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SARS-CoV-2 inactivation by supercritical carbon dioxide coupled with hydrogen peroxide

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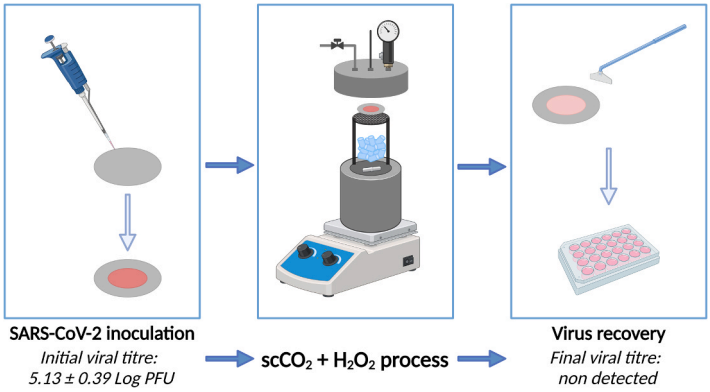
HIGHLIGHTS

- An experimental setup for supercritical CO_2 without pump was validated.
- SARS-CoV-2 sterilisation was achieved with a small amount of H_2O_2.
- Synergism between supercritical CO_2 and H_2O_2 was verified.

GRAPHICAL ABSTRACT



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ABSTRACT

The recent COVID-19 pandemic has underscored the need for innovative decontamination techniques capable of treating sensitive materials potentially contaminated. Combining supercritical carbon dioxide ($scCO_2$) with sterilant agents has shown promise in this regard. This study aimed at testing $scCO_2$ as a virus inactivation method for biomedical materials contaminated with the Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2). The virus was inoculated on a stainless-steel carrier and treated at 45 °C and 8 MPa. No inactivation was detected when only $scCO_2$ was used, even after long treatment times (60 min). The addition of 50 ppm of H_2O_2 to the process allowed the inactivation of more than 5 Log PFU (Plaque Forming Unit) of the virus by only pressurising and depressurising the vessel, while a 20-min process is needed by only using H_2O_2. Overall, the study demonstrates a synergistic effect when H_2O_2 is added to the $scCO_2$ process for the inactivation of SARS-CoV-2.

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1. Introduction

The recent pandemic caused by the diffusion of the Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) has resulted in 766.9 million confirmed cases and 6.9 million deaths globally [1]. Studies confirmed that infectious virions remain viable on various kinds of surfaces, especially plastic and steel, for several hours [2], representing a significant factor in virus transmission and human infection. This pandemic has also raised attention to the global production capacity of biomedical instruments, devices and personal protective equipment (PPE), especially face masks, which fell significantly short of meeting the demand of that particular period of time [3]. To address this challenge, the development and optimisation of decontamination technologies to facilitate the quick and safe sanitisation and reuse of biomedical instruments and PPE could not only prevent future unpreparedness in the healthcare industry but also mitigate the significant waste production and environmental impact associated with a similar event [4].

Standard sterilisation methods in the biomedical industry usually include radiation, steam, and disinfectant gases. Radiation-based decontamination techniques, such as gamma, electron beam (E-beam), X-ray, and UV-C, have been demonstrated effective against a large number of human pathogens, including corona viruses [5]. However, the detrimental effects and structural changes observed on different products (*i.e.* polymers and tissue allografts) treated with radiation-based sterilisation technology [6], together with the limited penetration capacity towards dense material or less-exposed sections of the products [7,8], represent a limitation for their application. On the other hand, the use of steam and disinfectant gases, namely hydrogen peroxide and ethylene oxide, may lead to the alteration in the performance of PPE, such as compromised filtration capacity of face respirators [9], or deposition of residues on the sterilised product. Moreover, a strong effort has been addressed in the last years by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) to reduce the worker and patient exposure to ethylene oxide, reported to be associated with various cancers, such as lymphoma and leukaemia [10].

In the context of the COVID-19 pandemic, different techniques have been explored to inactivate the virus and some indicator spores on protective masks and other matrices. Biffi et al. [11], for instance, achieved a 3-Log reduction of SARS-CoV-2 through the application of 280 nm UV LED irradiation in a plaque assay test. Hydrogen Peroxide Vapour (HPV) process has been tested for the sterilisation of masks obtaining a total inactivation of *Geobacillus stearothermophilus* [12] and some bacteriophages [9]; however, the cost for the industrial plant was estimated at around 6.9 million USD [13], thus very expensive for its common use on large scale.

Supercritical carbon dioxide ($scCO_2$) is a promising sterilisation agent that has been extensively studied in recent years. $scCO_2$ exhibits unique properties, including high diffusivity, low viscosity, and adjustable density, facilitating its penetration and dissolution into diverse materials. Moreover, $scCO_2$ is non-toxic, non-flammable, and it is considered environmentally friendly, making it a desirable alternative to conventional solvents, particularly in sensitive material processing [14].

The bactericidal effect of $scCO_2$ was first reported in the 1950s [15], and since then, many research papers have focused on the inactivation of bacteria, yeasts, and moulds using supercritical carbon dioxide for food and biomedical applications, demonstrating its effectiveness for a wide range of matrices and process conditions. In this case, as clearly shown by several authors [14,16,17], the interaction between $scCO_2$ and the cell membranes, together with the alteration of internal metabolisms, seem to be the main cause of cell death. However, $scCO_2$ alone is not able to inactivate bacterial spores and viruses because of their higher resistance to thermal and chemical penetration [14] and the use of high temperatures [18] or chemical additives is needed. The coupling effect of CO_2 with different sterilants, such as peracetic acid [19] and hydrogen peroxide [20], has been investigated obtaining promising results in the sterilisation of spores and viruses. Indeed, according to literature [19,20], the combination of CO_2 and chemical additives can strongly affect the permeability of the cells (outer envelope damage) and alter the internal metabolism by pH reduction, inactivation of proteins and enzymes and introduction of free radicals.

Regarding the sterilisation with $scCO_2$, the process has been recently applied on different spore strains (e.g., *Bacillus atrophaeus*, *GeoBacillus stearothermophilus, Bacillus pumilus*) inoculated onto respiratory protective equipment [3,23]. Inactivation to undetectable levels has been achieved using additives, especially hydrogen peroxide, peracetic acid, and ethanol, at concentrations ranging from 100 to 10,000 ppm. Utilising lower amounts of sterilants preserves the physical and chemical attributes of the tested face masks, including their filtration capacity. However, results on the SARS-CoV-2 strain treated with $scCO_2$ are scarcely present in the literature particularly due to the difficulties in safely performing these tests with high-pressure plants. To the best of the authors' knowledge, only Bennet et al. [21] have examined $scCO_2$ treatment on SARS-CoV-2 inoculated on N95 respirators, obtaining a complete inactivation after 90 min at 33–35 °C and 100 bar by using 1.25 % (v/v) of a solution composed of ethanol, peracetic acid and hydrogen peroxide in a 600-mL vessel. However, the efficacy of the vaporised solution alone was not verified making difficult to understand the role of $scCO_2$ in the inactivation mechanism.

This study aims to investigate the inactivation capacity of $scCO_2$ in synergism with a 50 ppm of H_2O_2 against SARS-CoV-2. This work permits to verify the inactivation behaviour obtained by previous studies on test microorganisms, such as bacterial spores or surrogate viruses, using, for the first time, a concentration of sterilant that has been shown to not alter the properties of the tested materials. Moreover, the inactivation was evaluated considering individual treatments ($scCO_2$ and H_2O_2 alone) which were overlooked in prior research, in order to demonstrate their synergistic effect.

2. Material and methods

2.1. Cells and virus preparation and inoculation

All the experiments, virus handling, and recovery procedures were carried out in a biosafety level 3 (BSL-3) facility at the Department of Molecular Medicine, University of Padova, Italy.

The African GreenMonkey Kidney Vero E6 (ATCC CRL-1586) cell lines were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM, Euroclone, Milano, Italy) supplemented with 10 % foetal bovine serum (FBS, Euroclone, Milano, Italy), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin sulphate (P/S, both from Life Technologies, Carlsbad, CA, USA). SARS-CoV-2 (Wuhan Hu-1 strain) was obtained from Istituto Superiore della Sanità (ISS) in Rome, Italy. The virus was propagated and titrated in Vero E6 cells following the method described in [24]. Briefly, each viral suspension was prepared and amplified on a large scale in monolayer cell cultures. After infection and multiplication of the virus, cell debris was removed by double centrifugation at low speed (699 × *g* for 10 min), and the supernatant containing the virus was collected to determine the viral titre (as described in Section 2.3). The supernatant was then aliquoted and stored at − 80 °C.

In order to evaluate the viricidal effect of the method, the virus suspension was tested following the standard test on non-porous surface without mechanical action EN 1677 [25]. Before each test, a volume of 50 μL of the viral suspension (around 10^7 PFU/mL, Plaque Forming Units per millilitre) was directly inoculated on sterile stainless-steel discs with a diameter of 35 mm, used as non-porous test carriers. The suspension was allowed to dry for around 30 min under a laminar flow hood. The control carriers were inoculated with the viral suspension (50 μL) but not subjected to any treatment.

2.2. Treatment procedure

The high-pressure tests were performed using a stainless-steel vessel with bolted closure, an internal volume of 330 mL and an internal height and diameter of 11 cm and 6 cm respectively, and able to hold up to 30 MPa. The vessel was equipped with a temperature probe (PT100, National Instruments, Milano, Italy) and a pressure gauge (EN837, Wika, Milano, Italy) to monitor the internal temperature and pressure during the treatments. The temperature probe was positioned near the inoculated disc at the top of the vessel. The vessel presented one outlet tube with a manual micrometric valve (Swagelok, Nordival, Milano, Italy) that was maintained closed until the depressurisation phase. A schematic representation of the setup is reported in Fig. 1.

The vessel was autoclaved and pre-heated to 45 °C before each test. The experimental procedure was performed in a BSL-3 facility, thus a specific setup was implemented and optimised for the study. Usually, $scCO_2$ processes are performed within a high-pressure setup, which includes several components (e.g. CO_2 tank, tubes, high-pressure pump, chiller). For safety reasons, it was not allowed to include a conventional setup in a BSL-3 facility. The supercritical conditions were achieved by using solid CO_2 followed by a change of state due to temperature increase and its consequent increase of pressure induced by a controlled volume. In particular, the amount of CO_2 inserted (115 g) was optimised to achieve the desired pressure conditions during the process considering thermodynamics calculation and practical arrangements (see Section 3). After the addition of CO_2, the inoculated disc was placed on a stainless-steel stand and the vessel was quickly closed. The reduction in temperature caused by the solid CO_2 was compensated by using a heating plate positioned under the vessel, leading to the achievement of supercritical conditions. Temperature and pressure were maintained at the target values of 45 ± 0.5 °C and 8.0 ± 0.1 MPa, respectively, ensuring the supercritical state of CO_2. The treatment time was defined as the duration from reaching the desired pressure until the initiation of depressurisation. The pressurisation step lasted approximately 2 min, while the depressurisation time was fixed at 3 min by manually opening the micrometric valve.

Mixing during the treatments was ensured by a magnetic stirrer positioned at the bottom of the vessel, operating at 400 rpm. High-pressure treatments were conducted with ($scCO_2 + H_2O_2$) or without ($scCO_2$) the addition of 15 µL (50 ppm) hydrogen peroxide (H_2O_2) (30 % wt in water, Sigma-Aldrich, Burlington, MA, USA). To evaluate the combined effects of CO_2 and H_2O_2, treatments with only temperature (CTRL-T) and treatments with both temperature and H_2O_2 (H_2O_2) were included.

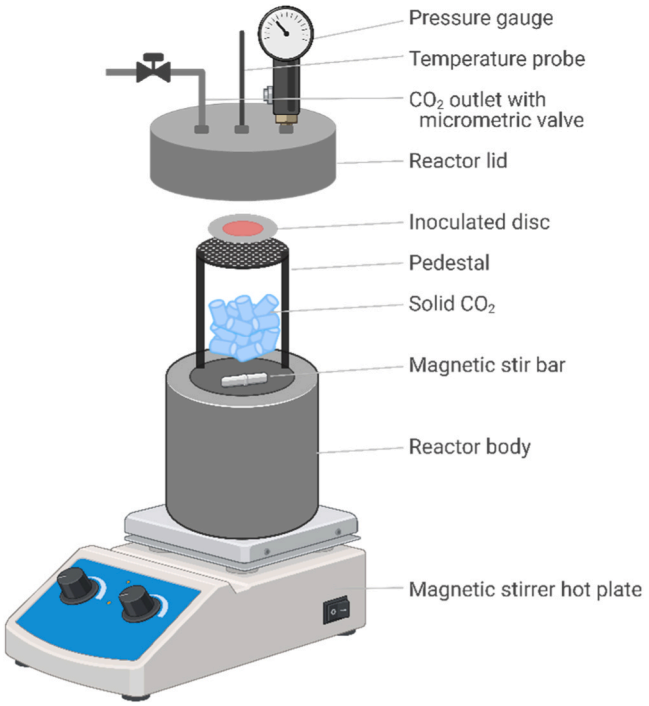

Fig. 1. Schematic representation of the experimental setup.

2.3. Determination of the viral titre

The infectivity titres of the virus for both untreated and treated samples were recovered by eluting the stainless-steel discs with 1 mL of culture medium and scraping them for 1 min. Serial dilutions ranging from 10^{-2} to 10^{-9} (1:10 dilutions) were prepared.

Briefly, 10^5 Vero E6 cells/well were seeded in a 24-well plate in 500 µL/well of DMEM 10 % FBS. The day after, when the confluence was roughly 90 %, cells were infected with 250 µL of 10-fold dilutions (10^{-2}–10^{-9}) of the virus of the untreated and treated samples in serum-free DMEM. After an incubation of 1 h at 37 °C, cells were washed with PBS (Phosphate Buffered Saline) (1X, pH 7.4) to remove the cell-free virus and incubated at 37 °C with 500 µL/well DMEM supplemented with 2 % v/v FBS and 0.75 % v/v carboxy-methyl-cellulose.

After the incubation at 37 °C for 72 h, plaque formation was assessed to determine PFU/mL. Cells were washed, fixed with 500 µL of 5 % formaldehyde for 10 min and stained with 0.1 % v/v crystal violet (for 50 mL: 50 mg crystal violet, 10 % methanol and deionized H_2O to volume). The plaques were counted at the optical microscope and the viral titre was calculated using the following equation:

$$X = n/(D * V) \quad (1)$$

where X is the virus titre in PFU/mL, n is the average number of plaques counted, D is the dilution factor and V is the volume of diluted virus/well. A decrease of ≥ 4 log units of viral titre was considered a viricidal effect.

2.4. Statistical analysis

Minitab® software was used to analyse statistical differences between the experimental and control groups. After verifying the independence of the observations, the normality of the data and the homogeneity of variances, ANOVA with post-hoc Tukey HSD analysis was performed, and a p-value of 0.05 was used as the threshold for significance. In order to verify the reproducibility, each treatment was repeated at least two times and the same sample was analysed twice.

3. Results and discussion

3.1. Experimental setup optimisation

In this study, the inactivation efficacy of $scCO_2$ in combination with hydrogen peroxide was assessed on stainless-steel discs inoculated with SARS-CoV-2. Preliminary trials were conducted to optimise and validate a new experimental setup that avoids the use of CO_2 tanks and pressurisation pumps by acting only on the temperature control. The protocol was optimised to ensure a robust experimental procedure using solid CO_2 and achieving the supercritical conditions in a short time. To compensate for the heat losses and temperature decrease during the CO_2 sublimation, the vessel was preheated and positioned on a hot plate. The temperature was fixed at 45 °C and monitored by a temperature probe located close to the inoculated disc. Different amounts of solid CO_2 were tested (data not shown) and 115 g of CO_2 was found to be the optimal initial amount to achieve a stable pressure of 8.0 MPa after only 2 min from the sealing of the vessel. Notably, the quantity used is higher than the estimation based on calculations by vessel internal volume (330 mL) and carbon dioxide density under the given conditions (250.46 kg/m^3, [26]). This discrepancy might be attributed to the loss of gaseous CO_2

through sublimation during the initial protocol phase (weight of solid CO_2, insertion inside the vessel, disc positioning and sealing of the vessel lid). Larger quantities of solid CO_2, which could have allowed the obtainment of higher pressure, were not considered due to constraints related to vessel volume and the need to avoid the direct contact of solid CO_2 with the inoculated disc.

The stirrer velocity was also optimised and fixed at 400 rpm to allow a quick distribution of CO_2 and the additive while ensuring its correct rotation inside the vessel. Fig. 2 reports the evolution of temperature and pressure overtime for the optimised protocol at 45 °C.

After the closure of the vessel, pressure rapidly increases to the desired value (8 MPa) in about 2 min with a slight deceleration near the critical pressure. The pressure set point is maintained during the whole treatment. At the end of the processing time, depressurisation can be achieved in about 3 min by opening the exit valve, corresponding to a depressurisation rate that remains roughly constant at around 2.67 MPa/min. The temperature, which was initially pre-heated at 45 °C, is partially cooled down due to the rapid change of phase of solid CO_2. However, the hot plate rapidly compensates for this decrease in temperature and mitigates heat losses during the treatment, ensuring a stable temperature profile. During depressurisation, the temperature quickly decreases due to the rapid decompression of CO_2.

The validated experimental setup allowed us to test the high-pressure technology inside a BSL-3 facility without the use of bulky and expensive equipment, such as high-pressure pumps, CO_2 tanks and chillers.

3.2. Inactivation tests

Once the operative protocols were optimised, stainless-steel discs were inoculated starting from an initial inoculum with a viral titre of 6.14 ± 0.29 Log PFU and the dried samples were subjected to treatments involving $scCO_2$ and/or hydrogen peroxide. Table 1 reports the obtained results for both control and experimental runs.

Temperature (45 °C) and supercritical carbon dioxide (45 °C and 8 MPa) alone did not result in a significant reduction of viral titre, even after a 60-min exposure period. These findings are in agreement with the previous literature studies. Indeed, different studies investigated the effect of low-temperature $scCO_2$ on various strains of viruses, such as vesicular stomatitis virus (VSV) [13]. Similar results were achieved also for the inactivation of bacterial spores, including *Bacillus subtilis* [18] and *Bacillus coagulans* [27], but the effects were always minimal unless high temperatures (> 60 °C) [18], and/or extended treatment times

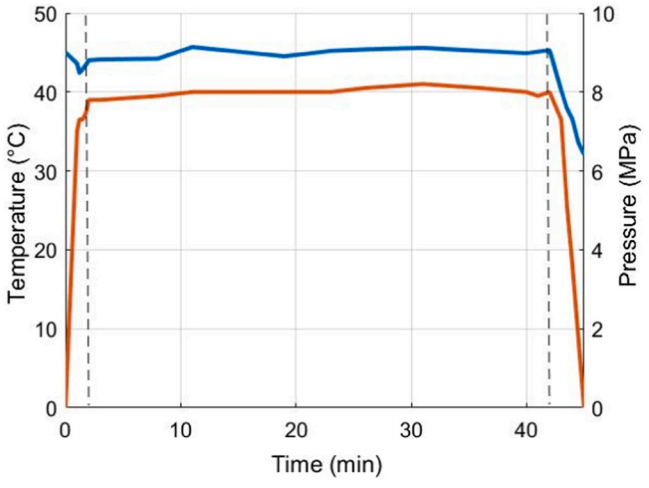

Fig. 2. Temperature (blue) and pressure (orange) profile of the optimised protocol. The vertical dashed lines represent the start and the endpoint of the treatment, respectively.

Table 1
SARS-CoV-2 viral titre on the control carrier (CTRL) and after treatment by only temperature at 45 °C (CTRL-T), by $scCO_2$ at 45 °C and 8 MPa ($scCO_2$), by H_2O_2 at 45 °C (H_2O_2), and by $scCO_2$ and H_2O_2 at 45°C and 8 MPa ($scCO_2 + H_2O_2$). Data are expressed in Log PFU (mean ± standard deviation). Each test was repeated at least twice. Means with different superscript letters are statistically different (p-value ≤ 0.05).

Sample	Time (min)	Viral titre (Log PFU)
CTRL	-	5.13 ± 0.39^{a}
CTRL-T	30	5.16 ± 0.15^{a}
	60	4.74 ± 0.09^{ab}
$scCO_2$	30	4.82 ± 0.17^{ab}
	60	4.37 ± 0.20^{ab}
H_2O_2 (50 ppm)	10	4.60 ± 0.16^{ab}
	15	4.00 ± 0.03^{b}
	20	n.d.
$scCO_2 + H_2O_2$ (50 ppm)	0	n.d.
	5	n.d.
	10	n.d.

n.d. = non-detected, viral titre < 1 Log PFU.

(days) were adopted [28].

Given its strong oxidising properties and its confirmed spore and virus inactivation capabilities, hydrogen peroxide was selected as an additional agent. Moreover, it is an environmentally safer agent with respect to other sterilants usually adopted, such as peracetic acid or ethylene oxide [29,30]. Firstly, experiments were conducted to assess the efficacy of hydrogen peroxide alone at 45 °C. In the selected experimental setup, a concentration of 50 ppm of hydrogen peroxide at 45 °C needed 20 min to completely inactivate the virus.

Similar tests have been conducted by Goyal et al. [31] on various virus strains, including Transmissible gastroenteritis coronavirus (TGEV) (a SARS-CoV surrogate) inoculated on a stainless steel disc, with a much higher concentration of hydrogen peroxide and longer treatment times. The authors exposed the samples to hydrogen peroxide vapour (HPV) in an environmental chamber with recirculation inactivating to undetectable levels with a minimum sterilant volume of 25 mL (2 mL/min for 1 min followed by 1.5 mL/min for 15 min) in a 4-L vessel and with a total exposure time, including injection and aeration, of approximately 2–3 h.

When $scCO_2$ treatment was combined with H_2O_2, the pressurisation and depressurisation of the vessel alone were sufficient to completely inactivate the virus on the stainless-steel disc, resulting in a substantial reduction in inactivation time. Similarly, Bennet et al. [21] achieved a complete inactivation of SARS-CoV-2 inoculated on N95 respirators by employing a combination of $scCO_2$ and sterilant additives. In their study, samples were subjected to treatment at 33–35 °C and 10 MPa for a duration of 90 min. Despite the lower temperature, their approach involved prolonged exposure and a considerably higher sterilant volume compared to this study: 7.5 mL of a solution composed of ethanol, peracetic acid and hydrogen peroxide in a 600-mL vessel (> 10, 000 ppm). Furthermore, the exclusive application of vaporised sterilant to evaluate the synergistic effect with $scCO_2$ was not assessed, making it challenging to ascertain the potential synergy between the agents.

To the best of the author's knowledge, there are no other published studies about SARS-CoV-2 inactivation by $scCO_2$ methods. Other virus strains have been tested, such as *Encephalomyocarditis virus* (EMCV). Qiu et al. [19] treated EMV-inoculated porcine acellular matrix samples with $scCO_2$ and peracetic acid obtaining a complete inactivation after 15 min at 10 MPa and 37–41 °C and 55 ppm of sterilant. The longer inactivation time can be attributed to the slightly lower temperature, the system setup and the strain used. ECMV is indeed a type of non-enveloped virus, usually more resistant with respect to enveloped ones, such as SARS-CoV-2. Thus, the fast inactivation obtained in this study could be imputed to the lipophilic nature of the $scCO_2$ and its interaction and solubilisation with the virus envelope, causing membrane disruption and, therefore, the virus inactivation [22].

4. Conclusions

This study focused on the inactivation of SARS-CoV-2 virus inoculated on stainless steel discs using supercritical carbon dioxide ($scCO_2$) coupled with hydrogen peroxide (H_2O_2). The virus was effectively inactivated after only pressurising and depressurising the vessel with 50 ppm of H_2O_2. The application of the $scCO_2$ or H_2O_2 alone were not effective even after 60 min. These findings hold significant promise for the development of systems for treating different matrices, particularly for biomedical applications such as the treatment of facial masks for reutilisation. Further studies are needed to optimise the process parameters. In particular, the sterilant amount could be further reduced in order to decrease the risks of side effects of the treatment on the treated matrices and increase the range of products that could be treated with this method, such as natural and synthetic materials for tissue engineering. Moreover, different strains of SARS-CoV-2 and/or other viruses, such as non-enveloped ones, could be tested with the experimental setup used in this study.

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CRediT authorship contribution statement

Riccardo Zulli: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Claudia Del Vecchio:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Pietro Andrigo:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Valeria Conciatori:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Fabio Santi:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Alessandro Zambon:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Enrico Lavezzo:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Sara Spilimbergo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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The graphical abstract and Fig. 1 have been created with BioRender.com.

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