Preliminary data on the efficacy of multi-wave (multi-wavelength) diode laser on bacteria in superficial canine pyoderma.

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INTRODUCTION

Superficial canine pyoderma is a common bacterial skin infection which affects the superficial portion of the hair follicle (Bajwa, 2016; Baumer, 2017). In dogs, this condition is associated with *Staphylococcus pseudintermedius* as predominant pathogen. Other bacteria can be also isolated, such as E. coli and others (Rantala, 2004). Antibiotic resistance is observed for *Staphylococcus pseudintermedius* for the presence of transposon-borne resistance gene which are incorporated into the chromosomal DNA; other mechanism of resistance in Staphylococci and Gram negative bacteria include plasmid-borne resistance genes. Resistance genes can be easily acquired and transferred in the bacterial population. Superficial bacterial folliculitis often tends to become a recurrent condition for inappropriate therapy (drugs used, duration of treatment), lack of diagnostics, development of methicillin resistance in the staphylococci population.

It is therefore of critical importance the application of a correct diagnostic protocol which include cytology, bacterial culture and antibiotic sensitivity evaluation.

Since the acquisition of antimicrobial resistance has become a common condition in the bacteria causing superficial canine pyoderma, therapeutic options usually require novel approaches.

At date, guidelines for antimicrobial therapy for canine superficial bacterial folliculitis include a combination of both topical and systemic antibiotic therapy (Hillier, 2014).

In the initial step of the therapeutic treatment, topical therapy is considered a good approach, when lesions are localized, or in the early stages of generalized superficial bacterial folliculitis (SBF) when lesions are mild. Moreover, local therapy can help prevent recurrence (Bajwa, 2016).

Laser therapy is an alternative treatment, since it has reported to be effective in the management of bacterial dermatitis. Laser therapy may help the homeostasis of host tissue, but beside that, a direct activity on pathogen survival, host inflammatory response and repair mechanisms have been demonstrated.

This study had a dual purpose: the first was to evaluate the ability of Near Infrared (NIR) laser emission (MLS® - Multiwave Locked System laser, Mphi, ASA Srl, Arcugnano, Italy) to decrease the bacterial load present in the skin lesions of the examined dogs, before and after the in vivo laser treatment; the latter was to evaluate the *in vitro* bactericidal activity of the laser treatment on the isolated bacterial strains, after direct irradiation of the bacterial suspension in log phase of growth.

MATERIAL AND METHODS

Experimental design

This preliminary study was carried out on a small group of patients (4 dogs). The study was conceived as a two-step work. In the first step, the bacterial load present in the superficial pyoderma lesions of the dogs was evaluated before and after laser treatment. Moreover, the bacterial agents collected from the superficial pyoderma lesions of the patients were identified. Skin swabs were collected before (T0) and after 30 minutes from multi-wave diode laser (MLS®) treatment (T30). Bacteriological culture, strain identification and CFU counts were performed. On each bacterial strain an evaluation of antibiotic sensitivity was performed by Kirby-Bauer agar disk

diffusion test.
The second step of the study was aimed to evaluate, on four of the previous bacterial isolates, the direct in vitro antimicrobial activity of NIR laser treatment (MLS®). This step was performed by evaluating the CFU count of a standardized bacterial suspension before and after laser irradiation.

Enrollment of patients
Dogs presenting clinical signs of superficial generalized pyoderma were examined by the clinician (Clinica Veterinaria Malpensa, Varese, Italy), who collected swabs from skin lesions to investigate the microbiological flora. Cytological examination of each lesion was also performed and only 4 patients with evidence of Gram positive cocci were included in the study.

Laser source
Details of the laser source have been previously described [Monici et al., 2013]. Briefly, the treatments were performed with a Multiwave Locked System (MLS®) Laser (Mphi, ASA Srl, Arcugnano, Italy), a commercial laser source built in compliance with EC/EU rules, cleared by the US Food and Drug Administration (FDA).
MLS laser is a class IV NIR laser with two synchronized sources (laser diodes). The two sources have different wavelength, peak power and emission mode. The first one is a pulsed 905nm laser diode with 25 W peak optical power. The pulse frequency may be varied in the range 1-2000 Hz, thus varying the average power delivered to the tissue. The second laser diode (808nm) may operate in continuous (power 1.1 W) or frequenced (repetition rate 1-2000 Hz, 550mW mean optical power) mode, with a 50% duty ratio independently of the repetition rate. The two sources emit radiation synchronously and the propagation axes of the two laser beams are coincident.

In vivo laser treatment and skin swabs collection
After collection of skin swabs (T0), each patient was treated by MLS® laser, using the specific program "contaminated wounds", with a radiant exposure of 2.05J/cm^2. The laser exposure in each of the 6 treated points was of 6 seconds. After 30 minutes from the laser exposure, other swabs were collected from the skin lesions for the microbiological cultures. All swabs were maintained at 4°C and conferred to the Infectious Diseases laboratory of the Department of Veterinary Science, University of Parma, within 24 hours.

Evaluation of antimicrobial activity of in vivo MLS® laser treatment
Each swab was vortexed in 1ml of sterile saline and this suspension further diluted 1:10 and 1:100. One hundred microliters of each dilution were plated on Columbia blood agar with 5% of bovine erythrocytes and MacConkey agar and incubated overnight at 37°C. After incubation, bacterial growth was evaluated and colonies were isolated and amplified when necessary. Identification of bacterial strains was based on colony morphology, Gram staining, catalase and oxidase reactions. Species identification was carried out using the API biochemical test system (bioMérieux, France), as well as conventional biochemical tests (Markey BK, 2013). Antimicrobial susceptibility test was performed by agar disk diffusion methods (Bauwe AW, 1966) on each isolated bacterial strain, according with the CLSI guidelines (Clinical Laboratory Standards Institute, 2015). Tested antibiotics were selected on the basis of the clinical use in the treatment of canine pyoderma. This list included Amikacin; Amoxicillin + Clavulanic acid; Ampicillin; Cephalexin; Cefovecin; Clindamycin; Chloramphenicol; Doxycycline; Enrofloxacin; Imipenem; Marbofloxacin; Oxytetracycline; Oxacillin; Sulfamethoxazole + Trimethoprim; Rifaximin; Rifampin.

To determine the antimicrobial activity of the MLS® laser treatment in vivo, samples collected before and after treatment were compared. For each sample and each isolated bacterial strain, CFU were counted.

Evaluation of antimicrobial activity of direct in vitro MLS® laser treatment
To evaluate the direct bactericidal activity of the MLS® laser treatment, in vitro tests were performed. Briefly, four of the isolated bacterial strains were inoculated in sterile Mueller Hinton Broth and incubated overnight at 37°C. The bacterial suspension was centrifuged 20 minutes at 2000 rpm and 4°C, then the pellet resuspended in phosphate buffer. The turbidity of the bacterial suspension was immediately measured and adjusted by spectrophotometry. At 600 nm absorbance, the OD range 0.08-0.13 was considered to correspond to a bacterial concentration of 108CFU/ml. The obtained suspension was further diluted 1:200 in phosphate buffer to obtain a final bacterial concentration of 5*105CFU/ml. Then, 500µl of the bacterial suspension were transferred in a 24 wells plate, according to Tab. 1. A black paper was placed between contiguous wells to avoid the transmission of laser radiation through the walls .
The bacterial suspensions were irradiated with two different exposure times (6 and 41 sec) with a radiant exposure of 2.05 J/cm^2. The laser source was placed in the biosafety cabinet and fixed by a mechanical arm, which maintained the laser source from a vertical distance of about 0.5 cm from the top of the plate well containing the bacterial suspension.
After the treatment, plates were incubated at room temperature for two different period (5 and 30 min). These incubation times were selected to understand if the direct application of laser treatment required some time to cause bacteria killing, depending on the bacterial structure and/or the parameters used in laser irradiation.

Then, for each incubation time, 10 µl of the irradiated bacterial suspension and its 1:10 dilution were plated on Mueller Hinton agar plates. Sterility and growth controls were also diluted and plated in the same way. Agar plates were incubated at 37°C overnight, then bacterial colonies (CFU) were counted. Each test was performed in triplicates and three independent experiments.

Table I - Scheme of the plate used for one experiment for the determination of the direct antimicrobial activity of MLS® laser on different bacterial strains. Each test was performed in three replicates and three independent experiments.

					Sterility control
	1st replicate		3rd replicate		1st growth control
		2nd replicate			2nd growth control
					3rd growth control

Table II - Bacterial isolates and CFU count before and after MLS® laser treatment.

		PRE- TREATMENT	POST- TREATMENT	Pre-treatment Total CFU count	Post-treatment Total CFU count	Overall inhibition percentage
Case 1	*Staphylococcus pseudintermedius*	59.8*10⁴ CFU/ml	3*10⁴ CFU/ml	**59,8*10⁴ CFU/ml**	**3*10⁴ CFU/ml**	**95%**
Case 2	*Staphylococcus pseudintermedius*	0,28*10⁴ CFU/ml	0.22*10⁴ CFU/ml	**6*10⁴ CFU/ml**	**0,56*10⁴ CFU/ml**	**90,6%**
	Proteus mirabilis swarming	4.5*10⁴ CFU/ml	0.035*10⁴ CFU/ml			
	Proteus mirabilis not swarming	1.1*10⁴ CFU/ml	0.28*10⁴ CFU/ml			
	Escherichia coli	0.125*10⁴ CFU/ml	0.02*10⁴ CFU/ml			
Case 3	*Staphylococcus pseudintermedius*	273*10⁴ CFU/ml	269*10⁴ CFU/ml	**2,75*10⁶ CFU/ml**	**2,69*10⁶ CFU/ml**	**2,2%**
	Pseudomonas fluorescens	2.29*10⁴ CFU/ml	0 CFU/ml			
Case 4	*Staphylococcus pseudintermedius*	28200*10⁴ CFU/ml	22700*10⁴ CFU/ml	**2,82*10⁸ CFU/ml**	**2,27*10⁸ CFU/ml**	**19,5%**

RESULTS

Evaluation of antimicrobial activity of *in vivo* MLS® laser treatment

Four clinical cases were collected.

Case n. 1

The first was a Bull Terrier male dog, eight years old, affected by a generalized superficial pyoderma with crusts and papules localized on legs, trunk and head. From each swab, a pure culture of *Staphylococcus pseudintermedius* was isolated.

Case n. 2

The second clinical case was a Great Dane male dog, seven years old, affected by a generalized superficial pyoderma. From each swab, *Staphylococcus pseudintermedius*, *Proteus mirabilis* (swarming and not) and *Escherichia coli* were isolated.

Case n. 3

The third case was a West Highland White Terrier male dog, four years old, affected by generalized superficial pyoderma with crusts and papules localized on legs, trunk and head. From the pre-treatment swab *Staphylococcus pseudintermedius* and *Pseudomonas fluorescens* were isolated, while from post-treatment swab only pure culture of *Staphylococcus pseudintermedius* was isolated.

Table III - Antimicrobial susceptibility test.

		Sensitive	Intermediate	Resistant
Case 1	*Staphylococcus pseudintermedius*	Amikacin; Amoxicillin + Clavulanic acid; Cephalexin; Cefovecin; Clindamycin; Chloramphenicol; Doxycycline; Enrofloxacin; Imipenem; Marbofloxacin; Oxytetracycline; Oxacillin; Rifaximin; Rifampin	Ampicillin	Sulfamethoxazole + Trimethoprim
Case 2	*Staphylococcus pseudintermedius*	Amoxicillin + Clavulanic acid; Cephalexin; Cefovecin; Chloramphenicol; Doxycycline; Enrofloxacin; Imipenem; Marbofloxacin; Oxacillin; Rifaximin; Rifampin	Amikacin; Ampicillin; Clindamycin	Oxytetracycline; Sulfamethoxazole + Trimethoprim
	Proteus mirabilis swarming	Amoxicillin + Clavulanic acid; Ampicillin; Cephalexin; Cefovecin; Chloramphenicol; Enrofloxacin; Imipenem; Marbofloxacin; Rifaximin; Sulfamethoxazole + Trimethoprim		Amikacin; Clindamycin; Doxycycline; Oxytetracycline; Oxacillin; Rifampin
	Proteus mirabilis not swarming	Amoxicillin + Clavulanic acid; Ampicillin; Cephalexin; Cefovecin; Chloramphenicol; Enrofloxacin; Imipenem; Marbofloxacin; Rifaximin; Rifampin; Sulfamethoxazole + Trimethoprim	Doxycycline	Amikacin; Clindamycin; Oxytetracycline; Oxacillin
	Escherichia coli	Amoxicillin + Clavulanic acid; Ampicillin; Cephalexin; Cefovecin; Chloramphenicol; Enrofloxacin; Imipenem; Marbofloxacin; Oxytetracycline; Sulfamethoxazole + Trimethoprim	Rifaximin	Amikacin; Clindamycin; Doxycycline; Oxacillin; Rifampin
Case 3	*Staphylococcus pseudintermedius*	Amoxicillin + Clavulanic acid; Ampicillin; Cephalexin; Chloramphenicol; Enrofloxacin; ; Marbofloxacin; Oxacillin; Rifaximin; Rifampin; Sulfamethoxazole + Trimethoprim	Amikacin; Cefovecin; Clindamycin; Imipenem	Oxytetracycline; Doxycycline
	Pseudomonas fluorescens	Enrofloxacin; Imipenem; Oxytetracycline	Amikacin; Amoxicillin + Clavulanic acid; Chloramphenicol; Marbofloxacin; Doxycycline	Ampicillin; Cephalexin; Cefovecin; Clindamycin; Oxacillin; Rifampin; Sulfamethoxazole + Trimethoprim
Case 4	*Staphylococcus pseudintermedius*	Amoxicillin + Clavulanic acid; Cephalexin; Cefovecin; Doxycycline; Enrofloxacin; Imipenem; Marbofloxacin; Oxacillin; Rifaximin; Rifampin	Amikacin	Ampicillin; Clindamycin; Chloramphenicol; Oxytetracycline; Sulfamethoxazole + Trimethoprim

Table IV - Antimicrobial activity of direct irradiation of bacterial isolates.

		Seconds of exposition	Minutes post- exposition	Laser (CFU/ml)	Growth control (CFU/ml)	% Inhibition
Bacterial strains	*Staphylococcus pseudintermedius* **Case 1**	6	5	1.17±0.20*10^5	1.31±0.18*10^5	11%
			30	1.05±0.30*10^5	1.05±0.30*10^5	0%
		41	5	1.04±0.05*10^5	1.13±0.13*10^5	8%
			30	1.51±0.99*10^5	1.51±0.99*10^5	0%
	Staphylococcus pseudintermedius **Case 2**	6	5	0.67±1.70*10^5	0.67±1.70*10^5	0%
			30	0.63±1.49*10^5	0.63±1.49*10^5	0%
		41	5	0.26±0.04*10^5	0.26±0.04*10^5	0%
			30	0.27±0.38*10^5	0.27±0.38*10^5	0%
	Escherichia coli **Case 2**	6	5	3.31±0.37*10^5	3.63±0.42*10^5	9%
			30	3.36±0.09*10^5	3.39±0.25*10^5	1%
		41	5	2.36±0.97*10^5	2.56±1.22*10^5	8%
			30	2.25±0.94*10^5	2.25±0.94*10^5	0%
	Pseudomonas fluorescens **Case 3**	6	5	1.64±0.17*10^5	1.67±0.14*10^5	2%
			30	1.59±0.45*10^5	1.59±0.45*10^5	0%
		41	5	2.28±1.63*10^5	2.35±1.71*10^5	3%
			30	2.05±1.61*10^5	2.21±1.56*10^5	7%

Case n. 4
The fourth case was a Poodle male dog, seven years old, affected by generalized superficial pyoderma with crusts and papules. From each swab *Staphylococcus pseudintermedius* was isolated.
CFU count was performed for each sample and each bacterial isolate. Results are shown in Table 2. and each bacterial isolate. Results are shown in Table 2.
Antimicrobial susceptibility test was performed on each isolated bacterial strain. Results are shown in Table 3.

Evaluation of antimicrobial activity of direct in vitro MLS® laser treatment
In the second step of the experimental design, four bacterial strains from clinical cases were selected for the evaluation of direct in vitro antimicrobial activity of MLS® laser, on the basis of their clinical importance (two Gram positive and two Gram negative).

Strain 1: *Staphylococcus pseudintermedius* isolated from Case 1. Five minutes after laser treatment (irradiation time of six seconds) growth inhibition percentage was 11%, while after thirty minutes inhibition was 0%. The same bacterial

strain subjected to a laser exposure of 41 seconds showed 8% of growth inhibition after incubation time of five minutes, while after thirty minutes inhibition was 0%.

Strain 2: *Staphylococcus pseudintermedius* isolated from Case 2. This strain did not show any growth inhibition, both after five minutes and thirty minutes of incubation from the irradiation step.

Strain 3: *Escherichia coli* isolated from Case 2. This strain showed growth inhibition after laser treatment of six seconds, both after five minutes and thirty minutes of incubation (9% and 1%, respectively), while laser irradiation of 41 seconds resulted in 8% inhibition of growth, only five minutes post-treatment.

Strain 4: *Pseudomonas fluorescens* isolated from Case 3. This strain showed an inhibition of growth equal to 2% in the 6 seconds laser-treatment mode, after five minutes incubation, while none inhibition was observed after thirty minutes. Furthermore, inhibition was of 3% and 7%, respectively, after five and thirty minutes of incubation from laser-treatment of 41 seconds.
All the results of the direct irradiation of bacterial strains are shown in Table 4.

DISCUSSION

As expected, from all the clinical cases of superficial pyoderma the most isolated strain was *Staphylococcus pseudintermedius*. Other bacterial isolates (Pseudomonas fluorescens, E. coli, Proteus spp.) can be considered opportunistic, co-infectious agents. Multi-drug resistance was observed mostly in Gram negative isolates (*Pseudomonas*, Proteus, E. coli).
Following in vivo laser treatment on the skin lesions, significant results in bacterial growth inhibition, demonstrated by CFU count, were observed on the bacterial flora load isolated after 30 minutes from laser application. In particular, a significant reduction of CFU count was observed for all isolates, except the three strains of *Staphylococcus pseudintermedius* isolated from cases 2, 3 and 4. A higher effectiveness on Gram negative isolates was observed, according to the literature, where it is reported that laser antimicrobial activity depends on bacterial species. This may be attributable to the peculiar structure of Gram positive and Gram negative bacterial membranes and wall (Schoop, 2004). Gram positive wall is thicker, being structured in an outer cytoplasmic lipid membrane, thick peptidoglycan layer, lipoteichoic acids and a smaller periplasm than in Gram negative bacteria. Other studies have confirmed that structural configuration of the cell wall affects bacterial susceptibility to laser irradiation. In different conditions, several cycles of laser irradiation are required to affect Gram-positive bacteria; whereas, Gram-negative bacteria are eliminated faster and more easily (Moritz et al, 2000). Moreover, it has been demonstrated that differences in the wavelengths, power, irradiation time, spot size and number of cycles are responsible for the variable efficacy of lasers reported in literature (De Paula, 2001).
The anti-bacterial effect of laser-therapy has been widely described in literature. IGAlAr laser emission was used against *Staphylococcus aureus* strains, and the results revealed reduction in the number of colonies (Wilson et al, 1995). In humans it was studied the effect of laser-therapy with the wavelengths 630, 660, 810 nm and 1-50 J/cm^2 fluence on *Staphylococcus aureus, Pseudomonas, and E. coli* which were collected from infected wounds (Nussbaum et al, 2002). A significantly reduction in the growth of bacteria was obtained in a study on diabetic wound healing in rats suffering from bacterial infection induced by *Staphylococcus aureus* (Ranjbar, 2016). A Diode laser source was used on dental implants in order to avoid microbial platelet aggregation (Kreisler et al, 2002). Moreover, an antibacterial effect on third-degree burn wounds was proved (Bayat et al, 2006). It has been also suggested that the anti-inflammatory action of laser therapy in wounds, skin lesions and infectious diseases could be partly due to the reduction of the bacterial load at the lesion site, thus reducing a major cause of inflammation (Meyerholz et al, 2009; Silva, 2013).
However, the results obtained in the second phase of the present study, where the antimicrobial effect of direct in vitro NIR laser (MLS®) treatment was evaluated, demonstrated a limited microbicidal activity. The growth inhibition was observed mostly with 6 sec exposure and 5 min incubation time. The findings suggest that there is no direct correlation between effectiveness and exposure time: in one case only a slight increase in growth inhibition was observed with increasing exposure time.
This result suggests that the damage produced by laser treatment on the bacterial population is slight, therefore it can be detected immediately after treatment but is easily diluted over a short time.
It was beyond the scope of this study to evaluate the effect of laser treatment on canine pyoderma from the clinical point of view. In fact, the clinical protocol for this application of MLS® therapy foresees several weekly sessions for a few weeks. Therefore, in this study, which was based on a single *in vivo* irradiation, the host biological response to laser treatment was not studied.
However, the higher antimicrobial activity observed *in vivo* in comparison with the in vitro irradiation suggests that *in vivo* laser irradiation induces a complex response which involves both microorganisms and host tissues. The final effects depend on the absorption of laser radiation by the host tissues and consequent biological response, which involves the immune cells

and their interaction with microrganisms (Clemente et al., 2015), the modulation of inflammation (Monici et al., 2013, Squarzoni et al, 2017) the enhancement of anabolic processes in the tissues and activation of repair mechanisms (Monici et al., 2013).

Beyond the complexity of the *in vivo* biological response, another explanation for the lower effectiveness of laser treatment in inhibiting bacterial growth *in vitro* could be the fluence (2.05J/cm^2), which had deliberately been kept the same as that used *in vivo*.

However, the absorption of laser radiation in the tissues is greater than that in bacterial suspension *in vitro*. So, higher fluences are probably required *in vitro*. This hypothesis is in agreement with what has been observed applying laser therapy to inhibit fungal growth. After trying different energy densities, the maximum effect in terms of mortality rate in *Candida albicans* suspensions treated *in vitro* was obtained with 7J/cm^2 fluence (Clemente et al., 2015). When considering the treatment of oral mucositis *in vivo*, very significant results are reported also with very low fluence (0.16J/cm2) (Squarzoni et al., 2017).

CONCLUSION

In conclusion, this study shows that a single application of laser therapy carried out by MLS device effectively reduced the microbial load of skin lesions in superficial canine pyoderma. The antimicrobial action was higher on the Gram negative bacteria than on the Gram positive ones.

This effect was not reproduced by *in vitro* irradiation of bacterial suspensions prepared from skin swabs collected before *in vivo* laser treatment of patients affected by superficial canine pyoderma.

In vitro, only a slight inhibition of bacterial growth was observed immediately after the treatment. The different effectiveness shown in *in vivo* and *in vitro* conditions could be explained with:

1) a higher absorption of laser radiation by the host tissues in comparison with bacterial suspensions (this implies that higher doses are required *in vitro*);

2) a more complex biological response induced by the *in vivo* irradiation, involving the interaction with the host tissues.

In conclusion, in-depth studies on the application of laser therapy in the management of canine skin infections are required and further investigations are necessary, considering a wider bacterial sample and varying lasers parameters to maximize their efficacy *in vitro*.

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