HAL open science

In Vitro Activities of Dermaseptins K4S4 and K4K20S4 against Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa Planktonic Growth and Biofilm Formation

A. Zairi, L. Ferrieres, P. Latour-Lambert, Christophe Beloin, Frédéric Tangy, Jean-Marc Ghigo, K. Hani

To cite this version:

A. Zairi, L. Ferrieres, P. Latour-Lambert, Christophe Beloin, Frédéric Tangy, et al.. In Vitro Activities of Dermaseptins K4S4 and K4K20S4 against Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa Planktonic Growth and Biofilm Formation. Antimicrobial Agents and Chemotherapy, 2014, 58 (4), pp.2221 - 2228. 10.1128/AAC.02142-13 . pasteur-01381816

HAL Id: pasteur-01381816

https://pasteur.hal.science/pasteur-01381816

Submitted on 21 Nov 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - ShareAlike 4.0 International License

In vitro activity of Dermaseptins K_4S_4 and $K_4K_{20}S_4$ against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* planktonic growth and biofilm formation

Amira Zaïri[*a], Lionel Ferrières[b], Patricia Latour-Lambert[b,d], Christophe Beloin[b], Frédéric Tangy[c], Jean-Marc Ghigo[b] and Khaled Hani[a]

[a] Laboratoire de Biochimie, Faculté de médecine de Sousse, Tunisia

[b] Institut Pasteur, Unité de Génétique des Biofilms. 25 rue du Dr. Roux, 75724 Paris cedex 15 France.

[C] Institut Pasteur, Unité de Génomique Virale et Vaccination. 28 rue du Dr. Roux, 75724 Paris cedex 15 France

[d] Present address: Institut Pasteur, Unité de Dynamique des Interactions Hôte-Pathogène, 25 rue du Dr. Roux, 75724 Paris cedex 15 France

Corresponding author: Amira ZAÏRI

Address: Department of Biochemistry, Faculty of Medicine Sousse, Tunisia

e-mail: zairi_amira@yahoo.fr

Phone: (+216) 98 82 53 28

Fax: (+216) 73 22 48 99

Running title: antibiofilm activity of dermaseptins

ABSTRACT

The rising number of infections caused by biofilm formation and the difficulties associated with their treatment by conventional antimicrobial therapies have led to an intensive search for novel antibiofilm agents. Dermaseptins are antimicrobial peptides with a number of attractive properties that might offer alternative therapeutics against resistant microorganisms. In this study, we synthesized a set of dermaseptin-derivative peptides and evaluated their activity against Gram-positive and Gram-negative bacterial biofilm formation. All dermaseptin-derivative peptides elicited concentration-dependant antibiofilm activity at microgram concentration and their activity was dependent on the nature of the peptide, with the highest levels of activity being exhibited by highly charged molecules. Fluorescent binding and confocal microscopy demonstrated that dermaseptin K_4S_4, a substituted derivative from the native molecule S_4, significantly decreased the viability of planktonic and surface attached bacteria and stopped biofilm formation under dynamic flow conditions. Cytotoxicity assays on HeLa cells showed that some of the tested peptides were less cytotoxic than current antibiotics. Overall, these findings indicate that dermaseptin derivatives might constitute a new lead structure for the development of potent antibiofilm agents.

Keywords: Biofilm, antimicrobial peptides, dermaseptins, *Pseudomonas aeruginosa*, *Escherichia coli*, S*taphylococcus aureus*.

67 **INTRODUCTION**

68

69 A biofilm is a microbial community of sessile microorganisms composed of cells that
70 are embedded in a matrix of extracellular polymeric substances, attached to a substratum or
71 interface. Biofilm bacteria are phenotypically and physiologically different from planktonic or
72 suspended cells (1, 2). Bacterial biofilms have been linked to a wide range of infections,
73 particularly in patients requiring indwelling medical devices, such as catheters and prostheses.
74 They have recently been associated with about 80% of all chronic human infections (3) and
75 described as important mediators of healthcare-associated infections (4).

76 Osteomyelitis, infective endocarditis, chronic wounds, and infections related to
77 indwelling devices are examples of infections that are often caused by biofilm-producing
78 strains. Staphylococci and enterobacteriaceae account for a large proportion of these
79 infections, with *Staphylococcus aureus* and *Escherichia coli* pathogens representing more
80 than 50 % of the species isolated from patients with medical device-associated infections (6,
81 7). Such infections typically exhibit increased tolerance to antimicrobial, biocidal and
82 immunological challenges, which makes their treatment with conventional chemotherapeutic
83 agents difficult, and sometimes impossible. These concerns have prompted a persistent search
84 for alternative therapies against planktonic microorganisms and biofilms. Of particular
85 interest, dermaseptins, antimicrobial peptides produced by the immune system of frogs, offer
86 a number of properties and attributes that might open promising opportunities for the
87 development of new antibiofilm agents.

88 Antimicrobial peptides are produced by both prokaryotic and eukaryotic cells and
89 have been extensively investigated in recent research because they are involved in several
90 host defense mechanisms and lend themselves to various applications as therapeutic agents.
91 (8–13). Dermaseptins are a family of eight closely related antimicrobial peptides that have
92 originally been isolated from the skin of a tree-dwelling, South American frog (*Phyllomedusa*
93 *sauvagei*). They are linear polycationic peptides composed of 28–34 amino acids that are
94 structured in amphipathic α-helices in apolar solvents (14, 15). They all have a conserved Trp
95 residue at position 3, an AG(A)KAAL(V/G)G(N/K)AV(A) consensus motif in the mid-
96 region, and a positive charge attributable to the presence of Lys residues that punctuate an
97 alternating hydrophobic and hydrophilic sequence (14). New members of the dermaseptin S
98 family (S_9–S_{11}) that do not resemble any of the so far characterized natural antimicrobial
99 peptides have recently been identified and cloned from a skin secretion-derived cDNA library
100 (16). In fact, some dermaseptins show marked abilities to inhibit microbial cells efficiently,

101 rapidly, and irreversibly without toxic effects on mammalian cells. They also display cytolytic
102 activity *in vitro* against a broad spectrum of host-free microorganisms, including bacteria
103 (Gram-positive and Gram-negative) (17–21), protozoa (22–24), yeasts and filamentous fungi
104 (19, 25) and viruses (26-28).

105 Dermaseptin S_1, from the skin secretion of *Phyllomedusa hypochondrialis* frogs, has
106 recently been reported to exhibit electroanalytical activity to dopamine (DA) oxidation. The
107 selectivity in the detection of DA is, in fact, a fundamental aspect for the development of
108 electrochemical sensors with potential applications in the biomedical and pharmaceutical
109 industries (29). Although the precise mechanism of action of antimicrobial peptides is not yet
110 fully understood, the antimicrobial action of dermaseptin is thought to be mediated by the
111 interaction of the amphipathic α-helix with the membrane phospholipids, resulting in the
112 permeation of the target cell by destabilizing the plasma membrane via either a 'barrel-stave'
113 mechanism or a 'non-pore carpet-like' mechanism (30,31).

114 Structure-activity relationship studies performed on native dermaseptin S_4 have
115 recently led to the identification of synthetic derivatives with improved antimicrobial
116 properties (32-34). When comparing the resistance emergence rates by propagating bacteria
117 under selective antibiotic pressure, both Gram-positive and Gram-negative bacteria were
118 noted to exhibit resistance to commercial antibiotics but not to the L-or D-isomers of the
119 dermaseptin derivatives that were tested (33). Overall, the data obtained from *in vitro* and *in*
120 *vivo* experiments indicate that some dermaseptin derivatives have a variety of potential
121 medical and antimicrobial applications (32). Nevertheless, no previous work has so far been
122 performed on the antibiofilm activity of dermaseptins. Accordingly, the present study was
123 undertaken to investigate the feasibility and gain effects of using dermaseptins against
124 biofilms formed by important biofilm-forming pathogens, including *Staphylococcus aureus*,
125 *Escherichia coli*, and *Pseudomonas aeruginosa*. It particularly focused on the evaluation of
126 the antibiofilm potential of dermaseptin S_4 derivatives. A number of chemically-modified
127 peptides were synthesized and investigated for antibiofilm activity. HeLa cultures were used
128 to evaluate their safety and toxicity, and fluorescent binding and confocal microscopy assays
129 were performed to assess the speed and irreversibility of their antibiofilm activity.
130

131 **MATERIALS AND METHODS**

132

133 **Synthesis, purification and preparation of peptides**

134 Peptides were prepared by stepwise solid phase synthesis using Fmoc polyamide-
135 active ester chemistry on Milligen 9050 pepsynthesizer. All Fmoc-amino acids were from
136 Milligen-Waters (France). 4-(Hydroxymethyl) phenoacetic acid-linked polyamide/kieselguhr
137 resin (pepsin kA), Fmoc-aminoacid pentafluorophenyl (Pfp), and 3-hydroxy-2, 3-dehydro-4-
138 oxo-benzotriazine (Dhbt) esters were from Milligen/Bioresearch. Cleavage of peptidyl-resin
139 and side chain deprotection were carried out using 5 mg of peptidyl-resin in 1 ml mixture
140 composed of trifluoroacetic acid, para-cresol, thioanisol, water, and ethyl methyl sulfide (82.5;
141 5.5; and 2.5% v/v) for 2 h at room temperature. After filtering to remove the resin and ether
142 extraction, the crude peptides were purified by a combination of Sephadex gel filtration, ion
143 exchange chromatography, and preparative high performance liquid chromatography (HPLC).
144 The homogeneity of the synthetic peptides was assessed by analytical HPLC, amino acid
145 analysis, solid phase sequence analysis, and mass spectrometry (35). All peptides were stored
146 frozen as stock solutions at 1 mg/ml and -20°C in double-distilled water. Prior to
147 experimentation, fresh solutions were diluted in the appropriate medium. For fluorescein-
148 labeled peptides, fluorescein was introduced at the N-terminus of the peptide using fluorescein
149 N-hydroxysuccinimide ester prior to Trifluoroacetic acid (TFA) treatment.

150

151 **Bacterial strains and growth media**

152 The strains used in this study are listed in Table 1. They were maintained as follows:
153 *Pseudomonas aeruginosa* was grown in Lysogeny Broth (LB) medium, *Escherichia coli* was
154 grown in Mueller Hinton (MH) broth (for antimicrobial assays) or in M63B1 medium
155 containing 0.4% glucose medium (for biofilm assay), and *Staphylococcus aureus* was grown
156 in Tryptic Soy Broth (TSB) containing 0.25 % Glucose.

157

158 **Antimicrobial activities against planktonic cultures**

159 To investigate the antimicrobial activities of dermaseptins (Table 2), we evaluated the
160 planktonic growth of different strains in the absence or presence of each peptide at different
161 concentrations. Accordingly, an overnight culture of each bacteria grown in the appropriate
162 medium was used to inoculate 100 µl of sterile MH medium in a 96-well plate to an optical
163 density at 600 nm (OD_{600}) of 0.0001. The peptides were added to final concentrations ranging
164 from 0 to 100 µg/ml. Polymyxin used at 6.25 µg/ml served as a positive control. Three

165 replicates were made for each condition. The cultures were incubated at 37°C, with shaking
166 for 24 h. The minimum inhibitory concentration (MIC) was determined from the lowest
167 concentration that induced 100% inhibition.
168
169 ***In vitro* biofilm susceptibility**
170 To quantify the biofilm formation of *E. coli* in the absence and presence of peptides, a
171 microplate-based assay was performed as follows. Strains were grown in M63 media
172 containing 0.4 % glucose for 24 h at 37°C in a shaking bath and then diluted to obtain a
173 suspension with an optical density (OD) at 600 of 0.01. 100 µl of the diluted suspension was
174 added into the wells of a Polyvinyl Chloride (PVC) microtiter plate and incubated for 24 h
175 without shaking to allow biofilm formation. Planktonic cells were carefully removed by
176 pipetting, and biofilms were then treated with 100 µl of different concentrations of each
177 peptide at indicated concentrations and incubated for 24 h at 37°C. Biofilm formation was
178 measured using crystal violet (CV) staining. After treatment, planktonic cells were gently
179 removed; each well was washed three times with water and pat dried with a piece of paper
180 towel in an inverted position. To quantify biofilm formation, each well was stained with 100
181 µl of 1 % crystal violet and incubated for 15 min at room temperature. The plates were then
182 washed three times with water again to remove extra dye. After that, 125 µl of mixed 80%
183 ethanol and 20% acetone were added to each well to dissolve all the absorbed dye. After 30
184 min of incubation at room temperature, OD_{570} was measured to quantify the total biomass of
185 biofilm formed in each well. The viability of biofilm cells was evaluated using the XTT
186 quantification method. After treatment, planktonic cells were removed by careful pipetting,
187 and the biofilms were washed with 125 µl of PBS 1X. Each well was supplemented with 125
188 µl of 0.05 mg/ml XTT/ 10 mM menadione and incubated at 37°C for 4 h in the dark. The
189 metabolic activity of biofilm cells reflecting their viability was assessed by determining the
190 amount of product resulting from the degradation of XTT into formazan, through OD_{492}
191 measurement.
192
193 **Confocal microscopy: Peptides effect on a biofilm in continuous flow**
194 *Growth of biofilm*
195 The bacterial strain used for this experiment was *E. coli* MG1655 F'Tet Δ*traD*:*apra* λ
196 ATT km-mars. Biofilms were grown at 37°C in M63B1 supplemented with 0.4% glucose as a
197 carbon source, in flow chambers with individual channel dimensions of 1 by 4 by 40 mm. The

198 flow system was assembled and prepared as described previously (36). Individual flow cells
199 were inoculated with fluorescent *E. coli* (see Table 1 and legend of Fig. 2) and adjusted to an
200 optical density at 620 nm of 0.1. To allow the adherence of bacterial cells to the substratum,
201 flow cells were left without flow for 1 h after inoculation at 37°C. A laminar flow was then
202 activated with a flow rate of 2µl/sec. (7,2ml/h calculated to eliminate unattached bacteria but
203 in planktonic phase in the channel).

204

205 *Exposure of biofilm to fluorescent dermaseptins*

206 After 5 h of biofilm growth in the flow cell, the flow was stopped, and 300 µl of each
207 fluorescein-tagged peptide sample at the following concentrations (0.78 µg/ml for K_4S_4 and
208 25 µg/ml for $D_4D_{20}S_4$) was added into the chamber by syringe injection. After 20 min, the
209 flow was restarted with a flow rate of 0.4µl/sec per section. Normal flow rate (2µl/sec) per
210 section was set up again 20 min later. Z-stack images were obtained every 1 h for at least 6 h.
211 Flow cells were imaged under confocal microscopy Leica SP5 using the following
212 parameters: Objective 63X oil, 512x512 pixels, line average 2, 1 µm z steps, 60 µm per stack,
213 excitation Filter Wavelengths: 475-490 nanometers and 540-565 nanometers, emission
214 wavenlengths: 500-535 nanometers and 570-620 nanometers. Side-view images of the biofilm
215 were converted into 3D structures using the Software Imaris. All microscopy observations
216 and image acquisitions were performed using methods described above. For quantification of
217 biofilm development with or without peptide, images from each sample were analyzed using
218 the computer program (Imaris MeasurementPro).

219

220 **Assay for eukaryotic cell viability**

221 The potential cytotoxicity of dermaseptin S_4 and its derivatives was measured in
222 normal human cervical HeLa cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl
223 tetrazolium bromide)-based assay. Briefly, cells were seeded into 96-well plates at a density
224 of 2 x 10^4 cells per well and incubated for 24 h at 37°C prior to drug exposure. On the day of
225 treatment, the culture medium was aspirated from the wells and replaced by the fresh medium
226 containing a drug concentration ranging from 0 to 500 µg/ml. Triplicate wells were used for
227 each treatment. Culture plates were then incubated for 30 min, 3 h or 6 h before the addition
228 of 10 µl of MTT solution (5 µg/ml in PBS) to each well. The wells containing only the
229 medium and MTT were used as controls for each plate. The tetrazolium/formazan reaction
230 was allowed to proceed for 4 h at 37°C, and then 100 µl of the solubilization buffer (10%

231 sodium dodecyl sulfate in 0.1% (v/v) HCL) was added to all wells and mixed thoroughly to
232 dissolve the dark-blue formazan crystals. After overnight incubation at 37°C, the optical
233 density at 540 nm was measured by a 96-well multiscanner auto-reader with the solubilization
234 buffer serving as a blank. To translate the OD_{540} values into the number of live cells in each
235 well, the OD_{540} values were compared with those of standard OD_{540}-versus-cell number
236 curves generated for each cell line. The percentage of cell survival was expressed as live cell
237 number in test group/live cell number in control group x 100.
238
239 **Statistical analysis**
240 Normality in this study was assessed using Proc Univariate in SAS software (37). All data
241 were statistically analyzed using the least-squares method with the procedure GLM for
242 General Linear Model. Effects were treatment, bacteria and the interaction treatment-bacteria.
243 The SNK multivariate test was used to compare means for bacteria and Dunnett's test to
244 compare each experimental mean of treatment (peptide) with the control mean. The following
245 equation was used for statistical calculations:
246 $$Yijk= \mu+ T_i+ B_j+ T*B_k+ e_{ijk}$$
247 Where:
248 Y_{ijk} is the response variable (peptides), μ is the overall mean, T_i is the fixed effects of
249 treatment, B_j is the fixed effect of bacteria, $T*B_k$ is the effect of treatment within bacteria and
250 e_{ijk} is the residual error
251
252
253

254 **RESULTS**

255

256 ***In vitro* toxicity of dermaseptin and derivatives against human cells**

257 We tested the potential *in vitro* cytotoxicity of dermaseptin S_4 and its derivatives
258 (described in Table 2) in confluent monolayers of human HeLa cells using the MTT cell
259 viability assay. Cells were exposed to increasing concentrations of peptides ranging from 0 to
260 128 µg/ml. Cytotoxicity of dermaseptins was concentration-dependent (data not shown).
261 Peptides concentrations that caused 50% cytotoxixity (CC_{50}) were determined (Table 3). The
262 highest cytotoxicity rates observed for all S_4 derivatives were recorded at concentrations
263 higher than 24.12 µg/ml (CC_{50}), except for S_4(5-28) whose toxicity was slightly lower than
264 the other peptides (CC_{50} > 100 µg/mL). Interestingly, results showed that shortening the
265 peptide in N- or C-terminal region (S_4(5–28) or K_4S_4(1–16)) and increasing its positive charge
266 by different substitutions ($K_4K_{20}S_4$ or K_4S_4) leads to peptides with low toxicity.

267

268 **Antimicrobial activity of dermaseptin S_4 derivatives on planktonic bacteria**

269 We next evaluated the ability of dermaseptin S_4 and its derivatives to inhibit
270 proliferation of Gram-positive *S. aureus*, and Gram-negative *P. aeruginosa* and *E. coli*
271 planktonic cells (Table 3). All peptides under investigation inhibited antimicrobial growth.
272 Their range of activity was, however, dependent on the nature of the peptide and the highly
273 positive charged molecules. In fact, the derivatives in which positive charge was increased
274 without shortening the length of the peptide, *i.e.* $K_4K_{20}S_4$ and K_4S_4, were the most potent
275 inhibitors, and the derivatives in which positive charge was reduced, such as $D_4D_{20}S_4$, were
276 less potent. Likewise, the substituted and deleted peptide K_4S_4(1–16) and the peptide with N-
277 terminal deletions S_4(5-28) displayed a nearly homogenous potency in all assays, with a
278 progressive loss of potency as compared to K_4S_4 particularly against *S. aureus*. However, the
279 truncation of the N-terminal carboxyl S_4(5-28), which potentially resulted in a less toxic
280 peptide (33), did not affect its potency.

281

282 **Effect of dermaseptin S_4 derivatives on *in vitro* static biofilm**

283 We first assessed the activity of the dermaseptin S_4 and its derivatives against mature
284 bacterial biofilms using biofilms formed in microtiter PVC plates. While exposure of bacterial
285 biofilms to the peptides at concentrations of 2 x MIC did not dissolve biofilms as measured by
286 crystal violet staining (data not shown), survival of biofilm cells was reduced (Fig. 1). K_4S_4

287 and $K_4K_{20}S_4$ were the most active peptides against all the strains tested, whereas $D_4D_{20}S_4$ was
288 the least active one. However, K_4S_4(1-16) and S_4(5-28), which strongly affected *E. coli* and *P.*
289 *aeruginosa* biofilm, had less activity against *S. aureus* biofilm.
290
291
292 **Effect of dermaseptin S_4 derivatives on *in vitro E. coli* biofilm formed in continuous**
293 **flow conditions**.
294 To further investigate the inhibiting effect of dermaseptin S_4 derivatives on pre-formed
295 biofilm, we evaluated their activity on *E. coli* biofilm developed under continuous flow
296 conditions using confocal imaging to visualize both bacterial cells labeled in red (red
297 fluorescent protein, Rfp) and fluorescent dermaseptins.
298 As mentioned earlier, since K_4S_4 and $K_4K_{20}S_4$ exhibited a similar activity against *E. coli*
299 biofilm and due to the lack of a fluorescent $K_4K_{20}S_4$ peptide, this experiment was performed
300 with fluorescein tagged K_4S_4 and $D_4D_{20}S_4$. Confocal images (Fig 2A and B) showed that,
301 under continuous culture conditions in flow cells for 6 h in minimal media with glucose as
302 carbon source, the fluorescent peptide K_4S_4 used at 2x MIC (0.78 µg/ml, in green), targeted
303 all biofilm cells, both in the interior part and in the upper layer of the biofilm. The presence of
304 K_4S_4 appeared to completely stop the growth of the biofilm with just a little detachment,
305 suggesting that this peptide has the ability to inhibit biofilm proliferation (Fig 2A). This was
306 verified by quantitative analysis of bacterial biomass that showed that biofilm proliferation
307 was stopped right after injection of K_4S_4 (Fig. 3). Co-localization measurements indicated that
308 the peptide penetrated bacterial cells, which confirmed its bacterial cell permeation ability
309 (data not shown). The fact that dermaseptin K_4S_4 activity was also discernable in the presence
310 of the continuous flow suggests a strong effect of K_4S_4 despite the dilution factor. In contrast,
311 when the biofilm was exposed to 2x MIC of dermaseptin $D_4D_{20}S_4$ (25 µg/ml), the established
312 biofilm continued to grow steadily, and red cells were still visible and continued to multiply
313 (Fig 2B). Interestingly, although $D_4D_{20}S_4$ showed both binding cell capacity and penetration,
314 it was not as effective as K_4S_4 (co-location measures), and appeared to be unable to stop the
315 growth of *E. coli* biofilm (Fig 3).
316 Among all peptides tested, we found that $K_4K_{20}S_4$ and K_4S_4 were the most potent to
317 inhibit biofilm formation with non toxic concentrations comparing to the others peptides. We
318 also showed that this inhibition of growth was dependent on the nature of the peptide, and the
319 highly charged molecules were the most active.
320

321 **DISCUSSION**

322

323 Infections in which bacteria are either slow growing, dormant, or within a biofilm,
324 pose a serious clinical challenge for therapy because the cells in these states exhibit tolerance
325 to the activity of antimicrobial agents. Many of these infections, including otitis media,
326 sinusitis, cholesteatoma, and tonsillitis, are caused by biofilm-forming mucosal pathogens,
327 such as *Staphylococcus aureus*, *Escherichia col* and *Pseudomonas aeruginosa.* Accordingly,
328 the discovery of anti-infective agents that are active against both planktonic microorganisms
329 and biofilms is of great public health significance. Antimicrobial peptides represent a
330 potential promising source for the development of new alternative agents to combat resistant
331 bacteria. These molecules are rapidly bactericidal, and since their action relates to physical
332 properties, it is all the more difficult for bacteria to develop resistance to such peptides.
333 Moreover, their structure can be engineered with relative ease, because peptide chemistry
334 allows a multitude of modifications that are relatively time and cost-effective. Nevertheless,
335 most of the agents undergoing clinical trials lack specificity and are examined only for topical
336 uses (31).

337

338 Dermaseptins are a specific class of antimicrobial peptides that have a broad range of
339 antimicrobial activity. Despite their promising properties and attributes, little work has so far
340 been performed to evaluate their effects on bacterial biofilm formation. The present study
341 aims to investigate the antimicrobial activity of five related synthetic peptides derived from
342 the natural peptide dermaseptin S_4. It evaluates the effects of dermaseptins and some of their
343 derivatives against a series of Gram-negative and Gram-positive strains, including *S. aureus*,
344 *E. coli* and *P. aeruginosa*, in planktonic and biofilm cells and describes their structure-
345 function relationship.

346

347 Our findings revealed that when used at high concentrations, this natural antimicrobial
348 peptide is cytotoxic *in vitro* for host cells. This toxicity can, however, be reduced through
349 introducing a number of modifications to the native sequence without altering its activity. To
350 increase the antimicrobial activity of S_4, four deletions and substitutions were tested based on
351 previous studies performed with *Escherichia coli* and human red blood cells. Our results
352 revealed that dermaseptins K_4S_4 and $K_4K_{20}S_4$ displayed a hundred times higher increase in
353 antibiofilm potency than the other derivatives. The results also showed that a positive charge

354 substitution led to a lower level of cytotoxicity without altering antibiofilm activity of the
355 peptides.
356
357 Bisubstituted derived peptide $K_4K_{20}S_4$ exhibited the best results. This dermaseptin
358 derivative combined two substitutions, the first substitution of methionine by lysine in
359 position 4 and the second asparagine by lysine in position 20. It was previously shown that
360 increasing the number of positive charges (6 versus 4 for dermaseptin S_4) and reducing its
361 hydrophobicity index (22.7 versus dermaseptin S_4 28.9) resulted in a reduction of hemolytic
362 activity (34). Other studies demonstrated that dermaseptin $K_4K_{20}S_4$ had a good antimicrobial
363 activity *in vivo* with no toxicity in mice (32). The combination of the deletion of the 12 C-
364 terminal residues with the substitution of methionine by lysine in position 4 K_4S_4(1-16), the
365 deletion of 5 N-terminal residues in dermaseptins S_4(5-28), or the reduction of positive
366 charges by substituting methionine in position 4 and 20 by aspartic acid were reported to
367 induce a marked decrease in its antimicrobial activity as compared to $K_4K_{20}S_4$. It can be
368 concluded from these observations that increasing the net positive charges of the peptide
369 without shortening its sequence would result in analogs that display potent antibiofilm activity
370 and low cytotoxicity. Besides, the selective activity of antimicrobial dermaseptins depends on
371 the membrane lipid composition of the microbe versus the host cell and its electrical potential
372 (33). For example, although the lipid compositions of host cell and *P. falciparum* membrane
373 are similar, the potential of the parasite membrane is higher than that of the host cell
374 membrane, leading to the discriminating effect of S_4 (34).
375
376 Furthermore, the literature shows that several other peptides isolated from different
377 sources have been investigated for their antibiofilm activity against several bacterial strains,
378 including cathelicidin (38, 39), histatin, mucins (40, 41), magainins (42, 445), pleurocidin
379 (43), and lactoferrin (44). Like dermaseptins, these peptides are cationic and unstructured and
380 fold into amphipathic α-helices upon contact with membranes. The data presented in this
381 work suggest that the antibiofilm activity of dermaseptins is significantly higher than that of
382 these peptides. For example, magainins II, which are 23-residue α-helical peptides isolated
383 from the skin of the frog *Xenopus laevis*, display antibiofilm activity against *P. aeruginosa*
384 and *E. coli* at 246.9 µg/ml and 15.4 µg/ml, respectively (40), whereas 0.38 µg/ml of
385 dermaseptin $K_4K_{20}S_4$ was enough to achieve the same effect. In addition, a novel peptide
386 mimetic, meta-phenylene ethylene (mPE), modeled after magainin was tested for its efficacy
387 against several biofilm forming strains, including *S. aureus*, and the results showed that the

388 eradication of biofilm was observed at 10xMIC (42, 45). Used at 2xMIC, on the other hand,
389 dermaseptin was sufficient to prevent the formation of *S. aureus* biofilm. Like the archetypal
390 dermaseptins, pyllospetins (PSN), a major family of phyllomedusine skin antimicrobials (45,
391 46), have recently been described to occur in some species in multiple isomeric forms. The
392 evaluation of the activity of these peptides' (PSN-1) against biofilms is, however, very
393 limited; only few strains have so far been evaluated, and no data have shown their ability to
394 inactivate bacterial strains (46). Comparatively, dermaseptins, with their broad range of
395 antibacterial activity, may certainly offer a new promising candidate for the inhibition of
396 biofilm formation. Taken together, the data indicate that the dermaseptin presented in this
397 work has a number of advantages over other antibiofilm cationic peptides tested.
398
399 Biofilms are known to constitute a diverse and complex aggregate of bacteria that
400 exhibit over 100-fold resistance to conventional antibiotics (47). Once a biofilm is
401 established, the live cells are typically buried beneath the surface or between layers of dead
402 cells and encased by a glycocalyx, an extracellular matrix of carbohydrates, proteoglycans,
403 DNA, and other cellular constituents. Not only does this complex of biological molecules
404 inhibit diffusion due to steric hindrance, but its constituents are believed to carry charges that
405 have been known to interfere with the diffusion of others antibiotics (42, 45). The confocal
406 microscopy experiments of this study showed that some dermaseptins such as K_4S_4 penetrate
407 well into the biofilm structure and inhibit biofilm formation.
408
409 The main question that remains is how these dermaseptins can cross a thick layer
410 established by exopolysaccharides, DNA, and proteins, to be able to reach their target cells. In
411 fact, Donlan and his coworkers showed that the matrix of exopolysaccharides also plays a
412 minor role in the antibioresistance properties of the biofilm by being directly bound to the
413 antimicrobial agents and by preventing them from penetrating within the biofilm (1). This
414 suggests that even if the matrix stops certain molecules of antibiotics, other molecules, such
415 as dermaseptins, can cross this barrier and penetrate within the biofilm. The penetration of
416 dermaseptins can stimulate exopolysaccharides production in the matrix and contribute to the
417 increase of its thickness. This increase does not seem to inhibit the peptide which remains
418 active against all the tested biofilm even at low concentrations, suggesting that the matrix may
419 not hold these peptides which are positively charged and that they can reach the target cells.
420 Once in contact with these cells, dermaseptins establish electrostatic interactions with the

421 bacterial membranes, leading to the formation of ionic channels in the lipid bilayers inducing
422 the death of the cell.
423
424 Finally, dermaseptin S_4 derivatives present a new lead structure for potent antibiofilm
425 agents. Nevertheless, new studies are needed to correlate the activity of S_4 in the *in vivo*
426 situation. The *in vitro* biofilm assays employed in this study are essentially preliminary
427 screens. They provide evidence that dermaseptins have a strong effect against biofilm strains,
428 and further studies on their mechanisms of action and activity against other biofilm sources
429 are warranted.
430

431 **Acknowledgments**

432 The authors would like to express their gratitude to Mr Anouar Smaoui and Mrs Hanen Ben
433 Salem from the English Language Unit at the Sfax Faculty of Science, Sfax, Tunisia for their
434 proofreading and language polishing services.
435
436

437 **References**

438

439 1. Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant
440 microorganisms. Clin Microbiol Rev. 15:167-193.
441 2. Yala JF, Thebault P, Héquet A, Humblot V, Pradier CM, Berjeaud JM. 2011.
442 Elaboration of antibiofilm materials by chemical grafting of an antimicrobial peptide.
443 Appl Microbiol Biotechnol. 89:623-634.
444 3. http://grants.nih.gov/grants/guide/pa-files/PA-03–047.html.
445 4. Dongari-Bagtzoglou A. 2008. Mucosal biofilms: Challenges and future directions.
446 Expert. Rev. Anti.Infect. Ther.6: 141–144.
447 5. Francolini I, Donelli G. 2010. Prevention and control of biofilm-based medical
448 device- related infections. FEMS. Immunol. Med. Microbiol. 59: 227–238.
449 6. Belley A, Neesham-Grenon E, McKay G, Arhin FF, Harris R, Beveridge T, Parr TR
450 Jr, Moeck G. 2009. Oritavancin kills stationary-phase and biofilm Staphylococcus
451 aureus cells in vitro. Antimicrob. Agents Chemother. 53: 918-925.
452 7. Schillaci D, Arizza V, Parrinello N, Di Stefano V, Fanara S, Muccilli V, Cunsolo V,
453 Haagensen JJ, Molin S. 2010. Antimicrobial and antistaphylococcal biofilm activity
454 from the sea urchin Paracentrotus lividus. J. Appl. Microbiol.17-24.
455 8. Salay LC, Nobre TM, Colhone MC, Zaniquelli ME, Ciancaglini P, Stabeli RG, Leite
456 JR, Zucolotto V. 2011. Dermaseptin 01 as antimicrobial peptide with rich
457 biotechnological potential: study of peptide interaction with membranes containing
458 Leishmania amazonensis lipid-rich extract and membrane models. J. Pept. Sci.
459 17:700-707.
460 9. Zampa MF, Araújo IM, Dos Santos Júnior JR, Zucolotto V, Leite JR, Eiras C. 2012.
461 Development of a novel biosensor using cationic antimicrobial Peptide and nickel
462 phthalocyanine ultrathin films for electrochemical detection of dopamine. Int. J. Anal.
463 Chem. 850969.
464 10. Krauson AJ, He J, Wimley WC. 2012. Determining the mechanism of membrane
465 permeabilizing peptides: Identification of potent, equilibrium pore-formers. Biochim.
466 Biophys. Acta. 1818: 1625-1632.
467 11. Nicolas P, El Amri C. 2009. The dermaseptin superfamily: A gene-based
468 combinatorial library of antimicrobial peptides *Biochim. Biophys. Acta* . 1788: 1537-
469 1550

470 12. Rivas L, Luque-Ortega JR, Andreu D. 2009. Amphibian antimicrobial peptides and
471 Protozoa: Lessons from parasites Biochim. Biophys. Acta. 1788: 1570-1581
472 13. Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:
473 389–395
474 14. Mor A, Nguyen VH, Delfour A, Migliore-Samour D, Nicolas P. 1991. Isolation,
475 amino acid sequence and synthesis of dermaseptin, a novel antimicrobial peptide of
476 amphibian skin. Biochemistry. 30: 8824–8830.
477 15. Mor A, Rouffaud MA, Montagne JJ, Nguyen VH, Nicolas P. 1993. Natural and
478 synthetic dermaseptins: in vitro large spectrum antimicrobial peptides. J. Mycol. Med.
479 3: 137–143.
480 16. Lequin O, Ladram A, Chabbert L, Bruston F, Convert O, Vanhoye D, Chassaing G,
481 Nicolas P, Amiche M. 2006. Dermaseptin S9, an alpha-helical antimicrobial peptide
482 with a hydrophobic core and cationic termini. Biochemistry. 17;45(2):468-480
483 17. Zaïri A, Tangy F, Hani K. 2013. Dermaseptin S4 derivative $K_4K_{20}S_4$: A potential
484 candidate for development of a new microbicide contraceptive agent - an in vitro
485 study..Eur. J. Contracept. Reprod. Health Care. 18(2):79-87
486 18. . Marynka K, Rotem S, Portnaya I, Cogan U, Mor A. 2007. In vitro discriminative
487 antipseudomonal properties resulting from acyl substitution of N-terminal sequence
488 of dermaseptin S4 derivatives. Chem. Biol. 14(1):75-85.
489 19. Zairi A, Tangy F, Ducos-Galand M, Alonso JM, Hani K. 2007. Susceptibility of
490 *Neisseria gonorrhoeae* to antimicrobial peptides from amphibian skin, dermaseptin,
491 and derivatives. Diagn. Microbiol. Infect. Dis. 57(3):319-324.
492 20. Porat Y, Marynka K, Tam A, Steinberg D, Mor A. 2006. Acyl-substituted
493 dermaseptin S4 derivatives with improved bactericidal properties, including on oral
494 microflora. Antimicrob. Agents. Chemother. 50(12):4153-4160.
495 21. Dagan A, Efron L, Gaidukov L, Mor A, Ginsburg H. 2002. In vitro antiplasmodium
496 effects of dermaseptins. Antimicrob. Agents. Chemother. 46: 1059–1066.
497 22. Efron L, Dagan A, Gaidukov L, Ginsburg H, Mor A. 2002. Direct interaction
498 of dermaseptin S4 aminoheptanoyl derivative with intraerythrocytic malaria parasite
499 leading to increased specific antiparasitic activityin culture. J. Biol. Chem.
500 277(27):24067-24072
501 23. Salay LC, Nobre TM, Colhone MC, Zaniquelli ME, Ciancaglini P, Stabeli RG, Leite
502 JR, Zucolotto V. 2011. Dermaseptin S1 as antimicrobial peptide with rich
503 biotechnological potential: study of peptide interaction with membranes containing

504 *Leishmania amazonensis* lipid-rich extract and membrane models. J. Pept. Sci.
505 17(10):700-707
506 24. Pérez-Cordero JJ, Lozano JM, Cortés J, Delgado G. 2011. Leishmanicidal activity of
507 synthetic antimicrobial peptides in an infection model with human dendritic cells.
508 Peptides. 32(4):683-690.
509 25. Morton CO, Dos Santos SC, Coote P. 2007. An amphibian-derived, cationic, alpha-
510 helical antimicrobial peptide kills yeast by caspase-independent but AIF-dependent
511 programmed cell death. Mol. Microbiol. 65(2):494-507
512 26. Bergaoui I, Zairi A, Tangy F, Aouni M, Selmi B, Hani K. 2013. In vitro antiviral
513 activity of dermaseptin S4 and derivatives from amphibian skin against herpes
514 simplex virus type 2. J. Med. Virol. 85(2):272-281.
515 27. Lorin C, Saidi H, Belaid A, Zairi A, Baleux F, Hocini H, Bélec L, Hani K, Tangy F.
516 2005. The antimicrobial peptide dermaseptin S4 inhibits HIV-1 infectivity in vitro.
517 Virology. 334(2):264-275.
518 28. Belaid A, Aouni M, Khelifa R, Trabelsi A, Jemmali M, Hani K. 2002. In vitro
519 antiviral activity of dermaseptins against herpes simplex virus type 1. J. Med. Virol.
520 66(2):229-34.
521 29. Zampa MF, Araújo IM, Dos Santos Júnior JR, Zucolotto V, Leite JR, Eiras C. 2012.
522 Development of a novel biosensor using cationic antimicrobial Peptide and nickel
523 phthalocyanine ultrathin films for electrochemical detection of dopamine. Int. J. Anal.
524 Chem. 850969.
525 30. Amiche M, Galanth C. 2011. Dermaseptins as models for the elucidation of
526 membrane-acting helical amphipathic antimicrobial peptides. Curr. Pharm.
527 Biotechnol. 12(8):1184-1193. Review.
528 31. William C. Wimley 2010. Describing the Mechanism of Antimicrobial Peptide
529 Action with the Interfacial Activity Model. ACS. Chem. Biol. 5(10): 905–917
530 32. Navon-Venezia SN, Feder R, Gaidukov L, Carmeli Y, Mor A. 2002. Antimicrobial
531 properties of dermaseptin S4 derivatives with in vivo activity. Antimicrob. Agents.
532 Chemother. 46: 689–694.
533 33. Kustanovich I, Shallev DE, Mikhlin M, Gaidukov L, Mor, A. 2002. Structural
534 requirements for potent versus selective cytotoxicity for antimicrobial dermaseptin S4
535 derivates. J. Biol. Chem. 277: 16941–16951

536 34. Efron L, Dagan A, Gaidukov L, Ginsburg H, Mor A. 2002. Direct interaction of
537 dermaseptin S4 aminoheptanoyl derivate with intraerythrocytic malaria parasite
538 leading to increased specific antiparasitic activity in culture. J. Biol. Chem. 277:
539 24067–24072.
540 35. Mor A, Nguyen VH, Delfour A, Migliore-Samour D, Nicolas P. 1991. Isolation,
541 amino acid sequence, and synthesis of dermaseptin, a novel antimicrobial peptide of
542 amphibian skin. Biochemistry. 30: 8824 – 8830.
543 36. Moller S, Sternberg C, Andersen JB, Christensen BB,. Ramos , Givskov M, and
544 Molin S. 1998. In situ gene expression in mixed-culture biofilms: evidence of
545 metabolic interactions between community members. Appl. Environ. Microbiol.
546 64:721-732.
547 37. SAS/STAT., 2000. User's Guide, Release 6.12 Ed., SAS Institute, Cary, NC.
548 38. Amer LS, Bishop BM, van Hoek ML. 2010. Antimicrobial and antibiofilm activity of
549 cathelicidins and short, synthetic peptides against Francisella. Biochem. Biophys.
550 Res. Commun. 396(2):246-251
551 39. Kanthawong S, Bolscher JG, Veerman EC, Van Marle J, De Soet HJ, Nazmi K,
552 Wongratanacheewin S, Taweechaisupapong S. 2012. Antimicrobial and antibiofilm
553 activity of LL-37 and its truncated variants against *Burkholderia pseudomallei*. Int. J.
554 Antimicrob. Agents. 39(1):39-44.
555 40. Wei GX, Campagna AN, Bobek LA. 2006. Effect of MUC7 peptides on the growth
556 of bacteria and on Streptococcus mutansbiofilm. J. Antimicrob. Chemother.
557 57(6):1100-1109
558 41. Da Silva BR, De Freitas VA, Nascimento-Neto LG, Carneiro VA, Arruda FV, De
559 Aguiar AS, Cavada BS, Teixeira EH. 2012. Antimicrobial peptide control of
560 pathogenic microorganisms of the oral cavity: a review of the literature. Peptides.
561 36(2):315-321
562 42. Beckloff N, Laube D, Castro T, Furgang D, Park S, Perlin D, Clements D, Tang H,
563 Scott RW, Tew GN, Diamond G. 2007. Activity of an antimicrobial peptide mimetic
564 against planktonic and biofilm cultures of oral pathogens. Antimicrob. Agents.
565 Chemother. 51(11):4125-4132
566 43. .Choi H, Lee DG. 2012. Antimicrobial peptide pleurocidin synergizes with antibiotics
567 through hydroxyl radical formation and membrane damage, and exerts
568 antibiofilm activity. Biochim. Biophys. Acta. 1820(12):1831-1838.

569 44. Dashper SG, Pan Y, Veith PD, Chen YY, Toh EC, Liu SW, Cross KJ, Reynolds EC.
570 2012. Lactoferrin inhibits *Porphyromonas gingivalis* proteinases and has sustained
571 biofilm inhibitory activity. Antimicrob. Agents. Chemother. 56(3):1548-1556
572 45. Neiva M, Vargas DC, Conceição K, Rádis-Baptista G, Assakura MT, Jared C,
573 Hayashi MA. 2013. Gene expression analysis by ESTs sequencing of the Brazilian
574 frog Phyllomedusa nordestina skin glands. Toxicon. 61:139-150
575 46. Zhang R, Zhou M, Wang L, McGrath S, Chen T, Chen X, Shaw C. 2010.
576 Phylloseptin-1 (PSN-1) from *Phyllomedusa sauvagei* skin secretion: a novel broad-
577 spectrum antimicrobial peptide with antibiofilm activity. Mol. Immunol. 47(11-
578 12):2030-2037
579 47. Mah TF, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial
580 agents. Trends. Microbiol. 9 (1):34-39. Review.
581 48. Valle J, Toledo-Arana A, Berasain C , Ghigo JM, Amorena B, Penades JR, Lasa I.
582 2003. SarA and not sigma B is essential for biofilm development by *Staphylococcus*
583 *aureus*. Mol. Microbiol. 48:1075–1087
584
585

Table 1. Different stains used in this study**.**

Strains	**Relevant characteristics**	**Reference**
E. coli		
MG1655_λatt_Km_*mars*_F'tetΔ*traD*	Red fluorescent biofilm forming *E. coli* strain producing the F pilus, Km^R, Tet^R	Laboratory collection
P. aeruginosa		
PAO1	Wild type, prototroph, *chl*-2	B. Holloway
S. aureus		
15981	Biofilm forming strain	(48)

Table 2 Dermaseptin S_4 derivatives used in this study

.

Dermaseptins	**Sequence**
K_4S_4	ALW**K**TLLKKVLKAAAKAALNAVLVGANA
$K_4K_{20}S_4$	ALW**K**TLLKKVLKAAAKAAL**K**AVLVGANA
$D_4D_{20}S_4$	ALW**D**TLLKKVLKAAAKAAL**D**AVLVGANA
K_4S_4 (1-16)	ALW**K**TLLKKVLKAAAK- - - - - - - - - - - - -
S_4 (5-28)	TLLKKVLKAAAKAALNAVLVGANA

Table 3. Antimicrobial activity and dose-dependent effect of different derivatives of dermaseptins

		MIC[b]		
Peptides	CC^{a}_{50}	*S. aureus*	*P. aeruginosa*	*E. coli*
K_4S_4	28.11**	12.5**	12.5***	0.39*
$K_4K_{20}S_4$	27.92**	0.19*	0.39*	0.19*
$D_4D_{20}S_4$	24.12**	25***	12.5***	12.5***
K_4S_4 (1-16)	28.51**	25***	12.5***	3.125**
S_4 (5-28)	103.11***	25***	12.5***	6.25***
Polymyxin	ND	25***	6.25**	6.25***

[a] Peptide concentration (µg/ml) that causes 50% cytotoxicity of dermaseptin S_4 and derivatives on HeLa cells,
[b] Minimal inhibitory concentration (MIC). Values *in vitro* expressed in µg/ml for all strains tested. ND: not determined. According to SNK multivariate test, we noticed a significantly difference ($p < 0.001$) between peptides, with the best activity, especially against *E. coli*, was reported in $K_4K_{20}S_4$ and K_4S_4 (0.39 µg/ml and 0.19 µg/ml respectively) compared to other peptides with a means activity (12.5 µg/ml, 6.25 µg/ml and 3.12 µg/ml), respectively for $D_4D_{20}S_4$, S_4(5-28) and K_4S_4 (1-16).

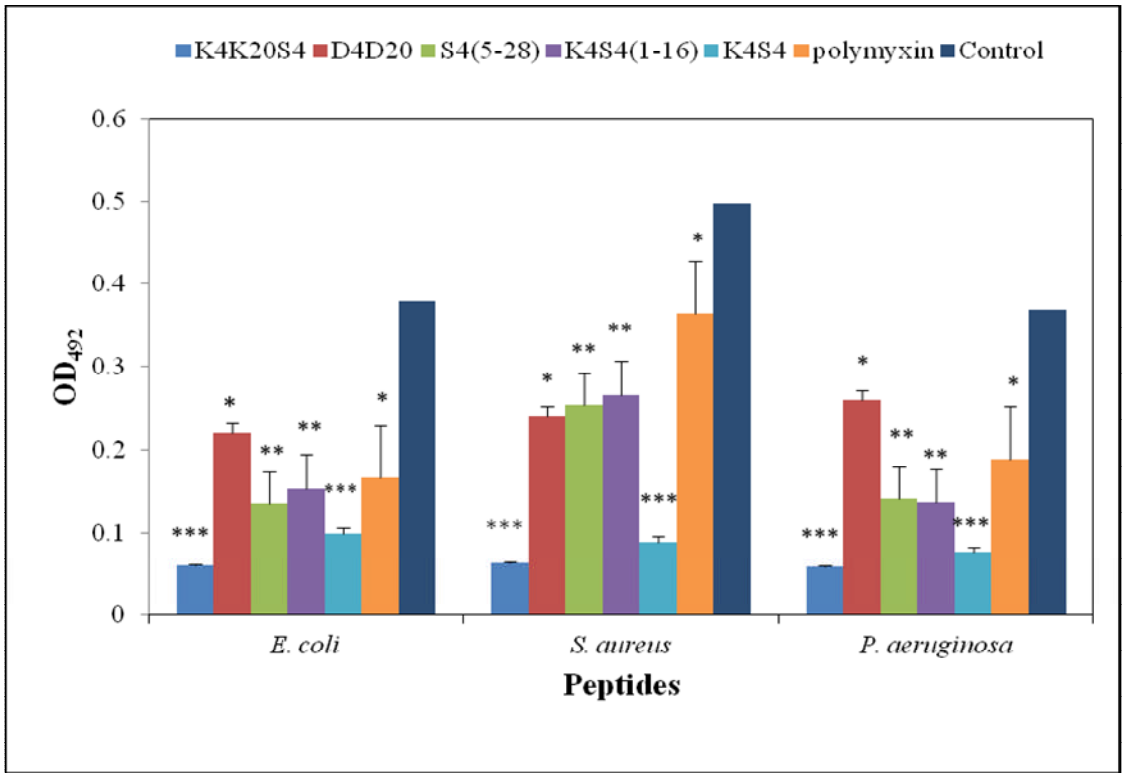


Figure 1. **Effects of dermaseptins S_4 derivatives on biofilm formation**. The viability of biofilm cells was evaluated using the XTT quantification method: Biofilm-forming *S. aureus*, *P. aeruginosa* and *E. coli* strains were grown in 96-well polystyrene plates and biofilms formed exposed to 2 x MIC concentrations of peptides during 24h. Control (negative control wells containing an appropriate medium for each strain only), Polymyxin (positive control wells). Statistical analysis was performed by using SNK multivariate test and Dunnett's test. $K_4K_{20}S_4$ and K_4S_4 were significantly different from control $p<0.001$, S_4(5-28) and K_4S_4(1-16) were different from control ($p<0.01$), and polymyxin is different from control ($p< 0.05$) ($^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$)

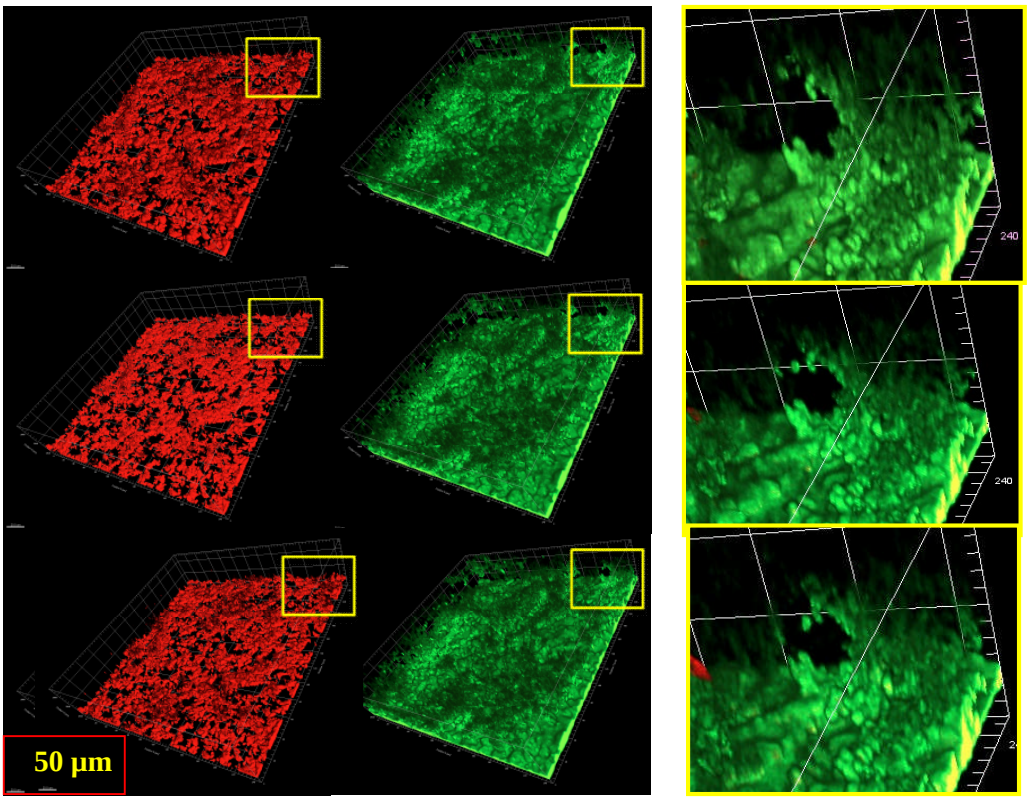
A

Time post-Injection | Bacterial Biofilm | K_4S_4 | MERGE X20

2h

4h

6h



B

Time post-Injection | Bacterial Biofilm | $D_4D_{20}S_4$ | MERGE X20

2h

4h

6h

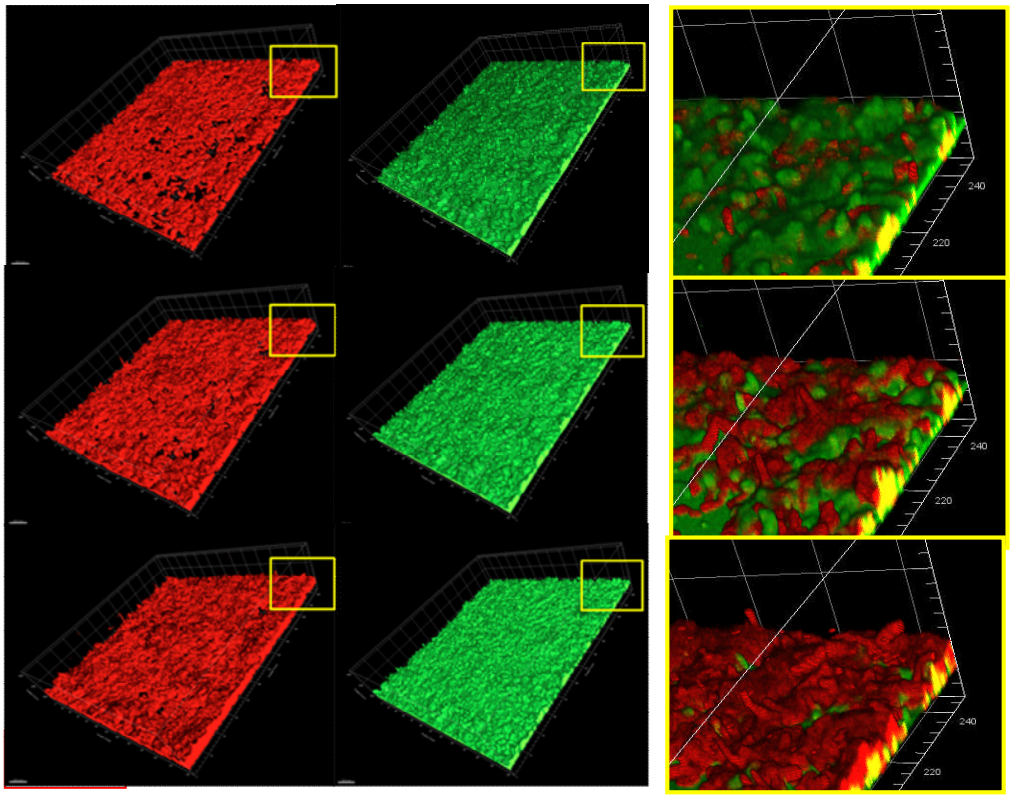


Figure 2 Effect of K_4S_4 on biofilms. To allow attachment of the bacterial cell to the substratum, flow cells were left without flow for 1h after inoculation at 37°C. Afterwards, a laminar flow with a flowrate of 2µl/sec was activated. After 5h of biofilm growth, the flow was stopped and 300 µl (0.78 µg/ml for K_4S_4 and 25 µg/ml for $D_4D_{20}S_4$) of each peptide sample was added. Confocal images acquisition showed that the fluorescent peptide **(A)**: K_4S_4 (green) has an instant effect on biofilm and stops its formation under dynamic flow conditions. Whereas, $D_4D_{20}S_4$, at 25µg/ml, (**B**), shows a less activity, biofilm cells exhibit high fluorescent signals (red) and survived the dermaseptin treatment.

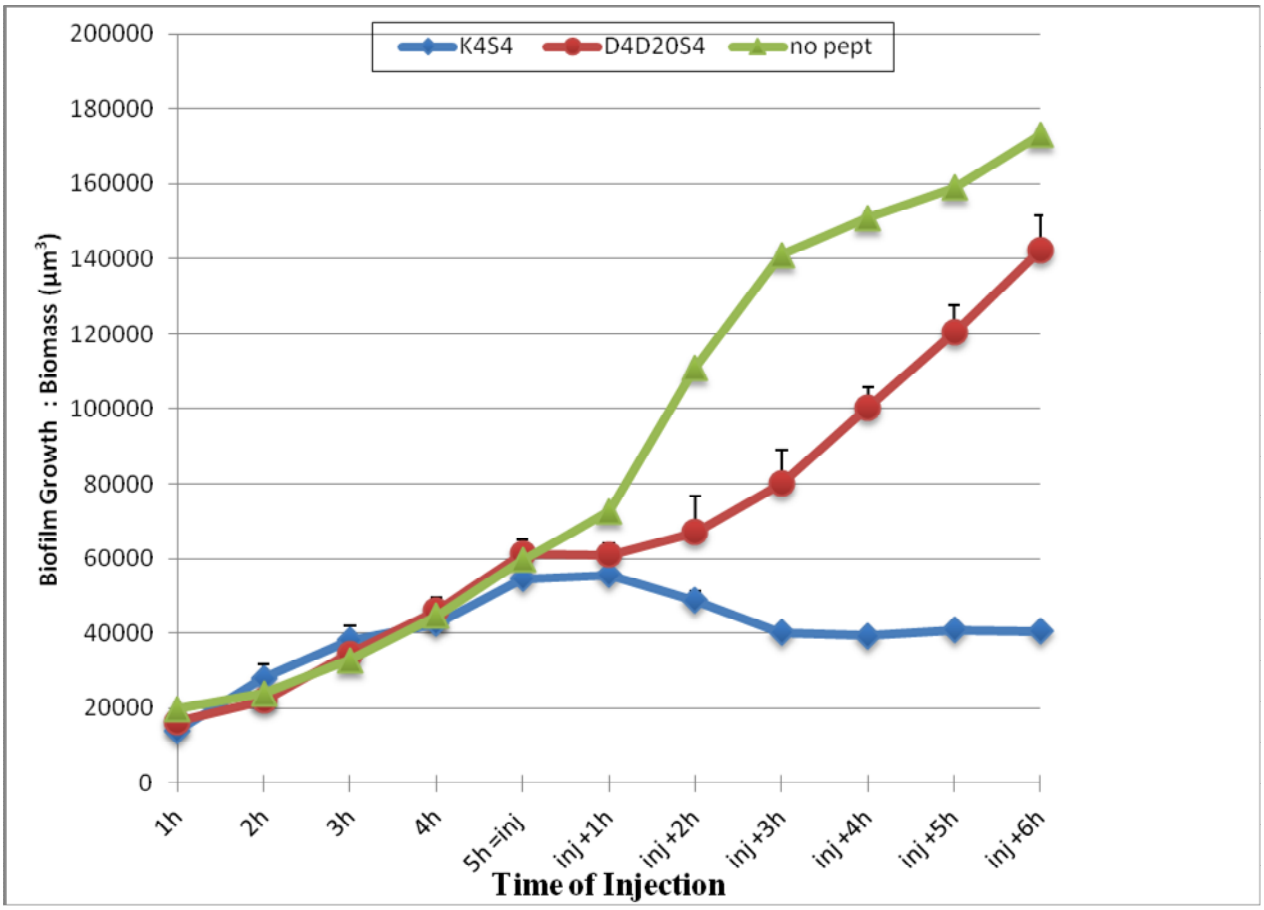


Figure 3. Confocal assays were confirmed by the quantification of biofilms. Differences in bacterial survival in the biofilms were quantified using the computer program Imaris MeasurementPro. Statistical analysis was performed by using SNK multivariate test and Dunnett's test. K_4S_4 was significantly different from control ($p<0.001$).