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Red pepper peptide coatings control *Staphylococcus epidermidis* adhesion and biofilm formation

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ABSTRACT

Medical devices (indwelling) have greatly improved healthcare. Nevertheless, infections related to the use of these apparatuses continue to be a major clinical concern. Biofilms form on surfaces after bacterial adhesion, and they function as bacterial reservoirs and as resistance and tolerance factors against antibiotics and the host immune response. Technological strategies to control biofilms and bacterial adhesion, such as the use of surface coatings, are being explored more frequently, and natural peptides may promote their development. In this study, we purified and identified antibiofilm peptides from *Capsicum baccatum* (red pepper) using chromatography-tandem mass spectrometry, MALDI-MS, MS/MS and bioinformatics. These peptides strongly controlled biofilm formation by *Staphylococcus epidermidis*, the most prevalent pathogen in device-related infections, without any antibiotic activity. Furthermore, natural peptide-coated surfaces dislayed effective antiadhesive proprieties and showed no cytotoxic effects against different representative human cell lines. Finally, we determined the lead peptide predicted by Mascot and identified CSP37, which may be useful as a prime structure for the design of new antibiofilm agents. Together, these results shed light on natural *Capsicum* peptides as a possible antiadhesive coat to prevent medical device colonization.

1. Introduction

Medical devices (indwelling) have made great contributions to health and quality of life evolution. (Lim et al., 2015; Shah and Goyal, 2008) However, infections related to the use of these medical devices and to widespread antibiotic misuse have led to a lethal health crisis with the increasing appearance of tolerant and resistant bacteria that do not respond to conventional antibiotics (Levin-Reisman et al., 2017; Heilmann et al., 2018; Menegueti et al., 2019). Associated with this phenomenon is the ability of microorganisms to form biofilms that confer evolutionary advantages that are phenotypically beneficial to increased survival and symbiotic relationships (Olsen, 2015). A biofilm is a complex matrix composed of assembled extracellular polymeric substances (EPSs) enwrapping microorganisms. This structure allows bacterial surface fixation on catheters, implants, probes, prostheses or damaged tissues and protects bacteria against antibiotics and host immune defenses. (Davies, 2003; Romling and Balsalobre, 2012; Haussler and Fuqua, 2013; Hall and Mah, 2017; Post et al., 2017).

Biofilm formation is considered a significant virulence factor and is present in 80% of difficult-to-treat human infections, such as endocarditis, osteomyelitis, urethritis, and periodontitis, strongly impacting patient outcomes and health system costs (Arciola et al., 2012;

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Lim et al., 2013; Madeo and Frieri, 2013; Travier et al., 2013; Macedo and Abraham, 2009).

In this context, *Staphylococcus epidermidis* is an opportunistic pathogen that is highly associated with medical device-related infections (Chusri et al., 2012; Laverty et al., 2013; Chessa et al., 2016; Vassallo et al., 2017; Oliveira et al., 2018; Lee et al., 2018). Current discussions suggest that this bacterium should be classified as pathogenic because disease-causing *S. epidermidis* strains represent a pathogenic subpopulation that has acquired genetic elements and related phenotypes that promote infection (Méric et al., 2018). Furthermore, the ability to form biofilms on diverse devices or surfaces is the major virulence factor of *S. epidermidis* (Otto, 2008). For instance, biofilm-forming *S. epidermidis* strains have been reported to include many strains that are resistant to ciprofloxacin and sulfamethoxazole, as well as to aminoglycosides (Li and Webster, 2018).

Some technological strategies for the development of biomaterials that have been proposed include functionalized or coated antiadhesive surfaces and intrinsically antibacterial or nanostructured materials (Campoccia et al., 2013; Ashton et al., 2019; Gao et al., 2017). However, among these strategies, the use of natural products to control bacterial adhesion and biofilm formation is rare (Trentin et al., 2015).

Peppers from the genus *Capsicum* comprise more than 200 varieties, and their fruits are broadly diverse in relation to size, shape, flavor and sensory heat (Qin et al., 2014). *Capsicum* species are native to the tropical and humid zones of Central and South America and include peppers of important economic value (Govindarajan, 1986; Menichini et al., 2009; Kim et al., 2014). They are commonly consumed as spice or food as well as used as remedies in Indian, Native American and Chinese traditional medicine to treat skin rashes, dog/snake bites and wounds (Meghvansi et al., 2010). *Capsicum* peppers display several biological activities, such as anti-inflammatory, hypoglycemic and hypocholesterolemic, though its pharmacological potential and chemical composition have been underexplored (Srinivasan et al., 2009; Zimmer et al., 2012a, b; Ahuja et al., 2007; Aizawa and Inakuma, 2009; Galvez Ranilla et al., 2010; Liu and Nair, 2010; Sharma et al., 2013). Recently, we demonstrated that an aqueous extract of *C. baccatum* seeds decreased *S. epidermidis* biofilm formation by 80%, indicating that this red pepper is a potential source of antibiofilm and antiadhesive compounds (Gomes Von Borowski et al., 2019). In the present study, we purified and identified antibiofilm and antiadhesive peptides from *C. baccatum* using chromatography-tandem mass spectrometry, MALDI-MS/MS and bioinformatics tools. We demonstrated the ability of *Capsicum* storage peptides (CSP) to prevent biofilm formation by methicillin-resistant *S. epidermidis* without antibiotic mechanisms. CSP cytotoxic profiles were assessed *in vitro*. Surface CSP coatings were developed, and their effects were examined and characterized. These results may corroborate the development of new natural strategies in the war against medical device-related infections.

2. Material and methods

2.1. Chemicals

All reagents, which were analytical or HPLC grade, were purchased from Sigma Chemical Company™. All solvents were purchased from Vetec™ AG (Rio de Janeiro, Brazil). The matrices 2,5-dihydroxybenzoic acid (DHB) and sinapinic acid (SA) were purchased from Bruker Daltonics. Synthetic peptides suitable for use in cell culture were purchased from China Peptides with > 95% purity.

2.2. Plant material and extraction

C. baccatum var. *pendulum* (Willd.) Eshbaugh (Solanaceae) fruits were obtained from a cultivated area in Turuçu, Rio Grande do Sul (RS), Brazil (Fig. S1, Supplementary Material). A voucher specimen (ICN 181469) was identified and deposited at the Herbarium of Universidade Federal of Rio Grande (UFRGS, Brazil). The seeds and extracts were obtained and prepared according to Gomes Von Borowski et al. (2019). The most active extract was lyophilized to obtain the residual aqueous extract from seeds (RAqS).

2.3. Purification of residual aqueous extract from seeds (RAqS)

RAqS (700 mg) were solubilized in ultrapure water and subjected to column chromatography (3 cm diameter) using Sephadex™ LH-20 (40 g). Water was used as the eluent, and fourteen fractions of 10 ml each were collected (F1-F14). These purified fractions were screened for antibiofilm and antibacterial activities against *S. epidermidis* at 4.0, 2.0, 1.0, 0.5 and 0.25 mg/mL. The selected active fraction, which presented 1.4% yield, was submitted to characterization by LC-DAD-ESI-MS and MALDI-MS/MS.

2.4. Bacterial strain and culture conditions

Staphylococcus epidermidis (ATCC 35984) was cultivated on Muller Hinton agar at 37 °C overnight. The inoculum concentration was adjusted to an optical density at 600 nm equivalent to 10^8 CFU/ml.

2.5. Sample preparation for microbiological assays

Samples were freshly dissolved in ultrapure water and filtered through a 0.2 μm pore size membrane before each assay. The red pepper active fraction was tested at 2.0 mg/ml and the synthetic peptide (CSP37) at 0.2, 0.4 and 0.9 mM.

2.6. Antibiofilm and antibacterial assays

To evaluate biofilm formation, an adapted protocol from Trentin et al. 2015). was applied using 0.4% crystal violet staining and standard sterile 96-well polystyrene flat-bottom microtiter plates. Finally, the absorbance (570 nm) was measured (Spectramax™ M2e Multimode Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Bacterial growth was monitored by absorbance at 600 nm. Biofilm formation in the presence of the water control was considered to represent 100% biofilm formation.

2.7. Kinetics of bacterial growth

Bacterial growth kinetics were evaluated by monitoring the optical density (600 nm) at different timepoints. Samples were monitored after 0, 1, 3, 6, 12, and 24 h of incubation (37 °C). The red pepper active fraction (2.0 mg/ml) was evaluated, and rifampicin (8 μg/ml) was used as a death control. The results were expressed as OD_{600nm} per incubation time.

2.8. Coatings preparation and characterization

Solutions of the red pepper active fraction were prepared using 70% methanol and filtered through a 0.2 μm pore size membrane. Permanox™ slides (30 × 25 mm) were coated once or three times with the active fraction solution at either 2.0 mg/ml or 10.0 mg/ml. Methanol was used to prepare nontreated control slides. In a Laurell model WS-650MZ-23NPP/LITE spin coater, 300 μl of the active fraction solution was deposited on each Permanox™ slide. It was accelerated for 5 s up to an angular velocity of 500 rpm and subsequently accelerated to 4000 rpm for 40 s. To remove the remaining solvent and to promote film annealing, slides were heat-treated at 40 °C in a stove for 4 h. After the coating process, slides were sterilized using UV light for 20 min, cut to 10 × 25 mm and used in antibiofilm assays and scanning electron microscopy evaluation. Permanox™ samples were characterized before and after coating by water contact angle (WCA), which was measured using the sessile drop technique and Milli-Q water. Drop images were

captured and analyzed by a Theta Lite optical tensiometer (Attension). The reported WCAs are the means of five measurements performed on different areas of each sample surface.

2.9. Scanning electron microscopy (SEM)

Biofilms of *S. epidermidis* were cultured as described above. One group received a piece of noncoated Permanox™ slide (as a nontreated and no solvent control), while others received a piece of red pepper active fraction-coated slide (once or three times at 2.0 mg/ml or 10.0 mg/ml) or methanol-coated slide (as a solvent control). Noncoated controls were incubated with TSB broth containing the active fraction and bacteria, and coated samples were incubated with only TSB broth and bacteria (24 h at 37 °C). Then, the slides were washed with sterile 0.9% NaCl and fixed with 2.5% glutaraldehyde (prepared in 100 mM cacodylate buffer, pH 7.2). Afterwards, they were washed with 100 mM cacodylate buffer (pH 7.2), dehydrated with increasing concentrations of acetone, dried with CO_2 as the critical point (Bal-Tec CPD 030 critical point dryer, Balzers, Liechtenstein) and placed on a mounting base. Finally, they were coated with carbon and examined under a scanning electron microscope (model JSM-6060, JEOL) at 10 kV.

2.10. Hemolysis test

Hemolysis assays were performed with human venous blood from healthy donors as previously described by Rocha et al. (2012). All consents, procedures and documents were approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul (UFRGS) (number 666.655/2014). The red pepper active fraction was tested at 2.0 mg/ml. A solution of 1% Triton™ was used to produce 100% hemolysis (reference, positive control), and phosphate buffered saline (PBS) was used as a negative control (no hemolysis). The test sample results were statistically compared with those of the positive and negative controls.

2.11. Lymphocyte cytotoxicity test and LDH release determination

Lymphocytes were obtained from heparin-treated human venous blood from healthy donors and isolated as previously described by Waechter et al. (2017). Briefly, the lymphocytes were separated by Histopaque™ gradient centrifugation, cultured at 100,000 cells/well in a 24-well plate in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), and treated with either the red pepper active fraction at 2 mg/ml, 1% Triton™ solution (cytotoxic control) or RPMI medium (nontoxic control) for 24 h, all in quintuplicate. Cell morphology was verified by optical microscopy, and viable cell counting was performed with a FACSVerse™ flow cytometer equipped with a 488 nm blue laser and a flow sensor (BD Biosciences, San Jose, CA, EUA). BD FacSuite™ software (BD Biosciences) was used for data analysis. All consents, procedures and documents were approved by the Ethics Committee of UFRGS (number 666.655/2014). To monitor lactate dehydrogenase (LDH) release due to lysis, the supernatants of the treated cells were assessed for LDH activity using the LDH enzymatic assay kit available from Laborclin™ (São José do Rio Preto, São Paulo, Brazil). The results are expressed as percentages, and the Triton treatment was considered to result in 100% LDH release.

2.12. High-throughput multiparameter cell image analysis

High Content Screening (HCS) and High Content Analysis (HCA) were performed with a robotic platform (ImPACcell, BIOSIT, Université de Rennes 1) using 7 representative human cell lines: HuH7 (liver), CaCo-2 (colon), MDA (breast), HCT116 (colon), PC3 (prostate), NCI-H727 (lung) and MCF7 (skin). The number of normal cells is presented as the residual cell percentage (%) compared to the DMSO control average, and the synthetic peptide (CSP37) was tested at 25 μM. (Richy et al., 2018; Rodriguez Castillo et al., 2016) The platform is equipped with an Olympus upright microscope (Spot NB camera and Simple PCI software, Compix), a Zeiss AxioImager M1 upright microscope (Marzhauser, Zeiss NB camera and AxioVision software), an ArrayScan VTI high-content imager (Thermo/Cellomics), Hamilton STARlet and Nimbus liquid handling systems, and a Scienion liquid spotter.

2.13. Liquid chromatography-tandem mass spectrometry analysis

Analyses were performed on a Shimadzu UFLC-20AD system coupled to a diode array detector (DAD) and an ESI-ion trap mass spectrometer (LC-DAD-ESI-MS) (AmaZon SL, Bruker Daltonics). A Luna C18 column (5 μm, 4,6 mm × 250 mm, Phenomenex) was used for chromatography analyses. The mobile phase consisted of acetonitrile (solvent B) and water (solvent A), each containing 0.1% formic acid. The flow rate was 0.6 ml/min, and the injection volume was 20 μl. The elution profile was the following: 2% B (0 to 3 min), 2 to 20% B (3 to 22 min), 20 to 30% B (22 to 30 min), 30 to 100% B (30 to 40 min), and 100% B (40 to 45 min). The LC flow was split 3:7. The MS conditions were the following: the capillary voltage was 3500 V, nebulizer gas (nitrogen) pressure was set to 50 psi, dry gas flow was 9 l/min, and dry temperature was 300 °C. MS data were acquired in negative and positive ion modes. The samples were analyzed over a mass range (m/z) of 0–1200.

2.14. MALDI-MS/MS analyses

UltrafleXtreme MALDI-TOF/TOF equipment (BrukerDaltonics, Bremen, Germany) was used for the analyses. For analyses of the fractions, the matrices 2,5-dihydroxibenzoic acid (DHB) and sinapinic acid (SA) were evaluated, and better results were obtained with DHB, which was used for all protein analyses. MS analysis: the samples were analyzed by reflector (700 to 5000 Da and 4 to 20 kDa) and linear modes (4–20, 20–50 and 50–100 kDa). The samples were dissolved in acetonitrile (ACN):H_2O (20:80, v/v) with 0.1% trifluoroacetic acid and the DHB matrix (20 mg/ml) in ACN:H_2O (30:70, v/v) with 0.1% trifluoroacetic acid. The sample solution and the matrix were mixed in equal amounts and spotted onto a ground stainless steel MALDI target (1 μl). The experimental conditions for MS analyses were as follows: pulsed ion extraction of 360 ns, laser frequency of 1000 Hz, and positive ion mode. In all, 5000 shots were averaged to record each mass spectrum. For the MS/MS analyses, the red pepper active fraction was resolved by electrophoresis gel and the obtained spots (Sp1, Sp2 and Sp3) were digested with trypsin. For these analyses, a saturated solution of α-cyano-4-hydroxycinnamic acid (CCA) prepared in ACN:H_2O (30:70, v/v) with 0.1% trifluoroacetic acid was used. The samples were dissolved in ACN:H_2O (30:70, v/v) with 0.1% trifluoroacetic acid. The sample solution and the matrix were mixed in equal amounts and spotted onto a ground stainless steel MALDI target (1 μl). The peptide fractions were analyzed by MS and MS/MS. For MS analyses, the experimental conditions used were as follows: pulsed ion extraction of 100 ns, laser frequency of 1000 Hz, reflectron mode, and positive ion mode. In all, 600 shots were averaged to record each mass spectrum. The selected ions were accelerated to 19 kV on the LIFT cell for MS/MS analyses. External calibrations were performed using peptide calibration standards (angiotensin II and I, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39 and somatostatin) and protein standards I (insulin, ubiquitin I, cytochrome *C* and myoglobin) and II (trypsinogen, protein A and bovine serum albumin (BSA)) from Bruker Daltonics.

2.15. Database identification and CSP37 prediction

Database identification: MS/MS spectra were analyzed and converted to mfg files using Mascot Distiller (MATRIX SCIENCES, London, UK). Protein identification was performed by database searches with a local Mascot Server v 2.3 (MATRIX SCIENCES, London, UK) against the

proteome sequence of *Capsicum anuum* (Kim et al., 2014). The parameters used for identification allowed a maximum of one missed tyrosine cleavage, the carbamidomethylation of cysteine, and the possible oxidation of methionine. The peptide MS/MS tolerance was 0.25 Da, and the significance threshold was set at $p < 0.05$. The sequences identified by MASCOT as significant hits were annotated using the NCBI BLAST program and the NCBI Conserved Domain Database. CSP37 prediction: since the genome/proteome of *C. baccatum* is not fully sequenced, we used an alternative strategy to identify the principal peptides predicted by Mascot, which led to the identification of CSP37. Proteins containing the predicted peptides were identified by using P-BLAST to search a database of annotated *Capsicum* sequences. This led to the identification of two proteins (XP_016559134.1, 2S albumin-like [*Capsicum annuum*] and XP_016567465.1, 2S sulfur-rich seed storage protein 2-like [*Capsicum annuum*]) with very strong sequence identity. We then realigned the two proteins and the Mascot peptides using Clustal Omega. We discovered that the two peptides were in fact two consecutive segments (separated by a single residue) of a single longer peptide (CSP37), whose mass indeed corresponded to the most intense peak of the MS spectrum.

2.16. Statistical analysis

The results are expressed as the mean ± standard deviation. Biofilm and growth data were analyzed using Student's *t*-test, and p-values < 0.05 were considered statistically significant. Cytotoxicity data were analyzed using ANOVA followed by Tukey's post hoc test, and p-values < 0.05 were considered statistically significant.

3. Results

3.1. Bioguided fractionation and identification of antibiofilm peptides from C. baccatum

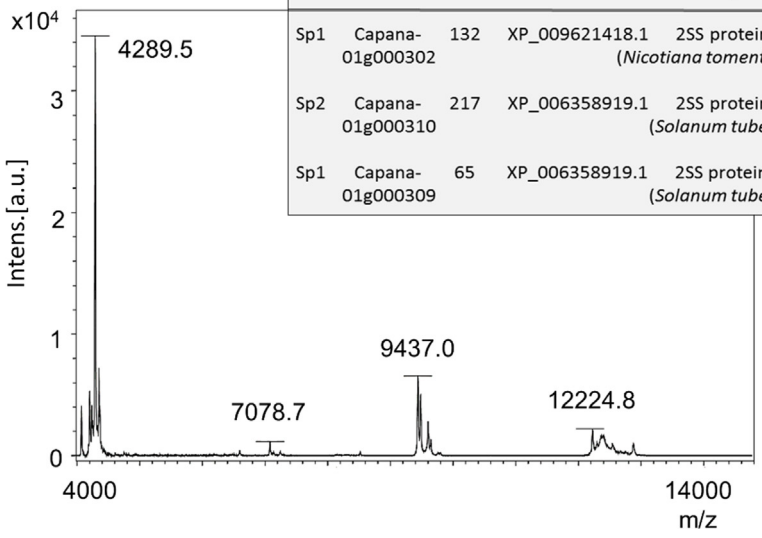
Previously, we obtained a protein extract from *C. baccatum* seeds (RAqS) with potential antibiofilm activity. (Gomes Von Borowski et al., 2019) Herein, further purification steps were performed in order to obtain the bioactive fraction from RAqS responsible for antibiofilm activity. After chromatographic collection of 14 fractions, the ninth fraction was determined to be the one containing the biological activity (Fig. S2). Once identified, this antibiofilm active fraction was subjected to chemical elucidation. Briefly, MALDI-MS analyses did not show ions up to *m/z* 4000, but some ions were observed in the range of 4 to 20 kDa (Fig. 1), characteristic of peptides abundant in *Capsicum* (Lee et al., 2008; Dias et al., 2013). The *m/z* 4305 ion showed the highest relative ion intensity in the mass spectrum of the red pepper active fraction, and less intense ions were observed at *m/z* 4220, 4364, 9372 and 9415. No ions were observed in the mass range 20–100 kDa, moreover, LC-DAD-ESI-MS analysis did not reveal secondary metabolites of low molecular weight. Thus, the red pepper active fraction was subjected to gel electrophoresis, and the obtained spots (Sp1, Sp2 and Sp3) were analyzed by MALDI-MS and MS/MS to identify the protein precursors. All spots were found to contain peptides belonging to the same protein family, the 2S sulfur-rich seed storage protein (2SS protein) family, according to the NCBI CDD and BLAST searches using the proteome of *C. annum* as the database (the *C. baccatum* proteome is unknown) (Fig. 1, Table 1). Therefore, the red pepper active fraction is composed of peptides derived from a 2SS protein (*C. baccatum)* and will be referred to herein as "*Capsicum* storage peptides (CSP)".

3.2. CSP control methicillin-resistant S. epidermidis biofilm formation

Staphylococcus epidermidis biofilm formation was completely prevented in the presence of CSP solution (2.0 mg/mL) (Fig. 2A). Remarkably, CSP did not influence bacterial growth at the same concentration (Fig. 2A). In addition, bacterial growth kinetics confirmed the absence of any antibiotic effect of CSP (Fig. 2B). Although biofilm formation was prevented, the planktonic cells remained viable, and the colonies were phenotypically normal.

3.3. Red pepper peptide coatings strongly prevent S. epidermidis adhesion

To verify the antiadhesive capability of CSP, we applied the spin-coat technique to coat Permanox™ slides with CSP (herein called "red pepper coat"), exposed the slides to *S. epidermidis* cultures and analyzed them by scanning electron microscopy (SEM) after 24 h of exposure (Fig. 3A–J). Red pepper-coated surfaces prevented bacterial adhesion, aggregation and accumulation (Fig. 3C–J). In addition, the noncoated control that received 2.0 mg/mL soluble CSP in the culture medium did not display any adherent bacteria (Fig. S8, Supplementary Material). Compared to controls, bacterial adhesion and aggregation profiles decreased on slides that had been coated once with 2.0 mg/ml CSP (Fig. 3C and D). Slides that had been coated three times with 2.0 mg/ml CSP show only a few individual cells, although extracellular matrix was



Peptide	MASCOT Identification* ID	Score*	Blast best Hit ID ID	Description	Pos.(%)	e-value
Sp1	Capana-01g000302	132	XP_009621418.1	2SS protein (*Nicotiana tomentosiformis*)	77	2,00E-64
Sp2	Capana-01g000310	217	XP_006358919.1	2SS protein (*Solanum tuberosum*)	83	3,00E-42
Sp1	Capana-01g000309	65	XP_006358919.1	2SS protein (*Solanum tuberosum)*	83	3,00E-42

Fig. 1. Identification of the peptides from *C. baccatum* active fraction. MALDI-MS mass spectra (linear, positive ion mode) of the antibiofilm active fraction peptides, range of 4000–20,000 *m/z*. Table 1. (Insertion) Mascot results for CSP spots according to the NCBI CDD and BLAST searches using the proteome of *C. annum* as the database. (2SS protein) is 2S sulfur-rich seed storage protein; *For the Capsicum proteome database in Mascot, scores > 28 indicate identity or extensive homology ($p < 0.05$).

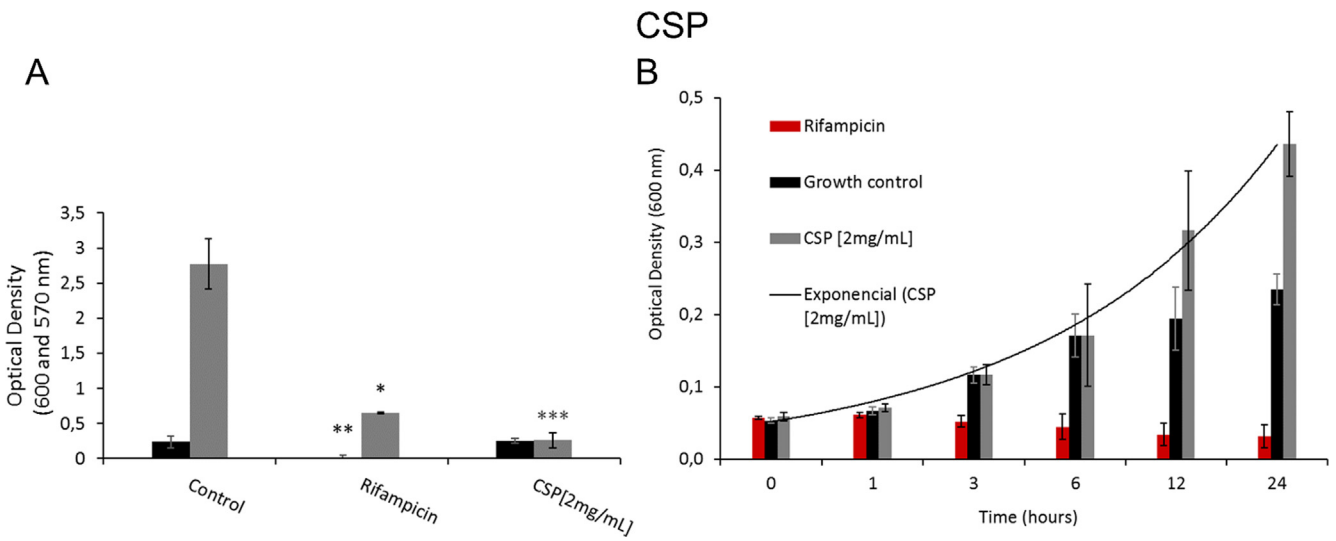


Fig. 2. Bacterial biofilm formation and growth by *S. epidermidis* (ATCC 35984) exposed to natural *Capsicum* storage peptides (CSP). A) Growth (black bars) and biofilm (gray bars), both after 24 h of CSP solution exposure at 2 mg/ml. B) Bacterial growth kinetics of *S. epidermidis* exposed to CSP solution at 2 mg/ml (0, 1, 3, 6, 12 and 24 h). Untreated samples were used as growth and biofilm controls, and rifampicin (8 μg/mL) was used as an antibiotic control. The results were monitored by optical density (OD_{570} for biofilm formation and OD_{600} for bacteria growth), and untreated samples were considered 100% growth and biofilm formation. (*) represents significant differences when compared to biofilm/growth control using Student's *t*-test; p-values < 0.05 were considered statistically significant.

not detected (Fig. 3G and H). Likewise, slides coated with a higher CSP concentration (10.0 mg/ml) displayed a complete absence of bacteria and extracellular matrix (Fig. 3E and F, I and J). Control slides (vehicle-coated and noncoated slides without CSP solution) were not able to prevent the bacterial adhesion, aggregation and accumulation that facilitate biofilm formation (Fig. 3A and B and S3, Supplementary Material).

3.4. Red pepper peptide coatings create a hydrophilic surface on coated material

To evaluate the hydrophilicity and the quality of red pepper coatings produced by spin coating (Fig. 3k), we used the water contact angle (WCA) image and measurement analysis. Red pepper coatings displayed hydrophilic characteristics in a concentration-dependent manner compared to controls. The surfaces singly and triply coated with 2.0 mg/mL CSP displayed WCAs of 75° and 81°, respectively. At 10.0 mg/mL, the WCA decreased to 26° and 18° for singly and triply coated surfaces, respectively. However, noncoated and methanol-coated surfaces exhibited hydrophobic characteristics, displaying WCAs of 87° and 92°, respectively.

3.5. CSP37 is the lead antibiofilm peptide from the CSP fraction

We postulated that the peptide detected with higher intensity in the CSP active fraction (*m/z* 4289, Fig. 1) would mediate CSP bioactivity. Thus, we performed sequence and structural predictions of this major peptide of CSP. For this purpose, we used the CSP MS/MS data and sequence alignment analysis to determine its sequence: RSCQQQIQQ-AQQLSSCQQYLKQRVQSEEGEDQISQRE (European Patent n EP19305205) (Fig. 4A–C). The predicted peptide was named "*Capsicum* storage peptide 37 (CSP37)" and was subsequently synthesized and experimentally tested. CSP37 prevented *S. epidermidis* biofilm formation independently of growth inhibition, equivalently to CSP (Fig. 4D). Their bioactivities (Antibiofilm, non-antibiotic and non-cytotoxic) support the correlation between them (Figs. 4D, 2A and 5).

3.6. Red pepper peptides are not cytotoxic

To evaluate CSP cytotoxicity, human erythrocytes and lymphocytes were used as model cells. CSP at 2.0 mg/ml displayed a slight hemolytic activity (11% hemolysis), preserving the erythrocytes integrity similarly to the PBS control (Fig. 5B). Equally, CSP did not present cytotoxic effects against lymphocytes after 24 h of exposure (7.7% decrease in cell counts) (Fig. 5A, C–E). Furthermore, the LDH assay indicated that CSP did not affect lymphocyte membrane integrity (LDH release by Triton treatment was considered 100% for quantification, the normal control showed 17.3%, and CSP 14.7%). Likewise, high content analysis (HCA) showed no CSP37 cytotoxicity against 7 different representative human cell lines (Fig. 5F).

4. Discussion

Biofilm-forming *S. epidermidis* is an important pathogen worldwide and is closely linked with medical device- and immunosuppression-associated infections. (Lourtet-Hascoët et al., 2016; Widerström, 2016; Gabriel et al., 2015) The present study demonstrates that natural *Capsicum* storage peptides (CSP) exhibit potent antibiofilm activity against methicillin-resistant *S. epidermidis* by a mechanism that does not involve cell killing. The study presents the identification of CSP37, the major peptide of CSP and a source of its antibiofilm activity. Strikingly, the results of our coating experiments support the antiadhesive applicability of these peptides.

Plants are constantly exposed to a large variety of pathogenic microorganisms, and they produce natural compounds as resistance factors, such as constitutive peptides and proteins. (Castro and Fontes, 2005; Silva et al., 2017; de Vries et al., 2017) Peptides are a very common chemical component of *Capsicum* seeds. (Dias et al., 2013; Lee et al., 2004) In this context, some studies have suggested that plant-derived peptide fractions exhibit antiadhesive properties. (Gomes Von Borowski et al., 2019; Lengsfeld et al., 2004; Wittschier et al., 2007; Bensch et al., 2011) Recent literature reviews have shown that peptides and peptidomimetics from different sources have been investigated for their prominent antibiofilm activity. (Von Borowski et al., 2017; Gomes Von Borowski et al., 2018; Batoni et al., 2016; de la Fuente-Nunez et al., 2015; Pfalzgraff et al., 2018)

There are no previous studies demonstrating a relationship between 2S sulfur-rich seed storage (2SS) proteins or peptides and antibiofilm activity. To the best of our knowledge, this is the first scientific report using *Capsicum* natural peptides as antibiofilm and antiadhesive agents,

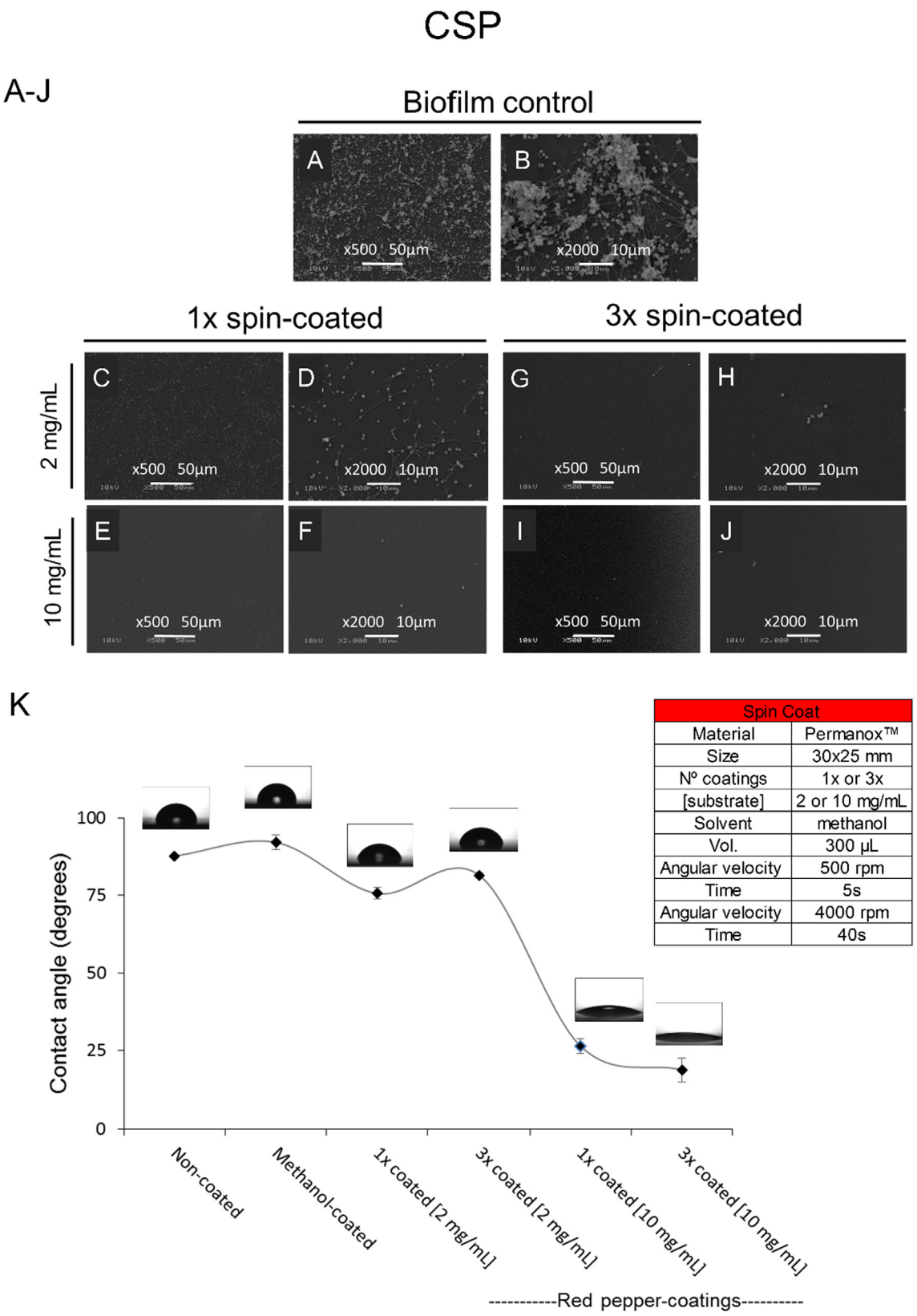


Fig. 3. Scanning electron microscopy (SEM) images of Red pepper-coated slides after exposure to biofilm-forming *S. epidermidis* and the water contact angle (WCA) of Permanox™ slides before and after spin coating. (A, B) Biofilm control: methanol (vehicle) was used to prepare untreated surface slides and anchored by spin coating three times. (C–J) Red pepper coatings: natural capsicum storage peptides (CSP) were anchored by spin coating once or three times at 2 or 10 mg/ml. Scale bars: 500 and 2000 × magnification. K) Graphic displaying the contact angle (degrees) results of noncoated and red pepper-coated Permanox™ slides. In the upper part, we show the photographs of the water drop and WCA, captured by a Theta Lite optical tensiometer and (Insertion) Spin Coat parameters. The WCAs are a mean of five measurements of different points of each sample.

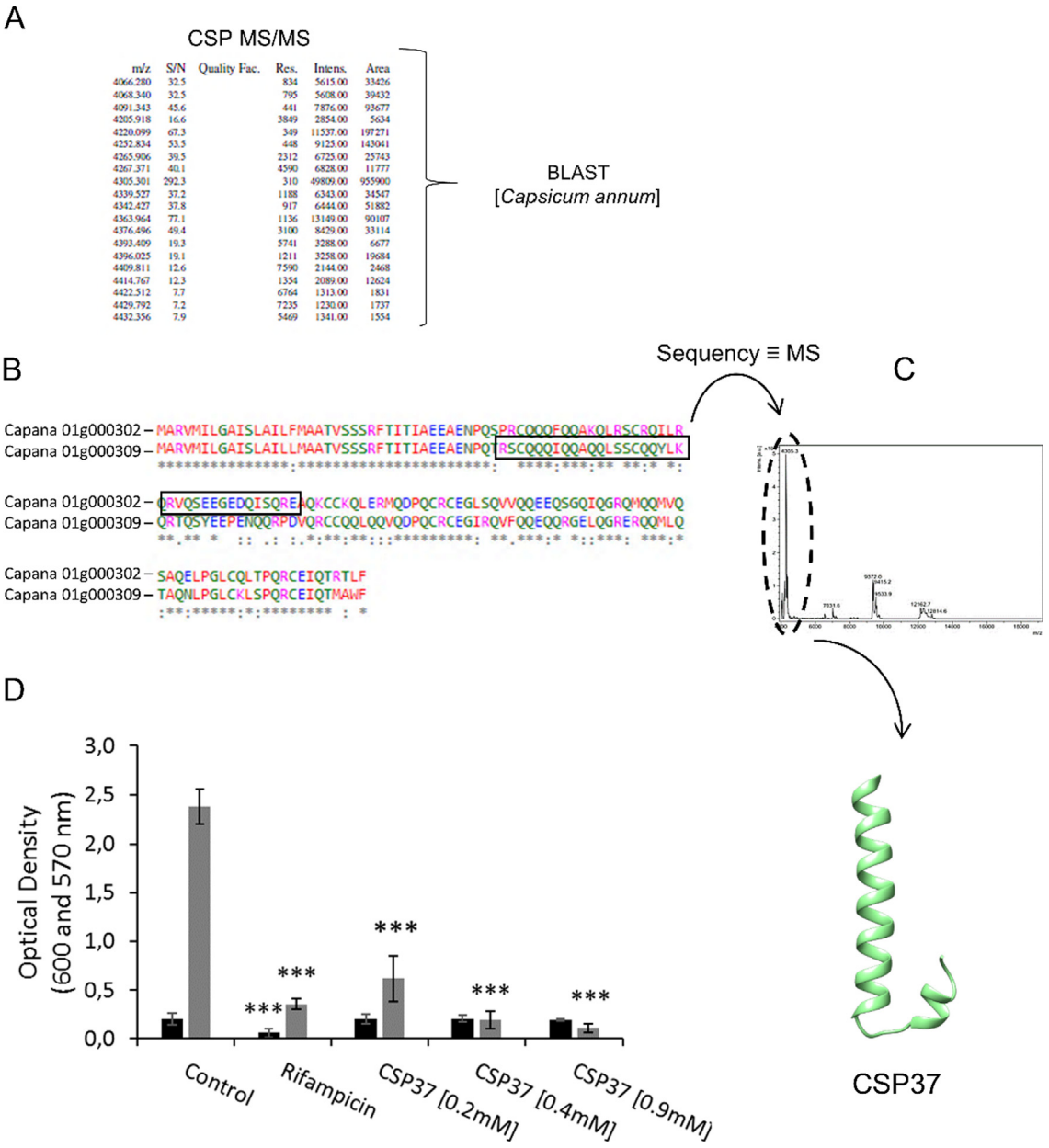


Fig. 4. CSP37 prediction and activity on biofilm formation and growth by *S. epidermidis* (ATCC 35984). (A) Predicted peptides by MASCOT were identified using BLAST (NCBI) and the annotated *Capsicum annuum* proteome. This lead to the identification of two separated proteins (Capana01g000302 = XP_016559134.1 and Capana01g000309 = XP_016567465.1). (B) Sequence alignment of the two proteins identified with BLAST. The two MASCOT peptides are highlighted in black. The position of the two peptides in the protein sequences and the strong sequence similarity between these two proteins suggest that the two peptides can be in fact two consecutives portion of a unique protein of *Capsicum baccatum*. (C) The mass of the CSP37 peptide (predicted from the union of the two precedent peptides) corresponds to the major MS pick of natural CSP fraction and, its 3D structural prediction by PEP-FOLD 3. (D) Growth (black bars) and biofilm formation (gray bars) of *S. epidermidis* exposed to CSP37 (synthetic) solution at 0.23, 0.46 or 0.92 mM for 24 h. Untreated samples were used as growth and biofilm formation controls, and rifampicin (8 μg/mL) was used as an antibiotic control. The results were monitored by optical density (OD570 for biofilm formation and OD600 for bacteria growth), and untreated samples were considered 100% growth and biofilm formation. (*) represents significant differences when compared to biofilm/growth controls using Student's *t*-test; p-values < 0.05 were considered statistically significant.

herein called *red pepper coat*. The purification of *C. baccatum* extract allowed the chemical identification of the active fraction able to prevent *S. epidermidis* biofilm formation. This antibiofilm fraction is composed of peptides from a *Capsicum* 2SS protein, herein named *Capsicum* storage peptides (CSP). Hence, the reconstitution of the major MS peak of the CSP spectrum allowed the identification of its main peptide (CSP37, European Patent n° EP19305205), consisting of a 37 amino acid sequence that is also present in the 2SS protein described in the *Capsicum* genus.

The NCBI database describes the 2SS protein and the 2S albumin-like protein as members of the Alpha-Amylase Inhibitors (AAIs) and Seed Storage (SS) Protein subfamilies. They are present in the seeds of a variety of plants and are widely distributed in dicot seeds, and they play an important role in plant natural defenses (NCBI accession: XP_016567465.1, CAA01774.1, NP_001295683.1, NP_001078429.1). Despite differences in their subunit structure and synthesis, all are compact globular proteins with conserved cysteine residues (Shewry et al., 1995).

Notably, both CSPs and CSP37 strongly decrease *S. epidermidis* biofilm formation without antibiotic effects. These results establish that

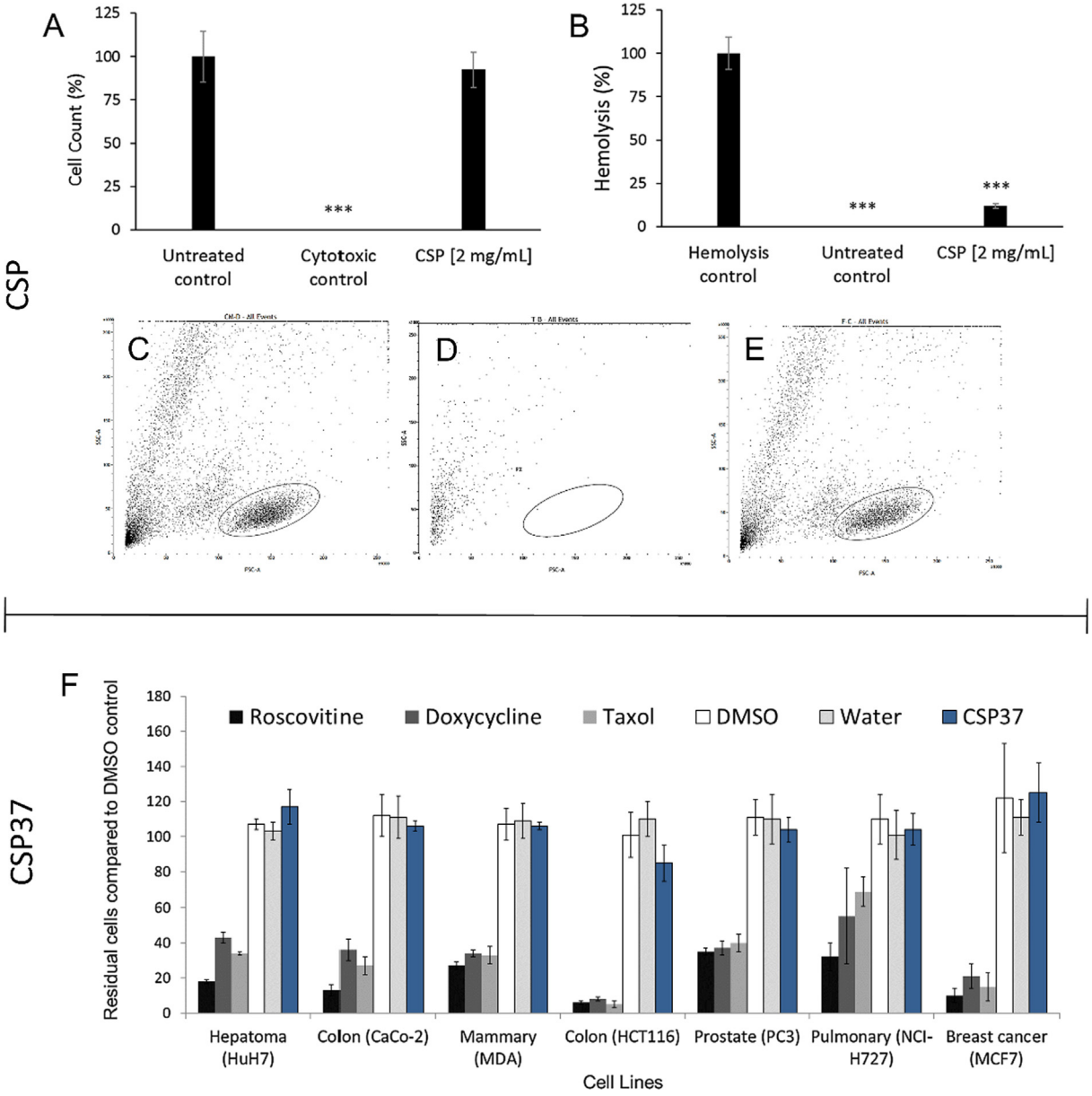


Fig. 5. Cytotoxicity of natural CSP and synthetic CSP37. (A) Human lymphocytes cells after 24 h of exposure to *Capsicum* storage peptides (CSP) at 2.0 mg/ml, 1% Triton solution (cytotoxic control), or RPMI 1640 medium (untreated control). (B) Hemolysis test with human erythrocytes exposed to CSP at 2 mg/ml, 1% Triton solution (100% of hemolysis), or phosphate buffered saline (PBS) (untreated control as nonhemolytic control). (C–E) Dot plots of lymphocytes obtained by flow cytometry. The circled areas highlight the cell populations of interest: (C) untreated control, (D) cytotoxic control and (E) cells exposed to CSP (2.0 mg/ml). (*) Represents a significant difference when compared to reference samples using ANOVA followed by Tukey's post hoc test; p-values < 0.05 were considered statistically significant. (F) CSP37 cytotoxicity evaluation in representative human cell lines at 25 μM. The number of normal cells is presented as the residual cell percentage (%) compared to the average of the control (DMSO, shown as white). The first three bars, which are black-gray, represent classic cytotoxic controls (roscovitine, doxycycline and Taxol), and the blue bar represents CSP37-exposed cells. Cells lines are described under the bars. An automated image-based cellular content analysis system (HCS/HCA) was used.

the antibiofilm activity of CSP and CSP37 utilizes a mechanism of action that is independent of cell killing and of antibiotic mechanisms. Accordingly, nonantibiotic strategies have emerged as alternatives to combat bacterial resistance and tolerance, such as the targeting of extracellular bacterial virulence factors. This strategy is very important because it is less likely to impact bacteria gene machinery, and it may include antibiofilm agents to reduce bacteria adhesion (Travier et al., 2013; Dostert and Belanger, 2018; Sun et al., 2018; Brannon and Hadjifrangiskou, 2016; Totsika, 2017; Silva et al., 2016). In this sense, the prototype red pepper coat (CSP spin-coated slides) prevented bacterial adhesion, aggregation, accumulation and consequently biofilm establishment. This activity seems to be concentration dependent.

The antiadhesive effect of red pepper coatings may be due to multiple causes, including electrostatic interactions and steric hindrance. Repulsion forces may act between the anionic Gram-positive bacterial surface and biofilm matrix and the electronegative field of the coated surface (Fig. 6). The film produced by CSP coating may also give rise to steric hindrance that blocks bacterium-substratum interactions (Trentin et al., 2015). Moreover, CSP are mainly composed of polar amino acids, leading to important hydrophilic characteristics. Supported by angle contact analysis, red pepper-coated surfaces presented increased hydrophilicity on the entire coated surface compared to controls (hydrophobic material), showing that CSP occupy and alter the hydrophobic characteristics of Permanox™. Bacterial attachment is initially

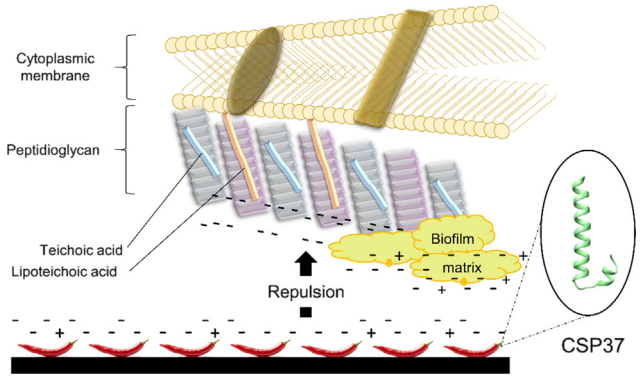


Fig. 6. Proposed mechanism of anti-adhesion and of biofilm formation inhibition by red pepper coating. The effects are due to repulsive forces between the polar material surface after CSP coating and the anionic *S. epidermidis* surface and biofilm matrix.

dependent on their hydrophobic and electrostatic physicochemical characteristics as well as on the surfaces involved; thus, the hydrophilic character of red pepper coat contributes to a reduction in these interactions (Trentin et al., 2015; Nostro et al., 2012; Otto, 2013).

In addition, both activities remain, even after 24 h under the incubation conditions. The spin-coating technique anchors substances on surfaces and is appropriate for the study of these coatings during transient or short-term exposure, which is sufficient for establishing proof of concept that natural products may act against pathogenic bacterial adhesion, although stronger interactions such as covalent attachment are required to extend coating lifetime for long-term studies.

Importantly, CSP was not cytotoxic to human erythrocytes and lymphocytes. CSP37, the proposed major peptide of CSP, showed no cytotoxicity against 7 representative human cell lines. Furthermore, previous studies with *C. baccatum* corroborate this favorable toxicological profile. For example, RAqS, the precursor of CSP, was not toxic to *Galleria mellonella larvae* (Gomes Von Borowski et al., 2019) which presents similarities to the innate immune response of mammals like humoral and cellular components, and other extracts from *C. baccatum* displayed a good level of pharmacological safety during 60 days of oral administration in mice (Zimmer et al., 2012). These results suggest that red pepper coatings do not affect either eukaryotic or prokaryotic membranes (Atefyekta et al., 2019; Strempel et al., 2015).

5. Conclusions

In conclusion, this study shows that red pepper peptide coatings are a promising antibiofilm and antiadhesive alternative to prevent methicillin-resistant *S. epidermidis* colonization. Importantly, components of red pepper coatings are not cytotoxic and act by a nonantibiotic mechanism of action. In addition, CSP37 may facilitate research for innovative molecular designs as a prime structure. Importantly, this study sheds light on the use of natural products and their derivatives, which could bring innovative solutions for materials science. The identification of novel peptides aims to overcome the lack of therapies against biofilm infections and to contribute to the development of anti-infective medical devices.

Authors contribution

AJM, ARZ, RG and SCBG contributed to scientific design of this project and advised the experimental/writing parts; CBO contributed to cytotoxicity assays; CCS contributed to peptide identification (database identification); DBD and NPL contributed to LC-DAD-ESI-MS and MALDI-TOF/TOF analysis; EG contributed to peptide prediction (bioinformatics analysis); MPB and KRZ contributed to biofilm analysis and RGVB contributed to all experimental tests and wrote the paper.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [R.G.V.B, S.C.B.G, A.R.Z, A.J.M and R.G. are co-inventors of a patent register of CSP37. Application number EP19305205.7 at European Patent Office. Specific aspect of manuscript covered in patent application: its amino acid sequence and bioactivity.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2019.118872.

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