Future MICROBIOLOGY

REVIEW

For reprint orders, please contact: reprints@futuremedicine.com

Low-level laser therapy as an antimicrobial and antibiofilm technology and its relevance to wound healing

Steven L Percival*[1,2], Iolanda Francolini[3] & Gianfranco Donelli[4]

ABSTRACT The biostimulative effect of low-level laser therapy (LLLT) in tissues has been noted in reference to the treatment of various diseases but little information exists on its effectiveness on chronic wounds and biofilm. The scope of this review was to identify literature reporting on LLLT alone, without photodynamic agents, as an antimicrobial/antibiofilm technology and determine its effects on wound healing. Overall the beneficial effects of LLLT in promoting wound healing in animal and human studies has been demonstrated. However, the lack of credible studies using reproducible models and light dosimetry restricts the analysis of current data. Efforts must be addressed to standardize phototherapy procedures as well as to develop suitable *in vitro* and *in vivo* biofilm models to test LLLT efficacy in promoting biofilm eradication and wound healing.

KEYWORDS
• biofilms • chronic wounds
• LLLT

Wound healing is a complex and dynamic process consisting of different integrated phases, namely hemostasis, inflammation, proliferation and tissue resolution [1]. Hemostasis begins immediately after injury and stops wound bleeding through vascular constriction and fibrin clot formation. Later, inflammatory cells migrate into the wound provoking inflammation characterized by the infiltration of neutrophils (responsible for clearance of invading microrganisms), macrophages and lymphocytes. Macrophages have the major role in wound healing. Indeed, in the initial phase they release cytokines to promote the inflammatory response, later they clear apoptotic cells to favor the resolution of inflammation and finally they undergo a phenotypic transition that stimulates keratinocytes, fibroblasts and angiogenesis to promote tissue regeneration [2]. Fibroblasts produce the major components of the extracellular matrix (ECM), such as collagen, glycosaminoglycans and proteoglycans.

There are many factors that can affect wound healing thus leading to delayed-healing in acute and chronic wounds. Apart from the presence of specific diseases such as diabetes, obesity and cardiovascular diseases, wound microbial colonization with subsequent infection development is recognized as the main cause for impaired wound healing [3,4].

There is increased evidence that microorganisms colonize the wound surface forming complex and polymicrobial communities known as biofilms. Biofilms are considered responsible for the chronic state of venous leg ulcers, diabetic foot ulcers and pressure ulcers as they are thought to induce chronic inflammation hampering wound healing [5]. Wounds are susceptible to infection due to impaired host immune response.

The role of biofilms in chronic wounds has been recently reviewed in detail [3–4,6–7]. One of the first investigations relating biofilms to wounds was published in 2003 by Harrison-Balestra

[1]Surface Science Research Centre, University of Liverpool, Liverpool, UK
[2]Institute of Ageing & Chronic Disease, University of Liverpool, Liverpool, UK
[3]Department of Chemistry, Sapienza University, Rome, Italy
[4]Microbial Biofilm Laboratory, IRCCS Fondazione Santa Lucia, Rome, Italy
*Author for correspondence: steven.percival@liverpool.ac.uk

Future Medicine part of fsg

et al. [8] who showed that *Pseudomonas aeruginosa* isolated from human burn wounds was able to develop a mature biofilm within 10 h of *in vitro* growth, suggesting that bacteria in wounds rapidly develop biofilms. The landmark studies reporting on direct microscopic evidence of bacterial biofilm involvement in chronic wounds were published some years later in 2008 [9–11]. Bjarnsholt *et al.* [9] analyzed sections from chronic wounds by fluorescence *in situ* hybridization and found bacterial aggregates of both *Staphylococcus aureus* and *P. aeruginosa*. The authors hypothesized that *P. aeruginosa* maintained the wound in a chronic state, due to the cytolytic effects of the rhamnolipids produced by *P. aeruginosa* itself. James *et al.* [10] examined chronic and acute wound specimens and characterized microorganisms inhabiting these wounds. Sixty percent of the 50 chronic wound specimens observed by scanning electron microscopy (SEM) contained biofilm, whereas only one of the 16 acute wound specimens (6%) was colonized by a microbial biofilm. These findings suggested that specific microbial biofilms might play a key role in hindering healing of chronic wounds. Davis *et al.* [11], using a porcine model, treated *S. aureus* contaminated wounds with either one of two topical antimicrobial agents (mupirocin cream or triple antibiotic ointment) within 15 min to represent planktonic bacteria or 48 h after initial inoculation to represent biofilm-associated wound infection. SEM and epifluorescence microscopy observations showed biofilm-like structures in wounds after 48 h of inoculation. Both the employed antimicrobial agents were effective against planktonic *S. aureus* but had a reduced efficacy against *S. aureus* biofilm.

A large variety of wound dressings are available nowadays for local wound treatment [12]. Recently a number of antimicrobial wound dressings have become available, most of them impregnated with silver [13–16]. Nondressing wound therapies are also currently used, including negative pressure wound therapy (NPWT), hyperbaric oxygen and low-level laser therapy (LLLT).

This review is focused on the use of LLLT to promote biofilm killing and wound healing. Particularly, the aim of this review is to provide a summary of studies that fitted into the search criteria of LLLT, biofilms and wounds, with the scope to identify literature reporting on the effect of LLLT alone, without photodynamic agents, as an antimicrobial/antibiofilm technology and determine its effects on wound healing.

Low-level laser therapy

The use of LLLT has a history of use, which can be traced back to the 1970s when it was found to have biostimulatory effects in animals [17]. This therapy involves the application of light, either a laser light with a specific wavelength or a light-emitting diode (LED), to stimulate processes at a cellular level. Wavelengths both in the visible region (400–700 nm) and in the infrared region (700 nm–1000 μm) are used in such a therapy.

In the visible light region, the photon energy is absorbed by a molecule (photoacceptor) that assumes an electronically excited state. Then, the excited molecule loses its extra energy in different ways, including re-emitting a photon of longer wavelength (fluorescence or phosphorescence), giving off heat, or transferring the energy to other molecules causing chemical reactions that may give rise to effects at a biological level (photochemistry) [18].

The adsorption of radiation in the infrared region causes molecular rotations and vibrations (bond stretching or bending) and is not expected to cause chemical changes, although the local heating generated by molecular movements can increase the rate of reactions [18].

Although visible radiation and infrared radiation differently stimulate molecules, they can produce similar biological responses. The exact mechanisms of actions following LLLT is not well understood, but a number of theories exist. The most accepted theory is that visible light irradiation provokes the photoactivation of enzymes. Specifically, Karu [19] proposed that light is absorbed by cytochrome-c oxidase, a terminal enzyme of the mitochondrial respiratory chain, which causes oxidation of the NAD pool leading to changes in the redox status of both the mitochondria and the cytoplasm. This affects membrane permeability (with changes in Na^+/H^+ ratio) and ATPase activity (increasing production of ATP) that in turn modulate the Ca^{2+} flux. The increase in intracellular Ca^{2+} stimulates DNA, RNA synthesis and cell proliferation (biostimulation). As for infrared irradiation, according to Smith [18] it leads to the same biological effects produced by visible light but initiates the response at the membrane level, probably through photophysical effects on Ca^{2+} channels provoked by bond rotations and vibrations.

A number of studies on various cell types have shown that the positive effects of LLLT depend on light parameters including wavelength (nm), energy dose (J/cm^2) and intensity (W/cm^2).

At a cellular and biochemical level LLLT has been shown to stimulate oxidative phosphorylation, reduce inflammation and increase cellular metabolism [20–23]. Of particular interest, in reference to wound healing, is the fact that at low dose (0.05–10 J/cm^2) LLLT is known to stimulate cellular activities. Particularly, near infrared laser light has been shown to have an effect on wound healing by enhancing the growth of fibroblasts and proliferation [24,25]. In contrast, at higher doses (generally more than 10 J/cm^2), LLLT has been shown to inhibit cellular proliferation [26].

As it will be discussed in the next sections, visible light especially in the wavelength 400–500 nm (blue light) and at specific energy doses, depending on the type of microorganism and its planktonic or biofilm state, has been documented to effect the growth of some fungal and bacterial species [27,28]. A number of studies have shown that blue light causes the inactivation of *Propionobacterium acnes*, *Helicobacter pylori* and other Gram-positive and Gram-negative bacteria including those associated with infected wounds. The effect of LLLT has been shown to be effective on both bacteria and fungi present in the oral field [29]. In contrast, low-power white light enhances bacterial proliferation.

The mode of action regarding the photoinactivation of bacteria still remains a speculative phenomenon. It is well documented that microorganisms are inactivated with ultra violet light (UV – at wavelengths of 240–280 nm) due to its ability to cause DNA damage and induce sub lethal damage [30]. This effect is presently not considered the mode of action regarding LLLT. The modes of action reported in the literature are related to both thermal and photo-disruption effects. LLLT is known to have sublethal effects on bacteria, in particular causing the denaturing of proteins and effects on the cell wall [31]. For bacterial infections most appropriate to wound healing, it seems logical that the light is having a stimulatory effect on the innate and adaptive immune system [32]. In addition, based on the literature to date, the damage to microbial cells caused by light seems to be due to the generation of reactive oxygen species (ROS), as proposed by Khaengraeng and Reed [33]. Indeed, light in the visible range is known to produce ROS.

In 1999, Hockberger *et al.* [34] showed that blue light stimulated H_2O_2 production in cultured mouse (3T3 fibroblasts), monkey (kidney epithelial cells) and human (foreskin keratinocytes) cells. Particularly, after irradiation of the cultured cells by blue light at several wavelength ranges (400–410, 445–455, 450–490 or 485–495 nm), H_2O_2 was found to originate in peroxisomes and mitochondria. The production of H_2O_2 was found to be enhanced in cells over expressing flavin-containing oxidases, supporting the hypothesis that photoreduction of flavoproteins stimulates H_2O_2 production in cells following light exposure. H_2O_2 is a ROS and can give rise to even more damaging hydroxyl radicals that can cause cellular damage.

A number of researchers have also documented that the inactivation of bacteria, which is considered to be oxygen dependent, is due to the photoexcitation of porphyrins that are thought to act as endogenous photosensitizers with the bacteria itself [35,36]. This is due to high levels of coproporphyrins which become photosensitized by blue light [37,38]. Principally, blue light leads to photosensitization of intracellular porphyrins, thus causing the production of reactive species, predominantly singlet deltaoxygen ($^1O_2$) and consequently, cell death. Several bacteria produce different porphyrins, each having its peak absorption wavelength. Therefore, optimum photostimulation will require different wavelengths.

This has also been shown to occur with *P. acnes* [39] that causes the disease acne vulgaris [40,41] which can be inactivated by photoexcitation of endogenous porphyrins. Ashkenazi *et al.* [39] studied the eradication of *P. acnes* by its endogenic porphyrins after illumination with blue light at 407–420 nm. The viability of 24 h cultures grown anaerobically in liquid medium was reduced by less than two orders of magnitude when illuminated once with a light dose of 75 J/cm^2. Better effects were obtained when cultures were illuminated twice or three-times consecutively with a light dose of 75 J/cm^2 and an interval of 24 h between illuminations. Indeed, the *P. acnes* viability decreased by four and five orders of magnitude after two or three illuminations, respectively.

Other than pulsed UV-rich light [42,43], recent studies [44,45] reporting the inactivation of *S. aureus* by super luminous diodes, further demonstrate that *S. aureus* can be inactivated by visible light without the use of exogenous

photosensitisers or d-ALA-induced porphyrins.

In the following section, the studies reporting the effects of LLLT on the viability of planktonic bacteria *in vitro* will be reviewed to find out the conditions (wavelength, energy dose) efficacious at a clinical level, especially for microorganisms involved in wound infections.

In vitro effects of LLLT on the viability of planktonic bacteria

Different light parameters, such as wavelength, intensity and dose, have been employed to evaluate the effects of irradiation on bacterial growth. The high variability of employed light parameters, in terms of intensity and energy dose, makes it difficult to extrapolate from these available literature studies a standard protocol to be applied for reduction of bacterial viability. Besides light parameters, the phototoxic effect was found to be strictly dependent on the type of employed bacterial strain. As stated by Kendric Smith in his review on laser and led photobiology [46], the magnitude of the photodynamic effect depends on the physiological state of cells at the moment of irradiation. This explains why the effect is not always detectable and the high variability of the results reported in the literature.

In order to better resume the available data, the different *in vitro* studies were classified in terms of the employed ranges of wavelength (infrared or blue light). In addition, a score (1, 2 or 3) was assigned to each cited paper according to the relevance of the antimicrobial effect (see **Tables 1–3**). According to the American Society of Microbiology any agent to be termed 'antimicrobial' must be able to kill at least three logs (99.9%) of planktonic cells. Scores were assigned as follows: marginal antimicrobial effect (≤2 log10 or 99.6% CFU reduction); good antimicrobial effect (= 3 log10 or 99.9% CFU reduction); optimal antimicrobial effect (>3 log10 or 99.9% CFU reduction).

• Irradiation by infrared light

In **Table 1**, the target microorganisms, the light parameters together with the main outcome are reported for the *in vitro* studies using infrared light. As reported there are clearly only just a few studies available. Nussbaum *et al.* [47] investigated the *in vitro* effects of LLLT on bacterial growth employing *P. aeruginosa*, *Escherichia coli* and *S. aureus*. Each bacteria was plated out onto agar and then exposed to infrared light at wavelengths of 810 and 905 nm (0.015 W/cm^2, 1–50xJ/cm^2). By counting the colony-forming units (CFUs) after 20 h of incubation post therapy, the authors found that there was an effect in reference to wavelength and species and also between wavelength and radiant exposure. Particularly, at 810 nm (0.015 W/cm^2 and 5 J/cm^2) reduction of *P. aeruginosa* viability (23%) was achieved but the growth of *E. coli* was increased. The growth of *S. aureus* increased at 905 nm (50 J/cm^2). The authors also exposed bacteria to visible light (630 nm, 1 J/cm^2), observing a reduction of the viability of both *P. aeruginosa* and *E. coli* but at a marginal level (27%).

Fonseca *et al.* [48] investigated the effects of low intensity (1, 4 and 8 J/cm^2) infrared laser exposure on the survival of *E. coli* and plasmid topological forms. Experimental models based on *E. coli* cultures both proficient and deficient in DNA repair mechanisms have been used to evaluate the effects of physical and chemical agents on DNA. Results showed that infrared laser had no effect on an *E. coli* wild-type, endonuclease IV, exonuclease III, formamidopyrimidine DNA glycosylase/MutM protein and endonuclease III deficient cultures, but decreased the survival of *E. coli* cultures deficient in nucleotide excision repair of DNA. In this latter case, the survival fraction, that was defined as the ratio between the number of viable cells exposed to laser light and the number of viable cells not exposed to laser, ranged from 0.4 to 0.6 depending on the laser intensity. No alteration in the electrophoretic profile of plasmids was observed.

Lipovsky *et al.* [49] failed in achieving any significant bactericidal effect after irradiation of *S. aureus* and *E. coli* strains at 780 nm at different energy doses (30, 60 and 120 J/cm^2).

From these few available studies we can conclude that the irradiation with red and infrared light (700–1000 nm) at doses ranging from 1 to 120 J/cm^2 seems to not have a significant effect on the viability of Gram positive (*S. aureus*) and Gram negative (*P. aeruginosa* and *E. coli*) bacteria.

• Irradiation by blue light

Blue light (400–500 nm) has been reported to be effective in eradication of a number of microorganisms due to accumulation of naturally occurring photosensitizers such as porphyrins and flavins [34,50]. Several researchers have shown that bacteria such as *S. aureus* can be inactivated at wavelengths ranging from 400 to 420 nm, with a maximum inactivation achieved

Table 1. Main outcome of the *in vitro* studies focused on the effects of infrared light on planktonic bacteria.

Study (year)	Target	Light parameters (wavelength and energy dose)	Main outcome	Score†	Ref.
Nussbaum *et al.* (2002)	*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*	810 nm 1–80 J/cm^2	Irradiation at 810 nm (5 J/cm^2) caused reduction of *P. aeruginosa* growth (23%) but increased *E. coli* growth. Irradiation at 630 nm (1 J/cm^2) reduced both *P. aeruginosa* and *E. coli* viability (27%). Irradiation at 905 nm (50 J/cm^2) increased *S. aureus* viability (27%). Marginal effect at 660 nm	1	[47]
Fonseca *et al.* (2012)	*E. coli* strains proficient and deficient in DNA repair mechanisms	700–800 nm 1, 4, 8 J/cm^2	Infrared laser had no effect on an *E.coli* wild-type but decreased the survival of *E. coli* deficient in nucleotide excision repair of DNA (survival fraction 0.4 at 4/J/cm^2). No alteration in the electrophoretic profile of plasmids was observed	1	[48]
Lipvsky *et al.* (2010)	*S. aureus*, *E. coli*	780 nm	No antimicrobial effects on both the tested bacteria	1	[49]

†Antibacterial effect was scored as follows: (1) marginal (≤2 log10 or 99.6% CFU reduction); (2) good (= 3 log10 or 99.9% CFU reduction); (3) optimal (>3 log10 or 99.9% CFU reduction).
CFU: Colony-forming unit.

at 405 nm [37–38,44–45,50–51]. The inactivation of *S. aureus* has been shown to be caused by a porphyrin-mediated effect.

In **Table 2**, the target microorganisms, the light parameters together with the main outcome are reported for the *in vitro* studies using blue light.

Feuerstein *et al.* [52] evaluated the effect of visible blue light irradiation (400–500 nm) without photosensitizers on the viability of oral microorganisms, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Streptococcus mutans* and *Streptococcus faecalis*. The authors studied the effect of light in either bacteria growing in suspension or on agar plates to evaluate the minimum inhibitory dose (MID) required to inhibit the bacterial lawn from growing into biofilm. Biofilm inhibition was defined as the absence of bacterial colonies in the halo exposed to the light. A 99.6% reduction of cell viability for suspended *P. gingivalis* was obtained after exposure to plasma-arc light for 1 min (62 J/cm^2). On the contrary, only a 40% reduction in viability was obtained for *F. nucleatum* in suspension after exposure to plasma-arc light for 3 min (206 J/cm^2). The survival rate of the Gram-positive *S. mutans* and *S. faecalis* in suspension was not affected by the various light sources and the length of exposure. The MID values for *P. gingivalis* and *F. nucleatum* were found to be 16 and 26 J/cm^2, respectively, obtained by employing a halogen lamp located at a distance of 1 mm from the agar surface. In the same conditions, higher doses (159 and 212 J/cm^2), obtained using a plasma arc, were needed to inhibit biofilm formation by *S. mutans* and *S. faecalis*.

Guffey and Wilborn [44] evaluated the bactericidal effect of 405 and 470 nm light on *S. aureus*, *P. aeruginosa* and the anaerobe *P. acnes*. The authors used 1, 3, 5, 10 and 15 J/cm^2 energy doses. Neither of the two wavelengths proved to be bactericidal against the anaerobic *P. acnes*. The 405-nm light produced a dose dependent reduction of viability on *P. aeruginosa* and *S. aureus*, achieving a reduction in the number of viable cells of 95.1% at 10 J/cm^{2}for *P. aeruginosa* and nearly 90% at 15 J/cm^2 for *S. aureus*, respectively. The 470-nm light was less efficient. Indeed, the number of colonies were reduced by an average of 60% at all dose levels for *P. aeruginosa* (1–15 J/cm^2), and by ca. 20 and 60% at 10 and 15 J/cm^2 for *S. aureus*. Doses of light at 1, 3 and 5 J/cm^2 had a stimulatory activity on *S. aureus*. The same research group also determined the *in vitro* effect of a combination of 405-nm blue light and 880-nm infrared light on *S. aureus* and *P. aeruginosa* [45]. Doses of 1, 3, 5, 10 and 20 J/cm^2 were used, and colony counts were performed and compared with untreated controls. With *P. aeruginosa*, the treatment reduced the number of bacterial colonies at all doses, the most effective dose being 20 J/cm^2 achieving a reduction as much as 93.8%. Also for *S. aureus* the maximum reduction (72%) in the number of bacterial colonies was achieved with 20 J/cm^2.

Enwemeka *et al.* [50] reported on the effect of irradiation at 405 nm and different energy

Table 2. Main outcome of the *in vitro* studies focused on the effects of blue light on planktonic bacteria.

Study (year)	Target	Light parameters (wavelength and energy dose)	Main outcome	Score†	Ref.
Feuerstein *et al.* (2004)	*P. gingivalis*, *F. nucleatum*, *S. mutans*, *S. faecalis*	400–500 nm 16–62 J/cm^2 159–212 J/cm^2	99.6% reduction in CFU for *P. gingivalis* at 62 J/cm^2, 40% reduction in CFU for *F. nucleatum* at 206 J/cm^2. No effect observed for *S. mutans* and *S. faecalis*	1	[52]
Guffey and Wilborn (2006) (*in vitro* study)	*S. aureus*, *P. aeruginosa*, *P. acnes*	405, 470 nm 1–15 J/cm^2	The 405 nm light produced a reduction in *P. aeruginosa* (95%) at 10 J/cm^2 and *S. aureus* (90%) at 15 J/cm^2. The 470 nm light was less efficient (60% *P. aeruginosa* and *S. aureus* reduction at 10 and 15 J/cm^2, respectively) No bactericidal effects vs *P. acnes* was found	1	[44]
Enwemeka *et al.* (2008) (*in vitro* study)	Two clinical isolates MRSA	405 nm 1–60 J/cm^2	The phototoxic effect was dependent on dose and irradiation time. A maximum CFU reduction (93.5%) was achieved with about 8.4 min of exposure at 50 J/cm^2	1	[50]
Maclean *et al.* (2008) (*in vitro* study)	Clinical wound isolates of *S. aureus* and MRSA obtained from the Royal Infirmary, Glasgow	400–500 nm 630, 1260 J/cm^2	Significant bactericidal effect on both sensitive and resistant *S. aureus*, with a 5-log$_{10}$ reduction being achieved at a dose of 630 J/cm^2 for the sensitive strain and 1260 J/cm^2 for the MRSA strain	3	[39]
Maclean *et al.* (2009) (*in vitro* study)	*S. aureus*, *MRSA*, *P. aeruginosa*, *E. coli*, *S. epidermidis*, *E. faecalis*, *C. perfingens*, *A. baumannii*, *P. vulgaris*, *K. pneumoniae*	405 nm 36–216 J/cm^2	5 log10 CFU reduction was obtained at 36 J/cm^2 for the methicillin sensitive *S. aureus* strain and at 45J/cm^2 for the MRSA strain. The *P. aeruginosa* and *E. coli* growth was reduced by 4.2 and 3.1 log$_{10}$ at 180 J/cm^2	3	[38]
Enwemeka *et al.* (2009) (*in vitro* study)	*Two clinical isolates MRSA*	470 nm 1–60 J/cm^2	About 90.4% of colonies of both strains was reduced with an energy dose of 55 J/cm^2	1	[51]
Lipovsky *et al.* (2009) (*in vitro* study)	Two clinical isolates of *S. aureus*, one of the two being a MRSA	400–800 nm (white light) 18, 90, 180 J/cm^2 400–500 nm (blue light) 7.2, 36, 72 J/cm^2	Both white light and blue light at low energy doses induced proliferation of the two strains. White light at high energy dose (180 J/cm^2) resulted in a 99.8 and 55% reduction in viable cells for the sensitive and resistant strain, respectively, Blue light at 72 J/cm^2 resulted in a 86% decrease in CFUs for the sensitive strain but in 15% increase in CFUs for the resistant strain	1	[53]
Lipvsky *et al.* (2010)	*S. aureus*, *E. coli*	415 nm 30, 60, 120 J/cm^2	99% CFU reduction for *E. coli* at 30 J/cm^2 90% CFU reduction for *S. aureus* at 120 J/cm^2	2 (*E. coli*) 1 (*S. aureus*)	[49]

†Antibacterial effect was scored as follows: (1) marginal (≤2 log10 or 99.6% CFU reduction); (2) good (= 3 log10 or 99.9% CFU reduction); (3) optimal (>3 log10 or 99.9% CFU reduction).
CFU: Colony-forming unit.

intensity (1–60 J/cm^2) on the viability of two clinical isolates (US-300 and the IS-853) of methicillin-resistant *S. aureus* (MRSA). Colony counts and the aggregate area occupied by bacteria colonies were used to compare the effect of light on both strains. The reduction in both the number of viable cells and the area of bacterial aggregates was dependent on dose and irradiation time. The maximum reduction of bacterial viability (93.5%) was achieved with 8.4 min of exposure, corresponding to 50 J/cm^2. The dose dependence of LLLT effect was nonlinear as increases of energy between 1.0 and 15 J/cm^2 resulted in more reduction of bacterial viability than increases between 15 and 60 J/cm^2.

Since a 405-nm superluminous diode (SLD) source may raise safety concerns in clinical practice, because of the trace of ultraviolet (UV) light within the spectrum, Enwemeka *et al.* [51] investigated the effect of 470-nm blue light, that has no trace of UV, on the same MRSA clinical isolates used in their previous study. Each strain was irradiated at doses ranging from 0 to 60 J/cm^2 using a 470 nm SLD device. 470

nm blue light was found to be effective in killing MRSA strains *in vitro* suggesting a possible application on both cutaneous and subcutaneous infections. As much as 90.4% of the CFUs of the two strains were killed with an energy density of 55 J/cm^2.

Also Maclean *et al.* [38] investigated the effect of high intensity blue light (400–500 nm) on three *S aureus* strains, two of which were MRSA, all suspended in broth. Light irradiation had a significant bactericidal effect on all the *S. aureus* strains, with a 5-log10 reduction being achieved after a dose of 630 J/cm^2 for the methicillin sensitive strain and after a dose of 1260 J/cm^2 for the two MRSA strains. The exposed *E. coli* suspensions demonstrated negligible inactivation over a 30-min exposure time (630 J/cm^2).

In the same study, in order to identify the narrow bandwidth of visible light between 400 and 500 nm inducing staphylococcal inactivation, *S. aureus* suspensions were exposed to each narrow 10 nm bandwidth between 400 and

Table 3. Main outcome of the *in vitro* studies focused on the low-level laser therapy effects (infrared or blue light) on microbial biofilms.

Study (year)	Target	Biofilm assay	Test parameters	Main outcome	Score†	Ref.
Steinberg *et al.* (2008)	*S. mutans* biofilms	Confocal laser scanning microscopy (CLSM) analysis	400–500 nm 68 J/cm^2	Visible light exposure showed no statistically significant effect on growth of *S. mutans*. Combination of H_2O_2 (3 mM) with light exposure for 60 s (68 J/cm^2) reduced bacterial growth by 2.3 logs compared with control	1	[54]
Basso *et al.* (2011)	*S. mutans* biofilm *C. albicans* biofilm *S. mutans* and *C. albicans* mixed biofilm	Scanning electron microscopy investigation	780 nm 5, 10, 20 J/cm^2	A 1 log10 CFU reduction was obtained for *S. mutans* at 20 J/cm^2 while no reduction for *C. albicans*	1	[55]
Baffoni *et al.* (2012)	24-h-old mono and polymicrobial biofilms produced by *S. aureus* and *P. aeruginosa*	Quantitative biofilm formation assay by crystal violet staining	980 nm 148 J/cm^2	No significant differences in terms of biomass reduction in both mono and polymicrobial biofilms was found when compared with the control	1	[56]
Krespi *et al.* (2011)	A biolumnescent strain of methicillin resistant *S. aureus*	Biofilm assay not specified	a) Shock wave (SW) laser at 300 nm b) NIR diode laser at 940 nm c) SW + NIR + ciproflaxicin	SW and NIR combined treatment caused a 88% reduction in live biofilm with respect to control. An ideal treatment suggested by the authors consists in SW laser irradiation (to disaggregate the biofilm) followed by NIR in combination with ciprofloxacin to kill the bacteria	1	[57]
Song *et al.* (2013)	*Aggregatibacter actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis*, in planktonic or biofilm state	CLSM analysis and live/dead viability assay	400–520 nm 7.5, 15, 30, 45, 60 J/cm^2	Only *P. gingivalis* showed a decrease (1 log10) in viable CFUs when irradiated with 60 J/cm^2. A decrease in the live/dead bacteria ratio in biofilm with increasing light exposure time was observed	1	[58]
McKenzie *et al.* (2013)	Single species *E. coli*, *S. aureus*, *P. aeruginosa* and *L. monocytogenes* biofilms grown on glass and acrylic surfaces	Biofilm assay not specified	405 nm 42–510 J/cm^2	4-h-old *E. coli* biofilm (monolayer), 3.41 log10 reduction at 170 J/cm^2. 4-h-old *P. aeruginosa* biofilm (monolayer), 3.72 log10 reduction at 170 J/cm^2. 4-h-old *S. aureus* biofilm (monolayer), 2.75 log10 reduction at 170 J/cm^2. 24-h-old *E. coli* biofilm, 5.7 log10 reduction at 340 J/cm^2. 48-h-old and 72-h-old *E. coli* biofilm, 3.5 log10 at 170 J/cm^2 and 7 log10 reduction at 510 J/cm^2	3	[59]

†Antibacterial effect was scored as follows: (1) marginal (≤2 log10 or 99.6% CFU reduction); (2) good (= 3 log10 or 99.9% CFU reduction); (3) optimal (>3 log10 or 99.9% CFU reduction).

CFU: Colony-forming unit; CLSM: Confocal laser scanning microscopy; NIR: Near-infrared.

500 nm and received an absolute dose of 23.5 J/cm^2. The maximum $\log_{10}$ reduction (2.4) of *S. aureus* cells resulted from exposure to 405 ± 5 nm wavelength light. Exposure to bandwidths of 430–500 nm did not cause significant inactivation of the bacteria.

Later, the same authors studied the effects of the exposure to 405 nm light on a number of different bacterial pathogens [37]. Interestingly, the authors reported the energy doses required to inactivate each tested microorganism. In particular, the lowest active dose was 36 J/cm^2 that was able to inactivate the methicillin sensitive *S. aureus* (5 $\log_{10}$ reduction). Higher doses were necessary to obtain a germicidal effect against MRSA (5 log10 reduction, 45 J/cm^2), *P. aeruginosa* (4.2 $\log_{10}$ reduction, 180 J/cm^2) and *E. coli* (3.1 $\log_{10}$ reduction, 180 J/cm^2) [37].

The higher resistance to photoinactivation of MRSA strains with respect to methicillin-resistant ones was also found by Lipovsky *et al.* [53], who studied the phototoxic effects of illumination with broadband visible light on the viability of two clinical isolates of *S. aureus*, one of the two being a MRSA strain. The bacteria were exposed either to white light (400–800 nm) at energy doses of 18, 90 and 180 J/cm^2 or to blue light (400–500 nm) at energy doses of 7.2, 36 and 72 J/cm^2. The researchers found that there was a difference in the light sensitivity of the two strains. Illumination with white light at 180 J/cm^2 resulted in a 99.8% reduction in the colony count in the 'sensitive strain', while only a 55.5% reduction for the 'resistance' strain was observed. Interestingly, at a low energy dose (18 J/cm^2) proliferation of both strains was detected. Illumination with blue light at a 72 J/cm^{2}dose resulted in a 86% decrease in the number of bacteria for the sensitive strain but a 15% increase of CFUs for the resistant strain. At low-energy doses (7.2 and 36 J/cm^2) both strains proliferated. The phototoxic effect was found to be dependent on production of oxy radicals (hydroxyl and superoxide radicals). Adaptation to oxidative stress was exhibited by the 'resistant' strain that produced twice as many carotenoids, giving protection from illumination, than the 'sensitive' strain. The 'sensitive' strain produced 10-times more endogenous porphyrins than the resistant strain.

Later, the same group [49] identified the most effective wavelengths in the visible range for inducing bactericidal effects on strains of *S. aureus* and *E. coli*. Both a halogen lamp equipped with appropriate filters for irradiation in the white (400–800 nm), blue (400–500 nm) and red (500–800 nm) regions and an LED arrays at 415 and 455 nm (100 mW/cm^2 each, fluencies of 30, 60 and 120 J/cm^2) were used in the experiments. The production of ROS following exposure to blue light was found to be higher than red light. Furthermore, the 415 nm wavelength induced more ROS than 455 nm. Interestingly at 415 and 455 nm, there was a significant reduced viability of *E. coli* (>99%) at all employed fluencies but a lower reduction for *S. aureus* reaching as much as ca. 90% at 120 J/cm^2. In addition, an enhanced proliferation of *S. aureus* was observed at low fluencies (30 J/cm^2). Illumination at 455 nm was less effective than 415 nm in the reduction of viability of both *E. coli* and *S. aureus*. Red light illumination at 780 nm did not produce any effect on the bacteria.

In conclusion, the application of blue light to planktonic bacteria can cause cell photoinactivation when proper wavelengths and energy doses are selected. In general, low energy doses (<30 J/cm^2) seems to enhance bacterial proliferation while higher doses must be applied to achieve a germicidal effect. Particularly, for the Gram-negative *P. aeruginosa* and *E. coli*, a dose of 180 J/cm^2 at 405 nm is needed [37]. In addition, the wavelength of 405 nm was shown in several studies to cause photo inactivation of both Gram positives and Gram negatives more efficiently than 415, 455 nm and broadband (400–500 nm) lights. In fact, a killing effect was achieved for methicillin-sensitive *S. aureus* strains at 36 J/cm^2 dose when irradiating at 405 nm light [37], but at a higher dose (630 J/cm^2) for 400–500 nm irradiation [38]. Finally, MRSA strains seemed to be more resistant to LLLT than the sensitive strains [37–38,53].

In vitro effects of LLLT on microbial biofilms

As described above, a large number of studies have shown that some Gram-positive and Gram-negative bacteria are susceptible to LLLT when they are grown as planktonic cultures. However, the majority of human infections, including non-healing chronic wounds, lung chronic infections in cystic fibrosis patients, medical device-related infections and so on, are caused and supported by microbial biofilms. Biofilms are responsible for the high antibiotic tolerance and the chronic state of these infections.

The light wavelengths and energy doses for which reduced cell viability was observed for planktonic cells could not be adequate for biofilm eradication. Indeed, the magnitude of the laser biostimulation effect depends on the physiological state of cells at the moment of irradiation. LLLT appears to work in presence of severe damage, while no light effects are observable, for example, on normally healing wounds. Indeed, light will stimulate cell proliferation in the cells that are growing poorly at the moment of irradiation. If a cell is fully functional, there is nothing for radiation to stimulate, and therefore no therapeutic benefits will be observed [46]. This opens interesting perspectives in the potential killing ability of LLLT toward microbial biofilms better than planktonic bacteria. Indeed, in the biofilms subpopulations of slowly growing or dormant cells are known to be present. These populations are responsible for the inefficiency of a number of antibiotics and antimicrobials in eradicating biofilms.

A pubmed search on LLLT and biofilms produces just a few results **(Table 3)**, mainly concentrated in the last 4 years, indicating that this is still a poorly investigated field. Most of those documented studies appear to utilize only a small diverse range of micro-organisms including *S. mutans* and *C. albicans*, with the focus specifically on oral biofilms. All the mentioned studies have followed appropriate protocols to obtain a mature biofilm to be irradiated, even if some authors have not provided details on the assays used to verify biofilm development and EPS matrix production. Information about this issue is provided in **Table 3**.

Basso *et al.* [55] investigated the effect of infrared light (780 nm) on biofilms formed by *S. mutans*, *C. albicans* or an association of both species. 48-h-old single-species or dual-species biofilms grown on the wells of a culture plate were exposed to doses of 5, 10 or 20 J/cm^2. Biofilm viability by the MTT assay and cell count showed that LLLT reduced cell viability as well as biofilm growth. For *S. mutans*, a 1 $\log_{10}$ reduction was obtained for the higher energy dose (20 J/cm^2). *Streptococcus mutans* exhibited even a more intense growth and greater resistance to LLLT when growing in association with *C. albicans*. *C. albicans* did not show a significant reduction of viability when exposed to all doses. These results were confirmed by SEM observations that evidenced a reduction in the number of cells adhering to glass with no altered morphology for *S. mutans* and unchanged number of cells adhering for *C. albicans*.

Similarly, Baffoni *et al.* [56] failed in demonstrating a positive effect of near-infrared (NIR) laser light on mono and polymicrobial biofilms produced by *S. aureus* and *P. aeruginosa* strains isolated from chronic wounds. Twenty-four hours old biofilms were treated with a NIR diode laser, at a wavelength of 980 nm, coupled to a 400-nm optical fibre (energy dose of 148 J/cm^2). The effectiveness of laser light was determined by biomass measurement, CFU count and cell viability. Results showed that the 24-h mature biofilms, grown under static conditions and submitted to laser treatment, showed no significant differences in terms of biomass reduction in both mono- and polymicrobial biofilms when compared with the control. The authors suggested the use of laser together with antimicrobial therapy to have better chances in eradicating wound infections instead of using pharmacological or laser therapies alone.

Krespi *et al.* [57] used a biolumnescent strain of methicillin resistant *S. aureus* to test the efficacy of visible and NIR light, alone or in combination with ciprofloxacin, against biofilm. The study included the treatment of 48-h-old biofilm with shock wave (SW) laser at 300 nm; NIR diode laser with a wavelength of 940 nm coupled to a 300-nm optical fiber; ciprofloxacin alone; SW and NIR lasers; SW laser and ciprofloxacin; and SW, NIR lasers and ciprofloxacin. The combination of SW and NIR caused a 43% reduction in optical density and a 88% reduction in live biofilm with respect to the control. Ciprofloxacin in combination with SW alone or SW + NIR lasers caused in both cases a 60% reduction in optical density and more than 80% reduction in live biofilm, much greater than ciprofloxacin alone (44%). The authors suggested to treat biofilm by a combined therapy consisting of SW laser (to disaggregate the biofilm) followed by NIR laser irradiation in combination with ciprofloxacin to kill the bacteria.

A combined treatment was proposed some years ago by Steinberg *et al.* [54], who studied the effect of broadband light at 400–500 nm on cell viability and gene expression of *S. mutans* in biofilms with or without the addition of hydrogen peroxide (H_2O_2, 3–300mM). Light exposure in the absence of H_2O_2 showed no statistically significant effect on growth of *S. mutans*. Combination of low H_2O_2 concentration (3 mM) with light exposure for 30 s (34 J/cm^2)

and 60 s (68 J/cm^2) reduced bacterial growth by 1.5 and 2.3 logs, respectively, compared with control. Real-time reverse transcription-PCR showed significant upregulation of expression of brpA, gtfB, smu630 and comDE following exposure to light for 60 s and H_2O_2 (15-, 10- and 10-fold, respectively, higher than that of the nontreated samples). The expression of relA and ftf was not affected by the treatment. Exposure to H_2O_2 alone did not affect the expression of the genes tested. The authors concluded saying that the combination of visible light and H_2O_2 may be applied in biofilm-related diseases as a minimally invasive antibacterial procedure.

Song *et al.* [58] evaluated the phototoxic effect of blue light on anaerobic periodontal pathogens both in the planktonic and biofilm state. Particularly, strains of *Aggregatibacter actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis*, in the planktonic or biofilm state, were exposed to a halogen lamp at wavelengths of 400–520 nm (500 mW/cm^2) for 15, 30, 60, 90 or 120 s corresponding to 7,5, 15, 30, 45 and 60 J/cm^2. In the planktonic state, for *F. nucleatum* and *P. gingivalis* viability reduction (3 $\log_{10}$) was obtained after light exposure for 60 s (30 J/cm^2). *Aggregatibacter actinomycetemcomitans* was not significantly affected by light irradiation. In the biofilm state, only *P. gingivalis* showed a decrease in CFU with increasing light exposure time, 1 $\log_{10}$ reduction being achieved after 120 s exposure (60 J/cm^2). For all strains, confocal scanning laser microscopy images showed a mixture of dead and live bacteria in the biofilm up to a 30–45 μm depth. However, only for *P. gingivalis* the live/dead bacteria ratio was found to decrease according to light exposure time (0 s vs 120 s). This finding suggests a higher resistance to photoinactivation of bacteria growing as biofilm with respect to the same bacteria in the planktonic state

Very recently, Mckenzie *et al.* [59] explored the possibility to use 405 nm light exposure to decontaminate glass and acrylic surfaces from *E. coli* biofilm. Samples were light exposed for 5–60 min with an average irradiance of 141.48 mW/cm^2, giving a range of average doses from 42 to 510 J/cm^2. When biofilms were grown on glass, the most rapid inactivation was observed with *E. coli* monolayer biofilms (4-h-old biofilm, starting population ca. 1 × 10^4 CFU/ml), with a 2.52 $\log_{10}$ CFU/ml reduction following 10 min exposure (85 J/cm^2) and a 3.55 $\log_{10}$ reduction obtained for 20 min exposure (170 J/cm^2). A similar viability reduction was found by the same authors when treating *E. coli* in suspension (3.1 log10 CFU/ml reduction, starting population ca. 1 × 10^5 $\log_{10}$ CFU/ml) [33], suggesting a similar behavior of planktonic bacteria and young biofilms. A less rapid decrease of viability was recorded for treatment of 24-h-old *E. coli* biofilms (initial biofilm populations of ca. 5.7 $\log_{10}$ CFU/ml), with significant reductions of 4.41 and 5.7 $\log_{10}$ CFU/ml following exposure to 30 (255 J/cm^2) and 40 min (340 J/cm^2), respectively. Fort-eight hours old and 72-h-old biofilms (starting populations between 7 and 8 $\log_{10}$ CFU/ml) possessed similar rates of inactivation, achieving 3.5 $\log_{10}$ reduction after 20 min and near-complete inactivation (<1 CFU/ml surviving) following 60 min exposure (510 J/cm^2). Similar results were found for *E. coli* biofilms grown on acrylic surfaces. The authors also found statistically significant biofilm inactivation employing other microorganisms (*S. aureus*, *P. aeruginosa* and *Listeria monocytogenes*). These positive results highlight the possible application of this technology for biofilm decontamination in food and clinical settings.

Interestingly, some authors have shown the potential of LLLT to affect the ability of microbes to form biofilm. Chebath-Taub *et al.* [60] tested the effect of the exposure of *S. mutans* to blue light (400–500 nm, 1–10 min, 68–680 J/cm^2) on the ability to re-form a new biofilm. The authors treated 24 h-old *S. mutans* biofilm with blue light (400–500 nm) for an increasing time period (1, 3, 5, 7, 10 min) corresponding to increasing doses (from 68 to 680 J/cm^2). Following exposure, the biofilm was detached; the bacteria were suspended in broth and immobilized again to re-form a biofilm by incubation for 2, 4 or 6 h. After 6 h incubation, the viability of the biofilm populations previously exposed to blue light for 7 or 10 min (equivalent to 476 and 680 J/cm^2) was lower than the control. Particularly, a 1 $\log_{10}$ reduction in the viable cell count was obtained. By confocal microscopy, the authors showed that the thickness of the biofilm formed 6 h after exposure of bacteria to blue light was similar to that of the control. In addition, in the samples exposed to blue light for 7 and 10 min there was an accumulation of dead bacteria in the outer layers of the biofilm. The authors explained their findings suggesting that presumably some bacteria were damaged by exposure to light and probably became more vulnerable to oxidative stress when reorganizing into the new

biofilm. This would explain the effect appearing mostly in the outer biofilm layers.

In conclusion, at present based on the available literature infrared light is confirmed to be inactive against either planktonic or biofilm growing bacteria, while blue light causes biofilm photoinactivation only at doses higher than 170 J/cm^2 [59].

In vivo effects of LLLT on wound infection & healing

Impaired wound healing has been associated with a decrease in cellular migration, proliferation of growth factors and collagen synthesis. The increase in oxidative stress also promotes cell death. It has been reported that low level light (doses ranging from 0.05 to 10 J/cm^2) in the red (620 nm) and near infrared (700–1000 nm) wavelength can enhance wound healing. This can occur through a number of processes [61]. Several studies have shown that LLLT provokes an increase in cell migration [62], the proliferation of cells such as fibroblasts [63], collagen synthesis [64,65], the production of growth factors [65] and ATP [66], human epidermal stem cell proliferation [67].

Very recently, Spitler and Berns [68], compared the efficacy of visible light at different wavelength in promoting wound healing. The authors found that laser light at 652 nm (10 J/cm^2) and 806 nm (2.3 J/cm^2) as well as LED at 637 nm (10.02 J/cm^2) and 901 nm (2.3 J/cm^2) induced comparable levels of cell migration and wound closure.

Dancakova *et al.* [69] demonstrated that infrared 810 nm laser (0.9 J/cm^2/wound/day) light was able to improve wound healing in diabetic rats with respect to the untreated group. Similarly, a positive effect of LLLT in promoting wound healing in rats with induced third-degree burns has been recently reported and related to decreased inflammatory cells and increased collagen deposition in the LLLT treated wounds [70].

In a mouse model, Gupta *et al.* [71] demonstrated that 635 nm and 810 nm light, delivered at a constant fluence (4 J/cm^2), was effective in promoting healing in dermal abrasions while 730 and 980 nm light showed no sign of stimulated healing.

A randomized clinical trial to evaluate the efficacy of LLLT on wound healing was performed by Kajagar *et al.* [72] on 68 patients with chronic diabetic foot ulcers having negative cultures. Thirty-four patients were treated just with the conventional therapy (systemic antibiotics) and the remaining 34 patients with LLLT (2 or 4 J/cm^2) combined with the conventional therapy. By recording healing and percentage reduction in ulcer area over a period of 15 day, the authors demonstrated the beneficial of LLLT in the treatment of diabetic foot ulcers. Unfortunately, the authors did not indicate the wavelength of irradiation.

Nussbaum *et al.* [73] compared in patients with spinal cord injury the effect on wound healing of LLLT alone and combined with either laser treatment or a regimen of ultrasound and UV-C (US/UVC). In total, 20 patients were randomly assigned to the treatment groups. The laser protocol consisted of three treatments weekly using a cluster probe with a 820-nm laser diode and 30 SLDs (10 each at 660, 880 and 950 nm), and energy density of 4 J/cm^2. The US/UVC regimen consisted of five treatments weekly, alternating the treatment modality daily. The pulsed US was applied at a frequency of 3 MHz and a spatial average-temporal average intensity of 0.2 W/cm^2 (1:4 pulse ratio) for 5 min per 5 cm^2 of wound area. The UVC dosage (95% emission at 250 nm) was calculated at each session according to wound appearance. By monitoring wound surface areas every 14 days, US/UVC treatment resulted to have a greater effect on wound healing than did nursing care, either alone or combined with laser.

More recently, the same group performed a double-blind randomized trial with stratification for ulcer location to buttock or lower extremity to compare the effects of UV-C (UVC) with placebo-UVC on pressure ulcer healing (stage 2 to 4 pressure ulcers) in individuals with spinal cord injury. Subjects were followed up for 1 year post intervention. Results showed that UVC was beneficial only for stage 2 buttock ulcers [74].

In view of the absence of randomized studies with sufficiently large sample sizes, Lucas *et al.* [75] performed a prospective, observer-blinded multicenter randomized clinical trial to assess the efficacy of LLLT in the treatment of stage III decubitus ulcers. A total of 86 patients were enrolled into the study. Treatment was the prevailing consensus decubitus treatment (n = 47); one group (n = 39) had LLLT in addition, five-times a week over a period of 6 weeks. During the treatment period, 11% of the patients in the control group and 8% of the patients in the LLLT group developed a stage IV decubitus

ulcer. The patients' Norton scores at 6 weeks did not change during the treatment period. Therefore, in this trial no evidence of low-level laser therapy as an adjuvant to the consensus decubitus ulcer treatment was found.

Contrary to other studies, Schubert [76] showed the efficacy of phototherapy on pressure ulcer healing in elderly patients (> or = 65 years) after a falling trauma. Phototherapy consisted of 9-min treatments with pulsed monochromatic infrared (956 nm) and red (637 nm) light. The ulcer surface area was traced weekly. Patients treated with pulsed monochromatic light had a 49% higher ulcer healing rate, and a shorter time to 50% and to 90% ulcer closure compared with controls. Their mean ulcer area was reduced to 10% after 5 weeks compared with 9 weeks for the controls.

Despite the variety of animal and human studies focused on LLLT application to promote wound healing, there have been only a small number of studies that have investigated the effect of LLLT on wounds that are infected **(Table 4)**.

Papageorgiou *et al.* [41] evaluated the efficacy of a combined treatment of blue (415 nm) and red (660 nm) light against acne vulgaris in a pilot human study. One hundred and seven patients with mild-to-moderate acne vulgaris were randomized into four treatment groups: blue light, mixed blue and red light, cool white light and 5% benzoyl peroxide cream. After 12 weeks of treatment a mean improvement of 76% in inflammatory lesions was achieved by the combined blue ± red light phototherapy; this was superior to that achieved by blue light (ca. 60%), benzoyl peroxide (ca. 60%) or white light (25%). The final mean improvement by using blue±red light was 58%, better than that achieved by the other active treatments used, although the differences were not statistically significant.

In 2006, Jawhara and Mordon [77] evaluated the bactericidal action of a 810 nm infrared diode laser on a wound infection by *in vivo* imaging of a bioluminescent *E. coli* strain. Two circular 14 mm diameter wounds (control and laser-irradiated) were induced in rats and a wound infection was established by inoculation with a 10^9 cells/ml suspension of bioluminescent *E. coli*. After 30 min, light irradiation was applied using a 810 nm diode laser at energy doses of 130, 195 and 260 J/cm^2. *In vivo* bioluminescence imaging showed thatat 4 h after laser irradiation and energy dose of 260 J/cm^2, the bioluminescence of *E. coli* was reduced (ca. 30%) when compared with the control. The reduction of *E. coli* bioluminescence was dose-dependent. At 48 h, bioluminescent bacteria were not detected in the wound irradiated at 260 J/cm^2. At this dose energy, the temperature reached 45°C at the end of the irradiation, that is a temperature that did not affect bacteria bioluminescence but caused a progressive desiccation of the wound. Therefore, the authors concluded with the hypothesis that laser irradiation dries out the wound making the wound an inhospitable place for bacteria suggesting this to be much more relevant than a direct effect of infrared light on chromophores inside bacteria.

Bornstein *et al.* [27] investigated NIR photoinactivation of bacteria (*S. aureus* and *E. coli*) and fungi (*C. albicans* and *Trichophyton rubrumat*) at physiologic temperatures by employing two wavelengths 870 and 930 nm. Using nonlethal dosimetry, the authors revealed a decrease in transmembrane potentials and an increase in ROS generation in methicillin-resistant *S. aureus*, *C. albicans* and human embryonic kidney cells. The authors postulated that these multiplexed wavelengths cause an optically mediated mechano-transduction of cellular redox pathways, decreasing transmembrane potentials and increasing ROS. Following live porcine thermal tolerance skin experiments, the authors also performed human pilot studies, examining photodamage to MRSA in the nose and fungi in onychomycosis. In the human onychomycosis pilot study, the great toe nails of seven patients with positive fungal cultures were irradiated four-times (days 1, 7, 14 and 60), with 870 nm/930 nm for 240 s (energy density of 408 J/cm^2), followed by 930 nm for 120 s (energy density of 204 J/cm^2). All seven patients reached a mycological negative through nail biopsy and culture at 60 days. The experimental temperatures did not exceed 38°C, well within the levels for human phototherapy and thermal tissue damage thresholds. As for the human MRSA pilot study in the nose, each patient underwent exposure with 870 nm/930 nm for 240 s (energy density of 110 J/cm^2) to each anterior nostril followed by 930 nm for 180 s (energy density of 83 J/cm^2) on day 1 and on day 3. MRSA was completely cleared by triplicate culture in all three carriers after the second laser treatment on day 3. In both cases, no observable damage to the nares or the nail matrix was observed.

Table 4. Main outcome of the *in vivo* studies on low-level laser therapy.

Study (year)	Target	Test parameters	Main outcome	Score†	Ref.
Papageorgiou *et al.* (2000) (*in vivo* study)	107 patients with *acne vulgaris* divided in four treatment groups: blue light, white light, mixed blue and red light and 5% benzoyl peroxide cream.	415 nm (320 J/cm^2) + 660 nm (202 J/cm^2)	The efficacy of the mixed blue and red light phototherapy was significantly superior than the other 3 treatments. After 12 weeks, a 76% improvement in inflammatory lesions was achieved by the mixed blue and red light phototherapy	1	[41]
Jawhara and Mordon (2006)	Bioluminescent *E. coli* infecting 14-mm diameter wounds in rats	810 nm 130, 195, 260 J/cm^2	Loss of *E. coli* viability was dose-dependent. At 260 J cm^{-2}, a 100% loss of viability was achieved at 48-h after laser irradiation. Temperature reached 45°C in the wound bed, supporting the hypothesis of bacterial death induced by drying	2	[77]
Bornstein *et al.* (2009)	*S. aureus, E. coli, C. albicans*	870 and 930 nm 83, 110, 204, 408 J/cm^2	Human onychomycosis pilot study, two treatments (day 1 and day 3) at 830/930 nm (408 J/cm^2) + 930 nm (204 J/cm^2) were able to clear *C. albicans* infection with no damages to nails. Human MRSA pilot study in the nose, two treatments (day 1 and day 3) at 830/930 nm (110 J/cm^2) + 930 nm (83 J/cm^2) were able to clear MRSA infection with no damages to nares	3	[27]
Lee *et al.* (2011)	MRSA, mouse model	1072 nm (12 J/cm^2)	Irradiation at 1072 nm did not show a reduction of MRSA CFUs	1	[78]
Dai *et al.* (2013)	One clinical isolate *MRSA*	415 nm (41, 108, 170 J/cm^2)	*In vitro* studies, 4.75-log10 MRSA inactivation was achieved after 170 J/cm^2 blue light had been delivered. *In vivo* studies, more than 2-log10 reduction of bacterial luminescence in the mouse skin abrasions was achieved by 41.4 (day 0) and 108 J/cm^2 (day 1) blue light treatment	2	[79]
Dai *et al.* (2013)	*P. aeruginosa* strain	415 nm (109.9, 55.8 J/cm^2)	When 109.9 J/cm^2 blue light was delivered *in vitro*, ca. 7.64-log10-cycle CFU inactivation of *P. aeruginosa* was achieved. *In vivo*, 55.8 J/cm^2 blue light, applied 30 min after *P. aeruginosa* inoculation to the infected mouse burns, caused an average 3.5-log10-cycle reduction of bacterial luminescence	3	[80]
Zhang *et al.* (2014)	Multidrug resistant *A. baumannii*	415 nm (70.2, 55.8 J/cm^2)	*In vitro*, >4 log10 *A. baumannii* CFUs were inactivated after a single exposure of 70.2 J/cm^2 blue light. In a mouse burn model, after a single exposure of 55.8 J/cm^2 blue light, an average reduction in bacterial luminescence of 4.4 log10 was achieved	3	[81]

†Antibacterial *in vivo* effect was scored as follows: (1) marginal (≤2 log10 or 99.6% CFU reduction); (2) good (= 3 log10 or 99.9% CFU reduction); (3) optimal (>3 log10 or 99.9% CFU reduction).
CFU: Colony-forming unit.

Recently, Dai *et al.* [79] evaluated the efficacy of blue light (415 ± 10 nm) therapy for eliminating community acquired methicillin-resistant *S. aureus* (CA-MRSA) infections in skin abrasions. Particularly, *in vitro* experiments showed that a MRSA strain was more susceptible to blue light inactivation at 170 J/cm^2 than human keratinocytes. A 4.75 $\log_{10}$ bacterial inactivation was detected versus a 0.29 $\log_{10}$ loss of viability in human keratinocytes. By developing a mouse model of skin abrasion infection and using bioluminescent bacteria, the authors also showed the ability of blue light to reduce the bacterial burden in both acute (30 min after bacterial inoculation) and established (24 h after bacterial inoculation) infection. Specifically, more than a 2 $\log_{10}$

reduction of bacterial luminescence in the mouse skin abrasion was achieved when 41.4 (day 0) and 108 J/cm^2 (day 1) blue light was applied.

The Dai's research group also demonstrated the efficacy of blue light at 415 nm for the treatment of *P. aeruginosa* infection [80] or multidrug resistant *Acinetobacter baumannii* infection [81] in a mouse burn model. In both studies, the susceptibilities of the strains and human keratinocytes (HaCaT) to blue light inactivation were studied *in vitro*. As for *P. aeruginosa* [80], when 109.9 J/cm^2 blue light was delivered *in vitro*, ca. 7.64 $\log_{10}$/cycle CFU reduction of *P. aeruginosa* was achieved. In contrast, the inactivation rate for HaCaT was much lower (0.16 $\log_{10}$/cycle). In the mouse model, by using an *in vivo* bioluminescence imaging technique, the authors showed that a single exposure of blue light (415 nm) at 55.8 J/cm^2, applied 30 min after *P. aeruginosa* inoculation to the infected mouse burns, reduced the area-under-the-bioluminescence-time-curve by approximately 100-fold in comparison with untreated infected mouse burns [80]. Survival analyses revealed that bluelight increased the survival rate of the infected mice from 18.2 to 100% [80]. As for the strain of *A. baumannii* [81], the authors found that the strain was significantly more susceptible than keratinocytes to bluelight inactivation. Fluorescence spectroscopy suggested the existence of endogenous porphyrins in *A. baumannii* cells. Blue light after a single exposure of 55.8 J/cm^2 (total illumination duration, 62 min; irradiance, 14.6 mW/cm^2) caused an average reduction in bacterial luminescence of 4.4 $\log_{10}$. In the untreated mice, a reduction of only approximately 0.14 $\log_{10}$ was observed during the same period. No resistance development to blue light inactivation was observed in *A. baumannii* after 10 cycles of sublethal inactivation of bacteria [81].

Lipovsky *et al.* [82] found that high-intensity broad-spectrum polychromatic light at 400–1000 nm (120 J/cm^2) was able to reduce by 62, 83 and 56% the colony counts of *E. coli*, *S. aureus* and *Serratia marcescens*, respectively, in infected diabetic ulcers. No reduction in the viability of *P. aeruginosa* was found.

Lee *et al.* [78] investigated the photomodulation effects of infrared light at a wavelength of 1072 nm on the immune response in relation to its antimicrobial action and its wound healing ability. For this study 30 mice were infected with MRSA on the skin. The treatment group was exposed to infrared light at 20 mW/cm^2 for 10 min (fluence: 12 J/cm^2) at 2, 4, 8, 12, 24 h, 3 and 5 days following inoculation). Changes in mRNA levels of numerous cytokines were investigated. It was found that IL-1β, TNF-α, IL-6 and MCP-1 increased significantly following exposure to 1072 nm which peaked at 12 and 24 h post inoculation. However, direct irradiation at 1072 nm over *S. aureus* colonies did not show a reduction of the colony count, suggesting that the shortened healing time of the infection might involve participation of immune cell functions rather than a direct antibacterial effect of the wavelength.

In conclusion, the *in vivo* literature data so far available are few to help define a standard protocol for eradication of infections caused by microbial agents growing in different conditions. However, even if a therapeutic approach is currently difficult to outline, it is possible to obtain useful suggestions from the clinical pilot study performed by Bornstein *et al.* [27].

Future perspective

Although there have been attempts to study the effect of LLLT on biofilms, the lack of credible studies using reproducible models and light dosimetry restricts the analysis of current data. There is a scarcity of 'activation spectra' for common bacterial pathogens. Although Dietel's study [83] does 'shed some light' on possible antimicrobial effects of visible light using endogenous porphyrins, it remains to be seen if the absence of exogenously applied 5-ALA would inhibit intracellular levels of bacterial porphyrins to an extent that there was only limited antimicrobial activity. In respect to the effect of NIR light alone, there are conflicting results and nonuniformity with regard to dosimetry. It is difficult to interpret the data with respect to any antimicrobial effect when dosimetry is so varied in the studies identified. LLLT by its very name refers to nonthermal (low intensity) light and therefore any studies that cite intensities of >110 mW/cm^2 should be reviewed with caution. In addition we found no mention of possible NIR absorbing chromophores. There is, however, real potential to conduct studies using reproducible biofilm models using both visible and NIR light, investigating the effect of differing wavelengths at sub thermal doses of light on common wound pathogens. Light has been shown to affect the regulation of gene expression causing genes to be either up- or downregulated depending on the environment and microorganism that is being evaluated [53,84–85].

Great progress has been made in the last 10 years, but many efforts must be addressed, in the future, to standardize phototherapy procedures as well as to develop suitable biofilm models in order to test the possible preventive and curative efficacy of visible and infrared light in wound healing. To this aim, the experimental steps listed by Smith [18] almost 15 years ago must be kept in mind when performing an experiment to evaluate LLLT efficacy. First of all an adsorption spectrum of the molecule that is absorbing the light should be recorded to identify wavelengths having a chance of producing a particular biological effect. Once observed a response at a determined wavelength, the optimum dose of radiation together with the number of treatments required to obtain an effect should be evaluated.

Finally, the number of clinical studies on LLLT application for eradication of wound infections and enhancement of wound healing is still limited. Therefore, well-designed, long-term, controlled randomized and double-blind clinical trials are needed for this type of therapy to become accepted and used as an adjuvant therapy for the treatment of chronic wounds, especially venous leg ulcers, diabetic foot ulcers and pressure ulcers.

Phototherapy can be beneficial in a number of clinical situations, especially for promoting healing processes as well as alleviating pain and inflammation. Therefore, extensive research efforts are needed to make it available for many patients who would benefit from it.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.

No writing assistance was utilized in the production of this manuscript.

EXECUTIVE SUMMARY

Background to low-level laser therapy

- Low-level laser therapy (LLLT) involves the application of light in the visible or infrared region to stimulate processes at a cellular level.
- LLLT is known to have lethal effects on bacteria due to the generation of reactive oxygen species causing denaturation of proteins and affecting microbial membrane.

The role of microbial biofilm on wound infection & healing

- Chronic and acute dermal wounds are susceptible to infection due to the loss of the barrier function of the skin.
- Certain microbial biofilms are responsible for wound infection and are thought to induce chronic inflammation hampering wound healing.

In vitro effects of LLLT on bacterial viability

- Irradiation with infrared light at doses up to 120 J/cm^2 seems to not have a significant effect on reducing viability of bacteria in planktonic or biofilm states.
- Blue light applied at 405 nm causes photoinactivation of planktonic bacteria at dose of 180 J/cm^2 for Gram-negatives (*Pseudomonas aeruginosa* and *Escherichia coli*) and 36 J/cm^2 for methicillin-sensitive *Staphylococcus aureus* strains.
- Biofilm photoinactivation by blue light can be obtained at doses higher than 170 J/cm^2.

In vivo LLLT effects on wound infection & healing

- The application of red and infrared light at doses ranging from 0.05 to 10 J/cm^2 was shown to enhance wound healing in animal models and in clinical trials.
- In animal models of wound infection, blue light irradiation at 415 nm and doses higher than 100 J/cm^2 was shown to be efficacious in eradicating infection and increasing survival rate of animals.
- Clinical trials are few to define a standard protocol for infection eradication by LLLT. From the available pilot studies, patient exposure with light at 870 nm/930 nm and energy doses higher than 100 J/cm^2 seems to be an efficacious therapeutic approach.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Guo S, DiPietro LA. Factors affecting wound healing. *J. Dent. Res.* 89, 219–229 (2010).

2 Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–969 (2008).

3 Percival SL, Hill KE, Williams DW *et al.* A review of the scientific evidence for biofilms in wounds. *Wound Repair Regen.* 20, 647–657 (2012).

•• **In this paper, the role of microbial biofilms in wound infection and delayed healing is extensively described.**

4 Scali C, Kunimoto B. An update on chronic wounds and the role of biofilms. *J. Cutan. Med. Surg.* 17, 371–376 (2013).

5 Wolcott RD, Rhoads DD, Dowd SE. Biofilms and chronic wound inflammation. *J. Wound Care* 17, 333–341 (2008).

6 Zhao G, Usui ML, Lippman SI *et al.* Biofilms and inflammation in chronic wounds. *Adv. Wound Care* 2, 389–399 (2013).

7 Bjarnsholt T. The role of bacterial biofilms in chronic infections. *APMIS Suppl.* 136, 1–51 (2013).

8 Harrison-Balestra C, Cazzaniga AL, Davis SC *et al.* A wound-isolated *Pseudomonas aeruginosa* grows a biofilm *in vitro* within 10 hours and is visualized by light microscopy. *Dermatol. Surg.* 29, 631–635 (2003).

9 Bjarnsholt T, Kirketerp-Moller K, Jensen PO *et al.* Why chronic wounds will not heal: a novel hypothesis. *Wound Repair Regen.* 16, 2–10 (2008).

• **One of the first studies reporting on direct microscopic evidence of bacterial biofilm involvement in chronic wounds**

10 James GA, Swogger E, Wolcott R *et al.* Biofilms in chronic wounds. *Wound Repair Regen.* 16, 37–44 (2008).

11 Davis SC, Ricotti C, Cazzaniga A *et al.* Microscopic and physiologic evidence for biofilm-associated wound colonization *in vivo*. *Wound Repair Regen.* 16, 23–29 (2008).

12 Valle MF, Maruthur NM, Wilson LM *et al.* Comparative effectiveness of advanced wound dressings for patients with chronic venous leg ulcers: a systematic review. *Wound Repair Regen.* 22, 193–204 (2014).

13 Li Z, Tang H, Yuan W *et al.* Ag nanoparticle–ZnO nanowire hybrid nanostructures as enhanced and robust antimicrobial textiles via a green chemical approach. *Nanotechnology* 25, 145702–145709 (2014).

14 Herron M, Agarwal A, Kierski PR *et al.* Reduction in wound bioburden using a silver-loaded dissolvable microfilm construct. *Adv. Healthc. Mater.* doi:10.1002/adhm.201300537 (2014) (Epub ahead of print).

15 Lin YH, Hsu WS, Chung WY *et al.* Evaluation of various silver-containing dressing on infected excision wound healing study. *J. Mater. Sci. Mater. Med.* doi:10.1007/s10856–014–5152–1 (2014) (Epub ahead of print).

16 Thomas JG, Slone W, Linton S *et al.* A comparison of the antimicrobial efficacy of two silver-containing wound dressings on burn wound isolates. *J. Wound Care* 20, 580–586 (2011).

17 Rocha JAM, Oliveira RG, Farias RE *et al.* Modulação da proliferação fibroblástica e da resposta inflamatória pela terapia a laser de baixa intensidade no processo de reparo tecidual. *An. Bras. Dermatol.* 81, 150–156 (2006).

18 Smith KC. The photobiological basis of low level laser radiation therapy. *Laser Ther.* 3, 19–24 (1991).

• **The mechanisms of cellular photoinactivation by either visible or infrared light are described.**

19 Karu TI. Molecular mechanism of the therapeutic effect of low-intensity laser radiation. *Lasers Life Sci.* 2, 53–74 (1988).

•• **In this paper, the most accepted theory on visible light phoactivation of enzymes is reported.**

20 Rocha JAM, Vieira BJ, Andrade LCF *et al.* Effects of low-level laser therapy on the progress of wound healing in humans: the contribution of *in vitro* and *in vivo* experimental studies. *J. Vasc. Bras.* 6, 266-(2007).

21 Karu TI. Photobiological fundamentals of low-power laser therapy. *J. Quantum Electron.* 23, 1703–1717 (1987).

22 Karu TI. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. *J. Photochem. Photobiol. B* 49, 1–17 (1999).

23 Hamblin MR, Demidova TN. Mechanisms of low level light therapy. *Proc. SPIE* 6140, 1–12 (2006).

24 Rodrigo SM, Cunha A, Pozza DH *et al.* Analysis of the systemic effect of red and infrared laser therapy on wound repair. *Photomed. Laser Surg.* 27, 929–935 (2009).

25 Posten W, Wrone DA, Dover JS *et al.* Low-level laser therapy for wound healing: mechanism and efficacy. *Dermatol. Surg.* 31, 334–340 (2005).

26 Henriques ACG, Cazal C, De Castro JFL. Ação da laserterapia no processo de proliferação ediferenciação celular: revisão da literatura. *Rev. Col. Bras. Cir.* 37, 295–302 (2010).

27 Bornstein E, Hermans W, Gridley S *et al.* Near-infrared photoinactivation of bacteria and fungi at physiologic temperatures. *Photochem. Photobiol.* 85, 1364–1374 (2009).

•• **The first human pilot studies demonstrating the efficacy of LLLT in the treatment of both onychomycosis and methicillin-resistant *S. aureus* infection in the nose.**

28 Hawkins-Evans DH, Abrahamse H. Efficacy of three different laser wavelengths for *in vitro* wound healing. *Photodermatol. Photoimmunol. Photomed.* 24, 199–210 (2008).

29 Maver-Biscanin M, Mravak-Stipetic M, Jerolimov V *et al.* Fungicidal effect of diode laser irradiation in patients with denture stomatitis. *Lasers Surg. Med.* 35, 259–262 (2004).

30 Blatchley ER, Peel MM. Disinfection by ultraviolet irradiation. In: *Disinfection, Sterilization and Prevention.* Block SS (Ed.). Lippinkott Williams & Wilkins, Philadelphia, PA, USA, 823–851 (1991).

31 Dworkin M. Endogenous photosensitization in a carotenoidless mutant of *Rhodopseudomonas speroides*. *J. Gen. Physiol.* 41, 1099–1112 (1958).

32 Fournier B, Philpott DJ. Recognition of *Staphylococcus aureus* by the innate immune system. *Clin. Microbiol. Rev.* 18, 521–540 (2005).

33 Khaengraeng R, Reed RH. Oxygen and photoinactivation of *Escherichia coli* in UVA and sunlight. *J. Appl. Microbiol.* 99, 39–50 (2005).

34 Hockberger PE, Skimina TA, Centonze VE *et al.* Activation of flavin-containing oxidases underlies light-induced production of H2O2 in mammalian cells. *Proc. Natl. Acad. Sci. USA* 96(11), 6255–6260 (1999).

• **A mechanism for inactivation of *S. aureus* by blue light is proposed in this study.**

35 Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem. Photobiol. Sci.* 3, 436–450 (2004).

36 Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *J. Antimicrob. Chemother.* 42, 13–28 (1998).

37 Maclean M, MacGregor SJ, Anderson JG *et al.* Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. *Appl. Environ. Microbiol.* 75, 1932–1937 (2009).

38 Maclean M, MacGregor SJ, Anderson JG *et al.* High-intensity narrow-spectrum light inactivation and wavelength sensitivity of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 285, 227–232 (2008).

39 Ashkenazi H, Malik Z, Harth Y *et al.* Eradication of *Propionibacterium acnes* by its endogenic porphyrins after illumination with high intensity blue light. *FEMS Immunol. Med. Microbiol.* 35, 17–24 (2003).

40 Morton CA, Scholefield RD, Whitehurst C *et al.* An open study to determine the efficacy of blue light in the treatment of mild to moderate acne. *J. Dermatolog. Treat.* 16, 219–223 (2005).

41 Papageorgiou P, Katsambas A, Chu A. Phototherapy with blue (415 nm) and red (660 nm) light in the treatment of acne vulgaris. *Br. J. Dermatol.* 142, 973–978 (2000).

42 MacGregor SJ, Rowan NJ, Mcllvaney L *et al.* Light inactivation of food-related pathogenic bacteria using a pulsed power source. *Lett. Appl. Microbiol.* 27, 67–70 (1998).

43 Anderson RR. Lasers in dermatology – a critical update. *J. Dermatol.* 27, 700–705 (2000).

44 Guffey JS, Wilborn J. *In vitro* bactericidal effects of 405-nm and 470-nm blue light. *Photomed. Laser Surg.* 24, 684–688 (2006).

45 Guffey JS, Wilborn J. Effects of combined 405-nm and 880-nm light on *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro*. *Photomed. Laser Surg.* 24, 680–683 (2006).

46 Smith KC. Laser and led photobiology. *Laser Ther.* 19, 72–78 (2010).

47 Nussbaum EL, Lilge L, Mazzulli T. Effects of 630-, 660-, 810-, and 905-nm laser irradiation delivering radiant exposure of 1–50 J/cm^2 on three species of bacteria *in vitro*. *J. Clin. Laser Med. Surg.* 20, 325–333 (2002).

48 Fonseca AS, Teixeira LB, Presta GA *et al.* Low intensity infrared laser effects on *Escherichia coli* cultures and plasmid DNA. *Laser Phys.* 22, 1635–1641 (2012).

49 Lipovsky A, Nitzan Y, Gedanken A *et al.* Visible light-induced killing of bacteria as a function of wavelength: implication for wound healing. *Lasers Surg. Med.* 42, 467–472 (2010).

50 Enwemeka CS, Williams D, Hollosi S *et al.* Visible 405 nm SLD light photo-destroys methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro*. *Lasers Surg. Med.* 40, 734–737 (2008).

51 Enwemeka CS, Williams D, Enwemeka SK *et al.* Blue 470-nm light kills methicillin-resistant *Staphylococcus aureus* (MRSA) *inxvitro*. *Photomed. Laser Surg.* 27, 221–226 (2009).

52 Feuerstein O, Persman N, Weiss EI. Phototoxic effect of visible light on *Porphyromonas gingivalis* and *Fusobacterium nucleatum*: an *in vitro* study. *Photochem. Photobiol.* 80, 412–415 (2004).

53 Lipovsky A, Nitzan Y, Friedmann H *et al.* Sensitivity of *Staphylococcus aureus* strains to broadband visible light. *Photochem. Photobiol.* 85, 255–260 (2009).

54 Steinberg D, Moreinos D, Featherstone J *et al.* Genetic and physiological effects of noncoherent visible light combined with hydrogen peroxide on *Streptococcus mutans* in biofilm. *Antimicrob. Agents Chemother.* 52, 2626–2631 (2008).

55 Basso FG, Oliveira CF, Fontana A *et al.* *In vitro* effect of low-level laser therapy on typical oral microbial biofilms. *Braz. Dent. J.* 22, 502–510 (2011).

56 Baffoni M, Bessa LJ, Grande R *et al.* Laser irradiation effect on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms isolated from venous leg ulcer. *Int. Wound J.* 9, 517–524 (2012).

57 Krespi YP, Kizhner V, Nistico L *et al.* Laser disruption and killing of methicillin-resistant *Staphylococcus aureus* biofilms. *Am. J. Otolaryngol.* 32, 198–202 (2011).

58 Song HH, Lee JK, Um HS *et al.* Phototoxic effect of blue light on the planktonic and biofilm state of anaerobic periodontal pathogens. *J. Periodontal. Implant Sci.* 43, 72–78 (2013).

59 McKenzie K, Maclean M, Timoshkin IV *et al.* Photoinactivation of bacteria attached to glass and acrylic surfaces by 405 nm light: potential application for biofilm decontamination. *Photochem. Photobiol.* 89, 927–935 (2013).

- **The reported *in vitro* activity of LLLT in reducing viability of *E. coli*, *P. aeruginosa* and *S. aureus* biofilms offers a new tool to decontaminate surfaces from biofilm.**

60 Chebath-Taub D, Steinberg D, Featherstone JD *et al.* Influence of blue light on *Streptococcus mutans* re-organization in biofilm. *J. Photochem. Photobiol. B* 116, 75–78 (2012).

61 Whelan HT, Smits RL Jr, Buchman EV *et al.* Effect of NASA light-emitting diode irradiation on wound healing. *J. Clin. Laser Med. Surg.* 19, 305–314 (2001).

62 Sekhejane PR, Houreld NN, Abrahamse H. Irradiation at 636 nm positively affects diabetic wounded and hypoxic cells *in vitro*. *Photomed. Laser Surg.* 29, 521–530 (2011).

63 Basso FG, Pansani TN, Turrioni AP *et al.* *In vitro* wound healing improvement by low-level laser therapy application in cultured gingival fibroblasts. *Int. J. Dent.* 2012, 719452 (2012).

64 Ayuk SM, Houreld NN, Abrahamse H. Collagen production in diabetic wounded fibroblasts in response to low-intensity laser irradiation at 660 nm. *Diabetes Technol. Ther.* 14, 1110–1117 (2012).

65 Hakki SS, Bozkurt SB. Effects of different setting of diode laser on the mRNA expression of growth factors and type I collagen of human gingival fibroblasts. *Lasers Med. Sci.* 27, 325–331 (2012).

66 Houreld NN, Masha RT, Abrahamse H. Low-intensity laser irradiation at 660 nm stimulates cytochrome c oxidase in stressed fibroblast cells. *Lasers Surg. Med.* 44, 429–434 (2012).

67 Liao X, Xie GH, Liu HW *et al.* Helium-neon laser irradiation promotes the proliferation and migration of human epidermal stem cells *in vitro*: proposed mechanism for enhanced wound re-epithelialization. *Photomed. Laser Surg.* doi:10.1089/pho.2013.3667 (2014) (Epub ahead of print).

68 Spitler R, Berns MW. Comparison of laser and diode sources for acceleration of *in vitro* wound healing by low-level light therapy. *J. Biomed. Opt.* 19, 38001 (2014).

69 Dancakova L, Vasilenko T, Kovac I *et al.* Low-level laser therapy with 810 nm wavelength improves skin wound healing in rats with streptozotocin-induced diabetes. *Photomed. Laser Surg* doi:10.1089/pho.2013.3586 (2014) (Epub ahead of print).

70 Fiorio FB, Albertini R, Leal-Junior EC *et al.* Effect of low-level laser therapy on types I and III collagen and inflammatory cells in rats with induced third-degree burns. *Lasers Med. Sci.* 29, 313–319 (2014).

71 Gupta A, Dai T, Hamblin MR. Effect of red and near-infrared wavelengths on low-level laser (light) therapy-induced healing of partial-thickness dermal abrasion in mice. *Lasers Med. Sci.* 29, 257–265 (2014).

72 Kajagar BM, Godhi AS, Pandit A *et al.* Efficacy of low level laser therapy on wound healing in patients with chronic diabetic foot ulcers-a randomised control trial. *Indian J. Surg.* 74(5), 359–363 (2012).

73 Nussbaum EL, Biemann I, Mustard B. Comparison of ultrasound/ultraviolet-C and laser for treatment of pressure ulcers in patients with spinal cord injury. *Phys. Ther.* 74, 812–823 (1994).

74 Nussbaum EL, Flett H, Hitzig SL *et al.* Ultraviolet-C irradiation in the management of pressure ulcers in people with spinal cord injury: a randomized, placebo-controlled trial. *Arch. Phys. Med. Rehabil.* 94, 650–659 (2013).

75 Lucas C, van Gemert MJ, de Haan RJ. Efficacy of low-level laser therapy in the management of stage III decubitus ulcers: a prospective, observer-blinded multicentre randomised clinical trial. *Lasers Med. Sci.* 18(2), 72–77 (2003).

76 Schubert V. Effects of phototherapy on pressure ulcer healing in elderly patients after a falling trauma. A prospective, randomized, controlled study. *Photodermatol. Photoimmunol. Photomed.* 17, 32–38 (2001).

77 Jawhara S, Mordon S. Monitoring of bactericidal action of laser by *in vivo* imaging of bioluminescent *E. coli* in a cutaneous wound infection. *Lasers Med. Sci.* 21, 153–159 (2006).

78 Lee SY, Seong IW, Kim JS *et al.* Enhancement of cutaneous immune response to bacterial infection after low-level light therapy with 1072 nm infrared light: a preliminary study. *J. Photochem. Photobiol. B* 105, 175–182 (2011).

79 Dai T, Gupta A, Huang YY *et al.* Blue light eliminates community-acquired methicillin-resistant *Staphylococcus aureus* in infected mouse skin abrasions. *Photomed. Laser Surg.* 31(11), 531–538 (2013).

80 Dai T, Gupta A, Huang YY *et al.* Blue light rescues mice from potentially fatal *Pseudomonas aeruginosa* burn infection: efficacy, safety, and mechanism of action. *Antimicrob. Agents Chemother.* 57(3), 1238–1245 (2013).

•• **Animal models were set up to treat infected burn wounds by blue light.**

81 Zhang Y, Zhu Y, Gupta A *et al.* Antimicrobial blue light therapy for multidrug-resistant *Acinetobacter baumannii* infection in a mouse burn model: implications for prophylaxis and treatment of combat-related wound infections. *J. Infect. Dis.* 209(12), 1963–1971 (2014).

82 Lipovsky A, Nitzan Y, Lubart R. A possible mechanism for visible light-induced wound healing. *Lasers Surg. Med.* 40, 509–514 (2008).

83 Dietel W, Pottier R, Pfister W *et al.* 5-Aminolaevulinic acid (ALA) induced formation of different fluorescent porphyrins: a study of the biosynthesis of porphyrins by bacteria of the human digestive tract. *J. Photochem. Photobiol. B* 86, 77–86 (2007).

84 Garcia-Dominguez M, Muro-Pastor MI, Reyes JC *et al.* Light-dependent regulation of cyanobacterial phytochrome expression. *J. Bacteriol.* 182, 38–44 (2000).

85 Schafer L, Sandmann M, Woitsch S *et al.* Coordinate up-regulation of carotenoid biosynthesis as a response to light stress in *Synechococcus* PCC7942. *Plant Cell Environ.* 29, 1349–1356 (2006).