NON-THERMAL PLASMA INACTIVATION OF *SALMONELLA* TYPHIMURIUM ON DIFFERENT MATRICES AND THE EFFECT OF SELECTED FOOD COMPONENTS ON ITS BACTERICIDAL EFFICACY

Klaas De Baerdemaeker[a,*], Inge Van der Linden[a], Anton Nikiforov[b], Sophie Zuber[c], Nathalie De Geyter[b], Frank Devlieghere[a]

[a] Ghent University, Department of Food Technology, Safety and Health, Research Unit Food Microbiology and Food Preservation, 9000 Ghent, Coupure Links 653, Belgium

[b] Ghent University, Department of Applied Physics, Research Unit Plasma Technology, 9000 Ghent, Sint-Pietersnieuwstraat 41, Belgium

[c] Nestlé Research, Institute of Food Safety and Analytical Science, Lausanne 26, Switzerland

*E klaas.debaerdemaeker@ugent.be
T +32 (0)9 264 61 77
A Coupure links 653
9000 Ghent
Belgium

Abbreviations

NTP : Non-thermal plasma;
slm : Standard litres per minute;
ROS : Reactive oxygen species

ABSTRACT

Non-thermal plasma (NTP) is known as an effective source of a variety of reactive species generated in the gas phase. Nowadays, NTP is gaining increasing interest from the food industry as a microbial inactivation technique. In the present study the effect of inoculation method and matrix on inactivation of *Salmonella* Typhimurium was examined by treating spread plated agar (2.2 log CFU/sample inactivation by NTP), spot inoculated agar (1.9 log CFU inactivation), glass beads (1.3 log CFU inactivation) and peppercorn (0.2 log CFU inactivation). Furthermore, multiple agar matrices supplemented with low and high concentrations of a certain food component (casein, starch, sunflower oil, vitamin C, sodium pyruvate or grinded peppercorns) were inoculated and treated to determine the effect of those components on NTP efficiency. Although starch, vitamin C and sodium pyruvate had no significant influence on the inactivation degree, the presence of 10% casein (2.1 log CFU/sample less inactivation compared to tryptone soy agar (TSA)), 10% pepper (2.1 log CFU less inactivation) or 1% and 10% sunflower oil (1.6 and 2.1 log CFU less inactivation, respectively) in TSA demonstrated the protective effect of these substances for NTP treatment. These experiments led to the conclusion that low inactivation on produce seemed not to arise from the inoculation method nor from the shape of the produce, but is the result of the food matrix.

KEYWORDS

Cold plasma, DBD, microbial inactivation, non-thermal processing, reactive species, food simulation media

1 INTRODUCTION

Plasma is a quasi-neutral ionized gas comprising a multitude of particles including photons, free electrons, ions, ground state and excited atoms/molecules and free radicals (De Geyter & Morent, 2012; Hati et al., 2012; Mandal et al., 2018). Non-thermal plasma (NTP) is created by applying an electrical field to a neutral gas resulting in the formation of a plasma with considerable differences in energy between electrons and heavy particles (Conrads & Schmidt, 2000). Such non-thermal plasmas can be operated at sub- and atmospheric pressure (Surowsky et al., 2015) and are thus advantageous for industrial application, since low pressure operations would pose the need of vacuum equipment, making it impossible to run as a continuous system and increasing overall operational costs (Niemira, 2012; von Woedtke et al., 2013).

The combination of active particles generated by NTP is capable of inactivating micro-organisms (MO), for instance leading to decimal reductions of 3.6, 6.7, and 6.5 on agar for *Escherichia coli* (60 sec), *Salmonella* Typhimurium (45 sec) and *Listeria monocytogenes* (420 sec) (Yong et al., 2015). The effectiveness of bacterial inactivation is related to three key factors: the target organism, the food matrix and the process technology (Van Impe et al., 2018). Industrial application of NTP will depend on inactivation achieved, on cost and on product quality after treatment. The operational cost of NTP treatment is correlated with gas composition, which can be air, common Modified Atmosphere Packaging (MAP) gasses or more expensive noble gasses such as argon mixed with oxygen which lead to a higher production of reactive oxygen species but drastic increase of the overall costs (Brandenburg et al., 2007). The gas composition also determines the category of main species (e.g. ions, radicals, photons and reactive oxygen or nitrogen species (ROS/RNS)) generated during the process (Schottroff et al., 2018). MO are inactivated as a result of biological mechanisms (DNA damage, protein denaturation, apoptosis, lipid peroxidation) and mechanical damage (electrostatic disruption and electroporation) (Liao, Liu, et al., 2017). This is influenced by the reactive species, which have different modes of action (Schottroff et al., 2018). Also the growth form of the organism is important, since spores are significantly less sensitive to NTP treatment than vegetative cells (Tseng et al., 2012). Furthermore, Gram-positive bacteria are more resistant against NTP treatment compared to Gram-negative bacteria (Lunov et al., 2016). The influence of pH and the initial concentration of MO has also been demonstrated to influence NTP efficacy, the latter showing a decreased inactivation at higher bacterial concentrations (Azharonok et al., 2009; Kayes et al., 2007; Yong et al., 2015). The studies have shown a clear influence of food matrix on microbial inactivation efficiency of NTP, however, the exact mechanism is not clearly understood. For solid food matrices, NTP is rather a surface treatment since reactive species have a penetration depth of a few µm only. It was reported that only 5% or less of the ROS are able to penetrate a biological tissue with 500 µm thickness (Duan et al., 2017). This can be beneficial, since it does not affect the majority of the nutrients in the product when applying a gentle decontamination. The large variety of the NTP treatment conditions and the complexity of correlating NTP effects with operational parameters of the electrical discharge and food products composition limit current progress in the field and transfer of lab scale tests to industrial level applications.

Considering the achieved progress in NTP based food treatment technology there are no doubts that non-thermal plasma is a promising technique as preservation method of food products. However, more research is needed before NTP can be successfully applied at industrial scale (Aguirre et al., 2019). In this study, we investigated how inoculation method and matrix influence the inactivation of *Salmonella* Typhimurium in NTP studies and explored the effects of food matrix components on inactivation efficiency.

2 MATERIALS AND METHODS

Preliminary research (see Figures A.1 and A.2 in Supplementary Data) has shown that, although NTP inactivation of *S.* Typhimurium on solid agar surfaces (spread inoculation) was quite high, its bactericidal effect on different types of dry produce (spot inoculated) was rather limited. Therefore, the influence of bacterial inoculation method (spread or spot inoculation) and product matrix (agar plates, glass beads or peppercorns) on NTP's treatment efficiency was investigated. Furthermore, the impact of different food components was examined by addition of these components (starch, casein, sunflower oil, vitamin C, pyruvate and grinded pepper) to the agar followed by inoculation and NTP treatment. Due to the importance of active species, the NTP was characterized in function of treatment time with respect to several ROS and RNS. An overview of these experiments is shown in Figure 1.

2.1 Plasma generation

A dielectric barrier discharge (DBD) reactor, shown in Figure 2A, was used for NTP generation. A set of electrodes made of tungsten were arranged in a planar array of 10x10 cm^2 whereas the plasma region was limited to an area of 7x7 cm^2. Each electrode was placed inside a capillary made of alumina with 99.9% purity and having a wall thickness of 1 mm. The gap in between the electrodes was also set at 1 mm. An AC power supply (50 kHz, Dipl.Ing.H.Bayerle, Germany) was used in a duty cycle of 10% to keep gas temperature below 45°C. The triggering of the generator was achieved by connection to a signal generator (TGP110, Aim-TTI, UK) generating predefined pulses. Air (Air Products) was used as inlet gas and a steady air flow was generated by a pump (Aqua Oxy 1000, OASE, UK). Air at a temperature of 24°C was washed through a saturated salt solution in order to obtain a relative humidity of ca. 60%. A sine wave-voltage with average Root Mean Square (RMS) value of 8 kV was used for all tests to generate a stable discharge covering the whole area of the electrodes. RMS current was 16.5 mA, and plasma on and off time defining the duty cycle were set at 1 and 9 milliseconds, respectively. A visual view of the plasma discharge is presented in Figure 2B. Voltage-current waveforms are depicted in Figure 3.

2.2 NTP treatment

Plates (without lid) were put on the plate holder in the vessel after which the vessel and protective cage were closed. Inoculated agar, glass beads, peppercorn and simulation media were treated for 180 sec at 2 slm and a distance of 3.2 – 3.3 cm from the electrodes, which were found to be the most favourable conditions for application on an industrial scale (see Figure A.2 in Supplementary Data). The plates were left in the vessel after the NTP treatment for an additional 5 minutes to remove the plasma activated air by means of the ventilation system. All samples and/or plates were analysed in at least technical triplicates.

2.3 Plasma characterization

To characterize the NTP, the amount of long living species was quantified using a detection system connected to the outlet of the reactor located at 1 m downstream. Ozone (O_3) was analysed by an ozone monitor (sensitivity 3 ppm, absolute error 1 ppm) measuring light absorption at 253 nm. Carbon monoxide (CO), total nitrogen oxides (NO_x), nitrogen dioxide (NO_2) and nitric oxide (NO) were monitored by a Testo 350 apparatus (sensitivity 1 ppm, absolute error 0.1 ppm). Measurements were conducted after multiple operational time intervals (60, 180, 300 and 600 sec) and results were converted to mg/L. Additionally, the emission spectrum of the NTP discharge was created by means of optical emission spectroscopy (OES).

2.4 Strain preparation

The protocol used for strain preparation (Butot et al., 2018) can be summarized as follows: *Salmonella enterica* serovar Typhimurium SL1344 obtained as cryobeads from the LFMFP (Ghent University) laboratory collection

were revived and subcultured in brain-heart infusion broth (BHI, Oxoid, England). Cells were harvested from tryptone soy agar (TSA, Oxoid, England) by adding 2 mL of sterile tryptone (Oxoid, England) salt solution (TS) to each plate and scraping the surface gently. The resulting slurry was transferred to a sterile test tube and stored at 4°C. Purity was checked on TSA (22°C) by means of the 4 quadrant streak method. Growth on xylose lysine desoxycholate agar (XLD, Oxoid, England) confirmed that *Salmonella* was the present species (37°C, 24h). Initial cell concentration was determined by plating on XLD and TSA (37°C). Cell suspensions used for inoculation were prepared on the day of use, aiming at around 4 log CFU/sample for inactivation on food simulation media, while this was 5 log CFU/sample for experiments investigating the effect of inoculation method. The solutions were stored at 4°C until further use (max 4h) and concentration N_0 was verified by spread plating on TSA at 37°C.

2.5 Effect of matrix and inoculation method on NTP treatment efficiency

INOCULATION OF THE MATRICES

Four different combinations of matrix and inoculation method were used. The first consisted of spread plating the inoculum on agar plates containing 16 mL TSA. The medium was first dried for 30 minutes in the BSC and inoculated by spreading 50 µL of the inoculum. A second method used spot inoculation on agar medium. Agar plates with 16 mL TSA, dried for 30 minutes in the BSC, were inoculated with 10 drops of 5 µL. Glass beads (3 mm diameter, VWR) were washed with a 10% solution of commercial bleach (5 min), followed by 70% ethanol (5 min) and rinsed 3 times with sterile distilled water. They were autoclaved (121°C, 15 min) after drying. A single layer of beads (8.6 g) was put into a sterile petri dish and dried for 30 minutes before inoculation with 10 drops (5 µL per drop) of the inoculum. Black peppercorns (5 g) were spot inoculated in the same way. All samples were subsequently dried for 1 – 2h in the BSC and stored at 4°C until treated or plated (max 4h).

ANALYSIS OF THE SAMPLES

Agar plates (both spread and spot inoculated) were incubated (37°C for at least 18h) and cells were counted visually. Glass beads and peppercorn samples were added to sterile filter stomacher bags and tenfold diluted in sterile buffered peptone water (BPW, Oxoid) + 0.01% Tween80 (Sigma-Aldrich). They were stomached (60 sec), diluted in TS and spread plated on TSA (1000 µL on 3 plates). From the next dilution, 100 µL was spread plated on TSA and all plates were incubated at 37°C for at least 18h and colonies were counted.

2.6 Effect of NTP on single component food simulation media

TSA solutions with low (1% w/v) and high (10% w/v) concentrations of food components were prepared. For simulation media containing proteins or starch, casein hydrolysate (Fluka) or starch (Sigma-Aldrich) was autoclaved, respectively. TSA was added and the solution was mixed while heating until homogeneous. The same sequence of operations was applied for vitamin C (ascorbic acid, Sigma-Aldrich), with lower concentrations (low: 0.1% w/v, high: 1% w/v), as vitamin C is an antioxidant which might scavenge reactive oxygen species (Fiorani et al., 2020). Sunflower oil (Carrefour) was autoclaved and TSA + 0.5% Tween20 (Acros organics) was added. The solution was homogenized by shaking it several times. Pyruvic acid sodium salt (Fluka), which helps sub-lethally injured cells to recover (Gurtler & Kornacki, 2009), was dissolved in sterile distilled water to obtain a concentration of 11 g/L. This solution was autoclaved, TSA was added and the solution was well mixed. Peppercorns were grinded in a sterilized house blender followed by sieving. Consequently, TSA was added to the grinded pepper. The suspension was swirled after pouring the plates during solidification to prevent the sinking of pepper particles. Plates of all different simulation media were left to dry and solidify for 30 minutes. Subsequently, they were inoculated with 50 µL of the inoculum, dried for 10 minutes in the BSC and stored at 4°C until further use (max 3h). Three plates of each media were NTP treated. Subsequently, all were incubated at 37°C without inversion to prevent leakage of the oil, and counted visually.

2.7 Statistical analysis

Average log differences were determined by calculating $\log_{10}(N_0) - \log_{10}(N_{plate}) = \log_{10}(N_0/N_{plate})$ or $\log_{10}(N_0) - \log_{10}(N_{sample}) = \log_{10}(N_0/N_{sample})$, with N_0, N_{plate} and N_{sample} being the number of CFU in the inoculum, on the plate or on the sample, respectively. SPSS Statistics 25 was used for the statistical analysis. A non-parametric Mann-Whitney U test or a Kruskal-Wallis test were performed when two or more than two groups were compared, respectively. Statistical significance was calculated with α = 0.05. Correction for multiplicity of tests was performed using the Bonferroni approach.

3 RESULTS

3.1 Plasma characterization

The concentration of long living species in the NTP which were found downstream of the reactor are presented in Table 1. Ozone reached a steady state concentration (around 0.44 mg/L) after 180 seconds. No nitric oxide was detected. The increase of NO_x (NO_2) was progressively smaller with increasing treatment times. CO, as indicator of NTP treatment intensity, on the other hand, decreased with prolonged exposure times, from 2.8×10^{-3} mg/L after 60 seconds to 1.1×10^{-3} after 600 seconds. The OES spectrum of the NTP discharge is shown in Figure 4. The first and second positive system of the nitrogen molecule (N_2 ($B^3\Pi_g - A^3\Pi_u$) and N_2 ($C^3\Pi_u - B^3\Pi_g$), respectively), the first negative system of nitrogen molecular ion (N_2^+($B^2\Sigma - X^2\Sigma$)) and the nitric oxide γ-system (NO($A^2\Sigma - X^2\Pi$)), which are the main components, are indicated on the spectrum. Inactivation of *S.* Typhimurium due to the observed plasma species is described in the following sections.

3.2 Effect of matrix and inoculation method on NTP treatment efficiency

Since preliminary research (see Figures A.1 and A.2 in Supplementary Data) showed that NTP treatment was effective on agar plates (spread plated) but not on dry produce like peppercorns (spot inoculated), the effect of inoculation method (spread vs spot) and the matrix (peppercorns vs glass beads) in NTP studies was investigated. The average differences between inoculum and cells recovered from the sample or plate are shown in Figure 5. The highest difference between treated and untreated samples was observed on spot and spread inoculated agar plates (1.9 and 2.2 log CFU, respectively). Treatment of inoculated glass beads and peppercorn resulted in a more limited effect (1.3 and 0.2 log CFU, respectively). Statistical analysis showed that all treated sample types except peppercorn showed a statistically significant decrease in *S.* Typhimurium numbers during the treatment compared to untreated samples ($p \leq 0.05$). Experiments were repeatable since the results of a second repetition were not significantly different from the first (all repeats are included in Figure 5).

Based on the results presented in Figure 5 it is obvious that the matrix effect might explain a notable part of the differences in the NTP treatment effect observed on solid agar surfaces versus dry produce. Despite variable recovery, the effect of NTP treatment was significant for all matrices and inoculation methods, except peppercorn samples with lowest statistical difference.

3.3 Effect of NTP on single component food simulation media

To check the influence of various food components on the NTP treatment efficacy, TSA plates were supplemented separately with six different food components. Protein (casein), starch and oil (sunflower oil) were added since they are major components of the majority of food products. Pyruvate helps sub-lethally injured cells to recover (Gurtler & Kornacki, 2009) and vitamin C is an antioxidative component that scavenges reactive oxygen species (Fiorani et al., 2020). Finally, addition of grinded peppercorns to the agar allowed us to relate to the previous experiment in the present study, in which inactivation of *S.* Typhimurium on peppercorn was investigated.

The average log reduction compared to the inoculum is shown in Figure 6. For all components, it was calculated whether *S.* Typhimurium reductions in the presence of two different concentrations of a compound were significantly different from each other. Starch, pyruvate and vitamin C had no significant effect on the log reduction ($p > 0.05$), regardless of their concentration. Addition of sunflower oil resulted in a reduction of inactivation efficiency ($p \leq 0.05$) for high and low concentrations: from 3.0 log CFU inactivation on pure TSA to 0.9 and 1.4 log CFU inactivation on TSA with high and low oil concentrations, respectively. Pepper and casein

had a strong effect ($p \leq 0.05$) only at high concentrations (10%), with a reduction of *S.* Typhimurium inactivation from 3.0 log CFU on pure TSA to 0.9 log CFU on both simulation media.

The presence of certain common food components as well as the grinded pepper resulted thus in a significantly lower and concentration dependent *S.* Typhimurium inactivation compared to when these components were absent.

4 DISCUSSION

The inactivation of *Salmonella* Typhimurium by means of NTP treatment was investigated on solid agar surfaces (two inoculation methods), glass beads, peppercorn and selected food simulation media. First, the NTP was characterized for multiple exposure times since the active chemical species, which might change over time, are largely responsible for bacterial inactivation (Vleugels et al., 2005).

Plasma species concentrations measured in the current study are shown in Table 1. The low concentration of the (although quite stable) products of NTP gas phase is explained by the relatively far position (one meter from the discharge active zone) of the monitoring equipment as well as by the low discharge power. The RMS voltage and current of the discharge used here were 8 kV and 16.5 mA at a duty cycle of 10% which corresponds to a very low input power of 6.6 W. No nitric oxide (NO) was detected during the whole experiment. Possibly, this is due to the long distance from the discharge active zone since most NO radicals will react very fast with ozone (O_3) forming NO_2. Therefore, the reaction $O_3 + NO \rightarrow O_2 + NO_2$ is responsible for NO loss, having a reaction rate (at 298 K) around 1.8×10^{-14} cm^3 s^{-1} (DeMore et al., 1997). This is confirmed by the emission spectrum (Figure 4), in which increased although low intensities are observed at wavelengths matching with nitric oxide. Another reactive oxygen species (ROS) detected in the plasma chamber is O_3, which is known to have bactericidal activity (Greene et al., 2012). Research has shown that treatment of multiple types of MO with 2 ppm gaseous O_3 (3.7×10^{-3} mg/L) for 4h resulted in significant reductions (Moore et al., 2000). Although the treatment time is much shorter when applying NTP, the concentration of O_3 observed in the present study was two orders of magnitude higher. Furthermore, the combined effect of O_3 and NO_2 in NTP is generally significantly higher than the effect of either individual component (Moldgy et al., 2020). Finally, Figure 4 shows high concentrations of nitrogen molecules and ionized species as N_2 and N_2^+ present in the plasma discharge.

It has to be emphasized that the observed NTP effects on food products are sometimes drastically different from one study to another. Bacterial reductions even for similar products ranged from 1.0 – 1.5 log up to 4 – 5 log with a change of the operational conditions, protocol or type of product. For example, Sharkey et al. (2015) showed that by treating *E. coli* with a helium plasma jet for 180 sec at 2 different frequencies (100 and 160 kHz), a reduction of approximately 0.3 log and 1.2 log CFU/mL was measured, respectively. They attributed this difference to the concentration of reactive species, which was minimal at 100 kHz (Sharkey et al., 2015). Sladek & Stoffels (2005) observed higher levels of *E. coli* inactivation at increasing dissipated power (saturation at 0.260 mW) or decreasing distance between the plasma needle and treated samples. These results indicate the strong effect of plasma power on bacterial inactivation. A 15 min treatment of *Salmonella* inoculated on lettuce, strawberries and potatoes with a nitrogen NTP jet resulted in 2.7 ± 0.3, 1.8 ± 0.7 and 0.9 ± 0.3 log reductions, respectively (Fernández et al., 2013), showing the differences in NTP efficacy depending on the treated products. Moreover, inactivation is also depending on the bacterial species, since it was reported that a 5 log reduction of *Salmonella* Typhimurium on Golden Delicious apples required a treatment time of ± 288 sec, while the same level of inactivation of *Escherichia coli* was obtained after only ± 180 sec of NTP treatment (Kilonzo-Nthenge et al., 2018). Additionally, the accessibility of bacterial cells can greatly affect the inactivation efficiency. Ziuzina et al. (2015) showed that planktonic populations of *Salmonella* (in lettuce broth) were inactivated completely after only 0.5 min of treatment, while an exposure time of 5 min was required to obtain substantial reductions in biofilms on lettuce surfaces. This protective effect of the biofilm can be attributed to the shielding properties of the three-dimensional network of polysaccharides (Vleugels et al., 2005).

Han et al. (2020) made a comparison between the roughness of some NTP treated products and the inactivation of a selection of pathogens on their surfaces. The authors noticed that there was a relationship between both factors. Nevertheless, this was only tested on real-life food products, in which inactivation levels may be influenced by a multitude of factors. The present study investigated a few of these factors separately, so its true effect could be understood.

In the present study, both methods of inoculation (spread and spot) led to a high inactivation on agar plates (see Figure 5). There was no significant difference between recovery for spread plated and spotted agar, and this was the case for both NTP treated and untreated plates. This implicates that the inoculation method does not have a large influence on the efficacy of the NTP treatment. Furthermore, the treatment was least efficient for peppercorn, leading to an average reduction of 0.3 log CFU/sample which is not statistically significant. This is in contrast with the 5 log/g reduction of *Salmonella* species, also on peppercorns, after 80 seconds of treatment, observed by Sun et al. (2014). However, no information of power nor energy input was provided and temperatures > 120°C were obtained at the end of treatment, which implicates an additional inactivation due to this thermal component (Sun et al., 2014). Glass beads, as a matrix, on the other hand, showed a significant effect. This demonstrates that the spherical shape, which could cause shadowing, also has a limited influence on efficiency. A possible explanation for the lack of reduction on peppercorns is the fact that bacteria might be located in protected places on the peppercorn (e.g. cracks, grooves and pits) causing strong shadowing, particularly against the UV-photons present in the plasma (Hertwig et al., 2017). Such protective places are not present on the uniform surface of glass beads. This is in agreement with the hypothesis of Fernández & Thompson (2012), saying that an increasing complexity of the surface results in a decreased inactivation effect of NTP treatment. However, the opposite was observed by Critzer et al. (2007), who noticed higher levels of recovery of *Salmonella* cells on nonselective agar plates compared to actual produce, at initial treatment (thus short treatment time) with non-thermal plasma. They attribute this observation to the cold storage of the produce in their experimental set-up and to the attachment of the *Salmonella* cells to the produce surface, hindering their recovery. Nevertheless, Critzer et al. (2007) recognized the fact that the rough surface of produce is expected to hinder bacterial inactivation, thus posing the need for longer treatment times. Apart from a rough surface, some types of produce (e.g. leafy vegetables) have stomata in which bacterial cells can congregate. Those stomata, from which the opening is subjected to light/dark conditions, reduced the antimicrobial efficacy of NTP treatment (Ziuzina et al., 2015).

Another hypothesis for the lower reduction on the food matrix compared to agar is the presence of organic materials that react with the active species of the NTP, thus reducing the concentration of those species or producing new active species and possibly protecting the microbial cells. Other mechanisms in which the substrate can influence NTP inactivation are dielectric heating of the substrate material or a catalytic effect of the substrate (Fernández & Thompson, 2012). The impact of different food components is presented in Figure 6. It can be concluded that the presence or absence of pyruvate, vitamin C (antioxidant) or starch does not have a significant effect on the inactivation by NTP. These results are surprising since it would be expected that vitamin C, as antioxidant, reacts with some active species, primary singlet oxygen $O_2(^1\Delta_g)$ (Bodannes & Chan, 1979), making them unavailable for reaction with bacteria. Reductions seem to be even higher after addition of this component. Although this enhanced bactericidal effect was not statistically proven in the present study, it has been shown before that vitamin C pre-treatment prior to NTP does result in higher levels of inactivation. This is caused by a negative influence of vitamin C against the membrane integrity of the bacterial cells, making them more susceptible to collapsing and thus disintegration during subsequent NTP treatment (Helgadóttir et al., 2017). Likewise, the lack of effect of sodium pyruvate is surprising at first since it normally aids in the recovery of sub-lethally injured *Salmonella* cells (Gurtler & Kornacki, 2009) and thus a lowered reduction would have been expected. However, nonselective TSA medium itself allows growth of both viable and damaged cells (Liao, Xiang, et al., 2017), making the use of sodium pyruvate as recovery aid redundant.

The presence of sunflower oil, casein or pepper, on the other hand, results in a significantly lower bacterial reduction compared to the control matrix. The oil showed this protective effect already at low concentrations (1%) and a higher protection was seen when high concentrations (10%) were applied. In contrast, the concentration of proteins (casein) or pepper needed to be high (10%) to have a significant impact. Casein has a protective effect towards *Salmonella*, which was previously shown in milk (Rubin, 1985). Since proteins and fat represent around 25% and 5% of pepper composition, respectively (Al-Jasass & Al-Jasser, 2012), their presence could explain a major part of the reduction in the bactericidal effect of NTP on the peppercorns and on the with grinded peppercorn enriched agar. The observed effect of the matrix composition clearly indicates

that NTP efficiency strongly depends on the chemical composition of the treated medium and a careful study of the plasma quenching food related compounds (vitamins, proteins, fats) is required for every NTP application.

A comparison of microbial inactivation levels on a whole range of food products was presented by Pankaj et al. (2018) and López et al. (2019). This proves that inactivation is not only determined by plasma parameters, but also by matrix form and composition, as shown in the present study.

Apart from the bioactive components present in the matrix, its moisture content also influences survival of bacteria during NTP treatment. In the present study, black peppercorns, a type of dry produce, were used as real-life food product, showing a reduction of 0.3 log CFU/sample due to the NTP treatment. It has been described that a higher water activity (e.g. fresh apples) resulted in a more effective reduction of NTP treatment (Lee et al., 2015; Yadav & Roopesh, 2020). A study in which dry figs with a_w 0.70, 0.80 and 0.93 were treated with NTP resulted in respective reductions of 0.5 ± 0.3, 0.7 ± 0.2, and 1.3 ± 0.3 log CFU/g for *E. coli* O157:H7 and 1.0 ± 0.1, 1.2 ± 0.1,and 1.6 ± 0.3 log CFU/g for *L. monocytogenes*. This higher inactivation at a_w 0.93 was significant ($p < 0.05$) (Lee et al., 2015). This might be the result of shrinkage of the microbial cell due to increased osmotic pressure, leading to a thicker cell membrane (Palou et al., 1997) that protects against reactive species (Lee et al., 2015).

5 CONCLUSION

The present study showed that differences in NTP inactivation efficiency between bacterial cells on agar plates and those on food matrices do not arise from the inoculation method (spotting for produce), nor from the shape of the produce. Meanwhile, it is more likely the result of either protective places (e.g. cracks, grooves and pits) causing some kind of shadowing or the composition of the food matrix, as inactivation is 1.1 log CFU higher on glass beads than on peppercorns. After all, low concentrations (1%) of sunflower oil or high concentrations (10%) of sunflower oil, casein or pepper have proven to be protective against NTP-mediated inactivation of the *S.* Typhimurium cells. Interestingly, addition of either vitamin C (antioxidative component) or sodium pyruvate (aids in recovery sub-lethally injured cells) did not result in an increased cell recovery hence a reduced inactivation. Further research is required to thoroughly investigate the effect of various components in food, the origin of their protective properties, and the impact of different active species in NTP.

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7 DECLARATION OF INTERESTS

None

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8 FIGURES

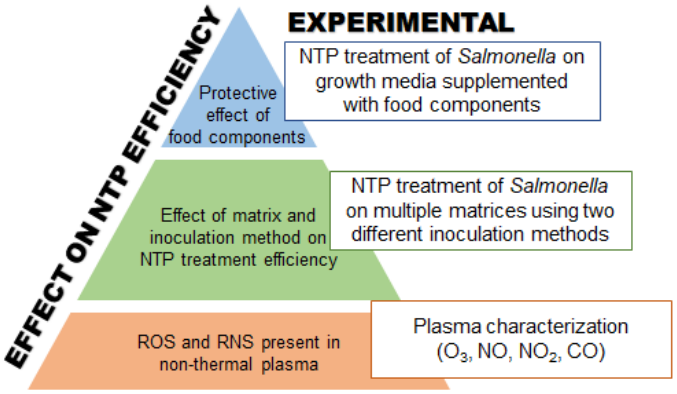


Figure 1: Experimental design: overview of the effects examined in this study, and the corresponding method of analysis.

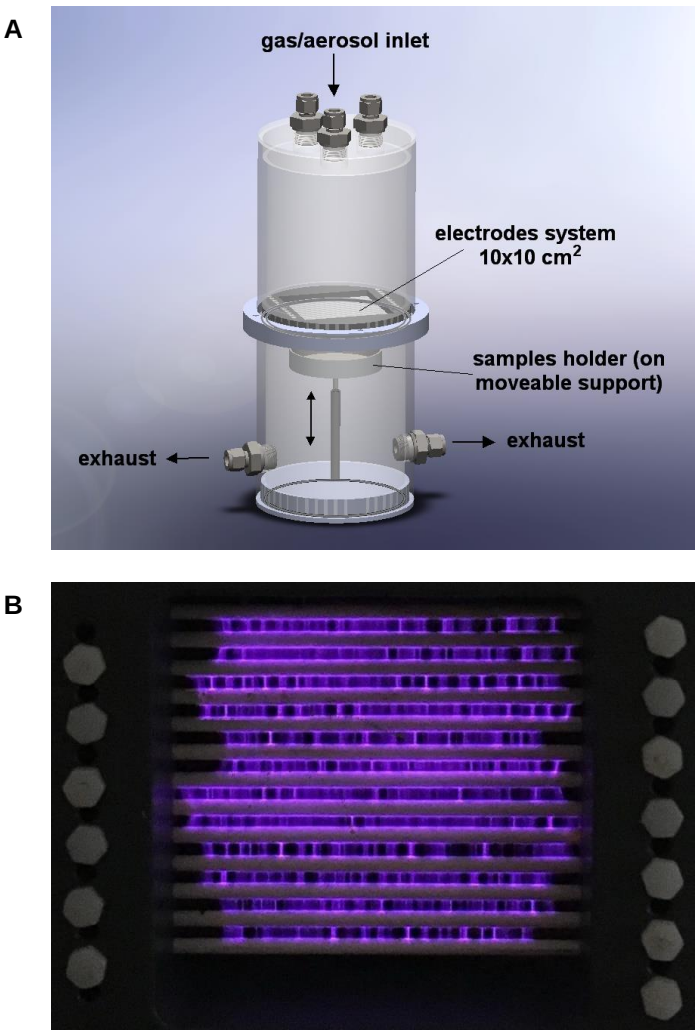


Figure 2: DBD plasma reactor used in the present study. A) 3D schematic of the reactor chamber. Control and diagnostics equipment is not shown. B) bottom view of the plasma discharge.

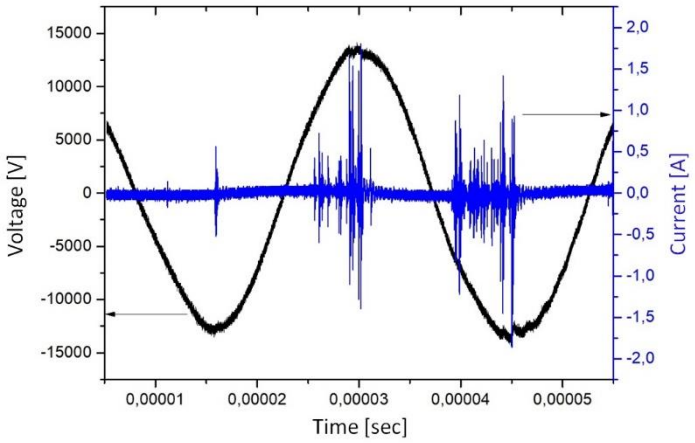
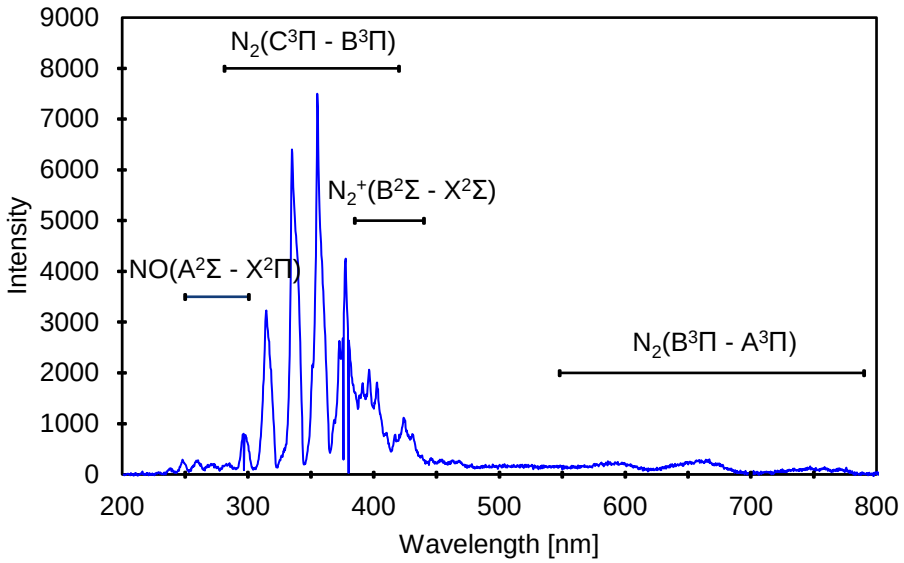


Figure 3: Voltage and current waveforms of the DBD plasma used in the present study. NTP was generated at an RMS voltage of 8 kV with an air flow (60% RH) of 2 slm.



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477 *Figure 4: Emission spectrum of the DBD plasma discharge. NTP was generated at an RMS voltage of 8 kV with an air flow*
478
479 *(60% RH) of 2 slm.*

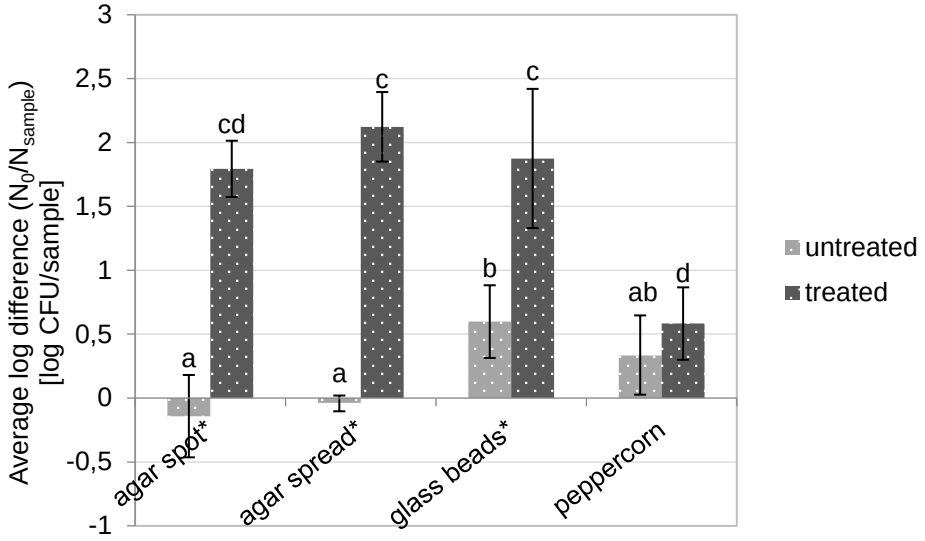

Figure 5: Effect of matrix and inoculation method on efficacy of NTP treatment (n = 6) and corresponding 95% confidence intervals. Each bar represents the average log difference between S. *Typhimurium counts in the inoculum and the concentration of cells recovered from the sample or plate, or* $log_{10}(N_0/N_{sample/plate})$*. Negative values indicate an increase in cells after inoculation. Results of untreated samples are displayed as grey bars, those of treated samples are black. Limits of quantification, which were never reached, were between -0.6 and +0.1 log CFU. An asterisk (*) indicates that NTP treatment resulted in a significant inactivation of* S. *Typhimurium (p ≤ 0.05). Different letters (a & b for untreated and c & d for treated) indicate significant differences (p ≤ 0.05) between methods within the type of treatment. NTP was generated at an RMS voltage of 8 kV with an air flow (60% RH) of 2 slm. The samples were treated for 180 sec at a distance of 3.2 – 3.3 cm from the electrodes.*

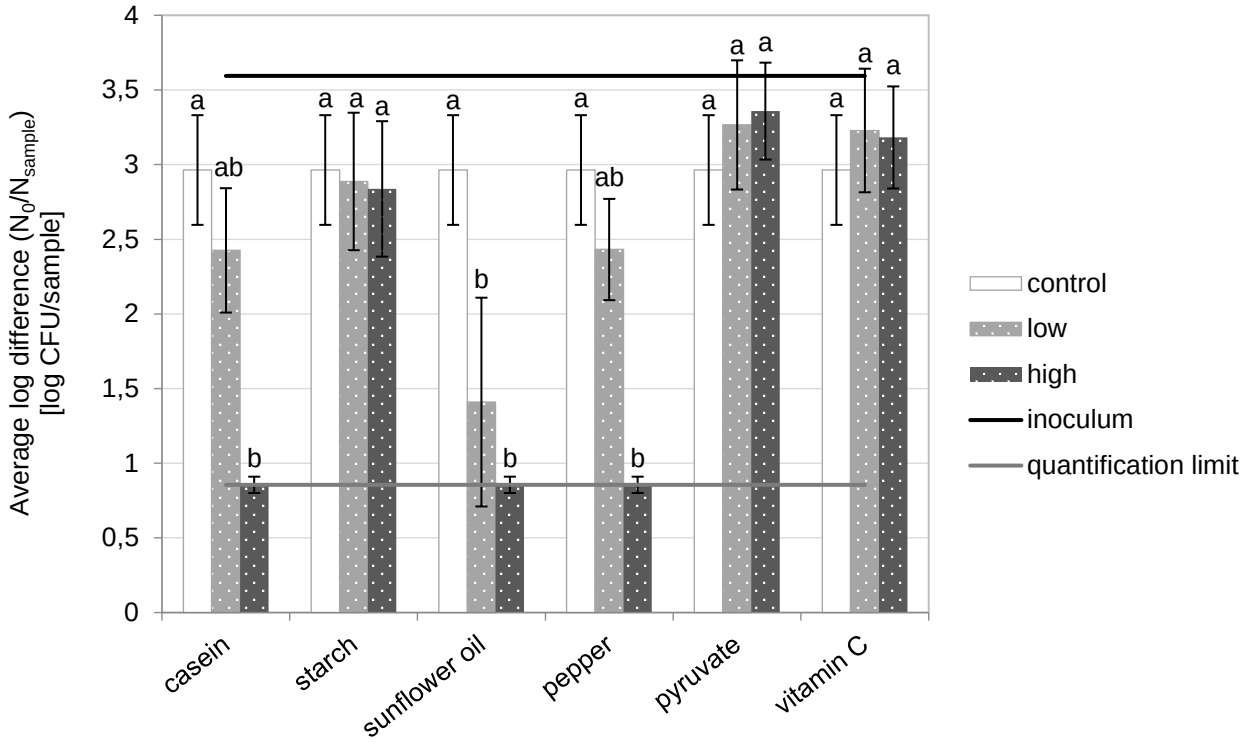

Figure 6: Average log reduction of S. Typhimurium SL1344 compared to inoculum on different food simulation media with one main component in low or high concentration (n = 6) and corresponding 95% confidence intervals. Control samples are regular TSA. All samples are NTP treated. Control = without addition; Low = 1% (except vitamin C: 0.1%); high = 10% (except vitamin C: 1%). Average applied inoculum (3.6 log CFU) and quantification limit (0.9 log CFU) are indicated by black and grey lines, respectively. Different letters indicate significant differences ($p \leq 0.05$) between concentrations within the type of simulation media. NTP was generated at an RMS voltage of 8 kV with an air flow (60% RH) of 2 slm. The samples were treated for 180 sec at a distance of 3.2 – 3.3 cm from the electrodes.

9 TABLES

Species	Operational time interval			
	60 sec	180 sec	300 sec	600 sec
O_3	0.16	0.48	0.41	0.43
NO	0	0	0	0
NO_2	0.069	0.193	0.215	0.222
NO_x	0.069	0.193	0.215	0.222
CO	2.8×10^{-3}	2.7×10^{-3}	1.9×10^{-3}	1.1×10^{-3}

Table 1: Concentration (mg/L) of long living species in the NTP, measured at 1 m from the outlet of the reactor and after different processing time intervals. NTP was generated at an RMS voltage of 8 kV with an air flow (60% RH) of 2 slm.