Cold atmospheric plasma inactivation of aerosolized microdroplets containing bacteria and purified SARS-CoV-2 RNA to contrast airborne indoor transmission

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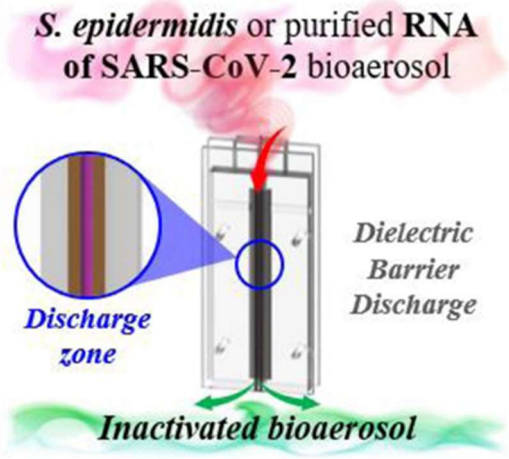
Abstract
One of the major concerns in the COVID-19 pandemic is related to the possible transmission in poorly ventilated spaces of SARS-CoV-2 through aerosol microdroplets, which can remain in the air for long periods of time and be transmitted to others over distances >1 m. Cold atmospheric pressure plasmas can represent a promising solution, thanks to their ability in producing a blend of many reactive species, which can inactivate the airborne aerosolized microorganisms. In this study, a dielectric barrier discharge plasma source is used to directly inactivate suitably produced bioaerosols containing Staphylococcus epidermidis or purified SARS-CoV-2 RNA flowing through it. Results show that for low residence times (<0.2 s) in the plasma region a 3.7 log R on bacterial bioaerosol and degradation of viral RNA can be achieved.



1 INTRODUCTION

Bioaerosols consist of airborne particles (from 0.001 to 100 μm) that originated biologically from plants and animals and can contain living organisms such as bacteria, viruses, fungi, and pollen.[1] In an indoor environment, human activity is the main cause for the formation of bioaerosols that could spread diseases such as influenza and respiratory syndromes. Bioaerosol exposure has become one of the major concerns for the residential, healthcare, and government sectors after the outbreaks of SARS in 2003 and influenza H1N1 viral infections in 2009 across the globe prompted worldwide attention for effective biological monitoring and control measures.[2,3] Indeed, many studies demonstrated that the pathogenic microorganisms can be transmitted, besides direct or indirect physical contact, also through air via respiratory droplets (>5 μm in diameter) and aerosols (from submicron to approximately 5 μm diameter).[4–6] A recent appeal of 239 experts in the field has drawn even more attention on the possible transmission of SARS-CoV-2 by means of small droplets (5–10 μm) in poorly ventilated spaces,[5] but the WHO has recently commented on the mounting evidence that viral particles floating indoors can be infectious, maintaining that related research is still inconclusive.[7]

Consequently, the spread of the infection might be significantly wider than the one associated with bigger droplets (traveling <1–2 m from the infected individual). In this perspective, new preventive solutions to mitigate the effects of airborne transmission might be needed, including suitably modified ventilation systems in synergy with powerful new filtering devices able to capture and/or inactivate the airborne microdroplets.[6,8–12] Indeed, several studies demonstrated that the transmission via aerosols is of great importance in the COVID-19 pandemic caused by the SARS-CoV-2.[9] In particular, the SARS-CoV-2 virus is primarily released from the respiratory tract as an aerosol with droplets generated, for example, by exhalation, talking, sneezing, coughing, and it can be increasingly released through intubation, bronchoscopy, rhinoscopy, or surgical interventions.[13–15] On this topic, van Doremalen et al.[9] showed that viruses can remain viable and infectious in indoor air as an aerosol for 3 hr.

While extraordinary efforts worldwide are devoted to searching for a vaccine for COVID-19, physicists and engineers together with microbiologists can explore efficient means for preventing the spread. As ventilation and recirculation systems have been shown to insufficiently reduce the indoor concentration of airborne pathogens or even to increase the risk of aerosol transmission, decontamination of air has become an important area of research with significant impact on several environments including hospitals[16] and other enclosed areas that are prone to microbial contamination. In this perspective, new control technologies capable of inactivating aerosolized bacteria and viruses are highly sought after; these new technologies must be economically convenient, should not be responsible for secondary pollution, and must be able to effectively inactivate different types of pathogens.[8,17] However, the efficacy of treatments depends on genome structure of the pathogens and their replication machinery[18]; furthermore, new technologies must take into account the possibility that both bacteria and viruses could develop harmful mutagenic outcomes associated with multiple transition mutations across the genome when they are underexposed or undergo insufficient inactivation treatments.[18,19]

Cold atmospheric pressure plasmas (CAPs) have been increasingly used as a tool for air sterilization and decontamination.[20,21] CAPs generate many reactive oxygen and nitrogen species as O_3, NO, NO_2, N_2O_5, HNO_2, HNO_3, $ONOO^-$, H_2O_2, •OH, and many other reactive species, that can react with the airborne particles and mediate various oxidative processes.[22–25] Due to the associated electric field, nonthermal plasmas (NTPs) can alter particle transport by inducing a negative charge on the particles, thus imparting charge-driven filtration as well as trapping of these particles. In this way, plasma can simultaneously act as a particle filter as well as a disinfection technology.[23,26,27]

Dielectric barrier discharge (DBD) is a popular approach to produce CAP at atmospheric pressure used for air treatment.[25] DBD produces many reactive species with antimicrobial efficacy, including ozone, and short-lived and stabilized species. Gallagher et al. use a dielectric barrier grating discharge plasma device to deactivate high concentrations of bacterial bioaerosol in flight at high flow rates in a ventilation system. It is shown that a 1.5- and 5.5-log reduction of the airborne *Escherichia coli* are achieved, respectively, after single plasma exposure of 10 s and 2 min.[19,28]

Another study demonstrated that NTP generated by a wire-to-plate type DBD reactor induces a significant inactivation of both *Bacillus subtilis*- and *Pseudomonas fluorescens*-containing bioaerosol.[2] Park et al.[1] used a different kind of DBD architecture to effectively inactivate *Staphylococcus epidermidis* bioaerosols using a short residence time (0.24 s). Romero-Mangado et al.[19] studied the effect of DBD on aerosolized *E. coli* concluding that the cell structure is damaged to a varying extent and severe oxidation of the cell membrane is found, establishing effective inactivation of the bacteria. Romero-Mangado et al.[29] also demonstrated the possibility to inactivate bioaerosol containing *S. epidermidis* or *Aspergillus niger*, respectively, a Gram-positive bacteria and fungal spores by means of CAP treatment. The morphology observed on the scanning electron microscope (SEM) micrographs shows deformations in the cellular structure of both microorganisms. Cell structure damage upon interaction with the DBD suggests leakage of vital cellular materials, which is a key mechanism for microbial inactivation.[29] Nayak et al.[23] shows that a volumetric DBD is effective in inactivating aerosolized porcine reproductive and respiratory syndrome (PRRS) virus in a wind tunnel within a few milliseconds, timescales relevant for typical heating, ventilation, and air conditioning (HVAC) conditions (few milliseconds). A 3.5-log reduction in the viable PRRS virus titer is achieved and the inactivation effect is independent of the discharge power and the sampling

time.[23] Moreover, similar values of log reduction is achieved by a packed-bed NTP reactor on bioaerosol containing infective bacteriophage MS2 and PRRS virus, 2.3 log and 5 log, respectively.[30,31]

The objective of the present preliminary study is to set the basis for supplementing state-of-the-art ventilation systems of indoor spaces with plasma-assisted portable air cleaners evaluating the lab-scale efficacy in the inactivation of aerosolized *S. epidermidis* and purified RNA of SARS-CoV-2 by a direct DBD plasma source, crucial to assess the potential efficacy of this technology toward the SARS-CoV-2 virus. aluminum electrodes (5 × 150 × 2 mm) fixed by epoxy resin onto two dielectric supports and covered by porcelain stoneware layers (with ϵ_r between 6 and 8); the interelectrode gap is 2 mm. As shown in Figure 1a, the plasma device was driven by a micropulsed high voltage generator (AlmaPULSE; AlmaPlasma s.r.l, Italy), applying a peak-to-peak voltage (V) of 56 kV and a frequency (f) of 14 kHz. A single-jet Blaustein Atomizer (BLAM; CH Technologies), fed with a volumetric flow rate of 0.6 slpm by a digital mass flow controller (EL-FLOW; Bronkhorst), is used to aerosolize 5-ml *S. epidermidis* (Gram-positive) or purified SARS-CoV-2 RNA suspensions Amplirun® SARS-CoV-2 RNA Control (Vircell Micriobiologists, Clearly, the use of purified SARS-CoV-2 RNA constitutes an introductory step to a more comprehensive analysis of the antiviral properties of CAPs, considering that viral proteins play a major role in establishing an infection. These preliminary results lay the foundation for the realization of a CAP system suitable for the control of the spread of this virus in indoor and poorly ventilated space.

2 MATERIALS AND METHODS

2.1 Plasma source and its electrical characterization

The plasma device employed in this study (Figure 1), a parallel-plate direct DBD configuration, consists of two Granada, Spain). Bioaerosols are flown through the interelectrode gap and exposed to the plasma discharge, with an estimated residence time of about 0.18 s, calculated as the ratio of the volume, which is delimited by the interelectrode gap and the volumetric flow rate. Figure 1c shows a representative picture of the microdroplets flowing out the plasma source. A laser pointer (YYLE; 532 nm, 5 mW) is used to illuminate the flow of aerosol at the exit of the plasma source; the influence of different levels of relative humidity (RH) is not taken into account since the RH of the compressed air line used to generate the aerosol feeding the plasma source is set stable around 7%.

The voltage (V) and the current (I) were measured by means of a high voltage probe (Tektronix P6015A) and a current probe (Pearson 6585), while the charge (Q) was evaluated measuring the voltage across a monitor capacitor of 0.94 nF (connected between the plasma source and the ground) by means of a low voltage probe (Tektronix P6139A). The corresponding waveforms were recorded using a digital oscilloscope (Tektronix DPO4034; 350 MHz, 2.5 GSa/s). The average discharge power (P) dissipated over the period (T) was determined from the voltage–charge (Lissajous) plots,[32] applying the following formula:

$$P = f \oint_T Q(V)dV. \quad (1)$$

Additionally, the real-time measurement of the aerosol particle size distribution at the outlet of the BLAM nebulizer was carried out using the laser diffraction system Spraytec (Malvern Panalytical, UK).[33,34] The measurements were performed 20 mm downstream from the outlet pipe of the BLAM nebulizer, to reduce the effects of the evaporation rate. The Sauter diameter (d_{32}), that is, surface-volume mean diameter, defined as the diameter with the same ratio of volume to surface area of the entire ensemble, and the De Brouckere diameter (d_{43}), that is, volume-weighted mean diameter, defined as the mean of a particle size distribution weighted by the volume were evaluated as follows:

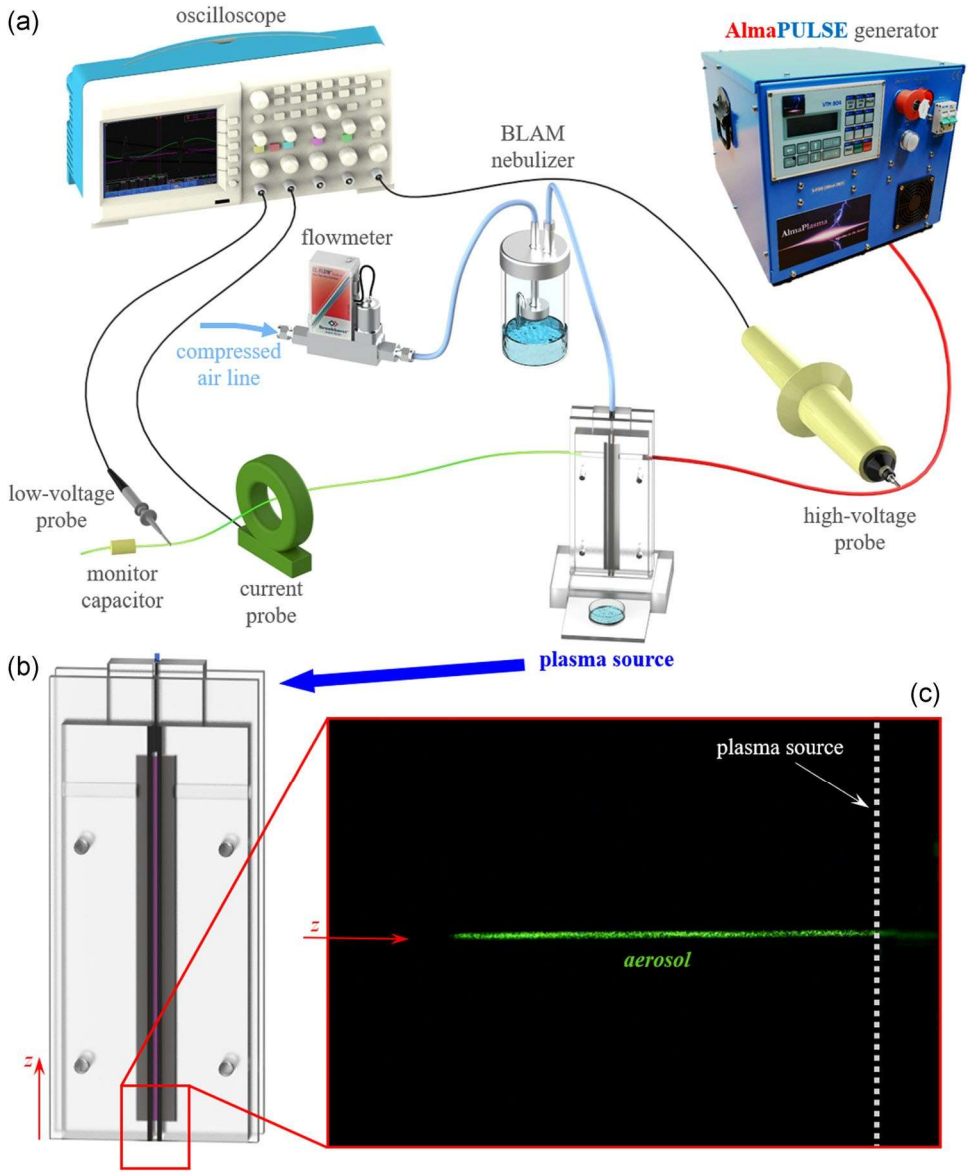


FIGURE 1 Schematic of the experimental setup for the CAP treatment of bioaerosols: (a) setup used for electrical characterization and (b) DBD plasma source. (c) Aerosol flowing out the DBD source during plasma treatment. CAP, cold atmospheric pressure plasma; DBD, dielectric barrier discharge

$$d_{32} = \frac{\sum_{i=1}^{N} x_i d_i^3}{\sum_{i=1}^{N} x_i d_i^2} \quad (2)$$

$$d_{42} = \frac{\sum_{i=1}^{N} x_i d_i^4}{\sum_{i=1}^{N} x_i d_i^{32}} \quad (3)$$

where x_i is the fraction of the total particle number associated with a diameter d_i, and N is the number of discrete size classes used.

2.2 Evaluation of the effect of CAP treatment on bacteria and purifiedSARS-CoV-2 RNA

2.2.1 *S. epidermidis* culture condition and CAP treatment

S. epidermidis is the most common bacteria on human skin and mucosal microbiota,[35] for example, nares,[36] typically studied in the field of bioaerosol research.[1] It is

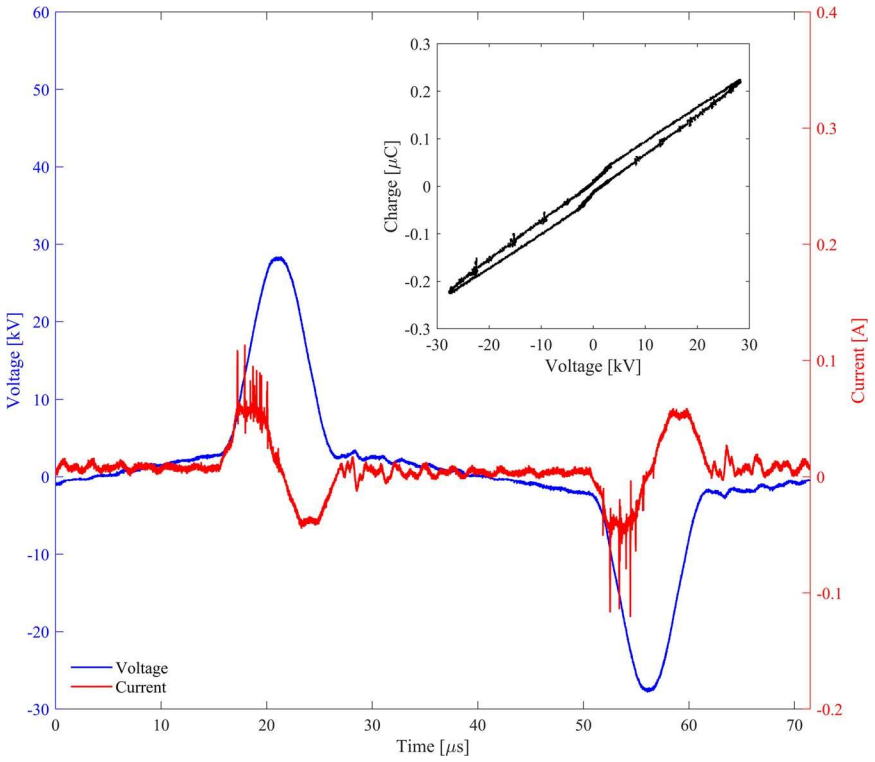


FIGURE 2 Current and applied voltage waveforms at operating conditions of 56 kV_{p-p} and 14 kHz. Inset: voltage–charge plot

not usually pathogenic but it is also a frequent cause of healthcare-associated infections.[37,38]

S. epidermidis (ATCC 12228) was cultivated on Tryptic Soy Agar plates and incubated at 37°C for 24 hr; colonies were used to prepare a standardized suspension in phosphate-buffered saline (PBS), a buffered solution commonly used in biological research, at $OD_{600\ nm} = 0.2$ (i.e., 10^7–10^8 CFU/ml). To evaluate the CAP antibacterial activity on bioaerosol, the nebulizer was loaded with 5 ml of standardized suspension. Bioaerosol was flowed through the plasma region for 150 s, collected in 1 ml of a liquid substrate, and plated. Control samples were collected using the same procedure while the plasma discharge was not generated. The liquid substrates for collecting samples were PBS or PBS-containing sodium thiosulphate, $Na_2S_2O_3$ (100 mM), as quenching solution for reactive species, which dissolve into liquid after plasma exposure, for example, O_3.[3,23] The plates were incubated at 37°C for 24 hr for viable colonies counting according to the following formula: $\log R = \log N_0 - \log N_t$ (N_0, viable colonies in control samples; N_t, viable colonies in treated samples). Results are presented as the mean $\log R \pm$ standard error of the mean of experimental data obtained from at least three independent experiments.[23,39,40]

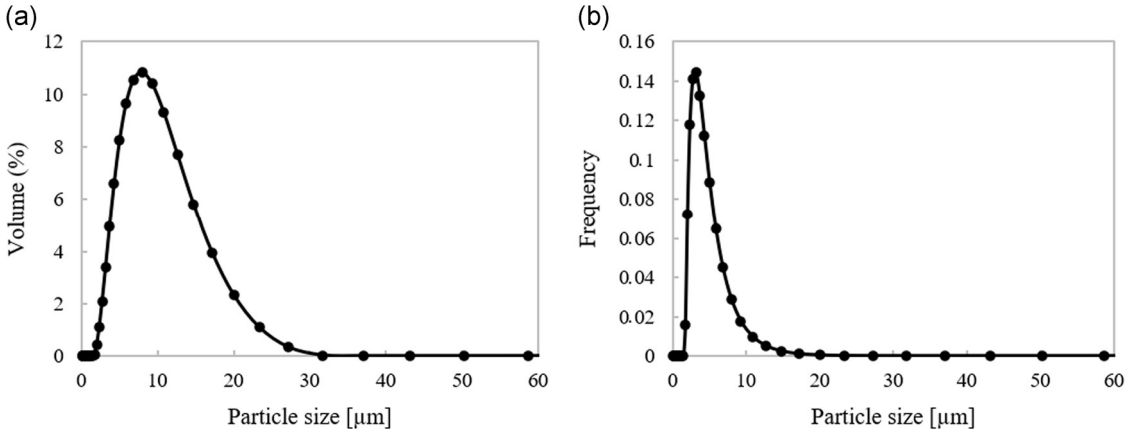


FIGURE 3 Size distribution of aerosol droplets: (a) distribution of the volumetric fraction and (b) distribution of the number fraction

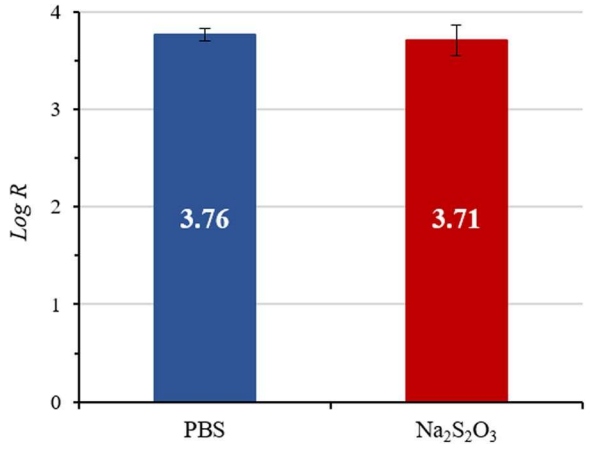


FIGURE 4 Log reduction (log *R*) of *Staphylococcus epidermidis* bioaerosol collected in phosphate-buffered saline (PBS) and PBS-containing $Na_2S_2O_3$ after cold atmospheric pressure plasma treatment. Results are presented as the mean log *R* ± standard error of the mean of three independent experiments

2.2.1 CAP treatment of bioaerosol containing purified SARS-CoV-2 RNA

Similarly, to evaluate the CAP effect on SARS-CoV-2 RNA, the nebulizer was loaded with 5 ml of RNA suspension and the bioaerosol collected in 1 ml of distilled water after flowing through the plasma region for 150 s, with or without (control) CAP treatment.

Successful plasma degradation of viral RNA was assessed by reverse-transcription polymerase chain reaction (RT-PCR) using Allplex 2019-nCoV assay (Seegene, Seoul, South Korea) targeting three viral genes: E (specific of the subgenus sarbecovirus), RdRp and N, both specific of SARS-CoV-2. The untreated RNA suspension was tested in parallel as positive control. In the RT-PCR assay, a positive reaction has been detected by the accumulation of a fluorescent signal using specific fluorophores: FAM, Cal Red 610, Quasar 670. The C_t (cycle threshold) has been defined as the number of cycles required for the fluorescent signal to cross the threshold. C_t levels were inversely proportional to the amount of target nucleic acid in the sample.

3 RESULTS AND DISCUSSION

3.1 Electrical characterization of the DBD plasma source

Figure 2 shows current and applied voltage waveforms representative of the electrical steady-state behavior of the plasma device operating at 56 kV_{p-p} and 14 kHz. The measured current contains both the one asso- ciated with active charge transfer in the gas gap (the discharge current) and the displacement one in the gas gap.[41] The presence of discharge activity can be distinguished by multiple discharge current spikes superimposed on the displacement current waveform. In the inset of Figure 2, the corresponding voltage− charge plot is reported. The average discharge power calculated from the area of this figure multiplied by the frequency is 17.23 W.

3.2 Characterization of the droplet distribution of the bioaerosol

The results from the real-time laser diffraction analysis show a log-normal droplet volume fraction distribution centered at 7.97 µm. Figure 3 shows that the droplet size distribution is characterized by a large number of very tiny particles with a diameter close to 6 µm (Figure 3b), but most of the volume of the liquid phase is due to droplets with a diameter close to 9 µm (Figure 3a).

TABLE 1 Results for SARS-CoV-2 RNA RT-PCR

Allplex™ 2019-nCoV assay							
	FAM		Cal Red 610		Quasar 670		
Name	E gene	C_t	RdRP gene	C_t	N gene	C_t	Interpretation
1	+	34.88	+	35.19	+	36.43	Positive
1-Bis	+	34.04	+	35.71	+	35.48	Positive
2	–	N/A	–	N/A	–	N/A	Negative
2-Bis	–	N/A	–	N/A	–	N/A	Negative
Negative control	–	N/A	–	N/A	–	N/A	Negative
Positive control	+	18.99	+	19.33	+	18.20	Positive

Abbreviation: RT-PCR, reverse-transcription polymerase chain reaction.

The Sauter (d_{32}) and the De Brouckere (d_{43}) diameters result equal to $d_{32} = 6.59$ and $d_{43} = 8.97$.

3.3 CAP effect on bacteria and purified RNA bioaerosols

The antibacterial activity of CAP on *S. epidermidis* bioaerosol was investigated and evaluated considering two liquid substrates for sample collections, that is, PBS and $Na_2S_2O_3$ in PBS. As summarized in Figure 4, CAP exerted an antibacterial activity on bioaerosol in a short residence time (<0.2 s) with a log R of 3.76 ± 0.06 and 3.71 ± 0.16, when samples were collected in PBS and PBS-containing $Na_2S_2O_3$, respectively.

Furthermore, CAP efficacy was evaluated on SARS-CoV-2 RNA and double-tested for specific SARS-CoV-2 targets, including a PCR-positive and negative control as a quality assurance of the entire amplification process (Table 1). The CAP-treated RNA suspensions resulted negative (samples, 2 and 2 bis), while the untreated ones showed positive results for all the three specific investigated genes (samples, 1 and 1 bis).

4 CONCLUSIONS

The presence of bioaerosol is generally correlated with human activity, particularly in indoor and poorly ventilated spaces, and it has been suspected as one of the causes of spreading diseases. Hence, increasing indoor air quality can play a pivotal role in human health. In this field, there is a need for innovative technology that could inactivate microbes and viruses in a short time. In this study, plasma technology is explored as a novel approach to inactivate bioaerosol containing *S. epidermidis* or purified SARS-CoV-2 RNA suspension using a lab-scale flow-through volumetric DBD reactor, a first unit of a future modular configuration. Results show that CAP can induce a log R around 3.76 on bacterial bioaerosol and degrade viral RNA in a short residence time (<0.2 s).

Further investigations are ongoing: the effects induced on bioaerosols containing viable SARS-CoV-2 virus, the analysis of the mechanisms behind the bioaerosol inactivation, to separate the filtering effect due to the electric field from the antimicrobial effect of reactive species produced by CAP, and the identification of the most suitable filter for the degradation of potentially harmful chemical species at the outlet of the DBD reactor. These preliminary results constitute a promising step toward the design and realization of a plasma-assisted portable air cleaner suitable for the containment of indoor airborne transmission of respiratory diseases such as COVID-19.

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