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Atmospheric cold plasmas for biofilm inactivation: does biofilm extracellular matrix limit the bactericidal process?

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ABSTARCT

Low Temperature Atmospheric Pressure Plasmas (LTAPPs) is a new promising sterilization and decontamination technology with a high potential to inactivate microbial biofilms. Conventional methods for inactivation of planktonic bacteria are often ineffective with biofilms that constitute a protected mode of bacterial growth. Microbial biofilms can be seen as a complex functional organization of microbial cells, water, and secreted extracellular substances of polysaccharides, proteins, nucleic acids and other cellular products. There is a lack of studies focused on the interaction of plasma with the organic matrix of biofilms that acts as a protective barrier against any physical or chemical stress. That is why the bactericidal action of LTAPPs on lactic acid bacteria organized or not in biofilm was measured. and the protective role of the exopolymeric matrix produced by this strain against cold plasma was highlighted. Therefore future strategies should focus on the interaction of matrix components toward reactive species generated by plasma.. Some relevant tools were thus proposed to follow the alteration of either the biochemical components or the physical properties of the matrix in order to provide new experimental approaches for the global analysis of plasma effect on the architecture of bacterial biofilms.

KeyWords: Low temperature atmospheric pressure plasma, microbial biofilm, exopolymeric matrix, sterilization decontamination

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INTRODUCTION

Plasma technology is one of the most promising technologies that may offer enhanced quality of care and food safety at a reduced cost. This technology providing absence of harmful residues, is a "white" technology that can be used as an alternative to classical chemical procedures. The advent of Low Temperature Atmospheric Pressure Plasmas (LTAPPs) technology has induced the

development of biomedical applications and the emergence of innovative processes in food industry. These considerations suggest that applications of plasmas are about to become a major field of research in many advanced technologies with a major economic impact and substantial industrial activity. For example, LTAPPs have been adopted for wide ranging applications including cell manipulation, oral health, tissue ablation, blood coagulation, wound healing, cancer treatment, sterilization and decontamination of inorganic and bio-materials (Yousfi et al. 2013). LTAPPs are also under intensive study as an alternative approach to control superficial wound and skin infections when the effectiveness of chemical agents is weak due to natural pathogens or biofilm resistance (Ermolaeva et al. 2011). LTAPP treatment is also generally considered to be a candidate method for ensuring food safety during processing (Maisch et al. 2012). Plasma technology thus represents a new state of the art sterilization and disinfection treatment for oral and environmental pathogens, heat-sensitive materials, contaminated medical waste, and decontamination of fresh foods without significantly affecting nutrient content and textural qualities.

Numerous studies have demonstrated the effectiveness of LTAPPs for inactivation of free-living (planktonic) microorganisms or adherent cells. Due to a mean low plasma temperature, plasma jets exhibit antimicrobial effects without any visible thermal damage on the treated bio-surfaces. Such low-temperature plasma jets and in particular those operating in open air, generate numerous active species (radicals, charged particles, long living excited species, UV photons) playing an efficient role for the inactivation of planktonic microorganisms (bacteria, yeasts) and even spores (Joshi et al. 2010). Involvement of reactive species such as ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) is suspected since inactivation is more efficient with plasma jets generated in the ambient air than those using rare gas flows (Sarrette et al. 2009). These species are formed in the plasma and might react with the liquid layers surrounding bacteria, forming antibacterial by-products such as peroxides or superoxides. These sterilization studies report that both Gram-negative and Gram-positive bacteria were successfully inactivated by LTAPPs. However, most of the treatments have been carried out on abiotic surfaces that discourage cell growth (Vleugels et al. 2005) and only single bacterial species models have been used.

Conventional methods of inactivation of planktonic bacteria by chemical, physical or biological ways are often ineffective with biofilms, because bacteria within biofilm show different properties from those in planktonic life and the matrix acts as a shield protecting cells (Stewart and William Costerton 2001). Biofilms are the default mode of life for many bacterial species and constitute a major proportion of the bacterial biomass present in the nature as complex multispecies ecosystems. Biofilms are present almost everywhere, and their presence leads to disease, prostheses colonization, product contamination, biofouling and equipment damage. According to the National Institutes of Health (Brelles-mariño 2012), 90% of infections in humans and 65% of nosocomial infections are due to biofilms.

Biofilms are highly organized communities of microbial cells embedded in a self-produced matrix composed of exopolymers such as proteins, polysaccharides and nucleic acids (Flemming and Wingender 2010). Biofilm formation begins when the free-living bacteria (planktonic) attach to a surface and recruit additional cells. This is followed by formation of cell clusters and further development and stabilisation of these microcolonies, which are occurring in extracellular matrix of variable density and composition. Dispersal mechanisms also facilitate colonization of surrounding area (Hall-Stoodley, Costerton, and Stoodley 2004).

The organic matrix of biofilms plays an essential role in the protection of biofilm cells against physical or chemical bactericidal stress; alteration and/or destruction of the matrix physical integrity appears thus indispensable to ensure efficient biofilm elimination. However, there is a lack of studies focused on the interaction of plasma with the organic matrix. In a first part of this study, the

bactericidal activity of LTAPP on an exopolymeric glucan-producing lactic acid bacteria organized or not in biofilm was measured in order to evaluate the involvement of the exopolymeric matrix in the biofilm resistance. In a second part, some relevant tools were proposed to follow the alteration of either the biochemical components or the physical properties of the matrix in order to provide new experimental approaches for the global analysis of plasma effect on the complex architecture of bacterial biofilms.

MATERIAL AND METHODS

Low temperature plasma generation for bacterial biofilm treatment

The low temperature plasma jet that look like to a flame of for instance of candle (but with a lower temperature) is produced by specific corona design is described elsewhere (Marchal et al. 2012). Plasma is produced by anodic needle inside a cylindrical cathode configuration driven by a DC high voltage power supply. This corona configuration gives a natural repetitive discharge current with a frequency of about 20 kHz under a high voltage DC power supply. The instantaneous discharge current for a given applied DC high voltage illustrating the dissipated power (about 100 mW) is displayed in figure 1.

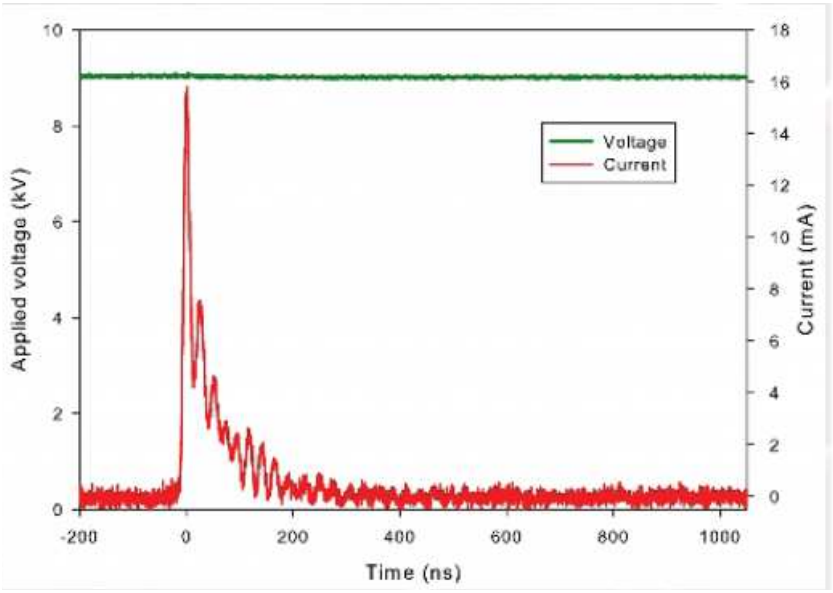

Fig. 1: Instantaneous discharge current generated by the plasma jet setup under a given DC high voltage (Marchal et al. 2012).

The plasma jet is generated directly in the ambient air at atmospheric pressure and launched by itself without any system of gas inlet feed. In fact, the plasma is ejected in open air with the help of physical forces that are the corona ionic winds having a subsonic velocity (close to 20 m/s) that are able to dry the treated biofilm during its exposure to plasma. The plasma temperature measured on the top of the plasma jet (at about 1 cm) using a thermocouple does not exceed 27°C. Bacterial samples are placed at the top of the air plasma jet at a distance *d* = 10mm of the jet nozzle (see figure 2). The sample temperatures measured after treatment do not differ from ambient temperature more than 2 ◦C. The UV-visible spectrum corresponding to the light emission of the top of the air plasma jet, already detailed elsewhere [12], shows classical emission bands of Nitrogen such as the Second Positive System (SPS) of $N_2(C_3\pi_u)_v \rightarrow N_2(B_3\pi_g)_{v'}$ (from about 290 nm up to 440) and the First Positive System (FPS) $N_2(B^3\Pi_g) \rightarrow N_2\ (A^3\Sigma_u^+)$ (between about 600 nm up to 900 nm).

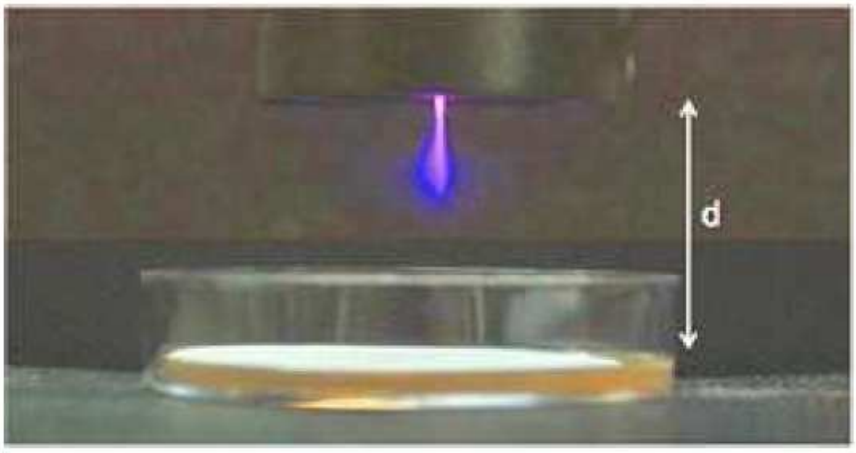

Fig. 2: View on the plasma jet ejected in open air with a bacteria sample positioned at a distance d from the nozzle of the plasma setup (Marchal et al. 2012).

There are also oxygen emissions $O_2(b^1\Sigma^+_g\ v=0) \rightarrow O_2(X^3\Sigma^-_g,v=0)$ at 759 nm and atomic line of the triplet state of O at 777.47 nm. This overview on plasma jet spectrum means that the excited species present at the top of jet are at least those involved by the emission bands such as $N_2(C_3\pi_u)_v$, $N_2(B_3\pi_g)_{v'}$, $O_2(b^1\Sigma^+_g\ v=0)$, $O_2(X^3\Sigma^-_g,v=0)$, $N_2\ (A^3\Sigma_u^+)$ and O.

To summarize, the different plasma species able to more or less inactivate bacteria and biofilms and present on the top of the plasma jet in open air are:

- Radicals and long lived neutrals such as NO, NO_2, NO_3, OH, HO_2, O_3, H_2O_2 but in a low concentration ($< 10^{13}$ cm^{-3})
- Metastable states storing internal energy such as: $N_2\ (A^3\Sigma_u^+)$, $O_2(a^1\Delta_g)$, ...
- Radiative states: $O_2(b^1\Sigma^+_g)$, N*, O*
-charged particles such as electrons, O_2^-,N^+, O^+, N_2^+,N_4^+, O_4^+, etc. in relatively low concentration ($< 10^{13}$ cm^{-3})

There are also photons and electric field on the top of the plasma jet:

-UV-A emitted by nitrogen bands N_2(SPS) while UV-C and UV-B are quenched since NOγ and OH(A-X) bands are not detected (see figure 3)

-Visible radiation emitted by N_2^+(FNS), N_2(FPS) and NO_2 continuum

-low electric field (around 10^4 V/cm)

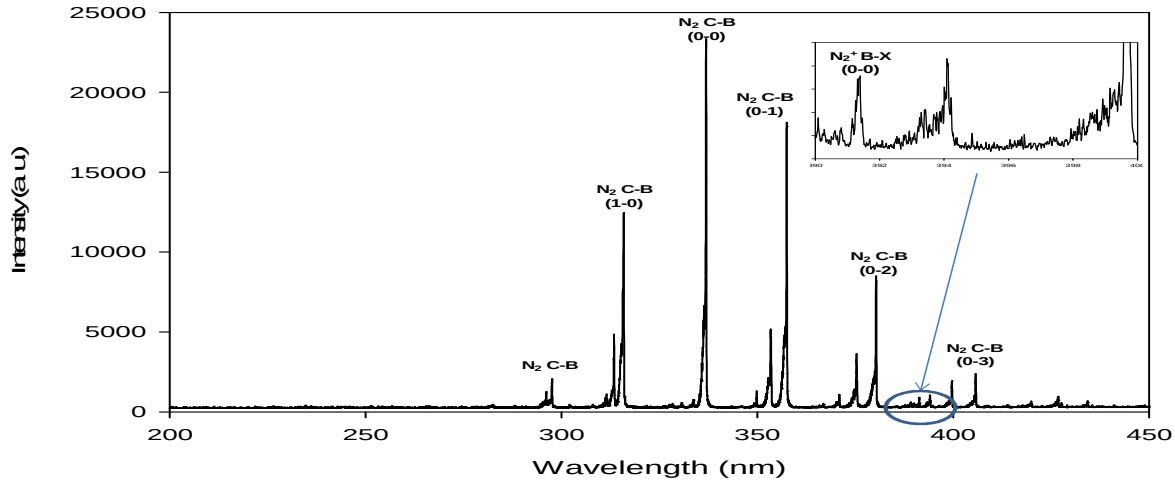

Fig. 3: **Spectra between 200nm to 450 nm at the top of the plasma jet**

showing some specific molecular nitrogen bands and also the quenching of NOγ (between 200 to 260nm) and OH(A-X) (between 306 to 310nm) bands synonymous of the absence of UV-C radiations by the plasma jet (Marchal et al. 2012)

Adherent cells and biofilm formation of Weisella confusa bacterial strain

The strain *Weissella confusa* LBAE C39-2 (Amari, Laguerre, et al. 2012) was pregrown in MRS (De Man, Rogosa and Sharpe) broth. Then 0.2 ml of standardized bacterial suspension was diluted in 20 ml of sterile MRS broth. A simple method modified from Cao *et al* (Cao, Y., Yang, P., Lu, X., Xiong, Z., Ye, T., Xiong, Q., Sun 2011) was developed to form biofilms on membrane after filtration. The diluted suspension was passed through a sterile filtration device containing a membrane Pall GN-6 Metricel® (17 cm^2, cut-off 0.45 μm). Thereafter, the membrane was recovered under sterile conditions and deposed on Petri dishes (55 mm diameter) containing MRS solid media or MRS solid media supplemented with sucrose (40 g l^{-1}) in order to trigger glucan production. There immediately proceeded to perform adherent cells plasma treatment. Otherwise, a uniform biofilm was formed on membrane during growth for 48 h at 30°C.

Evaluation of cell viability before and after plasma treatment by bacterial count

To evaluate the effect of plasma treatment on biofilm and adherent cells, a piece of membrane (1.5 cm^2) corresponding to the central treated surface by plasma was cut under sterile conditions. Then bacterial cells present on membrane were recovered in 1ml of sterile saline solution (sodium chloride 9 g l^{-1}) by pipetting extensively the colonized surface. Decimal dilutions in saline solution were performed to count viable bacterial population on MRS solid media after incubation at 30 °C for 48 h. All plasma treatments were performed in triplicate. Inactivation kinetics of adherent or biofilms cells were modelled with GInaFIT (Geeraerd, Valdramidis, and Van Impe 2005).

Analysis of the exopolymeric matrix of biofilms

Observations in laser scanning confocal microscopy (CLSM) were performed to visualize the main components of a biofilm of *Pseudomonas aeruginosa*, i.e. cells, proteins and polysaccharides of the matrix. Biofilms of the *P. aeruginosa* CIP103.467 strain were obtained after 48h of growth on glass slides in Tryptic Soy Broth (TSB) at 37°C. The CLSM observations were done after biofilm staining with the fluorescent DNA-binding stain 4',6-diamidino-2-phenyindole dihydrochloride (DAPI, Sigma-Aldrich), the protein stain Sypro Ruby (Molecular Probes), and the polysaccharide staining *Lycopersicon esculentum*-FITC (LE-FITC) lectin (Sigma-Aldrich), essentially as previously described (Doumèche et al. 2007). The stained biofilms were examined with a Leica SP2 upright confocal laser scanning microscope (CLSM) (DM RAX-UV) (Leica microsystems, Reuil-Malmaison, France).

For *in situ* determination of polysaccharides and proteins contents of the extracellular matrix, biofilms of *P. aeruginosa* were formed into polystyrene 96-well microtiter plates for 48 hours in TSB at 37°C and those of *Weissella confusa* in MRS medium at 30°C. Exopolysaccharides were quantified using Periodic Acid-Schiff staining (Kilcoyne et al. 2011)(PAS kit® from *Sigma-Aldrich)* with dextran solutions (dextran from *Leuconostoc spp.*T40, *Fluka Bioch*.) prepared in ultrapure water as standards. Extracellular proteins were determined using fluorescence staining with Epicocconone (Mackintosh, Veal, and Karuso 2005)(*Fluoroprofile Protein Kit® from Sigma-Aldrich*) with Bovine Albumin Serum (BSA) solutions, prepared in ultrapure water as standards.

The mechanical properties of a bacterial biofilm and of artificial matrices made of polysaccharides alone or polysaccharides and proteins have been evaluated by rheology using a cone-plate technology. For rheology analysis, biofilms of the mucoid *P. aeruginosa* NK125502 strain (Rebière-Huët et al. 1999) were obtained after 10 days of growth on glass slides at 26°C in TSB. Gels corresponding to artificial matrices were obtained by adding a protein solution (histones from calf thymus, Sigma-Aldrich, France) to an alginate solution (A2033, Sigma-Aldrich) or by using an alginate solution alone. Rheology measurements were performed with a MCR 301 from Anton Paar (Anton Paar, Courtaboeuf, France) in rotation mode.

RESULT AND DISCUSSION

Inactivation of bacteria biofilms by LTAPP

Gram-positive bacteria are considered more resistant than Gram-negative to plasma treatment. The Gram-positive bacteria cell wall (peptidoglycan) exposed to plasma is thicker than the cell wall of the Gram negative bacteria. Moreover, in Gram negative bacteria, the cell membrane is directly exposed to plasma because the thin peptidoglycan layer is localized under the outer membrane. Among Gram positive species, pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, are implicated in wound infection and implant, catheter biofilms whereas *Streptococcus mutans* is a cariogenic associated bacteria involved in dental plaque biofilm formation (Bowen and Koo 2011). It is noteworthy that, *in vivo*, biofilm is a very complex living structure of multiple species bacteria. To test the efficacy of cold plasma, a Gram-positive and acid resistant bacteria from *Weissella confusa* species was selected. This strain produces from sucrose a glucan exopolymer containing mainly α-(1,6) linked glucose (dextran) due to the activity of a single dextransucrase (Bounaix et al. 2010)(Amari, Arango, et al. 2012). Hence, this allows by a very simple way to obtain a monospecies biofilm model where matrix composition could be controlled by simple adjunction of sucrose.

Biofilms of *Weisella confusa* were cultivated for 48h on membranes deposited on Petri dishes containing MRS nutritive media with or without sucrose. Adherent cells (without any incubation period) were deposited on membrane just before plasma jet treatment. Figure 1 presents *W. confusa* viability counts after a 0, 10 and 20 min cold plasma treatment

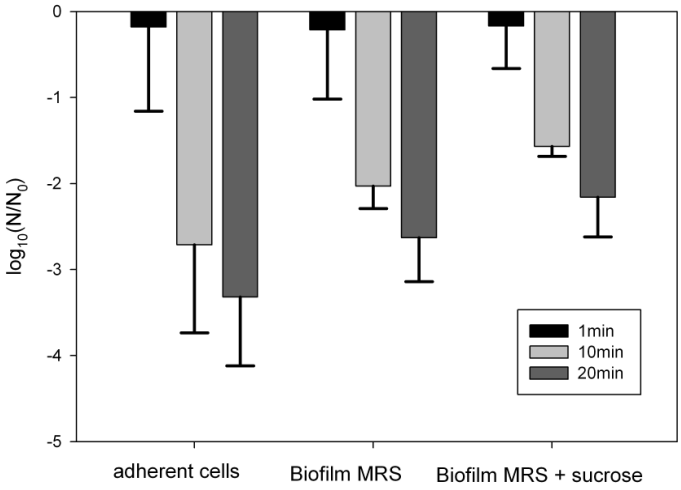

Fig 4 : Inactivation by LTAPP of the Gram positive bacteria *Weissella confusa* in adherent state and biofilms formed with or without sucrose

As illustrated in figure 4, *Weissella* biofilms were more resistant to cold plasma treatment than adherent cells. The time necessary to reduce by ten the population (D_{10}) is two-fold higher for biofilm conditions (D_{10}=5-6 min) than for adherent cells (D_{10}=3,5 min). Moreover, bacterial population embedded in dextran biofilm polymer (presence of sucrose) shows only a 2.16 log reduction after 20 min treatment. Bacterial population reduction is 10 fold greater for adherent cells for this time. Obviously, the increased thickness of biofilms grown with sucrose could explain the

obtained results. It was estimated that plasma penetration depth does not exceed a thickness less than 20 µm (Fricke et al. 2012)(Xiong et al. 2011).
Thus, after inactivation of the first layers, where the bacteria are more exposed to the plasma reactive species, the inactivation rate may decrease to nearly zero.
Typical biofilm survival curves obtained after plasma treatment often exhibits a biphasic behaviour, which is not true for planktonic cells treated on a surface or in a liquid suspension. During the first times of plasma treatment, bacteria seem to be more sensitive to reactive species generated by plasma. During the second part of curve, D10-value could be until 10 fold increased. Plasma have no additional significant lethal effect on biofilm after twenty minutes (results not shown).
In numerous studies, only a partial inactivation of Gram positive bacteria biofilm is obtained after a cold plasma treatment. For example a 2.19 Log reduction has been obtained for *Staphylococcus epidermis* biofilm by the kinpen 09 device after 5 min (Matthes, Koban, et al. 2013). Efficacy of surface discharge devices is higher for the same time in biofilm, with value near 3.5 log reduction in 10 min (Matthes, Bender, et al. 2013). For the same species cultivated in biofilm a greater inactivation has been obtained by Kampang et al., (Kamgang et al. 2007) using gliding discharge plasma but for a long time treatment of 70 min.
Considering all the available data for Gram positive bacteria, a maximum of 4-5 log reduction of bacterial viable population is generally observed, depending of plasma device, bacterial strain or biofilm growth conditions. However, this is clearly not enough to ensure a complete sterilization of abiotic surfaces or living tissues.
Results presented in these experiments are in favour of a protective role of polysaccharide (dextran) against cold plasma and highlighted the role of matrix components toward reactive species generated by plasma. They are also in agreement with previous observations that, after a single plasma treatment, re-growth of *Streptococcus mutans* biofilms (another lactic acid bacteria producing from sucrose a mixture of glucans) was depending on the presence of excreted polysaccharides (Sladek et al. 2007). Polysaccharides and proteins are the main components of matrix and form the backbone of a network where other components and bacteria can be included. Killing biofilm cells is insufficient to eradicate a biofilm; an action against the exopolymeric matrix is needed to decrease the regrowth of the structure.
Recently, some authors have postulated that dehydration of the first layers of biofilms could limit plasma efficacy (Matthes, Bender, et al. 2013). Indeed, they have observed by microscopy a "coagulation" state of the matrix after plasma treatment.. Future challenges in biofilm inactivation by LTAPP have thus to take into account the parameters (biological depending factors and plasma ones) that could counteract bactericidal effect of cold plasma. Thus, we propose to extensively study one key parameter that represents the matrix components of biofilms.

Characterization of the exopolymeric matrix of biofilms

The main extracellular components of the biofilm matrix are proteins and polysaccharides but their respective proportion and distribution are very dependent with the type of bacteria, the age of biofilm (initial adhesion or mature biofilm) and the environmental conditions (nutrients, temperature, flux forces). *Pseudomonas aeruginosa*, a Gram negative pathogenic bacteria highly efficient in biofilm formation, was chosen to illustrate the distribution of the matrix components in a model biofilm.

Organization of the extracellular matrix of Paeruginosa biofilms

The spatial organization of *P. aeruginosa* biofilm was analysed by CLSM. Multiple stains were applied and recorded simultaneously to visualize the different components of the biofilm: DAPI for bacterial cells (blue, Figure 5A) + LE-FITC for N-acetyl glucosamine $(glcNAc)_3$ -containing polysaccharides (green, Figure 5B) + Sypro Ruby for proteins (red, Figure 5C). The

polysaccharides stained with the lectin LEwere both located extracellularly and close to the bacterial cells surface in the biofilm matrix (Figure 5B). Some areas of the biofilm devoid of cells were stained by the LE-FITC lectin. Proteins were present all along the biofilm matrix where cells were present, but some areas of the matrix contained higher concentrations of proteins. Confocal images showing the Z-axis revealed that the biofilm varied in depth from 2 to 7 μm (Figure 5D). The microbial cells were organized as huge microcolonies localized in the entire depth of the biofilm. We have previously used this CLSM technology based on biofilm staining with fluorescent lectins and DAPI to analyse the biofilm growth and to determine the efficiency of anti-biofilm treatments (Di Martino et al. 2007) (Houari et al. 2013) (Marconnet et al. 2011). Nevertheless, this is only a semi-quantitative approach that needs to be used in conjunction with other analytical tools.

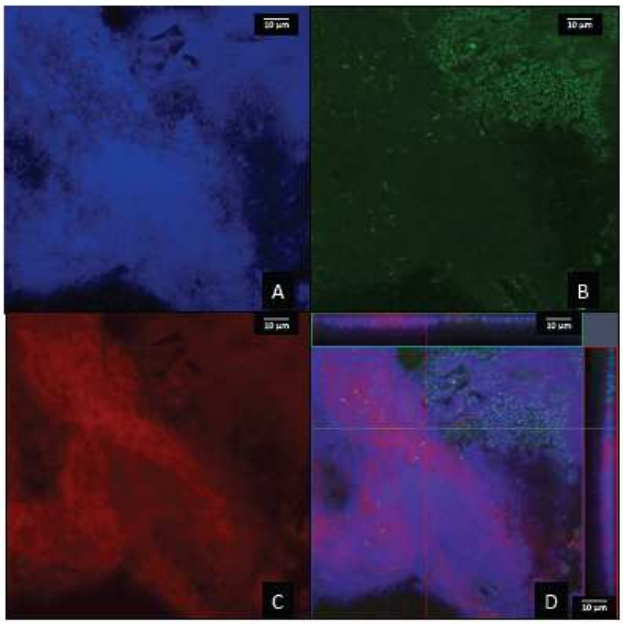

Fig. 5: CLSM observation of fluorescent signals from labeled microbial, exopolysaccharide and protein components of *Pseudomonas aeruginosa* 48h-biofilms.

The biofilm was stained with the fluorescent DNA-binding stain DAPI (A), the polysaccharide staining *Lycopersicon esculentum*-FITC lectin (B), and the protein stain Sypro Ruby (C). Fluorescent signals inside the *P. aeruginosa* biofilm corresponding to cells, exopolysachharides and proteins are shown together in panel D.

Quantification of the extracellular matrix components

Quantification of the matrix components is classically performed after extraction and *in vitro* determination of the exopolymeric substances using specific colorimetric methods. Various physical or chemical methods can be used to extract the exopolymers, some of them being tightly bound to the biofilm matrix (Marcato-Romain et al. 2012). Although efficient, these methods can be selective for one type of biopolymer and can induce alteration of the extracted EPS.

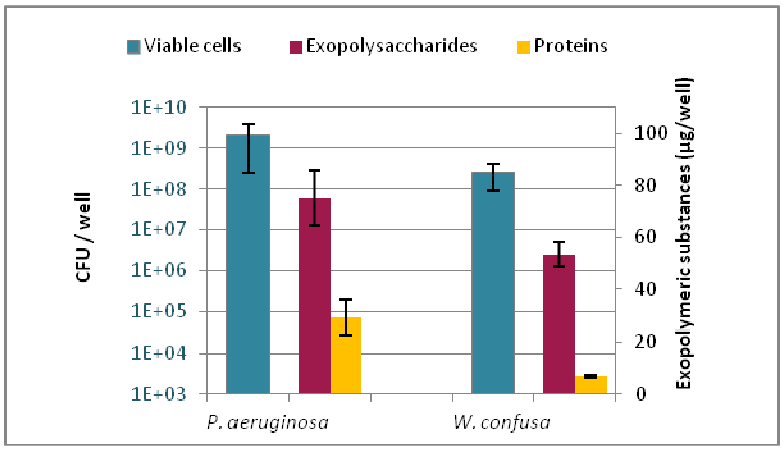

Fig. 6 : Viability and exopolymeric substances contents of *Pseudomonas aeruginosa* and *Weissella confusa* 48h-biofilms.

Carbohydrate content is expressed in microgram of dextran-equivalent and protein level is in microgram of BSA-equivalent. Bacteria growth is expressed in Colony Forming Unit (CFU) which represents the number of cells of the analysed well. Errors bars represent the standard deviations after six independent experiments.

An original method for "*in situ*" quantification of proteins and polysaccharides was adapted to avoid extraction or alteration of the matrix structure. Figure 6 shows the quantitative profiles of the proteins and polysaccharides measured in the biofilm of *Pseudomonas aeruginosa ATCC15442 (*a reference strain for the evaluation of disinfectants under the NF EN 1040) and in the biofilm of *Weissella confusa*.

A clear predominance of the polysaccharides towards the proteins was observed in both biofilm matrixes. After 48h, *P. aeruginosa* matrix is composed of about 2.5-fold (in weight) of exopolysaccharides (75.05 ± 10.57 µg/well) compared to proteins (29.16 ± 6.72 µg/well) for a cell-adhered viability of 2.09 $x10^9$ CFU/well.

As expected, *W. confusa* demonstrates here important sugar production within the biofilm, with an amount of 53.49 ± 4.82 µg per well of total polysaccharides against 6.80 ± 0.59 µg per well of proteins. Such biofilm, with a lower cell-adhered viability of 2.53 $x10^8$ CFU per well, shows a polysaccharide/protein weight ratio of 7.87.

The CLSM observations previously described in Figure 5 indicate a predominance of proteins in the biofilm matrix of *P. aeruginosa* while the quantitative data show a majority of polysacharrides. This may be explained by the fact that the polysaccharides observed in CLSM were only those stained by the LE-lectin, i.e. the polysaccharides containing $(glcNAc)_3$ residues. Thus, in the CLSM approach that generally employs lectins, exopolysaccharides may be underestimated. On the contrary, PAS assay was tested with several types of carbohydrates (data not shown) and seems to match with a great majority of them. This approach was selected because of its capacity to provide a global quantification of a wide variety of polysaccharides.. This example illustrates the importance to have the better knowledge as possible of the constitution and organization of the biofilm that needs to be inactivated by plasma.

Quantification of the physical properties of the matrix

Biofilms are considered to be highly porous polymer gels and laboratory-grown as well as natural biofilms are viscoelastic in nature. The ability of biofilms to deform in response to mechanical stress is thought to be a conserved strategy of defence to enable persistence on surfaces in different

flow conditions. The viscoelastic nature of the *P. aeruginosa* biofilm and of the artificial matrices and their deformation under shear stress has been studied by measuring the creep response to shear (Figure 7). The character of the strain response for the *P. aeruginosa* biofilm and for artificial matrices made of alginate or alginate and proteins under a shear stress of 2.5 Pa showed a typical viscoelastic nature. The curves exhibited an instantaneous elastic response, and a time-dependent viscous deformation. After removal of the shear stress, an instantaneous elastic partial recovery and a partial viscous relaxation were observed. The stress of the alginate matrix containing proteins was about three times higher than the value measured for the matrix made of pure alginate. The presence of proteins inside the alginate matrix enhanced its deformability, mimicking the viscoelastic response of *P. aeruginosa* biofilms under stress. Thus, artificial matrices (made of alginate and histone) can be used to study the effects of a cold plasma treatment onto the exopolymeric matrix of a *P. aeruginosa* biofilm.

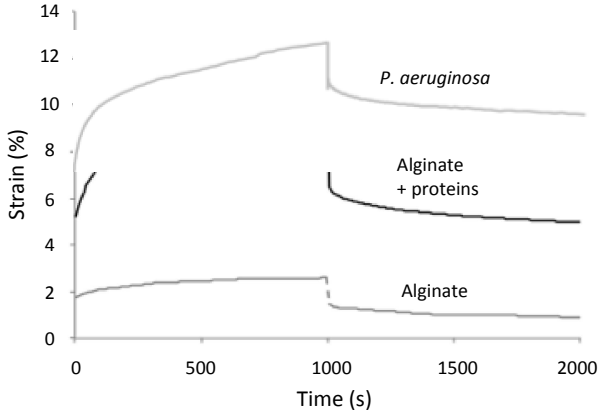

Fig 7. Creep test analysis showing the deformation and relaxation under stress of a 10 days old *Pseudomonas aeruginosa* biofilm, and of artificial matrices made of alginate or alginate and proteins.

A constant shear stress was held on the sample during 1000 sec and decreased to 0 during a second period of 1000 sec. The resulting deformation on gels was monitored by measuring the variations of shear strain with time**.**

CONCLUSION

The biofilm matrix is a key parameter to consider for efficient LTAPPs treatment against bacterial biofilms, in particular against biofilms formed by Gram positive bacteria such as *Weissella confusa.* Matrix components (polysaccharides, proteins and nucleic acids) can act as a physical barrier interacting with plasma reactive species, as ROS and RNS. Moreover ionic wind self-generated by plasma device has a dehydrating effect on biofilm matrix limiting reactive plasma species penetration and lowering water activity of biofilm. Hence apparent LTAPPs bactericidal power is lower on bacteria cluster located in the deeper layers of the biofilm.

In situ analytical tools (CLSM, proteins and polysaccharides quantification), matrix models (polysaccharides +/- proteins +/- nucleic acid) and biofilm models are now available to understand molecular interactions between LTAPPs and matrix components. This would greatly enhance our comprehension of bactericidal effect of LTAPPs and this is necessary to optimize plasma key parameters such as the gas composition (with for instance additional humidity), the choice of power supply (direct current or pulsed voltage or microwave source), the production rate of reactive species and the plasma device design to increase the biofilm surface treated by the plasma jet.

Combined treatments of biofilm should be a good solution to destroy matrix components before plasma treatment. For example, polysaccharides or proteins digestion by enzymatic treatments could be applied on biofilms to lower the biofilm cohesion before LTAPP treatment to get a better access to bacterial cells and enhance efficacy of plasma treatment.

Comparison of efficacy of different plasma devices is still challenging due to the diversity of biofilm models used in literature:

- Monospecies, multispecies or *ex vivo* biofilm models,
- Biofilm models growing conditions (liquid/semi-dried static or dynamic conditions, colonizing surfaces, maturity of biofilm)

Lack of common and well defined biofilm models could be a limiting factor to use plasma device in decontamination, our *Weissella confusa* biofilm model where the polysaccharide matrix component is easily modulated could be a good candidate for further experiments and researches.

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