

Negative Pressure Level and Effects on Bacterial Growth Kinetics in an *in vitro* Wound Model

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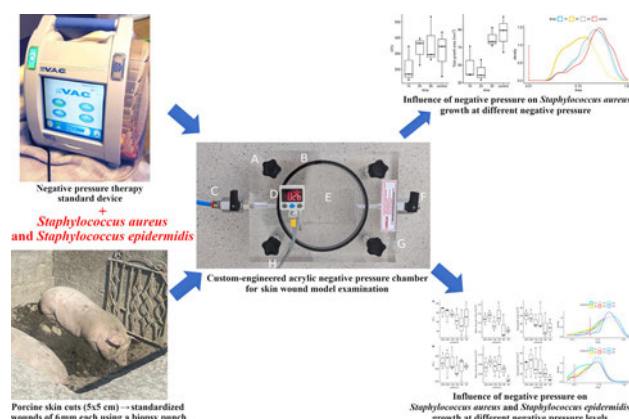
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Abstract

Negative Pressure Wound Therapy (NPWT) has been widely adopted in wound healing strategies due to its multimodal mechanism of action. While NPWT's positive impression on wound healing is well-established, its effect on bacterial load reduction remains equivocal. This study investigates NPWT's efficacy in reducing bioburden using an *in vitro* porcine skin model, focusing on the impact of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Custom-made negative pressure chambers were employed to apply varying negative pressures. Porcine skin was cut into 5 × 5 cm squares and three standardized wounds of 6 mm each were created using a biopsy punch. Then, wounds were infected with *S. aureus* and *S. epidermidis* bacterial suspensions diluted 1:10,000 to obtain a final concentration of 1.5×10^4 CFU/ml and were placed in negative pressure chambers. After incubation, bacterial counts were expressed as colony-forming units (CFU) per ml. For *S. aureus* at 120 hours, the median CFU, mean area per colony, and total growth area were notably lower at –80 mmHg when compared to –250 mmHg and –50 mmHg, suggesting an optimal negative pressure for the pressure-dependent inhibition of the bacterial proliferation. While analyzing *S. epidermidis* at 120 hours, the response to the negative pressure was similar but less clear, with the minor



CFU at –100 mmHg. The influence of intermittent negative pressure on the *S. epidermidis* growth showed notably lower median CFU with the interval therapy every hour compared to the *S. aureus* control group. This study contributes valuable insights into NPWT's influence on the bacterial load, emphasizing the need for further research to reformulate its role in managing contaminated wounds.

Key words: negative pressure wound therapy, bacterial growth, wound healing, bioburden

Introduction

Since its inception in 1997, Negative Pressure Wound Therapy (NPWT) has revolutionized wound-healing strategies (Argenta and Morykwas 1997). Its rapid adoption for variegated use in diverse clinical indications is attributed to its multimodal mechanism of action, which includes: macrodeformation (reduction in wound size), microdeformation (cellular

changes in the wound bed), edema reduction, maintenance of an optimal wound environment, and alterations in the hypoxic gradient within the microcirculation (Orgill et al. 2009; Back et al. 2013; Glass et al. 2014; Nie and Yue 2016). Numerous publications have since confirmed NPWT's positive impact on wound healing across diverse pathologies, complexities, and locations. Innovations such as NPWT with instillation, single-use NPWT, closed incision NPWT, and endoscopic NPWT

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have been developed to enhance efficacy and mitigate surgical complications (Back et al. 2013; Blackham et al. 2013; Bobkiewicz et al. 2020).

Despite its widespread use, the impact of NPWT on bacterial load reduction remains a contentious issue (Glass et al. 2017). Early studies, like Morykwas et al. (1997), demonstrated a decrease in wound bioburden in an experimental porcine model, sparking considerable interest. However, modern research presents no definitive conclusions on NPWT's effect on bacterial counts, accentuating discrepancies across experimental, randomized controlled trials, and clinical studies (Chester and Waters 2002; Weed et al. 2004; Mouës et al. 2007; Lalliss et al. 2010). Factors contributing to these variances include wound type (acute or chronic), measurement methods (tissue specimen vs. surface swab), timing, wound dressing types (with or without silver), instillation use, and debridement frequency and methods. Additionally, NPWT's differential effects on diverse bacterial species have been noted (Lalliss et al. 2010; Stinner et al. 2011; Liu et al. 2014b). Drawing from our previous experience with NPWT in contaminated and infected wounds, we found it a useful and safe option (Bobkiewicz et al. 2017; Borejsza-Wysocki et al. 2021). This study investigates NPWT's efficacy in reducing bioburden using an *in vitro* model, focusing on its potential impact on different bacterial species. Our preliminary findings contribute to the ongoing discussion about NPWT's effectiveness against bioburden, paving the way for further research that could redefine NPWT's role in managing contaminated wounds.

Experimental

Materials and Methods

Biological material. This study utilized a porcine skin model to assess the influence of Negative Pressure Wound Therapy (NPWT) on bacterial numbers. The experiments were conducted in a microbiology laboratory in Poznań, Poland. Porcine skin was chosen as a model because of its similarity to human skin in terms of thickness, structure, and bacterial colonization. The skin was shaved, washed with tap water, and disinfected with octenidine dihydrochloride solution. The skin was then cut into 5 × 5 cm squares and placed in a sterile container. The specimens were stored at -20°C until use. Before each experiment, the specimens were thawed at room temperature and washed with sterile saline. Using a biopsy punch (KAI Europe GmbH, Germany), three standardized wounds of 6 mm each were created on the porcine skin.

Pressure chambers. The experiments utilized 24 custom-made negative pressure chambers constructed

from thick acrylic glass. Each chamber was designed with a centrally machined main chamber, which ensured precise control over the experimental conditions. To maintain an airtight environment, a silicone gasket was fitted around each chamber. An additional thin channel was integrated into the design for accurate pressure monitoring, where an electronic pressure sensor was attached. This sensor was connected to a reading unit allowing continuous pressure tracking. The pressure within the chambers was supplied externally through a third channel equipped with a ball valve. This setup ensured that the interior remained sealed and allowed the chambers to maintain the pressure provided for a minimal duration of 72 hours, as verified through testing. The pressure chambers used in this study are presented in Fig. 1.

Bacterial strains. Two bacterial strains were used in this study: *Staphylococcus aureus* ATCC® 25923™ and *Staphylococcus epidermidis* ATCC® 12228™. The strains were purchased from the American Type Culture Collection (ATCC®, USA). The bacteria were cultured in Luria-Bertani (LB) broth (Sigma-Aldrich®, Merck KGaA, Germany), and incubated at 37°C for 24 hours. The bacterial concentration was adjusted to 0.5 McFarland standard (1.5×10^8 CFU/ml) using a spectrophotometer WPA Biowave II (Biochrom Ltd., UK). The bacterial suspension was diluted 1:10,000 in LB broth to obtain a final concentration of 1.5×10^4 CFU/ml.

Outcome measurements. The primary outcome of this study was the reduction in bacterial counts after NPWT using our custom-made negative pressure chambers. The bacterial counts were measured using the serial dilution method. To determine the target dilution, the wound specimens were washed with 100 µl of sterile saline to suspend the bacterial cells. After vortexing (Vortex-Genie 2T; Scientific Industries Inc., USA), the suspension was serially diluted in sterile saline and plated on LB agar plates (Sigma-Aldrich®, Merck KGaA, Germany). The plates were incubated at 37°C for 24 hours, and the bacterial colonies were quantified using automated image processing with the help of ImageJ software (Schneider et al. 2012). To that end, the plates were evaluated regarding the colony size, and colonies smaller than a cut of value for debris (less than 0.05 mm²) were removed from further analysis. The colony counts were multiplied by the respective dilution factor and expressed as colony-forming units (CFU) per ml.

Assessing the impact of varying negative pressure on bacterial growth. Twelve fresh, raw porcine skin samples were used. A suspension of coagulase-negative *S. aureus* or *S. epidermidis* was prepared to the density of 1.5×10^4 CFU/ml in LB broth, as described above. Before incubation, experimental chambers were rinsed with octenidine dihydrochloride. The prepared porcine

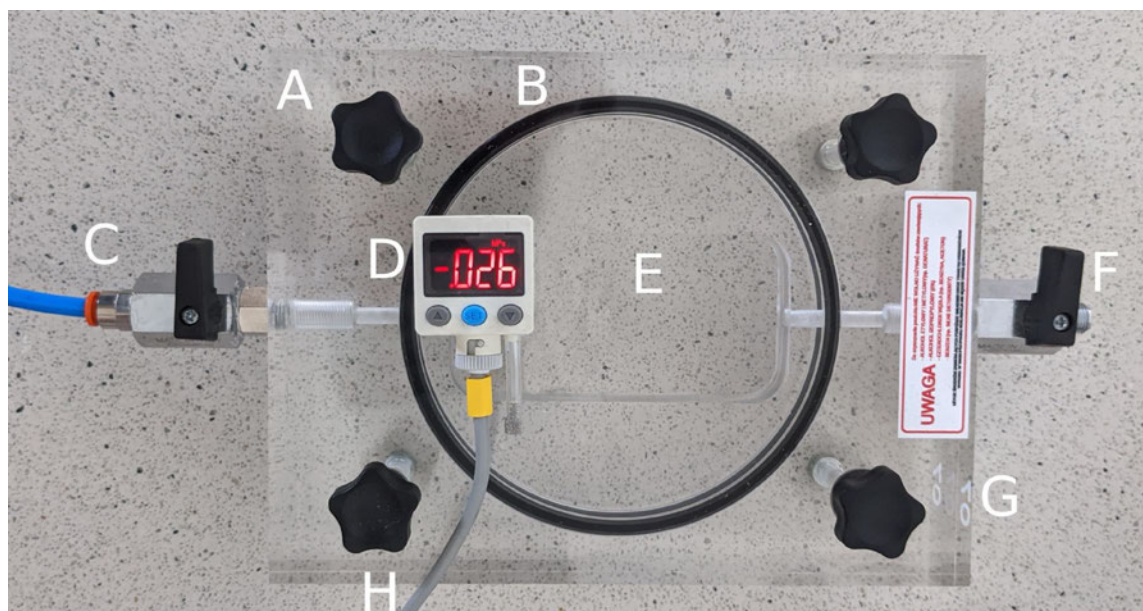


Fig. 1. Detailed representation of a custom-engineered acrylic negative pressure chamber for skin wound model examination.

The figure depicts an overhead view of an individually numbered negative pressure chamber designated for studying the impacts of vacuum conditions on bacterial colonization in a skin wound model. The principal compartment, 'E', serves as the receptacle for the skin wound model. The application of negative pressure is facilitated through the portal marked 'C', hermetically sealed with a silicone gasket 'B'. The electronic pressure sensor 'D' relays the internal pressure reading of -0.26 atm, signifying an operational negative pressure state. This sensor is linked via cable 'H' to an external device for continuous pressure monitoring. Port 'F' is utilized for instillation, permitting the administration or removal of substances without nullifying the vacuum environment. Access knobs 'A' allow for the manipulation of the internal contents. The device's identification is presented on the label 'G', indicating the unique chamber number ranging from 01 to 24, essential for distinguishing among multiple units in use during parallel experiments. This system is fundamental for the precise study of bacterial dynamics under negative pressure, a critical factor in optimizing NPWT protocols for enhanced wound healing outcomes.

wound models were placed in these chambers and contaminated with $50\ \mu\text{l}$ of the bacterial suspension. The chambers were sealed, and the following various negative pressures were applied with a syringe: -50 , -80 , -100 , -150 , -200 , -250 mmHg. The readouts were validated on a pressure sensor attached to the main chamber. A continuous mode of NPWT was utilized for all groups.

Assessing the impact of intermittent negative pressure on bacterial growth. Four fresh, raw porcine skin samples were used. A suspension of coagulase-negative *S. aureus* was prepared to the density of 1.5×10^4 CFU/ml in LB broth, as described above.

Before incubation, experimental chambers were rinsed with octenidine dihydrochloride. The prepared porcine wound models were placed in these chambers and contaminated with $50\ \mu\text{l}$ of the bacterial suspension. The chambers were sealed, and fluctuating negative pressures of -250 mmHg were applied. An intermittent mode of NPWT was used with the instillation of 0.9% saline solution over a 10-minute dwell time. With continuous pressure of -250 mmHg, the following incremental time intervals were tested: 1-hour, 2-hour and 3-hour. One group served as a control using the continuous mode of NPWT at -250 mmHg.

Incubation and sampling. After a 24-hour incubation period, $10\ \mu\text{l}$ of 0.9% saline solution was added to

the wounds to suspend the bacteria. Subsequently, $10\ \mu\text{l}$ of the solution was collected for bacteriological analysis.

Bacteriological analysis. Serial dilutions were prepared to a final volume of $100\ \mu\text{l}$. Dilutions of 1:10,000 were chosen for plating. A sample of $50\ \mu\text{l}$ from each group was plated on LB agar plates (LB Agar; A&A Biotechnology, Poland). The plates were incubated at 37°C overnight (approximately 16 hours).

Statistical analysis. As we observed a mostly positively skewed distribution of the colonies during culture, the CFU/ml values were compared in respective conditions using an inverse Gaussian regression model. One prerequisite for this statistical method is the removal of outlier observations, which were performed based on merit. Observations were removed where potential pipetting errors could not be excluded.

Results

Effects of negative pressure on bacterial growth kinetics. The application of varying negative pressure exhibited differential impacts on the growth kinetics of *S. aureus* and *S. epidermidis*, as depicted in Fig. 2.

For *S. aureus* at 120 hours (Fig. 2A), a trend towards reduced CFU was observed with higher negative pressures. At -80 mmHg, the median CFU was notably lower

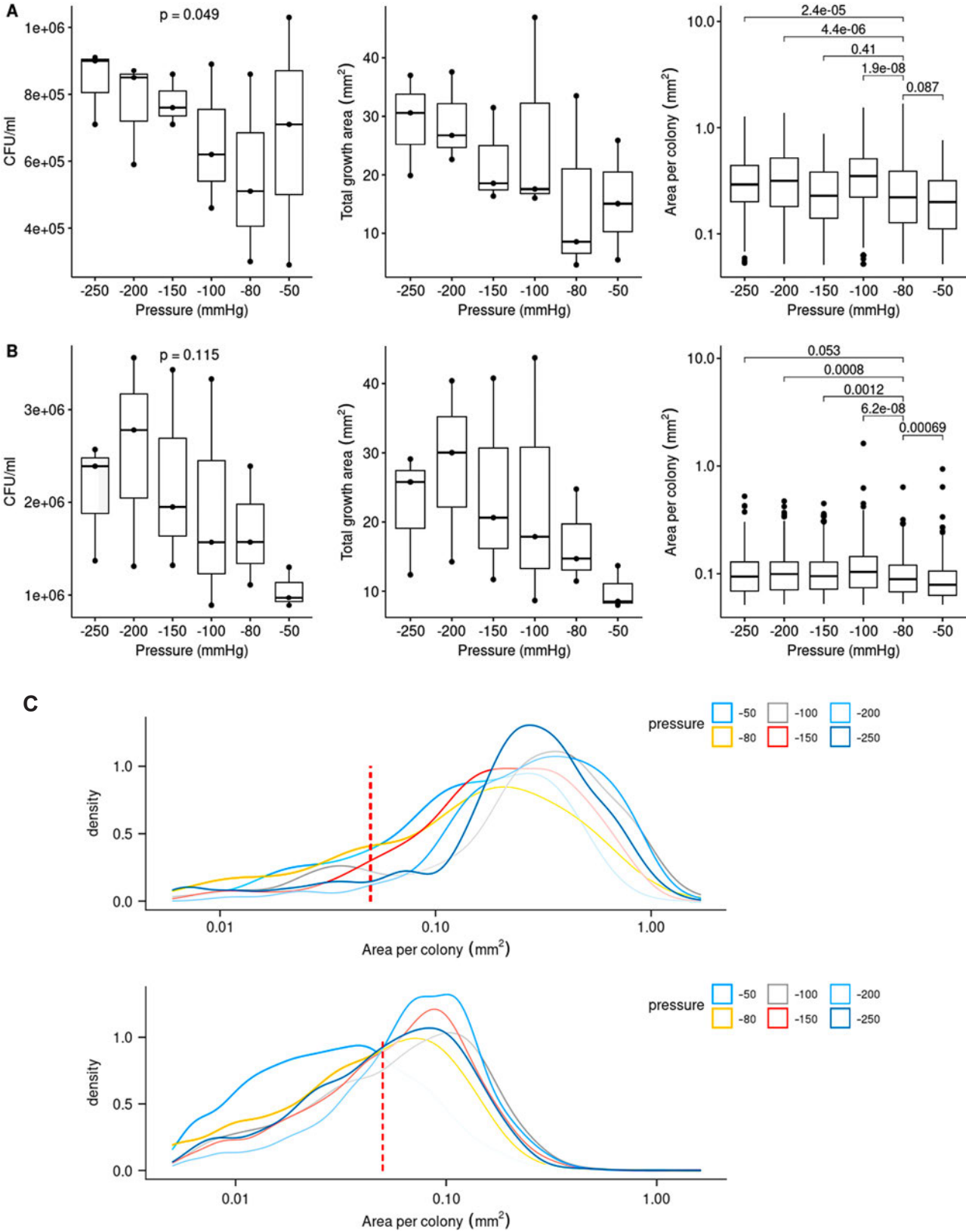


Fig. 2. Influence of negative pressure on *Staphylococcus aureus* and *Staphylococcus epidermidis* growth at different negative pressure levels.

This composite figure presents a series of box-and-whisker plots alongside density plots demonstrating the effects of varying levels of negative pressure on the growth characteristics of *S. aureus* and *S. epidermidis*. Each row represents a different combination of bacterial species and culture duration, with panel A corresponding to *S. aureus* at 120 hours culture, panel B to *S. epidermidis* at 120 hours culture. The first column of box plots in each panel depicts the colony-forming units (CFU), the second shows the mean area per colony, and the third illustrates the total growth area across a range of negative pressures from -50 to -250 mmHg. The accompanying density plots visualize the distribution of colony areas, with vertical dashed lines marking the cut-off values for debris.

compared to -250 mmHg and lower than -50 mmHg, which suggests it is an optimal negative pressure for the pressure-dependent inhibition of bacterial proliferation. The mean area per colony and the total growth area also followed a similar trend, indicating an optimal reduction in colony expansion at -80 mmHg.

In the case of *S. epidermidis* at 120 hours (Fig. 2B), the response to negative pressure was similar but less clear, with the fewest CFU around -100 mmHg. However, the variability in the mean area per colony and the total growth area was more pronounced.

The multiple regression analysis conducted for both bacterial species indicated a significant association between the growth of bacteria and negative pressure applied ($p = 0.0356$, Table I). The analysis restricted to

individual species indicated a significant association between pressure and CFU/ml in *S. aureus* culture ($p = 0.049$, Fig. 2A).

Although we observed that the *S. epidermidis* generally showed better growth than *S. aureus* in the negative pressure chambers ($p \leq 0.001$), there was no significant difference in the growth patterns of both of these species regarding negative pressure applied, as the interaction between pressure and species terms of the inverse Gaussian regression analysis showed $p = 0.076$ (Table I).

Effects of intermittent negative pressure on bacterial growth. The application of intermittent negative pressure exhibited differential impacts on the growth of *S. aureus*, as depicted in Fig. 3. After 24 hours, the median CFU was notably lower with the interval

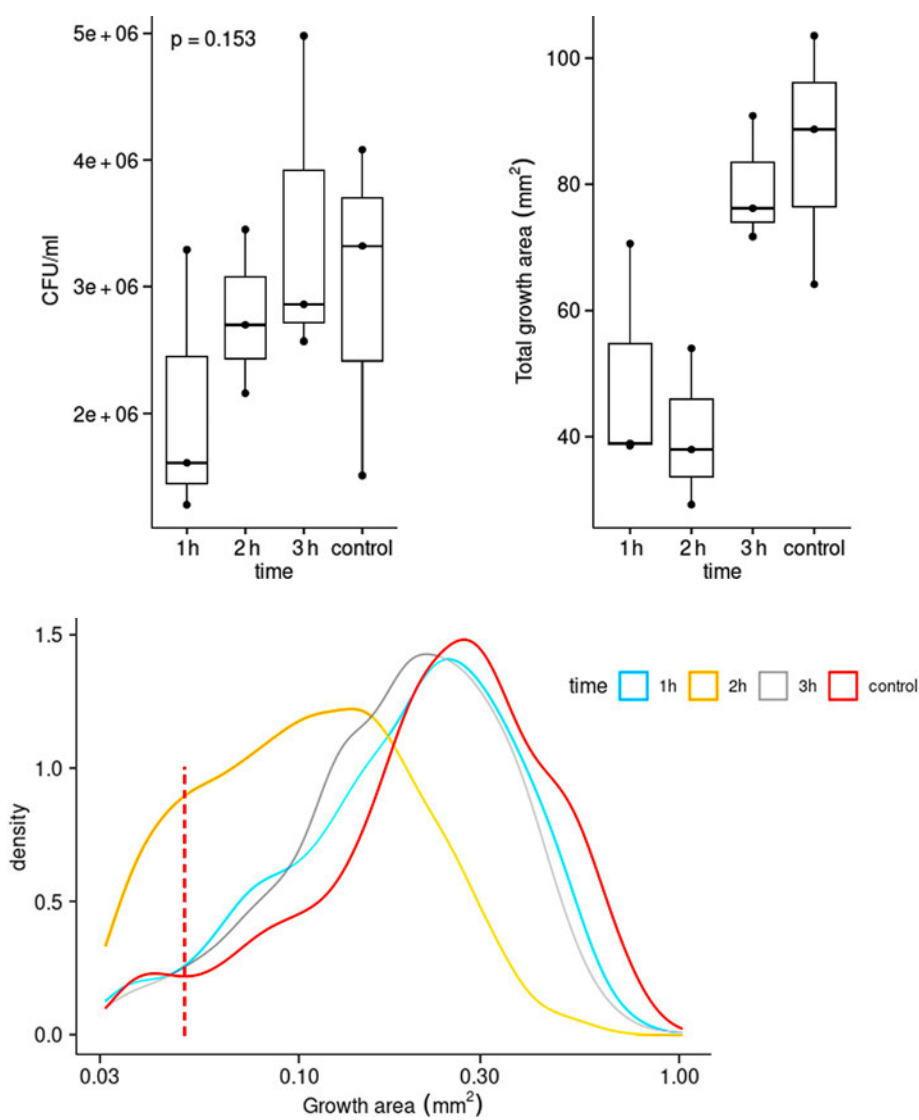


Fig. 3. Influence of negative pressure on *Staphylococcus aureus* growth at different negative pressure intervals. This composite figure presents a series of box-and-whisker plots alongside density plots demonstrating the effects of varying levels of negative pressure on the growth characteristics of *Staphylococcus aureus* using different modes of intermittent negative pressure intervals. The first column of box plots in each panel depicts the colony-forming units (CFU), and the second illustrates the total growth area, across an intermittent negative pressure intervals of 1–3 h and the second illustrates the total growth area across intermittent negative pressure intervals of 1–3 h. The pressure used was 250 mmHg. The accompanying density plots visualize the distribution of colony areas, with vertical dashed lines marking the cut-off values for debris.

Table I
Inverse Gaussian multiple regression analysis for the growth of *Staphylococcus aureus* and *Staphylococcus epidermidis* regarding negative pressure in an *in vitro* wound model.

	Dependent variable: CFU/ml
Pressure	9.21 ⁻¹⁵ (4.19 ⁻¹⁵) <i>p</i> = 0.0356
Species (<i>S. epidermidis</i> as reference)	-3.06 ⁻¹² (8.13 ⁻¹³) <i>p</i> = 0.0007
Pressure: species interaction	-7.85 ⁻¹⁵ (4.27 ⁻¹⁵) <i>p</i> = 0.0761
Constant	3.55 ⁻¹² (7.96 ⁻¹³) <i>p</i> = 0.0001
Observations	35
Log Likelihood	-502.335
Akaike Inf. Crit.	1,012.670

therapy every hour, compared to the control group, suggesting an optimal negative pressure for the pressure-dependent inhibition of bacterial proliferation. Nevertheless, the conducted regression analysis indicated an insignificant association of intermittent negative pressure therapy intervals and the bacterial growth in CFU/ml (*p* = 0.153, Fig. 3). The total growth area followed a similar trend, indicating an optimal reduction in colony expansion at -80 mmHg.

Discussion

The first study presenting the influence of negative pressure application on bacterial load reduction was conducted by Morykwas et al. (1997). In the porcine skin model contaminated with 10⁸ CFU/ml of *S. aureus* or *S. epidermidis*, authors revealed a significant reduction in bacterial numbers from 10⁸ CFU/g tissue to below 10⁵ CFU/g tissue using negative pressure. Later, Assadian et al. (2010) conducted a study analyzing bacterial overload in an *in vitro* wound model. Analogous to our research, they also used standardized wounds contaminated with *S. aureus* and analyzed the effect of negative pressure on bioburden. Surprisingly, they found no statistical differences in the reduction of the number of bacteria regardless of the intervention method. Thus, the potential effect on bacterial reduction might be achieved in a multimodal mechanism of action, mainly due to immune-modulating factors rather than macro- and microdeformation properties.

Although the clinical relevance of our results needs to be taken with caution, a recent study by Li et al. (2019) showed similar results in a more translatable animal full-thickness wound model over eight days of NPWT. The authors could visualize a reduction of

S. aureus strains as early as 48 h using fluorescence imaging with laser scanning confocal microscopy.

In general, several probable explanations for the reduction in bacterial numbers are observed in clinical practice. A combination of debridement as an obligatory first step of wound management can create optimal conditions for maintaining the NPWT mechanism of action (Birke-Sorensen et al. 2011). Applying NPWT to necrotic, non-viable, or eschar wounds is contraindicated; therefore, it should not be considered a method to replace extensive debridement (Birke-Sorensen et al. 2011). Next, increased blood flow within the surrounding tissues of the wound bed fosters a pro-inflammatory response, activating molecular cascades that may influence the reduction of bacterial numbers (Kilpadi et al. 2006; Labler et al. 2009). Further, alteration of oxygen concentration using NPWT results in either enhancement of neutrophil oxidative bursts or prevention of anaerobic growth (Hopf et al. 2001). Moreover, some NPWT dressings contain silver particles that may also impress the reduction in bioburden (Stinner et al. 2011). Lastly, some molecular alterations and modulations have been reported, such as VEGF-mediated neoangiogenesis; cytokines, growth factors, and metalloproteinases profile may suppress bacterial proliferation (Glass et al. 2014; Liu et al. 2014a).

It can be suggested that the mechanism of action in NPWT is rather multimodal and combinatory of all the rationales above and may theoretically result in a reduction of bacterial numbers (Boone et al. 2010). Based on recent studies, potential immunological and molecular mechanisms should be relatively responsible for the major effect on bacterial reduction, fueling the need for further investigation.

However, our intention in the presented study was to evaluate the potency of bacterial proliferation in an infected soft tissue *in vitro* model without the influence of the immune system. Recently, Glass et al. (2017) presented a comprehensive review regarding the impact of NPWT on bacterial growth. They analyzed experimental studies, randomized controlled trials, and clinical studies. When focusing only on experimental studies, eight of ten studies illustrated significant reductions in bacterial counts when comparing NPWT and control groups, whereas Boone et al. (2010) and Assadian et al. (2010) revealed no difference in bioburden. Lalliss et al. (2010) and Stinner et al. (2011) demonstrated the influence of NPWT on species selectivity favorably reduced the growth of *Pseudomonas* spp., but not *S. aureus* over the same period. According to a review by Glass et al. (2017), bacterial species-dependent influence was observed in five out of ten analyzed studies. The authors concluded that bacterial growth kinetics is exceedingly complex and is more likely bacterial species-dependent. However, in the presented study, a similar pattern

of bacterial decline was observed regarding varying negative pressure and bacterial strains. This may be explained by the fact that both investigated *Staphylococci* are facultative anaerobes, and their growth patterns might be similar in case of reduced oxygen availability due to the application of a sealed negative pressure chamber.

Independently, Bowler (2003) and Edwards and Harding (2004) showed that bacterial colonization greater than the traditional infection threshold of 10^5 CFU/g, did not affect wound healing when compared with standard bacterial overload. Instead, the authors deduced that this phenomenon might be associated with higher bacterial virulence or synergism. Furthermore, Fujiwara et al. (2013) conducted an *in vitro* study analyzing the influence of various types of negative pressure settings on the proliferation potency of non-pathogenic *Escherichia*. They revealed that the proliferation potency of *E. coli* happened to be higher when intermittent negative pressure was used, than continuous mode usage. Proliferation potency was also higher when a short cycle of intermittent negative pressure therapy was applied, compared to a long cycle of intermittent negative pressure. These outcomes are not aligned with our observation. Firstly, their examination used a different bacterial species – *E. coli*, whereas *S. aureus* or *S. epidermidis* were used in the presented study. Secondly, *E. coli* is a facultative anaerobe, which may incite different results from those conferred in our research. Thirdly, the methodology of the experiments was different. Thus, a straightforward comparison of available outcomes is challenging due to the heterogeneity of provided protocols.

Different *in vivo* animal models have been used to study wound healing. The porcine model seems to be the most convenient due to its anatomical and physiological similarity to humans. However, it is worth noting that the dermis of large and old animals is significantly thicker than in humans. Moreover, less vascular dermis and lack of eccrine sweat glands over almost every body surface are other limitations.

There are some disadvantages to using *in vivo* porcine model. Firstly, there are some ethical considerations with porcine *in vivo* models. Rodent wound models are more commonly used. However, the major disadvantage is the different wound healing patterns, which makes it difficult to extrapolate the outcomes to humans. In rodents, a wound heals through the contraction of the panniculus carnosus (contraction as a fundamental mechanism of action), while a human wound heals through re-epithelization. Secondly, a small sample size in the porcine model *in vivo* is used, which is another limitation. Although some researchers used multiple wounds on the same animal to decrease the number of animals, such a methodology may influence the poten-

tial bias of the study. Thirdly, cost is another limitation of this model. Fourthly, some potential technical problems are associated with maintaining the negative pressure chamber within the pig's back, as keeping the animals under anesthesia for multiple days is neither feasible nor ethical, and the risk of detaching the device in case of regular physical activity is high.

Based on current recommendations, a disparity between basic science favoring intermittent mode and clinical practice favoring continuous mode has been observed (Apelqvist et al. 2017). This finding is consistent with our observation and practice, using the preferable continuous mode of action (Bobkiewicz et al. 2017; Borejsza-Wysocki et al. 2021). Moreover, it is worth highlighting the continuous mode of NPWT as a method of choice in clinical scenarios consisting of peritoneum exposure, intestinal fistulas, tunneling wounds, and sternotomies, grafts, or skin flaps. According to the latest recommendation, the optimal negative pressure time phase is 2–3 hours with a 10-minute dwell time (Kim et al. 2020).

Conclusions

In the presented study, we assessed the bacterial kinetics of *S. aureus* and *S. epidermidis* as the most common pathogens influencing wound healing. We revealed that in different settings of negative pressure and mode of action, the bacterial proliferation potency might vary, favoring the decrease of bacterial load when using –80 mmHg for *S. aureus* and –100 mmHg for *S. epidermidis*. In the future, *in vivo* experiments are needed to confirm such outcomes by evaluating other mechanisms of negative pressure.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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