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Skin bacteria after chlorhexidine exposure—is there a difference in response to human β -Defensin-3?

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Title: Skin bacteria after chlorhexidine exposure - Is there a difference in response to human β -Defensin-3?

Article Type: Article

Keywords: time-kill study; chlorhexidine; human beta-Defensin; Staphylococcus spp.; sequence homology; gyr A genes

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Abstract: Purpose:

We investigated whether exposure to sub-lethal concentrations of chlorhexidine digluconate (CH) changed the response of 5 Staphylococcus spp. to human β -Defensin-3.

Methods:

The change in response for each strain was determined in-vitro with time-kill-experiments in suspension by comparing the mean log₁₀-reduction caused by hBD-3 at 1.5 and 3 h in exposed and non-exposed bacteria. Identity of staphylococcal species was verified by DNA sequence homology in the gyrA genes in comparison with reference strains.

Results:

Baseline sub-lethal concentrations allowing visible bacterial growth were between 0.0625 and 0.25 μ g/ml. Sub-lethal CH concentrations increased within 3 days in two isolates. For Staphylococcus capitis 19/2, CH-exposed cells were less susceptible to 0.5 μ g/ml hBD-3 (log₁₀-reduction 0.78 versus 2.06 at 1.5 h; p <0.001; t-test). For Staphylococcus aureus, however, CH-exposed cells were more susceptible to 1 μ g/ml hBD-3. The observed changes between CH-exposed and non-exposed cells did not indicate a general trend in response to hBD-3.

Conclusions:

Overall, we found no consistent evidence that 3 days of exposure to CHG changed the response of 5 Staphylococcus spp. to hBD-3. The use of CHG for skin antiseptics is, based on our data, unlikely to change the natural defence activity of hBD-3.

Response to Reviewers: REVIEWER #1:

Major points:

Reviewer comment:

Human beta-defensin-3 is a very sticky peptide. To minimize losses, cationic antimicrobial peptides are usually dissolved in weakly acidic solvents. The authors used water, which could influence losses of the peptide and thus might influence its concentration in the remaining solution.

Response:

We were aware that some studies were published with hBD-3 using acetic acid for dissolving the peptide, but the manufacturer (RELIATech) confirmed that their hBD-3 should be reconstituted in water, that it can also be diluted in water and that the use of water will not change the activity of the peptide which was used for the test. That was the reason why we used water (pH-value between 6.5 and 6.8).

Reviewer comment:

Did the authors pool the beta-defensin-3? If yes, did they use the material at exactly identical conditions (e.g. storage at ambient temperature or in the refrigerator after thawing). If not, one would expect variable results.

Response:

Whenever necessary, we pooled aliquots of the diluted peptide after thawing. We did not pool different batches because we only used one batch of the peptide. Nevertheless, we used cooled metal blocks for the storage of the peptide after thawing to ensure that the conditions did not change between tests.

The part in the Methods section of the manuscript was amended to clarify the questions of the reviewer.

Reviewer comment:

Could the authors reproduce in independent experiments (with another peptide sample) the results they found in the four cases where hBD-3 killed significantly fewer bacteria?

Response:

It is a very interesting question if the decrease of the sensitivity of the strain to hBD-3 could also be reproduced by using another peptide sample. In our study we carried out each test in triplicate but we did not use another peptide sample for these tests. We used the same batch of the peptide for all tests, because it was the only one available for us. The significant decrease in hBD-3-susceptibility (> 0.4 log₁₀-difference between the reduction of exposed and non exposed bacteria) was shown in all tests of the triplicate analysis. But we have now addressed this question in the Discussion section of the manuscript.

Minor points:

Reviewer comment:

Line 17: showis

Response:

The wording in Line 17 was corrected.

Reichel et al. Bacterial response to human β -Defensin-3 after chlorhexidine exposure

Skin bacteria after chlorhexidine exposure – Is there a difference in response to human β -Defensin-3?

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Keywords: time-kill study, chlorhexidine; human beta-Defensin; *Staphylococcus* spp.; sequence homology; gyr A genes

Abstract

Purpose:

We investigated whether exposure to sub-lethal concentrations of chlorhexidine digluconate (CH) changed the response of 5 *Staphylococcus* spp. to human β -Defensin-3.

Methods:

The change in response for each strain was determined *in-vitro* with time-kill-experiments in suspension by comparing the mean \log_{10} -reduction caused by hBD-3 at 1.5 and 3 h in exposed and non-exposed bacteria. Identity of staphylococcal species was verified by DNA sequence homology in the *gyrA* genes in comparison with reference strains.

Results:

Baseline sub-lethal concentrations allowing visible bacterial growth were between 0.0625 and 0.25 $\mu\text{g/ml}$. Sub-lethal CH concentrations increased within 3 days in two isolates. For *Staphylococcus capitis* 19/2, CH-exposed cells were less susceptible to 0.5 $\mu\text{g/ml}$ hBD-3 (\log_{10} -reduction 0.78 versus 2.06 at 1.5 h; $p < 0.001$; t-test). For *Staphylococcus aureus*, however, CH-exposed cells were more susceptible to 1 $\mu\text{g/ml}$ hBD-3. The observed changes between CH-exposed and non-exposed cells did not indicate a general trend in response to hBD-3.

Conclusions:

Overall, we found no consistent evidence that 3 days of exposure to CHG changed the response of 5 *Staphylococcus* spp. to hBD-3. The use of CHG for skin antiseptics is, based on our data, unlikely to change the natural defence activity of hBD-3.

1 **Background**

2 2 One of the main functions of the skin is to protect the host from microbial infections; however,
3 3 the skin itself is a potential source of endogenous infections. The skin has established differ-
4 4 ent physiological mechanisms to prevent infections, which can be caused by resident and
5 5 transient skin microorganisms. Firstly, it acts as a physical barrier. In addition, the skin shows
6 6 a diversity of immune-relevant processes to control the microbial ecology present on human
7 7 skin.

8 8
9 9 One important mechanism for controlling colonization of the skin is the secretion of constitu-
10 10 tively or inducibly expressed antimicrobial peptides (AMP) which are part of the innate im-
11 11 mune system. These peptides are effective against pathogenic microorganisms and some of
12 12 them e.g., defensins, are multifunctional signaling molecules that trigger adaptive immune
13 13 responses [1].

14 14
15 15 Human β -Defensin-3, a cationic, broad spectrum AMP of the defensin family, is one of the
16 16 AMPs with multiple biological functions [2, 3]. In contrast to human beta-Defensin-1 and -2,
17 17 hBD-3 shows potent antimicrobial activity against Gram-positive bacteria. These include *S.*
18 18 *aureus* [4, 5], which is one of the most common causes of nosocomial infections, such as
19 19 catheter-related bloodstream infections [6, 7], but also multidrug-resistant nosocomial patho-
20 20 gens, e.g. methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci [4,
21 21 8]. The hBD-3 peptide is inducibly expressed by keratinocytes of the human skin [9]. Various
22 22 stimuli which influence hBD-3 expression have been identified, e.g., the upregulation of hBD-
23 23 3 expression is associated with inflammatory skin diseases like psoriasis. Enhanced amount
24 24 of hBD-3 has been found after contact of the skin with bacteria [3, 4, 10]. In addition, current
25 25 research findings have shown that sterile wounding of the skin may also result in increased
26 26 hBD-3 expression [11].

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28 Maisetta *et al.* investigated synergisms in antimicrobial activity between hBD-3 and other
29 antibiotics against oral bacteria. They showed that a combination of chlorhexidine with hBD-3
30 had a beneficial antibacterial effect [12].

31
32 CH is used in skin antiseptics during the placement of central venous catheters (CVC); This is
33 in accordance with recommendations of the Centers for Disease Control (CDC) to use alco-
34 hol or a 2% chlorhexidine-based preparation for this procedure [7]. In a previous study, we
35 were able to show that chlorhexidine digluconate (CHG) in propan-1-ol slows down recoloni-
36 zation of the skin for at least 72 h [13]. We therefore assumed that CHG persists on the skin
37 for at least 3 days after application when the skin is covered with an adhesive dressing after
38 evaporation of the alcohol.

39
40 CVC may remain in place for an average of 6-28 days and dressings are changed several
41 times during this period [14]. Normally the skin around the catheter site is treated with a CH-
42 containing antiseptic repeatedly every time the catheter dressing is changed. Thus, the skin
43 bacteria at the catheter-site are permanently exposed to CH and the physical skin barrier,
44 which is essential for protection of the bloodstream against microorganisms, is permanently
45 disrupted for a long period of time. The results of Sørensen *et al.* showed that the concentra-
46 tion of hBD-3 increases within 4 days after sterile wounding [11]; therefore we assumed that
47 the bacteria are first exposed to CH for at least 3 days before the hBD-3 concentration on
48 skin increases.

49
50 It has been reported that different bacterial species are able to adapt to sub-lethal concentra-
51 tions of CH [15-20] and that this adaptation affects the susceptibility to different biocides [21]
52 and antibiotics in *Pseudomonas stutzeri* [16, 21, 22]. Moore *et al.* showed that different skin
53 bacteria were able to adapt to CH after repeated exposure [23].

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55 To the best of our knowledge, there are no studies on whether CHG exposure changes the
1 response of Gram-positive bacteria to antimicrobial peptides, such as hBD-3.
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6 58 The aims of this study were to analyze, using *in-vitro* time-kill-studies, if exposure to CHG at
7 sub-lethal concentrations for 3 days influences the response of different *Staphylococcus* spp.
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11 60 to hBD-3, and if this treatment reduces the potency of the natural defense.
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61 **Methods**

62 **Collection and growth of the bacterial strains**

63 Six different isolates of skin bacteria were taken from different skin sites of two healthy, male
64 volunteers (volunteer number 16 and 19). From volunteer 16, we obtained two isolates from
65 the upper back after application of 89.5% (v/v) propan-1-ol and one skin isolate from the ab-
66 domen. In addition, two isolates were taken from the forehead of volunteer 19 after applica-
67 tion of 70% (v/v) propan-2-ol or 89.5% (v/v) propan-1-ol and one isolate was collected from
68 the abdomen. The skin bacteria were collected using a swab method which has previously
69 been described [13]. The skin bacteria were grown aerobically on casein-peptone soymeal-
70 peptone agar (CASO agar) at 37°C for 48 h; thereafter, six single colonies were isolated,
71 plated separately on CASO agar and aerobically incubated at 37°C for 24 h. For susceptibili-
72 ty testing, isolate 19/3, 16/2 and 16/3 were excluded from the study due to clonal-identity
73 (see below; isolate); the chosen isolates are listed in Table 1. The bacteria were stored at -
74 74°C using the CRYOBANK-system (MAST Diagnostica Laboratoriumspräparate GmbH,
75 Reinfeld, Germany).

77 **Identification and characterization of skin isolates**

78 All isolates were Gram-positive; they were characterized by macroscopic and microscopic
79 analyses. They were plated on CASO agar, sheep blood agar and selective agar plates (ka-
80 namycin and mannitol-salt). The presence of clumping factor, protein A and capsular poly-
81 saccharide was analyzed with Staphytest™ plus test (Oxoid Deutschland GmbH; Wesel,
82 Germany). For genotypic characterization of the clonal relationship of the skin isolates
83 PFGE-analysis using the criteria of Tenover et al., 1995 [24] was used (data not shown).

85 Bacterial species

86 The following staphylococcal type strains were used to amplify the respective intragenic *gyrA*
87 fragment for sequence-based identification of skin isolates: *Staphylococcus aureus* ATCC
88 6538, *Staphylococcus warneri* ATCC 27836, *Staphylococcus warneri* ATCC 155, *Staphylo-*
89 *coccus capitis* subsp. *capitis* ATCC 27841, *Staphylococcus epidermidis* ATCC 14990.

91 Primers

92 Based on a multiple DNA sequence alignment of genes *gyrA* from different staphylococcal
93 species, regions of high homology were chosen for the design of a primer pair to be used for
94 PCR amplification and for cycle sequencing of the quinolone resistance-determining region
95 (QRDR). The primer pair includes 5'-primer *gyrA*_[A70V]_5'_:+73_Sau 5'-
96 TATGCGATG(A/T)(G/C)(A/C/T)GTTAT(A/C/T)GT -3' corresponding to nts. 73 to 82) and 3'-
97 primer *gyrA*_[A640R]_3'_+661_Sau 5'-CCGTTGG(A/G)AA(A/G)TC(A/T)GG(A/C/T)CC- 3'
98 complementary to nts. 661 to 680) giving rise to a 608 bp intragenic PCR fragment. For cycle
99 sequencing reactions primer *gyrA*-A70V was used.

101 PCR conditions

102 Template DNA was isolated from 3 to 5 individual colonies of a type strain of the respective
103 staphylococcal species and from skin isolates of volunteers using the QIAGEN Tissue Purifi-
104 cation Kit[®] according to the manufacturer's protocol. Using 2.5 μ l of template DNA in a total
105 volume of 50 μ l respective intragenic fragments of gene *gyrA* were amplified using the follow-
106 ing reaction conditions: 1.25 mM MgCl₂, 30 (40) pMol of each primer of *gyrA* 50 μ mol
107 dNTPs, 2.5 U Taq DNA polymerase (NE Biolabs, Germany) in reaction buffer supplied by the
108 manufacturer. After an initial denaturation at 94°C for 5 min. primer annealing followed for 45
109 s at 50°C, DNA synthesis at 72° for 1 min. and denaturation at 94°C for 30 s over 35 cycles.
110 PCR fragments were purified using the Macherey-Nagel NucleoSpin Extract II Kit[®] according
111 to the manufacturer's protocol. Samples were eluted with 50 μ l of a 1:10 diluted EB-Puffer[®]
112 (QIAGEN, Hilden, Germany).

113 Between 40 and 80 ng of purified PCR product were used for the subsequent cycle sequenc-
114 ing reactions, which were run for 30 cycles (denaturation at 96°C for 20 s; annealing at 50°
115 for 5 s and polymerisation at 60°C for 4 min, in a total volume of 20 μ l using 10 pMol of the
116 respective 5'-primer, Quick Start Kit (Beckman Coulter Krefeld, Germany) according to the
117 manufacturer's instructions.

118
119 After completing the cycle sequencing reactions DNA fragments were precipitated by adding
120 5 μ l of precipitation solution (2 μ l 3M sodium acetate solution pH 5,2; 2 μ l 100 mM EDTA pH
121 8,0 and 1 μ l Glycogen) and mixed with 60 μ l of absolute ethanol. Precipitated DNA was col-
122 lected by subsequent centrifugation (15 min, 15,000 rpm, room temperature) washed with
123 ethanol (70%). Samples were air-dried and resuspended in 30 μ l SLS[®] (Beckman-Coulter ,
124 Krefeld, Germany), and immediately cooled to 4°C before applying the DNA samples to the
125 capillary electrophoresis column. To assign an isolate to a staphylococcal species the DNA
126 sequence determined for the respective *gyrA* gene fragment was aligned to a database of
127 staphylococcal reference strains yielding homologies equal to or higher than 90%, whereas
128 homologies below 90% were found for *gyrA* gene fragments from different species.

129
130 To assign skin isolate 16/1 to a bacterial species without doubt, an API ID 32 Staph-test
131 (bioMérieux Deutschland GmbH, Nürtingen, Germany) was carried out for biochemical identi-
132 fication.

133

134 **Determination of oxacillin and chlorhexidine susceptibility (MIC)**

135 The MIC determinations were carried out by microdilution [25]. The tests were carried out
136 with the skin isolates, *S. aureus* ATCC6538 and *S. epidermidis* ATCC12228. *S. aureus*
137 ATCC29213 was used as the control strain and oxacillin (InfectoStaph, Infectopharm) was
138 used as the reference antibiotic. The oxacillin-containing Mueller-Hinton broth (DIFCO,
139 Augsburg, Germany) was supplemented with 3% NaCl.

140

141 **Preparation of test bacteria and time-kill-analyses with hBD-3**

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2 142 All tubes and plates were incubated aerobically at 37°C. The following procedure was carried
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4 143 out separately for each isolate.

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8 145 Day 0:

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11 146 The second passage of each test strain was generated by incubation on CASO agar for 24 h.

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15 148 Day 1:

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17 149 Preparation of the test suspension: A small number of single colonies taken from CASO agar
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19 150 were suspended in physiological saline to adjust the suspension to 10^6 to 10^7 bacteria (opti-
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21 cal density of 0.125-0.175 at 550 nm; measured with Eppendorf ECOM 6122 (Eppendorf AG,
22 151 Hamburg, Germany)).

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28 154 9.9 ml of tryptic soy broth (TSB) without any additional substances (control without CHG ex-
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30 155 posure) as well as 9.9 ml TSB containing different concentrations of CHG were inoculated
31
32 156 with 100 μ l of bacterial suspension. Both were incubated for 24 h.

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36 158 Day 2 and 3:

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38 159 After overnight incubation, each tube was shaken and screened for visible growth. 100 μ l of
39
40 160 the control sample were added to 9.9 ml of TSB without any additional substances (second
41
42 161 and third passage of control in broth). From the CHG-containing samples, the sample with
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44 162 the highest CHG concentration and visible growth was chosen. For inoculation of 9.9 ml TSB
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46 163 containing CHG (as described above), 100 μ l of the suspension were taken for each sample.

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48 164 The control and the CHG-dilution series were again incubated for 24 h.

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52 166 Day 4:

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167 100 μ l of the control sample and the sample with the highest CHG concentration with visible
168 growth were plated homogenously on CASO agar containing 0.1% L-histidine, 0.3% lecithin,
169 and 3% polysorbate 80 and incubated for 24 h.

170

171 Day 5:

172 The lyophilized recombinant hBD-3 (RELIATech, Braunschweig, Germany) was solubilized
173 and diluted in aqua bidest (pH 6.5-6.8) as recommended by the manufacturer of the peptide,
174 to obtain a 10 μ l solution containing the defined concentration of hBD-3 (Table 2). To ensure
175 identical conditions for each test, the reconstituted hBD-3 was stored in cooled metal blocks
176 until the test was performed. The tests were carried out within several minutes after prepar-
177 ing the diluted peptide solutions. Before the tests started, preliminary tests were carried out
178 to find suitable hBD-3 concentrations for each isolate, to enable us to carry out time-kill-
179 studies at 1.5 and 3 h. For each strain two specific hBD-3-concentrations were chosen, and it
180 was predefined that the high concentration must contain fourfold more hBD-3 than the low
181 concentration.

182

183 Colonies on each CASO agar plate were rinsed off with 10 ml phosphate buffer (10 mM), the
184 suspensions were transferred into sterile glass cylinders with glass pearls and homogenized
185 by mixing for at least 10 min. Each suspension was diluted with phosphate buffer (10 mM) to
186 an optical density of 0.125-0.175 as described above. A dilution series was plated in dupli-
187 cate to determine the baseline bacterial density.

188

189 All tests were carried out with the CHG-exposed and the non-exposed bacteria. Tests in so-
190 lution were carried out in triplicate as follows:

191

192 100 μ l of the 1:10 dilution of the adjusted suspension of bacteria were added to each of the
193 batch so that a total volume of 1 ml was obtained in all cases.

194

- control test: 900 μ l phosphate buffer

195 • susceptibility test with low hBD-3-concentration: 10 μ l low concentration of
1 hBD-3-solution in 890 μ l phosphate buffer (10mM)
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4 197 • susceptibility test with high hBD-3-concentration: 10 μ l high concentration of
5 hBD-3-solution in 890 μ l phosphate buffer (10mM)
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11 200 After the exposure time of 1.5 and 3 h, dilution series were prepared with TSB supplemented
12 with 3% polysorbate 80, 0.3% lecithin, 0.1% L-histidine, and 0.1% L-cysteine; 100 μ l-aliquots
13 201 were plated in duplicate on CASO agar containing 0.1% L-histidine, 0.3% lecithin, and 3%
14 202 polysorbate 80 and incubated for 48-72 h. The CFU were counted after incubation and trans-
15 203 ferred into \log_{10} -values.
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24 206 **Statistical analysis**
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26 207 All computer-based statistical calculations were performed with SPSS 13.0 or 15.0 (SPSS
27 Inc., Chicago, IL, USA).
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33 210 All plates, with CFU between 15 and 300 were evaluated. The results were evaluated as
34 arithmetic means of the \log_{10} -values of each duplicate analysis. When the results of two dif-
35 211 ferent dilution steps were within the defined CFU range, the weighted means were calcu-
36 212 lated.
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44 215 The \log_{10} -reduction for both exposure times was calculated by subtracting the means of the
45 triplicate analyses of \log_{10} CFU count for hBD-3 treated samples from the mean \log_{10} CFU
46 216 count of the phosphate buffer control. The change in response to hBD-3 was determined by
47 comparing the mean \log_{10} -reduction at 1.5 h as well as 3 h in exposed and non-exposed
48 217 (control) bacteria.
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57 221 A t-test was carried out to determine the differences between the \log_{10} -reduction of the CHG-
58 exposed and non-exposed samples after 1.5 h and 3 h for both test concentrations of hBD-3.
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223 **Results**

224 **Characterization of isolates and strains**

225 Two of the isolates obtained from the forehead of volunteer 19 (19/2 and 19/3), and all three
226 isolates from two different body sites of volunteer 16 (16/1 to 16/3) showed identical PFGE-
227 patterns (data not shown) although they were obtained from different body sites respectively
228 different test areas at the forehead.

229
230 The chosen isolates varied in their ability to obtain agglutination with the Staphylect plus™
231 test (Oxoid Deutschland GmbH; Wesel, Germany). Only isolate 16/1 showed a positive ag-
232 glutination reaction. All skin isolates grew on mannitol-salt agar and fermented mannitol.
233 None of the skin isolates showed hemolysis on blood agar. All skin isolates were susceptible
234 to oxacillin (MIC < 8 μ g/ml). The MICs for chlorhexidine were comparable for all tested
235 strains (MIC 0.5 - 2 μ g/ml).

236
237 The comparison of the resulting DNA sequences with those determined for the staphylococ-
238 cal reference strains revealed that isolates 19/1 and 19/2 should be assigned to *Staphylo-*
239 *coccus capitis* subsp. *capitis* (homology > 98 %). DNA sequencing results of an internal 284
240 bp PCR fragment of gene *gyrA* obtained from isolate 16/1 revealed sequence homologies of
241 87%, 88%, 90%, and 89% compared to the reference strains of *S. aureus*, *S. epidermidis*,
242 *S. warneri* and *S. capitis* subsp. *capitis*, respectively. The API 32 ID Staph (bioMérieux
243 Deutschland GmbH, Nürtingen, Germany) confirmed the *gyrA* gene sequence-based identifi-
244 cation of isolate 16/1 as *Staphylococcus warneri* with %id = 99.9% and t = 0.95; 0 biochemi-
245 cal results were found against the assignment to this species.

246
247 **CHG exposure followed by hBD-3 time-kill-test**

248 The concentrations of the CHG exposition as well as the results for the hBD-3-time-kill-tests
249 are presented in Table 2.

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251 A twofold decrease in CHG susceptibility was detected for two isolates (19/2 and 16/1) after
252 3 days exposure to CHG. No change in susceptibility was detected for 19/1 and *S. epider-*
253 *midis* ATCC 12228. However, we found only low turbidity after the first incubation of
254 *S. aureus* ATCC 6538 with 1 μ g/ml CHG and an increasing susceptibility in the second pas-
255 sage.

256
257 The results for hBD-3-time-kill testing were inconsistent (Table 2). In 13 out of 20 experi-
258 ments we found no statistically significant difference in response to hBD-3 with and without
259 previous CHG exposure. In four cases hBD-3 killed significantly fewer bacteria which had
260 been previously exposed to CHG, whereas in one of these the difference was less than 0.1
261 log-scale. We found a significant decrease in hBD-3 susceptibility for both exposure times
262 only for *S. capitis* 19/2 and after 3h exposure to hBD-3 for *S. warneri* 16/1. In three cases
263 more CHG-exposed bacteria were killed by hBD-3 compared to non-exposed bacteria, al-
264 though in one case the difference was less than 0.4 log-scales (Figures 1-5).

265 **Discussion**

1
2 266 We found no clear evidence that exposure to sub-lethal concentrations of CHG for 3 days
3
4 267 influenced the response of *Staphylococcus* spp. to hBD-3. Our results show variable
5
6 268 changes in time-kill-studies for different strains. In strains that showed a decrease in CHG
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8 269 susceptibility within the 3 day exposure period (*S. capitis* 19/2 and *S. warneri* 16/1) we found
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11 270 a significant decrease in response to hBD-3. This may indicate a link between CHG adapta-
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13 271 tion and a decrease in response to hBD-3. Further investigation is needed to verify this hy-
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15 272 pothesis.

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19 274 Exposure to CH has been reported to result in adaptation, e.g., in skin bacteria [23], and
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22 275 thus, changes susceptibility to different antibiotics and biocides [15-22]. Therefore an influ-
23
24 276 ence of previous exposure to CHG on susceptibility to hBD-3 is possible due to the structural
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26 277 properties of both substances and their comparable mode of action. A negatively charged
27
28 278 cell surface is essential for the affinity of both cationic antimicrobials to Gram-positive bacte-
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31 279 ria, to allow electrostatic interactions between the molecules and the cell surface [3, 26]. For
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33 280 hBD-3 as well as CHG and other cationic antibiotics, changes in susceptibility on the basis of
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35 281 alteration of the *dlt* operon and therefore the cell surface charge by D-alanylation of teichoic
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37 282 acids is described in the literature [27-29]. For example, *dltD* deficient mutants of *L. casei*
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39 283 were more susceptible to CH [29]. Peschel *et al.* demonstrated for *S. aureus* and *S. xylosus*
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42 284 that inactivation of the *dlt operon* resulted in enhanced susceptibility to defensins (human
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44 285 defensins HNP1-3) [28]. It is also described that Gram-positive bacteria are able to sense the
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46 286 existence of sub-inhibitory concentrations of antimicrobial peptides like hBD-3 to control dif-
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49 287 ferent resistance mechanisms. Li *et al.*, showed that exposure to sub-inhibitory concentra-
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51 288 tions of hBD-3 resulted in a significant upregulation of the expression of the *dlt* operon, and a
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53 289 subsequent increase in D-alanylation of teichoic acids in *S. epidermidis* [30]. A comparable
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55 290 mechanism with a change in the bacterial surface charge to resist human beta-defensins is
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58 291 also discussed for Gram-negative bacteria [31]. Therefore, a correlation between the mecha-
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292 nism of bacteria to circumvent the natural host defence of the skin caused by AMPs and the
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2 293 mechanism for resistance to cationic antimicrobials, e.g. CH is likely.

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6 295 Another mechanism which may also play a role in resistance to hBD-3 as well as CH is the
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9 296 expression of multidrug (MDR) efflux pumps. Huet *et al.* demonstrated that in *S. aureus*, sin-
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11 297 gle as well as multiple step-exposure to biocides, which were comparable to CH, resulted in
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13 298 an increase in the MIC for CH and multiple other biocides mostly due to an upregulation of
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15 299 the gene expression of different MDR efflux pumps like *mepA* and *norA* [32]. These results
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17 300 showed that single exposure to biocides may result in a change in cell regulatory mechan-
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20 301 isms and therefore a change in susceptibility. The role of an MDR efflux-related increase in
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22 302 MIC and skin disinfection with CH is also discussed by DeMarco *et al.* [33]. Efflux mechan-
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24 303 isms are also relevant for decreasing susceptibility, for example of *Treponema denticola*, to
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26 304 hBD-3 [34], although current research findings suggest that MDR efflux pumps do not play
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29 305 an important role in hBD-3-resistance [35].

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33 307 With regard to our results, we found considerable differences in response to different con-
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35 308 centrations of hBD-3 between the tested strains. Therefore, we assume that susceptibility is
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38 309 not only species-specific but also strain-specific. For *S. capitis* 19/2 and *S. epidermidis*
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40 310 ATCC12228, the difference in response to hBD-3 of CHG-exposed and non-exposed bacte-
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42 311 ria at a concentration of 0.125 $\mu\text{g/ml}$ at 1.5 h was statistically significant, but the \log_{10} -
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44 312 differences of the RF-values were less than 0.35 log-scales. Therefore, the biological relev-
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47 313 ance of these differences remains debatable.

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51 315 On the basis of our results, we could not find any evidence that residual CHG has a negative
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53 316 influence on the host immune defense e.g., when it is used for catheter-site-care. Therefore,
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56 317 a concluding evaluation regarding the clinical relevance of these findings is not yet possible.
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58 318 On the one hand, further discussion may be needed if studies verify that CHG-adapted bac-
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60 319 teria show a reduced response to hBD-3, however, it should be kept in mind that the tested

320 CHG concentrations were much lower than the concentrations used in clinical practice. On
1
2 321 the other hand, a beneficial effect of CHG has been shown in the prevention of catheter-
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4 322 related bloodstream infections, and a synergism with hBD-3 has been demonstrated. If a
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6 323 significant influence of CHG exposure on hBD-3-susceptibility can not be confirmed, the
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8 324 presence of both substances on the skin in combination may contribute to the positive effect
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11 325 seen in the prevention of catheter-related bloodstream infections due to a potentially syner-
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13 326 gistic effect against skin bacteria.

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17 328 However, the power of this study is limited due to the small number of strains tested. Moreo-
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19 329 ver, different results may be obtained with a longer CHG exposure time prior to hBD-3-time-
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21 330 kill-tests. It is possible that a time period of 3 days is not long enough to select bacteria which
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23 331 show changes in response to hBD-3. Further investigation with more test strains and other
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25 332 bacterial species is needed to clarify whether CHG-adapted bacteria are significantly less
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27 333 susceptible to hBD-3. Although we carried out all tests in triplicate and found comparable
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29 334 results in the tests for the strains which showed a decrease in susceptibility after CHG expo-
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31 335 sure, it would be interesting to see if the same results can be found with another sample of
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33 336 the same peptide. Our data, however, do not allow to give an answer because all tests were
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35 337 carried out with the same batch of hBD-3. If these findings can be verified by other studies, it
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37 338 would be interesting to determine if comparable or even identical resistance mechanisms are
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39 339 the cause.

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43 341 **Conclusions**

44
45 342 Apparent differences in the response to hBD-3 were found in bacteria which showed a de-
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47 343 crease in susceptibility after exposure to sub-lethal concentrations of CHG for 3 days. How-
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49 344 ever, we found no consistent evidence that 3 days of exposure to CHG changed the re-
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51 345 sponse of 5 *Staphylococcus* spp. to hBD-3. The use of CHG for skin antiseptis is, based on
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53 346 our data, unlikely to change the natural defence of hBD-3.

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348 **Acknowledgements**

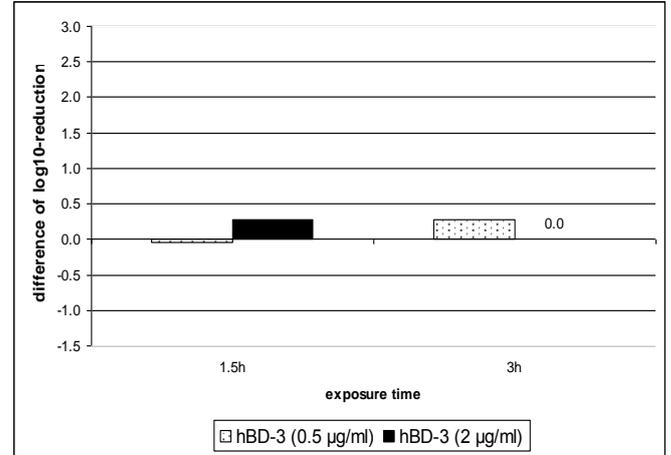
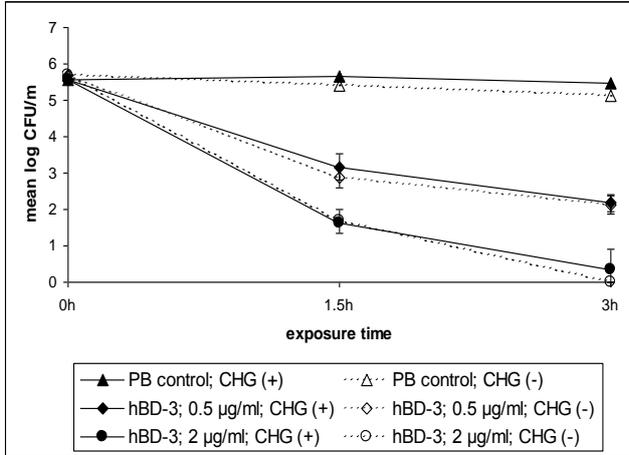
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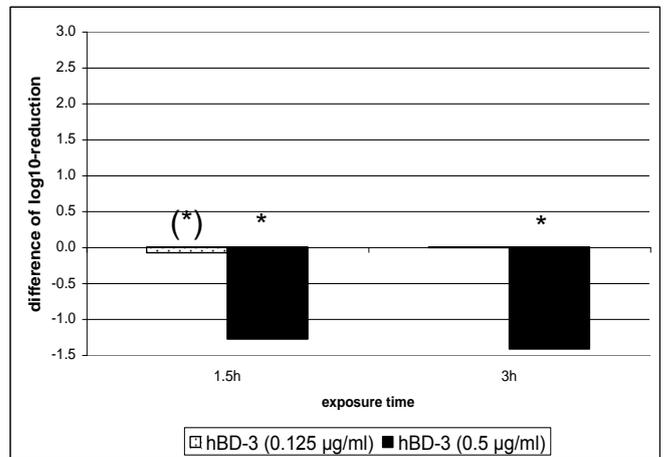
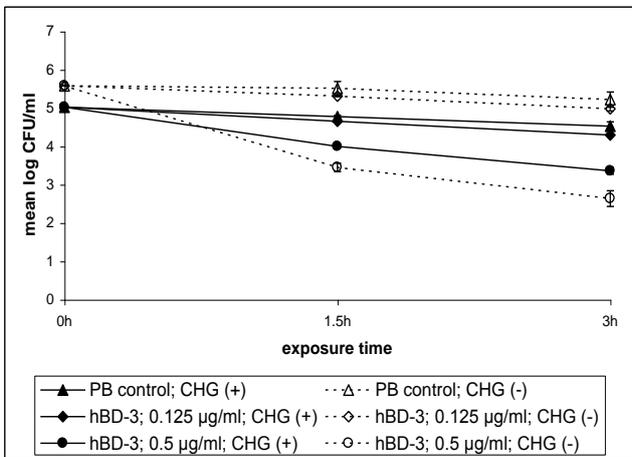
Figures

Figure 1 A-E – Reduction of CFU counts of five staphylococcal strains by hBD-3; previously exposed (closed symbols) or not-exposed (open symbols) for 3 days to sub-lethal concentrations of CHG

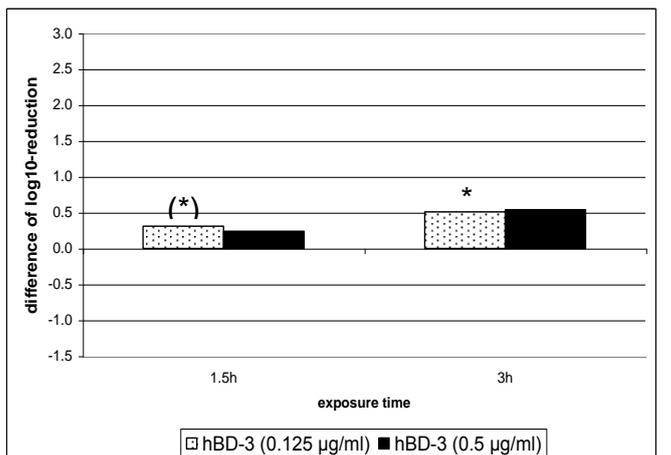
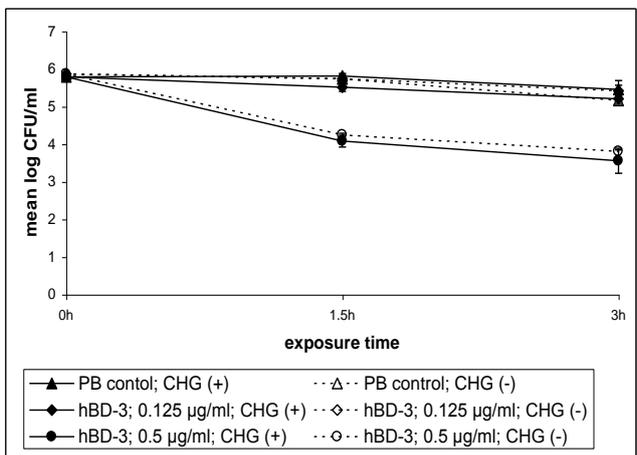
A. Isolate 19/1 (*Staphylococcus capitis*)



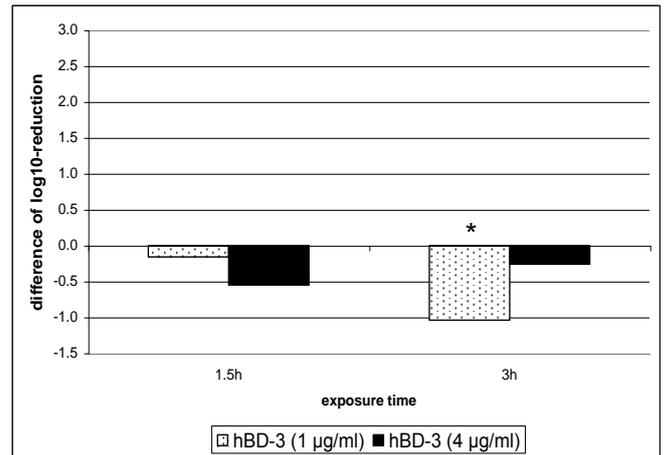
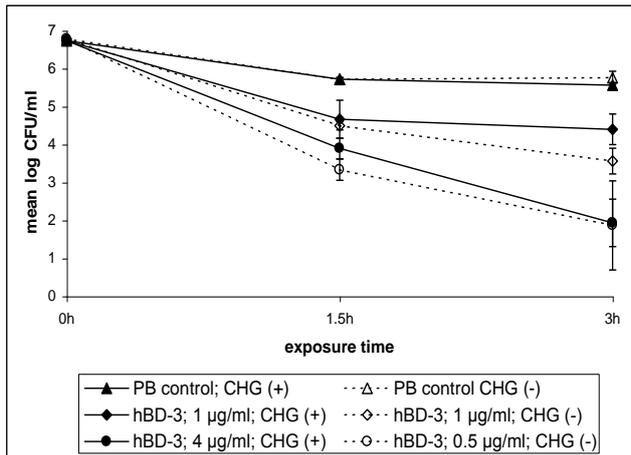
B. Isolate 19/2 (*Staphylococcus capitis*)



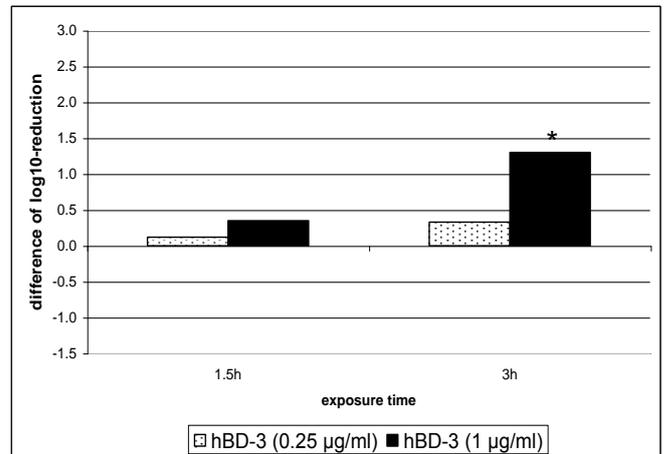
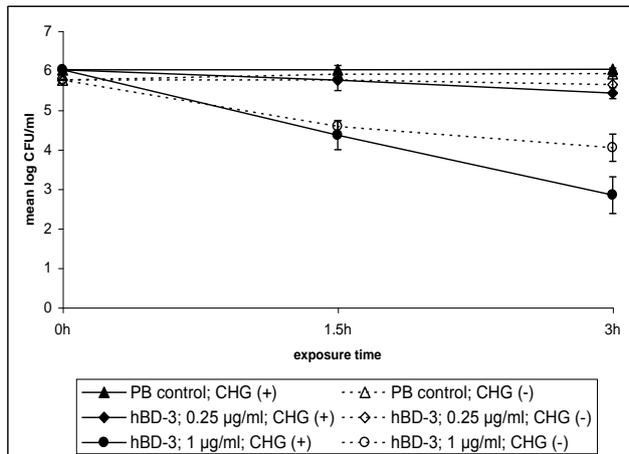
C. *Staphylococcus epidermidis* ATCC 12228



D. Isolate 16/1 (*Staphylococcus warneri*)



E. *Staphylococcus aureus* ATCC 6538



Left side: Mean log CFU/ml after 1.5 and 3 h exposure to different hBD-3 concentrations with and without previous exposure of bacteria to sub-lethal concentrations of CHG; PB = phosphate buffer; CHG (+) = CHG-exposed; CHG (-) = non CHG-exposed

Right side: Difference in log₁₀-reduction of CHG-exposed and non-exposed bacteria after 1.5 and 3h exposure to hBD-3; * significant difference; (*) = significant difference but log₁₀-difference < 0.4

Tables

Table 1 - Characterization of pre-selected isolates

Isolate	Species	Obtained from [after application of]	Growth on mannitol-salt agar / fermentation of mannitol	Growth on kanamycin agar	Hemolysis on blood agar	Staphylect plus™ test	MIC CHG [μ g/ml]	MIC OXA [μ g/ml]
19/1	<i>S. capitis</i>	Abdomen of a volunteer	+/+	inhibited growth / no change in the color of the agar	-	-	1	0.125
19/2	<i>S. capitis</i>	forehead of a volunteer [propan-1-ol 89.5% (v/v)]	+/+	inhibited growth / no change in the color of the agar	-	-	0.5	0.0625
16/1	<i>S. warneri</i>	abdomen	+/+	-	-	+	1	0.125
ATCC 12228	<i>S. epidermidis</i>	DSMZ Braunschweig, Germany	+/-	-	-	-	1	0.125
ATCC 6538	<i>S. aureus</i>	DSMZ Braunschweig, Germany	+/+	Strongly inhibited growth / no change in the color of the agar	+	+	2	<0.03 (low growth)
ATCC 29213	<i>S. aureus</i>	DSMZ Braunschweig, Germany	n.d.	n.d.	n.d.	n.d.	2	0.25

n.d. not determined

Table 2 – Comparison of log₁₀-reduction of five *Staphylococcus* spp. by hBD-3 with and without previous CHG-exposure for 3 days

Isolate	Turbidity after 24 h with CHG in broth (μg/ml)			concentration hBD-3 (μg/ml)	exposure time hBD-3; 1.5 h			exposure time hBD-3; 3 h		
	day 1	day 2	day 3		CHG exposed RF±SD	non CHG exposed RF±SD	P-value	CHG exposed RF±SD	non CHG exposed RF±SD	P-value
<i>S. capitis</i> 19/1	0.25	0.25	0.25	0.5	2.48 ± 0.36	2.53 ± 0.30	0.88	3.29 ± 0.23	3.01 ± 0.26	0.24
				2	4.02 ± 0.06	3.74 ± 0.32	0.28	5.13 ± 0.58	5.13 ± 0	0.99
<i>S. capitis</i> 19/2	0.0625	0.125	0.25	0.125	0.12 ± 0.03	0.20 ± 0.02	0.01	0.23 ± 0.03	0.24 ± 0.06	0.93
				0.5	0.78 ± 0.04	2.06 ± 0.11	<0.001	1.16 ± 0.09	2.58 ± 0.20	<0.001
<i>S. warneri</i> 16/1	0.25	0.5	1	1	1.06 ± 0.51	1.22 ± 0.11	0.62	1.16 ± 0.40	2.20 ± 0.34	0.03
				4	1.83 ± 0.27	2.38 ± 0.28	0.07	3.63 ± 0.63	3.89 ± 1.18	0.75
<i>S. epidermidis</i> ATCC 12228	0.25	0.25	0.25	0.125	0.30 ± 0.11	-0.01 ± 0.11	0.03	0.25 ± 0.08	-0.26 ± 0.28	0.04
				0.5	1.73 ± 0.16	1.49 ± 0.04	0.06	1.90 ± 0.34	1.36 ± 0.04	0.05
<i>S. aureus</i> ATCC6538	1	0.5	0.5	0.25	0.26 ± 0.26	0.15 ± 0.04	0.50	0.60 ± 0.14	0.28 ± 0.20	0.09
	(low turbidity)			1	1.65 ± 0.37	1.32 ± 0.15	0.22	3.18 ± 0.46	1.87 ± 0.35	0.02

RF = log₁₀-reduction

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