Maggot therapy´s modes of action : effect of maggot secretions on microbiological, haematological and immunological processes

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CHAPTER THREE

Synergistic actions of maggot excretions/secretions and antibiotics against biofilm-associated *Staphylococcus aureus*

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Submitted

Abstract

Objectives: Maggots of the blowfly *Lucilia sericata* are used for the treatment of chronic wounds. Earlier we reported that maggot excretions/secretions (ES) breakdown *Staphylococcus aureus* biofilms but do not kill the bacteria. As many antibiotics are not effective against biofilms and the associated bacteria we assessed the effect of combining ES and antibiotics on *Staphylococcus aureus* biofilms and on the survival of the bacteria released from the biofilms.

Methods: Combinations of ES and vancomycin, daptomycin or clindamycin on *S. aureus* ATCC 29213 biofilms and bacterial viability were tested using microtitre plates and *in vitro* killing assays.

Results: Vancomycin and daptomycin dose-dependently enhanced biofilm formation, whereas clindamycin reduced *S. aureus* biofilm size. Adding ES to antibiotic incubations caused a complete biofilm breakdown. There was a lag time before bacteria released from biofilms became susceptible to vancomycin and clindamycin, which was also dependent on refreshing medium and allowing time to restart bacterial replication. Daptomycin showed direct activity against biofilm-derived bacteria. In exponentially growing bacteria, ES did not affect the bactericidal activity of antibiotics whereas ES increased the activity of daptomycin against bacteria upon release from the biofilms.

Conclusions: Maggot excretions/secretions release biofilm-associated *S. aureus* into the surrounding milieu allowing them to become exposed to the action of antibiotics; the effect depends on the pharmacodynamic property of the specific antibiotic drug.

Introduction

Chronic wounds are common in patients with vascular insufficiencies and underlying chronic conditions such as diabetes mellitus, as well as patients suffering from acute, extended trauma[1,2]. These wounds and consequent amputations result in decreased physical, emotional and social function of patients, a reduced quality of life and major economic costs for patients, their families and society[3,4]. A severe complication of the healing process is bacterial colonization and subsequent infection of the wound surface[5-7], especially when the bacteria are residing in biofilms[8]. These bacteria exhibit altered growth characteristics and gene expression profiles, as compared to those present freely in the environment, the so called planktonic bacteria[9]. Importantly, biofilm formation and the consequences thereof for bacterial growth characteristics render microorganisms resistant to the action of many antibiotics[10,11] as well as cells and effecter molecules of the host's immune system[7,12]. Bacterial fragments/products released from biofilms continuously attract host cells to the wound. As phagocytes cannot ingest the biofilm-associated bacteria and therefore are unable to eliminate the cause of infection, the subsequent accumulation of inflammatory cells and enhanced release of pro-inflammatory cytokines, proteases and reactive oxygen species eventually lead to inactivation of growth factors and tissue destruction[13,14] thereby contributing to the establishment and/or maintenance of chronic wounds.

Sterile larvae -maggots- of the green bottle blowfly *Lucilia sericata* are used as a treatment of various types of chronic wounds[15-17]. Earlier we reported maggot excretions/secretions (ES) to breakdown *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms[18]. However, the bacteria released from these biofilms were not killed by ES. On the other hand, many antibiotics cannot break down bacterial biofilms but effectively kill planktonic bacteria. Therefore, we assessed the effect of combinations of maggot ES and antibiotics on *Staphylococcus aureus* biofilms and on the survival of the bacteria released from these biofilms.

Materials and methods

Maggots and maggot excretions/secretions

ES of sterile second- and third-instar larvae of *Lucilia sericata* (a kind gift from BioMonde GmbH, Barsbüttel, Germany) were collected as described[19]. In short, larvae were incubated in H_2O for 60 min. Sterile ES preparations were harvested, pooled and stored at -20°C.

Antibiotics

Vancomycin (Pharmachemie B.V., Haarlem, The Netherlands), daptomycin (Cubicin, Chiron Corporation Limited, Uxbridge, UK), and clindamycin (Upjohn GmbH, Heppenheim, Germany) were dissolved in distilled water to a final concentration of 10 mg/mL.

Staphylococcus aureus cultures

Staphylococcus aureus ATCC 29213 (Manassas, VA, USA) were grown in Tryptone Soya Broth (TSB) at 37°C under vigorous shaking. The MIC-values for this strain are 0.5-2 µg/mL for vancomycin, 0.25-1 µg/mL for daptomycin and 0.06-0.25 µg/mL for clindamycin[20].

Biofilm assay

Biofilm formation of *S. aureus* in 96-wells polyvinyl chloride (PVC) plates was conducted as described[18]. In short, bacteria from overnight cultures were diluted 1:1,000 and 5 µL of these bacterial suspensions were added to each well containing 100 µL of 'biofilm medium' consisting of 0.5x TSB supplemented with 0.2% (w/v) glucose. After 24 h, planktonic cells were removed and 100 µL of biofilm medium with or without antibiotics (1-400 µg/mL) and/or ES (20-200 µg/mL) were added to the biofilms. At the indicated time intervals, planktonic cells were harvested from these wells and the numbers of viable bacteria were determined microbiologically using serial dilutions of these suspensions plated in six-fold onto agar plates. The reliable lower detection limit of this method is 100 cfu/well. In addition, after washing the wells with tap water, biofilms were exposed to a 1% (w/v) crystal violet solution for 15 min, washed and then incubated in absolute ethanol for 15 min to extract the crystal violet retained by the cells. Next, this solution was transferred to 96-wells plates (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) and used to quantify the amount of biofilm by measuring the absorbance at 590 nm.

Furthermore, we investigated the effect of antibiotics on bacteria derived from the biofilms and subsequently transferred to fresh biofilm medium. For this purpose, the planktonic cells were removed from 24 h old biofilms and fresh biofilm medium was added to the wells containing ES (20 - 200 µg/mL) or H_2O as a control. After an additional 24 h, the bacteria released from the biofilms were harvested and 25 µL of these bacterial suspensions were transferred to wells of a PVC plate containing 75 µL of TSB medium supplemented with antibiotics; the final concentrations of the medium was 0.5 x TSB and 0.2% glucose. After 3 h and 24 h, the numbers of surviving bacteria were determined microbiologically as described above.

Influence of maggot ES on the concentration-effect relationship for antibiotics on exponential growing S. aureus

To further determine the concentration-effect relationship for antibiotics on planktonic *S. aureus* in the presence or absence of ES, *in vitro* killing assays were conducted as described[21] with minor modifications. Bacteria in mid-log phase were centrifuged at 2,000xg for 10 min, washed twice with PBS and resuspended in biofilm medium supplemented with antibiotics (0.005 - 500 µg/mL) and/or ES (20 - 200 µg/mL) to a concentration of 1 x 10^7 bacteria/mL. Subsequently, 100 µL aliquots of these bacterial suspensions were transferred

to wells of a 96-wells PVC plate and incubated at 37°C. After 1, 2 and 3 h, the numbers of surviving bacteria were determined microbiologically as described above.
Next, the differences between the logarithms (base 10) of the numbers of CFU in the absence and presence of antibiotics and/or ES were calculated for each time point[22]. For further calculations, the highest value of the net killing rate during the 3 h of exposure was used (E_R). The concentration-effect relation was established by using the Hill Equation:

$$E_R = E_R,max \times C / (EC_{50} + C)$$

where E_R,max is the estimated maximal killing rate, C the antibiotic concentration (µg/mL), and EC_{50} the estimated antibiotic concentration at which 50% of the maximal killing is reached. The parameters of this pharmaco-dynamic model were calculated in SPSS using non-linear regression analysis.

Statistical analysis
Statistical analyses were performed using Graphpad Prism version 4.02. Statistical differences between the values for ES-incubated and control-incubated bacteria were analyzed using a paired t-test. The level of significance was set at p-values < 0.05.

Results

Effect of antibiotics and ES on S. aureus biofilms
Planktonic cells from 24 h biofilms were removed and mixtures of fresh biofilm medium containing antibiotics (1 – 400 µg/mL) were added to the wells for 3 h or 24 h.
The results showed a dose-dependent increase in biofilm size by vancomycin and daptomycin already within 3 h (Table 1). This effect persisted over the next 21 h. In contrast, clindamycin dose-dependently decreased the amount of biofilm; after 3 h of incubation the biofilm partly vanished although total breakdown was not observed (Table 1). As reported earlier[18] within 3 h ES degraded the *S. aureus* biofilms completely and this effect was not counteracted by any of the antibiotics (data not shown).

Effect of combining ES and antibiotics on the viability of S. aureus released from biofilms
To investigate whether the antibiotics eradicate the bacteria released from the biofilms and if ES influence this process, planktonic cells were harvested from the wells and their viability tested. Preliminary experiments revealed no reduction in the number of viable bacteria when using ≤10 µg/mL of vancomycin and daptomycin.

Table 1 Effect of antibiotics on established biofilms of *Staphylococcus aureus*

	Vancomycin		Daptomycin		Clindamycin	
µg/mL	3 h	24 h	3 h	24 h	3 h	24 h
0	0.18 ± 0.02	0.30 ± 0.02	0.21 ± 0.01	0.30 ± 0.02	0.21 ± 0.01	0.29 ± 0.02
1	0.19 ± 0.03	0.26 ± 0.04	0.20 ± 0.03	0.27 ± 0.03	0.20 ± 0.03	0.24 ± 0.04
5	0.21 ± 0.03	0.29 ± 0.05	0.20 ± 0.03	0.26 ± 0.05	0.17 ± 0.03	0.24 ± 0.04
10	0.27 ± 0.04*	0.38 ± 0.06*	0.23 ± 0.04	0.35 ± 0.07	0.17 ± 0.04	0.24 ± 0.04
50	0.26 ± 0.04*	0.36 ± 0.05*	0.26 ± 0.04*	0.45 ± 0.10*	0.16 ± 0.01*	0.24 ± 0.01*
100	0.26 ± 0.04*	0.40 ± 0.03*	0.27 ± 0.01*	0.39 ± 0.03*	0.16 ± 0.01*	0.22 ± 0.01*
200	0.26 ± 0.04*	0.35 ± 0.02*	0.28 ± 0.02*	0.36 ± 0.03*	0.16 ± 0.01*	0.21 ± 0.02*
400	0.22 ± 0.01*	0.43 ± 0.03*	0.33 ± 0.02*	0.38 ± 0.04*	0.15 ± 0.01*	0.21 ± 0.02*

Results (Abs at 590 nm) are means ± SEM of ≥ 4 experiments. *Values are significantly (*$p<0.05$) different from those for control biofilms. For all samples, the addition of ES resulted in total breakdown of the biofilms (Abs < 0.10).

Vancomycin at concentrations of 50 µg/mL or higher significantly reduced the number of viable bacteria by 24 h (Figure 1A), but not yet after 3 h (data not shown). Daptomycin dose-dependently reduced the number of biofilm-derived bacteria within 3 h, up to 99.9 ± 0.02% at a concentration of 400 µg/mL (data not shown). This reduction in bacterial numbers continued the next 21 h (Figure 1B). After 3 h of incubation, the number of viable bacteria was 90% lower in the presence of clindamycin compared with control incubations of bacteria derived either from ES-treated or untreated biofilms (data not shown). The following 21 h, no bacterial outgrowth was observed in the presence of clindamycin (Figure 1C). Furthermore, a dose-dependent effect of clindamycin was observed at the lowest concentrations used in the experiments (i.e., 1, 5 and 10 µg/mL, resulting in a reduction of viable bacteria by 53 ± 9%, 78 ± 4% and 80 ± 14%, respectively), whereas at clindamycin concentrations above 10 µg/ml a maximal inhibition had been reached.

Of note, at all conditions chosen, ES (200 µg/mL) did not affect the antibiotic-induced bacterial killing. Using 20 µg of ES/mL yielded identical results (data not shown).

Effect of ES and antibiotics on biofilm-derived bacteria transferred to fresh biofilm medium

As large numbers of bacteria derived from the biofilms remained viable in the presence of antibiotics, we considered the possibility that this resistance was caused by phenotypic variation. Therefore, bacteria were transferred from ES-incubated or control-incubated biofilms to fresh biofilm medium supplemented with antibiotics.

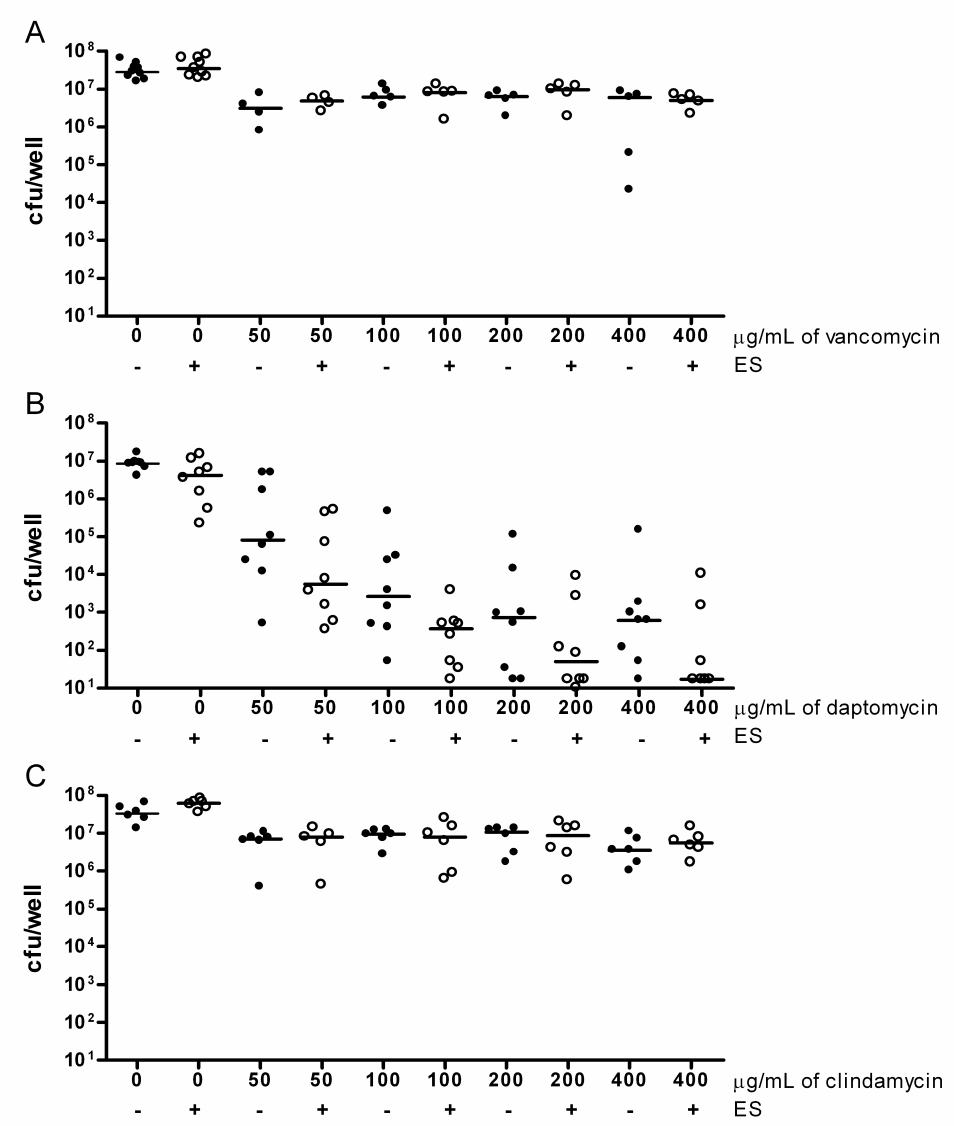

Figure 1 Effect of antibiotics and/or ES on biofilm-derived *Staphylococcus aureus*.
Biofilms were incubated with increasing concentrations of vancomycin (A), daptomycin (B) or clindamycin (C) in the absence or presence of 200 µg of ES/mL for 24 h. Results of 4-6 experiments are shown with the median (line). The values from the antibiotic samples are significantly different from the control wells.

Vancomycin failed to affect the number of viable bacteria at 3 h but reduced the number by 99% at 24 h. This effect was independent of the chosen dose of antibiotics or the presence of ES (Figure 2A). Daptomycin dose-dependently reduced the number of bacteria within 3 h. This antimicrobial effect was further enhanced by 200 µg of ES/mL (Figure 2B) but not by 20

µg of ES/mL (data not shown). After 24 h, all bacteria were killed by the used concentrations of daptomycin independent of the presence of ES (data not shown). Clindamycin prevented outgrowth of the bacteria at 3 h of incubation at all antibiotic concentrations used (data not shown) and this effect remained constant during the following 21 h (Figure 2C); ES did not affect the activity of clindamycin on the bacteria.

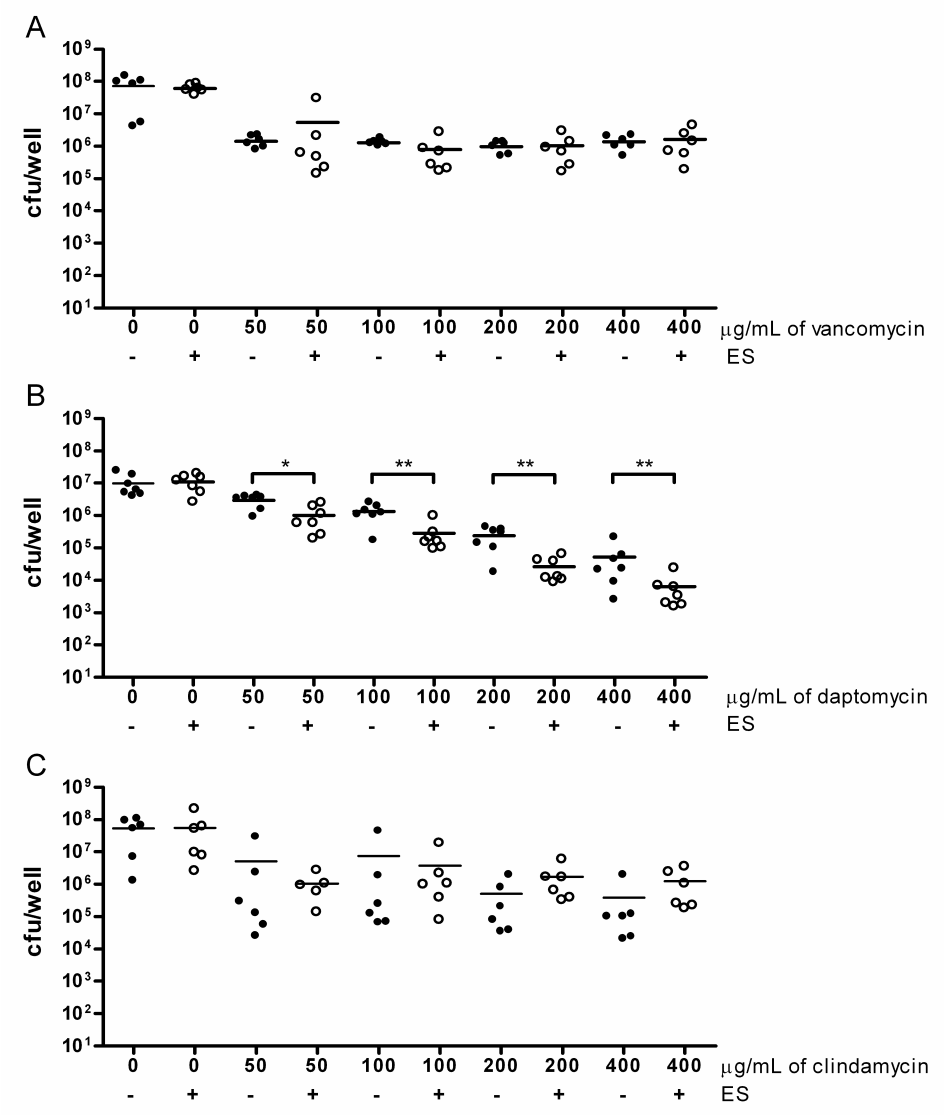

Figure 2 Effect of antibiotics on *Staphylococcus aureus* derived from ES-incubated and control-incubated biofilms. Results of 6-7 experiments with the median (line) are shown after 24 h incubation for vancomycin (A) and clindamycin (C) whereas the effect of daptomycin (B) was obtained after 3 h. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for bacteria derived from control-incubated biofilms supplemented with antibiotics.

Effect of ES on the concentration-effect relationship of antibiotics on exponentially growing S. aureus

To investigate the activity of the antibiotics on exponentially growing bacteria, we determined the growth curves at various antibiotic concentrations using the Hill equation. The results showed a dose-dependent reduction of viable *S. aureus* through the actions of all three antibiotics, although the maximum effect of daptomycin was higher than that of clindamycin and vancomycin, which were equally effective against the bacteria (Figure 3 A-C). The estimated EC_{50} and E_R, max values are given in Table 2. Additionally, we determined the effect of ES on these parameters of the antibiotics. The effects of vancomycin (Figure 3A) and clindamycin (Figure 3C) on exponential growing *S. aureus* were not significantly affected by ES although the E_R,max was enhanced somewhat and the EC_{50} decreased (Table 2). The E_R,max of daptomycin was enhanced in the presence of both 20 µg and 200 µg of ES/mL, although this effect did not reach a level of significance except for incubation with 100 µg of daptomycin (Figure 3B). Of note, 500 µg of daptomycin/mL was sufficient to kill all bacteria within 1 h in 4 out of 5 experiments under all conditions. Furthermore, the maximum effect of clindamycin was observed at 1 µg/mL, that of vancomycin at approximately 10 µg/mL, whereas of daptomycin 500 µg/mL was required to reach a maximal effect.

Table 2 Pharmacodynamic parameters of the antibiotics and ES

	Clindamycin		Vancomycin		Daptomycin	
ES (µg/mL)	E_R,max/h	EC_{50} (µg/mL)	E_R,max/h	EC_{50} (µg/mL)	E_R,max/h	EC_{50} (µg/mL)
0	1.64	0.036	1.65	0.167	5.41	46.24
20	1.57	0.025	1.77	0.080	7.25	57.99
200	2.30	0.024	1.98	0.110	5.77	39.30

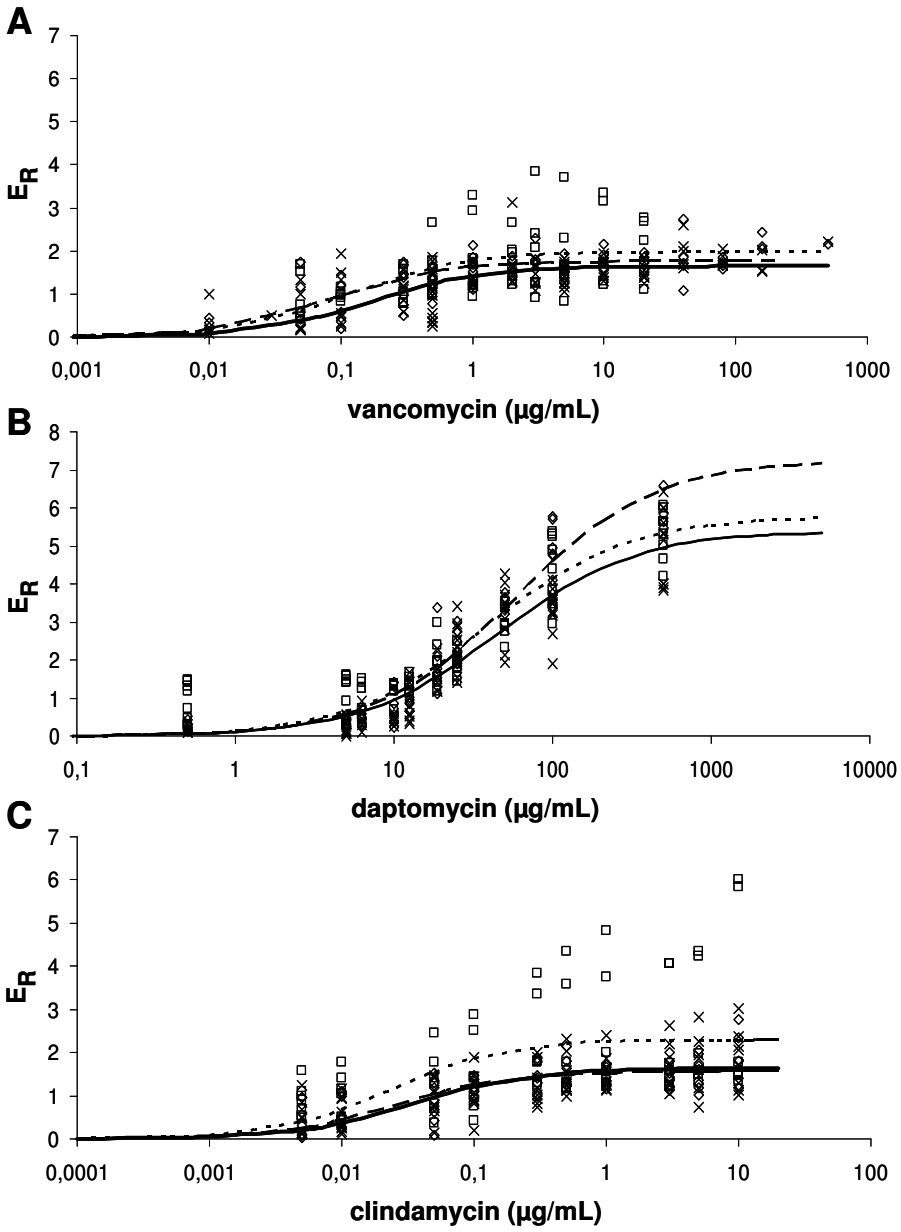

Figure 3 Effect of ES on the concentration-effect relationships of the antibiotics against exponential growing *Staphylococcus aureus*.
Bacteria were incubated with increasing concentrations of vancomycin (A), daptomycin (B) or clindamycin (C) in the presence or absence of ES (20-200 µg/mL). Results of 6-8 experiments are shown independently and as a line after processing the data using the Hill equation (x/— = no ES; ◊/— — = 20 µg of ES/mL; □/– – – = 200 µg of ES/mL).

Discussion

The main conclusion from the present study is that after maggot excretions/secretions breakdown *Staphylococcus aureus* biofilms, the released bacteria become susceptible again to antibiotics that fail to affect the viability of biofilm-associated microorganisms. The conclusion is based on the following observations. First, ES broke down established biofilms within 3 h and this effect was not negatively or positively affected by the antibiotics. In the absence of ES, samples containing vancomycin or daptomycin, antibiotics whose activity depends on their action on the bacterial cell-envelop, lacked activity against biofilms; similar findings were observed for the betalactam antibiotic flucloxacillin (unpublished observations). In contrast, clindamycin and linezolid (unpublished data) decreased the amount of biofilm, albeit they were unable to completely eradicate it in the 24 hour incubations applied here. Second, biofilm-derived bacteria became susceptible to the action of antibiotics after addition of fresh medium, as compared to their overall lack of activity against released bacteria kept in the biofilm wells. An explanation for these results is that the bacteria derived from biofilms are in a static/dormant state and are therefore not susceptible to antibiotics that solely target growing bacteria[9]. In agreement, we found no increase in bacterial numbers in the wells in which the biofilm had been exposed to ES, whereas increased numbers of *S. aureus* were observed after transferring the bacteria to fresh medium. Daptomycin showed direct activity against biofilm-derived bacteria. Third, ES did not alter the activity of the antibiotics against exponential growing and biofilm-derived bacteria. An exception was our observation that 200 µg of ES/mL enhanced the antibacterial activity of daptomycin against biofilm-derived *S. aureus* transferred to fresh medium. Although we cannot explain these results, it likely depends on the specific pharmacodynamic mechanism of this antibiotic.

In the interpretation of the current findings, the following points need be considered. First, we tested a single strain of the Gram-positive *S. aureus*. Although the strain is an ATCC reference strain, we cannot exclude that our findings are not generalisable to other *S. aureus* strains, and/or other bacterial species. However, in agreement with our result, several reports describe that daptomycin is one of the most active antibiotic in the control of biofilm-related *S. aureus* whereas clindamycin and vancomycin are less effective[23,24]. Second, the concentrations of antibiotics used in the *in vitro* biofilm assay are relatively high compared to the free, active antibiotic concentrations generally achieved in patients (vancomycin 10-40 mg/L, daptomycin 1-15/20 mg/L, clindamycin 1-20 mg/L). Therefore, at clinically relevant concentrations, the antibiotics used in this study are not expected to differ much in their activity against exponential growing *S. aureus*. However, much higher concentrations of antibiotics can be attained in wounds, through topical application, which are similar to those tested here. Third, at MIC values vancomycin or daptomycin did not affect the biofilm size, whereas at higher concentrations biofilm formation was enhanced.

However, MIC concentrations of antibiotics did not reduce the number of viable biofilm-derived bacteria whereas the higher, biofilm-enhancing concentrations did. In agreement, supra-MIC concentrations of antibiotics are reported to be effective against killing of bacteria released from biofilms, whereas sub-MIC and MIC levels were not[25]. Fourth, in contrast to the above mentioned reports[23,24], we did not observe a reduction in biofilm size when using low levels of antibiotics. The explanation for this inconsistency could be the method of quantification. We used CV staining to quantify the amount of biomass whereas many reports describe the use of redox indicators to measure the metabolic activity of the bacteria. However, reduced metabolic activity does not exclude similar or even increased biomass. In agreement, it is reported that several antibiotics, including vancomycin, reduce the redox potential of bacteria without reducing the matrix[26]. This may lead to bacterial re-growth from the remaining matrix and may even contribute to the development of resistance against the antibiotics. Clearly, more research should be done to investigate the effect of antibiotics on bacterial biofilms and the bacteria derived from these structures.

What is the clinical relevance of our findings? The failure to affect biofilms and the associated bacteria parallels the overall lack of activity of antibiotics against bacterial colonization and infection of chronic wounds where biofilm formation may be prominent[7,8,10,27]. Therefore, biofilm matrices and the associated bacteria have to be targeted simultaneously to eradicate chronic infections. Earlier we found that maggot excretions/secretions break down biofilms of *Staphylococcus aureus*[18]. Here we report that the released bacteria become exposed and susceptible to the actions of antibiotics that fail to affect biofilm-associated microorganisms. Additionally, these bacteria will be subjected to the effector mechanisms of the immune system and ingestion by maggots[28,29]. Thus, addition of maggots or maggot ES to antibiotics for the treatment of chronically colonized/infected wound surfaces may become a promising approach in inert and unresponsive chronic wounds. Of note, antibiotics including vancomycin and clindamycin have no detrimental effects on maggot growth and survival[30]. Based on our results and other reports[23,24], daptomycin and ES combined appear particularly promising in the treatment of biofilm-related *S. aureus* wound infections. Daptomycin, in contrast to vancomycin and cationic antimicrobial peptides, kills bacteria without inducing bacterial lysis[31-33]. As chronic wounds often are marked by a prolonged and dysregulated inflammatory responses[13,34-36], decreased bacterial lysis may reduce excessive pro-inflammatory responses to bacterial products by immune cells thereby contributing to the healing process[32].

In conclusion, maggot therapy and antibiotics could be used together to combat chronically colonized inert wounds. This would ensure complete breakdown of the biofilms, thereby preventing bacterial re-growth from the remaining matrix, and prompt antibiotic action against the bacteria released from the biofilms. In this respect, it should be realized that some current treatment modalities, where maggots apparently are used as replacement instead of adjunct to antibiotics, often overestimate bacterial killing by ES when applied in

therapeutically relevant amounts[18]. The combination of antibiotics and maggots will most likely lead to faster healing as maggots, besides affecting biofilm-associated microorganisms, affect other processes in wounds as well[19,37,38].

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