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1 **TITLE PAGE**

2 **Title:**

3 Antibiotic Efficacy against Methicillin-Susceptible *Staphylococcus aureus* Biofilms on  
4 Synthetic and Biological Vascular Grafts

5

6 **Running title:**

7 Antibiotics vs. Staph Biofilms on Grafts

8

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76 **MAIN BODY**

77

78 **ABSTRACT**

79

80 **Objective:** To compare the efficacy of five antibiotics against methicillin-susceptible  
81 *Staphylococcus aureus* (MSSA) biofilms on the surface of four vascular grafts.

82

83 **Methods:** *In vitro* study of two clinical MSSA strains (MSSA2 and MSSA6) and four  
84 vascular grafts (Dacron, Dacron-silver-triclosan, Omniflow-II, and bovine pericardium).  
85 After a 24-hour incubation period, the graft samples were divided into six groups:  
86 growth control (no treatment), ciprofloxacin 4.5mg/L, cloxacillin 100 mg/L, dalbavancin  
87 300 mg/L, daptomycin 140 mg/L, and linezolid 20 mg/L. Quantitative cultures were  
88 obtained and results expressed as log<sub>10</sub> colony-forming units per milliliter (CFU/mL).  
89 Analysis of variance was performed to compare biofilm formation between the  
90 different groups.

91

92 **Results:** Mean  $\pm$  SD MSSA2 count on the growth control Dacron graft was  
93  $10.05 \pm 0.31$  CFU/mL. Antibiotic treatment achieved a mean reduction of 45%;  
94 ciprofloxacin was the most effective antibiotic (64%). Baseline MSSA2 counts were very  
95 low on the Dacron-silver-triclosan ( $0.50 \pm 1.03$  CFU/mL) and Omniflow-II ( $0.33 \pm 0.78$   
96 CFU/mL) grafts. On the bovine pericardium patch, the count was  $9.87 \pm 0.50$  CFU/mL,  
97 but this was reduced by a mean of 45% after antibiotic treatment (61% for  
98 ciprofloxacin). Mean MSSA6 count on the growth control Dacron graft was  $9.63 \pm 0.53$   
99 CFU/mL. Antibiotics achieved a mean reduction of 48%, with ciprofloxacin performing

100 best (67% reduction). Baseline MSSA6 count on the Dacron-silver-triclosan graft was  
101  $8.54 \pm 0.73$  CFU/mL. Antibiotics reduced biofilm formation by 72%; cloxacillin was the  
102 most effective treatment (86%). The MSSA6 count on the untreated Omniflow-II graft  
103 was  $1.17 \pm 1.52$  CFU/mL. For the bovine pericardium patch, it was  $8.98 \pm 0.67$  CFU/mL.  
104 Mean reduction after antibiotic treatment was 46%, with cloxacillin achieving the  
105 greatest reduction (68%).

106

107 **Conclusion:** In this *in vitro* study, ciprofloxacin and cloxacillin performed best at  
108 reducing biofilms formed by clinical MSSA strains on the surface of biological and  
109 synthetic vascular grafts.

110

111 **KEYWORDS**

112 Biofilms, *Staphylococcus aureus*, Vascular graft, Infections, Antibiotics.

113

114

115 **1. INTRODUCTION**

116

117 Vascular grafts are a fundamental part of routine vascular surgery. They are used for  
118 arterial reconstruction in different areas of the body and are essential when  
119 autologous material is not available. They do, however, carry a risk of infection.<sup>1</sup>  
120 Vascular graft infections (VGIs) are multifaceted, complex, and potentially  
121 devastating.<sup>2,3</sup> Their management requires a multidisciplinary approach, often entailing  
122 tailored surgical removal of prosthetic material and antibiotic therapy, which is the  
123 cornerstone of VGI treatment.<sup>4,5</sup> Biofilm formation is one of the greatest challenges  
124 encountered in prosthetic graft infections, as these films hamper prevention, diagnosis,  
125 and treatment. Understanding the intricate mechanisms underlying biofilm formation<sup>6-</sup>  
126 <sup>10</sup> and associated antibiotic susceptibility is crucial for effective VGI management.

127

128 As a follow-up to a previous study by our group in which we quantified the formation  
129 of methicillin-susceptible *Staphylococcus aureus* (MSSA) biofilms on the surface of  
130 vascular grafts routinely used in vascular surgery,<sup>11</sup> we designed a new experiment to  
131 further test the grafts that had shown the greatest resistance to biofilm formation. The  
132 aim of the present study was to study the efficacy of five antistaphylococcal antibiotics  
133 commonly used for VGIs (ciprofloxacin, cloxacillin, dalbavancin, daptomycin, and  
134 linezolid) against biofilms formed on synthetic two biological vascular grafts.

135

136 **2. MATERIALS AND METHODS**

137

138 **2.1. Setting and grafts**

139 This study was conducted in the antimicrobial resistance laboratory at Vall d’Hebron  
140 Research Institute (VHIR) as a follow-up to a previous study by our team<sup>11</sup> where  
141 almost all manufactured grafts available for vascular reconstruction were included. For  
142 the present study we chose the grafts that had shown greater resistance to biofilm  
143 formation, in order to study how they could interfere on biofilm formation in  
144 combination with antibiotics. We studied two synthetic grafts—a standard Dacron  
145 polyester vascular graft (Intergard, Le Ciotat, Cedex, France) and a Dacron graft  
146 combining silver acetate and triclosan (Dacron-Silver-Triclosan) (Intergard Synergy,  
147 Maquet, Orleans, France)—and two biological grafts—a vascular prosthesis combining  
148 a polyester mesh and denatured ovine collagen (Omniflow II; LeMaitre Vascular, Inc.,  
149 Burlington, Massachusetts, USA) and a bovine pericardium patch (XenoSure; LeMaitre  
150 Vascular, Inc.). The synthetic grafts were sterilized with gas at a low temperature, while  
151 the biological grafts were prepared with 70% alcohol under sterile conditions.

152

153 Using the standardized method described in the previous study,<sup>11</sup> the grafts were cut  
154 into circular pieces under sterile conditions to fit the diameter of a 10-mL calibrated  
155 loop (Deltalab SL, Barcelona, Spain). Each piece was attached to a loop using three  
156 stitches of nonabsorbable monofilament polypropylene (Prolene 6-0; Ethicon Inc.;  
157 Johnson & Johnson, Somerville, New Jersey, USA). The cap of a 10-mL tube was then  
158 pierced, and a handle fitted so that the graft could be suspended within the tube,  
159 1.5 cm from the bottom, in order to submerge the samples in the inoculum and  
160 prevent them from floating.

161

162 **2.2. Strains**

163 We studied two clinical MSSA strains (MSSA2 and MSSA6) obtained from patients with  
164 a catheter-related bacteremia at Vall d'Hebron University Hospital. The strains were  
165 selected because they were the strongest biofilm producers in a previous study by our  
166 group<sup>11</sup>.

167

### 168 **2.3. Biofilm formation on vascular graft surfaces**

169 A modification of the protocol developed by Chandra et al<sup>12</sup> was used for biofilm  
170 formation. First, the MSSA strains were grown overnight in tryptic soy broth (TSB;  
171 Becton, Dickinson, and Company, Le Pont-de-Claix, France) at 37°C with agitation at  
172 60 rpm for 24 hours. Then, the suspension was washed three times with sterile  
173 phosphate-buffered saline (PBS), pH 7.2 (Merck, Germany) and centrifuged at  
174 2500 rpm for 5 minutes at 4°C and resuspended with 10 mL of PBS Ph 7.2. An inoculum  
175 of  $1 \times 10^7$  colony-forming units per milliliter (CFU/mL) was prepared with PBS pH 7.2.  
176 The graft was submerged in a 10-mL tube previously filled with 4 mL of the inoculum  
177 containing  $1 \times 10^7$  CFU/mL. The tubes were incubated for 90 minutes at 37°C (adhesion  
178 step). Then, the vascular graft was gently transferred to a new tube containing 4 mL of  
179 TSB, taking care not to add any drops containing unattached microorganisms. The  
180 grafts were incubated for 24 hours at 37°C with agitation at 60 rpm (growth step).

181

### 182 **2.4. Microbiological assays**

183 At 24 hours, once the biofilms had formed on the vascular grafts, the grafts were  
184 randomly assigned to six groups: 1) no treatment (growth control), 2) ciprofloxacin  
185 4.5 mg/L, 3) cloxacillin 100 mg/L, 4) dalbavancin 300 mg/L, 5) daptomycin 140 mg/L,  
186 and 6) linezolid 20 mg/L. The grafts were transferred to new tubes prepared with 4 mL

187 of TSB medium containing the corresponding antibiotic at the test concentration (no  
188 antibiotics were added to the growth control tubes) and incubated overnight at 37°C  
189 without agitation. The experiments were carried out in triplicate (three graft pieces for  
190 each group). Sterility controls were used in all experiments.

191

192 After 24 hours of incubation, the suture attaching the graft piece to the loop handle  
193 outside the tube was cut in a sterile field, with care taken not to transfer any bacterial  
194 cells from the loop. The graft was then gently transferred to a sterile 12-well plate  
195 containing 1 mL of TSB using a sterile needle, again with care not to add any drops  
196 containing unattached microorganisms. Finally, both surfaces of the graft samples were  
197 gently scraped into the 12-well plate using the blunt edge of a no. 11 scalpel (Swann-  
198 Morton Ltd., Sheffield, UK). The suspension and graft were transferred to new tubes,  
199 vortexed for 1 minute, and sonicated for 10 minutes at 59 MHz and vortexed for  
200 another minute. The samples were then serially diluted in physiological serum; 50 µL of  
201 this serum were then plated in tryptic soy agar (TSA; bioMérieux SA, Marcy l'Etoile,  
202 France) for quantitative culture. Finally, the bacterial cells were quantified and  
203 expressed as  $\log_{10}$  CFU/mL. The biofilm quantification method is depicted in Figure 1.

204

## 205 **2.5. Statistical analysis**

206 The untreated growth control biofilms were used to establish a baseline for comparing  
207 the efficacy of the antibiotic agents tested. Analysis of variance was used to compare  
208 biofilm formation on each graft according to the antibiotic used and biofilm formation  
209 for each treatment between all grafts. Bar graphs with error bars were built to  
210 represent the means and standard deviation for each graft and treatment. A *P* value of

211 < .05 was considered significant. For the comparison between pairs of grafts or  
212 treatments, a post-hoc analysis of multiple comparisons was carried out using the  
213 Tukey test. IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, N.Y.,  
214 USA) was used for the statistical analyses.

215

### 216 **3. RESULTS**

217 The biofilm quantification results are summarized in Tables I (MSSA2) and Table II  
218 (MSSA6) and for each graft show the counts for the growth control group (baseline  
219 growth) and the five antibiotic groups. These results are described in more detail  
220 below. Tables with statistical analyses can be found in Supplementary Material 1.

221

#### 222 **3.1. MSSA2**

223 Mean  $\pm$  SD MSSA2 count on the Dacron graft was  $10.05 \pm 0.31$  CFU/mL. Using this as a  
224 baseline value, biofilm formation was significantly reduced by all the antibiotics tested  
225 ( $P < .001$ ), with a mean reduction of 45% (Supplementary Material 1). The greatest  
226 reduction was observed for ciprofloxacin (64%), which was the only antibiotic to show  
227 significant differences with the other treatments. Mean MSSA2 count was very low on  
228 the Dacron-silver-triclosan graft ( $0.50 \pm 1.03$  CFU/mL). All the antibiotics eradicated the  
229 biofilms on this graft, but given the low baseline growth, no significant differences  
230 were observed. The count was also low on the Omniflow-II graft ( $0.33 \pm 0.78$  CFU/mL),  
231 and in this case, all the antibiotics except cloxacillin eradicated the biofilms. Mean  
232 MSSA2 count on the bovine pericardium patch was  $9.87 \pm 0.50$  CFU/mL. All the  
233 antibiotics achieved a significant reduction in biofilm formation ( $P < .001$ ). The mean  
234 reduction, 45%, was the same as that observed with the Dacron graft. Ciprofloxacin

235 was the most effective antibiotic (61% reduction) and showed the strongest differences  
236 in relation to the other treatments. Biofilm formation overall was significantly less  
237 extensive on the Dacron-silver-triclosan and Omniflow-II grafts than on the Dacron and  
238 bovine pericardium grafts ( $P < .001$ ).

239

### 240 **3.2. MSSA6**

241 Mean MSSA6 count on the growth control Dacron graft was  $9.63 \pm 0.53$  CFU/mL. A  
242 significant reduction in biofilm formation was achieved by all the antibiotic treatments  
243 ( $P < .001$ ), with a mean reduction of 48%. Similarly to with the MSSA2 strain,  
244 ciprofloxacin achieved the greatest reduction (67%) and was significantly more  
245 effective than all the other antibiotics except cloxacillin, which achieved a mean  
246 reduction of 57.5%. Mean MSSA6 count on the Dacron-silver-triclosan graft was  $8.54 \pm$   
247  $0.73$  CFU/mL, and compared with baseline growth, all five antibiotics effectively  
248 reduced the biofilms formed ( $P < .001$ ), with a mean reduction of 72%. Cloxacillin was  
249 the most effective treatment, with a reduction of 86%. The only significant differences  
250 between treatments were observed for cloxacillin and ciprofloxacin. Baseline biofilm  
251 growth on the Omniflow-II graft was very low ( $1.17 \pm 1.52$  CFU/mL). All the antibiotics  
252 eradicated the biofilms formed, but given the low baseline count, no significant  
253 differences were found. Mean MSSA6 count on the bovine pericardium patch was  
254  $8.98 \pm 0.67$  CFU/mL. The antibiotics achieved a mean reduction of 46% ( $P < .001$  vs.  
255 growth control). Cloxacillin was the most effective antibiotic (reduction of 68%). The  
256 MSSA6 biofilm formation was significantly reduced on the Omniflow-II graft compared  
257 with all the other grafts ( $P < .001$ ). The combined results of the experiments, with SD  
258 values, are shown in Figure 2 and Figure 3.

259

260

261 **4. DISCUSSION**

262 The main findings of this experimental study indicate that ciprofloxacin and cloxacillin  
263 were the most effective antibiotics for reducing MSSA2 and MSSA6 biofilm formation  
264 on the vascular grafts tested. The antibiotics reduced MSSA2 and MSSA6 biofilms on  
265 the Omniflow-II graft and MSSA2 biofilms on the Dacron-silver-triclosan graft. None of  
266 the antibiotics, all applied as monotherapy for 24 hours, was able to completely  
267 eliminate the biofilms formed on the Dacron or bovine pericardium grafts.

268

269 The five antibiotics tested—ciprofloxacin, cloxacillin, dalbavancin, daptomycin, and  
270 linezolid—are commonly used to treat VGIs. Cloxacillin has shown *in vivo* bactericidal  
271 activity against staphylococcal infections.<sup>13,20</sup> Ciprofloxacin is a broad-spectrum  
272 fluoroquinolone with good *in vitro* activity against *S. aureus* (including methicillin-  
273 resistant *S. aureus* [MRSA]) and other staphylococcal species.<sup>15</sup> It has also produced  
274 good clinical responses in patients with prosthetic joint infections.<sup>16</sup> In our study,  
275 ciprofloxacin monotherapy effectively reduced biofilms formed by both MSSA2 and  
276 MSSA6, supporting previous findings.<sup>18</sup> Daptomycin and linezolid both have gram-  
277 positive coverage.<sup>4</sup> Daptomycin has been described as an effective treatment for the  
278 prevention and eradication of biofilm formation in an *in vivo* staphylococcal infection  
279 model,<sup>20</sup> and has shown particularly promising results in the treatment of VGIs.<sup>21</sup>  
280 Nonetheless, like us, other authors have found that daptomycin alone is ineffective at  
281 eradicating biofilms in *S. aureus* prosthetic VGIs.<sup>2</sup> Dalbavancin is a novel, long-acting  
282 lipoglycopeptide that is active against gram-positive pathogens.<sup>23,24</sup> It has proven

283 effective at reducing MRSA biofilm formation *in vitro*.<sup>27,28</sup> We, however, did not observe  
284 any striking effects for this antibiotic on MSSA2 or MSSA6 biofilm formation. In line  
285 with reports by Žiemyte et al.,<sup>29</sup> who also studied immature biofilms (produced by  
286 MRSA), we found that dalbavancin reduced but did not completely eradicate biofilm  
287 growth. Other studies have highlighted the need to combine dalbavancin with other  
288 antibiotics to improve MRSA eradication.<sup>30,31</sup>

289

290 Although the main aim of the present study was to evaluate the efficacy of five  
291 commonly used antibiotics against biofilm formation, our experiments also provided  
292 insights into biofilm growth on synthetic and biological grafts before the administration  
293 of antibiotics. The CFU counts for MSSA2 and MSSA6 on the Omniflow-II graft were so  
294 low that it was not possible to analyze the efficacy of the different antibiotics tested.  
295 Few *in vitro* studies have been performed using Omniflow-II. Most have focused on the  
296 clinical use of this graft for aortic reconstruction in infected fields<sup>32,33</sup> as it has shown  
297 strong resistance to reinfection.<sup>34</sup> As far as we know, there is just one *in vivo* study,  
298 performed in rats, where MRSA counts were higher on Omniflow-II than  
299 polytetrafluoroethylene grafts.<sup>35</sup> These results are in stark contrast to the clinical  
300 experience with Omniflow-II and the results of this and another recent study by our  
301 group.<sup>11</sup>

302

303 DST was the other graft that showed the greatest resistance to biofilm formation prior  
304 to antibiotic treatment, and its protective effect was particularly evident in the case of  
305 MSSA2. This finding is consistent with previous findings by our group<sup>11</sup> and other  
306 recent studies.<sup>36</sup> Dacron-silver-triclosan grafts have been shown to have superior

307 antiseptic properties to grafts coated with silver only.<sup>37,38</sup> In our study, all the  
308 antibiotics eradicated the scant MSSA2 biofilms formed on the Dacron-silver-triclosan  
309 graft. The results for this graft were also better for MSSA6 reduction.

310 The findings of this study have relevant implications for both the prevention and  
311 treatment of VGIs. Although MSSA biofilms adhered weakly to Omniflow-II, this graft  
312 has some technical and commercial limitations. Preparation and rinsing protocols must  
313 be carefully followed, and in addition, the Omniflow-II graft is currently only available  
314 as a straight prosthesis (in varying sizes). When chosen for aortic reconstruction, it may  
315 be necessary to manually create a bifurcated prosthesis. If it is decided to use a more  
316 available synthetic graft (for emergency repairs, for example), it should be noted that  
317 the Dacron-silver-triclosan graft appears to offer greater resistance to biofilm  
318 formation. In addition, it was associated with greater reductions in bacterial counts  
319 after antibiotic treatment than either the Dacron or bovine pericardium graft. The  
320 Dacron-silver-triclosan graft thus may be useful for the prevention and treatment of  
321 VGIs, as it seems to be associated with enhanced antibiotic susceptibility after biofilm  
322 formation.

323

324 This study has some limitations. First, we investigated the efficacy of antibiotics against  
325 MSSA biofilm formation using the conventional CFU counting method, which only  
326 measures cultivable cells in the biofilm system. In future studies, it would be beneficial  
327 to use microscopy techniques, such as confocal laser scanning microscopy, to gain a  
328 more thorough understanding of the role of biofilms in antimicrobial resistance.  
329 Second, we used just two clinical strains of MSSA. Although *S. aureus* is the most  
330 common cause of graft infections, future studies should evaluate other gram-positive

331 and gram-negative microorganisms and fungi. Also, it should be recalled that we  
332 performed *in vitro* experiments with immature biofilms (formed within a 24-hour  
333 incubation period), and that these models may not reproduce what occurs in clinical  
334 practice. In addition, the antibiotics were administered for just 24 hours. Much remains  
335 to be learned about which grafts offer the greatest protection against infection.<sup>39</sup> More  
336 *in vivo* and clinical studies are needed to better understand VGIs and to guide  
337 recommendations on the use of certain materials over others.

338

339

## 340 **5. CONCLUSIONS**

341 Ciprofloxacin and cloxacillin performed best at reducing biofilms formed by MSSA2 and  
342 MSSA6 on the surface of biological and synthetic vascular grafts. The Dacron-silver-  
343 triclosan and Omniflow-II grafts were associated with the least extensive MSSA biofilm  
344 formation and Dacron-silver-triclosan showed the highest antibiotic susceptibility  
345 following formation. None of the monotherapy antibiotics tested over a 24-hour period  
346 was able to completely eliminate the biofilms formed on either the Dacron or bovine  
347 pericardium grafts.

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361

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364 commercial, or not-for-profit sectors.

365

366 **8. CONFLICTS OF INTEREST**

367 None of the authors have conflicts of interest to declare. The grafts were supplied by  
368 the respective companies, but these did not participate in any of the stages of the  
369 study.

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546 **10. TABLES**

547

548 **Table I**

549 Quantification of MSSA2 biofilms formed on four vascular grafts before (growth  
 550 control) and after five antibiotic treatments. Results expressed as mean log<sub>10</sub> CFU/mL  
 551 and SD.

**MSSA 2**

TREATMENT	GRAFT							
	Dacron		DST		Omniflow-II		Bovine pericardium	
	Mean (log <sub>10</sub> CFU/mL)	SD						
Growth control	10.05	0.31	0.50	1.03	0.33	0.78	9.87	0.50
Ciprofloxacin 4.5 mg/l	3.61	0.93	0	0	0	0	3.82	1.26
Cloxacillin 100 mg/l	5.49	1.14	0	0	0.21	0.63	5.31	0.73
Dalbavancin 300 mg/l	6.85	0.66	0	0	0	0	6.75	0.45
Daptomycin 140 mg/l	5.33	2.22	0	0	0	0	5.24	1.63
Linezolid 20 mg/l	6.17	0.93	0	0	0	0	6.03	1.03

CFU, colony-forming units; DST, dacron-silver-triclosan; log, logarithm; MSSA, methicillin-susceptible *Staphylococcus aureus*; SD, standard deviation.

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555 **Table II**

556 Quantification of MSSA6 biofilms formed on four vascular grafts before (growth  
 557 control) and after five antibiotic treatments. Results expressed as mean log<sub>10</sub> CFU/mL  
 558 and SD.

559

## MSSA 6

TREATMENT	GRAFT							
	Dacron		DST		Omniflow-II		Bovine pericardium	
	Mean (log <sub>10</sub> CFU/mL)	SD						
Growth control	9.63	0.53	8.54	0.73	1.17	1.52	8.98	0.67
Ciprofloxacin 4.5 mg/l	3.15	0.62	3.30	1.15	0.18	0.53	3.67	0.68
Cloxacillin 100 mg/l	4.10	0.94	1.23	1.37	0.71	1.10	2.87	0.57
Dalbavancin 300 mg/l	7.40	0.36	2.74	2.69	0.20	0.59	7.11	0.97
Daptomycin 140 mg/l	4.73	1.58	2.09	1.69	0	0	5.72	1.36
Linezolid 20 mg/l	5.90	0.64	2.51	1.77	0.71	1.08	4.99	0.84

CFU, colony-forming units; DST, Dacron-silver-triclosan; log, logarithm; MSSA, methicillin-susceptible *Staphylococcus aureus*; SD, standard deviation.

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563 **11. LEGENDS FOR FIGURES**

564

565 **Figure 1**

566 Biofilm quantification.

567 CFU, colony forming units; DST, Dacron-silver-triclosan; MSSA, methicillin-susceptible

568 *Staphylococcus aureus*; PBS, phosphate-buffered solution; TSA, tryptic soy agar; TSB,

569 tryptic soy broth.

570

571 **Figure 2**

572 Quantification of MSSA2 biofilms formed on four vascular grafts after no treatment

573 (GC) and treatment with five antibiotics. The GC results are used as baseline values to

574 evaluate the efficacy of the tested antibiotics, shown in different colors. The error bars

575 indicate the standard deviation. Results are expressed as mean  $\log_{10}$  CFU/mL and SD.

576 CFU, colony-forming units; DST, Dacron-silver-triclosan; GC, growth control; log,

577 logarithm; MSSA, methicillin-susceptible *Staphylococcus aureus*.

578

579 **Figure 3**

580 Quantification of MSSA6 biofilms formed on four vascular grafts after no treatment

581 (GC) and treatment with five antibiotics. The growth control results are used as

582 baseline values to evaluate the efficacy of the tested antibiotics, shown in different

583 colors. The error bars indicate the standard deviation. Results are expressed as mean

584  $\log_{10}$  CFU/mL and SD. CFU, colony-forming units; DST, Dacron-silver-triclosan; log,

585 logarithm; MSSA, methicillin-susceptible *Staphylococcus aureus*.