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

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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**

24 **components in *Capsicum* peppers (CP). Here, we describe the development of CP bioinspired**
25 **peptide “capsicumicine”. We demonstrate that capsicumicine strongly prevents methicillin-**
26 **resistant *S. epidermidis* biofilm via a new extracellular “matrix anti-assembly” mechanism of**
27 **action. Catheters pre-coated with capsicumicine decreased *S. aureus* colonization leading to the**
28 **attenuation of infection, decreasing mice systemic infection. Capsicumicine is the first-in-class non-**
29 **antibiotic, 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).

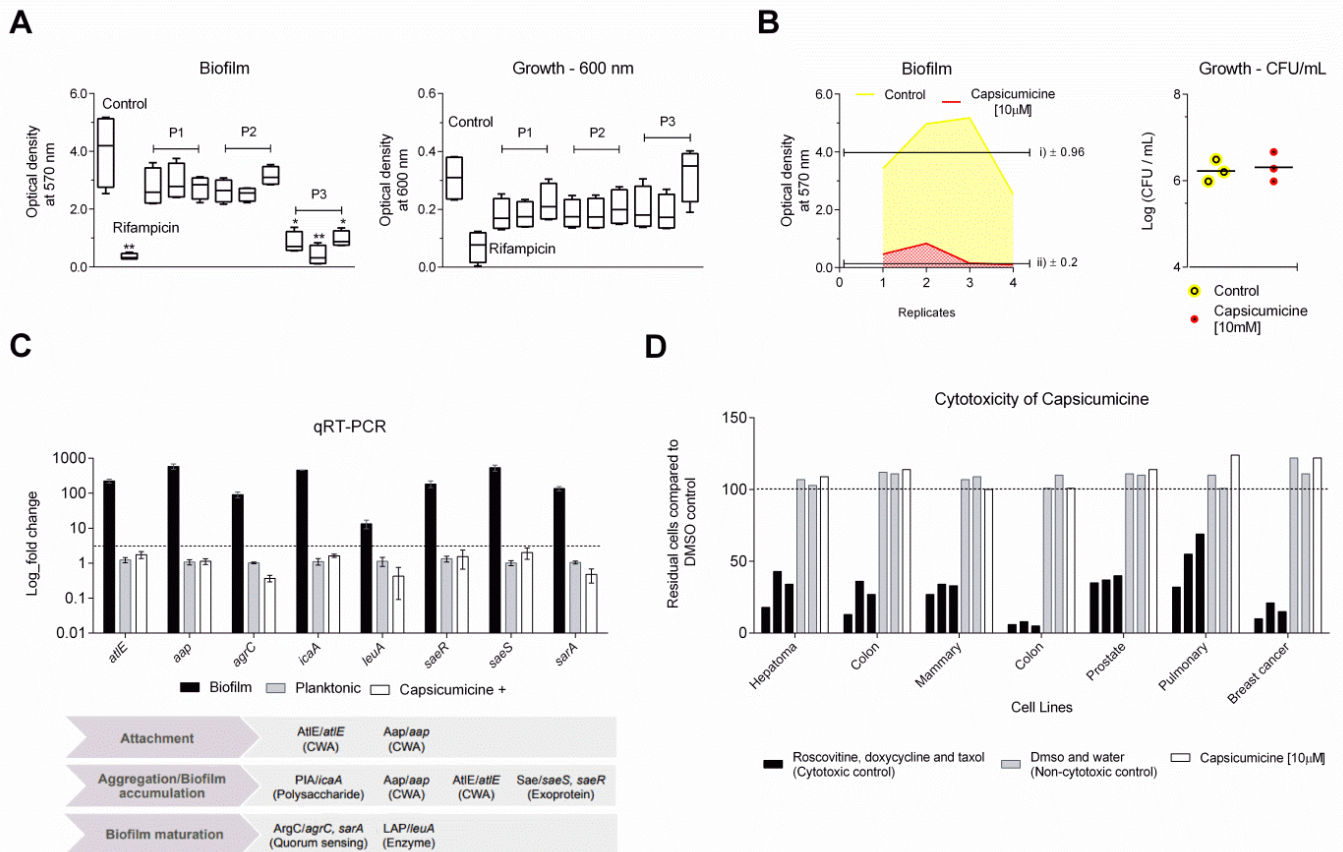
60 Here, we describe the discovery of the antibiofilm peptide capsicumicine, inspired by natural
61 peptides from the seeds of the red pepper *Capsicum baccatum*. We elucidated its mechanism of
62 action (MoA), the matrix anti-assembly (MAA). Capsicumicine is the first-in-class non-antibiotic
63 peptide displaying this extracellular MoA. Notably, we report an *in vivo* 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 (RSCQQIQQAQQLSSCQQYLKQ). 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 antibiofilm activity 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).



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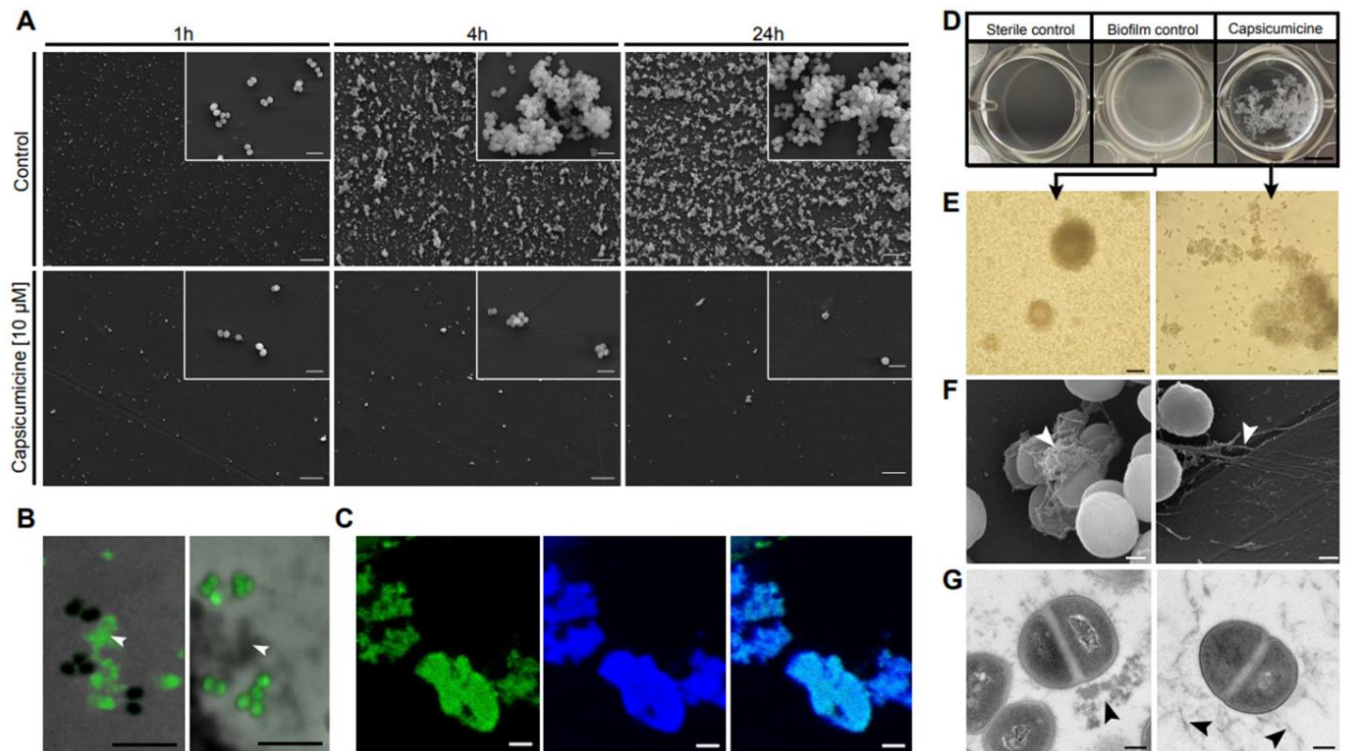
82 **Fig. 1.** Antibiofilm activity of bioinspired peptides and cytotoxicity. (A) Antibiofilm activity of peptides
 83 P1, P2, and P3 (capsicumicine) at 1, 10, and 100 μ M. Quantification of *Staphylococcus epidermidis*
 84 (ATCC 35984) biofilm and growth were done at an optical density; bacteria without peptide exposure
 85 (Control) and the antibiotic control, 16 μ g/mL rifampicin (Rif.). Student’s *t*-test: *, $p \leq 0.05$; **, $p \leq$
 86 0.01. (B) Antibiofilm activity and growth of capsicumicine at 10 μ M. Yellow represents 100% of
 87 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 \pm
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 μ M 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. 1C).

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. 1D).

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
113 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. 2B).



118

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 μ M capsicumicine. Magnification x500,
122 with insets at x5,000; scale bars, 10 μ m. (B) CFM images: (*left*) *S. epidermidis* after exposure to 10 μ M
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 μ m. (C) CFM images: (*left*) *S.*
127 *epidermidis* after exposure to 10 μ M capsicumicine-FITC; (*center*) after exposure to concanavalin A
128 conjugates; and (*right*) their colocalization. Scale bars, 5 μ m. (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.

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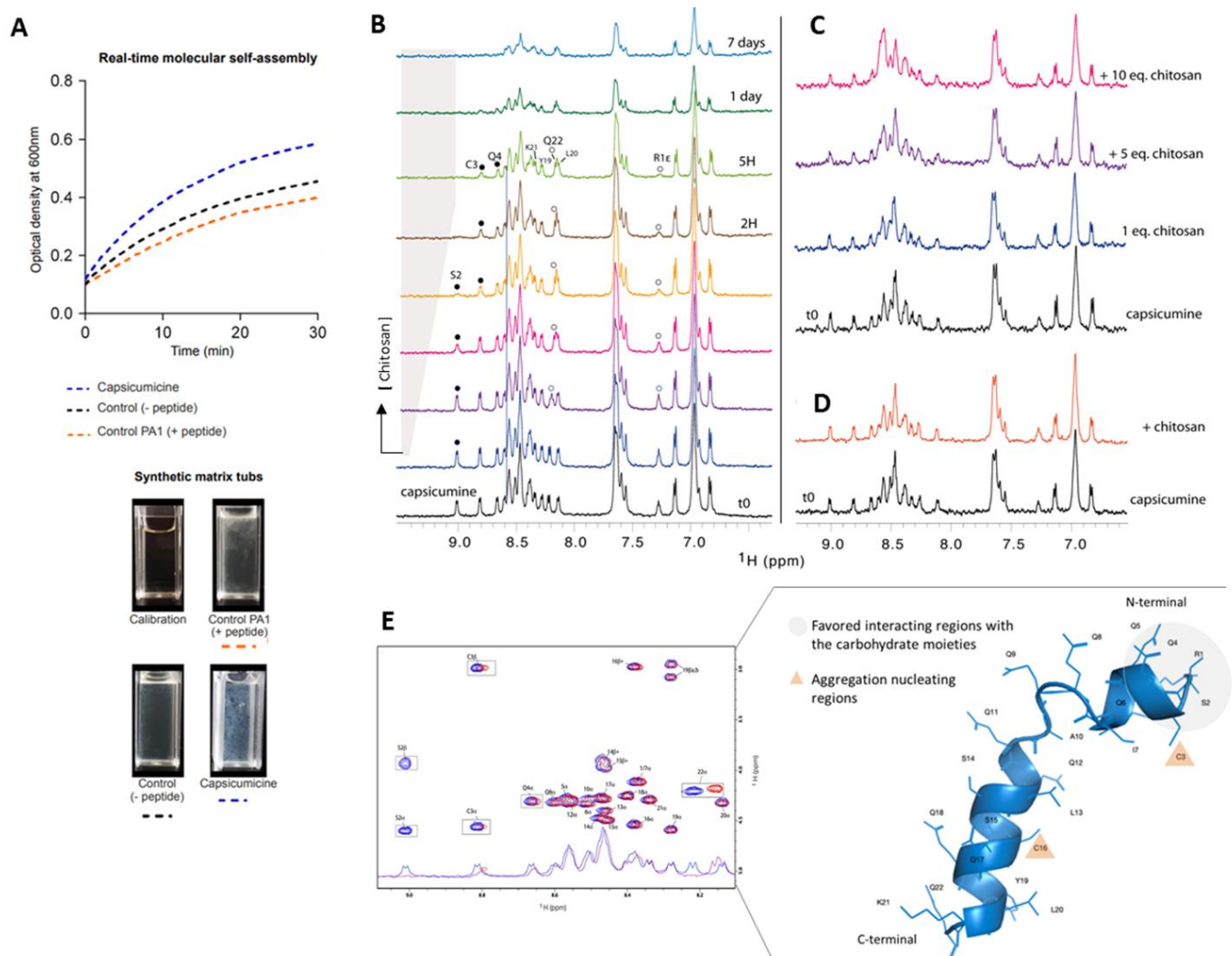
140 **Capsicumicine disturbs *S. epidermidis* matrix assembly.** Since capsicumicine’s antibiofilm activity
141 was not associated with direct bacterial interactions or gene expression modulation, we used various
142 microscopic approaches to investigate the interactions between the peptide and the extracellular

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

168 measuring the optical density at 600 nm (OD_{600}) as a function of time with or without capsicumine.
 169 OD increases when capsicumine 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 capsicumine, 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. 2D).



174

175 **Fig. 3.** Molecular interactions between capsicumine 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 capsicumine (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 capsicumine 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 capsicumine 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)
186 Superposition of capsicumicine 1D NMR and TOCSY spectra immediately after sample preparation
187 (blue) and chitosan [10 eq.] 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).

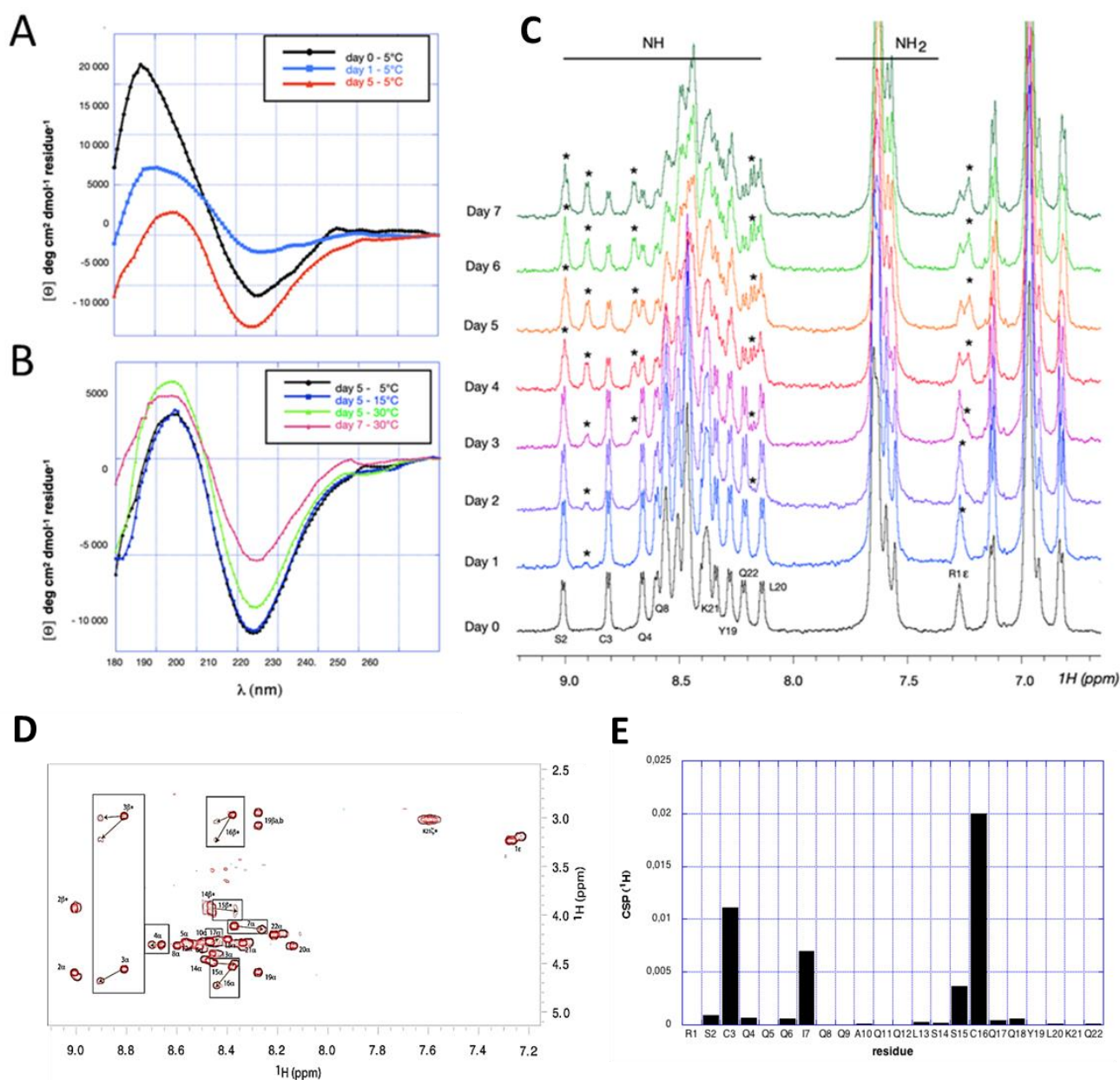
209 **Structural requirements toward to molecular interactions with target-saccharides.** To better
210 understand the mechanisms involved in capsicumicine bioactivity we step toward the knowledge of
211 its conformation in solution and molecular interactions with matrix representative saccharides. To

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, I7 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

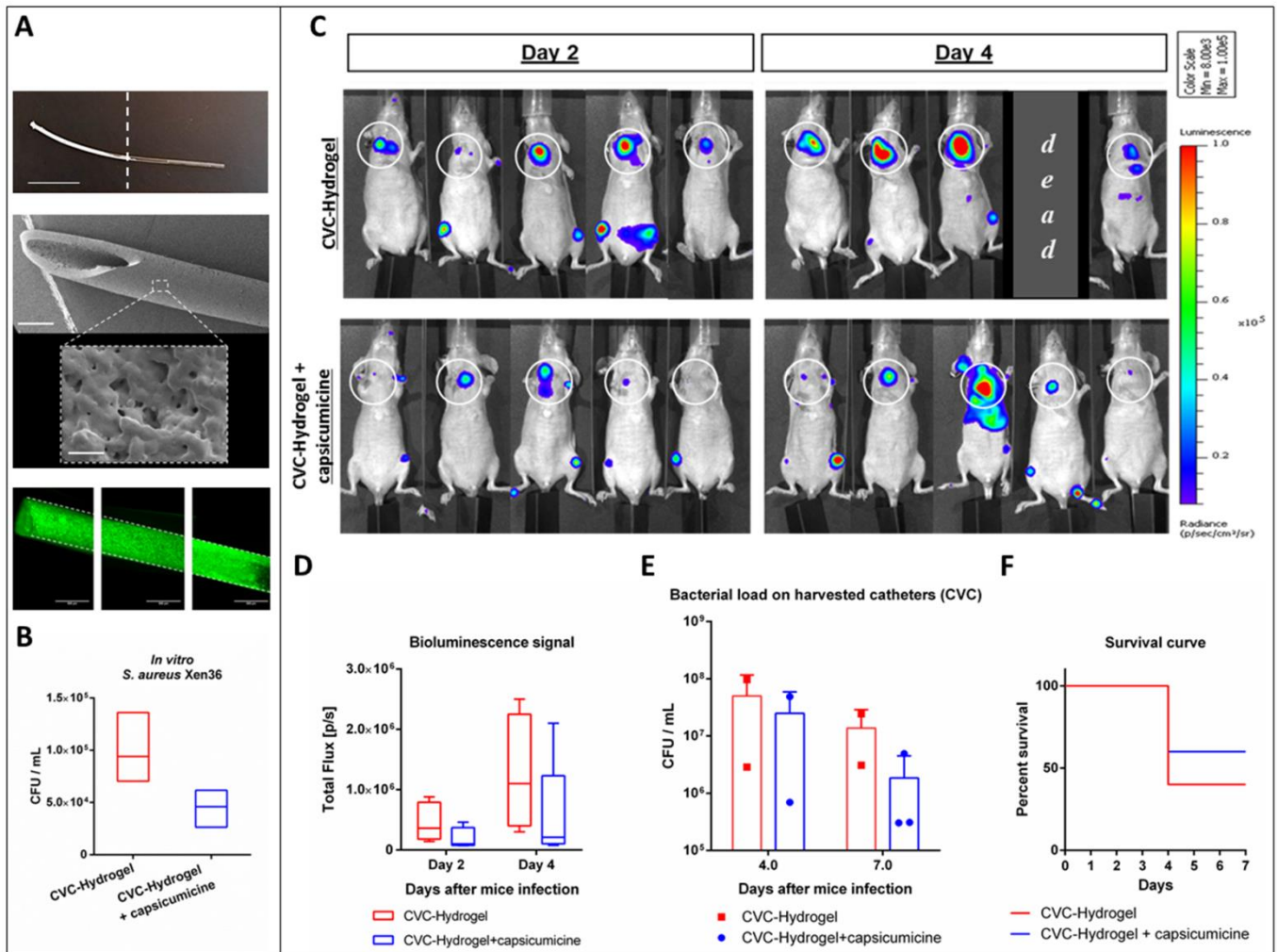
237 reaches more than 50% after 7 days (Fig. 4C, top). The solution remained clear overall the spectra
 238 recording time.



239
 240 **Fig. 4** Capsicumicine conformational changes. CD spectras (A) Evolution at 5°C over 5 days; (B)
 241 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
 243 time corresponding to the second set of amide resonances (stars). (D) Superposition of capsicumicine
 244 TOSY spectra immediately after sample preparation (red) and at day 5 (brown). The most important
 245 chemical shift perturbations between the original and the new conformations are boxed and
 246 clustered around the two cysteines. (E) Proton chemical shift perturbations (CSP) computed from the
 247 TOSY 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 ≥ 51 % of *S. aureus* colonization (Fig. 5B). 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. 5E). 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. 5E). 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. 5E).



268

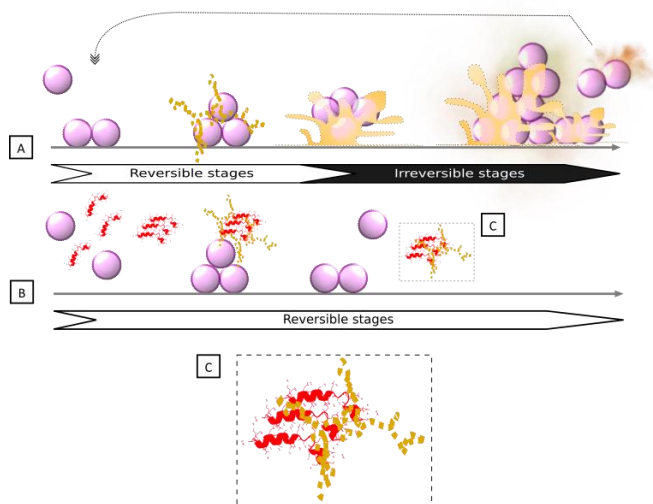
269 **Fig. 5.** Assessment of capsicumicidine pre-coated central venous catheter (CVC) in mice (SKH1) infected
 270 by *Staphylococcus aureus* Xen36. (A-B) *In vitro* CVC development; (A) From the top to the bottom: a
 271 picture of a up to half capsicumicidine pre-coated CVC (whitish), scale bar 1 cm; SEM image of pre-
 272 coated CVC, scale bar 0.5 mm and its magnification, scale bar 1 μ m and; a compilation of images of
 273 fluorescence microscopy of pre-coated CVC (capsicumicidine-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 + capsicumicidine) in the
 277 bottom; white circles delimitate the CVC localization; the luminescence scale is presented in
 278 radiance. (D) Bioluminescence quantification (ROI mean signals \pm 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).



304

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

337 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 (AtIE,
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 3C,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).

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

401 Finally, we demonstrated that capsicumicine strongly prevents biofilm formation for the most
402 frequent bacteria related to nosocomial infections, *S. epidermidis* and *S. aureus*. We first confirmed
403 that capsicumicine was not cytotoxic thus being safe for *in vivo* tests. Then, we trialed the peptide in
404 a translational pre-clinical model that mimics medical device-related infection. As a result, pre-
405 coated CVCs enhanced mice survival decreasing *S. aureus* colonization and consequently attenuated
406 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.

411

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 μ M 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 μ M 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.

451 **Scanning electron microscopy (SEM):** Sterile 10x4mm polystyrene coupons were inserted into
452 bacterial cultures in the presence or absence of capsicumicine for 1, 4, and 24 h. The coupons were
453 then washed with sterile 0.9% NaCl and fixed with 2.5% glutaraldehyde, 2% paraformaldehyde, and
454 0.1 M cacodylate buffer (pH 7.2). Afterwards, they were washed with 0.1 M cacodylate buffer and
455 0.2 M sucrose, then dehydrated with increasing concentrations of ethanol. A Leica EM CPD300 was
456 used for critical point drying of the dehydrated samples. These were then sputtered with palladium
457 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 μM) 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 18M² 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 ($[\theta]$) per residue and molar ellipticity
506 per residue ($[\theta]$, 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)

508 and different reference protein data sets provided by the DICHROWEB facility (64). MER data curves
509 were smoothed for presentation, using the interpolate and weighted data (5%) routines provide by
510 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 $+21 \pm 3^{\circ}\text{C}$, 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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552 R.G.V.B, S.C.B.G, A.J.M, S.N-L. and R.G. designed research; R.G.V.B, S.C., R.S., S.N-L. and S.B.

553 performed research; R.G.V.B, A.R.Z., E.G., S.C.B.G, A.J.M, S.N-L., S.B. and R.G. analyzed data; and

554 R.G.V.B, A.J.M, S.N-L. and R.G. wrote the paper. **Competing interests:** R.G.V.B, S.C.B.G, A.R.Z, A.J.M

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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
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560 requested from the authors.

561

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714

715 **Supplementary Materials**

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

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon
<i>atlE</i>	TACCAGGGTTTGCAGGATTC	GCGCTAAATTCATTGGAAA	85 pb
<i>aap</i>	AGGCCGTACCAACAGTGAAT	ATGGGCAAACGTAGACAAGG	100 pb
<i>agrC</i>	TCATCAATATCGCATTTCATCG	CCTAAACCGCGATTATCACC	136 pb
<i>icaA</i>	TTATCAATGCCGCAGTTGTC	CCGTTGGATATTGCCTCTGT	104 pb
<i>leuA</i>	GATGATCTCGGAATGGCAGT	TGAGGCATTTCTGCTCTTT	108 pb
<i>saeR</i>	GCTAACACTGTCAATGTCCACA	AGGCCCCACACAGTTGTAAT	92 pb
<i>saeS</i>	GGCGTCAATTTGTTGTGCTA	AGGGCATAGGTATCGTTCCA	140 pb
<i>sarA</i>	TTTGCTTCTGTGATACGGTTGT	CGTAATGAACACGATGAAAGAACT	107 pb
<i>gyrB</i>	ATCAACATCGGCATCAGTCA	GCATTTGGTACGGGTATTGG	87 pb
<i>rrsA</i>	AAGCAACGCGAAGAACCTTA	ATGCACCACCTGTCACTCTG	95 pb

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719

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.

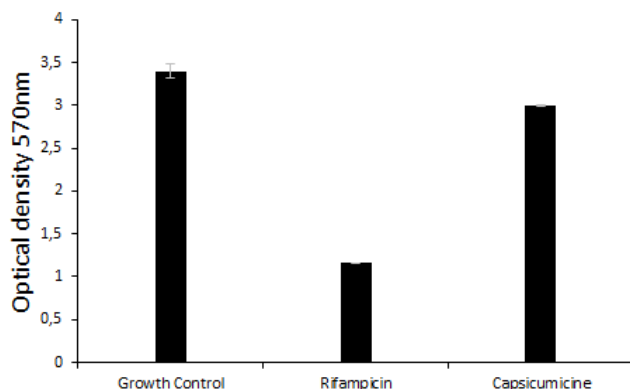
Entry	Name	Accession ID
ICAA_STAEQ	Intercellular adhesion protein icaA	Q5HKQ0.1
ICAD_STAEQ	Poly-beta-1,6-N-acetyl-D-glucos. synthase prot. icaD	Q5HKP9
A7Z8H9_BACVZ	Chitosanase	A7Z8H9
A0A0N0MLT5_9ACTN	Chitosanase	A0A0N0MLT5
CBP2_MOROL	Chitin-binding protein 2	COHKC5
A0A1R0GTZ5_9FUNG	Chitin synthase 8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

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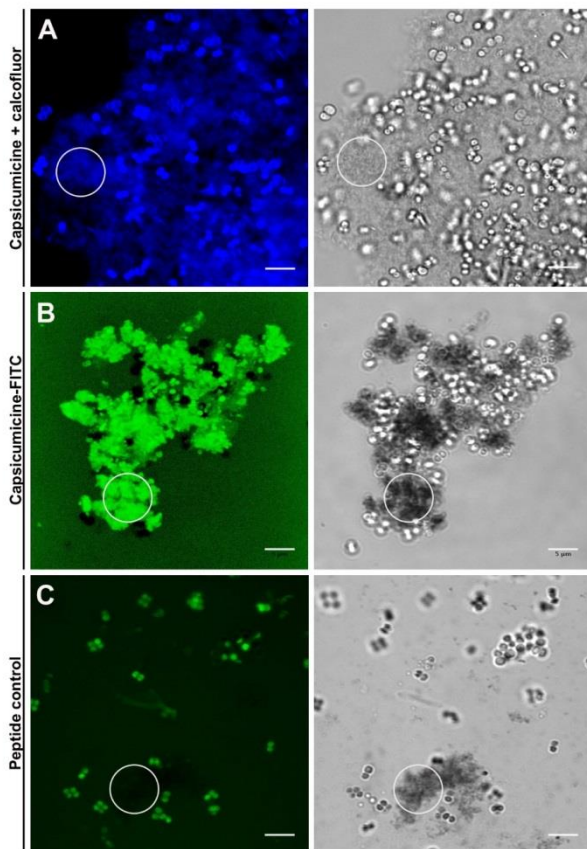
Table S3. Results of the CD spectra deconvolution using CDSSTR algorithm.

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
731 exposure to the rifampicin antibiotic control, and after 24 h treatment with 100 µM capsicumicine.
732



733

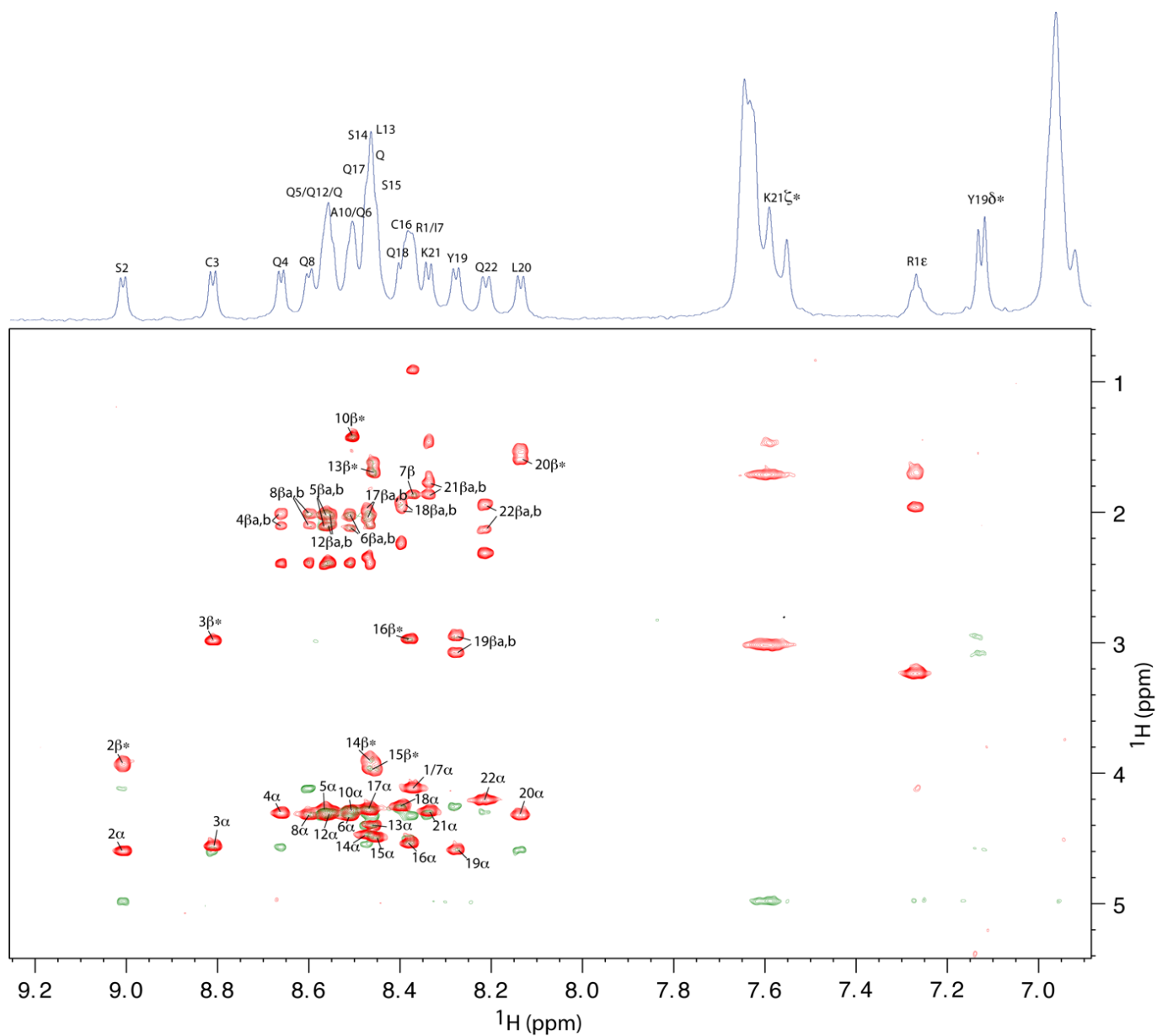
734 **Fig. S2.** Confocal fluorescence microscopy (CFM) of *Staphylococcus epidermidis* (ATCC 35984).
735 Calcofluor was used to highlight matrix polysaccharides (blue), and FITC used for the peptides
736 (green). Visualization was done by fluorescence (left) and transmitted light (right) microscopy. (A)
737 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 matrix is highlighted (circles). Scale bars, 5 μm .

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741

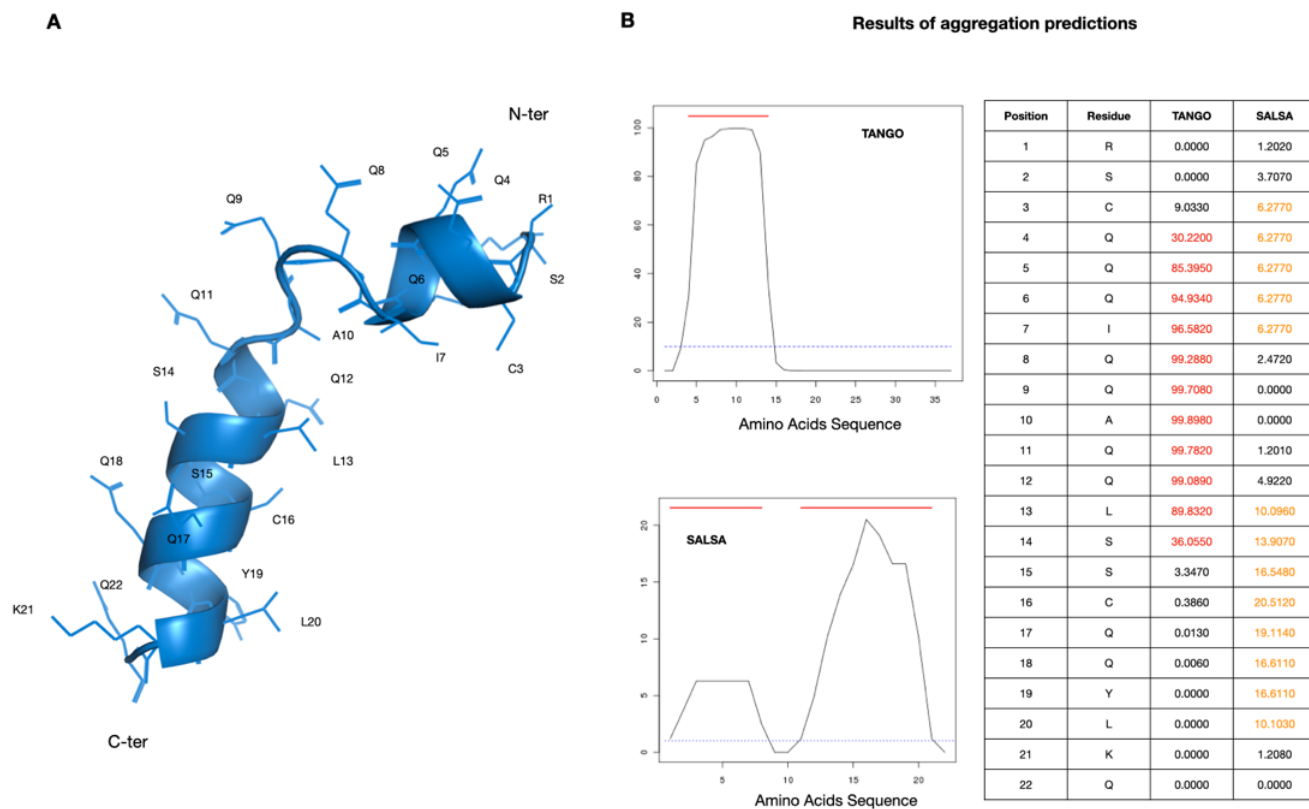
742 **Fig. S3.** Amino acid alignment of capsicumicine and its carbohydrate-binding domain protein
743 homologs. (a) The icaA (protein accession number Q5HKQ0.1) fragment from 1 to 60. (b)
744 Capsicumicine fragment from 1 to 21. (c) Chitosanase (A7Z8H9) fragment from 1 to 60 and from 61
745 to 120. (d) Capsicumicine fragment from 1 to 15 and from 16 to 22. (e) Chitin synthase 8
746 (AOA1R0GTZ5) fragment from 1801 to 1860 and from 1921 to 1967. (f) Capsicumicine fragment from
747 1 to 14 and from 16 to 22. Equal (* and grey highlighting), similar (.), and highly similar (:)
748 amino acids are indicated, as well as amino acid polar characteristics (purple). Support for this analysis is
749 available at UniProt.
750



751

752 **Fig. S4.** Superposition of the TOCSY (red), NOESY (green) and 1D (blue) spectra of capsicumicine
753 recorded at pH 5.0 and 10°C. All amide NH resonances were assigned using nNH-(n-1)H α NOESY
754 cross-peaks, except for Q9 and Q11 residues due to poor resolution. For clarity, only NH-H α and NH-
755 H β labels were reported. The spectra display 22 spin systems as expected for a single conformation.
756 According to the spectral dispersion, capsicumicine is mainly folded.

757



758

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