Innovative Food Science and Emerging Technologies 95 (2024) 103674

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Impact of species and strain variability on non-thermal plasma decontamination efficacy

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ARTICLE INFO

Keywords:
Surface decontamination
Plasma-processed air
Oxidative stress
Microbial inactivation
Strain variability

ABSTRACT

Microwave non-thermal plasma (NTP) is a promising preservation technology for surface decontamination, alternative to conventional methods such as heat treatments and chemical additives. This study explores the impact of plasma processed air (PPA) exposure on 50 strains from five diverse microbial species relevant to food quality and safety. Bacterial vegetative cells were effectively inactivated in an agar plate model system. The average reduction after 60 s of treatment was 4.9 $\log_{10}$, 5.8 $\log_{10}$, and 5.9 $\log_{10}$ for *Listeria monocytogenes*, *Escherichia coli*, and *Lactiplantibacillus plantarum* respectively, with limited strain variability. *Saccharomyces cerevisiae* reached an average of 5.3 $\log_{10}$ reduction after 30 s of treatment, with one resistant strain (AD1890). *Bacillus subtilis* spores were inactivated by 2.0 $\log_{10}$ on average after 30 min, with two distinct strain groups. These results showed that PPA can be used as a mild decontamination treatment applicable to dry and heat-sensitive surfaces, suitable for various food applications.

1. Introduction

Conventional thermal treatments such as pasteurization and sterilization are commonly used for the inactivation of pathogenic and spoilage microorganisms in food products to ensure safety and prolong shelf life. However, thermal processing can affect the overall quality of the product by changing appearance, taste, or texture, and by degrading nutrients such as thermosensitive vitamins (Awuah, Ramaswamy, & Economides, 2007). Moreover, thermal processes are energy-intensive and responsible for a large portion of the energy consumed in food processing (Ladha-Sabur, Bakalis, Fryer, & Lopez-Quiroga, 2019). For this reason, in recent years great attention has been paid to the development of innovative non-thermal technologies such as high-pressure processing, pulsed electric field, ultrasound, and non-thermal plasma (NTP). These methods allow to obtain minimally processed food products that have high quality, are safe and nutritious, and meet the consumers' demand for products that are natural and free from additives and preservatives (Khouryieh, 2021). NTP has gained interest as a promising technology for surface decontamination of fresh or ready-to-eat food at short treatment times (Thirumdas, Sarangapani, & Annapure, 2015).

Plasma is an ionized gas and it is considered the fourth state of matter next to solid, liquid, and gas states. Non-thermal plasmas are generated by the application of an electromagnetic field to a gas, causing the acceleration of free electrons and subsequent ionization of gas atoms and molecules (Mandal, Singh, & Pratap Singh, 2018). The resulting high-energy plasma is composed of free radicals, electrons, positive and negative ions, UV radiation, and different reactive chemical species depending on the carrier gas. The combination of these particles shows antimicrobial activity against a wide range of microorganisms, including bacteria, molds, yeasts, and even bacterial and fungal spores (Klämpfl et al., 2012).

Plasma properties are related to the reactive species produced, which are determined by intrinsic factors of the plasma source, as well as the carrier gas composition. Different configurations of plasma sources are currently available on the market, and the most common are dielectric barrier discharge, plasma jet, corona discharge, radiofrequency, and microwave. Commonly used gases are argon, helium, and air, where air

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https://doi.org/10.1016/j.ifset.2024.103674
Received 30 January 2024; Received in revised form 9 April 2024; Accepted 17 April 2024
Available online 21 April 2024

has the clear advantage of being cost-effective for food processing applications and easier to use (Laroque, Seó, Valencia, Laurindo, & Carciofi, 2022).

Although several studies tried to elucidate the mode of action of microbial inactivation upon NTP treatment, the specific mechanisms leading to cell death are not precisely known yet and could differ based on the setup and the application. For instance, it is known that UV light is capable of inhibiting microbial growth by inducing the formation of DNA thymine dimers. However, the contribution of UV radiation to the anti-microbial effect of NTP in most setups is debated (López et al., 2019). The most generally accepted hypothesis involves the primary role of reactive chemical species, and in particular reactive oxygen species (ROS) and reactive nitrogen species (RNS) for plasma produced with atmospheric air. Various microbial structures are affected by the action of these highly energetic chemical species, including membranes, structural proteins, enzymes, and DNA. ROS and RNS interact with biological macromolecules, affecting both outer membranes and intracellular structures (Dobrynin, Fridman, Friedman, & Fridman, 2009). In this study, the decontamination efficacy of plasma processed air (PPA) treatment was assessed using 50 strains of five pathogenic or spoilage species, namely *Listeria monocytogenes*, *Lactiplantibacillus plantarum, Escherichia coli*, *Bacillus subtilis* spores, and *Saccharomyces cerevisiae*, representing model species for Gram+ bacteria, Gram- bacteria, bacterial spores, and yeasts, respectively. This study provides information on the decontamination potential of NTP on diverse microbial targets, as well as the impact of strain variability on the overall resistance.

2. Materials and methods

2.1. Experimental set-up

A schematic representation of the NTP device and setup is shown in Fig. 1. NTP was generated by MidiPLexc, a microwave-driven plasma torch previously described by Handorf, Below, Schnabel, Riedel, and Ehlbeck (2020). The device produces microwaves with a frequency of 2.45 GHz and an input power of 80 W. It was operated under atmospheric pressure, using compressed air as working gas at a flow rate of $2.25*10^{-5}$ m^3/s. The produced PPA is collected in the glass bottle containing ~20 mL of distilled water to stabilize the humidity of the gas. The bottle is connected to an in-house designed polytetrafluoroethylene (PTEF) treatment chamber with dimensions 15 cm × 15 cm × 15 cm, where the inoculated agar plates (either round with a diameter of 9 cm or square 12 cm × 12 cm petri dishes, or standard well plate 12.8 cm × 8.5 cm) can be easily inserted and treated in a closed environment. PPA rapidly cools down and the treatment itself is carried out at room temperature. The plasma source was ignited 30 min before the treatment, to ensure a stable effluent production. To expose the samples to PPA, the lid was briefly removed from the opening of the treatment chamber to manually insert the plate. After the required treatment time, the sample was removed and the chamber was left for 3 min to equilibrate before the next treatment.

2.2. PPA characterization by Fourier Transformation infrared spectroscopy and concentration estimation

Fourier Transformation infrared spectroscopy (FTIR) (Vertex 70v; Bruker, Billerica, MA, USA) was connected 1.5 m downstream from the effluent of the PTEF treatment chamber. The measurements were carried out with an absorption path length of 0.25 m and a resolution of 0.6 cm^{-1}. A wavenumber range of 400–4000 cm^{-1} was selected to generate simulated standard spectra. The absolute concentrations of NO and NO_2 were determined, when no additional water was introduced in the system, via the calibration curve constructed with standard reference gases (Linde GmbH, Gases Division, Pullach, Germany). The measurements were carried out with an absorption path length of 0.15 m, a resolution of 1 cm^{-1}, and a wavenumber range of 400–4000 cm^{-1}. Due to the non-linearity phenomenon of NO_2 in gas-phase FTIR at high concentration when quantified by the principal strong band at 1630 cm^{-1} (Ahro &

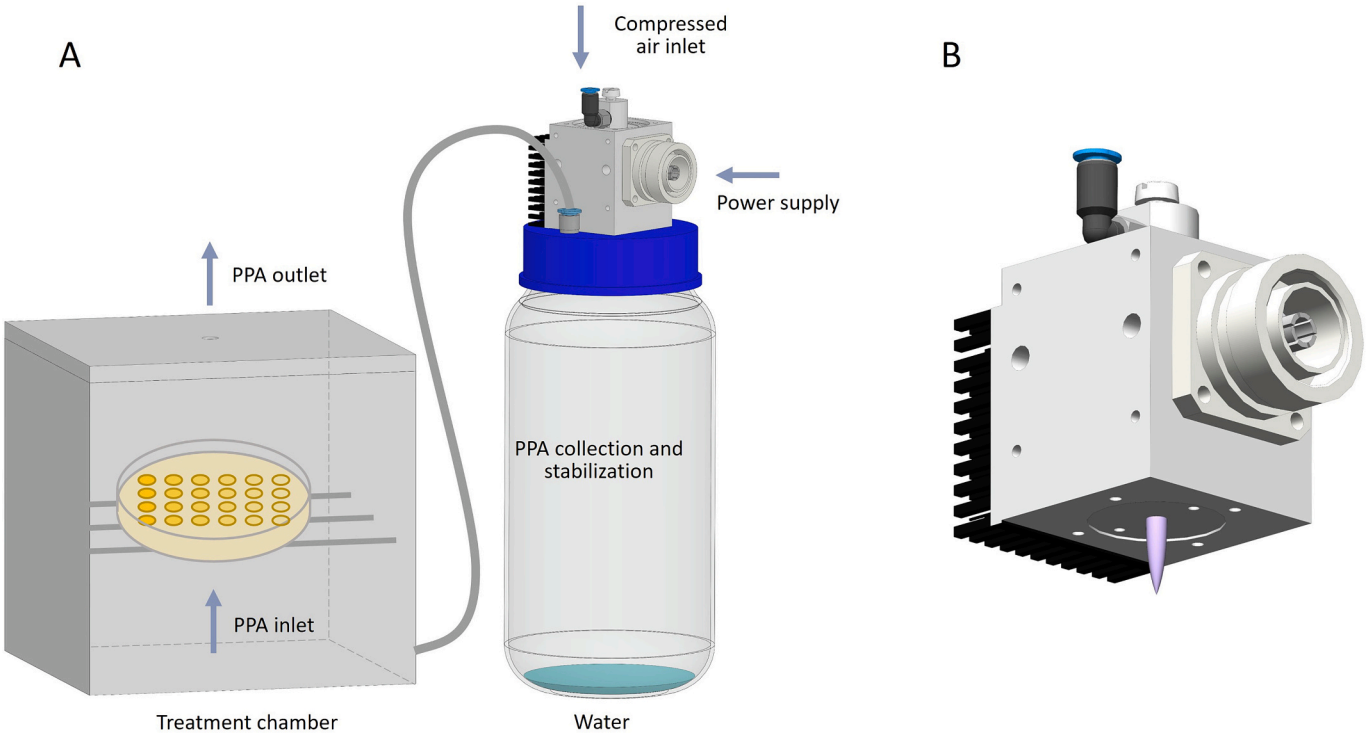

Fig. 1. Schematic representation of the experimental set-up used. In panel A, the full set-up is shown, including the plasma source, the glass bottle for plasma processed air stabilization, and the treatment chamber where the samples are inserted. The agar spot plate assay is represented inside the treatment chamber. In panel B, a close-up of the MidiPLexc plasma source and visible plasma flame.

Kauppinen, 2001), the vibrational band of the second order of energy absorption at 2919 cm^{-1} was used. For the quantification of reactive species, the FTIR spectrum was taken when the plasma source was operated for 35 min under 80 W and 2.25*10^{-5} m^3/s. NO_2 was characterized by the peaks centering at 1630 and 2919 cm^{-1}, whose concentration was determined by the vibrational band at 2919 cm^{-1}. NO was characterized and further quantified by the peak center at 1876 cm^{-1}.

2.3. Vegetative cells cultures

In this study, two strains for each microbial species (*L. monocytogenes*, *E. coli*, *L. plantarum, S. cerevisiae*, and *B. subtilis* spores) were tested for their susceptibility to NTP treatment at different treatment times, and a total of ten strains per species were used to evaluate the strain variability. The list of the used strains can be found in supplementary data.

Cultures of the bacterial and yeast strains were stored at −80 °C in Tryptic Soy Broth without dextrose (TSB-d; Scharlau Chemie, Spain) for *L. monocytogenes* and *E. coli*, De Man, Rogosa and Sharpe Broth (MRSB; Oxoid LTD, England) for *L. plantarum*, and Malt Extract Broth (MEB; Oxoid LTD, England) for *S. cerevisiae*, supplemented with 30% (v/v) glycerol (Sigma-Aldrich, Germany). From the stock, strains were grown on agar plates (corresponding broth supplemented with bacteriological agar 1.5% (w/w); Oxoid, England) for 24 h to 48 h. A single colony was then used to prepare the first overnight culture, incubated at the appropriate conditions (30 °C with shaking at 160 rpm for *L. monocytogenes* and *E. coli*, 30 °C with microaerobic conditions for *L. plantarum*, and 25 °C with shaking at 160 rpm for *S. cerevisiae*). Microaerobic conditions were generated with the use of Anoxomat WS9000 (Mart Microbiology, Drachten, the Netherlands) and airtight jars, where air was substituted with a microaerophilic gas mixture composed of 5% O_2, 10% CO_2, and 85% N_2. After 24 h, a second culture was prepared by diluting 1:500 (v/v) the first overnight in fresh broth, and incubation at the same conditions until the early stationary phase was reached. Experiments were performed using this second overnight culture as working culture. At least three independent biological replicates were tested on different days.

2.4. Spore preparation

B. subtilis spores were produced following the procedure described by Berendsen, Zwietering, Kuipers, and Wells-Bennik (2015). Briefly, a −80 °C stock culture was used to inoculate Luria Bertani broth (LB broth; Oxoid LTD, England) and incubated at 37 °C with shaking at 160 rpm for 16 h. The overnight cultures were diluted 1:100 (v/v) in sporulation media consisting of Nutrient Broth 8 g/L (NB, Oxoid LTD, England), supplemented with 1 mM $MgSO_4$, 13 mM KCl, 0.13 mM $MnSO_4$, 1 mM $CaCl_2$, and a final pH of 7.0, and cultured at 37 °C until an OD_{600} of 0.6 was reached. Then, 200 µL of culture was spread-plated on three sporulation agar plates prepared with Nutrient Agar (NA, Oxoid LTD, England) 23 g/L supplemented as described above, with a final pH of 7.0. The plates were incubated at 37 °C for 7 days, and spore formation was confirmed by microscopy analysis. After spores formation, they were collected by adding 2 mL of sterile demineralized water to the agar plate and gently scraping off the bacterial layer with a 10 µL loop. The suspensions from the three plates were combined in one tube and washed three times in sterile water. The spore suspensions were stored in sterile water at 4 °C for at least a month to allow spore maturation. Two independent spore batches were produced for each strain.

2.5. Cells treatment and enumeration

For vegetative cells, the working culture was serially diluted in a buffered Peptone Physiological Salt solution (PPS, Tritium Microbiologie B.V., the Netherlands). Up to six consecutive decimal dilutions were spot-plated in 5 µL drops on the corresponding nutrient-rich agar plates in quadruplicates (Fig. 1). When the microbial suspension drops were absorbed after approximately 5 min, the plate was exposed to PPA treatment. The negative control was inserted in the treatment chamber with only the airflow but without plasma ignition, to exclude any possible inactivation given by the drying effect. Subsequently, samples and controls were incubated at the appropriate conditions (30 °C for *L. monocytogenes* and *E. coli*, 30 °C with microaerobic conditions for *L. plantarum*, and 25 °C for *S. cerevisiae*). Bacterial growth was assessed after two and five days, to allow damaged cells to recover and form colonies. To evaluate the decontamination effect of PPA on the investigated microorganisms, the Most Probable Number (MPN) method was used, where each spot was considered as an independent unit. When the growth of at least one colony in a spot was observed, it was considered positive. Then, the results were converted to $\log_{10}$ CFU/mL using the method described by Blodgett (2023), with a detection limit of 1.7 $\log_{10}$ CFU/mL.

For spores, the initial concentration was measured after the inactivation of vegetative cells by heating a 1.5 mL tube containing 1 mL of spore suspension to 80 °C for 10 min in a water bath with shaking conditions at 60 rpm. Then, the spore suspensions were decimally diluted and spot-plated on NA plates. Colonies were counted after 3 days of incubation at 30 °C. To avoid spore germination before the plasma treatment, the spore suspension was spot-plated on nutrient-free agar. In a petri dish, 1.5% (w/v) agar in distilled water was poured into single drops and after solidification each drop was singularly inoculated by spot plating 10 µL of spore suspension. After the treatment, spores were retrieved by placing each inoculated agar drop in a tube with 5 mL 1% (v/v) Tween80 (Merck Schuchardt oHG, Germany) in sterile water and vigorously shaking for 5 min. The suspension was then serially diluted, and the appropriate dilution was spot-plated on NA plates for enumeration. Colonies were counted after two and five days of incubation at 30 °C.

2.6. Statistical analysis

Student's *t*-test was used in the kinetic analysis to make pair-wise comparisons between the two strains used for each species at the different treatment time points. One-way analysis of variance (ANOVA) was used to assess the statistically significant difference between the 10 strains within each species. When a difference was found, the post-hoc

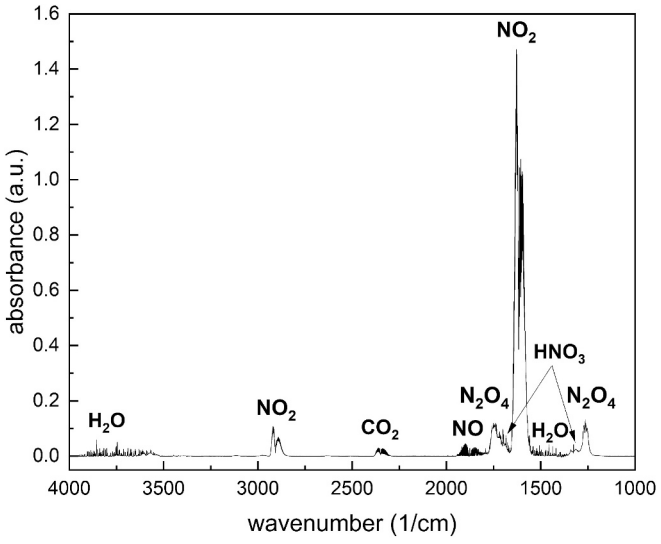

Fig. 2. FTIR spectrum of plasma processed air generated by MidiPLexc. The measurement was performed 35 min after ignition of the plasma source. Peaks corresponding to the main components of plasma processed air are indicated. See text for details.

Tukey test was used to identify the strains that differed. Also, the average strain reduction per species was compared between the three bacterial species *L. monocytogenes*, *L. plantarum*, and *E. coli* using the 95% confidence interval and the 95% prediction interval. Differences were considered statistically significant when the *p*-value was lower than 0.05. The relative standard deviation was calculated as the percentage of the standard deviation divided by the mean. All calculations were carried out with Microsoft® Excel® for Microsoft 365 MSO (Version 2302).

3. Results

3.1. Chemical characterization of PPA

Traceable, long-living chemical species in the PPA were characterized by FTIR after passing though the experimental set-up (PPA outlet in Fig. 1). The resulting absorption spectrum is reported in Fig. 2. Predominantly, nitrogen oxides were generated by MidiPLexc with air as the working gas. NO_2, NO, and N_2O_4 were detected with the peak center at 1630 and 2919 cm^{-1}, 1876 cm^{-1}, 1751 and 1263 cm^{-1}, respectively. The humidity present in the system was visible in the peak clusters in the ranges of 1260–2092 cm^{-1} and 3393–4047 cm^{-1}. The peaks of HNO_3, whose centers were at 1700 and 1325 cm^{-1}, were partially overlapping with the ones of N_2O_4. The concentration of NO_2 and NO measured in the dry system was 6035 ppm and 2668 ppm, respectively.

3.2. Bacterial vegetative cell inactivation

The inactivation of bacterial vegetative cells exposed to PPA treatment was measured at five different time points, ranging from 15 to 90 s (Fig. 3 A-C). Two strains for each species were tested, including one reference strain (EGDe, WCFS1, K12 for *L. monocytogenes*, *L. plantarum,* and *E. coli* respectively). For all three species, the fastest inactivation was achieved during the first 15 s of treatment, followed by a relatively linear inactivation region and tailing effect at a longer treatment time. For *L. plantarum*, no difference was observed between the inactivation of the two strains at any time point. For *E. coli*, a difference between K12 and BL21 was observed after 15 s and 30 s, and the inactivation then converged from 45 s of exposure onwards. A different trend was observed for *L. monocytogenes*, where the inactivation at the initial and final treatment time points was comparable between EGDe and 10403S.

Comparing the three species, the highest variability can be found in the first 60 s of treatment, in particular *L. monocytogenes* being more resistant than *L. plantarum* and *E. coli*. At 90 s of treatment though, the inactivation for the three species was comparable (an average reduction of 6.1 log_{10} for *L. monocytogenes*, 6.2 log_{10} for *L. plantarum*, and 6.9 log_{10} for *E. coli*) and close to the detection limit.

To evaluate the strain variability in PPA resistance, eight additional strains per species were selected from various isolation origins and exposed to 60 s treatment (Fig. 3 D-F). One-way ANOVA analysis showed no significant difference between any strain in each of the three species, independently from the origin or serotype. Under the treatment

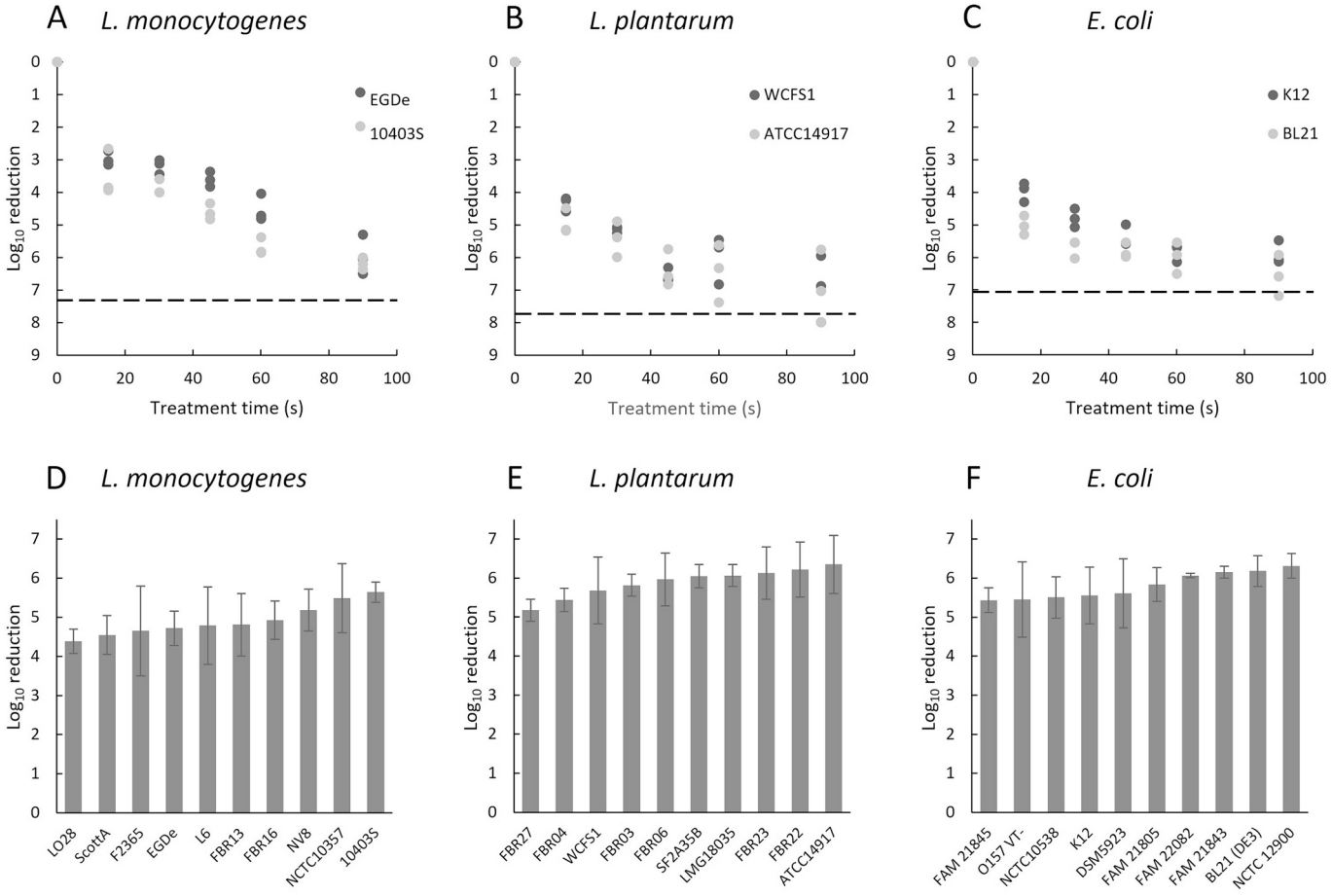

Fig. 3. Bacterial inactivation after NTP treatment. Panels A, B, and C show the inactivation kinetics of the two strains reported in the legend for each species, and the average detection limit is indicated as a dashed line. Each data point represents one biological replicate. In panels D, E, and F, the log_{10} reduction after 60 s of treatment is reported for ten strains per species. Each bar is the average of at least three biological replicates and the error bars indicate the standard deviation.

condition tested, the inactivation ranged between 4.4 $\log_{10}$ and 5.6 $\log_{10}$ for *L. monocytogenes*, with an average of 4.9 $\log_{10}$. Higher inactivation was found for *L. plantarum* and *E. coli*, with values between 5.4 $\log_{10}$ and 6.3 $\log_{10}$, and between 5.2 $\log_{10}$ and 6.3 $\log_{10}$ respectively. The average inactivation of these two species was comparable, 5.8 $\log_{10}$ and 5.9 $\log_{10}$ respectively. When comparing the mean reduction of the ten strains between the different species, *L. monocytogenes* was significantly more resistant than both *E. coli* and *L. plantarum*, however, the 95% prediction intervals of the means of the species were overlapping and not significantly different. The relative standard deviation of the species inactivation was low (8.3% for *L. monocytogenes*, 5.9% for *E. coli,* and 4.0% for *L. plantarum*), indicating a limited strain variability in PPA resistance within the species.

3.3. Spore inactivation

To investigate the inactivation kinetic of bacterial spores, spores derived from two *B. subtilis* strains (4062 and 4067) were exposed to NTP treatment. Considering their extreme resistance to stresses, the inactivation was measured throughout a longer treatment time compared to vegetative cells. Two technical replicates for each independent spore crop were tested, and the average is reported in Fig. 4A.

Negligible inactivation was detected for the first 10 min (between 0.0 $\log_{10}$ and 0.4 $\log_{10}$ reduction) for both strains, followed by a more linear inactivation region. The two strains showed no significant difference at all time points except at 20 mins, where strain 4062 was more susceptible. After 30 mins of treatment, the average inactivation for strain 4062 was 2.2 $\pm$ 0.8 $\log_{10}$ and 1.2 $\pm$ 0.6 $\log_{10}$ for strain 4067. Eight additional strains were then tested and compared after being exposed for 30 mins to PPA (Fig. 4C). The ANOVA test indicated significant differences between two groups of spores composed of resistant strains (4071 and 4067), with an average inactivation of 1.2 $\log_{10}$, and susceptible strains (4070 and 4146), with an average inactivation of 3.1 $\log_{10}$. The majority of the strains showed an intermediate inactivation and no significant differences between each other.

3.4. Yeast inactivation

The effects of PPA treatment were tested using *S. cerevisiae* as a model eukaryotic organism, as well as a spoilage organism. The inactivation of two strains (AD1890 and 077.0001) was measured between 5 s and 45 s of PPA treatment (Fig. 4B). The differences between the two kinetic curves were significant from 10 s onwards. Strain AD1890 was more resistant to the treatment and had a concave downward trendline, while strain 077.0001 was more susceptible and had a concave upward kinetic curve. The maximum reduction of 6.1 $\log_{10}$ was achieved with counts below the detection limit at 30 s for strain 077.0001, while strain AD1890 was reduced by 4.7 $\log_{10}$ after 45 s of exposure. Eight additional strains were screened for PPA susceptibility after 30 s of treatment (Fig. 4D). On average, *S. cerevisiae* was reduced by 5.3 $\log_{10}$, with values

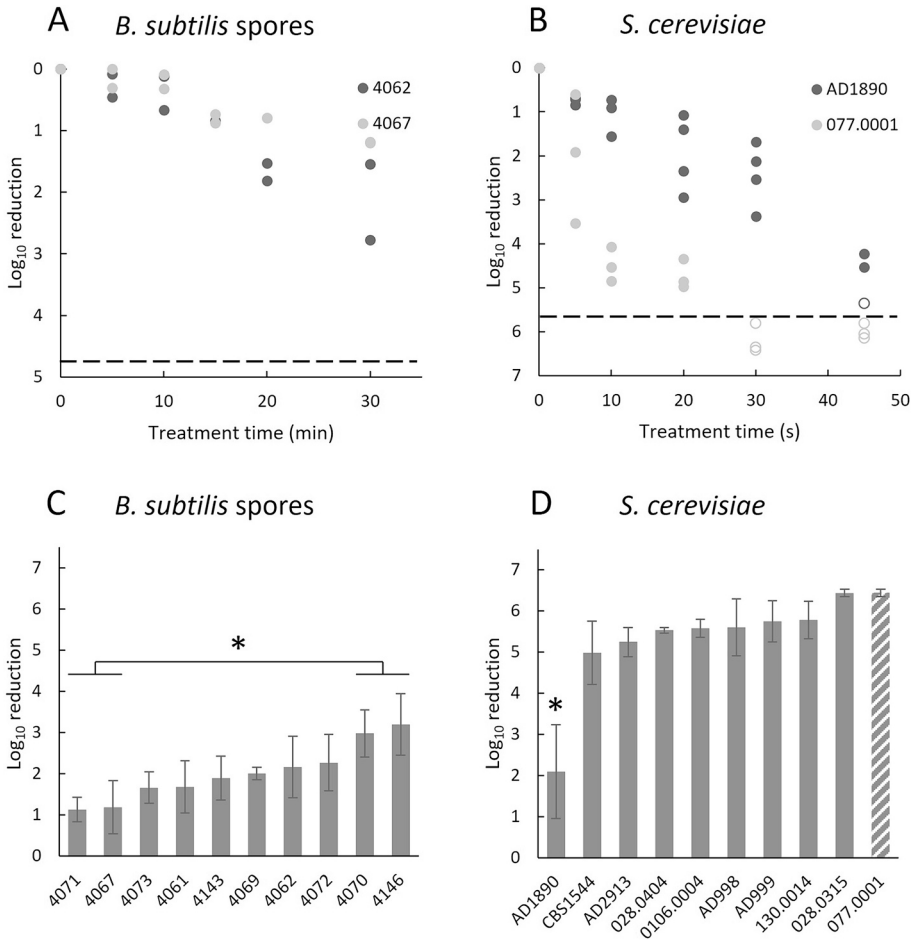

Fig. 4. Spores and yeast inactivation after NTP treatment. Panels A and B show the inactivation kinetics of the two strains reported in the legend for each species, and the average detection limit is indicated as a dashed line. Measurements below the detection limit are represented by open circles. In panel A, each data point represents one spore crop (average of two technical replicates), in panel B, each data point represents one biological replicate. In panels C and D, the $\log_{10}$ reduction after 30 min or 30 s of treatment respectively is reported for ten strains per species. Each data point is the average of at least three biological replicates, and the error bars indicate the standard deviation. Dashed columns represent measurements below the detection limit and asterisks a significant difference.

ranging from 2.1 $\log_{10}$ (strain AD1890) to below the detection limit (strain 077.0001). ANOVA analysis confirmed higher resistance of strain AD1890 to the treatment compared to all other yeast strains.

4. Discussion

In this study, the effect of indirect NTP treatment was tested on *Listeria monocytogenes*, *Lactiplantibacillus plantarum, Escherichia coli*, *Bacillus subtilis* spores, and *Saccharomyces cerevisiae*. The inactivation potential was assessed on the surface of agar plates as a simplified model food system. A modified, miniaturized version of the classical MPN method described by Blodgett (2023) was used, similar to what was described by Pavic, Groves, Bailey, and Cox (2010). Instead of using the conventional 10 mL tubes, serial decimal dilutions were spot plated on agar plates and treated with PPA. After treatment, plates were incubated, and each individual spot was checked for microbial growth and scored as positive or negative. Using this method, it was possible to adapt the MPN count to this NTP surface treatment and to treat multiple strains at the same time, reducing experimental time and material use compared to a classical plate count method. The five species tested were selected to include phenotypically different microorganisms, adapted to a wide variety of ecological niches and external stress. NTP produces several different chemical species with antimicrobial properties, but with the set-up used in this study, microorganisms are only exposed to long-living species. After the microwave plasma source was ignited using air as a working gas, reactive atoms (O, N), excited molecules, and ions were generated (Meichsner, Schmidt, Schneider, & Wagner, 2013). A previous study showed that around 3% of the working gas was converted by the plasma torch into reactive nitrogen and oxygen species (Schnabel et al., 2019). Subsequently, PPA cooled down during the transit from the nozzle to the treatment chamber. The resulting chemical species were in a metastable state and were then determined via FTIR. NO_2 was formed by the reaction of NO and O_2. N_2O_4 results from a dimerization reaction of NO_2 and is in equilibrium ($N_2O_4 \rightleftharpoons 2NO_2$) with an equilibrium constant of 0.146 at the temperature of 25 °C (Chao, Wilhoit, & Zwolinski, 1974). A small amount of nitric acid was produced by the reaction of NO_2 and H_2O, which was expected considering the 100% relative humidity in the system. Plasma composition and the possible interactions between plasma, microbial cells, and substrate are highly complex systems. As the most abundant compound (more than 70%) in PPA produced by microwave plasma, NO_2 is expected to play a critical role in microbial inactivation, which is capable of initiating lipid peroxidation (Kappus, 1987). In parallel, NO can react with superoxide radicals, which is a crucial molecule in metabolic processes (Hao et al., 2014). These reactive nitrogen species can both directly or indirectly affect the biological systems, resulting in cell damage, and disturbance in biochemical processes (Patel et al., 1999). RNS and in particular NO_2 are expected to exert their bactericidal activity through intense oxidative stress, which can result in different effects. For Gram- bacteria, the membrane integrity is irreversibly compromised, with subsequent leakage of intracellular components and cell death. For Gram+ bacteria on the other hand, cell death is mostly caused by reactive chemical species that penetrate the cell envelope and affect DNA, enzymes, and other cytoplasmic component (Laroque et al., 2022). It is however difficult to predict bacterial susceptibility based only on Gram characterization. In our study, two Gram+ and one Gram- species were analyzed, and results seem to confirm the role of additional factors other than the membrane structure. *L. monocytogenes, L. plantarum,* and *E. coli* showed a comparable inactivation after 60 s of treatment, namely 4.9 $\log_{10}$, 5.9 $\log_{10}$ and 5.8 $\log_{10}$ on average, respectively. Moreover, no statistically significant differences between the ten strains was detected for each species after 60 s of PPA treatment. The limited variability among bacterial strains is a promising observation for industrial application. It indicates a low probability of having resistant populations in the initial product, that would reduce dramatically the effect of the process and reduce the shelf-life.

Bacterial spores are protected by robust outer layers, such as the cortex and the spore coat, and by different components in the inner core, such as small acid soluble proteins, making them able to withstand extreme stress conditions. Despite their defense structures, spores have been reported to be susceptible to NTP treatment. In this study, the exposure of *B. subtilis* spores to intense PPA treatment was tested using ten strains. After 30 min of exposure, the reduction obtained varied significantly depending on the strain, ranging from 1.1 ± 0.3 $\log_{10}$ for strain 4071 to 3.2 ± 0.7 $\log_{10}$ for strain 4146. Although affected by the treatment, spore reduction was limited and with a wide dispersion (i.e., 1.9 ± 0.9 $\log_{10}$ considering all ten strains). Keener et al. (2012) reported that it is possible to achieve up to 6 $\log_{10}$ inactivation of *B. subtilis* spores with 15 s of direct air NTP treatment in a closed package followed by 24 h incubation with the ionized gas. A direct comparison between NTP setups is challenging due to the many variables at play, and differences in inactivation efficacy could be explained by a range of factors including the type of reactive species involved, the different technical applications, and the type of spores and/or strains used. Liao et al. (2019) suggested a possible mechanism of inactivation for spores following NTP treatment including DNA damage by UV radiation, and outer membrane disruption and intracellular damage by reactive species. Being an indirect NTP treatment (Ehlbeck et al., 2010), UV radiation is not present in our sample treatment chamber and can be excluded as a factor. Moreover, in the present study, no incubation time in contact with PPA was provided, and the spores were plated directly after the exposure. It is possible that the density of the reactive species was lower in this study, or that the inactivated spore fraction corresponds to those whose membranes were immediately disrupted by the external radicals and that more time was required for further inactivation and possibly penetration in the inner spore layers. This might also indicate that susceptibility to the treatment might depend on the thickness and structure of the protective membranes. This is an example of why the understanding of the inactivation mechanisms is crucial to guarantee an appropriate application of emerging technologies, and it should be further investigated with the use of transcriptomic or proteomic analysis.

In our study, *S. cerevisiae* was the most susceptible of the four vegetative species analyzed, with inactivation levels between 2.1 $\log_{10}$ and 6.4 $\log_{10}$ after 30 s of treatment and close to the detection limit for most strains. These results though are in contrast with what was reported in previous studies (Baldanov, Ranzhurov, Semenov, & Gomboeva, 2019; Kye-Nam, Kwang-Hyun, Won-Tae, & Yeon-Hee, 2006; Scholtz, Julák, & Kříha, 2010) where yeast species showed comparable or lower sensitivity to air NTP than bacterial species. The discrepancies could be explained by the different exposure and quantification methods used. In this study, microbial cells are exposed to PPA on agar surfaces and not in liquid media, and liquid matrices showed protective effects on the suspended cells. Moreover, in this study, surviving cells were enumerated with the MPN count method, while in the aforementioned publication, the susceptibility was quantified by measuring the inhibition area after NTP treatment. This can be limiting when the reactive species are very concentrated and are not able to diffuse from the direct exposure area.

Stress sensing and stress responses are regulated by multiple molecular pathways in bacterial cells, as well as by general stress response systems that are able to deal with different environmental conditions and possibly confer cross-protection. In *L. monocytogenes* for instance, multiple stress signals converge in the activation of σ^B, a transcriptional factor that controls the general stress response and is involved in the resistance to acidity, osmotic stress, thermal stress, oxidative stress, and high hydrostatic pressure (Bucur, Grigore-Gurgu, Crauwels, Riedel, & Nicolau, 2018). In *B. subtilis* and *B. cereus,* heat stress triggers the upregulation of general stress response genes including σ^B, proteases, and chaperones, and can also induce secondary oxidative stress defense mechanisms with the activation of catalases and production of thioredoxin (Mols & Abee, 2011). It could thus be interesting to compare the

heat resistance pattern with PPA inactivation data for the species tested in this study, to evaluate if a correlation is present between the two stress responses. Most strains of *L. monocytogenes*, *L. plantarum,* and *B. subtilis*-derived spores used in this study have been characterized previously for their thermal resistance (Aryani, Den Besten, Hazeleger, & Zwietering, 2015; Aryani, Den Besten, & Zwietering, 2016; Berendsen et al., 2015; Den Besten, Berendsen, Wells-Bennik, Straatsma, & Zwietering, 2017). Although some molecular response pathways are supposedly involved in both stress responses, a clear correlation between the two sets of data is not present. For *L. monocytogenes* and *L. plantarum*, thermal resistance varied dramatically between strains (D-values are reported in supplementary data), while no significant strain difference emerged in PPA susceptibility. It is possible that the treatment is so intense that the cells die before being able to activate any defense mechanism, or that the molecular processes involved in heat resistance do not overlap with PPA resistance. According to Berendsen et al. (2015), *B. subtilis* spores can be divided into two distinct groups based on heat sensitivity. However, the strains in this study that showed PPA sensitivity (strains 4070 and 4176) or resistance (strains 4071 and 4067) all belong to the heat-resistant group (D-values are reported in supplementary data), indicating that heat and NTP resistance do not appear to be correlated for *B. subtilis* spores as well. This information could be particularly interesting for applications that involve the inactivation of highly heat-resistant spores.

The comparison of inactivation data from different NTP setups is complicated, due to the amount of parameters to take into consideration. The variables include but are not limited to: the type of plasma source (double barrier discharge, microwave, and plasma jet are the most commonly used), the power and gas used for plasma production, the treated microorganism, and the treatment matrix. For example, Lis et al. (2018) achieved an inactivation of 4.2 $\log_{10}$ for *L. monocytogenes* after 10 min using a Surface Micro Discharge plasma source, while Guo, Li, Huang, Li, and Wang (2017) treated *E. coli* with a Plasma Jet reaching around 7 $\log_{10}$ inactivation after 3 min. Both procedures used ambient air and agar plates as in this study, although the treatment involved direct plasma exposure resulting in possibly different reactive species composition. While the latter study has similar inactivation values to this study, the former is much less effective. *E. coli* and *L. monocytogenes* though, showed highly comparable inactivation at longer treatment time (6.2 and 6.1 $\log_{10}$ after 90 s, respectively) in this study. This observation suggests that the experimental set-up might play a crucial role in determining the effectiveness of the treatment and should thus be carefully evaluated for an appropriate application in the food industry. In this study, agar plates were used as a food surface model system to assess the efficacy of microbial inactivation, hence application on real food matrices should be tested in future studies. It is necessary to evaluate the protective effect of the surrounding material on the microorganisms, as well as undesired effects on the food organoleptic properties. For instance, food components such as casein and sunflower oil reduced drastically the inactivation of *Salmonella* Typhimurium (De Baerdemaeker et al., 2022). It is thus important to carefully determine the most suitable product and application. Possible targets for plasma treatment are dry and thermo-sensitive foods with a smooth surface, such as some spices and grains. As a gas, PPA allows to treat products homogeneously in contrast to direct NTP treatments such as double barrier discharge or plasma jet, which have localized areas of action. Its effectiveness is however limited by the penetration ability, and microbial cells can be shielded in porous or rough matrices. NTP can also be used in combination with other mild technologies as a hurdle process, but further studies are needed to assess whether possible cross-protection effects occur. The microwave NTP setup used in this study operates at room temperature and atmospheric pressure using air as a carrier gas, making it relatively inexpensive and easy to use for food processing applications. Although not currently present on the market, these properties could also allow the adaptation of this plasma source to forms of continuous products treatment. The use of NTP in the form of PPA allows for the treatment of thermosensitive product surfaces, which can be otherwise challenging to process and can pose a risk to food safety. Moreover, PPA resistance does not show a correlation with thermal resistance and it thus offers an alternative method to deal with particularly resilient microorganisms.

5. Conclusion

The NTP set-up presented in this study resulted in the effective inactivation of vegetative cells on the surface of agar plates at short treatment times. The inactivation ranged between 4.4 and 6.3 $\log_{10}$ after 60 s for bacteria, and between 2.1 and 6.4 $\log_{10}$ after 30 s for yeasts. For *L. monocytogenes*, *E. coli*, and *L. plantarum*, no significant differences were detected between the tested strains, while for *S. cerevisiae*, strain AD1890 was more resistant. The differences between the three bacterial species were limited, while yeast strains were substantially more susceptible compared to bacteria. The effects of PPA on bacterial spores were less intense compared to vegetative cells, and it is thus necessary to evaluate the most appropriate treatment condition and target product. Air-based NTP is a promising technology for the surface decontamination of dry and heat-sensitive products. In-depth evaluation and understanding of the molecular mechanisms leading to microbial inactivation is crucial to ensure an optimal application in the food production chain.

CRediT authorship contribution statement

Domiziana Battaggia: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. **Yijiao Yao:** Data curation, Formal analysis, Writing – original draft. **Masja N. Nierop Groot:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Tjakko Abee:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Heidy M.W. den Besten:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

We would like to acknowledge Dr. Uta Schnabel (Leibniz Institute for Plasma Science and Technology, Department of Plasma Biotechnology, Greifswald, Germany) for technical and supervisory support in plasma air characterization and visualization.

The authors would like to acknowledge Remco Hamoen (Wageningen Food & Biobased Research, Wageningen University & Research, Wageningen, The Netherlands) for the design of the treatment chamber and the technical support, and Hannah Sperlich (Food Microbiology, Wageningen University & Research, Wageningen, The Netherlands) for her contribution to data collection.

The authors would like to thank and acknowledge Dr. Nicolas Nguyen Van Long from ADRIA Food Technology Institute (FR), Dr. Elissavet Gkogka from Arla Foods amba (DN), Dr. Jörg Ehlbeck from Leibniz Institute for Plasma Science and Technology (DE), Dr. Jörg Hummerjohann from Agroscope (CH), Dr. Marjon Wells-Bennik from NIZO Food Research (NL), Dr. Kimon-Andreas Karatzas from the University of Reading (UK), and Dr. Vasilis Valdramidis from the University of Malta (MT) for providing microbial strains from their collections.

This study is part of the TRANSIT project and has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement N°955431.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103674.

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