Garlic ointment inhibits biofilm formation by bacterial pathogens from burn wounds

Pushpalatha Nidadavolu,¹ Wail Amor,² Phat L. Tran,³ Janet Dertien,⁴ Jane A. Colmer-Hamood¹ and Abdul N. Hamood¹

1Department of Microbiology & Immunology, Texas Tech University Health Sciences Center, Lubbock, TX, USA
2Honors College, Texas Tech University, Lubbock, TX, USA
3Department of Ophthalmology & Visual Sciences, Texas Tech University Health Sciences Center, Lubbock, TX, USA
4Department of Pharmacology & Neurosciences, Texas Tech University Health Sciences Center, Lubbock, TX, USA

When thermal injury damages the skin, the physical barrier protecting underlying tissues from invading micro-organisms is compromised and the host’s immune system becomes suppressed, facilitating colonization and infection of burn wounds with micro-organisms. Within the wound, bacteria often develop biofilms, which protect the bacteria from the immune response and enhance their resistance to antibiotics. As the prophylactic use of conventional antibiotics drives selection of drug-resistant strains, the use of novel agents to prevent biofilm formation by wound pathogens is essential. In the present study, we utilized our recently developed in vitro wound biofilm model to examine the antibiofilm activity of garlic (Allium sativum). Wound pathogens were inoculated on sterile cellulose discs, exposed to formulated garlic ointment (GarO) or ointment base, and incubated to allow biofilm development. Biofilms were quantified and visualized microscopically. GarO prevented biofilm development by Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Acinetobacter baumannii and Klebsiella pneumoniae, and caused a 2–5 log reduction of the bioburden within Enterococcus faecalis biofilms. Additionally, GarO disrupted partially developed biofilms produced by S. aureus, S. epidermidis and A. baumannii. The antistaphylococcal activity of GarO was stable for over 3 months at room temperature. Thus, GarO could be used as a prophylactic therapy to prevent wound biofilms caused by both Gram-negative and Gram-positive bacteria from forming, and may be a potential therapy for disrupting established staphylococcal biofilms.

INTRODUCTION

Damage to the skin and its underlying connective tissues breaches its innate physical and chemical protective mechanisms (Church et al., 2006; Poslusny et al., 2011). Immediately following wounding, whether it is caused by a severe burn, trauma or surgery, the affected area is at risk of colonization by a variety of micro-organisms, which proliferate within the damaged tissues and establish an infection (Church et al., 2006; Kooistra-Smid et al., 2009; Poslusny et al., 2011; Rafla & Tredget, 2011). These micro-organisms may come from the host’s skin, respiratory or gastrointestinal flora (Church et al., 2006; Poslusny et al., 2011; Rafla & Tredget, 2011). Alternatively, they may reach the wound through contact with health care workers or come from environmental sources (Church et al., 2006; Poslusny et al., 2011; Rafla & Tredget, 2011). Micro-organisms proliferating within the tissue may gain access to the bloodstream, the result of which can be sepsis, multi-organ failure and death (Church et al., 2006; Rafla & Tredget, 2011). Such infections are responsible for ~75% of mortality in burn patients (Rafla & Tredget, 2011). The two most common bacteria causing burn wound infections are Staphylococcus aureus and Pseudomonas aeruginosa (Church et al., 2006; Kooistra-Smid et al., 2009; Poslusny et al., 2011; Rafla & Tredget, 2011).

Within an infected wound, bacterial pathogens frequently exist within protective structures termed biofilms (Bjarnsholt et al., 2008; James et al., 2008). Biofilms are embedded in a glycocalyx, which is a combination of bacterial secreted extracellular polysaccharides that form a protective matrix adhering to the host’s surrounding cells.
tissues (Stoodley et al., 2002; Sutherland, 2001). Biofilms are, therefore, highly resistant to host immune responses and antibiotics (Costerton et al., 1999; Donlan & Costerton, 2002; Donlan, 2011) and failure to eradicate biofilms leads to persistent infections (Costerton et al., 1999; Edwards & Harding, 2004). Previous studies have documented the production of bacterial biofilms at a range of infection sites and on a variety of different medical devices (Donlan & Costerton, 2002; Donlan, 2011; Macleod & Stickler, 2007). James et al. (2008) reported the formation of polymicrobial biofilms within chronic wounds such as foot ulcers, venous leg ulcers and pressure ulcers. Additionally, using the murine model of thermal injury, Schaber et al. (2007) described the formation of a P. aeruginosa biofilm within injured/infected tissues. Furthermore, Kennedy et al. (2010) recently analysed multiple biopsies from numerous burn wounds and reported the presence of bacterial biofilms within the ulcerated areas of these wounds.

Previous studies have demonstrated the effectiveness of garlic (Allium sativum) or garlic extract (especially allicin) in inhibiting the growth of different bacterial pathogens (Al-Waili et al., 2007; Rattanachaikunsopon & Phumkhachorn, 2009; Sarkar & Chakraborty, 2008; Tsao et al., 2003). Garlic extract has also been shown to increase the susceptibility of P. aeruginosa to tobramycin and to phagocytosis (Bjarnsholt et al., 2005; Rasmussen et al., 2005), and, in the murine model of lung infection, it has been shown to significantly reduce mortality rate and promote rapid clearing of P. aeruginosa from the lung (Bjarnsholt et al., 2005). Furthermore, garlic extract blocks P. aeruginosa and Acinetobacter baumannii quorum sensing systems (Bjarnsholt et al., 2005; Harjai et al., 2010; Rasmussen et al., 2005; Sarkar & Chakraborty, 2008), which have been shown to control biofilm development (Brackman et al., 2011; Davies et al., 1998).

Due to the nature of burn injuries, topical antimicrobial agents are effective in significantly reducing the wound bioburden as well as preventing the systemic spread of infecting pathogens (Church et al., 2006; Murphy et al., 2003). However, several studies have reported the emergence of strains that are resistant to most of these antimicrobial agents (Keen et al., 2010; Silver et al., 2006). Therefore, the use of novel antimicrobial agents to prevent biofilm formation by wound pathogens is essential.

Using our in vitro wound biofilm model, we have recently shown that gentamicin sulfate ointment, mupirocin ointment and triple antibiotic ointment (bacitracin, neomycin and polymixin B) all prevented biofilm development by P. aeruginosa strain PAO1 by inhibiting growth and/or the colonization process (Hammond et al., 2011). In addition, we used the model to assess the effectiveness of these antibiotic ointments in eliminating established (24 h) P. aeruginosa PAO1 biofilms, as well as biofilms produced by other P. aeruginosa and S. aureus burn wound isolates (Hammond et al., 2011). Thus, in the present study, we aimed to assess the effectiveness of a formulated garlic ointment (GarO) in inhibiting biofilm development by both Gram-positive and Gram-negative bacteria in our in vitro wound biofilm model, using strains isolated from burn wounds. GarO prevented biofilm development by S. aureus, Staphylococcus epidermidis, P. aeruginosa, A. baumannii and Klebsiella pneumoniae. GarO also disrupted partially developed biofilms produced by S. aureus, S. epidermidis and A. baumannii.

METHODS

Bacterial strains and media. Strains utilized in this study are listed in Table 1. Clinical isolates were obtained from burn patients in the Timothy J. Harnar Burn Center at University Medical Center, Lubbock, TX, USA, under a protocol approved by the Institutional Review Board. Strains were grown in Luria–Bertani (LB) broth or on LB agar plates.

Preparation of GarO. Pure garlic powder was obtained from a commercial supplier (McCormick) and mixed with petroleum jelly (Vaseline; Unilever) at w/w ratios of 1:1 or 3:1 to produce sufficient GarO for each experiment. GarO was applied to sterile 1 inch gauze squares (cut from sterile 2 inch cotton eight-ply gauze sponges; Kendall Curity) in 260 mg aliquots using sterile spatulas. The formulation ratios yielded GarO with two different strengths: 130 or 195 mg pure garlic per 260 mg aliquot (designated GarO-130 and GarO-195, respectively). Equivalent amounts of petroleum jelly without additive (CtlO) were spread on gauze squares and were used as controls. GarO was freshly prepared for each experiment, except those involving multiple applications over time, and that testing the stability of the active compound of GarO over time.

Zone of inhibition assay. Overnight bacterial cultures were diluted to produce a confluent lawn of growth (~1 × 10⁸ c.f.u. in 100 μl). Inocula were spread evenly on the surface of LB agar plates and a gauze square spread with either CtlO or GarO-130 was placed in the middle of each plate. The plates were incubated at 37 °C for 24 h and inspected for a zone of inhibition around the gauze square.

In vitro wound biofilm model. The in vitro wound biofilm model was used as previously described (Hammond et al., 2011). Briefly, overnight cultures were pelleted, washed and resuspended in fresh LB broth. The cultures were then serially diluted tenfold to obtain a concentration of 1 × 10⁵ to 1 × 10⁷ c.f.u. in 10 μl. Four sterile 6 mm cellulose discs (Becton Dickinson) were placed on the surface of freshly prepared LB agar plates. Ten microtitre aliquots of diluted culture were applied to each disc and the plates were incubated at room temperature for 30 min. A gauze square spread with either CtlO or GarO was then placed over the four discs and the plates were incubated at 37 °C for 24 h. To examine the effect of GarO on partially formed biofilms, plates with the inoculated discs were first incubated at 37 °C for 8 h, then gauze squares spread with either CtlO or GarO were applied to the discs and the plates were incubated for an additional 24 h.

Analysis of the biofilms. Quantitative analysis was done by determining the numbers of c.f.u. per disc (Hammond et al., 2011). Gauze squares were carefully removed and the discs were gently rinsed in PBS to remove any loosely attached planktonic cells. Each disc was placed in an individual 1.5 ml centrifuge tube containing 1 ml PBS and vigorously vortexed to disrupt the biofilm and detach the bacteria. The bacterial suspension was then serially diluted tenfold and 10 μl aliquots of each dilution were spotted on LB agar plates.
performed using NIS-Elements 2.2 software (Nikon). Using the Olympus 1X71 Fluoview 300 confocal laser scanning microscope visualized by confocal laser scanning microscopy (CLSM) using an Biofilms formed by formula: c.f.u. per disc The number of c.f.u. per disc was calculated using the following

All burn wound isolates were obtained from patients in the Timothy J. Harnar Burn Unit at University Medical Center, Lubbock, TX, USA.

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. baumannii</td>
<td>ABCI-1</td>
<td>Burn wound isolate</td>
<td>This study</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>EFCI-1</td>
<td>Burn wound isolate</td>
<td>This study</td>
</tr>
<tr>
<td>EFCI-2</td>
<td>Burn wound isolate</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>EFCI-3</td>
<td>Burn wound isolate</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>KPCLI-1</td>
<td>Burn wound isolate</td>
<td>This study</td>
</tr>
<tr>
<td>KPCLI-2</td>
<td>Burn wound isolate</td>
<td>This study</td>
<td></td>
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<tr>
<td>KPCLI-3</td>
<td>Burn wound isolate</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>KPCLI-4</td>
<td>Burn wound isolate</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>PAO1-GFP</td>
<td>PAO1 carrying plasmid pMRP9-1 in which the GFP is constitutively expressed</td>
<td>Davies et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>PA14</td>
<td>Prototrophic, biofilm-producing laboratory strain</td>
<td>Rahme et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>PACI-1</td>
<td>Burn wound isolate</td>
<td>Hammond et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>PACI-2</td>
<td>Burn wound isolate</td>
<td>Hammond et al. (2011)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>AH133</td>
<td>RN4220 carrying plasmid pCM11 in which the GFP is constitutively expressed</td>
<td>Malone et al. (2009)</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>SECI-1</td>
<td>Burn wound isolate</td>
<td>Hammond et al. (2011)</td>
</tr>
</tbody>
</table>

After 24 h of incubation at 37 °C, the numbers of c.f.u. were counted. The number of c.f.u. per disc was calculated using the following formula: c.f.u. per disc = c.f.u. counted × dilution factor × 100.

Biofilms formed by S. aureus AH133 or P. aeruginosa PAO1-GFP were visualized by confocal laser scanning microscopy (CLSM) using an Olympus IX71 Fluoview 300 confocal laser scanning microscope (Olympus). Three-dimensional biofilm image reconstructions were performed using NIS-Elements 2.2 software (Nikon). Using the COMSTAT program (Heydorn et al., 2000), 20 image stacks were analysed for the following parameters: biomass or volume (μm³) of the biofilm per μm², mean thickness (μm) and surface area (μm²), which provides an indication of the efficiency with which the strain colonizes the surface.

**Stability of the GarO.** Cellulose discs were inoculated with the test strain as described above. To determine if the effect of garlic was stable over time, gauze squares prepared with CtlO or GarO (single application) were applied to three sets of inoculated discs and the plates were incubated at 37 °C in a humid chamber for 3, 6 or 9 days.

To determine the effect of repeated applications of GarO, three sets of inoculated discs were covered with gauze squares spread with CtlO or GarO and incubated at 37 °C for 3 days. The gauze squares were then removed gently so as not to dislodge any biofilm present on the disc. One set of discs was analysed and the other two sets of discs were transferred to fresh LB agar plates and covered with fresh gauze squares spread with GarO or CtlO. The plates were incubated for a further 3 days and the process was repeated so that one set of biofilms were exposed to GarO three times over 9 days.

**Statistical analyses.** Results of the c.f.u. assays were statistically analysed using GraphPad InStat 3.06. Significance between pairs of values (CtlO versus one treatment group) was calculated using an unpaired two-tailed t test when SD was not significantly different and when a Gaussian distribution was observed. If SD was significantly different, the Welch correction was applied to the unpaired two-tailed t test. When non-Gaussian distribution was observed (Kolmogorov–Smirnov test), significance was calculated by a non-parametric Mann–Whitney test. Two treatment groups were compared using the Kruskal–Wallis test (non-parametric ANOVA) with Dunn’s multiple comparisons test for significance. Differences were considered significant when the P-value was <0.5.

**RESULTS**

**GarO inhibits planktonic growth of P. aeruginosa and S. aureus**

Previous studies have reported the inhibitory effect of garlic and garlic extract against planktonic cells of Gram-positive and Gram-negative bacteria (Al-Waili et al., 2007; Rattanachaikunsopon & Phumkhachorn, 2009; Sarkar & Chakraborty, 2008; Tsao et al., 2003). Our goal was to develop a GarO that would inhibit the colonization of wounds and the development biofilms by different bacterial pathogens. Using the zone of inhibition assay, we initially compared the potency of fresh garlic, pure garlic powder and garlic extract in inhibiting planktonic growth of P. aeruginosa and S. aureus. Fresh garlic and pure garlic powder consistently produced the largest zones of inhibition (data not shown). Based on these results, we developed a GarO containing pure garlic powder in petroleum jelly. Petroleum jelly alone (CtlO) did not inhibit the growth of S. aureus and P. aeruginosa (data not shown). We applied GarO containing different amounts of pure garlic to gauze squares and examined their ability to inhibit S. aureus by using the zone of inhibition assay. GarO-130 produced a strong inhibitory effect against S. aureus (Fig. 1) and similar results were obtained against P. aeruginosa (data not shown). Based on these findings, we used GarO-130 to examine the ability of garlic to inhibit...
biofilm development by different Gram-positive and Gram-negative wound isolates.

**GarO inhibits biofilm development by Gram-positive wound pathogens**

To confirm that *S. aureus* forms a mature biofilm in our recently developed *in vitro* wound biofilm model (Hammond *et al.*, 2011), rather than simply colonizing the surface of the disc, cellulose discs were inoculated with *S. aureus* AH133 (Table 1), covered with CtlO and incubated at 37 °C for 24 h. Visualization by CLSM showed that *S. aureus* AH133 colonized the surface of the cellulose disc and formed a biofilm-like structure in the presence of CtlO (Fig. 2a). Three dimensional analysis revealed the presence of a well-developed mature biofilm that was 172 μm thick (Fig. 2b). Quantitative analysis of the biofilm using the COMSTAT program revealed a biomass (35.35 μm³ per μm²), mean thickness (120.79 μm) and surface area (2.15 × 10⁷ μm²) similar to *P. aeruginosa* biofilms in the flow-through continuous-culture system (Schaber *et al.*, 2007) and *S. aureus* AH133 biofilms on haemodialysis catheters *in vivo* (Tran *et al.*, 2012). Quantification of the bacteria present showed a 4–5 log increase in the number of micro-organisms from 10³–10⁴ c.f.u. per disc in the initial inoculum to 10⁸ c.f.u. per disc in the mature biofilm (data not shown). This increase is comparable to the c.f.u. recovered using the murine model of chronic wound infection, in which we recovered approximately 10⁹ c.f.u. from the infected wound (Tran & Hamood, unpublished results). Together, these data indicate that the model not only supports the growth of *S. aureus* AH133 but allows development of a mature biofilm within 24 h post-inoculation.

We then used the *in vitro* wound biofilm model to examine the effect of GarO against biofilm development by *S. aureus* AH133. Based on c.f.u. counts, in the presence of CtlO, *S. aureus* AH133 colonized the surface of the cellulose disc and formed a mature biofilm; however, in the presence of GarO-130, *S. aureus* AH133 did not form a biofilm and scarcely colonized the surface of the disc (Fig. 3a). Examination by CLSM confirmed these results in that a well-developed biofilm was observed on discs treated with CtlO but only scattered organisms were observed on discs treated with GarO-130 (Fig. 3b). To extend these findings, we examined the effect of GarO-130 on biofilm development by clinical isolates of *S. aureus* (SACI-1), *S. epidermidis* (SECI-1) and *Enterococcus faecalis* (EFCI-1, EFCI 2 and EFCI 3), obtained from infected burn wounds (Table 1). As seen with *S. aureus* AH133, GarO-130 inhibited biofilm development by both staphylococcal clinical isolates (Fig. 3a). GarO-130 did not inhibit biofilm formation by the *E. faecalis* isolates but did cause a 2–3 log reduction of the bioburden within the biofilms for all three strains (Fig. 3c). To determine if an increased amount of pure garlic in the GarO would inhibit *E. faecalis* biofilm development, we repeated the experiments using GarO-195. Despite the increased amount of garlic, *E. faecalis* biofilm formation was not significantly reduced compared to GarO-130 (Fig. 3c). However, GarO-195 did cause an additional 2 log reduction in biofilm formation by *E. faecalis* EFCI-3 (Fig. 3c).
3c). These results suggest that GarO interferes with biofilm development by diverse Gram-positive wound pathogens, although GarO appears to be more efficient in inhibiting biofilm formation by *S. aureus* and *S. epidermidis* than by *E. faecalis*.

GarO inhibits biofilm development by Gram-negative wound pathogens

We first examined the effect of GarO-130 against *P. aeruginosa* PAO1-GFP (Table 1). As seen with *S. aureus* AH133, GarO-130 completely inhibited the development of a *P. aeruginosa* PAO1-GFP biofilm (Fig. 4a), a finding that was supported by CLSM (Fig. 4b). We obtained comparable results in tests using the extensively studied *P. aeruginosa* laboratory strain PA14 as well as *P. aeruginosa* clinical isolates PAC1-1 and PAC1-2 (Table 1) (Fig. 4a). GarO-130 was also effective in preventing biofilm formation by the *A. baumannii* clinical isolate ABC1-1 (Fig. 4a). Finally, we tested the ability of GarO-130 to prevent biofilm formation by four *K. pneumoniae* clinical isolates (KPCI-1, KPCI-2, KPCI-3 and KPCI-4) (Table 1). While GarO-130 prevented biofilm development by *K. pneumoniae* strains KPCI-2, KPCI-3 and KPCI-4, strain KPCI-1 was resistant to GarO-130 (Fig. 4c); however, unlike our observation with the *E. faecalis* isolates, increasing the amount of garlic in the ointment to 195 mg per dose was effective in preventing biofilm formation by this strain (Fig. 4c). These results suggest that different dosages of GarO may be necessary to prevent biofilm development by Gram-negative bacteria.

GarO disrupts partial biofilms formed by wound pathogens

In experiments described above, we showed that GarO prevents biofilm development by different wound pathogens. We then determined if the application of GarO would eliminate or reduce already established biofilms. *S. aureus* strains AH133 and SACI-1 and *S. epidermidis* SECI-1 were allowed to develop biofilms for 8 h (partial biofilm). We then applied CtlO, GarO-130 or GarO-195 to the partial biofilms and continued the incubation for an additional 24 h. Biofilms were quantified after the initial 8 h of incubation and after the total incubation time of 32 h. By 8 h all three strains had produced substantial biofilms (~1 \(6\times10^8\) c.f.u. per disc) (Fig. 5a). The application of CtlO had no effect on the development of the biofilms; however, the application of GarO-130 or GarO-195 either eliminated the biofilms, or, in the case of *S. epidermidis* SECI-1, reduced the bioburden significantly (Fig. 5a). CLSM analysis of *S. aureus* AH133 biofilm formation supported these findings. At 8 h on the disc treated with CtlO, *S. aureus* AH133 produced a partial biofilm that became thick and dense by 32 h, but discs treated with GarO-130 or GarO-195 showed only a few individually attached cells (data not shown).

Using the same approach, we tested whether GarO disrupts partial biofilms formed by the Gram-negative strains *P. aeruginosa* PAO1-GFP, *K. pneumoniae* KPCI-4 and *A. baumannii* ABC1-1. After 32 h, the number of *P. aeruginosa* PAO1-GFP cells within the biofilms treated with CtlO and GarO-130 were similar, whereas application of GarO-195 only resulted in a reduced bioburden (Fig. 5b). As with *S. aureus* AH133, these results were supported by CLSM analysis (data not shown). Similar results were obtained with *K. pneumoniae*
KPCI-4 (Fig. 5b). However, the biofilm developed by A. baumannii ABCI-1 showed a ~7 log reduction after treatment with GarO-130 and was disrupted completely by treatment with GarO-195 (Fig. 5b). These results suggest that unlike the effect GarO had in disrupting partial biofilms of the Staphylococcus strains, its ability to disrupt partial biofilms developed by Gram-negative pathogens is highly variable.

GarO inhibits biofilm development by S. aureus AH133 over extended periods

One benefit of treating burn wounds with antimicrobial ointments is the continuation of the antimicrobial effect over an extended period. The aforementioned experiments tested the efficiency of GarO in inhibiting the development of biofilms for 24 h. We expanded the incubation period of our experiments to determine if GarO would maintain this effect for several days. Biofilms were initiated with S. aureus AH133 on three sets of discs and treated with GarO-195 or CtlO. The biofilms were examined at 3, 6 and 9 days post-application. Analysis by CLSM revealed that GarO-195 prevented the development of AH133 biofilm at all time points (Fig. 6). These results were confirmed by quantitative analysis, which showed an 8 log reduction in c.f.u. at all three time intervals examined (data not shown).

The treatment of burn patients frequently involves numerous dressing changes. To simulate this situation, S. aureus AH133 biofilms were initiated on three sets of discs and treated with GarO-195 or CtlO. One set of discs was analysed for biofilm formation at 3 days post-application. For the other sets of discs, the gauze squares were removed, carefully replaced with new gauze squares spread with GarO-195 and incubated for a further 3 days, after which...
another set of discs was analysed and the process repeated. Similar to the single applications, repeated applications of GarO-195 inhibited biofilm development over the course of 9 days, while robust biofilms developed with CtlO (Fig. 7). Again, the results of quantitative analysis supported the CLSM observations (data not shown). Further experiments will need to be conducted to determine if the prolonged inhibitory effect of GarO occurs with biofilms produced by other wound pathogens.

**The antibiofilm component of GarO is stable**

For the 24 h experiments, we formulated GarO fresh for each use. This process is cumbersome and would not be feasible in a clinical setting. For the extended incubation experiment, we prepared sufficient GarO-195 to last for 9 days. The results suggested that the active component of GarO is stable at room temperature for at least 9 days. Therefore, we stored freshly formulated GarO-195 for 3 months in a sterile container and then tested its ability to inhibit *S. aureus* AH133 biofilm formation for up to 24 h. Compared to CtlO, the 3-month-old GarO-195 inhibited biofilm development by *S. aureus* AH133 as effectively as the freshly formulated product (Fig. 8).

**DISCUSSION**

An important attribute of GarO as a topical antibiofilm treatment is the broad spectrum activity it has against...
Garlic inhibits biofilm formation by wound pathogens

Gram-positive and Gram-negative pathogens, which is demonstrated here. Immediately after thermal injury, burn wounds are predominantly colonized by, and can become infected with, Gram-positive pathogens such as *S. aureus* or *S. epidermidis* (Church *et al.*, 2006; Kooistra-Smid *et al.*, 2009; Poslusny *et al.*, 2011). Several days after the trauma, wounds are likely to become colonized with Gram-negative pathogens such as *P. aeruginosa* or *A. baumannii* (James *et al.*, 2008; Keen *et al.*, 2010). In our *in vitro* wound model, discs treated with CtlO produced biofilms containing approximately 10⁸ c.f.u. per disc (Figs 3 and 4). In contrast, discs treated with GarO yielded 0–20 c.f.u. for most strains (Figs 3 and 4). While neither GarO-130 nor GarO-195 inhibited biofilm development by the three *E. faecalis* isolates, both dosages reduced the biofilm burden within the biofilm. One factor contributing to the efficient antibiofilm property of GarO, in this model, is the direct contact between the ointment and the colonizing bacteria. In a burn wound, the presence of necrotic tissue and the avascular eschar increases the susceptibility of the wound to bacterial infection by reducing the delivery of immune cells and systemically administered antibiotics (Church *et al.*, 2006; Kooistra-Smid *et al.*, 2009). Therefore, direct application of GarO could prevent the development of wound biofilms and reduce the risk of systemic infection.

The variation in GarO inhibition of biofilm development by the tested isolates is consistent with results of other studies. Al-Waili *et al.* (2007) showed that *Escherichia coli*, *P. aeruginosa*, *Streptococcus pyogenes* (haemolyticus A), *Streptococcus agalactiae* (haemolyticus B), *Klebsiella* species and *Candida albicans* were sensitive to garlic juice at varying levels. Similarly, Bachrach *et al.* (2011) demonstrated that the garlic component allicin was effective against oral pathogens, including *Streptococcus mutans*, *Streptococcus sobrinus* and *Actinomyces oris* as well as *Porphyromonas gingivalis*, which was the least sensitive. Whether different genera utilize a common mechanism in their resistance to garlic is not known at this time. Clues to the mechanisms of bacterial resistance to garlic may originate from the analysis of *E. faecalis* and *K. pneumoniae* isolates. Even GarO-195 did not prevent *E. faecalis* biofilm formation, unlike the three *Staphylococcus* strains tested, in which biofilm development was prevented by GarO-130. Thus, examination of the cytoplasmic membrane and cell wall of these strains may reveal the mechanism involved. Similarly, testing of additional *K. pneumoniae* isolates may yield a strain that is impervious to the effects of GarO. Further analysis of such strains, including the profile of their outer-membrane proteins, could shed light on the mechanisms of resistance to garlic. Theoretically, treating infected wounds with GarO-130, or lower concentrations of GarO, may indirectly enhance the growth of *E. faecalis* by eliminating other competing wound pathogens. However, the efficacy of GarO against mixed-species biofilms may vary from its effectiveness against single-species biofilms.

One of the obvious limitations of GarO is its failure to disrupt partial biofilms formed by *P. aeruginosa* and *K. pneumoniae*, whereas it did eliminate partial biofilms formed by *S. aureus*, *S. epidermidis* and *A. baumannii* (Fig. 5). It is unlikely that this variation resulted from differences in the cell-wall structure between Gram-positive and Gram-negative bacteria. Not only did GarO inhibit biofilm development by both Gram-positive and Gram-negative pathogens, the zone of inhibition assays revealed that GarO also effectively inhibited the growth of planktonic *S. aureus* (Fig. 1) and *P. aeruginosa* cells (data not shown). Instead, this variation may be related to the susceptibility of established biofilms to GarO. Regardless of the potential cause of this difference, our results suggest that, in infected wounds, GarO is effective in preventing biofilm development by Gram-negative pathogens but may not eliminate already existing ones. Consequently, in infected wounds in which Gram-negative pathogens are predominant, GarO would be more effective if applied immediately after wound debridement when the bioburden is low.

Despite its close resemblance to *in vivo* conditions, our *in vitro* model examines the effectiveness of antimicrobial agents in inhibiting biofilm development on the surface of the infected burn wound only. In infected burn wounds, bacterial pathogens form biofilms on the surface of the wound and grow within the underlying connective tissues. Therefore, it is important to examine the effectiveness of GarO as a treatment for biofilm prevention *in vivo*. Previous *in vivo* studies examining the effectiveness of orally administered garlic produced mixed results. Tsao *et al.* (2003) showed that oral garlic extract reduced bioburden within different internal organs of mice intravenously infected with *S. aureus*. However, Smyth *et al.* (2010) recently showed that oral garlic did not significantly reduce the level of *P. aeruginosa* quorum sensing molecules within the plasma and sputum of cystic fibrosis patients. We plan to examine GarO using our murine model of thermal injury and infection.

Another attribute of GarO as a topical antibiofilm ointment is the durability of its effect. GarO inhibited *S. aureus* biofilm formation for 9 days, whether it was applied only once or every 3 days, which suggests that garlic is bactericidal rather than bacteriostatic. The bactericidal effect of garlic has been reported previously (Al-Waili *et al.*, 2007; Bachrach *et al.*, 2011). To confirm that GarO is bactericidal, we initiated and treated *S. aureus* biofilms for 24 h with GarO. We then replaced GarO with CtlO and incubated for an additional 24 h. Despite the garlic-free conditions, no *S. aureus* biofilm developed (data not shown). The lack of emerging *S. aureus* garlic-resistant mutants clearly contributed to the observed durability of the GarO antibiofilm effect. Although we recovered a few colonies (0–15 per disc) from discs that were exposed to GarO for 3, 6 or 9 days, when we used these colonies to initiate biofilms, they produced robust biofilms with CtlO but failed to develop biofilms in the presence of GarO (data not shown). Thus, these colonies most likely represent bacteria that were not fully exposed to GarO, rather than a
form of resistance. At this time, and in the absence of a complete understanding of the mechanism of GarO inhibition of biofilm formation, it is difficult to speculate about the lack of S. aureus garlic-resistant mutants. We also do not know if the observed durability of the GarO antibiofilm effect is limited to S. aureus or if it extends to other wound pathogens.

In summary, formulated garlic ointment prevented biofilm development by a number of different Gram-positive and Gram-negative bacteria commonly found in infected wounds. Our results suggest that GarO could be used as a prophylactic therapy to prevent wound biofilms caused by both Gram-negative and Gram-positive bacteria, as well as a potential therapy for established biofilms due to staphylococci and species of Acinetobacter. Finally, the stability of the GarO active component for at least 3 months makes its use as an antibiofilm treatment more practical.

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REFERENCES


Garlic inhibits biofilm formation by wound pathogens


