	-	-	3.57	-
CZG 	-	-	-	3.46
Ferulic Acid 	4.46	3.48	5.66	3.90
Sinapic Acid 	3.27	3.14	4.63	3.65

were found in either strain (Fig. S13 and S14), suggesting that the observed effects on virulence factor production are not related to changes in bacterial growth.

3.9. Impact of the cinnamic acid derivatives on motility

The effect of ferulic and sinapic acids on motility was also investigated for the two strains PA14-WT and PAO1-WT (Fig. 14 and S15). No significant changes in twitching motility were observed in either strains when treated with ferulic or sinapic acids, indicating that these compounds do not affect this type of motility ($p < 0.05$). For swimming motility, only sinapic acid led to a significant reduction in the halo diameter from 16 mm to 14 mm in PA14-WT strain ($p < 0.05$, Fig. 14). For swarming motility, sinapic acid treatment resulted in a significant decrease in PA14-WT strain (21–20 mm). In contrast, both ferulic and sinapic acids caused a significant decrease in swarming motility in PAO1-WT, with halos decreasing from 20 mm to 18 mm and 17 mm, respectively ($p < 0.05$, Fig. S15).

4. Discussion

Although the direct inhibition of QS by ferulic and sinapic acids in *P. aeruginosa* has not yet been investigated, there is evidence of their potential as QS inhibitors [48,49]. Various studies indicate that these

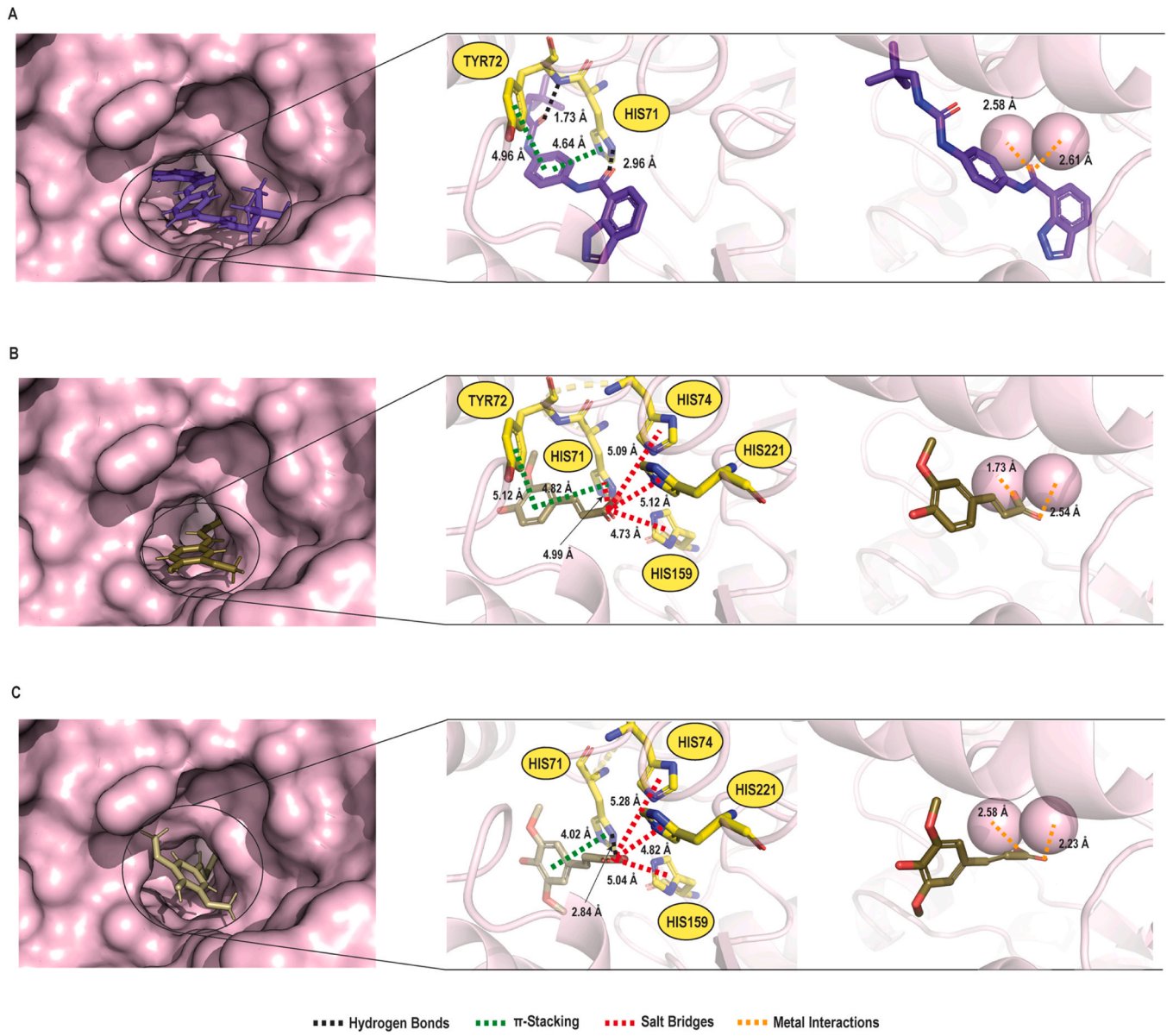


Fig. 8. Position and interaction map of the reference ligand of PqsE (KYX) in the PqsE pocket, highlighting important residues and metal ions (A). Putative positions of ferulic acid (B) and sinapic acid (C) and their interaction maps with key residues and metal ions in the PqsE pocket. The surface representation of 7TZA, showing the pocket shape and residues, was created using Pymol 2.3.0. The interactions between ligands and residues were determined using the PLIP [36].

type of hydroxycinnamic acids, significantly impair QS-regulated processes such as cell adhesion, motility and biofilm formation in bacterial pathogens such as *Staphylococcus aureus*, including methicillin-resistant (MRSA), *Escherichia coli, P. aeruginosa, Listeria monocytogenes, Bacillus cereus* and *Pseudomonas fluorescens* [22,28,48–50]. For example, Lemos *et al.* [48] found that ferulic acid (at 100 μg mL^{-1}) reduced the swimming motility of *B. cereus* clinical isolate and *P. fluorescens* ATCC 13525. In addition, Ugurlu and colleagues [49] reported that plant-derived hydroxycinnamic acids, including caffeic, cinnamic, and ferulic acids, reduced biofilm formation in *P. aeruginosa* clinical isolate by approximately 45 % (biomass reduction).

These results suggest that the QS-inhibitory activity of ferulic and sinapic acids may be closely related to their structural features, particularly the presence of hydroxyl and methoxy substituents in the aromatic ring. Indeed, the number and position of these functional groups have been shown to significantly influence their biological activities, with the hydroxyl groups playing a crucial role in disrupting QS, possibly through hydrogen bond formation [51]. For example, Li *et al.* [52] investigated the influence of hydroxyl groups on QS modulation in *P. aeruginosa* by analysing three cyclic dipeptides, Cyclo(L-Pro-L-Tyr), Cyclo(L-Hyp-L-Tyr) and Cyclo(L-Pro-L-Phe). The authors found that Cyclo(L-Pro-L-Tyr) and Cyclo(L-Hyp-L-Tyr), both of which contain hydroxyl groups, significantly inhibited QS by targeting genes in the *las* and *rhl* systems. In contrast, cyclo(L-Pro-L-Phe), which does not contain hydroxyl groups, mainly acted on the *rhl* and *pqs* system by down-regulating the expression of *rhlI* and *pqsR*. These results could explain the strong anti-QS activity of the positive controls FC-30 and Furvina used in this study, which lack hydroxyl groups but effectively inhibit QS-regulated processes. However, the exact role of the functional groups of ferulic and sinapic acids in QS modulation remains unclear. In this context, our study provides, for the first time, direct evidence that ferulic and sinapic acids disrupt the *las* and *pqs* QS systems of *P. aeruginosa*. While previous studies [19,20,22,48,51,53] have only shown the inhibition of processes regulated by QS, such as virulence factor production, motility and biofilm formation, our results establish a mechanistic link by showing the direct effect of ferulic and sinapic acids on the *las* and *pqs*

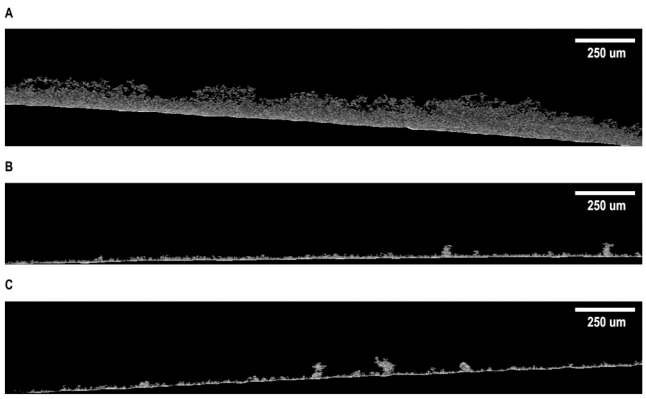


Fig. 9. Representative images of the structure of 24 h old *P. aeruginosa* PAO1-WT biofilms formed in polystyrene coupons without the presence of cinnamic acid derivatives (A), in the presence of ferulic acid (B) and sinapic acid (C). The visualisation field was 3.66 × 2.98 mm3 in the X–Z section, which corresponds to 1024 pixels × 1024 pixels.

regulatory pathways.

In the first phase, the antibacterial tests showed that ferulic and sinapic acids did not significantly inhibit or kill the bacteria at the concentrations tested, so bacterial growth remained largely unaffected. As QS inhibitors should not interfere with growth by more than 20 % to minimise non-specific effects of growth interference on the QS response, the data can be looked as relevant [18]. The results are in accordance with Ugurlu *et al.* [49], who found that ferulic acid did not affect the cellular growth of a *P. aeruginosa* clinical isolate when tested at approximately 780 μg mL^{-1}. Similarly, Song and colleagues reported an MIC of 1000 μg mL^{-1} for ferulic acid against *P. aeruginosa* ATCC 27853 [21]. Conversely, Chimi *et al.* [54] reported a much lower MIC of 27 μg mL^{-1} for sinapic acid against 37 *P. aeruginosa* clinical isolates from infected wounds. On our experimental conditions sinapic acid has an MIC higher than 1000 μg mL^{-1} (Fig. 3). This significant discrepancy may be attributed to differences in the specific strains of *P. aeruginosa* used in the studies. Furthermore, there is a lack of other studies comparing or confirming the MIC of sinapic acid in *P. aeruginosa*, which supports the need for further research.

Following the evaluation of the antimicrobial activity, the QS inhibitory potential of ferulic and sinapic acids on the *las* system of *P. aeruginosa* was investigated at subinhibitory concentrations. The *las* system consists of the LasR regulator and LasI which is responsible for the synthesis of the 3-Oxo-C12-HSL [55]. When this autoinducer reaches a critical concentration, it binds to the LasR and leads to the activation of QS target genes. In strain PA14-R3, the presence of 3-Oxo-C12-HSL, produced by the PA14-WT strain or artificially added, leads to the emission of bioluminescence, which is used as an indicator of the activity of the *las* system and the efficacy of potential inhibitors [30]. An effective QS inhibitor should reduce bioluminescence by at least 50 % without affecting significantly the cell growth (ideally below 20 % as mentioned previously) [13]. The anti-QS activity of ferulic and sinapic acids is comparable to that of benzaldehydes (4-hydroxybenzaldehyde, vanillin and syringaldehyde) previously studied recently by our research

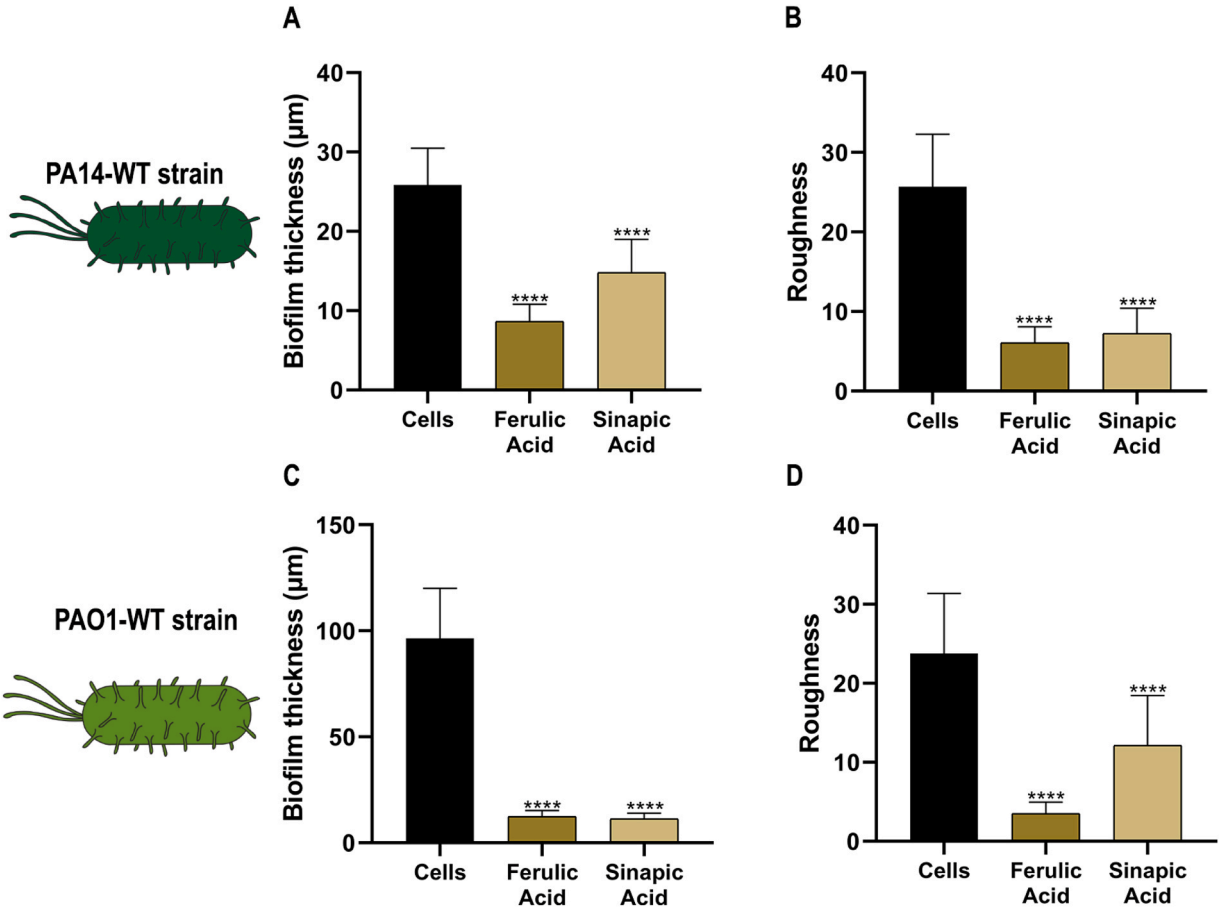


Fig. 10. Influence of ferulic and sinapic acids (at 1000 μg mL^{-1}) on biofilm thickness (**A** and **C**) and roughness (**C** and **D**) in *P. aeruginosa* strains. Panels A and B represent the PA14-WT strain, while C and D represent the PAO1-WT strain. Biofilm thickness and roughness were measured in the presence and absence of cinnamic acid derivatives. Mean values ± standard deviations of three independent experiments are shown. Bars with '* ' indicate statistically significant differences from the control group (untreated cells) at a confidence level of 95 % ($p < 0.05$).

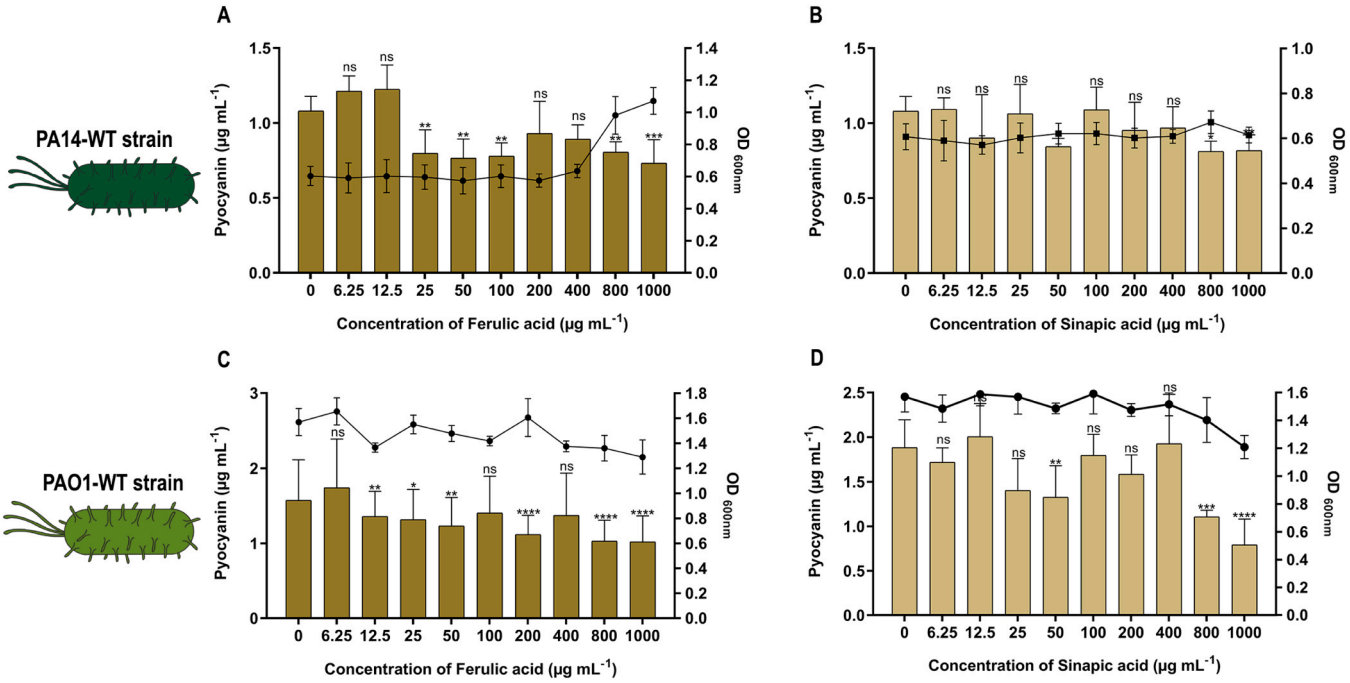


Fig. 11. Influence of ferulic acid (A and C) and sinapic acid (B and D) (6.25–1000 µg mL^{-1}) on pyocyanin production (bars) and growth inhibition ($OD_{600\ nm}$, line). Pyocyanin content was measured in cultures of *P. aeruginosa* PA14-WT strain (A and B) and *P. aeruginosa* PAO1-WT (**C** and **D**) in cell-free supernatants ($OD_{520\ nm}$). Mean values ± standard deviations of three independent experiments are shown. Bars with '* ' are statistically different from the control group (cells without cinnamic acid derivatives) at a confidence level of more than 95 % ($p < 0.05$).

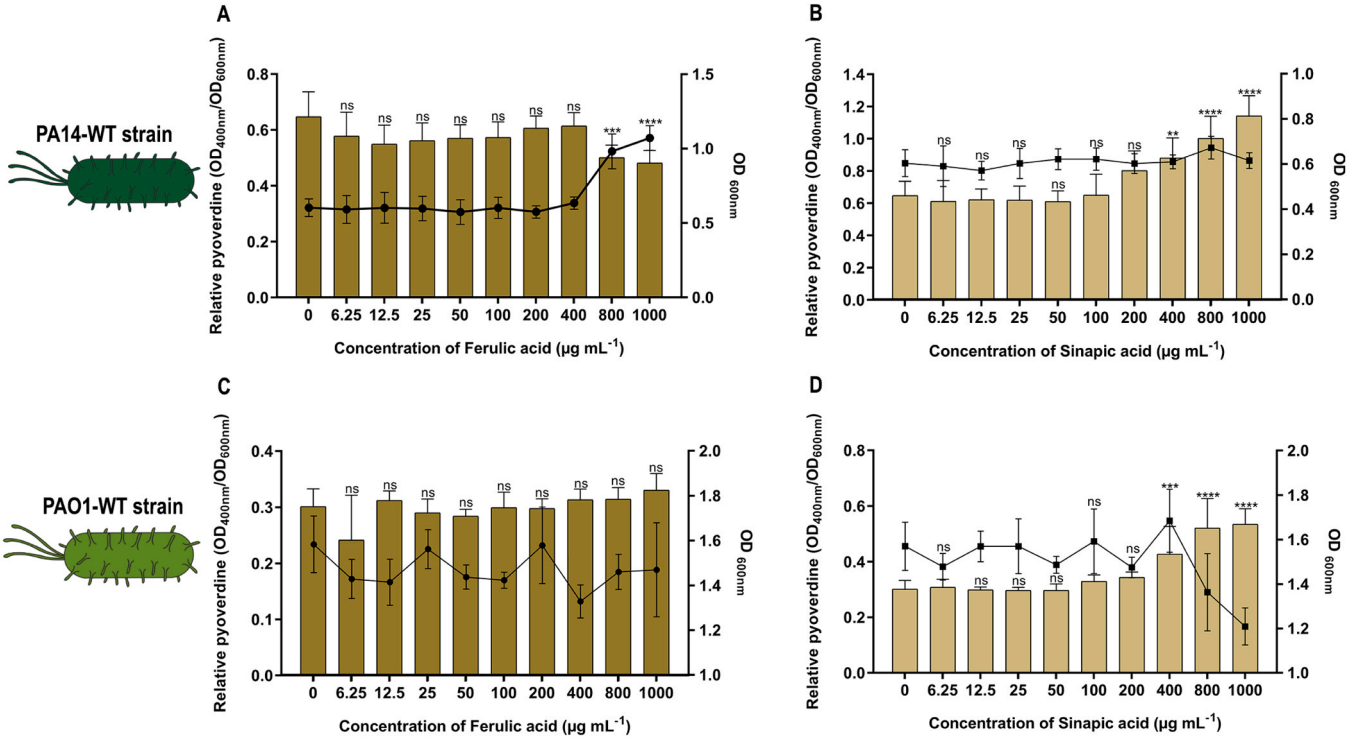


Fig. 12. Influence of ferulic acid (A and C) and sinapic acid (B and D) (6.25–1000 µg mL^{-1}) on pyoverdine production (bars) and growth inhibition ($OD_{600\ nm}$, line). Pyoverdine content was measured in cultures of *P. aeruginosa* PA14-WT strain (A and B) and *P. aeruginosa* PAO1-WT (C and D) in cell-free supernatants (OD_{400} values). The total amount of pyoverdine was calculated and normalized per cell density (OD_{600} values) and expressed as relative pyoverdine production. Mean values ± standard deviations of three independent experiments are shown. Bars with '* ' are statistically different from the control group (cells without cinnamic acid derivatives) at a confidence level of more than 95 % ($p < 0.05$).

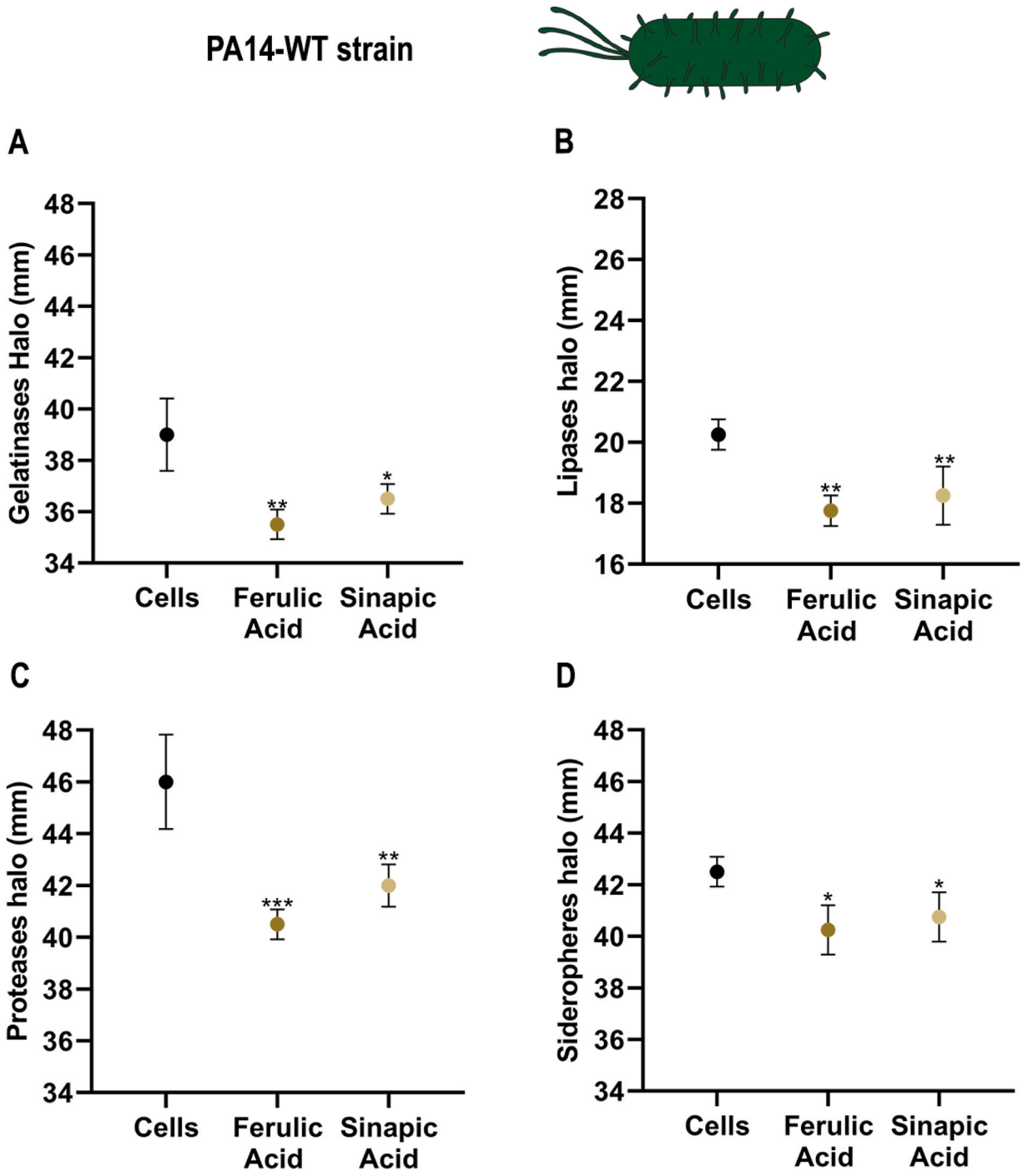


Fig. 13. The inhibitory activity of ferulic and sinapic acids (at 1000 μg mL^{-1}) on the production of virulence factors by *P. aeruginosa* PA14-WT biofilm-derived cells was evaluated by measuring the total halo diameter (in mm) on agar plates. The halo includes both the virulence factor and bacterial growth. Virulence factors tested include gelatinases(A), lipases(B), proteases (C), and siderophores (D). Mean values ± standard deviations of three independent experiments are shown. Balls with '* ' are statistically different from the control group for a confidence level greater than 95 % ($p < 0.05$), ns = non-significant.

group (Fig. 4) [13]. These type of benzaldehydes, when applied at concentrations of 800 and 1000 μg mL^{-1}, resulted in an almost complete reduction of bioluminescence in the *las* system of *P. aeruginosa*. As the cinnamic acid derivatives under study have a similar aromatic substitution pattern, it is expected that they can act as QS inhibitors in a similar way.

The efficacy of QSI in the *las* system can be evaluated at multiple levels, including synthesis, detection, and response to autoinducers [55, 56]. In this context, further studies were carried out to clarify the specific mode of action of ferulic and sinapic acids on the production of 3-Oxo-C12-HSL by LasI and its detection by LasR. As LasR protein plays a key role in coordinating the QS communication in *P. aeruginosa*, this regulator acts not only as a receptor for the autoinducers produced by LasI but also as a regulator for all signalling systems in this bacterium [9]. LasR can influence the *rhl* and *pqs* systems by positively regulating the proteins RhlI and RhlR of the *rhl* system and PqsH and PqsR of the *pqs* system [57]. LasR also regulates the *las* system by inducing the expression of RsaL, a transcriptional repressor of LasI. When RsaL binds to the LasI promoter, a negative feedback loop is created that balances the positive feedback loop triggered by the binding of the autoinducer to

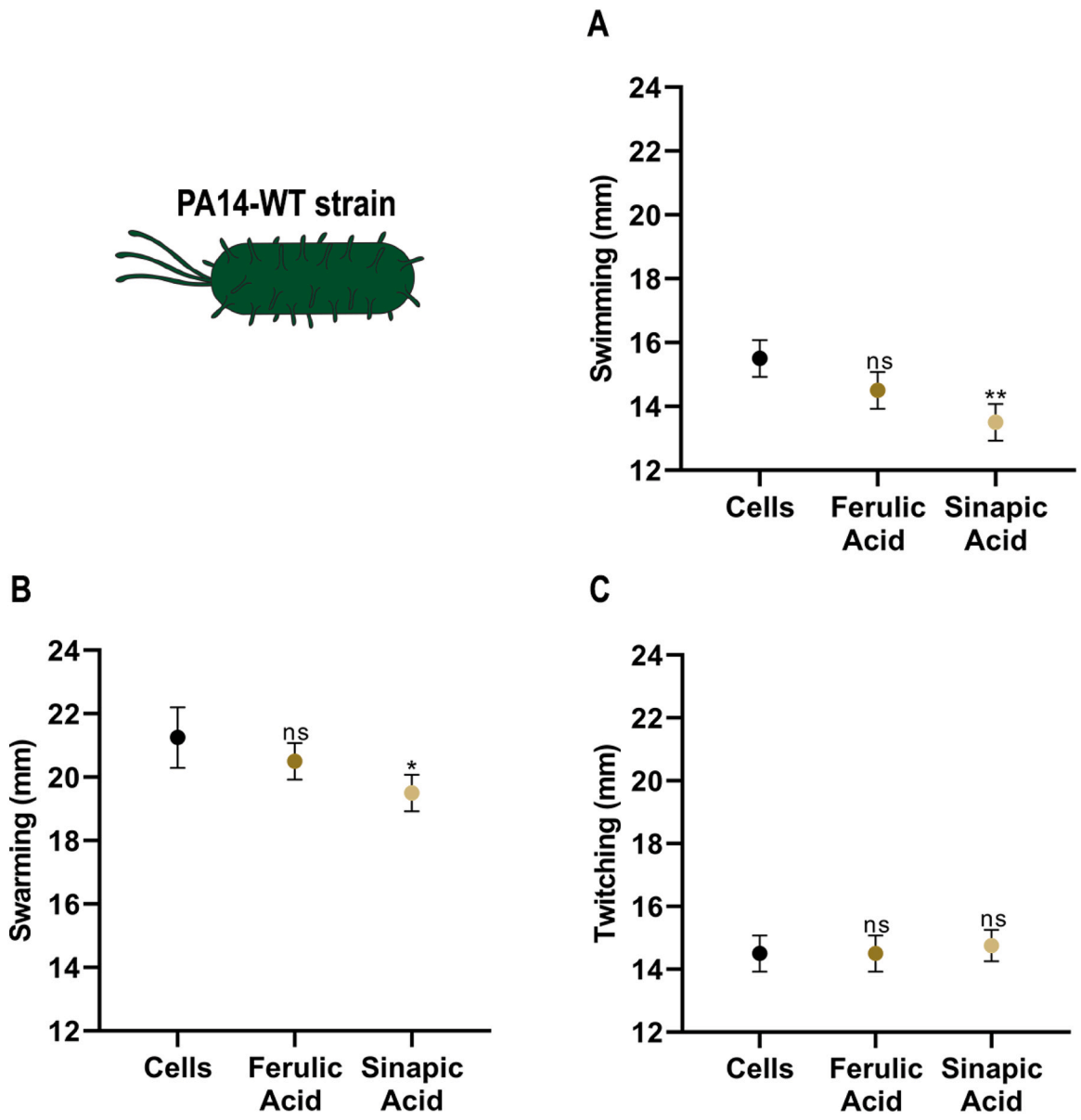


Fig. 14. Influence of ferulic and sinapic acids (at 1000 μg mL^{-1}) on the swimming (A), swarming (B) and twitching (C) motilities of *P. aeruginosa* PA14-WT. Mean values ± standard deviations of three independent experiments are shown. Balls with '* ' indicate statistically significant differences from the control group (untreated cells) at a confidence level of 95 % ($p < 0.05$).

LasR, allowing control over the amount of autoinducer [9].

In our study, the cinnamic acid derivatives influence the production of autoinducers by AHL synthase more strongly than their detection by receptor proteins (Figs. 5 and 6). Flavonoids like naringenin, which are phenolic compounds, were able to inhibit the *las* system in *P. aeruginosa*. For example, Hernando-Amado *et al.* [58] observed that naringenin inhibits the QS response in *P. aeruginosa* by competing with 3-Oxo-C12-HSL for binding to LasR. When naringenin was added at the early growth phase (when the levels of 3-Oxo-C12-HSL are low), it effectively inhibited the expression of QS-regulated genes and virulence factors such as elastase and pyocyanin. However, when added during the stationary phase, where LasR is already activated by 3-Oxo-C12-HSL, naringenin was unable to inhibit the QS response.

The *pqs* system also plays a central role in regulating virulence and biofilm formation in *P. aeruginosa* [1,3]. PQS is a byproduct of the precursor 2-heptyl-4-hydroxyquinoline (HHQ), which in turn, its production is regulated by genes in the *pqsABCDE* operon. This operon is activated by the transcriptional regulator PqsR, also known as MvfR [59]. Once produced, HHQ is converted to PQS by the enzyme PqsH through hydroxylation of HHQ at position 3 [9]. During and after the exponential growth phase of the bacteria, PQS levels increase rapidly due to a positive autoinduction cycle triggered by the activation of PqsR [3]. Furthermore, activation of PqsR acts as a transcriptional activator for the regulator RhlR. Remarkably, regulation of the *pqs* system also involves negative regulatory mechanisms, such as inhibition by the *rhl* system. In *P. aeruginosa* PA14 specifically, it is known that the regulator RhlR represses expression from the *pqsA* promoter. This process occurs since RhlR binds to a *las/rhl* regulatory box, located before the start of *pqsA* transcription, and induces the production of a longer transcript that forms a secondary structure in the 5′ untranslated region. This structure hinders the binding to the ribosomes and thereby reduces translation efficiency [59]. These types of regulations in *P. aeruginosa* show the interdependence between different QS systems in regulating bacterial pathogenicity and virulence [15].

As with the evaluation of the *las* system, bioluminescence tests were also carried out to assess the *pqs* system. In these assays,

bioluminescence emission occurs when PQS or HHQ molecules, produced by the PAO1-WT strain, are detected by the PAO1-CTX bioreporter strain [26]. Our results showed that cinnamic acid derivatives led to a reduction in bioluminescence by more than 80 % after 4 h of incubation, indicating a significant decrease in the synthesis of autoinducers (Fig. 7). These results are similar to those obtained with vanillin, a known inhibitor of the *pqs* system previously studied by our group [13].

Computational approaches were used to corroborate the *in vitro* data and to infer the possible binding modes of ferulic and sinapic acids in different protein pockets of the *las* and *pqs* systems. In the present study, molecular docking was performed to investigate the interactions of these cinnamic acid derivatives with the LasR, PqsR, PqsA, and PqsE proteins. The docking results showed that cinnamic acid derivatives could effectively bind to the target proteins, indicating their anti-QS activity (Table 1). The findings obtained are consistent with those of Escobar-Muciño [53], who conducted a molecular docking analysis of ferulic acid on the LasR regulator of *P. aeruginosa*. Using Mcule software, the study revealed that ferulic acid had a binding Gibbs energy value of -7.13 ± 0.21 kcal mol^{-1}. On the other hand, the author found that the natural autoinducer 3-Oxo-C12-HSL and the commercial inhibitor FC 30 showed values of -8.00 ± 0.10 kcal mol^{-1} and -4.20 ± 0.05 kcal mol^{-1}, respectively. These data suggest that ferulic acid binds more strongly to the LasR domain than FC-30 and similarly to the 3-Oxo-C12-HSL. Comparable effects were also observed for the RhlR protein.

In addition, the cinnamic acid derivatives were found to have key interactions with residues TRP60, TYR64 and TRP88 in the LasR protein (Fig. S5). This is in agreement with the results of Annapoorani *et al.* [60] who identified these residues together with others such as LEU36, TYR56, ASP73, THR75, VAL76 and ALA127 as crucial for the binding of the selected compounds and the natural ligand 3-Oxo-C12-HSL. The overlaps in these interactions suggest that the cinnamic acid derivatives may mimic the binding behaviour of the natural autoinducer. Furthermore, Kiratisin and colleagues [61] found that proteins containing TYR64 (pLXRY64H), TRP88 (pLXRW88Y) modifications cannot multimerise even in the presence of 3-Oxo-C12-HSL.

With respect to PqsR, Klein *et al.* [62] identified residue GLN194 as the only polar residue within the PQS binding pocket that plays a crucial role in ligand interactions through hydrogen bonds and CH/π interactions. In our study, we found that sinapic acid interacts with this residue specifically through hydrogen bonds (Fig. S6). Since the PqsR protein has 2 subunits in the pocket, we also verified that the selected cinnamic acid derivatives fit into the region where the aliphatic chain of the reference ligand is linked in the PqsR protein [33]. Regarding the PqsA protein, Witzgall *et al.* [40] showed that GLY279 and ASP299 are crucial for stabilizing the binding of anthraniloyl-AMP, through hydrogen bonds. Specifically, GLY279 interacts with the adenine ring and ASP299 forms hydrogen bonds with the 6-amino group of the purine base. It is worth noting that ASP299 is a highly conserved residue in other aryl-CoA ligases. In our study, both ferulic and sinapic acids were found to interact with these same residues and show a preference for positioning in the binding pocket associated with the adenine ring in anthraniloyl-AMP (Fig. S7). As far as PqsE is concerned, this protein is known for its interactions with ferrous ions. Soheili *et al.* [63] found that oxicams, which are non-steroidal anti-inflammatory drugs, interact with key residues such as TYR72 through hydrogen bonds. In addition, hydrophobic interactions with the residues HIS271 and HIS221 and polar interactions with HIS71 were observed. Electrostatic interactions with Fe^{2+} in the binding site of PqsE were also reported. Similarly, our study revealed that selected cinnamic acid derivatives interact with HIS71 and TYR72 through hydrogen bonds and also bind with Fe^{2+}, showing a comparable binding pattern to oxicams (Fig. 8). These findings suggest that cinnamic acid derivatives may modulate the activity of key QS proteins, which are crucial in regulating various processes, such as biofilm formation.

The formation of biofilms plays a central role in bacterial persistence and resistance to antibiotics and immune responses [1]. These structured bacterial communities provide bacteria a protected environment that enhances their ability to withstand external stressors, making them an important factor in chronic infections [16]. It is estimated that around 90 % of bacteria live predominantly in biofilm communities, emphasising their importance in various clinical and industrial contexts [11,64]. The QS systems are the main regulators of cells in biofilms and ensure the collective and orchestrated action of various cellular processes [65]. Ferulic and sinapic acids distressed the structure of the biofilms formed by PAO1-WT and PA14-WT, possibly due to their anti-QS activity. Clear differences were observed, with 2D-OCT imaging showing thinner and less rough biofilms compared to those formed without treatment (Figs. 9, S9 and 10). Our data is consistent with the results of Shih *et al.* [66], who showed that LasI mutants have lower EPS production, leading to the formation of thinner biofilms. In addition, Sakuragi *et al.* [67] showed that transcription of the *pel* operon, which is essential for the production of a glucose-rich exopolysaccharide matrix, is strongly reduced by LasI and RhlI mutants. Their results establish a link between QS signalling and the transcription of genes for biofilm matrix biosynthesis. It is plausible that the cinnamic acid derivatives under study can suppress the activity of the *pel* operon and alter the composition of the biofilm matrix by influencing the production of autoinducers in both the *las* and *pqs* systems. Another possible explanation is that the cinnamic acid derivatives can influence the biofilm structure by affecting the rhamnolipids. These molecules are crucial for biofilm architecture maintenance through cell-cell interactions and bacterial adhesion to surfaces, being their production regulated by the *las* and *rhl* in *P. aeruginosa* [68]. The effects on biofilm architecture could also be related to the inhibition of the *pqs* system. Allesen-Holm *et al.* [69] demonstrated that pqsA mutants of *P. aeruginosa* exhibit reduced extracellular DNA production, leading to significant changes in biofilm structure compared to the wild type.

Interestingly, despite these biofilm structural changes, the cinnamic acid derivatives did not lead to a reduction in the bacterial cell culturability (Fig. S8). This indicates that the biofilm cells retained their ability to regrow on a solid medium despite the observed alteration in the biofilm structure. Therefore, the cinnamic acid derivatives appear to influence the matrix and structural properties of the biofilm but do not significantly alter the culturability of the bacteria within the biofilm. Since cinnamic acid derivatives can modulate the QS signalling pathways in *P. aeruginosa*, their ability to inhibit virulence factors has also been investigated.

The QS systems play a crucial role in the virulence of *P. aeruginosa* by regulating the expression of various virulence factors such as exotoxins, proteases, alginate and siderophores. Pyocyanin, a bioactive pigment produced by about 95 % of *P. aeruginosa* strains, is secreted in significantly higher amounts during the bacterial colonisation phase [12,70]. Its synthesis begins with chorismate and is mediated by the *phz* operons, with the enzymes PhzM and PhzS playing a crucial role in the final stages [71]. Pyocyanin production is regulated by the *las*, *rhl* and *pqs* systems, with the role of regulators PqsR and RhlR being particularly important [72]. Although pyocyanin acts as an electron acceptor, which is relevant for maintaining the bacterial redox balance, it can trigger oxidative stress in the host cells during infection [72]. This leads to increased levels of reactive oxygen species and hydrogen peroxide, which contribute significantly to tissue damage, especially in chronic lung infections [71]. In addition, its zwitterionic properties facilitate the penetration of bacteria into the membranes of host cells. Pyocyanin also promotes the release of eDNA through cell lysis triggered by oxidative stress [72]. The decrease in pyocyanin levels observed after incubation of PA14-WT and PAO1-WT strains with ferulic and sinapic acids is a key indicator of their role in reducing virulence and decreasing the activity of QS systems in *P. aeruginosa*. Sinapic acid (at MTC) was most effective in reducing pyocyanin levels in the PAO1-WT strain, with a decrease of about 50 % (Fig. 11). This result is consistent with the study by Chimi *et*

al. [73], which observed a reduction in pyocyanin production in *P. aeruginosa* ATCC 27853 by approximately 90 % when the strain was treated with sinapic acid at 13.90 μg mL^{-1}. In another study, Pattnaik and colleagues [50] reported an approximately 40 % reduction in pyocyanin production in PAO1 after incubation with 250 μg mL^{-1} of ferulic acid, while in our work, an approximately 35 % reduction was observed with the same phenolic compound (at 1000 μg mL^{-1}, Fig. 11).

Pyoverdine is a siderophore produced by *P. aeruginosa* that is essential for iron capture. This compound binds to iron in a highly efficient way [74]. During infection, pyoverdine plays a dual role: in addition to its function as a siderophore, it also acts as a signalling molecule for the production of two critical virulence factors, the endoproteinase PrpL and exotoxin A [75]. The regulation of pyoverdine production could be related to the *pqs* system [76]. Our data showed that only ferulic acid was able to reduce the relative pyoverdine production (Fig. 12). However, the selected cinnamic acid derivatives were found to slightly inhibit the production of total siderophores in both stains investigated (Fig. 13 and S12).

In addition to siderophores, other virulence factors of *P. aeruginosa*, such as proteases, including elastase A, elastase B (pseudolysin) and protease IV, play a decisive role in evading the immune system during infection [77]. In particular, elastase B (encoded by the *lasB* gene) targets structural proteins such as collagen and immunoglobulins and disrupts wound healing and host defence. Protease IV, which is regulated by the *prpL* gene, further impairs host immunity by degrading fibrinogen and complement proteins, thereby exacerbating tissue damage [78]. Studies on QS mutants (LasI and RhlI) have shown significantly reduced proteolytic activity, demonstrating the role of QS in controlling their production [79]. In our study, ferulic and sinapic acids modulated QS activity, resulting in a significant decrease in total protease production in the PA14-WT strain (Fig. 13). Remarkably, only sinapic acid specifically reduced protease production in the PAO1-WT strain (Fig. S12). These findings highlight the potential of this type of phytochemicals in targeting virulence through QS modulation. Interestingly, Ahmed *et al.* [80] demonstrated that elastase A activity was inhibited by 31 % and 65 % by salicylic acid and *trans*-cinnamaldehyde, respectively. Similarly, the production of elastase B was reduced by 22 % and 28 % for salicylic acid and trans-cinnamaldehyde, respectively.

Based on the general role of proteases, we have also focused our study on other specific types, such as lipases and gelatinases, as QS mechanisms are often involved in the regulation of these enzymes [81]. In particular, lipases are controlled by two-component regulatory systems (such as CbrA-CbrB), *las* and *rhl* systems [82]. Ferulic and sinapic acids led to a reduction in lipase production, which in turn may influence the biofilm structure (Fig. 13 and S12). This observation is in line with the results of Rosenau *et al.* [83], who showed that a lipase C mutant of *P. aeruginosa* exhibits an altered biofilm architecture due to impaired lipase activity. In addition, the authors found that the mutants exhibited significant impairments in type IV pilus-dependent twitching and swarming motility and a decrease in extracellular rhamnolipid production.

The motility of *P. aeruginosa* is intricately regulated by QS systems that coordinate the expression of genes responsible for various forms of bacterial movement, including twitching, swarming, and swimming motilities. The *las* and *rhl* are central to the control of genes associated with flagellar and pilus functions [84]. In particular, RhlR regulates the *rhlAB* genes that promote swarming motility and contribute to biofilm development. In addition, the *las* and *rhl* systems are critical for twitching motility as they modulate the production of functional type IV pili that are important for surface-associated movement [84]. Motility plays an important role in the lifestyle of *P. aeruginosa* influencing attachment, biofilm formation, evasion of host immune responses, resistance to antimicrobial agents and biofilm dispersal [85,86]. In our study, ferulic and sinapic acids showed an inhibitory effect on swarming motility (Fig. 14 and S15), a result consistent with that of Ugurlu *et al.* [49], who observed a 50 % reduction in swarming motility associated with ferulic acid. Other phenolic compounds have shown a similar effect. For example, Zeng *et al.* [87] demonstrated that a derivative of caffeic acid (4-bromophenethyl-3-(3,4-dihydroxy-phenyl)acrylate) effectively inhibited swarming motility and exhibited strong binding affinity for LasR and PqsR by molecular docking and binding energy calculations. Interestingly, ferulic and sinapic acids only slightly reduced swarming motility, but did not affect twitching motility. A similar result was observed by O'May and Tufenkji [88], who investigated the motility of *P. aeruginosa* using polyphenolic compounds (proanthocyanidins) from cranberry extract. These authors found that the proanthocyanidins completely blocked swarming motility but did not affect swimming and twitching motilities. This selective inhibition of swarming motility supports the hypothesis that dissimilar phenolic compounds may have different effects on the types of motility regulated by QS systems.

5. Conclusions

The present study showed that ferulic and sinapic acids have an inhibitory effect on the *las* and *pqs* systems of *P. aeruginosa*, showing a significant reduction in bioluminescence. The phenolic compounds interfered with the production of the natural autoinducers of these QS systems. *In silico* approaches showed significant interactions between cinnamic acid derivatives and key proteins of the *las* and *pqs* systems. The high docking scores as well as the similarity of pocket binding of ferulic and sinapic acids compared to natural autoinducers and reference inhibitors emphasise their potential as robust QS regulators.

Ferulic and sinapic acids altered the structure of the biofilms, resulting in thinner and less rough biofilms of PAO1-WT and PA14-WT. In addition, the cinnamic acid derivatives showed the ability to affect the virulence of the *P. aeruginosa* strains tested by inhibiting the production of pyocyanin, pyoverdine, total proteases, siderophores, gelatinases, and lipases. The motility of the PA14-WT and PAO1-WT strains was also affected, with swarming motility decreasing significantly when treated with the cinnamic acid derivatives. The similar anti-QS activity observed for the cinnamic acid derivatives suggests that the difference in the aromatic pattern (additional methoxy group in sinapic acid compared to ferulic acid) is not directly related to their anti-QS activity in *P. aeruginosa*. However, the presence of the hydroxyl group is relevant for the observed anti-QS effects in both the *las* and *pqs* systems.

As these compounds present low toxicity, as demonstrated in previous studies, they can work as a safer option for potential therapeutic applications [89,90]. Their ability to modulate bacterial virulence and biofilm formation, as well as their favourable safety profile, suggest that they could serve as effective adjuvants in the treatment of *P. aeruginosa*-related infections.

CRediT authorship contribution statement

Borges Anabela: Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Simões Manuel:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition. **Gonçalves Ariana S.C.:** Validation, Methodology. **Leitão Miguel M.:** Writing – original draft, Validation, Methodology, Conceptualization. **Borges Fernanda:** Writing – review & editing, Supervision. **Sousa Sérgio F.:** Writing – review & editing, Resources, Methodology.

Compliance with ethics requirement

This article does not contain any studies with human or animal subjects.

Declaration of Competing Interest

There are no conflicts to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118090.

Data availability

Data will be made available on request.

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