

First-in-class matrix anti-assembly peptide prevents staphylococcal biofilm in vitro and in vivo

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| 1 | First-in-class matrix anti-assembly peptide prevents staphylococcal biofilm in vitro and in |
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| 16 | |
| 17 | Abstract |
| 18 | Staphylococci are pathogenic biofilm-forming bacteria, source of multidrug-resistance and/or – |
| 19 | tolerance causing a broad spectrum of infections. These bacteria are enclosed in a matrix that |
| 20 | allows them to colonize medical devices such as catheters and tissue, and which protects against |
| 21 | antibiotics and immune systems. Advances in antibiofilm strategies for targeting this matrix are |
| 22 | therefore extremely relevant. Plants are constantly attacked by a wide range of pathogens, and |
| 23 | have protective factors such as peptides to defend themselves. These peptides are common |

components in *Capsicum* peppers (CP). Here, we describe the development of CP bioinspired peptide "capsicumicine". We demonstrate that capsicumicine strongly prevents methicillinresistant *S. epidermidis* biofilm *via* a new extracellular "matrix anti-assembly" mechanism of action. Catheters pre-coated with capsicumicine decreased *S. aureus* colonization leading to the attenuation of infection, decreasing mice systemic infection. Capsicumicine is the first-in-class nonantibiotic, carbohydrate-binding peptide.

- 30 **Keywords:** antibiofilm, biofilm, matrix, peptides, anti-assembly, resistance, tolerance.
- 31

32 Introduction

33 Antimicrobial failure is a worldwide challenge, currently addressed by a WHO global action plan (1). A 34 lack of new antibiotics and the inappropriate use of older treatments mean that multidrug-resistant 35 strains are increasing (2). This process is favored by biofilm development, and microorganisms 36 enclosed in biofilm matrix have antibiotic resistance that is up to 1000 times higher than planktonic 37 ones (3). This makes the matrix itself an important target for biofilm control. Biofilms are organized 38 microbial clusters made of a self-assembled matrix that usually attaches to a surface, whether abiotic 39 (medical devices such as catheters, teeth, etc.) or biotic (host tissues, mucus, chronic wounds, etc.) 40 (4, 5). Since the bacteria are embedded into this matrix, they are harder to treat because of their 41 increased tolerance and resistance to antibiotics, disinfectants, and host defenses (6, 7). Other 42 advantages over planktonic forms include physiological and biochemical changes, beneficial quorum 43 sensing, higher (up to 100 times) mutation rates, and persister cell development (8-10). 44 Staphylococci are the most frequent sources of nosocomial infections, particularly S. epidermidis and 45 S. aureus. While S. aureus expresses many virulence factors such as toxins and proteases, for S. 46 *epidermidis* the formation of biofilm is the most important mechanism in infection development (11) 47 . Staphylococcus epidermidis is the most frequent coagulase-negative staphylococcal (CoNS)

| 48 | infection-causing disease (12), surviving on various surfaces for months (13). It is present in 30% of |
|----|---|
| 49 | health care-involving bloodstream infections, and is significantly associated with medical device |
| 50 | infections, including 15-40% of prosthetic valve endocarditis (14) and 30-43% of prosthetic |
| 51 | orthopedic device infections (15). More than 150 million intravascular catheters are used per year in |
| 52 | the USA, and there are about 250,000 catheter-related infections (16, 17). These bacteria are |
| 53 | developing antibiotic multi-resistances such as elevated glycopeptide minimal inhibitory |
| 54 | concentrations (18), and 73-88% of isolates display resistance to oxacillin, fluoroquinolones, |
| 55 | macrolides, clindamycin, and trimethoprim/sulfamethoxazole (19-21). |
| 56 | In this circumstance, the extracellular matrix is a complex physicochemical barrier representing one |
| 57 | of the biggest challenges in microbial treatments (4). Therefore, the development of antivirulence |
| 58 | strategies such as antibiofilm agents is crucial for the current antibiotic crisis, and peptides are an |
| 59 | increasingly arsenal for controlling pathogenic biofilms (22-24). |
| 60 | Here, we describe the discovery of the antibiofilm peptide capsicumicine, inspired by natural |
| 61 | peptides from the seeds of the red pepper Capsicum baccatum. We elucidated its mechanism of |
| 62 | action (MoA), the matrix anti-assembly (MAA). Capsicumicine is the first-in-class non-antibiotic |
| 63 | peptide displaying this extracellular MoA. Notably, we report an <i>in vivo</i> anti-infective proof of |
| 64 | concept towards to the use of capsicumicine for complementary treatment of infectious diseases. |
| 65 | |
| 66 | Results |

67 **Capsicumicine prevents biofilm formation without antibiotic activity.** We synthesized three

68 peptides inspired by a natural antibiofilm fraction previously identified from *Capsicum baccatum* var.

- 69 pendulum pepper seeds (25): P1 (RVQSEEGEDQISQRE), P2 (RAEAFQTAQALPGLCRI), and P3
- 70 (RSCQQQIQQAQQLSSCQQYLKQ). To find the most active one, we exposed these compounds to
- 71 strong biofilm-forming *S. epidermidis* RP62A (ATCC 35984). After 24 h, crystal violet was used to

72 quantify the remaining biofilm (Fig. 1A). The P3, named "capsicumicine," was the most active with 73 particularly strong antibiofilm activity. Biofilm decreases were observed at all tested concentrations, 74 but especially at 10 μ M. There, biofilm was reduced by over 91%, independently of cell growth 75 inhibition (Fig. 1B). To examine the effects of capsicumicine on growth, we checked S. epidermidis 76 colony-forming unit (CFU) counts after peptide exposure. As expected, the CFUs were unchanged by 77 capsicumicine, so the peptide's biofilm inhibition is not due to bactericidal activity (Fig. 1B). To verify 78 the interactions between capsicumicine and established matrices, we exposed a pre-existing S. 79 epidermidis biofilm to a single concentration (100 μ M) of the peptide. At that concentration, 80 capsicumicine accounts for 15% of the disruption of pre-existing biofilm (Fig. S1).



81

Fig. 1. Antibiofilm activity of bioinspired peptides and cytotoxicity. (*A*) Antibiofilm activity of peptides P1, P2, and P3 (capsicumicine) at 1, 10, and 100 μ M. Quantification of *Staphylococcus epidermidis* (ATCC 35984) biofilm and growth were done at an optical density; bacteria without peptide exposure (Control) and the antibiotic control, 16 μ g/mL rifampicin (Rif.). Student's *t*-test: *, *p* ≤ 0.05; **, *p* ≤ 0.01. (*B*) Antibiofilm activity and growth of capsicumicine at 10 μ M. Yellow represents 100% of formed biofilm and red represents the remaining biofilm after exposure to capsicumicine. Growth 88 was verified using colony-forming units (CFUs). (C) Gene expression (mean log fold changes ± 89 standard errors of the means) of the encoding genes involved in S. epidermidis biofilm formation as 90 compared to the planktonic (grey) and biofilm controls (black), with the ssrA gene used as a 91 reference. The group exposed to capsicumicine is shown in white. CWA, cell wall-anchored proteins. 92 D) Capsicumicine cytotoxicity evaluation in representative human cell lines shown via automated 93 image-based cellular content analysis. Cell counts are presented as residual cell percentages (%) 94 compared to the average of the DMSO control, with water control also shown (grey bars). The black 95 bars on the left show cytotoxic controls (roscovitine, doxycycline, and taxol), while the white bars are 96 cells exposed to 10 µM of capsicumicine. 97

- 98 To study the peptide's possible mechanisms of action, we selected several genes involved in different
- 99 stages of biofilm development (*atlE*, *aap*, *agrC*, *icaA*, *leuA*, *saeR*, *saeS*, and *sarA*); primers are listed in
- 100 Table S1. Fold changes were analyzed by quantitative real-time PCR (qRT-PCR). Since exposed
- 101 bacteria remain planktonic, we compared their relative gene expressions to planktonic control cells.
- 102 For all tested genes, capsicumicine-exposed cells show the same fold changes as the control (Fig. 1*C*).
- 103 Capsicumicine is not cytotoxic in mammalian cells. To ensure that capsicumicine is safe before
- 104 propose *in vivo* trials, we verified its cytotoxicity in seven different representative human cell lines.
- 105 We used automated image-based cellular content analysis and found that capsicumicine-treated
- 106 cells perform exactly the same as untreated controls, displaying no cytotoxicity (Fig. 1*D*).

107 Independently of cell interactions, capsicumicine impairs biofilm attachment, aggregation, and

- 108 **accumulation.** To explore its activity during the first stages of biofilm development, we analyzed
- 109 biofilm cultures on polystyrene coupons with and without capsicumicine after 1, 4, and 24 h.
- 110 Scanning electron microscopy (SEM) analysis shows that bacterial attachment decreases after 1 h of
- 111 capsicumicine exposure, with biofilm accumulation and cell aggregation profiles strongly reduced
- 112 after 4 and 24 h (Fig. 2A). This demonstrates that capsicumicine prevents *S. epidermidis* coupon
- adhesion, and notably this activity still occurs after 24 h incubation. In order to localize the peptide,
- 114 we used capsicumicine conjugated to fluorescein isothiocyanate (capsicumicine-FITC) and confocal
- 115 fluorescence microscopy (CFM). Analysis of the CFM images showed that capsicumicine-FITC stays
- 116 associated with extracellular matrix components, not entering into bacterial cells or the walls or
- 117 membranes (Fig. 2*B*).

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119 Fig. 2. Biological characterization of capsicumicine by microscopic approaches. (A) SEM images of 120 polystyrene coupons after 1, 4, or 24h of culture with Staphylococcus epidermidis (ATCC 35984). Top: 121 peptide-less biofilm control; bottom: cultures exposed to 10 µM capsicumicine. Magnification x500, 122 with insets at x5,000; scale bars, 10 µm. (B) CFM images: (left) S. epidermidis after exposure to 10 µM 123 capsicumicine-FITC, with the matrix in green (fluorescence), and the bacterial cells in black (no 124 fluorescence); (right) control, S. epidermidis after exposition to pseudonajide-FITC, an antibacterial 125 peptide, this time showing the matrix in gray (no fluorescence) and bacterial cells in green 126 (fluorescence). White arrows indicate matrix content. Scale bars, 5 µm. (C) CFM images: (left) S. 127 epidermidis after exposure to 10 µM capsicumicine-FITC; (center) after exposure to concanavalin A 128 conjugates; and (right) their colocalization. Scale bars, 5 μm. (D-G) These images explore the 129 organizational state of S. epidermidis biofilm after 24 h in the presence (right) or absence (control, 130 *left*) of capsicumicine. (*D*) Macroscopic examination by pictures from the bottom of 24-well plates. 131 The "sterile control" shows no bacteria or biofilm formation; the "biofilm control" shows 132 homogenous adhered layers of bacteria; and "capsicumicine" shows non-adhered bacteria but 133 agglutinates; scale bar, 5 mm. (E) TLM images show the biofilm control with overlapping attached 134 bacterial clusters, while the capsicumicine-exposed culture shows agglutinated non-adhered cells; 135 scale bar, 20 μ m. (F) SEM images show the control with dense globular-like matrix features, while the 136 capsicumicine-exposed culture shows fibrillary branch-like oligomer structures; scale bar, 200 nm. 137 (G) TEM images of the biofilm control show denser assembled structures, while the capsicumicine-138 exposed displays thin fibrillary oligomer structures; scale bar, 200 nm. Arrows indicate the matrices. 139 140 Capsicumicine disturbs S. epidermidis matrix assembly. Since capsicumicine's antibiofilm activity

141 was not associated with direct bacterial interactions or gene expression modulation, we used various

142 microscopic approaches to investigate the interactions between the peptide and the extracellular

143 matrix. In the biofilm control, macroscopic observation shows a homogenous whitish adhered layer 144 covering the walls and bottoms of the well (Fig. 2D, middle), but when capsicumicine is present, we 145 see whitish flocculent non-adhered heterogeneous agglutinates (Fig. 2D, right). Transmitted light 146 microscopy (TLM) images match with these macroscopic observations (Fig. 2E). Crucially, SEM and 147 TEM technique yield ultra-structural descriptions that support these results, with the control biofilm 148 matrix showing denser assembled globular-like structures, while the capsicumicine-exposed matrix is 149 less dense and has thin fibrillary branch-like structures (Fig. 2F and G). However, cellular 150 morphologies are not different from the controls. Therefore, different imaging techniques prove that 151 matrix assembly changes when capsicumicine is present. 152 Capsicumicine interacts with matrix polysaccharides. To explore the peptide's affinity with these 153 major matrix components, we exposed S. epidermidis cultures to capsicumicine (-FITC; green) and 154 saccharides staining, then analyzed them all by CFM. We used concanavalin A and calcofluor to 155 selectively target matrix saccharides (blue). Amounts of capsicumicine-FITC appear exclusively on the 156 matrix (Fig. 2B left; S2B) and its colocalization indicates the interaction between both marked 157 elements (Fig. 2C, right; S2A). Visualization was done by individual (Fig. 2C, left and center) and 158 colocalization fluorescence (Fig. 2C, right). The peptide control was pseudonajide-FITC, an 159 antibacterial peptide; it showed green fluorescence in the cells but not in the matrix (Fig. 2B right; 160 S2C). To explore whether capsicumicine features are compatible with carbohydrate-binding modules 161 (CBMs), we performed a BLAST and amino acid alignments between capsicumicine and chitin-, 162 chitosan-, and polysaccharide intercellular adhesin (PIA), PIA-binding proteins (Table S2). UniProt 163 tools (26) and CAZy information crossing (27) showed that capsicumicine does in fact present CBM 164 homology with all tested proteins (Fig. S3). 165 Capsicumicine shifts Staphylococcal synthetic matrix. To confirm these interactions in the absence

166 of bacteria metabolic or regulatory influences, we adapted a model of artificial staphylococcal biofilm

assembly to test it. Briefly, we monitored the real-time molecular self-assembly (RTMSA) reaction by

- 168 measuring the optical density at 600 nm (OD₆₀₀) as a function of time with or without capsicumicine.
- 169 OD increases when capsicumicine is present, which shows that the molecular self-assembly reaction
- 170 is quicker overall (Fig. 3A). The profiles of assembled matrices are visually different, with larger
- 171 agglutinates in the presence of capsicumicine, although for both controls are similar (Fig. 3A).
- 172 Remarkably, these profiles are comparable to those previously observed in the presence of bacteria
- 173 (Fig. 2*D*).



174

175 Fig. 3. Molecular interactions between capsicumicine and target-saccharides. (A) RTMSA curves of 176 artificial staphylococcal biofilm assembly. Optical densities (OD_{600}) were recorded as a function of the 177 time in the presence of capsicumicine (blue dots) or PA1 as peptide control (orange dots) and the 178 synthetic matrix without peptides (black dots); each reaction tub is shown under the graph. (B-D) 179 NMR titration of capsicumicine by chitosan at 600MHz, 10°C. (B) From the bottom to the top: 180 increasing concentrations of soluble chitosan [< 0.5 eq. and 8% of volume variation] were gradually 181 added to capsicumicine water solution. Specific line broadenings and frequency shifts are shown in 182 the NMR spectrum for some amide protons upon titration; white bullets for terminal R1 and Q22 and 183 black bullets for S2, C3 and Q4. (C) Assembled chitosan gradually added to soluble capsicumicine [0.5 184 eq.]; an overall line broadening was observed. (D) Assembled chitosan suddenly added to soluble 185 capsicumicine [0.5 eq.]; the orange spectrum was recorded after few minutes of stirring. (E)

Superposition of capsicumicine 1D NMR and TOCSY spectra immediately after sample preparation

- 186
- 187 (blue) and chitosan [10 eg.] addition (red) and; capsicumicine structure predicted by Phyre2 server;
- 188 according to NMR results the aggregation nucleating regions are shown as orange triangles and
- 189 favored interacting region with sugar moieties is shown in grey.
- 190

191 To evidence the interactions between capsicumicine and target-saccharides we performed NMR 192 titration experiments using chitosan as a mimic of the matrix poly-N-acetyl glucosamine (PNAG). The 193 evolutions of the capsicumicine NMR spectra upon chitosan addition were monitored. Reference 1D 194 proton NMR spectra were recorded at all tested conditions. Chitosan was gradually added from a 195 concentrated solution and spectra changes were monitored. This solution remained clear during the 196 7 days of recording. The addition of soluble chitosan drives noticeable changes of the resonance 197 frequency of some of the amide protons mainly in the N-terminal part of the peptide < S2, C3, Q4 198 and R1 (not shown) > (Fig. 3B). Conversely, to < Y19, L20 and K21 NH and the Y19 > aromatic proton 199 resonances, between 6.8 and 7.2 are not sensitive to the presence of chitosan (Fig. 3E). The 200 concentration variation of chitosan was estimated to less than 0.5eq at the end of the titration. This 201 small quantity of chitosan drives spectral modifications in the N-terminal part of capsicumicine and 202 strongly supports the interaction between both partners. The important broadening observed after 1 203 day shows that the peptide structure keeps evolving over time and this is not due to cysteines' 204 oxidation (Fig 4D, E). These experiments were repeated adding gelled chitosan instead of soluble 205 chitosan, mimicking an assembled matrix. It does not drive any significant spectral changes, except 206 for a general line broadening which probably arises from an increased viscosity of the solution (Fig. 207 3C). The suddenly addition of gelled chitosan pellets in the NMR solution, does not modify the 208 spectrum either (Fig 3D).

209 Structural requirements toward to molecular interactions with target-saccharides. To better 210 understand the mechanisms involved in capsicumicine bioactivity we step toward the knowledge of 211 its conformation in solution and molecular interactions with matrix representative saccharides. To

212 monitor the conformation of free capsicumicine in response to time and temperature we first used 213 Circular Dichroism (CD) and Nuclear Magnetic Resonance (NMR) spectroscopies. Spectral 214 deconvolution using the CDSSTR algorithm discloses a helical structure (about 43-44%) with a non-215 negligible proportion of β strands (31-33%) and unfolded (about 20%) (Table S3). The structure 216 stability was assessed over 5 days recording the CD spectra at 5°C (Fig. 4A). The main structure 217 observed over the first 24h is helical. After 5 days, deconvolutions show a slight decrease of the 218 helical content. Furthermore, on the fifth day, CD spectra were also recorded at 15 and 30°C (Fig. 219 4B). The helical content decreases to 18-23% while the β strand and turn mean proportions rose 220 around 37% and 23%, respectively. The proportion of unfolded structures also increased. After 2 221 days at room temperature, a new CD spectrum was recorded at 30°C. The helix proportion became 222 less than 15 % in favor of β strand and unfolded conformations (respectively 38-41% and 31-35%). 223 During these days, the peptide solution remained clear, showing no macroscopic signs of 224 aggregation. 225 The 1D and 2D NMR spectra of a 0.3mM freshly prepared solution of capsicumicine were recorded at 226 10°C, pH 5.0. All amide resonances were unambiguously assigned using TOCSY and NOESY spectra, 227 except for Q9 and Q11 residues (Fig. S4). The spectral dispersion and the spreading of the amide 228 proton resonances disclose that the peptide is folded with one conformation in these conditions. In 229 accordance with CD spectra, a second set of NH resonances appears as a function of time (Fig. 4C, D), 230 revealing conformational changes in the slow exchange regime on the NMR time scale. After 5 days, 231 the most important chemical shift perturbations observed on the TOCSY spectrum are clustered 232 around the two cysteines apart from the two terminal residues (boxed cross-peaks, Fig. 4E). 233 Interestingly, 17 located one helix turn apart C3 is among the most sensitive residues to the 234 conformational perturbation. This is due to a disulfide bridge (DB), since no reducing agent was 235 added. Consequently, large proton chemical shift perturbations (CSP) would be expected for every -236 NH amino acid and this was not detected (Fig. 4D, E). The prevalence of the second conformation

reaches more than 50% after 7 days (Fig. 4*C*, top). The solution remained clear overall the spectra

238 recording time.



239



Evolution vs time and temperature. (C) NMR spectra recorded at pH 5.0, 0.3mM and 10°C

242 immediately after extemporaneous preparation (bottom); a second conformation appears over the

time corresponding to the second set of amide resonances (stars). (D) Superposition of capsicumicine TOCSY spectra immediately after sample preparation (red) and at day 5 (brown). The most important

TOCSY spectra immediately after sample preparation (red) and at day 5 (brown). The most important chemical shit perturbations between the original and the new conformations are boxed and

- clustered around the two cysteines. (E) Proton chemical shift perturbations (CSP) computed from the
- 247 TOSCY spectra are displayed in the box.
- 248

249 Capsicumicine attenuates the dynamics of *S. aureus* (Xen36) infection on central venous catheter

250 (CVC). We performed a translational proof-of-concept evaluating long-term bacterial biofilm and 251 related infection in mice implanted with capsicumicine pre-coated CVC. These CVCs were previously 252 coated using an immobilization polymer ("hydrogel") encompassing capsicumicine. This coating 253 confers amorphous and biocompatible surfaces to CVC (Fig. 5A). They were first validated in vitro, 254 decreasing \geq 51 % of *S. aureus* colonization (Fig. 5*B*). Then, bacterial development was evaluated *in* 255 vivo by bioluminescence imaging and bacterial load of harvested CVC (Fig. 5C-F). Two days (D2) after 256 S. aureus systemic infection, 40% of the control group (CVC-Hydrogel) presented high 257 bioluminescence signal (red zones) related to ROI against none in the treated group (CVC-Hydrogel + 258 capsicumicine), (Fig. 5C). Four days (D4) after infection, 75% of the control presented high ROI red 259 zones against 20% in the treated group (Fig. 5C). In addition, at D4, 1 animal was found dead in the 260 control group. Therefore, bioluminescence quantifications show that the treated group decreased 261 56% (D2) and 54% (D4) of the total flux compared to the control (Fig. 5D). This trend was also 262 observed on the CFU load at D4 (Fig. 5*E*). After image acquisitions (D4), 2 animals / group were 263 euthanized due to ethical criteria. At the end of the experiment, 7 days after infection (D7), the 264 treated group showed a decrease of 86% of bacterial load compared to control (Fig. 5*E*). Macroscopic 265 observation revealed that one animal from the control presented several organs with necrosis (liver, 266 spleen, intestine, kidneys and bladder). Finally, the treated group increased survival rate of 50% at 267 D7 compared to control (Fig. 5*E*).



268

- 269 Fig. 5. Assessment of capsicumicine pre-coated central venous catheter (CVC) in mice (SKH1) infected 270 by Staphylococcus aureus Xen36. (A-B) In vitro CVC development; (A) From the top to the bottom: a 271 picture of a up to half capsicumicine pre-coated CVC (whitish), scale bar 1 cm; SEM image of pre-272 coated CVC, scale bar 0.5 mm and its magnification, scale bar 1 µm and; a compilation of images of 273 fluorescence microscopy of pre-coated CVC (capsicumicine-FITC in green); (B) Bacterial load on CVC. 274 (C-F) In vivo CVC assessment; (C) IVIS images (radiance-thresholded and smoothed with a 275 bidimensional Gaussian filter) of ventral position for each mouse at each time point; the control 276 group (CVC-Hydrogel) is shown in the top and the treated (CVC-Hydrogel + capsicumicine) in the 277 bottom; white circles delimitate the CVC localization; the luminescence scale is presented in 278 radiance. (D) Bioluminescence quantification (ROI mean signals ± SD) is shown as photons total flux 279 [p/s] for each group at each time point. (E) Bacterial load (CFU/mL) on harvested CVC for each mouse 280 at each time point (day 4 and 7 after infection) and; (F) The Kaplan-Meier survival curve (% of 281 survival) at each group, during 7 days after infection. All bioluminescence data and analysis were 282 performed using IVIS Spectrum of Perkin Elmer. All experiments were conducted in accordance with 283 ethical committee and the French authorities (n = 5 / group). Bioluminescence images are presented 284 with a defined pseudo-color scale to visualize the intensity of signals emitted.
- 285

286 Discussion

287 Bioinspired peptides are increasingly being explored as alternative biofilm controls, and become 288 important allies in the fight against bacterial tolerance and resistance (28, 29). As shown here, 289 capsicumicine, a peptide derived from *Capsicum baccatum* red pepper seeds, possesses strong 290 antibiofilm activity in vitro and in vivo. Capsicumicine prevents the establishment and maintenance 291 of biofilm architecture through a new mechanism of action the "matrix anti-assembly" (MAA). MAA 292 differs from matrix disassembly (30) as instead of de-structuring pre-established matrices it acts on 293 the initial phase of assembly preventing its correct structuration. In fact, established biofilms are 294 harder to treat than initial biofilms, because they have more complex structures (31), and increased 295 structural complexity means that more energy is required for disassembly (32). 296 Bacterial surface proteins can passively interact with surfaces such as medical devices, generating an 297 initial and reversible adhesion after electrostatic and hydrophobic interactions, Van der Waals forces 298 and others (33). Bacteria will then require extracellular matrix production in order to remain 299 attached after these weak interactions (34). During this process, physicochemical interactions drive 300 molecular and colloidal matrix self-assembly, establishing a chain of dense architecture that results in 301 stable adhesion (Fig. 6A) (35). In contrast, capsicumicine interacts with matrix saccharides and 302 modifies the self-assembly chain, resulting in a less dense and nonfunctional matrix, and impaired 303 biofilm formation (Fig. 6B).



305 Fig. 6. The matrix anti-assembly (MAA), antibiofilm mechanism of action (MoA) identified in 306 capsicumicine. (A) Untreated biofilm development on abiotic surfaces: planktonic cells interact 307 passively with the surface and start the extracellular matrix production. Initial adhesion is 308 established, after which matrix self-assembly occurs, leading to irreversible biofilm structuring and 309 adhesion. (B) Biofilm development in the presence of capsicumicine shows the MAA MoA: planktonic 310 cells are still able to interact passively with the surface. At that point, capsicumicine acts, attracting 311 extracellular saccharides and shifting the matrix self-assembly; (C) Capsicumicine (red) tends to 312 organize itself in beta leaves favoring the interactions with matrix target-saccharides (yellow). These 313 interactions occur between the non-catalytic carbohydrate binding modules of the peptide and the 314 saccharides. This peptide supra organization can compete with matrix assembly, 315 preventing/destroying intra matrix h-bonds over a large range of residues. Consequently, the MAA 316 decreases adhesion and aggregation, forcing bacteria to remain planktonic. The arrows indicate 317 reversible (white background) and irreversible stages (black background) during biofilm formation. 318 319 Capsicumicine's amino acid sequence contains several residue characteristics for recognizing 320 putative chitin-binding domains, including polar and hydrophobic residue (45% of Q) and cysteines 321 (36-39). The chitin-binding domain is a well-conserved amino acid stretch that binds specifically to N-322 acetyl glucosamine, a homologous structure of PIA (27, 40, 41). Additionally, non-catalytic 323 carbohydrate binding modules (CBMs) are contiguous amino acid sequences with a discreet fold 324 displaying carbohydrate-binding activity. In this context, capsicumicine displays homologies with all 325 tested CBM-proteins, notably with icaA protein that is a PIA synthetase from the same S. epidermidis 326 strain studied here (Fig. S3 and Table S2). 327 According to the CAZY web site, there are 86 CBMs defined families up to now 328 (http://www.cazy.org/Carbohydrate-Binding-Modules.html). CBMs exhibit different folds, with 329 secondary structures ranging from mainly β -sheet based organization to a mixture of α -helices and β -330 sheets and usually display a high content of loops and unfolded parts. Likewise, capsicumicine 331 secondary fold is a mixture of α -helix, β -sheet and unfolded regions, with conformational time and 332 temperature interconversion in favor of β -sheet and unfolded segments. The β -sheet sub-structure 333 observed in the CD concerns the Nter part of the peptide according to the chemical shifts of the 334 corresponding amino acids (between 8.6 and 9.0). The TOCSY spectra analyses disclose that the 335 conformational changes observed through the time are originated around the two cysteines, possibly

involved in intermolecular disulfide bonds. This disulfide bond is possibly intermolecular since the

- formation of an intramolecular disulfide bond is only possible by bending the structure to get both
- 338 cysteines close to each other. The size of different conformers and potential polymers are limited as
- 339 shown by the NMR peaks of free peptides which are not broadened at day 7 (Fig. 4C). As a result,
- 340 these conformational changes may correspond to structural requirements toward to interactions
- 341 with staphylococcal matrix.
- 342 Antibiofilm, matrix anti-assembly (MAA) mechanism of action
- 343 This proposed mechanism is based on a set of intermolecular (i) and cooperative (ii) forces triggered
- 344 by capsicumicine which then disrupt matrix assembly:
- 345 *(i) Intermolecular forces*
- 346 The staphylococcal matrix is composed mainly of polysaccharides, but also contains proteins (AtlE,

347 Aap, Empb), teichoic acids, and extracellular DNA (42). These saccharides are PIA/PNAG,

348 homoglycans of beta-1,6-linked 2-amino-2-deoxy-D-glucopyranosyl residues. The matrix contains

- 349 positively-charged amino groups (PNAG), as well as negative charges from O-succinylation, which
- 350 confers electrical charge lability on the matrix.

351 Supported by NMR observations, capsicumicine interacts with free chitosan, a PNAG mimetic, and 352 then switches to a higher molecular weight organization (Fig 3B). The line broadening observed with 353 the peptide-chitosan mixture after one day evidence the aggregation of chitosan particles with 354 capsicumicine over the time as schematized on Fig 6. Likewise, CSPs analysis reveals that chitosan is 355 able to bind preferentially capsicumicine N-terminus (Fig 4E) before intermolecular disulfide bridges 356 formation and the relative conformational transition. This is supported by the fact that chitosan is 357 sub-stoichiometric at the end of the NMR titration and because the capsicumicine spectrum of the 358 mixture is different from the spectra of the free peptide (Fig 4C). These interactions may proceed by 359 both hydrogen-bonding between the hydrophilic Q's lateral chains and hydrophobic/stacking 360 interactions involving I and L residues. According to NMR, the N-terminal part of the peptide is

361 probably the favored interacting region with the carbohydrate moieties. Thus, these data support 362 the MAA model in which the peptide supra organization would compete with matrix self-assembly 363 preventing intra h-bonds over a large range of residues. However, the NMR results obtained with 364 assembled chitosan, mimetizing a preformed biofilm, demonstrate that capsicumicine does not 365 interact with pre-assembled matrix (Fig 3*C*,*D*) such as demonstrated in the eradication test. 366 Additionally, the amino acids in capsicumicine are mostly neutral, counting 13 Q and 3 S although, 367 due to Q's amide group and S's hydroxyl group, they can generate electronegative (dipole-dipole) 368 zones. This high electronegativity suggests that capsicumicine may interact with the positive free 369 charges of the PIA/PNAG. Thus, these polar non-ionic forces perform moderate interactions with the 370 polysaccharides. Since strong interactions such as ionic forces may trigger unwanted effects including 371 matrix repulsion or sequestration (43, 44), the moderate interactions of capsicumicine seems to be 372 ideal.

373 (ii) Cooperative polymerization forces

374 In living systems, biomolecules perform their functions in the presence of various macromolecules of 375 different shapes and sizes, and these interactions can include cooperative polymerization forces such 376 as depletion forces (DFs) and subsequent molecular crowding (MC) (45, 46). These forces are 377 noncovalent and non-specific physicochemical interactions leading to bridging, aggregation, and 378 rheological variations (47, 48), as is observed in the presence of capsicumicine (Fig. 2D-G and 3A), 379 and also may contribute to MAA. In a suspension containing different molecules, DFs are the 380 pressures exerted by small particles, which in turn cause attractive forces between the 381 macromolecules. DFs are only expressed in crowded environments like biofilms, driving the assembly 382 and final shape of these structures (49). Taking together NMR and CD experimental results as well as 383 the TANGO and SALSA analysis (Fig. S5B), we can picture that capsicumicine can self-organize to form 384 extended structures both by disulfide bridges and β -sheet mediated fibrils formation. We propose 385 that capsicumicine-associated DFs may coagulate in polymer solutions forming fibers and parallel

386 bundles (50), explaining the observed branch-like profile (Fig. 2D-G) (51). This higher organization 387 should facilitate the interaction with the saccharides of the matrix preventing their pattern assembly 388 and resulting in flocculation (52). In a same way, MC in macromolecule solutions is characterized by 389 a decrease in accessible volume due to high macromolecule concentrations, as well as attractive and 390 repulsive forces between them (45, 53). Some molecular crowders modulate refolding kinetics and 391 decrease competing aggregation and segregation (54). In this way, capsicumicine enhances the 392 aggregation kinetics (Fig. 3A), acting as an agent of MC, decreasing entropic forces and leading to 393 segregation (55).

Thus, capsicumicine' structures are chemically enable to interact with staphylococcal matrices saccharides. These combined intermolecular and cooperative forces disturb matrix self-assembly at both molecular and colloidal levels. Consequently, matrix functionality shifts and antibiofilm activity occurs. In other hand, planktonic microorganisms are more available for the innate immune system to recognize and clear them (*56*). Furthermore, non-antibiotic activity appears less susceptible to the development of bacteria resistance because these microorganisms are under less evolutionary pressure then when exposed to conventional antibiotics (*57, 58*).

Finally, we demonstrated that capsicumicine strongly prevents biofilm formation for the most frequent bacteria related to nosocomial infections, *S. epidermidis* and *S. aureus*. We first confirmed that capsicumicine was not cytotoxic thus being safe for *in vivo* tests. Then, we trialed the peptide in a translational pre-clinical model that mimics medical device-related infection. As a result, precoated CVCs enhanced mice survival decreasing *S. aureus* colonization and consequently attenuated the infection.

407 Although there are no antibiofilm drugs available here we relate the discovery of the first-in-class

408 carbohydrate-binding peptide as a promising candidate for complementary drug/treatment of

409 infectious diseases. In particular, we elucidated its antibiofilm mechanism of action, matrix anti-

410 assembly (MAA) and validated a proof of concept towards to an *in vivo* application.

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412 Materials and Methods

- 413 **Peptides.** All peptides were synthesized by Biomatik and ProteoGenix at purity grades over 95% in
- 414 salts suitable for cell culture. For the assays, the peptides were all solubilized in ultra-pure sterile
- 415 water.

416 **Bacterial strains and growth conditions.** *Staphylococcus epidermidis* ATCC 35984 was grown

417 overnight on blood agar (Thermo Scientific Oxoid PB5039A) at 37 °C. Oxoid LB agar was used for the

418 colony-forming unit (CFU) assay. The other assays were done using a bacterial suspension of 3×10^8

419 CFU/mL in tryptone soya broth (TSB, Oxoid) or 0.9% NaCl.

420 Biofilm formation. At least three technical and biological replicates were done for each assay of 1,

421 10, or 100 μM peptide concentrations. *Biofilm formation inhibition:* A protocol adapted from Trentin

422 et al (59) was used, with crystal violet in 96-well BD Falcon polyvinyl chloride (PVC) microtiter plates.

423 The cell-bound stains were solubilized with absolute ethanol (Sigma-Aldrich), and absorbance was

424 measured at 570 nm using a BIO-TEK PowerWave XS plate reader. The biofilm formation control

425 represents 100% of biofilm formation. *Biofilm eradication:* Biofilm was pre-formed as described

426 above for 24 h at 37 °C without treatment. Afterwards, the wells were washed to remove planktonic

427 cells, peptide solutions and controls were added, and all were incubated for 24 h. Biofilm eradication

428 was verified by evaluating the remaining content by crystal violet.

429 **Bacterial growth assays.** *Microtiter plates:* Bacterial growth was evaluated by comparing OD₆₀₀

430 values at the start and end of incubation in 96-well PVC microtiter plates. *Colony-forming units:* After

431 incubation at 37 °C for 24 h, CFU/mL was calculated to determine the peptide solution's bactericidal

- 432 effects. The untreated growth control was considered to be 100% planktonic cells. At least three
- 433 technical and biological replicates were performed for all assays.

434 Quantitative reverse transcription PCR (qRT-PCR): After culturing for 24 h, RNAs were isolated from 435 planktonic controls, biofilm controls, and from total cells exposed to 10 µM capsicumicine. An 436 Invitrogen TRIzol Max bacterial RNA isolation kit and an Ambion TURBO DNase treatment were used 437 as per manufacturer instructions. Total RNA concentrations and purities were assessed using a 438 Biochrom SimpliNano spectrophotometer, and PCR reactions was done to ensure the complete 439 absence of DNA. Each qRT-PCR reaction was then subjected to previously established quantities of 440 cDNA (10 ng) and primers (0.2 μ M). Reactional volumes were calculated using SYBR Select Master 441 Mix (Applied Biosystems), as per the manufacturer's instructions. Primers (see Table S1) were 442 designed using the Primer3 program, then produced by Eurofins Genomics. Applied Biosystems 443 StepOnePlus equipment and software were used. Relative transcript levels were determined by the 444 $2^{-\Delta\Delta ct}$ method (60).

445 Cytotoxicity assays: Cytotoxicity assays were performed on the ImPACcell robotic platform (BIOSIT,
446 Université de Rennes 1). Multiparameter high-content screening (HCS) and high-content analysis
447 (HCA) were done on 7 different mammalian lines: HuH7, CaCo-2, MDA, HCT116, PC3, NCI-H727, and
448 MCF7. The number of normal cells is presented as residual cell percentage compared to the DMSO
449 control average.

450 **Microscopic analysis.** *S. epidermidis* ATCC 35984 biofilm was cultured as described above.

Scanning electron microscopy (SEM): Sterile 10x4mm polystyrene coupons were inserted into bacterial cultures in the presence or absence of capsicumicine for 1, 4, and 24 h. The coupons were then washed with sterile 0.9% NaCl and fixed with 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M cacodylate buffer (pH 7.2). Afterwards, they were washed with 0.1 M cacodylate buffer and 0.2 M sucrose, then dehydrated with increasing concentrations of ethanol. A Leica EM CPD300 was used for critical point drying of the dehydrated samples. These were then sputtered with palladium in a Leica EM ACE200, and analyzed with a JEOL JSM-7100F microscope with EDS and EBSD at 10 kV. 458 Transmission electron microscopy (TEM): All well content was carefully detached at 1, 4, and 24 h, 459 centrifuged at 10,000 g for 15 min at 4 °C, then washed with sterile 0.9% NaCl. Fixation was 460 performed at 4 °C with sodium 0.1 M cacodylate, 2% paraformaldehyde, 2.5% glutaraldehyde, and 75 461 mM lysine. Samples were washed with 0.1 M sodium cacodylate and 0.2 M sucrose and contrasted 462 with 1% osmium tetroxide and 1.5% potassium ferrocyanide. Dehydration was done with a gradual 463 solution of ethanol and infiltration of increasing concentrations of LR White resin (Delta 464 Microscopies, France). LR White resin inclusion and polymerization were then performed over 24 h 465 at 60 °C in the absence of O₂. Thin 80 nm sections were collected onto carbon grids, and visualized at 466 200 kV with a FEI Tecnai Sphera microscope equipped with a Gatan 4k x 4k CCD UltraScan camera. 467 Confocal fluorescence microscopy (CFM): Capsicumicine-fluorescein isothiocyanate (capsicumicine-468 ITC, 10 μ M) was used to detect the capsicumicine peptide, whose antibiofilm activity was previously 469 verified. After incubation for 1, 4, or 24 h, the well contents were carefully detached, centrifuged at 470 11,000g for 2 min at 4 °C, then washed with sterile 0.9% NaCl. The suspension was visualized directly 471 or after adding 0.1µg/µL concanavalin A conjugates (Alexa Fluor[®] 633, Invitrogen) or 2 mg/mL 472 Calcofluor White dye (Fluorescent Brightener 28, Sigma-Aldrich). To find bacterial cells permeated by 473 the peptide control, we used pseudonajide FITC-labelled antimicrobial peptide (Ref). Images were 474 acquired via resonant scanner with a Leica SP8 DMI 6000 CS confocal microscope with hybrid 475 detector, and ImageJ software was used for image analysis.

476 Real-time molecular self-assembly (RTMSA) assay: After checking the starting point (pH 7.2) of the 477 assembly reaction for the synthetic staphylococcal matrix (*35*), we recorded the OD₆₀₀ as a function 478 of the time every 30 sec until 30 min. Molecular self-assembly reactions were calculated to a final 479 volume of 4 mL, with 0.3% chitosan (medium molecular weight, 75-85% deacetylation), 0.15% bovine 480 serum albumin, and 0.015% lambda DNA (all from Sigma) in TSB. The concentration (µM) of tested 481 peptides was calculated for a final volume of 4 mL. Before getting the assembly reaction pH starting 482 points, a calibration record was done using the same reactional tube containing all reagents (auto 483 zero). Acetic acid and NaOH were used to adjust pH, and the reaction temperature was about 30 °C.

484 As a negative control a similarly sized peptide was used, PA-1 (61).

485 NMR: NMR spectra were recorded in 3mm tubes on a Bruker Avance III 600MHz spectrometer 486 equipped with a TXI (1H,13C,15N) probe and a Z-gradient unit. Spectra were processed with Topspin 487 4.0.8 (Bruker Biospin) and CcpNmr Analysis (62). TOCSY and NOESY experiments were respectively 488 recorded at 10°C and pH5.0 with a 70ms and 300ms mixing time. Capsicumicine concentration in 489 water was set at 0.3mM and the pH adjusted either 5.0 or to 3.5 with few microliters of deuterated 490 HCl 0.1M and/or NaOH 0.1M solutions. Chitosan powder (purchased from Sigma) was dissolved in 491 pure water and the pH adjusted to either 5.0 or 3.5. Reference 1D proton NMR spectra were 492 recorded at 10°C and either at pH3.5 and 5.0. Chitosan was stepwise added from a concentrated 493 solution at pH 5.0 or 3.5 and spectra changes were monitored. We first titrated capsicumicine 494 solution with chitosan solution at pH 5.0. The concentration variation of chitosan was estimated 495 using the peaks intensities, resulting in 3.6 and 3.8 ppm (not shown) in the beginning to less than 496 0.5eq at the end of the titration. These experiments were repeated with a starting capsicumicine 497 solution at pH 3.5 and the gradually addition of gelled chitosan, at pH 3.5. The suddenly addition of 498 gelled chitosan pellets in the NMR solution, at pH3.5.

499 **Circular Dichroism (CD):** The peptide was dissolved in 18M[®] water at a concentration of 50µM, at pH 500 5.0. The ultraviolet CD spectra were recorded at 5, 15 and 30°C, in a 0.1cm path length quartz cell on 501 a Jobin Yvon CD6 spectrometer equipped with a temperature controller unit, over a 180-260 nm 502 range, with a 2nm bandwidth, a step size of 1 nm and an integration time of 2s per point. The 503 samples were conserved at 5°C between each recording. Spectra were averaged over 5 records. 504 Water CD contributions were subtracted from CD spectra before processing. Spectra were processed 505 using Kaleidagraph (Synergy Software). Molar circular dichroism (22) per residue and molar ellipticity 506 per residue (22), MER) were computed from the difference of the delta absorbance recorded by the 507 spectrometer. Raw delta epsilon per residue spectra were analyzed using the CDSSTR program (63)

and different reference protein data sets provided by the DICHROWEB facility (64). MER data curves
were smoothed for presentation, using the interpolate and weighted data (5%) routines provide by
Kaleidagraph.

511 TANGO (65) and SALSA (66) predictions: Tango predictions were run at 298K. Predictions were
512 identical for pH set either to 5.0, 7.0 or 9.0. SALSA predictions were run with a window size dynamic
513 of 4-20 residues, a cutoff of 1.2 and a minimal hot spot length of 5.

514 **CVC coatings:** We adapted an approach to immobilize peptides based on poly(ethylene

515 glycol)diacrylate (PEGDA) hydrogel (67). Briefly, polyurethane (PU) tubes (Instech) of 2Fr or

516 25G equivalent were used as coating framework. Hydrogel base was prepared using

517 pentaerythritol tetrakis(3-mercaptopropionate) - PTMP (4.1 mmol), PEGDA (10 mmol), PEG-

518 600 (20mM), 2,2-Dimethoxy-2-phenylacetophenone - DMPA (0.1 wt%), THF soluble PU (10

519 wt%) and methanol (qs). Peptides were solubilized in DMSO and added to hydrogel base

520 under vortex agitation. PU tubes were first internally coated by suction using a needle-

521 syringe and then externally by immersion. The reaction and polymerization conditions such

522 as room temperature and oxygen tolerance were convenient. After polymerization,

523 successive washes with methanol under agitation were used to eliminate undesirable

524 monomers. All reagents were purchased from Sigma Aldrich.

525 **CVC infection assay**: This study was conducted by Voxcan s.a.r.l. The ethical agreement is part of the 526 project n° APAFiS# 10756-2017072522272676 v4, approved by Voxcan ethical committee (CEAA-129) 527 and the French authorities (ministry of national education, the higher education and research). This 528 study used SKH1 mice, females, immunocompetent and specific pathogen free (SPF) provided by 529 Charles River Laboratories. Animals were acclimated at least 2 days, housed collectively in disposable 530 standard cages in ventilated racks A3, at +21 ± 3°C, 30-70% of humidity, 12 hours of dark and light 531 cycles, with filtered water and autoclaved standard food provided *ad libitum*. Catheters were blind 532 implanted (n = 5 / group) in mice jugular vein following by an intravenous (IV) inoculation of S. 533 *aureus* Xen36, 5 days days later, which was in turn to colonize the device from the blood circulation. 534 The bacterial development was evaluated and compared between the different catheters by *in vivo* 535 bioluminescence imaging (IVIS Spectrum, acquisition and analysis with Living Image 4.5.5 version) 536 performed 2 and 4 days after mice infection. In addition, 7 days after inoculation or at the time of 537 mice euthanasia for ethical reason, catheters were harvested and bacterial load were evaluated by 538 CFU counting (SCAN500). All along the study, mice clinical state was evaluated using a scoring grid 539 and body weight measurements 3 times a week. At each timepoint, a bioluminescence acquisition 540 was also performed on the background (BKG) mouse to measure the flux level corresponding to the 541 auto-bioluminescence. Images were radiance-thresholded with respect to the background radiance 542 level and smoothed with a bidimensional Gaussian filter (3x3).

543

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- 556 A1 at European Patent Office. Specific aspect of manuscript covered in patent application: its amino
- 557 acid sequence and bioactivity.
- 558 **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present
- in the paper and/or the Supplementary Materials. Additional data related to this paper may be
- 560 requested from the authors.
- 561

562 **References**:

- 563 1. WHO, W. H. Organization. (2015).
- 564 2. WHO, W. H. Organization. (2018).
- 5653.A. Penesyan, M. Gillings, I. T. Paulsen, Antibiotic discovery: combatting bacterial resistance in566cells and in biofilm communities. *Molecules* **20**, 5286-5298 (2015).
- 567 4. H.-C. Flemming, J. Wingender, The biofilm matrix. *Nature Reviews Microbiology* 8, 623-633
 568 (2010).
- 5695.A. Taglialegna *et al.*, Staphylococcal Bap Proteins Build Amyloid Scaffold Biofilm Matrices in570Response to Environmental Signals. *PLoS Pathog* **12**, e1005711 (2016).
- 571 6. N. Hoiby, T. Bjarnsholt, M. Givskov, S. Molin, O. Ciofu, Antibiotic resistance of bacterial
 572 biofilms. *International Journal of Antimicrobial Agents* **35**, 322-332 (2010).
- 573 7. C. Beloin, S. Renard, J. M. Ghigo, D. Lebeaux, Novel approaches to combat bacterial biofilms.
 574 *Curr Opin Pharmacol* 18C, 61-68 (2014).
- 5758.D. Davies, Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov 2,576114-122 (2003).
- 577 9. A. Brauner, O. Fridman, O. Gefen, N. Q. Balaban, Distinguishing between resistance,
 578 tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol* 14, 320-330 (2016).
- 57910.V. Defraine, M. Fauvart, J. Michiels, Fighting bacterial persistence: Current and emerging580anti-persister strategies and therapeutics. Drug Resist Updat 38, 12-26 (2018).
- 581 11. M. Otto, Physical stress and bacterial colonization. *FEMS Microbiol Rev* **38**, 1250-1270 (2014).
- 12. I. Uçkay *et al.*, Meticillin resistance in orthopaedic coagulase-negative staphylococcal
 infections. *J Hosp Infect* **79**, 248-253 (2011).
- 584 13. M. Otto, Staphylococcal biofilms. *Bacterial Biofilms* **322**, 207-228 (2008).
- 585 14. Y. Nishizaki *et al.*, Japanese features of native valve endocarditis caused by coagulase-586 negative staphylococci: case reports and a literature review. *Intern Med* **52**, 567-572 (2013).
- 587 15. C. L. Abad, A. Haleem, Prosthetic Joint Infections: an Update. *Curr Infect Dis Rep* **20**, 15 (2018).
- 58916.M. E. Rupp, Clinical characteristics of infections in humans due to Staphylococcus590epidermidis. *Methods Mol Biol* **1106**, 1-16 (2014).
- 59117.CDCP, Center for Disease Control and Prevention. Vital signs: central line-associated blood592stream infections -United States, 2001, 2008, and 2009. MMWR Morb Mortal Wkly Rep 60,593243-248 (2011).
- 59418.J. Y. H. Lee *et al.*, Global spread of three multidrug-resistant lineages of Staphylococcus595epidermidis. *Nat Microbiol*, (2018).

| 596 597 508 | 19. | J. M. Streit, R. N. Jones, H. S. Sader, T. R. Fritsche, Assessment of pathogen occurrences and resistance profiles among infected patients in the intensive care unit: report from the |
|-------------------|-----|---|
| 598 599 | | 24 , 111-118 (2004). |
| 600 601 | 20. | R. K. Flamm <i>et al.</i> , Linezolid Surveillance Results for the United States (LEADER Surveillance Program 2014). <i>Antimicrob Agents Chemother</i> 60 , 2273-2280 (2016). |
| 602 603 | 21. | R. Hope <i>et al.</i> , Non-susceptibility trends among staphylococci from bacteraemias in the UK and Ireland, 2001-06. <i>J Antimicrob Chemother</i> 62 Suppl 2 , ii65-74 (2008). |
| 604 | 22. | N. Strempel, J. Strehmel, J. Overhage, Potential Application of Antimicrobial Peptides in the |
| 605 606 | 23. | Treatment of Bacterial Biofilm Infections. <i>Current Pharmaceutical Design</i> 21 , 67-84 (2015). C. de la Fuente-Nunez <i>et al.</i> , D-Enantiomeric Peptides that Eradicate Wild-Type and |
| 607 | | Multidrug-Resistant Biofilms and Protect against Lethal Pseudomonas aeruginosa Infections |
| 608 | ~ ~ | (vol 22, pg 196, 2015). <i>Chemistry & Biology</i> 22 , 1280-1282 (2015). |
| 609 | 24. | O. M. El-Halfawy <i>et al.</i> , Discovery of an antivirulence compound that reverses β -lactam |
| 610 | | resistance in MRSA. <i>Nat Chem Biol</i> 16 , 143-149 (2020). |
| 611 612 | 25. | adhesion and biofilm formation. <i>Int J Pharm</i> 574 , 118872 (2020). |
| 613 614 | 26. | The UniProt Consortium, UniProt: the universal protein knowledgebase. <i>Nucleic Acids Res</i> 45 , D158-D169 (2017) |
| 615 | 27. | V. Lombard, H. Golaconda Ramulu, F. Drula, P. M. Coutinho, B. Henrissat, The carbohydrate- |
| 616 | | active enzymes database (CAZy) in 2013. <i>Nucleic Acids Res</i> 42 . D490-495 (2014). |
| 617 | 28. | C. Feuillie <i>et al.</i> . Molecular interactions and inhibition of the staphylococcal biofilm-forming |
| 618 | | protein SdrC. <i>Proc Natl Acad Sci U S A</i> 114 . 3738-3743 (2017). |
| 619 | 29. | S. Schmitt <i>et al.</i> , Analysis of modular bioengineered antimicrobial lanthipeptides at nanoliter |
| 620 | | scale. Nat Chem Biol 15, 437-443 (2019). |
| 621 | 30. | R. Roy, M. Tiwari, G. Donelli, V. Tiwari, Strategies for combating bacterial biofilms: A focus on |
| 622 | | anti-biofilm agents and their mechanisms of action. Virulence 9, 522-554 (2018). |
| 623 | 31. | S. Jabbouri, I. Sadovskaya, Characteristics of the biofilm matrix and its role as a possible |
| 624 | | target for the detection and eradication of Staphylococcus epidermidis associated with |
| 625 | | medical implant infections. FEMS Immunol Med Microbiol 59, 280-291 (2010). |
| 626 | 32. | D. Fleming, K. P. Rumbaugh, Approaches to Dispersing Medical Biofilms. <i>Microorganisms</i> 5, |
| 627 | | (2017). |
| 628 629 | 33. | C. R. Armbruster, M. R. Parsek, New insight into the early stages of biofilm formation. <i>Proc</i> <i>Natl Acad Sci U S A</i> 115 , 4317-4319 (2018). |
| 630 | 34. | M. Otto, Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as |
| 631 | | Critical Determinants of Pathogenicity. Annual Review of Medicine, Vol 64 64, 175-188 |
| 632 | | (2013). |
| 633 | 35. | E. J. Stewart, M. Ganesan, J. G. Younger, M. J. Solomon, Artificial biofilms establish the role of |
| 634 | | matrix interactions in staphylococcal biofilm assembly and disassembly. Sci Rep 5, 13081 |
| 635 | | (2015). |
| 636 | 36. | H. Hemmi, J. Ishibashi, T. Tomie, M. Yamakawa, Structural basis for new pattern of conserved |
| 637 | | amino acid residues related to chitin-binding in the antifungal peptide from the coconut |
| 638 | | rhinoceros beetle Oryctes rhinoceros. J Biol Chem 278, 22820-22827 (2003). |
| 639 | 37. | T. Suetake et al., Chitin-binding proteins in invertebrates and plants comprise a common |
| 640 | | chitin-binding structural motif. J Biol Chem 275, 17929-17932 (2000). |
| 641 | 38. | H. T. Wright, G. Sandrasegaram, C. S. Wright, Evolution of a family of N-acetylglucosamine |
| 642 | | binding proteins containing the disulfide-rich domain of wheat germ agglutinin. J Mol Evol |
| 643 | | 33 , 283-294 (1991). |
| 644 | 39. | J. Yin, S. Yang, K. Li, W. Guo, Y. Cao, Identification and molecular characterization of a chitin- |
| 645 646 | | binding protein from the beet webworm, Loxostege sticticalis L. <i>Int J Mol Sci</i> 15 , 19147-19161 (2014). |

| 647 648 | 40. | S. El-Gebali <i>et al.</i> , The Pfam protein families database in 2019. <i>Nucleic Acids Res</i> 47 , D427-D432 (2019). |
|------------|------------|---|
| 649 | 41. | W. Suginta, P. Sirimontree, N. Sritho, T. Ohnuma, T. Fukamizo, The chitin-binding domain of a |
| 650 | | GH-18 chitinase from Vibrio harvevi is crucial for chitin-chitinase interactions. <i>Int J Biol</i> |
| 651 | | Macromol 93 , 1111-1117 (2016) |
| 652 | 42 | A E Paharik A R Horswill The Stanhylococcal Biofilm: Adhesins Regulation and Host |
| 653 | 12. | Response Microhiol Spectr 4 (2016) |
| 654 | 13 | G Batoni G Maisetta S Esin Antimicrobial pentides and their interaction with biofilms of |
| 655 | 45. | medically relevant hacteria. <i>Biochimica Et Biophysica Acta-Biomembranes</i> 1858 , 10/4-1060 |
| 656 | | |
| 657 | 11 | A Sharma P. Gunta R. Kumar A. Bhardwai, dPARBs: A Novel in silico Approach for |
| 658 | | Predicting and Designing Anti-hiofilm Pentides Sci Ren 6 21839 (2016) |
| 659 | <i>4</i> 5 | S E Banani H O Lee A A Hyman M K Rosen Biomolecular condensates: organizers of |
| 660 | 45. | cellular hiochemistry. Nat Rev Mol Cell Riol 18 , 285-298 (2017) |
| 661 | 46 | C Rest R Kandanelli G Fernández Strategies to create hierarchical self-assembled |
| 662 | 40. | structures via cooperative pon-covalent interactions. <i>Chem</i> Soc Rev 11 , 2543-2572 (2015) |
| 663 | 47 | A Kudlay M S Cheung D Thirumalai Influence of the shape of crowding particles on the |
| 66A | 47. | structural transitions in a polymer <i>I Phys Chem B</i> 116 , 8512-8522 (2012) |
| 665 | 10 | C Even et al. Recent advances in studying single bacteria and hiofilm mechanics. Adv Colloid |
| 666 | 40. | Interface Sci 247 572-588 (2017) |
| 667 | 10 | D. Marenduzzo, K. Einan, D. P. Cook. The depletion attraction: an underappreciated force |
| 668 | 49. | driving cellular organization <i>L Cell Biol</i> 175 , 681-686 (2006) |
| 660 | 50 | H X Zhou Effect of mixed macromolecular crowding agents on protein folding. Proteins 72 |
| 670 | 50. | 11.0, $2100, 2100, 2100, 2100, 200, 200, 200, 2$ |
| 671 | 51 | A Sakaguchi K Higashiguchi K Matsuda Bundle formation of supramolecular fibers of |
| 672 | 51. | amphinhilic diarylethene by depletion force. <i>Chem Commun (Camb)</i> 54 A298-A301 (2018) |
| 673 | 52 | R Fantoni A Santos Depletion force in the infinite-dilution limit in a solvent of nonadditive |
| 674 | 52. | hard spheres. J Chem Phys 140 , 244513 (2014). |
| 675 | 53. | W. M. Aumiller, B. W. Davis, C. D. Keating, Phase separation as a possible means of nuclear |
| 676 | | compartmentalization. Int Rev Cell Mol Biol 307 , 109-149 (2014). |
| 677 | 54. | S. Biswas, J. Kundu, S. K. Mukherjee, P. K. Chowdhury, Mixed Macromolecular Crowding: A |
| 678 | | Protein and Solvent Perspective. ACS Omega 3, 4316-4330 (2018). |
| 679 | 55. | J. M. Polson, D. R. Kerry, Segregation of polymers under cylindrical confinement: effects of |
| 680 | | polymer topology and crowding. Soft Matter 14, 6360-6373 (2018). |
| 681 | 56. | C. de la Fuente-Núñez, F. Reffuveille, L. Fernández, R. E. Hancock, Bacterial biofilm |
| 682 | | development as a multicellular adaptation: antibiotic resistance and new therapeutic |
| 683 | | strategies. Curr Opin Microbiol 16, 580-589 (2013). |
| 684 | 57. | A. M. Krachler, K. Orth, Targeting the bacteria-host interface: strategies in anti-adhesion |
| 685 | | therapy. Virulence 4, 284-294 (2013). |
| 686 | 58. | L. Travier, O. Rendueles, L. Ferrieres, JM. Herry, JM. Ghigo, Escherichia coli Resistance to |
| 687 | | Nonbiocidal Antibiofilm Polysaccharides Is Rare and Mediated by Multiple Mutations Leading |
| 688 | | to Surface Physicochemical Modifications. Antimicrobial Agents and Chemotherapy 57, 3960- |
| 689 | | 3968 (2013). |
| 690 | 59. | D. S. Trentin <i>et al.</i> , Natural Green Coating Inhibits Adhesion of Clinically Important Bacteria. |
| 691 | | Scientific Reports 5, (2015). |
| 692 | 60. | K. J. Livak, T. D. Schmittgen, Analysis of relative gene expression data using real-time |
| 693 | | quantitative PCR and the 2(-Delta Delta C(T)) Method. <i>Methods</i> 25 , 402-408 (2001). |
| 694 | 61. | P. Liu et al., Targeting Inhibition of SmpB by Peptide Aptamer Attenuates the Virulence to |
| 695 | | Protect Zebrafish against. Front Microbiol 8, 1766 (2017). |
| 696 | 62. | S. P. Skinner et al., CcpNmr AnalysisAssign: a flexible platform for integrated NMR analysis. J |
| 697 | | <i>Biomol NMR</i> 66 , 111-124 (2016). |

- 63. N. Sreerama, R. W. Woody, Estimation of protein secondary structure from circular dichroism
 spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference
 set. Anal Biochem 287, 252-260 (2000).
- 70164.L. Whitmore, B. A. Wallace, DICHROWEB, an online server for protein secondary structure702analyses from circular dichroism spectroscopic data. *Nucleic Acids Res* **32**, W668-673 (2004).
- A. M. Fernandez-Escamilla, F. Rousseau, J. Schymkowitz, L. Serrano, Prediction of sequencedependent and mutational effects on the aggregation of peptides and proteins. *Nat Biotechnol* 22, 1302-1306 (2004).
- 5. Zibaee, O. S. Makin, M. Goedert, L. C. Serpell, A simple algorithm locates beta-strands in
 the amyloid fibril core of alpha-synuclein, Abeta, and tau using the amino acid sequence
 alone. *Protein Sci* 16, 906-918 (2007).
- R. T. C. Cleophas *et al.*, Convenient Preparation of Bactericidal Hydrogels by Covalent
 Attachment of Stabilized Antimicrobial Peptides Using Thiol–ene Click Chemistry. *ACS Macro Letters* 3, 477-480 (2014).
- 71268.L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, M. J. Sternberg, The Phyre2 web portal for713protein modeling, prediction and analysis. *Nat Protoc* **10**, 845-858 (2015).
- 714

715 Supplementary Materials

716 **Table S1.** Primers used in this study. These were previously designed using the Primer3 program and717 standardized by our team.

| Gene | Forward primer 5'-3' | Reverse primer 5'-3' | Amplicon |
|------|------------------------|--------------------------|----------|
| atlE | TACCAGGGTTTGCAGGATTC | GGCGCTAAATTCATTGGAAA | 85 pb |
| аар | AGGCCGTACCAACAGTGAAT | ATGGGCAAACGTAGACAAGG | 100 pb |
| agrC | TCATCAATATCGCATTCATCG | CCTAAACCGCGATTATCACC | 136 pb |
| icaA | TTATCAATGCCGCAGTTGTC | CCGTTGGATATTGCCTCTGT | 104 pb |
| leuA | GATGATCTCGGAATGGCAGT | TGAGGCATTTCCTGCTCTTT | 108 pb |
| saeR | GCTAACACTGTCAATGTCCACA | AGGCCCCACACAGTTGTAAT | 92 pb |
| saeS | GGCGTCAATTTGTTGTGCTA | AGGGCATAGGTATCGTTCCA | 140 pb |
| sarA | TTTGCTTCTGTGATACGGTTGT | CGTAATGAACACGATGAAAGAACT | 107 pb |
| gyrB | ATCAACATCGGCATCAGTCA | GCATTTGGTACGGGTATTGG | 87 pb |
| rrsA | AAGCAACGCGAAGAACCTTA | ATGCACCACCTGTCACTCTG | 95 pb |

718

720 Table S2. Carbohydrate-binding domain proteins that are capsicumicine homologs. These proteins

- 721 are available in the UniProtKB database and were used for BLAST and amino acid alignments to
- 722 perform similarity analysis.

| Entry | Name | Accession ID |
|------------------|--|--------------|
| ICAA_STAEQ | Intercellular adhesion protein icaA | Q5HKQ0.1 |
| ICAD_STAEQ | Poly-beta-1,6-N-acetyl-D-glucos. synthase prot. icaD | Q5HKP9 |
| A7Z8H9_BACVZ | Chitosanase | A7Z8H9 |
| A0A0N0MLT5_9ACTN | Chitosanase | A0A0N0MLT5 |
| CBP2_MOROL | Chitin-binding protein 2 | COHKC5 |
| A0A1R0GTZ5_9FUNG | Chitin synthase 8 | A0A1R0GTZ5 |
| A0A194V113_9PEZI | Chitin biosynthesis protein CHS5 | A0A194V113 |

⁷²³

724

725 Table S3. Results of the CD spectra deconvolution using CDSSTR algorithm.

| Day | Temp (°C) | Helix | Bêta | Turn | Unfolded |
|-----|-----------|-------|-------|-------|----------|
| | | (%) | (%) | (%) | (%) |
| 0 | 5 | 42-46 | 29-34 | 5-8 | 16-21 |
| 1 | 5 | 42-45 | 30-32 | 6-8 | 20-21 |
| 5 | 5 | 38-42 | 24-31 | 8-12 | 17-23 |
| 5 | 15 | 22-26 | 36-45 | 13-14 | 20-26 |
| 5 | 30 | 16-24 | 33-40 | 18-20 | 20-25 |
| 7 | 30 | 6-17 | 38-41 | 10-23 | 31-35 |

726

727



728

- 729 Fig. S1. Biofilm eradication test. Shown are Staphylococcus epidermidis (ATCC 35984) biofilm
- 730 quantifications at OD₅₇₀ for the bacterial biofilm without peptide exposure ("Growth Control"), after exposure to the rifampicin antibiotic control, and after 24 h treatment with 100 µM capsicumicine.





734 **Fig. S2.** Confocal fluorescence microscopy (CFM) of *Staphylococcus epidermidis* (ATCC 35984).

735 Calcofluor was used to highlight matrix polysaccharides (blue), and FITC used for the peptides

736 (green). Visualization was done by fluorescence (left) and transmitted light (right) microscopy. (A)

Cultures exposed to capsicumicine and calcofluor. (*B*) Cultures exposed to capsicumicine-FITC. (*C*)

738 Peptide negative control cultures exposed to an antimicrobial peptide-FITC (Pseudonajide). The

 $739 \qquad \text{matrix is highlighted (circles). Scale bars, 5 \ \mu\text{m}.}$

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- 742 **Fig. S3.** Amino acid alignment of capsicumicine and its carbohydrate-binding domain protein
- homologs. (a) The icaA (protein accession number Q5HKQ0.1) fragment from 1 to 60. (b)
- 744 Capsicumicine fragment from 1 to 21. (c) Chitosanase (A7Z8H9) fragment from 1 to 60 and from 61
- to 120. (d) Capsicumicine fragment from 1 to 15 and from 16 to 22. (e) Chitin synthase 8
- 746 (A0A1R0GTZ5) fragment from 1801 to 1860 and from 1921 to 1967. (f) Capsicumicine fragment from
- 1 to 14 and from 16 to 22. Equal (* and grey highlighting), similar (.), and highly similar (:) amino
- acids are indicated, as well as amino acid polar characteristics (purple). Support for this analysis is
- available at UniProt.
- 750

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- 759 **Fig. S5.** (*A*) Predicted capsicumicine structure using the Phyre² prediction server. (*B*) Aggregation
- 760 structure predictions using TANGO (68) and SALSA algorithms (66).