Wound healing potential of topical bacteriophage therapy on diabetic cutaneous wounds

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ABSTRACT

Chronic wounds that fail to heal are a common complication of diabetes mellitus and the most common precipitating reason for nontraumatic lower limb amputation. Unfortunately, the bacterial species that cause these infections are becoming more resistant to antibiotics, making them increasingly difficult to treat. We assessed the feasibility of combating chronic bacterial infections with a topically delivered bacteriophage cocktail in two animal models of diabetes mellitus. Microbiological, planimetric, and histological parameters were compared in debrided infected wounds with or without topical bacteriophage treatment. We determined that bacteriophage treatment effectively decreased bacterial colony counts and improved wound healing, as indicated by smaller epithelial and dermal gaps, in *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections but was not as effective against *Acinetobacter baumannii*. Although the improvements were more significant in the rodent model than in the porcine model, our results suggest that topically administered bacteriophage treatment may be effective in resolving chronic infections, especially when applied in conjunction with wound debridement. These findings have important implications for the feasibility of using topical antimicrobial therapies to safely treat chronic infections in diabetes mellitus patients.

INTRODUCTION

Diabetic foot infections (DFIs) are a frequent and serious complication of diabetes mellitus (DM) and are the world's leading cause of nontraumatic lower limb amputation.[1] In current clinical practice, DFI treatment includes debridement and systemic antibiotics.[2] However, because of deficient vascularization and insufficient local antibiotic concentrations, these treatments are often ineffective.[3] In addition, multidrug-resistant organisms, such as methicillin-resistant *Staphylococcus aureus* and pan-drug-resistant nonfermenting Gram-negative bacilli, threaten the efficacies of these therapies in both community-dwelling and hospitalized patients.[4,5] Thus, it is necessary to identify new therapeutic strategies for DFIs. Topical treatment is advantageous because it avoids adverse effects from systemic treatment, increases target site concentration, and allows the use of agents that are not suitable for systemic therapy. Mechanical debridement remains pivotal to this strategy because it significantly reduces the bioburden, and as elegantly demonstrated by Wolcott et al.,[6] opens a time-dependent therapeutic window for topical antimicrobial therapy (TAT). Nevertheless, no TAT agent has been proven to effectively treat DFI to date.[7]

Bacteriophages are small viral entities, existing as nucleic acids packaged within a protein capsid, that specifically infect bacteria. Depending on their nature, after injection of their nucleic acids inside the bacteria, bacteriophages can either reside as a stable element called prophage inside the host cell as a free plasmid molecule or integrated into the host chromosome (temperate bacteriophages), or induce lysis of the bacterial host with the release of newly formed viral particles (lytic bacteriophages).[8] If appropriately complemented by adequate mechanical debridement, lytic bacteriophages could be efficient TAT agents in selected clinical environments because of their specificity and efficiency in lysing pathogenic bacteria, including those associated with multidrug resistance.[9] Moreover, they are not pathogenic in animals or humans,[10] effectively eliminate bacteria in biofilms, and are active even in microaerophilic environments with high bacterial loads.[11]

DFI	Diabetic foot infection
DG	Dermal gap
DM	Diabetes mellitus
EG	Epithelial gap
i.p.	Intraperitoneal
i.v.	Intravenous
TAT	Topical antimicrobial therapy
TSA	Tryptone soy agar

Bacteriophage therapy is widely used and generally accepted as safe and beneficial in some parts of the world,[12] and recent trials in animal models have shown their potential to improve or heal bacterial skin infections following both internal[13] and external application.[14,15] However, there is little experimental evidence demonstrating that bacteriophages can cure chronic infections established for more than several hours.[16]

The aim of this study was to investigate the antimicrobial activity and wound-healing capability of topically delivered bacteriophage solutions against wounds with chronic *S. aureus, Pseudomonasaeruginosa*, and *Acinetobacter baumannii* infections in two animal models of DM (rodent and porcine).

MATERIALS AND METHODS

This study was approved locally by the Animal Ethics Committee of the Instituto de Medicina Molecular and nationally by the Portuguese General Directorate of Veterinary Services (Direcção Geral de Veterinária), in accordance with Portuguese law. All animals were maintained in accordance with European Directive 86/609/EC,[17] Portuguese law (Portaria 1005/92),[18] and the *Guide for the Care and Use of Laboratory Animals (NRC 2011)*.[19]

Bacterial strains

S. aureus, *P. aeruginosa*, and *A. baumannii* strains were isolated from clinical skin wound samples that were collected from patients and identified in Lisbon area hospitals. All host strains were stored in tryptone soy broth (Biokar Diagnostics, Pantin Cedex, France) with 15% glycerol (w/v) at −70 °C until needed. For all experiments, single colonies were grown overnight on tryptone soy agar (TSA, Biokar Diagnostics) at 37 °C. After 24-hour incubation, bacterial cells were suspended in saline (NaCl 0.9%, Applichem, Darmstadt, Germany) and adjusted to McFarland's scale 0.5 (bioMérieux, Craponne, France) with a subsequent 1 : 10 dilution, producing a final solution concentration of 2.0×10^7 cfu/mL.

Bacteriophages

S. aureus F44/10 and F125/10, *P. aeruginosa* F770/05 and F510/08, and *A. baumannii* F1245/05 lytic bacteriophages were isolated from sewage water from the Lisbon area. Standard methods[20] for bacteriophage isolation and amplification were employed using the host strains described above. To produce bacteriophage stocks in sufficient quantities for experiments, a previously described protocol of amplification, concentration by high-speed centrifugation, and purification on a cesium chloride gradient[21] was used. Final concentrations were determined with double agar overlay plaque assays.[22]

Three primary cocktails (*S. aureus* cocktail, *P. aeruginosa* cocktail, and *A. baumannii* cocktail) and one final cocktail were prepared using different concentrations and relative proportions of purified bacteriophages (Figure 1).

Rodent model

A previously optimized rodent wound infection model in chemically induced diabetic Wistar rats was employed to investigate new approaches to TAT.[23] Briefly, specific pathogen-free male Wistar rats [Crl:WI(Han)], weighing 250–350 g (8–10 weeks old) were obtained from Charles River Laboratories (L'Arbresle Cedex, France). The animals were housed in an approved animal care center, and all surgical procedures were performed in a sanitized surgery room using autoclave-sterilized instruments. DM was chemically induced as described by Wu and Huan.[24] Eight days later, following DM confirmation, 42 diabetic rats were anesthetized. Their dorsal surface hair was trimmed with an electric clipper, remaining hair removed using cold wax strips. Four days later, the animals were again anesthetized, and a round wound was inflicted by making a 6-mm diameter single full-thickness incision extending through the panniculus carnosus muscle in the interscapular region of the upper back of each rat using a punch biopsy instrument. Immediate-bonding cyanoacrylate glue was used to fix an oval-shaped silicone splint to the skin, and interrupted 3-0 nylon sutures were placed to maintain its position. Before dressing, wounds were photographed from a standard 1.5-cm distance using a mounted digital microscope (SuperEyes 200 × USB Digital Microscope, Shenzhen Tak and Assistive Technology, Shenzhen, China). Wound and surrounding area were covered with a previously tailored, semi-occlusive, nonwoven polyester dressing and maintained in place throughout the entire course of the experiment with a jacket made from adhesive tape.

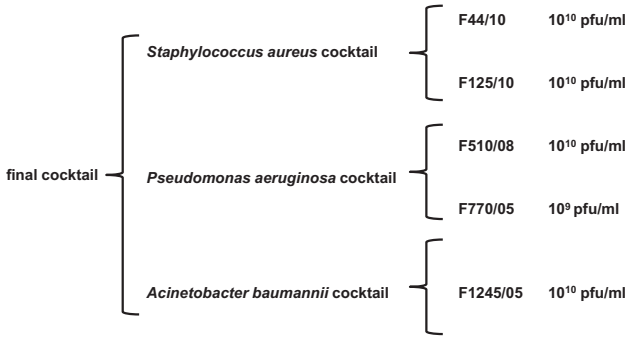

Figure 1. Schematic depicting bacteriophage cocktail preparation.

After applying the dressing but before the animals were conscious, they were randomly divided into seven groups: negative control ($n = 6$), *S. aureus*–inoculated control ($n = 6$) and test ($n = 6$), *P. aeruginosa*–inoculated control ($n = 6$) and test ($n = 6$), and *A. baumannii*–inoculated control ($n = 6$) and test ($n = 6$). Wounds of the animals in the negative control group were injected with 100-µL sterile saline, whereas wounds of the inoculated groups (test and control) were injected with 100-µL cultured *S. aureus*, *P. aeruginosa*, or *A. baumannii* resuspended in sterile saline by inserting a 27G/19-mm needle attached to a 1-mL disposable syringe through the silicon splint at a 45° angle. On days 4, 5, and 8 post-wounding, the semi-occlusive dressing was cut off, and the wounds were debrided. Debridement consisted of the strict, aseptic, mechanical removal of the scab, defined as a crust of dried blood, serum, and exudate.

All test groups underwent a bacteriophage treatment protocol that consisted of an induction phase and a maintenance phase. The induction phase occurred after the first debride-

ment (postwounding day 4) and comprised of six 100-μL primary bacteriophage cocktail administrations (every 4 hours). The maintenance phase was from day 5 to day 8 and consisted of twice-daily (every 12 hours) 100 μL primary bacteriophage cocktail administrations. If debridement was performed, bacteriophage administration followed. The control groups received 100-μL sterile saline with the same frequency.

On days 4, 5, and 8 postwounding and after debridement, a liquid Amies elution swab (eSwab Collection and Preservation System, Copan, Corona, CA) was used to collect and transport swab cultures. Bacteria collection was performed using the one-point method described by Sullivan et al.[25] Briefly, a sterile swab was used to scrub the center surface of each wound by rotating the swab three times clockwise with enough manual pressure to produce a small amount of exudate. The swab was then inserted into the tube and transported to the laboratory for immediate processing. The swab collection tube was vortexed (with the swab inside) for 5 seconds, and a 100-μL aliquot of the resulting suspension was used for serial dilutions. Quantification was performed using the 10-fold serial dilution method.[26] In the inoculated groups, 100 μL of each dilution was plated onto the respective selective media plates: Chapman mannitol salt agar (Biokar Diagnostics) for *S. aureus*, cetrimide agar (Merck Chemical) for *P. aeruginosa* and CHROmagar Acinetobacter for *A. baumannii* (CHROmagar, Paris, France). In the *A. baumannii*-inoculated and negative control groups, 100 μL of each dilution was simultaneously inoculated onto TSA media plates (Biokar Diagnostics). The plates were incubated under aerobic conditions at 37 °C for 24 hours, at which time colony counts were performed. The isolates grown on Chapman mannitol salt agar were presumptively identified as *S. aureus* based on colony morphology and mannitol salt agar fermentation.[27] The isolates grown on cetrimide agar were presumptively identified as *P. aeruginosa* based on colony morphology.[28] The isolates grown on CHROmagar Acinetobacter were identified as *A. baumannii* based on red colony color.[29] Prior to sacrifice on postwounding day 9, wounds were photographed from a standard 1.5-cm distance using a mounted digital microscope as previously described. Wound kinetics were quantified using image-processing software (ImageJ, National Institutes of Health, Bethesda, MD) to measure the wound area by planimetry, and wound area was expressed as a percentage of the initial wound area. All animals were sacrificed by an i.p. injection of pentobarbital (200 mg) on day 9 postwounding, and each ulcer and the surrounding 0.5-cm skin border was harvested with sterile surgical scissors and placed in a tube. The samples were fixed in 10% buffered formalin solution, and after overnight fixation, they were trimmed and cut through at the widest margin, embedded in paraffin, and sectioned in 3-μm increments. Sections were made perpendicular to the anterior–posterior axis and perpendicular to the wound surface. For each wound, two serial sections were placed on a slide and stained with hematoxylin & eosin. The sections were photographed using a motorized inverted bright-field microscope (Zeiss Axiovert 200M, Göttingen, Germany) equipped with a color camera (Leica DM2500, Leica Microsystems GmbH, Wetzlar, Germany) at 50× magnification. Panoramic cross-sectional digital images of each wound were prepared with automated microscopy software (MetaMorph, MDS Analytical Technologies, Sunnyvale, CA) and image-processing software (ImageJ). Each image was analyzed for epithelial gap (EG) and dermal gap (DG) using the same image-processing software. EG was defined as the distance between the advancing edges of clear, multilayered neoepidermis,[30,31] and its size was measured in millimeters; a completely reepithelialized wound corresponded to an EG score of zero. DG was defined as the distance between uninjured dermis on both sides of the wound[30,31] and was measured in millimeters. All wound kinetics and histological measurements were done with the investigator blinded as to sample origin (test or control).

Pig model

A previously optimized pig wound infection model in animals with chemically induced DM[32]was modified to fit our needs. Three animals (negative control, inoculated-control, and inoculated-test) with a total of 48 excisional wounds (12 negative control wounds, 12 *S. aureus*–inoculated wounds, 12 *P. aeruginosa*–inoculated wounds, and 12 *A. baumannii*–inoculated wounds) were used in this study.

Three female Yorkshire pigs weighing approximately 60 kg at arrival were allowed to acclimatize for 1 week prior to the experiment. Animals were housed individually in cages, had free access to water, and were fed twice daily with a standard diet. Pigs were kept in a containment device during all procedures. Pigs were fasted for 12 hours before DM induction. On the day of the procedure, the animals were weighed and given intramuscular anesthesia with xylazine hydrochloride and ketamine hydrochloride. While they were under anesthesia, a 21-gauge intravenous (i.v.) catheter was inserted into an ear vein. Streptozotocin (150 mg/kg body weight diluted in 10 mL/g sterile saline and sterilized by filtration) was administered through the catheter over 1 minute. After recovering from anesthesia, postprocedural antiemetic therapy with metoclopramide was administered. Pigs were continuously observed for the first 3 hours, and then food was offered ad libitum to prevent hypoglycemia. Blood glucose was measured on a daily basis, and pigs received daily, subcutaneous injections of 16 IU premixed neutral suspension of neutral (30%) and isophane insulin (70%) (Mixtard 30, Novo Nordisk, Bagsværd, Denmark) to keep blood glucose concentrations between 250 and 400 mg/dL. Fourteen days after DM induction, pigs received induction anesthesia as previously described. They underwent endotracheal intubation and were mechanically ventilated with a volume-limited, time-cycled ventilator (Mark 9; Bird Corporation, Palm Springs, CA) on a mixture of room air and titrated isoflurane (0.5% to 1.5%). The tidal volume was set at 12 mL/kg, and the ventilator rate was 12 breaths/minute. Prior to surgery, the dorsal surface hair was trimmed with an electric clipper, the remaining hair was removed with cold wax strips, and the paraspinal area was thoroughly disinfected using 10% povidone-iodine paint and washed with 70% isopropanol 15 minutes later. For the inoculated pigs, nine full-thickness excisional wounds (6-mm diameter, 6-mm depth) were created on each side of the paraspinal area (18 in total) using a 6-mm diameter biopsy punch. For the negative control pig, only six excisional wounds were created on each side of the paraspinal area (12 in total). Subsequently, sterile forceps and a surgical blade were used to remove the full-thickness skin flap, and sterile gauze was used to remove coagulated blood and control bleeding. The wounds were photographed from a standard height using a mounted digital microscope. Afterward, a

modified adhesive chamber made from a colostomy bag (two-piece 35-mm Ostomy, Hollister Inc., Libertyville, IL) was placed over each wound, covered with a semi-occlusive, non-woven polyester dressing, and secured in place with surgical staples (Manipler AZ, B. Braun, Tuttlingen, Germany) and adhesive bandages. In the inoculated animals, wounds were divided into three subgroups: *S. aureus* (2×6 ulcers), *P. aeruginosa* (2×6 ulcers), and *A. baumanni* (2×6 ulcers). To immerse the enclosed surface, wounds were respectively inoculated with 2×10^6 cfu of *S. aureus, P. aeruginosa*, or *A. baumanni*in 100 μL total solution (sterile 0.9% saline). In the negative control group (12 ulcers), wounds were injected with 100-μL sterile saline. After recovering from anesthesia, post-procedural anesthesia (buprenorphine 0.005 mg/kg) and antiemetic therapy was given every 12 hours for 48 hours. On days 4, 5, and 8 postwounding, the semi-occlusive dressing was cut off, and the wound debrided as described for the rodent model.

A two-part bacteriophage treatment protocol similar to that employed in the rodent model was used. The induction phase began after the first debridement (postwounding day 4) and consisted of 100 μL final bacteriophage cocktail administrations every 4 hours for 24 hours. The maintenance phase took place between days 5 and 8 and consisted of twice-daily (every 12 hours) 100 μL final bacteriophage cocktail administrations. Bacteriophage administration followed every debridement session. The control groups received 100-μL sterile saline at the same time.

We employed a microbiological analysis protocol similar to the rodent study. On days 4, 5, and 8 postwounding and after debridement, a liquid Amies elution swab was used to collect and transport swab cultures, which were processed using the same methods described for the rodent study. Bacteria collection was performed using the one-point method described by Sullivan et al.[25] The swab was then inserted into the tube and transported to the laboratory for immediate processing. Quantification was performed using the 10-fold serial dilution method.[26] In the inoculated group samples, 100 μL per dilution was plated onto their respective selective media plates: Chapman mannitol salt agar, cetrimide agar, and CHROmagar Acinetobacter. In the negative control group samples, 100 μL of each dilution was inoculated onto TSA media plates. The plates were incubated under aerobic conditions at 37 °C for 24 hours, after which colony counts were performed. The isolates were presumptively identified as previously described. In the negative control group, ulcers with more than 10^3 cfu/swab on any given day were considered to be critically colonized and were excluded from further analysis. Wounds were again photographed on postwounding day. Wound kinetics were quantified using image-processing software as described above. Wound area was expressed as a percentage of the initial wound area. All animals were sacrificed by i.v. injection of pentobarbital on day 9 postwounding, and each ulcer (including a 0.5-cm skin border) was entirely harvested using sterile surgical scissors and placed in a tube. For histological studies, the samples were processed and photographed, and the images were analyzed for EG using the same methods as described above.

Statistical analysis

All quantitative microbiological results are presented as the mean ± standard deviation and expressed as logarithm-transformed values [log(cfu/swab) for swab samples and log(cfu/ulcer) for tissue samples]. The data were compared using a logarithmic scale because of wide variations in cfu/swab among samples. Planimetric and histological results are expressed as the mean ± standard deviation. For all data sets, comparisons between groups were performed using two-tailed Student's *t* tests, and *p*-values < 0.05 were considered significant. All data were entered into a spreadsheet program (Excel, Microsoft, Redmond, WA) for statistical analysis. Analytical statistics were performed by Analyse-it, version 2.21 Excel 12+ (Analyse-it Software, Leeds, United Kingdom), a statistical add-in program for Excel.

RESULTS

Rodent model

The results of the microbiological study are presented in Figure 2A. Before treatment (*t0*), the average swab colony count in selective media for the *S. aureus*–inoculated, *P. aeruginosa*–inoculated, and *A. baumannii*–inoculated groups were 5.62 ± 0.28 log(cfu/swab), 5.55 ± 0.34 log(cfu/swab), and 2.80 ± 0.68 log(cfu/swab), respectively. The average colony count in nonselective media for the *A. baumannii*–inoculated groups was 5.95 ± 0.23 log(cfu/swab). There were no statistically significant differences between the test and control subgroups. After induction therapy (*t1*), there was a statistically significant difference in colony count in selective media between control and test subgroups in *S. aureus*–inoculated (control, 5.43 ± 0.49 log[cfu/swab]; test, 2.47 ± 1.41 log[cfu/swab]; $p < 0.01$), *P. aeruginosa*–inoculated (control, 4.91 ± 0.55 log[cfu/swab]; test, 0.69 ± 0.67 log[cfu/swab]; $p < 0.01$), and *A. baumannii*–inoculated groups (control, 2.70 ± 0.77 log[cfu/swab]; test, 0.79 ± 0.95 log[cfu/swab]; $p = 0.01$). There was no statistically significant difference in average colony count in nonselective media between the *A. baumannii*–inoculated test and control subgroups. On day 4 after treatment initiation (*t4*), there was a statistically significant difference in colony count in selective media between control and test subgroups for *S. aureus* inoculation (control, 5.02 ± 0.62 log[cfu/swab]; test, 2.40 ± 0.50 log[cfu/swab]; $p < 0.01$), *P. aeruginosa* inoculation (control, 2.56 ± 0.94 log[cfu/swab]; test, 0.00 log[cfu/swab]; $p < 0.01$), and *A. baumannii* inoculation (control, 3.03 ± 0.45 log[cfu/swab]; test, 0.00 log[cfu/swab]; $p < 0.01$). There was no significant difference between the *A. baumannii*–inoculated test and control subgroups. From *t0* to *t4* in the *S. aureus*– and *P. aeruginosa*–inoculated control subgroups, there was a tendency for microbial load reduction, but statistical significance was only obtained in the *P. aeruginosa*–inoculated group ($p < 0.01$).

The wound closure kinetics (planimetric) results are presented in Figure 2B. There was a statistically significant difference in wound area between the negative control group and all inoculated control subgroups (negative control, 24.3 ± 1.8%; *S. aureus*–inoculated control, 68.2 ± 14.6%, $p < 0.01$; *P. aeruginosa*–inoculated control, 77.3 ± 6.4%, $p < 0.01$; *A. baumannii*–inoculated control, 62.3 ± 13.3%, $p < 0.01$). There was a statistically significant difference between control and test subgroups wound areas in the *S. aureus*–inoculated (test, 33.7 ± 6.7%, $p < 0.01$) and *P. aeruginosa*–inoculated groups (test, 38.7 ± 11.1%, $p < 0.01$). Although

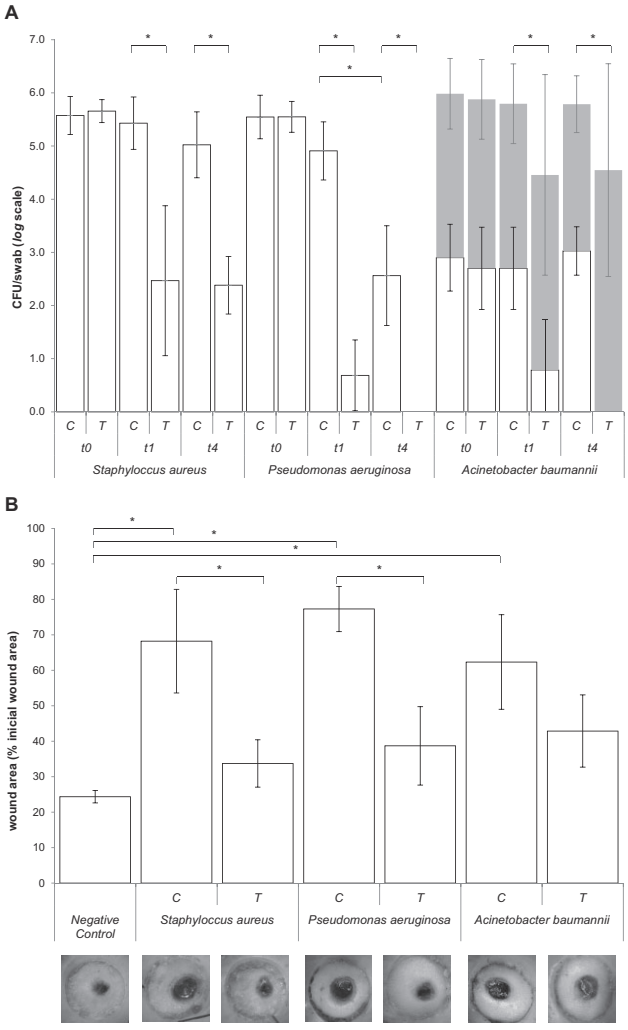

Figure 2. (A) Average swab colony count in infected rats. Wounds were swabbed at *t0*, *t1*, and *t4*, and the number of bacterial colony-forming units were compared between control and test conditions for each group. The bacteriophage-treated animals showed significantly lower counts than the control animals in all three groups on *t1* and *t4*. C, control, T, test; White bars, selective media; gray bars, nonselective media; $*p < 0.05$; (B) Wound closure kinetics in rats. Wound area was assessed on *t1* and t9, and the differences between the two timepoints were calculated. Bacteriophage treatment only reduced wound size in *S. aureus*– and *P. aeruginosa*–infected wounds. C, control, T, test; $*p < 0.05$.

there was a tendency for wound area reduction between control and test subgroups, it did not reach statistical significance in the *A. baumannii*–inoculated group (test, 42.9 ± 10.2%).

The results of the histological study are presented in Figure 3. There was a statistically significant difference between the negative control group and all inoculated control subgroups for both EG (negative control, 0.11 ± 0.13 mm; *S. aureus*–inoculated control, 1.65 ± 0.36 mm, $p < 0.01$; *P. aeruginosa*–inoculated control, 1.90 ± 0.23 mm, $p < 0.01$; *A. baumannii*–inoculated control, 1.60 ± 0.64 mm, $p < 0.01$) and DG (negative control, 1.67 ± 0.32 mm; *S. aureus*–inoculated control, 2.97 ± 0.52 mm, $p < 0.01$; *P. aeruginosa*–inoculated control, 3.30 ± 0.23 mm; *A. baumannii*–inoculated control, 2.85 ± 0.39 mm, $p < 0.01$). There was a statistically significant difference for EG between control and test subgroups in the *S. aureus*–inoculated (test, 0.14 ± 0.13 mm, $p < 0.01$) and *P. aeruginosa*–inoculated groups (test, 0.30 ± 0.25 mm, $p < 0.01$). In DG, the difference between test and control subgroups only achieved statistical significance in the *P. aeruginosa*–inoculated group (test, 42.9 ± 10.2%, $p = 0.02$). There were no significant differences between control and test subgroups with regard to EP or DG in the *A. baumannii*–inoculated group.

Pig model

The results of the microbiological study are presented in Figure 4A. Before treatment (*t0*), the average swab colony count for the *S. aureus*–inoculated, *P. aeruginosa*–inoculated, and *A. baumannii*–inoculated groups were 5.94 ± 0.69 log(cfu/swab), 4.81 ± 0.18 log(cfu/swab), and 3.61 ± 1.87 log(cfu/swab), respectively. There were no statistically significant differences between the test and control subgroups. After induction therapy (*t1*), there was a statistically significant difference in colony count in selective media between control and test subgroups in the *S. aureus*–inoculated (control,

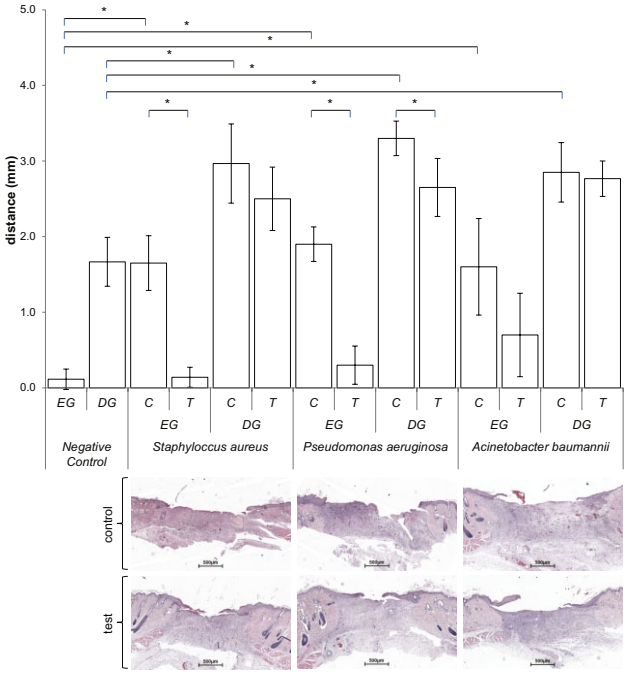

Figure 3. Histological wound analysis in rats. Epithelial gap (EG) and dermal gap (DG) were measured in harvested ulcers. Significant differences were only observed in bacteriophage-treated wounds infected with *S. aureus* and *P. aeruginosa*. C, control, T, test; $*p < 0.05$.

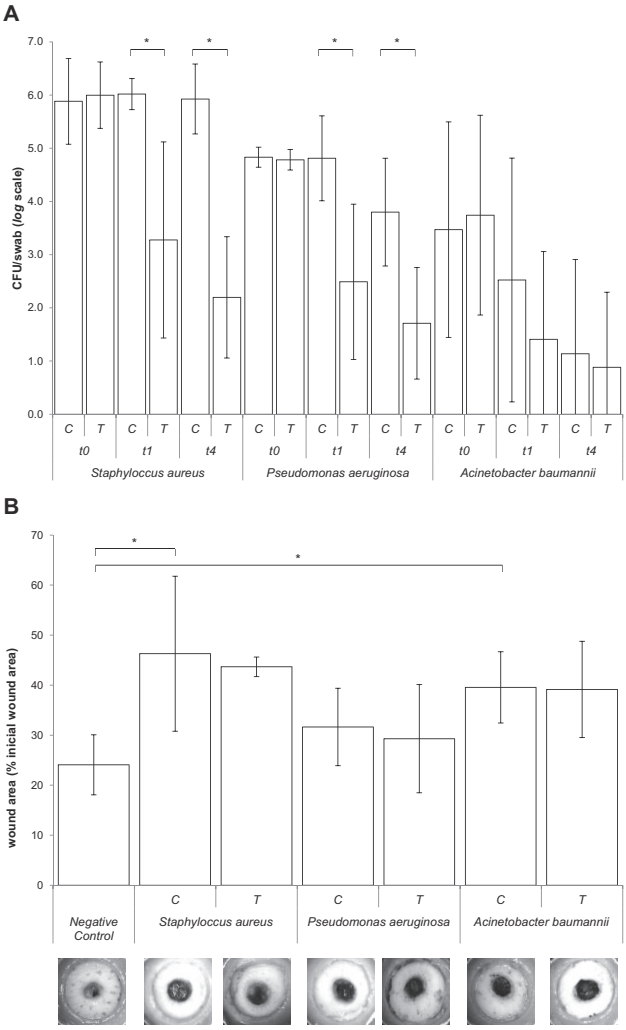

Figure 4. (A) Average swab colony count in infected pigs. Wounds were swabbed at *t0, t1*, and *t4*, and the number of bacterial colony-forming units were compared between control (C) and test (T) conditions. The bacteriophage-treated wounds showed significantly lower counts for wounds infected with *S. aureus* and *P. aeruginosa*. $*p < 0.05$; (B) Wound closure kinetics in pigs. Bacteriophage treatment did not significantly decrease wound size for any of the three conditions. However, *S. aureus* and *P. aeruginosa* infections both resulted in larger significantly larger wounds. $*p < 0.05$.

6.02 ± 0.29 log[cfu/swab]; test, 3.28 ± 1.84 log[cfu/swab]; $p = 0.02$) and *P. aeruginosa*–inoculated groups (control, 4.81 ± 0.80 log[cfu/swab]; test, 2.49 ± 1.46 log[cfu/swab]; $p = 0.03$). Although there was a tendency for microbial load reduction in average colony count for the *A. baumannii*–inoculated test and control subgroups (control, 2.52 ± 2.29 log[cfu/swab]; test, 1.41 ± 1.65 log[cfu/swab]), the difference was not statistically significant. At day 4 after treatment initiation (*t4*), there was a significant difference in colony count between control and test subgroups in the *S. aureus*–inoculated (control, 5.93 ± 0.66 log[cfu/swab]; test, 2.20 ± 1.14 log[cfu/swab]; $p < 0.01$) and *P. aeruginosa*–inoculated (control, 4.81 ± 0.80 log[cfu/swab]; test, 1.71 ± 1.05 log[cfu/swab]; $p = 0.02$) groups. No such difference was observed for the *A. baumannii*–inoculated test and control subgroups.

The wound closure kinetics (planimetric) results are presented in Figure 4B. Wound closure kinetics statistically differed between the negative control group (24.1 ± 6.0%) and the *S. aureus*–inoculated (46.3 ± 15.5%, $p = 0.02$) and *A. baumannii*–inoculated (39.6 ± 7.1%, $p = 0.01$) control subgroups, but this was not verified for the *P. aeruginosa*–inoculated control subgroup. Wound closure kinetics were not significantly different between any inoculated control and test subgroups for the three bacteria strains.

The results of the histological study are presented in Figure 5. The analysis revealed a statistically significant difference with regard to EG between the negative control group and all the inoculated control subgroups (negative control, 1.80 ± 0.33 mm; *S. aureus*–inoculated control, 4.23 ± 0.14 mm, $p < 0.01$; *P. aeruginosa*–inoculated control, 3.02 ± 0.23 mm, $p < 0.01$; *A. baumannii*–inoculated control, 3.43 ± 0.60 mm, $p < 0.01$). We also found statistically significant differences for EG between control and test subgroups in the *S. aureus*–inoculated (test, 3.38 ±0.59 mm, $p = 0.02$) and *P. aeruginosa*–inoculated groups (test, 2.20 ± 0.05 mm, $p = 0.02$). No such difference was observed for *A. baumannii*–inoculated wounds.

DISCUSSION

Current DFI treatment protocols employ debridement and systemic antibiotics,[2] which are often ineffective in promoting

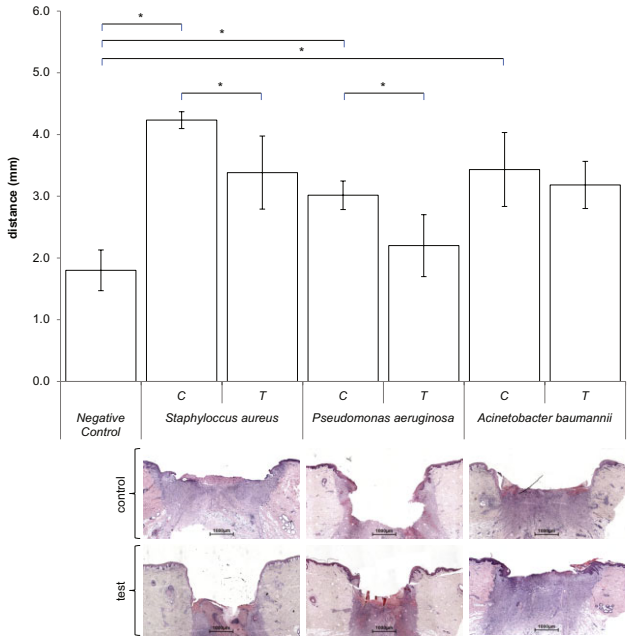

Figure 5. Histological wound analysis in pigs. Epithelial gap (EG) values were measured in harvested wounds. Significant differences were only observed in *S. aureus* and *P. aeruginosa* bacteriophage-treated wounds. C, control, T, test; $*p < 0.05$.

wound healing.[3] In addition, bacterial resistance to antibiotics renders these therapies less effective. Novel therapeutic regimens are needed to successfully treat DFIs. Although TATs are an attractive alternative, current formulations have not been successfully implemented for DFIs.[7]The addition of lytic bacteriophages may enhance the utility of TAT agents; they are safe for human use[10] and can effectively combat drug-resistant bacteria.[9] Although these bacteriophages have been used to treat acute bacterial infections,[16] no study has assessed their ability to ameliorate chronically infected wounds.

Previously published studies have not assessed the effects of bacteriophage cocktails in chronic wounds; most examined outcomes after only a few hours of infection.[16] In addition, most results came from burn models.[33] We have investigated the ability of bacteriophages combined with debridement to improve microbiological, planimetric, and histological wound parameters in diabetic animal models.

Based on previous rodent studies[23] we knew that the bacterial colony counts in tissue cultured from infected wounds at *t4* were, on average, 7.54 ± 0.19 log(CFU) per ulcer. We used high bacteriophage doses (10^8 to 10^9 pfu per administration), which yields a multiplicity of infection of 10 to 100. This initial dose is sufficiently in excess of the target bacterium population to cause reductions without the need for bacteriophages to replicate and complete their life cycle. This is in contrast with previous bacteriophage therapy studies[34] that employed relatively low bacteriophage doses and mainly relied on active therapy, which involves phage infection/replication cycles to reduce the target bacterium. These processes of active and passive bacteriophage therapy have been well described for in vitro and in vivo studies.[35,36]

All three outcomes were improved by bacteriophage treatment in animals that were infected with *S. aureus* and *P. aeruginosa*, but only bacterial reduction was observed in those infected with *A. baumannii*. This is potentially justified by a study[37] in which the presence of *Acinetobacter* spp. in a biofilm community was found to facilitate surface colonization by other species, namely *Staphylococcus* spp. Indeed, our microbiological data are in line with this finding. We determined that excess bacteria growing in nonselective media in *A. baumannii*–inoculated groups were primarily *Staphylococcus* spp.

Bacterial counts were assessed at *t4*, and colony counts were significantly different for *S. aureus* and *P. aeruginosa* test conditions compared with control. This difference was particularly pronounced for the latter. This finding is in agreement with our previously published optimization study.[23] It is also in line with results reported by Fazli et al.,[38] who used confocal laser scanning microscopy of clinical wound-biopsy specimens to demonstrate that the distance from *P. aeruginosa* aggregates to the wound surface was significantly greater than that of *S. aureus* aggregates, which led to an underestimation of the former in swab samples. This observation supports the possibility that factors intrinsic to each pathogenic bacterial strain can contribute to differences among studies that compare cultures grown from swabs and tissue samples.

Planimetric assessments revealed statistically significant differences between the control and test groups treated with *S. aureus* and *P. aeruginosa*. Although the same trend was observed for *A. baumanni*, the difference was not significant. These results were similar to the EG and DG measurements in harvested histological specimens. However, only the *P. aeruginosa* test group showed a significant difference. This was likely due to the smaller standard deviation value in that group.

Although rodent models are useful, we wanted to assess the utility of bacteriophage treatment in pigs, which are considered the ideal large animal model for studying cutaneous disease.[39] The results obtained in the rodent model were largely corroborated by experiments in swine. In both models, there was a significant reduction of bacterial counts at both time points (*t1* and *t4*) for *S. aureus* and *P. aeruginosa* infections, but this was not verified for *A. baumannii* infections. A possible explanation for this difference is the low initial (*t0*) bacterial counts, which limited evaluation power. An alternative possibility for discrepant results between the two models is different host–microbe interactions between species, which may limit the establishment of bacterial infection in swine.[40]

Although the planimetric results were not significantly different in the swine model, we did find differences among the negative control and *S. aureus* and *A. baumannii* control groups. Notably, the *P. aeruginosa* test group was not significantly different from the control group. It is possible that this was because that bacterial strain causes a deeper infection, and the damage was localized further beneath the dermis, resulting in a smaller ulcerated surface area.[38] Despite the discrepant bacterial counts, there was no statistically significant difference between the *A. baumannii* and *S. aureus* group planimetries. However, both were significantly different from the negative control group. This may also be justified by the coaggregation of *Acinetobacter* spp. and other species, namely *Staphylococcus* spp.

We did observe significant results in the *S. aureus*–inoculated and *P. aeruginosa*–inoculated test animals with regard to EG measurements. However, the results for the *A. baumannii*–inoculated test group were not different from those of the control. A possible reason for this result is that the bacteria failed to successfully infect the wound.

There are several limitations inherent to the study design. We used swabs rather than biopsies to quantify bacterial counts. This method was chosen because we also evaluated planimetry and histology, both of which would have been influenced by biopsies. Moreover, the swab technique was previously optimized and shown to correlate with the invasive method for the rodent model.[23] Second, we did not investigate resistance of bacterial strains to the bacteriophage cocktail, which may have played a role in the *A. baumannii*–inoculated groups. This variable will be studied in future investigations. Finally, we did not grow all the samples in nonselective media cultures; it was not feasible to simultaneously grow such a large number of samples in the lab without risking cross-contamination. Finally, the porcine experiments only included a single pig per group. Although this decreased the power of the statistical analysis, previously published findings suggest that the large degree of interindividual variability makes it necessary to make comparisons within individual animals.[41]

To our knowledge, this is the first study to assess the microbiological, planimetric, and histological efficacies of topical bacteriophage therapy in animal models. It is important that the treatment was performed in conjunction with sharp debridement, as there is currently no clinical or experimental rationale for using TAT products in its absence.[23] Debridement leads to a significant decrease in the resistance of the bioburden to TAT for up to 24 hours and enhances

cicatrization.[6] Collectively, the results of this study suggest that bacteriophage-containing TAT may be a viable treatment for DFIs, including infections caused by drug-resistant bacteria. Although additional studies are necessary, it may be an effective and novel therapeutic approach for addressing the serious problems associated with DFIs and other chronic skin and soft tissue infections.

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Conflicts of Interest: The authors would like to disclose that: JJM, CL, RB, and MG have pending patent applications related to the field of bacteriophage therapy; CL, RB and SCR are paid employees, and PCS and MG are board members and stock owners of TechnoPhage S.A., which holds a patent on the bacteriophages used in this study; and AG is co-inventor of a patent related to bacteriophage therapy not related to this study.

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