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Original article

# *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* Lubbock Chronic Wound Biofilm (LCWB): a suitable dual-species model for *in vitro* studies

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ABSTRACT

Antimicrobial treatment of methicillin-resistant *Staphylococcus pseudintermedius* associated with canine wounds represents an important challenge. The aim of this study was to create a canine wound infection model, Lubbock Chronic Wound Biofilm (LCWB), with a focus on *S. pseudintermedius*, drawing inspiration from the established human model involving *Staphylococcus aureus*. Methicillin-resistant *S. pseudintermedius* 115 (MRSP) and *Pseudomonas aeruginosa* 700 strains, isolated from dog wounds, were used to set up the LCWB at 24, 48 and 72 h. The LCWBs were evaluated in terms of volume, weight, and microbial CFU/mg. The microbial spatial distribution in the LCWBs was assessed by SEM and CLSM imaging. The best incubation time for the LCWB production in terms of volume (3.38 $cm^3 \pm 0.13$), weight (0.86 gr ± 0.02) and CFU/mg (up to $7.05 \times 10^6$ CFU/mg $\pm 2.89 \times 10^5$) was 48 h. The SEM and CLSM images showed a major viable microbial colonization at 48 h with non-mixed bacteria with a prevalence of MRSP on the surface and *P. aeruginosa* 700 in the depth of the wound. The obtained findings demonstrate the capability of *S. pseudintermedius* to grow together *P. aeruginosa* in the LCWB model, representing the suitable model to reproduce the animal chronic wound *in vitro*.



The antimicrobial resistance (AMR) represents a global health challenge affecting the traditional treatments by increasing the severity, the incidence and the cost of infections [1]. The selective pressure due to the use and abuse of antimicrobials in different fields such as in human medicine, for prevention, control and/or treatment, in agriculture and in animals to fight the frequent outbreaks in intensive farming, has significantly contributed to this worrying phenomenon. It has been estimated that approximately 75% of human infectious diseases, that have emerged or re-emerged in recent decades, are of zoonotic origin [2] and the dynamic animal-human transmission of resistant microorganisms increases the spread of antimicrobial resistance [3]. In particular, the antimicrobial use in animals is an important contributor to antimicrobial resistance among human pathogens, such as *Salmonella* spp., *Campylobacter* spp., *Enterococcus* spp., and *E. coli* and, in some cases, other bacteria that can also be zoonotic, e.g., *Staphylococcus aureus* [3] and *Staphylococcus pseudintermedius*. The One Health approach is necessary to effectively address the AMR problem, embracing the environmental health as well as human and animal health [4].

*Staphylococcus pseudintermedius*, a coagulase-positive bacterium, is a member of the *Staphylococcus intermedius* group (SIG), together with *Staphylococcus intermedius*, *Staphylococcus delphini*, *Staphylococcus ursi*, and *Staphylococcus cornubiensis* [5]. This strain, routinely isolated in the normal microbial community of dogs and cats, is also considered an emerging zoonotic pathogen, responsible for several diseases, especially skin lesions and soft-tissue infections in dogs and cats, representing the major strain isolated in wound infections [6]. Moreover, methicillin-resistant *S. pseudintermedius* (MRSP) strain is commonly found on the skin of dogs and cats, as well as of humans that often harbour methicillin-resistant *S. aureus* (MRSA) strain on their skin [5]. Methicillin resistance in these strains is mediated by altered form of

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penicillin binding protein (PBP), encoded by *mecA* gene [7,8]. While *S. aureus* and *S. pseudintermedius* share a relationship, it is crucial to note that MRSA and MRSP are distinctly different entities. MRSP and MRSA, referred to as "superbugs," exhibit resistance to many commonly used antibiotics, making them more difficult to effectively treat.

While humans are not the primary hosts for *S. pseudintermedius*, transient colonization, including MRSP, can occur, and humans colonised with *S. pseudintermedius* may serve as a vehicle for transmission between animals. In particular, individuals with compromised immune systems, such as those undergoing cancer treatment or suffering from immune diseases, face a higher risk. MRSP and MRSA pose significant concerns as zoonotic opportunistic pathogens in both veterinary medicine and public health. Hospital-acquired infections and zoonoses are on the rise, an example being the worrying number of MRSP cases among dogs, pet owners and veterinary staff [9]. Therefore, the clinical importance of *S. pseudintermedius* or MRSP transmission from dogs to humans should not be underestimated [10]. The increasing prevalence of multidrug-resistant *S. pseudintermedius* strains poses a significant challenge in the field of veterinary medicine, and considering the intimate connection between dogs and humans, *S. pseudintermedius* emerges as a significant candidate for One Health initiatives taking into account that the MRSP multidrug resistance profiles have been reported to be more severe than those of MRSA [11,12].

In our previous works, we validated an *in vitro* wound infection model, the Lubbock Chronic Wound Biofilm (LCWB), to study the poly-microbial sessile colonization in human wound infections. The LCWB is a complex inter-kingdom poly-microbial biofilm, including the main pathogens isolated in wound infections, such as *S. aureus, Pseudomonas aeruginosa,* and *Candida albicans*, that mimics the real microbial spatial distribution in wounds. The presence of red blood cells, plasma and nutrients, mimicking the wound bed environment, promotes microbial growth, reproducing the spatial distribution in a human-like environment [13–15]. This *in vitro* model is widely recognized as more closely resembling the *in vivo* human-like-wound environment due to the wound simulating medium, the fibrin network produced by *S. aureus,* the 3D structure, the realistic nutrient, host matrix, several chosen species, flow, grown on a solid surface and the oxygen gradient [13,16]. This suitable *in vitro* model represents a pivotal preliminary screen useful to translate into *in vivo* evaluations [16].

Based on these considerations, this study aimed to create a canine wound infection model with a focus on *S. pseudintermedius,* drawing inspiration from the established human model involving *S. aureus*. Furthermore, the selection of the *S. pseudintermedius* and *P. aeruginosa* combination for creating a canine wound infection model was based on the frequent isolation of these two bacteria in most canine skin infections. To the best of our knowledge, this is the first study that sets up the LCWB with canine *S. pseudintermedius* strain to propose an *in vitro* animal wound model that, prior to *in vivo* analysis, offers a suitable model for the evaluation of new antimicrobial strategies.

## 1. Materials and methods

### 1.1. Bacterial strains

Clinical isolates of *S. pseudintermedius* and *P. aeruginosa* identified respectively as #115 and #700 were selected from the bacterial culture stock stored at −80 °C in Microbank™ vials (Pro-lab Diagnostics, Richmond Hill, ON, Canada) of the Microbiology Laboratory of the Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Italy. The strains were isolated from dogs, which were referred for skin disorders to the University Veterinary Teaching Hospital of the above-mentioned department. The strains were recovered by plating *S. pseudintermedius* 115 on Columbia Nalidixic Acid (CNA) with 5% sheep blood agar medium and *P. aeruginosa* 700 on Mac Conkey agar plate. The plates were purchased from Liofilchem (Teramo, Italy). After the overnight incubation at 37 °C, colonies recovered in pure cultures, were firstly evaluated for their morphologic and fermentative features, and then subjected to Gram's Staining and to the standard, rapid screening tests of catalase (BioMérieux, Marcy l'Etoile, France) and oxidase (Liofilchem, Teramo, Italy). Staphylocoagulase test was also performed on *Staphylococcus* spp. presumptive colonies grown on CNA plate. Afterwards, the collected isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonik, Germany) following the manufacturer's instructions. Precisely, according to Bruker biotyper's guidelines, a score of ≥2.0 designated highly probable species-level identification, a score from 1.70 to 1.99 indicated a secure identification to the genus level, while a score of <1.7 was considered as no identification.

*S. pseudintermedius* ATCC®49444™ and *P. aeruginosa* ATCC® 27853 were used as quality control strains.

Furthermore, confirmation of *S. pseudintermedius* identification was carried out by single PCR for the species-specific thermonuclease *nuc* [17] and β-haemolysin *hlb* [18] genes. *S. pseudintermedius* ATCC®49444™ was used as positive control.

### 1.2. Antimicrobial susceptibility testing

Antimicrobial resistance profiles were examined by Kirby Bauer disc diffusion method on Mueller Hinton agar plates (Liofilchem, Teramo, Italy). For *S. pseudintermedius* the following antimicrobials were tested: amoxicillin-clavulanate (AMC, 20/10 μg), ampicillin (AMP, 10 μg), cefoxitin (FOX, 30 μg), cephalothin (KF, 30 μg), ceftriaxone (CRO, 30 μg), clindamycin (CD, 2 μg), ciprofloxacin (CIP, 5 μg), erythromycin (E, 15 μg), enrofloxacin (ENR, 5 μg), gentamicin (CN, 10 μg), imipenem (IMI, 10 μg), linezolid (LNZ, 30 μg), penicillin (P, 10 IU), streptomycin (S, 10 μg), sulfamethoxazole-trimethoprim (SXT, 23.75/1.25 μg), tetracycline (TE, 30 μg), vancomycin (VA, 30 μg). Moreover, susceptibility to oxacillin (OX, 1 μg) was assessed as marker for methicillin resistance. *S. pseudintermedius* strain was classified as susceptible (S), intermediate (I) or resistant (R) according to the Clinical and Laboratory Standards Institute guidelines [19]. For streptomycin and vancomycin, breakpoints employed were those recommended by the French Society for Microbiology.

*P. aeruginosa* antimicrobial resistance profile was evaluated against 12 antimicrobials agreeing with the European Committee on Antimicrobial Susceptibility Testing criteria [20]: amikacin (AK, 30 μg), aztreonam (ATM, 30 μg), ceftazidime (CAZ, 30 μg), ceftriaxone (CRO, 30 μg), ciprofloxacin (CIP, 5 μg), gentamicin (CN, 10 μg), imipenem (IMI, 10 μg), marbofloxacin (MAR, 5 μg), meropenem (MRP, 10 μg), piperacillin-tazobactam (TPZ, 110 μg), sulfamethoxazole-trimethoprim (SXT, 23.75/1.25 μg), tobramycin (TOB, 5 μg).

Multidrug resistance in selected *S. pseudintermedius* and *P. aeruginosa* was characterized based on criteria defined by Magiorakos et al. [21], when showing resistance to at least three different antimicrobial classes.

### 1.3. Genotypic characterization of antibiotic resistance

DNA extraction was done from *S. pseudintermedius* 115 and *P. aeruginosa* 700 strains by using the commercial Isolate II Genomic DNA Kit (Bioline, London, UK) following the manufacturer's

instructions. *S. pseudintermedius* 115 was characterized in our previous work [22].

The presence of extended spectrum-β-lactamase $bla_{CTX-M}$, $bla_{TEM}$, $bla_{SHV}$, $bla_{PER}$ genes [23–26] and metallo-β-lactamase $bla_{OXA-48}$, $bla_{IMP}$, $bla_{VIM}$, $bla_{NDM}$, $bla_{GES}$ genes [26–28] was investigated by PCR in selected *P. aeruginosa* 700 strain. *P. aeruginosa* ATCC® 27853 was used as positive control strain for ESBL and MBL genes.

### 1.4. LCWB set up

LCWB was prepared according to Di Giulio et al. [13] with *S. pseudintermedius* 115 and *P. aeruginosa* 700 that were isolated from dog wounds. The strains were standardized at optical density (OD) at 600 nm = 0.125 and diluted to obtain the final concentration of $10^6$ CFU/ml and $10^5$ CFU/ml *S. pseudintermedius* 115 and *P. aeruginosa* 700, respectively. For the LCWB preparation, 5 ml of medium containing Brucella Broth (BB, Oxoid, Milan, Italy) with 0.1% agar bacteriological, 50% porcine plasma (Sigma Aldrich, Milan, Italy), 5% horse erythrocytes (BBL, Microbiology System, Milan, Italy) and 2% Fetal Calf serum (Biolife Italiana, Milan, Italy) were distributed into glass sterile tubes. Then, 10 μl of each diluted broth culture were inoculated into glass tubes with sterile pipette tips and incubated at 37 °C in aerobic condition for 24, 48 and 72 h. After each contact time, the biofilm was harvested from glass tube, the pipette tip was removed and the LCWB volumes ($V = \pi \times r^2 \times h$), the LCWB weight and the *S. pseudintermedius* 115 and *P. aeruginosa* 700 CFU/mg were determined. For CFU/mg determination in the LCWB, harvested and dried biofilm was vortexed for 2 min, sonicated for 3 min (with ultrasound bath) and vortexed for other 2 min. Live/Dead staining was used to confirm the effect of this procedure in terms of disaggregating action and the cell viability retaining. The CFU/mg was determined by spreading the serial dilution on Mannitol Salt Agar (MSA, Oxoid, Milan, Italy) and Cetrimide Agar (CET, Oxoid, Milan, Italy) and incubated at 37 °C for 24–48 h.

### 1.5. Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) observations

To evaluate the spatial microbial distribution and the LCWB structure, Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) observations were performed.

For the SEM analysis, the biofilms were fixed in glutaraldehyde solution, dehydrated in ethanol solutions and then immersed in hexamethyldisilazane (HMDS, Sigma–Aldrich, Milan, Italy). The dried samples were subjected to the gold-sputtering with a Desk Sputter Coater (Phenom-WorldB.V., Dillenburgstra at 97 Eindhoven, 5652, AM, Netherlands) and then observed under SEM (Phenom-WorldB.V., Dillenburgstra at 97 Eindhoven, 5652, AM, Netherlands) at different magnifications [14].

For CLSM, the samples were washed in PBS and then stained with Live/Dead Bac Light viability kit (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min at room temperature. Samples were then examined using a Zeiss LSM800 microscope (CarlZeiss, Jena, Germany) coupled to an inverted microscope Axio-observer D1 (CarlZeiss, Jena, Germany) equipped with a Plan Neofluaroil-immersion objective (100×/1.45NA). The green and red emissions (SYTO9 and propidium iodide) were excited using the 488 nm setting (4% of potency) of an argon laser and a helium/neon 543 nm source (2.5% of potency). To separate the fluorescence emissions, HTF 488/543 and NTF 545 as primary and secondary dichroic mirrors, respectively, were used. Detector band-pass filters were set over 505–530 and 565–615 ranges for the green and red emissions, respectively. Images were alternatively recorded using the multitrack acquisition [15].

### 1.6. Statistical analysis

Statistical evaluation was performed by using SPSS for Windows version 21 (IBM SPSS Inc., Chicago, IL, USA). Analysis of variance (One-way ANOVA) and LSD test were used to compare the parameters analyzed in the study. The significance threshold was set at 0.05. Data are shown as the mean value ± standard deviation (S.D.). All data were obtained from three independent experiments performed at least in triplicate.

## 2. Results

### 2.1. Strains identification and antimicrobial resistance profiles

The identification performed by MALDI-TOF-MS gave a log (score) of ≥2.3 and 2.0 for *S. pseudintermedius* 115 and *P. aeruginosa* 700, respectively. *S. pseudintermedius* 115 proteomic identification was further confirmed by molecular investigation by the presence of species-specific *nuc* and *hlb* genes. Moreover, *S. pseudintermedius* 115 resulted to be a methicillin-resistant strain (MRSP), as its phenotypic resistance to oxacillin was supported by the detection of *mec*A gene [22]. The antimicrobial susceptibility result revealed that the strain was resistant to all tested antimicrobials except for vancomycin and linezolid. In addition, *S. pseudintermedius* 115 harbored *tet*M and *erm*B, which were found to be the genes responsible for the resistance to tetracycline and erythromycin, respectively. *P. aeruginosa* 700 resulted to be resistant to aztreonam, ceftazidime, ceftriaxone, imipenem, meropenem, piperacillin-tazobactam, and sulfamethoxazole-trimethoprim. For this strain, ESBL genotypic resistance was determined by $bla_{PER}$, while for MBL-genotypic resistance, $bla_{VIM}$ and $bla_{GES}$ were detected simultaneously. Both the selected strains showed worrying antimicrobial resistance profiles. In particular, *S. pseudintermedius* 115 resulted to be an extensively drug-resistant strain, while *P. aeruginosa* 700 was classified as a multidrug-resistant strain.

### 2.2. LCWB set up

The dual-species biofilm composed of *S. pseudintermedius* 115 and *P. aeruginosa* 700 in Lubbock Chronic Wound Biofilm (LCWB) model was performed following the experimental steps shown in Fig. 1. The formed biofilm was harvested from glass tube and placed on an artificial wound bed.

The LCWB volumes at 24, 48 and 72 h are showed in Fig. 2A. The volumes of harvested and washed biofilms at different contact times were evaluated by using the following formula: $V = \pi \times r^2 \times h$. The best-obtained volume was detected after 48 h (3.38 $cm^3$ ± 0.13) in respect to the LCWBs recorded at 24 (2.33 $cm^3$ ± 0.36) and 72 h (1.86 $cm^3$ ± 0.23) ($P = 0.0150$).

The harvested and washed biofilms were also analyzed by weights determination at each detected contact time. Fig. 2B confirms the trend of LCWB volumes with the major weight detected at 48 h.

The *S. pseudintermedius* and *P. aeruginosa* CFUs for mg of LCWBs at different contact times were determined by spreading on selective media after the detachment of the cells from the obtained biofilms (Fig. 3). The major amount of bacterial CFU/mg was detected at 48 h for each tested strain. For *S. pseudintermedius,* the best values of CFU/mg were recorded at 48 h ($6.11 \times 10^6$ CFU/mg ± $3.83 \times 10^5$), although no statistical differences ($P > 0.05$) were registered in respect to 24 ($3.08 \times 10^6$ CFU/mg ± $2.09 \times 10^5$) and 72 h ($2.33 \times 10^6$ CFU/mg ± $1.15 \times 10^5$). A statistical significance was obtained with *P. aeruginosa* CFU/mg detected at 48 h ($7.05 \times 10^6$ CFU/mg ± $2.89 \times 10^5$) in respect to 24 ($4.84 \times 10^6$ CFU/mg ± $1.09 \times 10^5$) ($P = 0.0267$) and 72 h ($2.67 \times 10^6$ CFU/

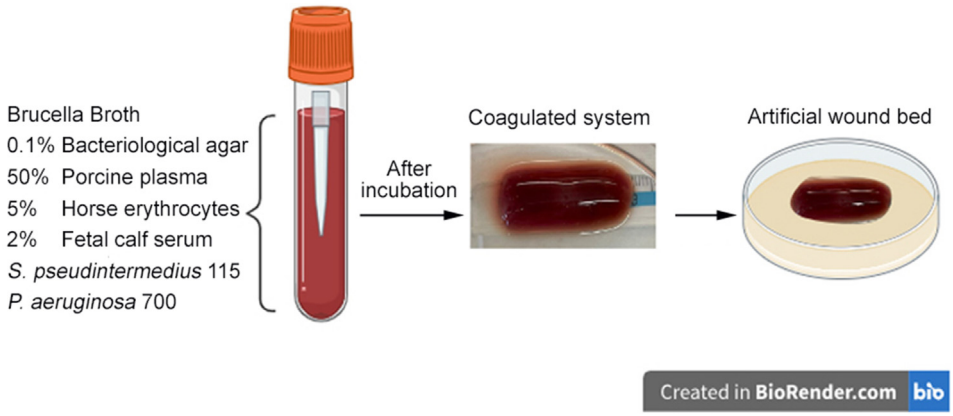


**Fig. 1.** LCWB preparation after 48 h of mixed medium incubation. The 48 h coagulated system was transferred on artificial wound bed to mimic the real chronic wound and analysed for the volume, weight and CFU/mg detections. Fig. 1 was created by using Biorender.

mg ± 1.53 × $10^5$) ($P = 0.0217$). A significant *P. aeruginosa* CFU/mg reduction was detected at 72 h in respect to 48 h determining a decreasing of this bacterium load. A statistically difference ($P = 0.0102$) between *S. pseudintermedius* and *P. aeruginosa* CFUs/mg was detected at 48 h. Considering the inter-group analysis, statistically significant CFU/mg was detected between *S. pseudintermedius* at 24 h in respect to *P. aeruginosa* at 48 h ($P = 0.0061$) and between *S. pseudintermedius* at 72 h and *P. aeruginosa* at 48 h (P = 0.0068).

The SEM images (Fig. 4) confirmed the major bacterial colonization after 48 h in respect to the other contact times. In addition, the images displayed an unmixed spatial distribution of bacterial species in the LCWBs at all tested times. *S. pseudintermedius* tended to colonize the upper surface of the LCWB and *P. aeruginosa* in the deeper area, as demonstrated by the first and third columns of Fig. 4. A complex fibrin network was observed at 48 h, while at 24 h, it was evident that there was a loose fibrin and a disaggregated fibrin at 72 h. At 72 h, there was a reduction of *P. aeruginosa* in respect to the other times and the fibrin appeared more stressed.

The CLSM images (Fig. 5) showed remarkable green cells at 24 and 48 h except for 72 h with relevant red *S. pseudintermedius*. At 72 h, the *P. aeruginosa* colonization was more in the middle area in respect to the down one as shown in other sections of the LCWB at 24 and 48 h.

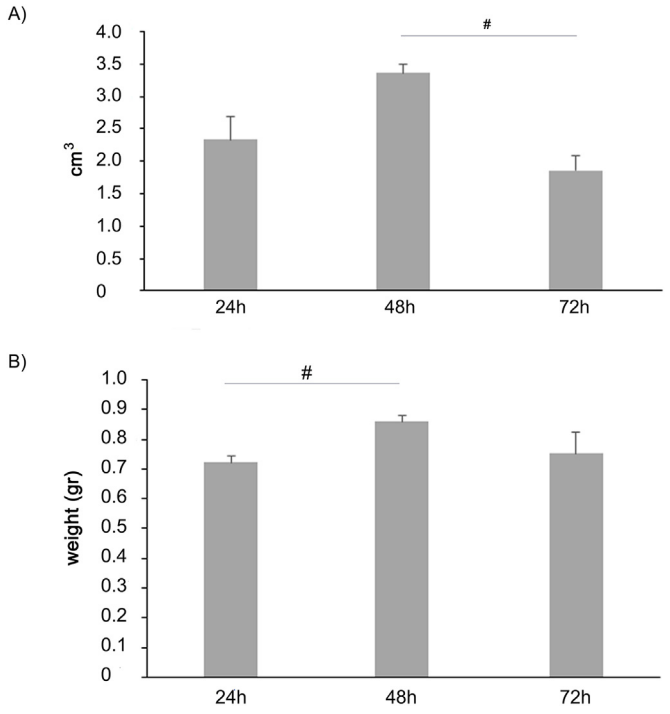


**Fig. 2.** LCWB volumes (A) and weights (B) obtained at 24, 48 and 72 h of incubation. The 24–48–72 h volume was determined by using the following formula $V = \pi \times r^2 \times h$ after each contact time. The harvested biofilms were weighted, and the weight was expressed in grams after each contact time. All data are expressed as the mean value ± standard deviation (S.D.). # indicates statistically significant ($P \leq 0.05$. In particular, $P = 0.0150$ between 48 h volume and 72 h volume and $P = 0.0163$ between 24 h weight and 48 h weight).

## 3. Discussion

The AMR phenomenon is a global challenge involving human, animal, and environmental health in a circular way. The dynamic animal-human transmission of the opportunistic pathogen *S. pseudintermedius*, resistant to antimicrobial drugs, plays an important role in pet and human health. In the last decade, the emergence of MRSP has become a substantial health challenge in companion animals [29]. The prevalence of MRSP has been consistently rising exhibiting global multidrug resistance and

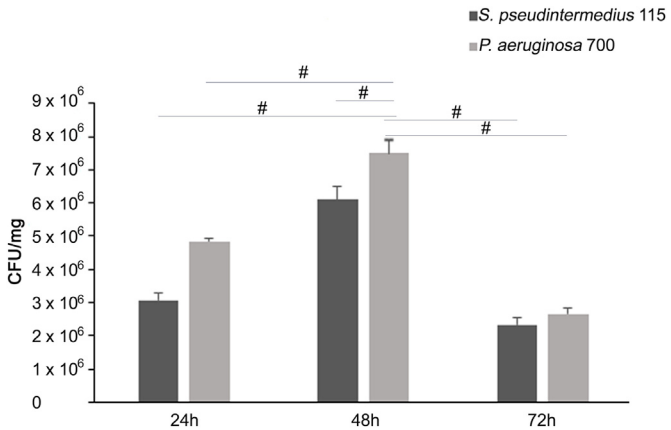


**Fig. 3.** *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* CFUs for mg of LCWB obtained at 24, 48 and 72 h of incubation. After each contact time, the microbial CFU/mg was determined by spreading on selective media. ° indicates statistically significant intra-group ($P \leq 0.05$). # indicates statistically significant inter-group ($P \leq 0.05$).

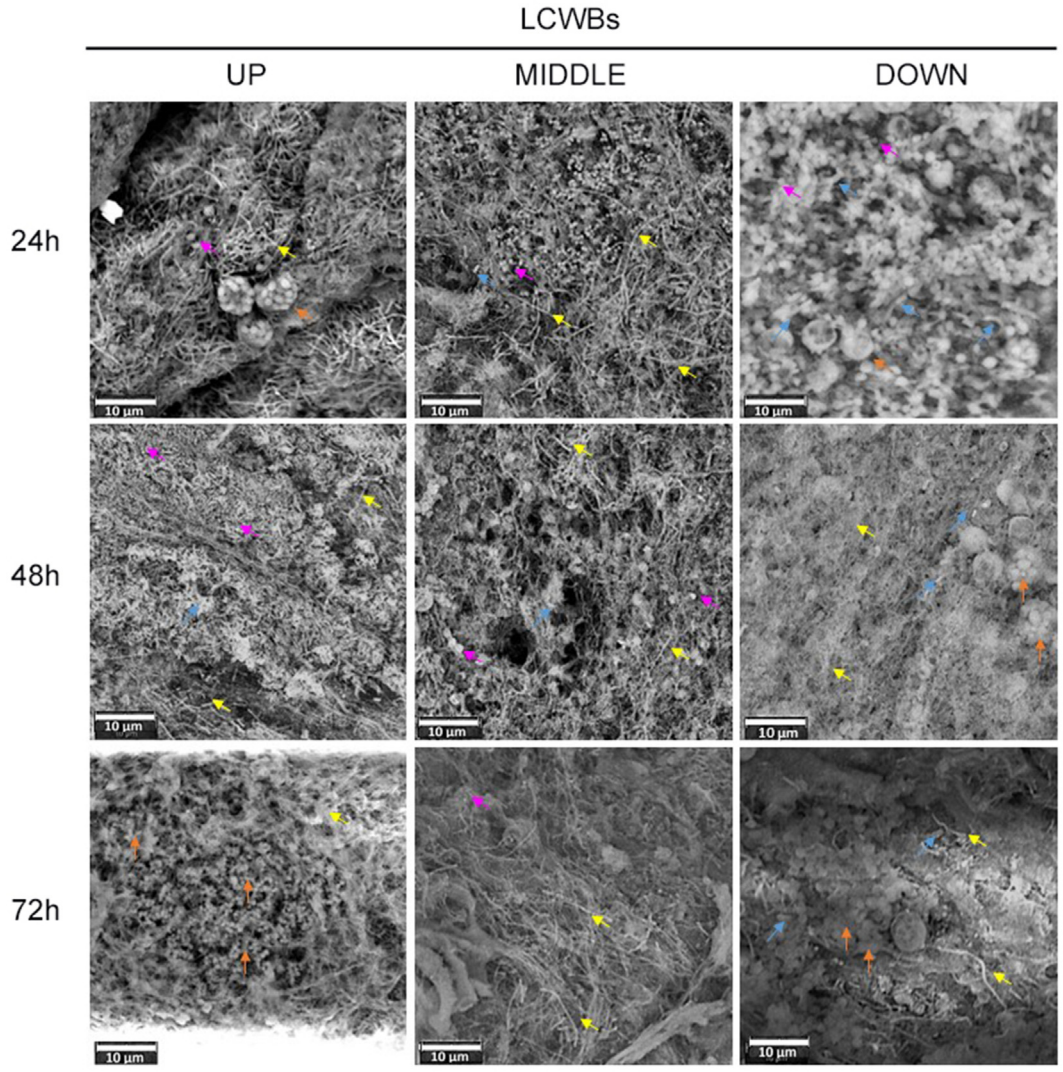


**Fig. 4.** Representative SEM images of up, middle and down of LCWBs at 24, 48 and 72 h.The SEM images allowed to evaluate the microbial spatial distribution in the 24–48–72 h LCWBs. Some representative elements observed in the pictures have been indicated with the arrows, as follows: *S. pseudintermedius* (pink arrow); groups of *S. pseudintermedius* (orange arrow); fibrin (yellow arrow); *P. aeruginosa* (light blue-arrow). Original magnification, 3600×.

encompassing resistance to various classes of antimicrobial drugs [30]. MRSP infections are difficult to treat, representing a challenge for the eradication and they are typically recalcitrant to traditional antimicrobial therapy [31]. *S. pseudintermedius* is responsible for wound infection in pet animals, and it is often associated with a poly-microbial biofilm with other microorganisms [32,33].

The complex treatment of MRSP strains in poly-microbial wound biofilms underlines the need for study a suitable *in vitro* model to reproduce microbial spatial colonization by mimicking an *in vivo* chronic wound. It is characterized by high levels of pro-inflammatory cytokines, proteases, reactive oxygen species (ROS), senescent cells, as well as the existence of persistent infections. In fact, the microbial colonization in biofilm growth mode, is responsible of the wound chronicity [16]. In this scenario, the LCWB is a useful *in vitro* model for evaluating the effect of new therapeutic approaches because it mimics the spatial microbial colonization in chronic wounds and their clots [13–15] respecting the 3R principles. In fact, according to the 3R principles (Replacement, Reduction and Refinement), the LCWB limits the use of animal for the experiments and their suffering, underling an ethical and sustainable research [34].

In this study, it has been reproduced an *in vitro* LCWB model including animal isolates to create a canine wound infection model with a focus on *S. pseudintermedius*, drawing inspiration from the established human model involving *S. aureus*. *S. pseudintermedius,* thanks to coagulate system, produces a fibrin network that represents a scaffold on which bacteria can adhere to forming a biofilm.

The decision to use *S. pseudintermedius* and *P. aeruginosa* combination for developing a canine wound model was based on the frequent isolation of these two bacteria, either individually or together, in most canine skin infections. The LCWB environment allows *S. pseudintermedius* to stick up for the killing action of *P. aeruginosa,* co-aggregating and growing together [35]. In terms of volume, weight, and microbial CFU/mg, the LCWB at 48 h was the major detected sample chosen for the best time to allow the *S. pseudintermedius* and *P. aeruginosa* poly-microbial biofilm formation. Twenty-four hours is the first step for the biofilm formation with a few fibrins network produced by *S. pseudintermedius,* few cells and produced biofilm. In the beginning, *P. aeruginosa* prevails on the *S. pseudintermedius* growth, probably for the expression of virulence factors such as PYO, LasA or rhamnolipids to outcompete *S. pseudintermedius* [36–39]. At 48 h, an *S. pseudintermedius* and

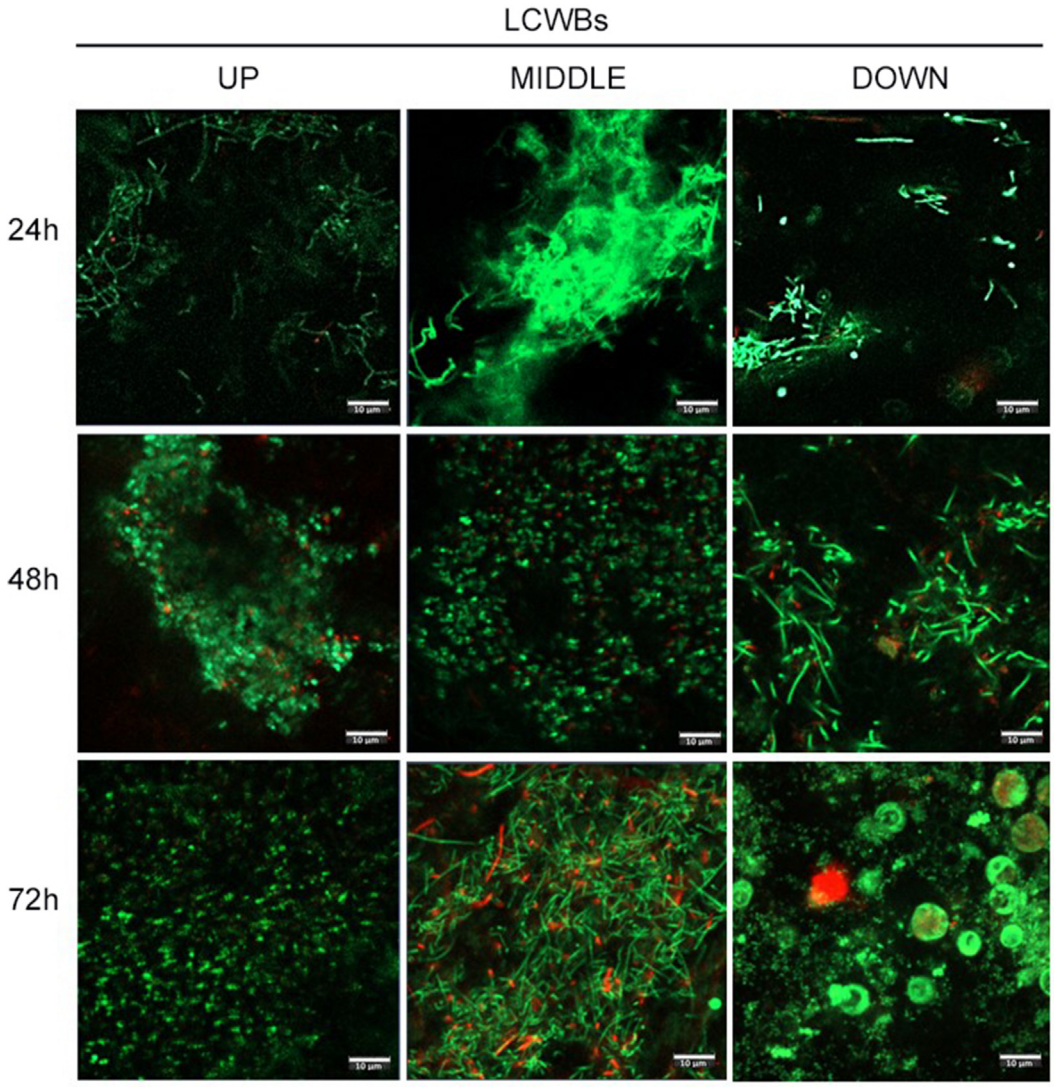


**Fig. 5.** Representative CLSM images of up, middle and down of LCWBs at 24, 48 and 72 h. The viable green (SYTO 9) and dead red (Propidium iodide) cells are shown in the 24–48–72 h LCWBs. Propidium iodide shows a damaged membrane whereas green stained bacteria represent viable cells. The images observed at Zeiss LSM800 microscope (CarlZeiss, Jena, Germany) coupled to an inverted microscope Axio-observer D1 (CarlZeiss, Jena, Germany) equipped with a Plan Neofluaroil-immersion objective (100×/1.45NA) were recorded at an emission wave length of 500 nm for SYTO 9 and of 635 nm for Propidium iodide and more fields of view randomly were examined. Original magnification.

*P. aeruginosa* adaptation was observed, and the bacterial growth rate was the same as at 24 h, with the best-produced biofilm. *S. pseudintermedius* produces traceamines (TAs) like phenethylamine, tyramine, or tryptamine, and their urea derivatives suppress the *P. aeruginosa* growth [38,40]. Probably, at 72 h, the increase of these metabolites is the cause of the *P. aeruginosa* reduction associated with a general decrease in microbial growth. In addition, the *P. aeruginosa* reduction could be related to the use of the closed system in which no additional nutrients were added to the system and wastes were not removed.

The SEM and CLSM images showed the same microbial spatial distribution of the human-chronic wound with *S. aureus* in the up of the surface and *P. aeruginosa* in the deep of the wound [38]. In this study, *P. aeruginosa* is able to colonize the deeper region of the canine LCWB model thanks to its capability to migrate via type IV pili and flagellum-mediated motility in biofilms [15]. The SEM observation permits us to appreciate the 3D organization of the fibrin network and the relationship between the two species of bacteria. The CLSM images displayed that at 72 h, a major amount of *P. aeruginosa* cells was detected in the middle section with respect to the down area. Probably, the 72 h-LCWB was less thick and serried, and the bacterium was able to move more freely and could reach the surface parts more easily. Matching the SEM and CLSM images, the LCWB microbial spatial distribution reveals *S. pseudintermedius* on the upper layers, in the middle a mixture of the two bacteria and on the lower layers a higher presence of *P. aeruginosa*.

The obtained findings demonstrate the capability of *S. pseudintermedius* to grow together with *P. aeruginosa* in the LCWB model, representing a suitable model to reproduce the animal chronic wound *in vitro*. This new model represents the first validation line for the novel treatment of animal skin lesions. LCWB allows to reproduce the *in vitro* wound biofilm condition, keeping the most important factors intact. The use of the *in vitro* model is low cost, easy to replicate, quick to manage and ethically sound, since it does not pose the risk for animals or humans. However, the limitation of the LCWB lies in its closed system and the absence of an immune response. The innovative aspect of this research was the

validation of an *in vitro* model of canine wound infection, aimed at studying alternative approaches for treating zoonotic multidrug-resistant bacteria and preventing their spread in veterinary facilities and community.

## CRediT authorship contribution statement

**Silvia Di Lodovico:** Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Morena Petrini:** Writing – original draft, Methodology, Investigation. **Paola Di Fermo:** Writing – original draft, Methodology, Data curation. **Valeria De Pasquale:** Visualization, Funding acquisition, Conceptualization. **Luisa De Martino:** Writing – review & editing, Validation. **Simonetta D'Ercole:** Writing – review & editing, Investigation, Formal analysis. **Francesca Paola Nocera:** Writing – review & editing, Writing – original draft, Resources, Investigation, Conceptualization. **Mara Di Giulio:** Writing – review & editing, Validation, Resources, Data curation, Conceptualization.

## Declaration of competing interest

Authors declare no competing interests.

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