Cutaneous wound biofilm and the potential for electrical stimulation in management of the microbiome

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Cutaneous wound biofilm and the potential for electrical stimulation in management of the microbiome

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Abstract

Infection contributes significantly to delayed cutaneous wound healing which impacts patient care. External application of electrical stimulation (ES) has beneficial effects on wound repair and regeneration. The majority of studies to date have explored ES in relation to planktonic microorganisms, yet evidence indicates that bacteria in chronic wounds reside as antibiotic resistant polymicrobial biofilms, which contribute to impairing wound healing. Culture-independent sequencing techniques have revolutionised our understanding of the skin microbiome and allowed a more accurate determination of microbial taxa and their relative abundance in wounds allowing a greater understanding of the host-microbial interface. Future studies combining the fields of ES, biofilm and microbiome research are necessary to fully elucidate the use of ES in the management of wound infection.

Keywords: biofilm; electrical stimulation; microbiome; wound healing; cutaneous wounds

Introduction

Chronic cutaneous wounds have a major impact on patients' quality of life and mobility and present a huge burden on the healthcare system. Persistent wound infection is a major contributor to delayed wound healing [1,2]. Chronic wounds can be ~~are predominantly~~ poorly managed with the prolonged use of antibiotics, leading to growing antibiotic resistance [3,4]. There is an unmet need for better non-pharmacological anti-microbial technologies in the management of chronic wound infections.

Skin is electrically charged, termed the "skin battery" [5]. Cutaneous wounds generate large and persistent endogenous electric currents and fields named the "current of injury" [5,6]. The current of injury is involved in numerous wound healing processes [7,8]. These observations support the concept ~~have led to the hypothesis~~ that applied electrical stimulation (ES) could promote wound healing by imitating the natural electrical current that occurs in cutaneous wounds ~~[8]~~. Human studies have elucidated that ES affects all the stages of wound healing ~~and its mechanism of action is likely multifactorial~~ [9,10] and specific mechanisms include ~~Based on human in vitro, ex vivo and in vivo studies, the specific modes of action include:~~ anti-bacterial effects [11], down regulation of inflammation [12], increase of tissue oxygenation [13], wound blood flow [14], haemoglobin levels [14], angiogenesis [9,15], fibroblast proliferation [16] and galvanotaxis-electrotaxis [17,18]. ES also affects collagen synthesis and wound strength ~~impacts the remodelling phase of wound healing via collagen effects (up-regulation of mature collagen synthesis and increased collagen production) leading to accelerated wound contraction and greater wound tensile strength~~ [12].

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Evidence indicates that bacteria in chronic wounds reside as highly antibiotic resistant polymicrobial biofilms rather than in their planktonic state [19-23]. Culture based techniques have significantly underestimated the number and abundance of microbial species present in these wounds [24]. Culture independent sequencing techniques have allowed more accurate determination of microbial taxa and their relative abundance in cutaneous wounds allowing a greater understanding of the host-microbial interface [25].

This comprehensive review introduces the concepts of wound biofilm and the microbiome and how these are shaping our current knowledge of cutaneous wounds. It further details the *in vitro* and *in vivo* evidence supporting the anti-microbial effects of ES on planktonic bacteria, biofilms and the microbiome. Finally, we discuss potential future perspectives in light of the above in the field of cutaneous wound infection, healing and ES.

Cutaneous wound biofilms and microbiome

Biofilms

Acute wound infections are generally as a result of planktonic bacteria that undergo a four phase growth cycle consisting of a lag, log, stationary and death phase. However, it is the presence of microbial biofilms that are fundamental in impairing cutaneous wound healing [26-28]. In fact, 60% of chronic wounds exhibit biofilms [29]. Biofilms are defined as complex microbial communities embedded in a protective self-produced biopolymer matrix, which provides protection against antimicrobial agents and host defense mechanisms [30-32]. The ~~biofilm~~ matrix is composed of an extracellular polymeric substance, which includes bacterial carbohydrates, proteins, lipids and extracellular DNA [33,34]. This structure segregates microbes from the external environment. The components of the ~~biofilm~~ matrix provide stabilisation [35], adhesive properties [36], nutrition, hydration [37], integrity [38] and antimicrobial effects [39]. Bacteria within the biofilm communicate through quorum sensing. Quorum sensing is a cell-to-cell communication method in which bacterial cells synthesise and react to small signalling molecules. This communication coordinates the biofilm architecture, enzyme production, microbial growth rates, toxin production, species interactions, bacterial virulence and antimicrobial resistance [40-45]. Biofilm formation involves reversible followed by irreversible attachment and binding of microbes to the wound. These bacteria produce and secrete the extracellular polymeric substance, which encases the microbial colonies. As the biofilm grows and matures focal areas are freed allowing spread and further colonisation [40,46-48].

The two main propositions suggesting the pathogenic role of wound biofilms are the specific and non-specific bacteria hypothesis. There are currently two main hypotheses regarding the pathogenic role of wound biofilms [49]. The former implies specific bacteria hypothesis suggests that only a few species of bacteria within the heterogeneous polymicrobial biofilm are involved in the infectious process; whereas conversely, the non-specific bacteria hypothesis latter considers the bacterial composition of biofilm as a whole to constitute a functional unit, and does not examine the role of individual pathogenic bacteria alone. This postulates that certain bacterial species that usually behave in a non-pathogenic manner, or at least are not capable of maintaining a chronic infection when present on their own, may co-aggregate symbiotically in a pathogenic biofilm and act synergistically to cause an infection [50].

Skin microbiome

Microbes outnumber our own cells by 10 fold and the human microbiome is 100 times larger than the human genome [51]. The human cutaneous microbiome refers to the entire collection of microorganisms (bacteria, archaea, fungi, viruses, and mites) that occupy our skin. It was first defined by Ledeberg as the "ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" [52]. The four most dominant bacterial phyla are *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. The distribution of these four phyla varies significantly from tissue to tissue with *Proteobacteria* and *Actinobacteria* abundant in the skin [53]. In total at least 19 phyla are known to be part of the skin bacterial microbiome. In 2007 the National Institute of Health in the USA initiated the Human Microbiome Project. The objective was to survey and identify the microbial

diversity that resides at different body sites, including the skin [25,54]. This influential project from 18 body sites in over 240 healthy individuals was completed in 2012 and has revealed the complex nature of the human microbial inhabitants and the incredible amount of spatial, temporal and individual variations [55]. Limited information is available regarding the skin mycobiome and virome. *Malassezia spp.* have been identified as the most abundant fungal genus in healthy skin [56]. *Propionibacterium* and *Staphylococcus* phages, human papillomaviruses, and Merkel cell polyomaviruses have been identified as common viral skin inhabitants [57-59].

Culture based methods are frequently used to isolate, identify, and study skin microbes. The sensitivity of these methods is limited ~~thus whereby~~ data using them underestimate the diversity of bacteria present [24]. Limitations of culture techniques include inability to reproduce *in vivo* conditions in the provided *in vitro* environment, and the overgrowth of rapidly dividing non-fastidious bacteria in the *in vitro* environment. Therefore, when using culture-based methods, fastidious difficult-to-grow anaerobes are often under-reported and easily cultured bacteria such as *Staphylococcus spp.* over-reported [60]. It is estimated that ~~more than~~up to 60% of bacterial species in the human microbiome are not culturable using standard methods highlighting the need for better alternative detection and identification methods [54].

Culture independent genomic sequencing techniques employed for the examination of the entire skin microbiome revolutionised our understanding of its complexity. It also highlighted the significant risk for experimental bias when using less sensitive methods [61]. For bacterial microbiome identification, these methods utilise the 16S

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ribosomal RNA (rRNA) gene, which is present in the genome of all bacteria but not in eukaryotes and amplify it by polymerase chain reaction (PCR) [62]. The 16S rRNA gene is ideal as it is present in all bacteria and it contains both conserved and hyper-variable regions, which allows binding sites for PCR primers and taxonomic classification [63-66]. This provides a more robust approach in terms of identification and relative quantity of bacteria present compared to culture based techniques [67-71]. Mycobiome identification involves DNA sequencing based on the fungal 18S rDNA and internal transcribed spacer (ITS). As with 16S, 18S rDNA has conserved and variable regions, which allow species identification. ITS allow fungal identification at the species or subspecies level [72].

Initial culture independent methods employed Sanger sequencing which has been the standard method used to sequence the human genome [73]. The advantage of Sanger sequencing is that it allows sequencing the full length of the 16S rRNA or the 18S gene. However, it has proven to be expensive and time consuming with low throughput to completely explore the diversity of the human microbiome. Next generation sequencing platforms provide more precise identification of the microbiota and cost effectiveness. These platform families include 454 [74], Illumina [75], SOLiD, Ion Torrent [76] and PacBio [65].

The first step for all microbiological diagnostics is sampling and the quality of the report depends on the quality of this step. Sampling is a significant dilemma and potential cause for bias also in microbiome research. Sampling types used range from superficial skin swabs, curettage, debridement and biopsies. Superficial skin swabs may not catch the full diversity of bacteria found in deeper layers [67].

Curettage and debridement includes mostly non-viable tissue and will give an inaccurate picture of the more superficial microbiota. Skin biopsies would seem to provide the optimum representation of the skin microbiome. However, studies have shown comparable level of wound microbial burden between the non-invasive and invasive sampling techniques [67,77], which is promising as biopsies are not always feasible and can be traumatic to the patient.

The culture independent approaches described above are not without limitations. They are not able to distinguish between live and dead organisms, they can be costly and time consuming and although they provide a thorough taxonomic classification of microbial species present they provide no information on function. Shotgun metagenomic sequencing allows examination of both the taxonomy and functionality of the microbiome [78,79]. It involves a minimal need for DNA amplification, which provides greater accuracy in determining the relative proportions of each microbe. It has provided a deep insight into the functional role of microbes in the gut [80]. However the small amount of DNA provided by skin sampling and the lack of reference genome sequences limits its current use in skin microbiome studies.

Cutaneous wound microbiome

Next generation sequencing has enabled characterisation of the microbiome based on three dimensions which are important in understanding the role of microbes on wound outcomes: total microbial load, microbial diversity, and presence of pathogenic organisms [81]. Over 60 different bacterial genera have been identified to

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be present in chronic wounds (table 1) [69,82-84]. No studies as of yet have characterised the cutaneous wound mycobiome or virome.

The four dominant phylotypes identified in chronic wounds are *Staphylococcus*, *Corynebacterium*, *Clostridiales* and *Pseudomonas* [85] (figure 1). In a recent large study of over 2000 wounds, Wolcott et al reported a high proportion of *Staphylococcus species* in wounds of differing aetiologies [86]. In line with this, Han et al found varying amounts of *Staphylococci* in almost all wounds they studied [87]. Dowd et al identified *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Fingoldia*, and *Serratia spp.* as common findings in pooled diabetic foot, venous leg and pressure wound samples [68]. A more recent study by the same group focused on the bacterial microbiome of diabetic foot wounds and *Corynebacterium spp.* was found to be the most prevalent bacterial genus although this was not present in all samples [50]. They also showed that anaerobes including *Bacteroides*, *Peptoniphilus*, *Fingoldia*, *Anaerococcus*, and *Peptostreptococcus spp.* are present in diabetic foot wounds. This has been confirmed in a number of other studies since [69,86]. Price et al identified a mean of 10 different bacterial families present in chronic wounds with anaerobic *Clostridiales family XI* amongst the most prevalent bacteria [88].

Due to methodological limitations the bacterial load and diversity was underestimated in some of the earlier studies [50,68]. In a more recent study by Gontcharova et al, the skin microbiome was shown to be significantly more diverse compared to wounded skin [89]. However, they also identified anaerobic bacteria, *Corynebacterium* and *Staphylococcus* the most prevalent species in wounds.

Gardner et al studied neuropathic diabetic foot wounds and noted that the wound microbiome profile was dependent on ulcer depth and duration [90]. Deeper wounds and those of a longer duration had a greater microbial diversity and a higher relative abundance of anaerobic bacteria and gram-negative *Proteobacteria* than those of shorter duration whereas shallower wounds contained a greater abundance of *Staphylococci* [90]. Tuttle et al showed a higher bacterial abundance and diversity in chronic wounds that failed to heal in 6 months compared to similar aetiological wounds that healed completely [91]. They also found that the proportion of *Actinomycetales* was increased in wounds that had not healed, and that of *Pseudomonadaceae* was increased in wounds that healed.

Horton et al identified an association between certain skin microbiomes and skin abscess formation [92]. They showed that the peri-abscess skin sample microbiome was similar to that of the contralateral skin samples but ~~different~~ differed from control patients. This highlights the potential role of the individual's skin microbiome in determining risk of developing skin disorders.

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Antimicrobial effects of electrical stimulation

Mechanism of action of electrical stimulation

ES has a bacteriostatic and bactericidal effect. However, the exact mechanism still remains unknown with both direct and indirect mechanisms suggested (figure 2). Liu et al proposed ES may directly disrupt the integrity of the bacterial membrane [93].

The exact mechanism of bacterial membrane disruption is unknown; however, one suggestion is that ES produced electrons repeatedly exciting the bacterial cell membranes cause eventual leakage of cellular constituents [94]. There are many indirect mechanisms, which have been proposed which include ~~production of substances secondary to electrolysis,~~ pH changes, temperature variations and ~~galvanotaxis~~electrotaxis. ~~ES leads to the production of gas and toxic substances such as hydrogen peroxide and chlorine secondary to electrolysis, which may explain its indirect antibacterial effect [94-97]. However, it seems unlikely to be a predominant factor in bacterial inhibition as ES without the production of these substances still demonstrates antimicrobial effects [98] and neutralisation of these bi-products would be expected in human wounds [95].~~ The acidity and alkalinity is pole dependent, with low pH at the anode and high pH at the cathode [95,96]. The ES modality also seems to be important with significant alterations in pH noted with DC compared to HV~~M~~PC [95]. Although pH changes secondary to ES seem to effect bacterial growth, their transient nature makes it unlikely to be the major mechanism of bacterial inhibition [97]. Temperature variations have been found to be minimal during the application of ES and this seems unlikely to affect bacterial viability [97]. Galvanotaxis-Electrotaxis occurs secondary to ES [98] and we agree with Asadi and Torkaman [11] that this migratory flow of cells may include attraction of antimicrobial

cells to the wound, which may explain the indirect antimicrobial activity of ES. One of the key processes of biofilm formation includes attachment and adherence which have been shown to be reduced with ES [99]. More recent findings suggest ES may affect gene expression with repression of quorum sensing genes identified following the application of ES [100]. The above evidence proposes both direct and indirect antimicrobial mechanisms of ES with direct mechanisms appearing more predominant [7,11].

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Planktonic

In vitro

Various ES modalities including direct current (DC), ~~low intensity direct current (LIDC)~~, alternating current (AC), high voltage monophasic pulsed current (HVMPC) ~~and~~, low voltage monophasic pulsed current (LVMPC) ~~and low voltage biphasic pulsed current (LVBPC)~~ have been studied *in vitro* with respect to their antimicrobial effects on planktonic bacteria (~~figure 3~~; table 2). These have varied in protocols with regards to current type, intensity, duration and polarity. The majority of studies involved initial growth of the microorganisms in various broths followed by spread of the suspension into specifically prepared agar media-filled (solidified agarose, nutrient, Mueller-Hinton and eosin methylene blue) petri plates containing the electrodes for ES. There have been a few exceptions where bacteria were grown on cotton patches or specially designed Teflon coupons.

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Barranco et al investigated the effect of DC delivered at different intensities in a petri dish (0.4, 4, 40 or 400 μ A) using four different types of electrodes (silver, gold, platinum and stainless steel) on *S. aureus* [101]. Overall findings suggested the greater the intensity of current, the greater the antibacterial effect. However, the increased current intensity was associated with gas formation, electrode corrosion and significant pH shifts. The use of silver positive polarity electrode was far superior at lower currents and at this intensity had negligible detrimental effects [101]. The benefits of silver electrode use were supported by Spadaro et al who showed a significant bacteriostatic effect on four bacterial species when used as the anode at low current intensity [102]. The beneficial antimicrobial effects of silver are well documented [103] and electrically stimulated release of silver ions has been shown to significantly improve this effect [103-105].

A weak DC of 100 μ A delivered via a silver anode electrode had a bactericidal effect on gram negative bacteria and a bacteriostatic effect on gram positive micro-organisms [94]. On the contrary the same group had earlier found the opposite [106]. Liu et al found even weaker DC (10 μ A) had a bactericidal effect at the cathode on *S. epidermidis* and *S. aureus* [93]. The effects of ES on *Candida albicans* were investigated by Karba et al [107]. They applied $\pm$ DC at 0.2-1 mA for between 2 to 18 hours using either the electrodes directly immersed in the culture medium or over agar bridges. They found that the inhibitory action of the ES was proportional to the magnitude and application time.

Rowley was the first to investigate the antibacterial effects of AC and DC [108]. He found growth rates of *Escherichia coli* were affected very little or not at all by AC while a significant bacteriostatic effect occurred with DC. Interestingly Petrofsky et al investigated the effects of AC and DC ES on *S. aureus*, *E. coli* and *P. aeruginosa* and showed AC had a positive antibacterial effect over DC stimulation against *P. aeruginosa* [109]. They applied AC at 5 and 20 mA for 30 minutes and showed a significant growth reduction of *P. aeruginosa*. DC stimulation at 100 μ A over the same time period had no bacteriostatic effect. In contrast Maadi et al ~~who~~ found AC had no inhibitory effect on growth of *P. aeruginosa* [110].

Daeschlein et al investigated the inhibitory effect of LVMPC on common gram positive and negative pathogens of chronic wounds [111]. They found a significant decrease in all microorganisms irrespective of polarity when compared to controls. However they did find, as did Barranco et al [101], that positive polarity has a higher antibacterial effect than negative polarity [111].

Merriman et al investigated the antimicrobial effect of four different types of ES stimulation current [95]. They found application of microampere direct current and HVMP had a time and polarity independent effect on bacterial growth. Whereas low voltage monophasic and biphasic pulsed current had no antibacterial effect. Guffey and Asmussen found a difference between the antimicrobial effects of HVMP and DC [112]. They applied HVMP at 50 to 800 mA and 100 pulses per second at a maximum 160 V for 30 minutes and showed it had no effect on the levels of *S. aureus*, while DC applied at 1, 5 and 10 mA inhibited its growth. In contrast Kincaid

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and Lavoie showed HVMPC (150-350 V, 1-4 hour duration and 120 pulses per second) had an inhibitory effect against *S. aureus*, *E. coli* and *P. aeruginosa*, which was dose and exposure duration dependent [113]. However they used a higher voltage intensity and longer duration compared to Guffey and Asmussen [112]. Similarly Szuminsky et al found HVMPC applied at 500 V for 30 minutes at 120 pulses per second had bactericidal effects against *S. aureus*, *E. coli*, *Klebsiella sp.* and *P. aeruginosa* at both polarities [97]. These observations suggest the effect of HVMPC may be more dependent on intensity rather than treatment duration. It is important to note that extrapolation of these findings to human subjects needs to be considered cautiously as these parameters of ES delivery would not be clinically possible. Gomes et al were the first to investigate the effect of fixed diphasic – Bernard current on bacterial inhibition [114]. Cultures of *S. aureus*, *P. aeruginosa* and *E. coli* were stimulated with either fixed diphasic – Bernard current at 3, 6 and 9 mA for 15 and 30 minutes, or HVMPC at 32, 64 and 95 V for 30 and 60 minutes. There was a decrease in bacterial counts for the two current types, but the most effective reduction was in fixed diphasic – Bernard where there was no pole dependent effect. However, the different currents, the different application times and stimulation intensities produced results indicating a time dependent or a voltage dependent response.

In vivo

There is limited data on the antimicrobial effects of ES on planktonic bacteria *in vivo* than *in vitro* (table 3). ES must be charge-balanced in order to be safe for tissue health. Wolcott et al, were the first to investigate this *in vivo* [115]. They applied

negative polarity ~~LD~~DC to chronic human wounds colonised with *Pseudomonas* and *Proteus* species and observed pathogen free wounds within several days. Rowley et al found that application of negative polarity DC at 1 mA for 3 days to rabbit wounds infected with *P. aeruginosa* had a bacteriostatic effect [116]. Bolton et al noted DC stimulation of intact human skin at varying current intensities ranging from 0-100 μ A led to bactericidal effects at the anode with no effect noted at the negative electrode [96]. They found that the longer the duration of stimulation and the higher the current density the greater the degree of antimicrobial effect. The antimicrobial effect of electrically stimulated silver has been investigated only once *in vivo*. Chu et al showed an enhancing antimicrobial effect of DC stimulated silver nylon dressings [117].

Biofilm

In vitro

Only two studies have assessed the effect of an electric wound dressing on biofilm [100,118] (table 4). The electric dressing utilised was Procellera™ (Vomaris Innovations, Inc) which is a single layer dressing consisting of a matrix of alternating silver and zinc that are held in position on a polyester substrate. The dressing system is unlike the other modalities described and is activated in the presence of a conductive fluid and produces a DC voltage of 0.5-0.9 V. The electric field produced is topographical, uncontrollable and potentially provides an unbalanced ion flux.

Banerjee et al successfully showed biofilm disruption in *P. aeruginosa* biofilm within 24 hours of dressing application with the use of imaging and gene expression studies [100]. Kim et al applied the dressing to a variety of antibiotic sensitive and

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multi-drug resistant bacterial strains and found a bactericidal effect, except in the case of *Enterococcus* species, where a bacteriostatic effect was noted [118].

Ten studies have investigated the bioelectric effect, which is the phenomenon whereby ES increases the efficacy of antibiotics. All except the study by Caubet et al [119] who utilised both DC and an alternating radio frequency electric current, assessed the bioelectric effect of DC [120-128]. Bacterial biofilms of multiple species were assessed including *E. coli* [119], *P. aeruginosa* [120,121,123,124,127], *S. epidermidis* [121,122,125], *S. aureus* [121,128], *Streptococcus gordonii* [126] and *Klebsiella Pneumoniae* [127]. The majority of studies found an enhancing effect of ES on the efficacy of antibiotics against the biofilms [119,120,122,123,126-128]. However, Del Pozo et al found varying degrees of antibiotic enhancement by ES across different bacteria and antibiotics and concluded that the bioelectric effect is not generalizable across all microorganisms and antimicrobial agents [121]. Jass et al found the ES enhancement was antibiotic dependent [124] and Sandvik et al found no additional benefit to biofilm disruption with the addition of antibiotics to ES biofilms [125]. The relationship between current intensity, biofilm disruption and the bioelectric effect has also been investigated. Del Pozo et al found a direct relationship between current intensity and antimicrobial effect, however, this direct relationship was not evident with the addition of various antibiotic agents [121]. On the contrary, Haddad et al found a greater electrical current intensity increased the effectiveness of Vancomycin against *S. epidermidis* biofilm, although they only investigated one bacterial species and one antibiotic [122]. Supporting Del Pozo et al, both Jass et al [123] and Sandvik et al [125] found no benefit of increased current intensity on the enhancement of the bioelectric effect.

The materials on which the biofilms are formed vary widely between the studies. Metals have been commonly used such as titanium [122,129-131], steel [120,132,133] and platinum [134] to resemble prosthetic models. Other materials used include a dialysis membrane [123,124], Teflon [121,135,136], polyvinyl chloride urinary catheter model [137], carbon fabric conductive scaffold [138], polycarbonate [100,125-127], glass [119] and a composite consisting of synthetic hydroxyapatite and zinc oxide [139].

In vivo

The investigation into the effect of ES on biofilms is sparse. Only 6 *in vivo* studies have assessed the effect of ES on biofilms with the majority showing positive outcomes (table 5). Although none of the studies to date have directly investigated the effect of ES on cutaneous wound bacterial biofilms, the outcomes presented below are promising and potentially translatable.

A study in goats assessing the effect of 100 μ A of DC ES applied to metal pins inoculated with *S. epidermidis* inserted into the tibia showed a marked reduction in clinical signs of infection compared to controls. However, sample numbers were relatively low and there were no techniques used to quantify the biofilm [140]. A similar study was conducted by Del Pozo et al where they applied a higher DC current (200 μ A) to *S. epidermidis* inoculated stainless steel implanted into the tibia of rabbits [141]. They had a second treatment group which received intravenous doxycycline as well as a third control group for comparison. Bacterial load quantification showed a significant reduction in counts for both ES and doxycycline

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treated rabbits compared to controls and also found ES treatment was significantly more efficacious than antibiotic treatment [141]. Post inoculation incubation time is an important factor in biofilm development and in the latter study [141] treatment did not commence until after 4 weeks post inoculation allowing time for the biofilm to develop, however, there was no imaging available to confirm biofilm formation. Conversely, Paryavi et al showed no benefit of DC ES in reducing bacterial load compared to control in an instrumented rabbit model [142]. However, they did investigate a different microbe (*S. aureus*) and used a lower intensity current (60 μ A).

Using a capacitive coupling ES device, Gilotra et al evaluated the bioelectric effect in a rabbit spine infection model [143]. Rabbits with spinal rods implanted were infected with *S. aureus* following a single dose of intravenous antibiotics. After 7 days of ES, instrumentation related infection was significantly reduced compared to controls (36% vs. 81%), although there was no difference in soft tissue infection burden and total bacterial load [143]. Ehrensberger et al found application of cathodic voltage-controlled ES at 1.8 V to titanium for 1 hour implanted into a rodent model with preformed methicillin-resistant *S. aureus* biofilm led to a significant reduction in bacterial counts both on the implant and the surrounding bone tissue [131]. Using a similar rodent model, the same group found ES enhanced the activity of vancomycin in reducing the implant bacterial burden but had no benefit in reducing bone bacterial burden compared to antibiotic treatment alone [144].

Microbiome*In vitro*

There are no studies to date investigating the effect of ES on the microbiome *in vitro*.

In vivo

There are no studies to date investigating the effect of ES on the microbiome *in vivo*.

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Future perspective

Cutaneous wound infections play a pivotal role in delayed wound healing. The growing need for non-pharmacological management of such infections is growing with the increasing rate of anti-microbial resistance. ES has the potential to bridge this current predicament.

The majority of studies presented above utilise ES in the *in vitro* environment in both planktonic and biofilm bacterial states. Although studies on planktonic bacteria are helpful in establishing potential ES mechanism of action, their translatability to biofilm and clinical use is very limited. Also the varying use of different ES modalities and parameters also provide no consensus yet as to the best ES device for bacterial management. The use of ES in inhibiting bacterial biofilms is far more clinically relevant. Although there is a good body of *in vitro* studies developing, only a couple of studies have assessed the impact of a clinically useable electric dressing [100,118]. Both these studies have shown promising results with regards to biofilm disruption. However, the ES Procellera™ delivers to the wound is not controllable or quantifiable. Also, as with planktonic bacterial models, the varying use of different ES parameters and study protocols makes concluding which is the best form of ES in the management of biofilms currently difficult.

Although *in vivo* studies on planktonic bacteria are useful and have shown positive outcomes, we feel the role of ES is more clinically suited to the management of chronic highly resistant wound biofilms. There are only a handful of *in vivo* studies assessing the impact of ES on bacterial biofilms. These studies have shown promising results, however, the majority of studies were assessing the effect of ES

on biofilm formed on metallic implants. None of the studies were directly assessing the effect of ES on cutaneous wound biofilms and with the delicate nature of human cutaneous wounds, these findings are unlikely to be directly translatable.

The use of ES in the management of cutaneous wound biofilms may not be clinically successful when used in isolation. This possibility makes the bioelectric effect of significant interest. Both *in vitro* and *in vivo* studies have shown in the majority of cases an enhancing effect of ES on antibiotic efficacy against biofilms. However, again only a limited number of bacterial species and antibiotic agents have been investigated and all in non-cutaneous wound models.

The majority of studies to date have assessed the antimicrobial efficacy of ES against single species biofilms and easily culturable species. However, microbes reside in chronic wounds in polymicrobial biofilms and culture independent techniques have allowed the identification of a wider microbial community residing in chronic wounds. No studies to date have assessed the impact of ES on the microbiome profile. Next generation sequencing techniques are providing a greater insight not only into the diversity of microbes that reside in cutaneous wounds but also the functional potential of the microbial community. There is currently a need to investigate the effect of ES on the microbiome to provide a deeper insight into its role in the management of infections and wound healing.

ES has the potential to be an effective antimicrobial tool specifically against microbes that may delay cutaneous wound healing either used in isolation or as an adjunct to antibiotics and we agree with Korzendorfer and Hettrick that current evidence is

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limited and lacking and relevant clinical studies are needed [145]. In order to investigate this, revolutionary studies over the next decade are needed focusing on key specific areas. Firstly, standardised protocols are necessary to develop robust evidence supporting the use of ES in cutaneous wound management. Secondly, clinically relevant biofilm models are necessary which would have more translatability to clinical practice. Finally, the use of next generation sequencing techniques to identify how ES modifies the wound microbiome to enhance wound healing is a vital and currently unexplored area.

There is currently a disconnect between cutaneous wound infections, biofilms, microbiome and ES. Future studies combining the fields of ES, biofilm and microbiome research are necessary and an exciting prospect to fully elucidate the use of ES in the management of cutaneous wounds.

Executive summary

Introduction

- Chronic cutaneous wounds have a major impact on patients' quality of life and mobility and present a huge burden on the healthcare system.
- Due to major challenges in current management of cutaneous wound infections, new non-pharmacological approaches are urgently required.
- ES could promote cutaneous wound healing by imitating the natural electrical current that occurs in wounds.

Cutaneous wound biofilms and skin microbiome

- Bacteria in chronic wounds reside as highly antibiotic resistant polymicrobial biofilms, which are fundamental in impairing cutaneous wound healing.
- Culture based methods are frequently used to isolate, identify, and study skin microbes. The sensitivity of these methods is limited whereby data using them underestimate the diversity of bacteria present.
- Culture independent sequencing techniques have allowed comprehensive identification of the cutaneous wound microbiome.

Antimicrobial effects of electrical stimulation

- The majority of studies have investigated with positive outcomes the use of ES *in vitro* in regards to both planktonic and biofilm bacterial states.
- There is a lack of high quality *in vivo* data specifically related to cutaneous wound infection.
- There are currently no studies investigating the ability of ES to alter the microbiome both *in vitro* and *in vivo*.

Future perspective

- Current evidence suggests ES is a successful anti-microbial treatment.

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- However, there is currently a disconnect between cutaneous wound infections, biofilms, microbiome and ES.
- Future studies combining the fields of ES, biofilm and microbiome research are necessary and an exciting prospect to fully elucidate the use of ES in the management of cutaneous wounds.

For Review Only

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* Review of next generation sequencing techniques critical to wound microbiome research.

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Table 1. Studies using next generation sequencing technology to analyse microbiome of chronic wounds

Reference	Wound type and location	Sampling method	Microbial identification methods	Principle findings
[50]	Diabetic	Debridement	bTEFAP	Most prevalent bacterial genus was <i>Corynebacterium spp.</i>
[68]	Diabetic	Debridement	Culture methods	Each wound type revealed marked differences
	Pressure			
	Venous		PRAPS	in bacterial populations
			FRACS	
[69]	Diabetic	Debridement	Culture methods	145 genera identified with molecular methods compared to only 14 identified by culture techniques
	Post-surgical			
	Pressure		Pyrosequencing (454 Roche)	
	Venous			
[82]	Venous	Debridement	bTEFAP and bTEFAP titanium	Predominant organisms include <i>Bacteroidales</i> , various anaerobes, <i>Staphylococcus</i> , <i>Corynebacterium</i> and <i>Serratia</i>
[83]	Pressure	Debridement	bTEFAP	Polymicrobial in nature with no single bacterium exclusively colonising the wounds

[84]	Neuropathic	Curettage	Pyrosequencing	58 bacterial families and 91 bacterial
	Other		(Roche 454)	genera characterised
	Post-surgical			
	Pressure			
[85]	Venous			
	Arterial	Curettage	Culture methods	Individual wounds contained 4-22
	Diabetic			phylotypes
	Scalp	Surface	MegaBACE 1000 DNA	
[86]	Venous	swabbing	sequencer	Different diversity and dominance
				information given between molecular
			ABI3730 DNA	and culture methods
			sequencer	
[87]	Diabetic	Debridement	Pyrosequencing (454	High proportion of <i>Staphylococcus</i>
	Post-surgical		Roche)	and
	Pressure			<i>Pseudomonas spp.</i>
	Venous			
[87]	Arterial	Biopsy	Culture methods	High prevalence
	Diabetic			of anaerobic bacteria and bacteria
	Other	Curettage	Pyrosequencing (454	traditionally considered commensal
	Pressure		Roche)	
[87]	Traumatic			Pyrosequencing revealed increased
	Venous			bacterial diversity with an average of
				17 genera in each wound

[88]	Diabetic	Curettage	Culture methods	Gene analyses revealed
	Post-surgical			approximately 4 fold more bacterial
	Pressure		Pyrosequencing (454	families in wounds than estimated by
	Venous		Roche)	culture
[89]	Diabetic	Debridement	bTEFAP	Intact skin significantly more diverse
	Normal skin			than wounds
		Surface		
		swabbing		Wounds show heightened levels of
				anaerobic bacteria
[90]	Diabetic	Surface	Culture methods	Wound depth positively correlated
		swabbing		with abundance of anaerobic
			Pyrosequencing (454	bacteria, and negatively correlated
			Roche)	with abundance of <i>Staphylococcus</i>
[91]	Venous	Debridement	Culture methods	Significantly higher bacterial
				abundance and diversity in wounds
			Ibis T5000 universal	that had not healed
			biosensor	
				<i>Actinomycetales</i> was increased in
			bTEFAP and bTEFAP	wounds that had not healed
			titanium	
				<i>Pseudomonadaceae</i> was increased
				in wounds that had healed

bTEFAP - bacterial tag encoded FLX amplicon pyrosequencing; PRAPS - partial ribosomal amplification and pyrosequencing; FRACS - full ribosomal amplification, cloning and Sanger sequencing; PRADS – partial ribosomal amplification, density gradient gel electrophoresis and Sanger sequencing.

Table 2. Planktonic *in vitro* studies

Reference	ES	Pathogens	Treatment Parameters	Principal findings
[93]	DC	<i>S. epidermidis</i>	CI: 10-100 μ A	Antimicrobial effect at low CI around
		<i>S. aureus</i>	<u>Voltage: 9 V</u>	cathode
			<u>Duration: 16 h</u>	
			P: anode and cathode	
			ET: carbon	
[101]	DC	<i>S. aureus</i>	CI: 0.4-400 μ A	CI directly proportional to
			<u>Voltage: 0.4-9.3 V</u>	bacteriostatic effect
			<u>Duration: 24-48 h</u>	
			P: anode	
			ET: silver, gold, platinum	
			and stainless steel	

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[95] DC *S. aureus* CI: 500 μ A (DC); Antibacterial effect with DC and
HVPC 30 mA (LV MPC + LVBPC); HV MPC irrespective of polarity
LV MPC NS/A (HV MPC)
LVBPC Voltage: 0-102 V (DC);
HV MPC NS/A (LV MPC + LVBPC);
250 V (HV MPC);
Duration: 1 h/d for 3 d
P: anode and cathode
ET: stainless steel
PW: 120 μ s (LV MPC +
LVBPC); NS/A spaced 70
 μ s apart (HV MPC)
IPI: 7.69 ms (LV MPC); 7.47
ms (LVBPC); 9.93 ms
(HV MPC)
PF: 128 pps (LV MPC +
LVBPC); 100 pps (HV MPC)

[97] HV MPC *S. aureus* CI: NS/A Antibacterial effects irrespective of
E. coli Voltage: 500 V polarity
Klebsiella Duration: 30 m
P. aeruginosa Voltage: 500 V
P: anode and cathode
ET: stainless steel
PW: 7 μ s spaced 70 μ s
apart
IPI: 8-9 ms
PF: 120 pps

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[104]	DC	<i>S. aureus</i>	CI: 0.4-400 μ A	Antibacterial effect at all ET at high
		<i>E. coli</i>	<u>Voltage: 0.086 - $\geq$ 9.5 V</u>	CI
		<i>P. vulgaris</i>	<u>Duration: 24 h</u>	
		<i>P. aeruginosa</i>	P: anode and cathode ET: silver, platinum, stainless steel, gold and copper	Only silver electrode used as anode had bacteriostatic effect at low CI
[94]	DC	Gram +ve	CI: 100 μ A	Bacteriostatic effect on gram +ve
		Gram -ve	<u>Voltage: NS/A</u>	
			<u>Duration: NS/A</u>	Bactericidal effect on gram -ve
			P: <u>a</u> Anode and cathode ET: silver	
[106]	DC	<i>S aureus</i>	CI: 26-800 μ A	Bacteriostatic effect on P.
		<i>P aeruginosa</i>	<u>Voltage: NS/A</u>	aeruginosa
			<u>Duration: NS/A</u>	
			P: <u>a</u> Anode ET: silver	Bactericidal effect on <i>S. aureus</i>
[107]	DC	<i>C. albicans</i>	CI: 200-1000 μ A	Antimicrobial effect proportional to
			<u>Voltage: NS/A</u>	CI and application time
			<u>Duration: 12-18 h</u>	
			<u>P: cathode</u> <u>ET: platinum-iridium alloy</u>	

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[108]	DC	<i>E. coli</i>	CI: 1-140 mA (DC);	DC showed bacteriostatic effect
	AC		15-30 mA (AC)	
			<u>Voltage: NS/A</u>	AC showed no effect
			<u>Duration: NS/A</u>	
			P: cathode	
[109]			ET: platinum	
			<u>PW: NS/A (AC)</u>	
			<u>PF: NS/A (AC)</u>	
	DC	<i>S. aureus</i>	CI: 100 µA (DC);	AC reduced growth of P.
	AC	<i>E. coli</i>	5-20 mA (AC)	aeruginosa
		<i>P. aeruginosa</i>	<u>Duration: 30 m</u>	
			<u>Voltage: NS/A</u>	
[110]			<u>P: NS/A</u>	
			<u>ET: carbonised rubber</u>	
			<u>PW: 250 µs (AC)</u>	
			<u>PF: 30 pps (AC)</u>	
	DC	<i>P. aeruginosa</i>	<u>CI: NS/A</u>	DC inhibited growth
	AC		Voltage: 1.5-10 V	
			<u>Duration: up to 19 h</u>	AC had no effect
			P: <u>an</u> ode and cathode	
			<u>ET: Stainless steel</u>	
			<u>PW: 20 ms</u>	
			<u>PF: 50 pps</u>	

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[111]	LVMP	<i>S. aureus</i>	CI: 42 mA	Antibacterial effect on all microorganisms irrespective of polarity
		<i>S. epidermidis</i>	<u>Voltage: NS/A</u>	
		<i>E. faecium</i>	<u>Duration: 30 m</u>	
		<i>E. coli</i>	P: anode and cathode	
		<i>P. aeruginosa</i>	ET: hydrogel	
		<i>Klebsiella</i>	<u>PW: 140 μs</u>	
[112]	DC HVMP	<i>S. aureus</i>	CI: 1-10 mA (DC); 50-800 mA (HVMP)	DC had antibacterial effect at all CI HVMP had no antibacterial effect
			<u>Voltage: NS/A (DC); <160 V (HVMP)</u>	
			<u>Duration: 30 m</u>	
			<u>P: anode and cathode</u>	
			<u>ET: NS/A</u>	
			<u>PW: NS/A</u>	
			<u>IPI: NS/A</u>	
			<u>PF: NS/A</u>	
[113]	HVMP	<i>S. aureus</i>	<u>CI: NS/A</u>	Antibacterial effect against all organisms
		<i>E. coli</i>	Voltage: 150-300 V	
		<i>P. aeruginosa</i>	<u>Duration: 1-4 h</u>	
			P: anode and cathode	
			ET: stainless steel	
			<u>PW: NS/A spaced 55 μs apart</u>	
			<u>IPI: NS/A</u>	
			<u>PF: 120 pps</u>	

[114]	FDBC	<i>S. aureus</i>	CI: 3-9 mA (FDBC); <u>NS/A</u>	FDBC had a more effective
	HV <u>M</u> PC	<i>P. aeruginosa</i>	<u>(HVMPC)</u>	reduction in bacterial counts
		<i>E. coli</i>	Voltage: <u>2.72-6.97 V²</u> irrespective of polarity	
			<u>(FDBC); 32-95 V (HVMPC)</u>	
			<u>Duration: 0-30 minutes</u>	
			<u>(FDBC); 0-1 h (HVMPC)</u>	
			P: <u>a</u> A node and cathode	
			<u>ET: stainless steel</u>	
			<u>PW: 10 ms (FDBC); 15 μs</u>	
			<u>(HVMPC)</u>	
			<u>IPI: NS/A</u>	
			<u>PF: 100 pps</u>	

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ES – electrical stimulation; DC – direct current; AC – alternating current; ~~LVPC – low voltage pulsed current~~; HVMPC – high voltage monophasic pulsed current; LVMPC – low voltage monophasic pulsed current; LVBPC – low voltage biphasic pulsed current; FDBC - fixed diphasic Bernard current; CI – current intensity; P – polarity; ET – electrode type; A – ampere; V – volt; PW – pulse width; IPI – inter pulse interval; PF – pulse frequency; pps – pulses per second; NS/A – not stated/available; -

Table 3. Planktonic *in vivo* studies

Reference	ES / model	Pathogens	Treatment Parameters	Principal findings
[96]	DC	<i>S. epidermidis</i>	CI: 10-100 μ A	Bactericidal effect at anode
	Human		<u>Voltage: 6-9 V</u>	
	(n=28)		<u>Duration: 4 or 18 h</u>	No effect at cathode
			P: anode and cathode	
[115]	DC	<i>P. aeruginosa</i>	CI: 200-800 μ A	Cathodal treated wounds
	Human	<i>Proteus</i>	<u>Voltage: NS/A</u>	pathogen free in few days
	(n=75)		<u>Duration: 6 h/d for 0.8-15.4 weeks</u>	
			P: anode and cathode	
			<u>ET: NS/A</u>	
[116]	DC	<i>P. aeruginosa</i>	CI: 1 mA	Bacteriostatic effect
	Rabbit		<u>Voltage: NS/A</u>	
	(n=140)		<u>Duration: 72 h</u>	
			P: cathode	
[117]	DC	<i>P. aeruginosa</i>	CI: 0.4-40 μ A	Anodal enhancing antimicrobial
	Rat		<u>Voltage: NS/A</u>	effect
	(n=360)		<u>Duration: 5 d</u>	
			P: anode and cathode	
			ET: silver	

ES – electrical stimulation; DC – direct current; CI – current intensity; P – polarity; ET – electrode

type; A – ampere; V – volts; NS/A – not stated/available.

Table 4. Biofilm *in vitro* studies

Reference	ES / model	Pathogens	Treatment Parameters	Principal findings
[125]	DC <u>Antibiotics</u> Polycarbonate	<i>S. epidermidis</i>	<u>CI: 2-5 mA</u>	Addition of ES to antibiotic treated biofilms had no significant benefit in reducing biofilm viability
			<u>Voltage: NS/A</u>	
			<u>Duration: 24 h</u>	
			<u>P: anode and cathode</u>	
			<u>ET: platinum</u> <u>Antibiotics</u>	
[121]	DC <u>Antibiotics</u> Teflon	<i>P. aeruginosa</i> <i>S. aureus</i> <i>S. epidermidis</i>	<u>CI: 0-2000 mA</u>	Bioelectric effect was not observed for all bacteria and antibiotic combinations
			<u>Voltage: NS/A</u>	
			<u>Duration: 24 h</u>	
			<u>P: anode and cathode</u>	
			<u>ET: stainless steel and graphite</u> <u>Antibiotics</u>	
[100]	Procellera™ Polycarbonate	<i>P. aeruginosa</i>	<u>CI: 2-10 µA</u>	Markedly disrupted biofilm integrity using SEM
			<u>Voltage: 0.3-0.9 V</u>	
			<u>Duration: 24 h</u>	
			<u>P: anode</u>	
			<u>ET: silver and zinc</u>	
				Reduced biofilm thickness and number of live bacteria
				Repressed quorum sensing genes

[118]	Procellera™	<i>A. baumannii</i>	<u>CI: 2-10 µA</u>	Procellera™ had a bactericidal
	Textile	<i>A.</i>	<u>Voltage: 0.3-0.9 V</u>	effect against all study organisms
	materials	<i>calcoaceticus</i>	<u>Duration: 24-48 h</u>	except Enterococcus species
		<i>E. faecalis</i>	<u>P: anode</u>	where a bacteriostatic effect was
		<i>E. coli</i>	<u>ET: silver and zinc</u>	noted
		<i>K. pneumoniae</i>		
		<i>P. aeruginosa</i>		
		<i>S. aureus</i>		
		<i>S. aureus</i>		
		(MRSA)		
		<i>S. epidermidis</i>		
		<i>E. Faecium</i>		
		<i>E. raffinosus</i>		
[119]	DC + RF	<i>E. coli</i>	<u>CI: 200 mA + 20 V</u>	Synergistic effect between ES
	<u>Antibiotics</u>		(DC);	and antibiotics
	Glass		150 mA + 10 MHz (RF)	
			<u>Antibiotics</u>	DC produces a more pronounced
			<u>Voltage: ~ 20 V (DC)</u>	bioelectric effect than RF
			<u>Duration: 24 h</u>	
			<u>P: NS/A (DC)</u>	
			<u>ET: stainless steel</u>	
			<u>PF: 10000000 pps (RF)</u>	
[120]	DC	<i>P. aeruginosa</i>	<u>CI: 50 mA</u>	Combined ES and antibiotic
	<u>Antibiotics</u>		<u>Voltage: 10V</u>	treatment was more successful
	Steel		<u>Antibiotics</u>	in reducing bacterial count
			<u>Duration: 24-48 h</u>	compared to either treatment
			<u>P: anode and cathode</u>	used in isolation
			<u>ET: stainless steel</u>	

[122]	DC	<i>S. epidermidis</i>	<u>CI: 22-333 µA</u>	Bioelectric effect was enhanced
	<u>Antibiotics</u>		<u>Voltage: 4 V</u>	with higher current intensity
	Titanium		<u>Antibiotics</u>	
			<u>Duration: 24 h</u>	
			<u>P: anode and cathode</u>	
			<u>ET: stainless steel</u>	
[123]	NS/A	<i>P. aeruginosa</i>	<u>CI: 0-20 mA/cm²</u>	ES alone had no effect on biofilm
	Dialysis		<u>Antibiotics</u>	formation
	membrane		<u>Voltage: NS/A</u>	
			<u>Duration: 12 h</u>	Addition of ES to antibiotic
			<u>P: NS/A</u>	treatment enhanced anti-
			<u>ET: NS/A</u>	microbial activity of antibiotic
[124]	NS/A	<i>P. aeruginosa</i>	<u>CI: 0-9 mA/cm²</u>	Addition of ES to antibiotic
	<u>Antibiotics</u>		<u>Antibiotics</u>	treatment did not enhance anti-
	Dialysis		<u>Voltage: NS/A</u>	microbial activity of all antibiotics
	membrane		<u>Duration: 12 h</u>	used
			<u>P: NS/A</u>	
			<u>ET: NS/A</u>	
[126]	DC	<i>S. gordonii</i>	<u>CI: 2 mA</u>	ES enhanced the antibiotic
	<u>Antibiotics</u>		<u>Antibiotics</u>	efficacy against biofilm formation
	polycarbonate		<u>Voltage: 1-8 V</u>	
			<u>Duration: 24 h</u>	
			<u>P: NS/A</u>	
			<u>ET:stainless steel</u>	

[127]	DC	<i>P. aeruginosa</i>	<u>CI: 0-10 mA</u>	Bioelectric effect against biofilm
	<u>Antibiotics</u>	<i>K. pneumoniae</i>	<u>Antibiotics</u>	formation noted
	polycarbonate		<u>Voltage: 4-7 V @ 1 mA</u>	
			<u>Duration: 24 h</u>	
			<u>P: anode and cathode</u>	
			<u>ET: platinum</u>	
[128]	DC	<i>S. aureus</i>	<u>CI: 200 µA</u>	ES significantly enhanced
	<u>Antibiotics</u>		<u>Antibiotics</u>	antibiotic killing efficacy of biofilm
	Polypyrrole/chitosan		<u>Voltage: NS/A</u>	
			<u>Duration: 4 h</u>	
			<u>P: anode and cathode</u>	
			<u>ET: copper</u>	
[129]	DC	<i>S. aureus</i>	<u>CI: 3645 A</u>	ES coupled with anodised
	Titanium		<u>Voltage: 20 V</u>	nanotubular titanium led to a
			<u>Duration: 6 m</u>	significant decrease in biofilm
			<u>P: anode and cathode</u>	formation compared to controls
			<u>ET: platinum and titanium</u>	
[130]	<u>LVBPC</u>	<i>P. aeruginosa</i>	<u>CI: NS/A</u>	Greatest effect of ES against
	Titanium		<u>Voltage: 0.5-5 V</u>	biofilm formation (50% reduction)
			<u>Duration: 6 d</u>	was seen with ES at 5 V and 200
			<u>P: anode and cathode</u>	Hz
			<u>ET: titanium</u>	
			<u>PW: NS/A</u>	
			<u>IPI: 0-0.5 s</u>	
			<u>PF: 200-10000 ppsHz</u>	

[131]	CVCES Titanium	<i>S. aureus</i> (MRSA)	<u>CI: NS/A</u> <u>Voltage: 1.8 V</u> <u>Duration: 1 h</u> <u>P: cathode</u> <u>ET: silver and graphite</u> <u>1.8 V</u>	92% reduction in bacterial count with the use of CVCES compared to control
[132]	DC + BC Steel	<i>S. epidermidis</i>	<u>CI: 60-100 µA (DC); 60 100 µA +1Hz (BC)</u> <u>Voltage: NS/A (DC + BC)</u> <u>Duration: 400 m</u> <u>P: anode and cathode</u> <u>ET: stainless steel and indium tin oxide</u> <u>PF: 1 pps</u>	Detachment of biofilm from steel is higher with the use of DC compared to BC
[133]	BC Steel	<i>S. epidermidis</i>	<u>CI: 15-100 µA</u> <u>Voltage: 1.5–1.7 V</u> <u>0.1-2 Hz</u> <u>Duration: 150 m</u> <u>P: anode and cathode</u> <u>ET: stainless steel and indium tin oxide</u> <u>PF: 0.1-2 pps</u>	Percentage of bacterial detachment from steel was frequency dependent

[134]	DC	<i>K. pneumoniae</i>	<u>CI: 50 μA</u>	Biofilm viability was polarity
	Platinum	<i>P. fluorescens</i>	<u>Voltage: 1.3 V</u>	dependent with anodic polarity
		<i>P. aeruginosa</i>	<u>Duration: NS/A</u>	reducing biofilm thickness by
			<u>P: anode and cathode</u>	26%
			<u>ET: platinum</u> <u>0.016-20</u> <u>Hz</u>	
[135]	DC	<i>S. epidermidis</i>	<u>CI: 2000 mA</u>	Reduced bacterial count with ES
	Teflon		<u>Voltage: NS/A</u>	application compared to controls
			<u>Duration: 4-24 h</u>	at 4 and 24 hours
			<u>P: anode and cathode</u>	
			<u>ET: stainless steel and</u> <u>graphite</u>	
[136]	DC	<i>S. epidermidis</i>	<u>CI: 200-500 μA</u>	ES effect on species is time
	Teflon	<i>P. aeruginosa</i>	<u>Voltage: NS/A</u>	dependent
		<i>E. coli</i>	<u>Duration: 4-48 h</u>	
		<i>P. acnes</i>	<u>P: anode and cathode</u>	ES was not effective in reducing
		<i>C. albicans</i>	<u>ET: platinum</u>	<i>P. acnes</i> biofilm
[137]	DC	<i>S. epidermidis</i>	<u>CI: 0-500 μA</u>	Biofilm reduction was time and
	Polyvinyl	<i>E. coli</i>	<u>Voltage: NS/A</u>	current intensity dependent
	chloride urinary	<i>P. aeruginosa</i>	<u>Duration: 24 h – 4 d</u>	
	catheter model	<i>C. parapsilosis</i>	<u>P: anode and cathode</u>	
			<u>ET: platinum</u>	
[138]	DC	<i>A. baumannii</i>	<u>CI: NS/A</u>	80% decrease in biofilm surface
	Carbon fabric		<u>Voltage: 400-800 mV</u>	coverage within 24 hours
	conductive		<u>Duration: 24 h</u>	
	scaffold		<u>P: cathode</u>	
			<u>ET: carbon and silver</u>	

[139]	DC	<i>S. aureus</i>	<u>CI: 0 A</u>	Time dependent decrease in
	HA-ZnO	(MRSA)	<u>Voltage: 1 V/cm</u>	bacterial viability and biofilm
			<u>Duration: 6-24 h</u>	formation
			<u>P: anode and cathode</u>	
			<u>ET: stainless steel</u>	

ES – electrical stimulation; V – volts; SEM – scanning electron microscopy; DC – direct current;

HAZno – Hydroxyapatite zinc oxide; RF – radio frequency; A – ampere; Hz – hertz; CVCES –

Cathodic voltage controlled electrical stimulation; NS/A – not stated/available/specified; ~~PC~~—~~pulsed~~
~~current~~; BC – block current; MRSA – methicillin resistant *staphylococcus aureus*. LVBPC – low
voltage biphasic pulsed current; CI – current intensity; P – polarity; ET – electrode type; PW – pulse
width; IPI – inter pulse interval; PF – pulse frequency; pps – pulses per second.

Table 5. Biofilm *in vivo* studies

Reference	ES / model	Pathogens	Treatment Parameters	Principal findings
[131]	CVCES	<i>S. aureus</i>	<u>CI: NS/A</u>	ES significantly reduced bacterial
	Rat <u>(n=16)</u>	(MRSA)	<u>Voltage: 1.8 V</u>	load by 87% in the bone and 98% in
			<u>Duration: 1 h</u>	the titanium implant
			<u>Polarity: cathode</u>	
[140]			<u>ET: silver and platinum</u>	
	DC	<i>S. epidermidis</i>	<u>400-μACI: 100 μA</u>	Implant infection rate reduced in ES
	Goat <u>(n=9)</u>		<u>Voltage: 9 V</u>	treated animals compared to
			<u>Duration: 24 h/d for 21 d</u>	controls (11% vs. 89%)
			<u>Polarity: anode and</u>	
			<u>cathode</u>	
[141]			<u>ET: stainless steel and</u>	
			<u>platinum</u>	
	DC	<i>S. epidermidis</i>	<u>200-mA</u>	Bacterial load significantly reduced
	<u>Antibiotics</u>		<u>6VCI: 200 μA</u>	in ES and antibiotic only groups
	Rabbit		<u>Voltage: 6 V</u>	compared to controls
	<u>(n=39)</u>		<u>Duration: Treatment</u>	
			<u>initiated 4 weeks after</u>	ES more efficacious than antibiotic
			<u>inoculation and</u>	treatment
			<u>administered for 21 d</u>	
			<u>Polarity: anode and</u>	
			<u>cathode</u>	
			<u>ET: stainless steel</u>	

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[142]	DC	<i>S. aureus</i>	<u>60 μA</u> <u>CI: 60 μA</u>	No significant reduction in biofilm
	Rabbit	(MRSA)	<u>Voltage: NS/A</u>	observed with ES compared to
		<u>(n=17)</u>	<u>Duration: 24 h/d for 7 d</u>	controls
			<u>Polarity: cathode</u>	
			<u>ET: stainless steel</u>	
[143]	AC	<i>S. aureus</i>	<u>CI: 6 mA (max 14 mA)</u>	Overall bacterial burden was not
	Rabbit		Antibiotics	reduced with ES
		<u>(n=30)</u>	<u>Voltage: NS/A</u>	
			<u>Duration: 24 h/d for 7 d</u>	
			<u>ET: - hydrogel</u>	
			<u>PW: NS/A</u>	
			<u>PF: NS/A</u>	
[144]	CVCES	<i>S. aureus</i>	<u>1.8 V</u>	ES had a significant bioelectric
	<u>Antibiotics</u>	(MRSA)	<u>AntibioticsCI: NS/A</u>	effect on implant bacterial burden
	Rat <u>(n=20)</u>		<u>Voltage: 1.8 V</u>	ES had no significant bioelectric
			<u>Duration: treatment</u>	effect on bone bacterial burden
			<u>initiated 1 week after</u>	
			<u>inoculation and</u>	
			<u>administered for 1 h</u>	
			<u>either on d7 or on d7 +</u>	
			<u>d21</u>	
			<u>Polarity: cathode</u>	
			<u>ET: titanium</u>	

ES – electrical stimulation; DC – direct current; A – ampere; V – volts; CVCES – Cathodic voltage controlled electrical stimulation; AC – alternating current; MRSA – methicillin resistant *staphylococcus aureus*. CI – current intensity; P – polarity; ET – electrode type; PW – pulse width; PF – pulse frequency; pps – pulses per second; NS/A – not stated/available;

Figure Legend

Figure 1. Common bacteria identified in chronic wounds by sequencing methods.

The commonest bacteria based on phylum, family, genus or species identified from 13 studies assessing the microbiome of chronic wounds using sequencing techniques.

Figure 2. Proposed antimicrobial mechanism of action of electrical stimulation.

Figure highlights the direct (bacterial membrane disruption and inhibit bacterial proliferation) and indirect (pH ~~and~~, temperature alterations, ~~production of toxic substances~~ and ~~galvanotaxis~~electrotaxis) antimicrobial effects of electrical stimulation.

~~**Figure 3.** Electrical stimulation waveforms.~~

Figure 1. Common bacteria identified in chronic wounds by sequencing methods. The commonest bacteria based on phylum, family, genus or species identified from 13 studies assessing the microbiome of chronic wounds using sequencing techniques.

254x190mm (96 x 96 DPI)

Figure 2. Proposed antimicrobial mechanism of action of electrical stimulation. Figure highlights the direct (bacterial membrane disruption and inhibit bacterial proliferation) and indirect (pH, temperature alterations and electrotaxis) antimicrobial effects of electrical stimulation.

137x74mm (300 x 300 DPI)