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# Inactivation Kinetics of *Salmonella typhimurium* and *Staphylococcus aureus* in Different Media by Dielectric Barrier Discharge Non-Thermal Plasma

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**Abstract:** A study was conducted to determine the effect of dielectric barrier discharge non-thermal plasma (DBD-NTP) on *Salmonella typhimurium* and *Staphylococcus aureus* populations on solid surfaces and in liquid suspensions. Our results showed that inactivation kinetics of *S. typhimurium* and *S. aureus* by DBD-NTP treatments can be well predicted with mathematical models. The survival curves of both *S. typhimurium* and *S. aureus* showed a log-linear phase followed by tailing behaviors on solid surfaces, and shoulder behaviors followed by a log-linear phase in liquid suspensions. The *D* values (decimal reduction time) for *S. typhimurium* and *S. aureus* in suspension were higher than those on solid surfaces ($p < 0.05$). Additionally, the maxima of sublethal injury values under low NaCl concentration and neutral pH condition were higher than those under high NaCl and low pH condition. In addition, mathematical modeling was evaluated to predict the final inactivation result for potential industrial applications. This study indicates that different microbial supporting matrices significantly influence the inactivation effect of DBD-NTP; it also provides useful information for future applications of NTP in enhancing food shelf life and safety.

**Keywords:** DBD plasma; *Salmonella typhimurium*; *Staphylococcus aureus*; solid media; liquid media; inactivation kinetics model

## 1. Introduction

Fresh food becomes more prevalent in the global market due to their nutritional benefits. However, fresh food has strict requirements for decontamination, which need to be effective in preservation of fresh food quality and extending shelf-life without causing unacceptable changes in nutritional or organoleptic properties of food. These requirements have spurred scientists to develop novel non-thermal methods to reduce microbial populations in fresh food [1]. Non-thermal plasma (NTP) is an emerging decontaminating technology. Under ambient pressure and temperature conditions, it generates energetic electrons that collide with gas molecules and result in dissociation, excitation and ionization of gas molecule [2]. NTP consists of not only partially ionized gases but also highly bio-reactive components including UV light, free radicals, and oxidative molecules. Dielectric barrier discharge (DBD) is one of the simplest ways for generating NTP.

Numerous studies have been conducted to investigate the effects of NTP on microorganisms on food polymer and medical utensil surfaces, and in liquids and gases [1,3,4]. For example, Perni et al. [5]

examined efficiency of NTP at 8 kV in reducing the *Escherichia coli* type 1, *Saccharomyces cerevisiae*, *Gluconobacter liquefaciens*, and *Listeria monocytogenes* Scott A on the surfaces of filter membranes. Ziuzina et al. [6] investigated the in-package inactivation efficacy of DBD-NTP on *Escherichia coli* suspended in different media and the diffusion of reactive species into liquid media. Rothrock et al. [7] reported the effects of DBD-NTP on *Pseudomonas*, *Campylobacter*, and *Salmonella* in liquid media. A wide range of microorganisms, including spores, bacterial, fungi, virus, can be inactivated by NTP treatment, and has been reviewed by Scholtz [8]. Technological parameters, such as input power, gas composition and treatment time, influence inactivation efficiency of plasma. However, food intrinsic properties will also affect the resistance of microorganisms towards plasma treatment [2]. Food intrinsic factors such as osmolarity and pH also affect the efficacy of plasma treatment, while lipid and protein content and antioxidant state can diminish the activity of plasma reactive species [9]. Kayes et al. reported that *S. Enteritidis* cells were more susceptible treated by plasma at pH 5 as compared to pH 7 [10]. *S. typhimurium* and *Listeria monocytogenes* cells were more resistant towards plasma treatment at salt concentration 0% (*w*/*v*) compared to 6% (*w*/*v*) [11]. In addition, these observation also suggest the complexity of NTP treatment effects on microbes and the importance of further investigation of microbial survival curves to elucidate the advantages and limitations of this technology [12]. In food, inactivation kinetics analysis is a useful tool to describe microbial survival curves, since quantitative surviving of microorganisms is essential to maintain food quality [13].

Sublethal injury is a consequence of exposure to a chemical and/or physical antimicrobial process. In sublethal injury, microbes are damaged but not killed by the antimicrobial treatment [14]. The significance of the sublethal injury in foods is twofold and somewhat contradictory. Sublethal injury causes false negative results, since injured cells do not develop in selective media and therefore escape detection. But the potential for hazard is still present because injured cells are still capable of repairing themselves and producing toxins [14]. In order to estimate sublethal injury after an antimicrobial treatment, the difference in plate counts between nonselective media, which support cell recovery and represent both uninjured and injured cells, and the corresponding selective media, to which injured cells become sensitive, is used to indicate a proportion or percentage of sublethal injury in the entire microbial population [14]. To our knowledge, very little literature is available regarding the sublethal injury of microbes by DBD-NTP treatments.

The objectives of this study are: (1) to study the effects of environmental conditions, such as osmotic pressure, pH, and nutrients, in media on survival of gram-positive bacterium *S. aureus* and gram-negative bacterium *S. typhimurium*; (2) to investigate the bactericidal mechanisms of DBD plasma against different bacterial type by a kinetics approach; and (3) to estimate sublethal injury of *S. aureus* and *S. typhimurium* by DBD-NTP.

## 2. Materials and Methods

### 2.1. Bacterial Strains and Culture Conditions

Strains of *S. typhimurium* (CMCC (B) 50115) and *S. aureus* (ATCC 6538) were provided by Guangdong Huankai Microbial Sci. and Tech. Co., Ltd. (Guangzhou, China). Freeze-dried stocks were revived in Brain Heart Infusion broth (BHI, Hopebio, Qingdao, China) at 37 °C for 24 h followed by streaking onto BHI agar (Hopebio, Qingdao, China). BHI agar plates were also incubated at 37 °C for 24 h. An isolated colony of *S. typhimurium* and *S. aureus* were inoculated into 10 mL tubes containing tryptic soy broth (TSB; Hopebio, Qingdao, China) and incubated overnight at 37 °C. The overnight culture (18 h) was harvested by centrifugation at 8000× *g* for 10 min.

### 2.2. Sample Preparation

#### 2.2.1. Preparation of Bacteria Embedded on Solid Media

The bacterial cell pellet was washed twice with sterile phosphate buffered saline (PBS; Oxoid, Basingstoke, UK) and re-suspended in 10 mL of PBS.

With the appropriate amount of NaCl, gelatin at 5% (*w*/*v*) (gelatin from bovine skin, type B, Sigma-Aldrich, Saint-Louis, MO, USA) was added to BHI. After gelatin-BHI heating for 20 min at 60 °C in a thermostatic water bath (GR150-S12, Grant, Shepreth, UK), the gelled medium was filter-sterilized using a 0.2 mm filter (0.22 µm, Whatman, TPP, Schaffhausen, Switzerland) and transferred into sterile petri dishes (diameter 5.0 cm). The plates were then surface-inoculated with 100 µL of appropriately diluted cell samples. For glass slide surface (diameter 2.0 cm), 16 µL of the diluted cell sample was transferred and spread on the glass. The cells were allowed to dry for 40 min in the laminar flow cabinet before DBD-NTP treatments.

#### 2.2.2. Preparation of Bacterial Suspensions

The bacterial cell pellet was washed twice with sterile PBS and then re-suspended in 10 mL of physiological saline (0.9, 2% (*w*/*v*) NaCl), PBS (pH values 6.0, 7.3), or bovine serum albumin (BSA; Sigma-Aldrich, MO, USA) (1, 12% (*w*/*v*)) buffer. A working concentration (3.00 mL) of $1 \times 10^8$ CFU $mL^{-1}$ (CFU stands for colony forming unit) *S. typhimurium* and *S. aureus* in NaCl, PBS and BSA were pipetted into 6-well microtitre plates (Sigma-Aldrich, MO, USA).

#### 2.2.3. Sample Packaging

Samples were placed in polypropylene trays (HS-6; Chuo Kagaku, Shanghai, China) with dimension being 178 mm × 126 mm × 35 mm, oxygen transmission rate being 10 $cm^3/m^2/24$ h at 23 °C, and water vapor transmission rate being 10 $g/m^2/24$ h at 23 °C. The trays were sealed with laminated barrier film polyamide/polyethylene (oxygen transmission rate of 3 $cm^3/m^2/24$ h). A Senrui H 360 modified atmosphere packaging machine was used to pack samples in mixed gas (30% $O_2$ + 70% $N_2$). The gas combination in the sealed packages was checked by a gas analyzer (Check Point-Handheld Gas Analyzer, Dansensor, Ringsted, Denmark). Samples were placed at 25 °C for 2 h to let relative humidity reach more than 80% in packages (RH/Temp data logger R-4HC, Elitech, Hangzhou, China) before DBD-NTP treatments.

### *2.3. Plasma Treatment*

The DBD system consisted of an AC Dielectric Test Set (BK-130, Phenix Technologies, Accident, MD, USA), aluminum cyclic annular electrodes (150 mm diameter), and two dielectric barriers layers (polypropylene sheets). We placed the package between two aluminum electrodes with a gap of 40 mm and the two polypropylene layers (upper: 400 × 400 × 2 mm; bottom: 400 × 400 × 3 mm) above and below the package as additional dielectric barriers. The sample was treated at 85 kV (peak-to-peak) for pre-defined durations (0, 20, 40, 60, 80, 100, 120, 140 s). Experiments were repeated 3 times to ensure the stability and accuracy of the equipment. Microbial recovery was conducted after NTP treatment immediately.

### *2.4. Microbiological Recovery and Analysis*

For microbial recovery from glass slides, the inoculated surface with 5 mL of phosphate buffered saline containing 0.05% triton X-100 were repeatedly washed to remove microorganisms. The buffer was then serially diluted in PBS containing 0.1% peptone.

For microbial recovery from a gelatin-BHI surface, the gelatin-BHI was transferred to a stomacher bag, liquefied in a thermostatic water bath at 37 °C, and homogenized in the stomacher for 30 s. 1 mL was taken from the bag, and serial decimal dilutions were prepared with PBS containing 0.1% peptone [11].

For microbial recovery from the liquid media, serial decimal dilutions were prepared with 1 mL of NaCl, PBS or BSA containing microorganism depending upon the bacterial suspensions. The initial suspensions were diluted 10 fold in PBS containing 0.1% peptone.

After series dilutions, 0.1 mL of the initial and diluted suspensions were spread onto BHI-Agar plates (general media) and Salmonella Shigella (SS, *S. typhimurium*) Agar (selective media) or

Baird-Parker (BP, *S. aureus*) Agar plates (selective media). Plates with general media were incubated at 37 °C for 24 h before counting, while selective plates were incubated up to 48 h at 30 °C. Cell counts for each replication in each treatment were the mean of all countable plates.

### 2.5. Bacterial Inactivation Model and Sublethal Injury Estimation

Various different models of kinetic were applied to describe plasma inactivation curves, including log-linear model with shoulder or tailing [15], Weibull type model [16], and Biphasic model [17]. The model developed by Geeraerd et al. [18] was used to fit experimental data. This model for a microbial inactivation curve consists of a shoulder, a log-linear inactivation, and a tail phase. It is described as follows:

$$N(t) = (N_0 - N_{res}) \times exp(-k_{max} \times t) \times \left( \frac{\exp(k_{max} \times t_l)}{1 + (exp(k_{max} \times t_l) - 1) \times \exp(-k_{max} \times t)} \right) + N_{res} \quad (1)$$

where $N(t)$ [CFU/mL] is the cell density at time $t$ [s]; $N_0$ [CFU/mL] is the initial cell density, $N_{res}$ [CFU/mL] is a resistant subpopulation of cells; $k_{max}$ [1/s] is the maximum specific inactivation rate; and $t_l$ [s] is the length of the shoulder.

In order to calculate the percentage of sublethal injury, actual counts of bacteria obtained from selective and non-selective media were used. The percentage of injured survivors after exposure to cold atmospheric plasma (CAP) treatment was determined using the following equation [19].

$$\text{sublethal injury } \% = \frac{N_{nonselective}(t) - N_{selective}(t)}{N_{nonselective}(t)} \times 100 \quad (2)$$

where $N_{nonselective}(t)$ [CFU/mL] is counts on nonselective medium; and $N_{selective}(t)$ [CFU/mL] is counts on selective medium.

### 2.6. Statistical Analysis

Statistical significance was assessed with analysis of variance (ANOVA) of SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Significant mean differences were determined with Fisher's least significant difference (LSD) test at $p < 0.05$. All the experiments were independently repeated three times with duplicate samples.

## 3. Results and Discussion

### 3.1. Bacterial Inactivation Effect and Kinetics Model Analysis

The inactivation effect on *S. typhimurium* and *S. aureus* by DBD-NTP are presented in Figures 1 and 2, respectively. All survival curves consist of three phases: shoulder or tailing phase, a log-linear phase, and a transition phase between the shoulder and log-linear phases. As shown in Figure 1a and Table 1, survival curve of *S. typhimurium* (total population) on the surface of glass showed a log-linear phase (from 8.05 ± 0.07 to 4.91 ± 0.61 log (CFU/cm$^2$)) followed by a long tail. As shown in Figure 1b and Table 1, survival curve of *S. typhimurium* (total population) in 0.9% NaCl solution showed a shoulder phase (treated time from 0 to 73.3 ± 4.08 s) followed by a log-linear phase (treated time after 73.3 ± 4.08 s). In this study, the inactivation efficiency of DBD plasma was evaluated 5 different parameters. In addition, a log-linear shoulder or tailing model was also used to analyze the plasma-induced inactivation. The analysis was $R^2$ (0.9761–0.9993) and RMSE (0.0393–0.7184), which determined the goodness of fit of the models.

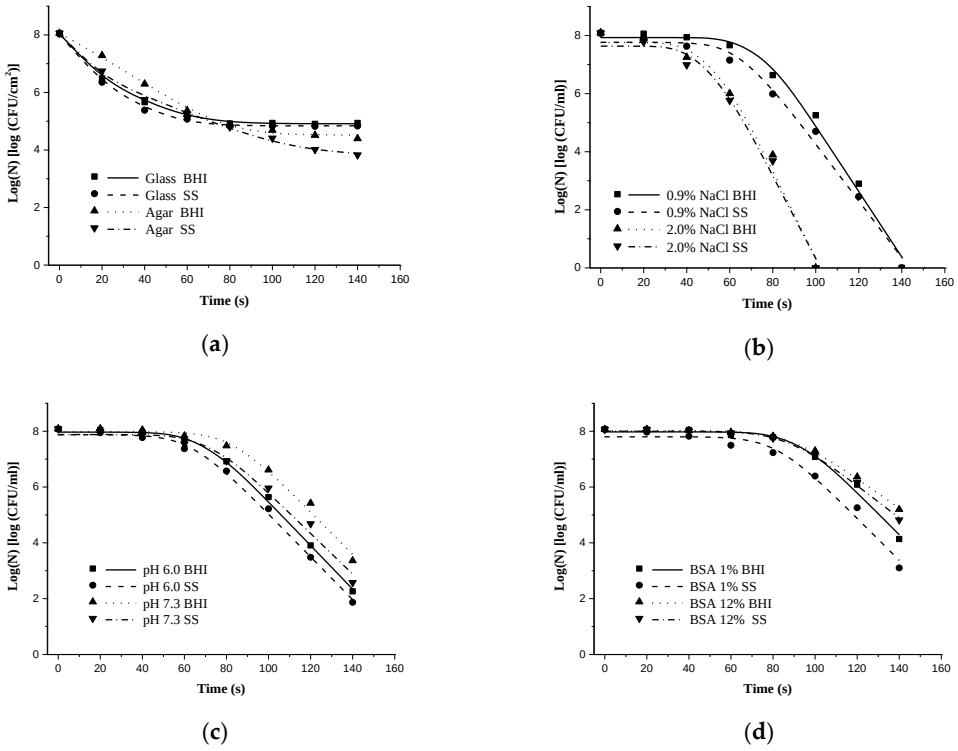


**Figure 1.** Survival curves of *S. typhimurium* after exposure to dielectric barrier discharge (DBD) plasma. Cells were inactivated on solid(like) surface of glass and gelatin (**a**) and in liquid carrier of NaCl (**b**), phosphate buffered saline (PBS) (**c**) and bovine serum albumin (BSA) (**d**). All points were actual values and all lines were fitted values of the model. BHI: Brain Heart Infusion Agar; SS: Salmonella Shigella Agar.

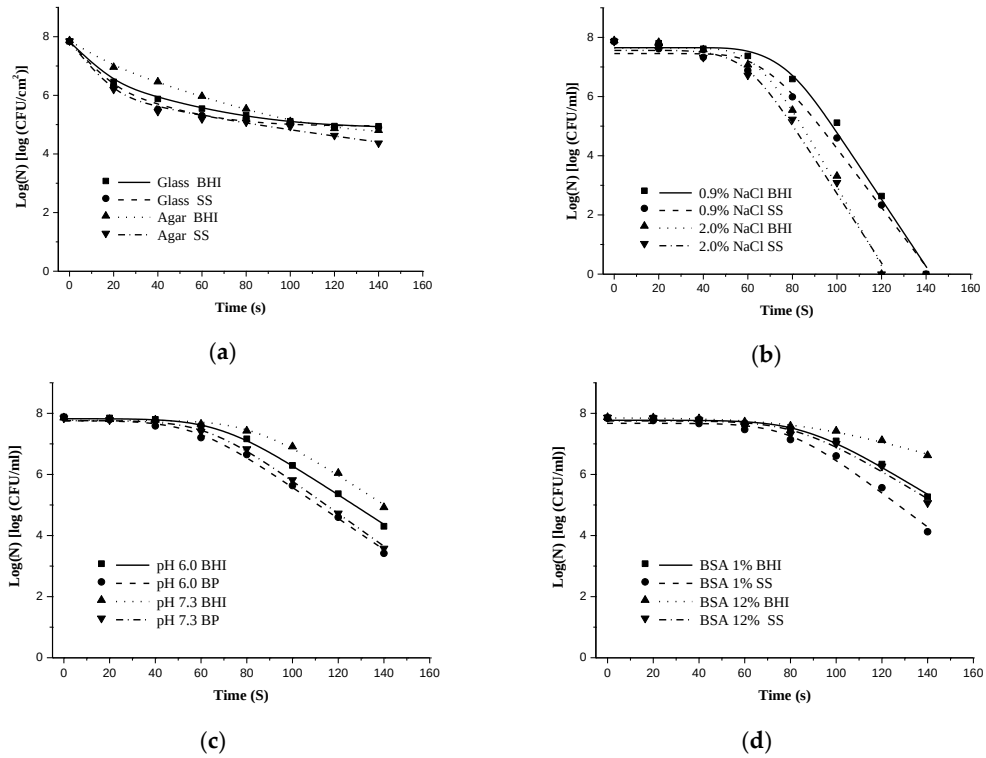


**Figure 2.** Survival curves of *S. aureus* during DBD-NTP (non-thermal plasma treatment). Cells were inactivated on solid surface of glass and gelatin (**a**) or in a liquid suspension of NaCl (**b**), PBS (**c**) or BSA (**d**). All points were actual values and all lines were fitted values of the model.

3.1.1. Effect of Solid Surface on Inactivation of *S. typhimurium* and *S. aureus* by DBD-NTP

For inactivation of the solid surface (*S. typhimurium* for Figure 1a and *S. aureus* for Figure 2a), a steep drop in the slope of inactivation regression lines was noted within the first 20 s. Thereafter, the increment of the pathogen lethality slowed down, following an exponential relationship with treatment time. After 80 s, slopes became mostly flat for most data, indicating that the increase in treatment time does not affect the germicidal efficacy much. Populations of *S. typhimurium* and *S. aureus* on the solid surface were reduced to 2.83 and 2.29 $\log/cm^2$ in 60 s. However, treatments for an additional 80 s merely reduced them by 0.29 and 0.6 $\log/cm^2$, respectively. Similar survival curves were found when low-pressure plasma (1–10 Torr) and UV irradiation were evaluated for their bacteria inactivation kinetics [20]. The kinetics of UV irradiation was ascribed to suppression due to reabsorption by emitting gas from plasma. Similarly, the reduction in inactivation of microbes in kinetic curves with DBD-NTP could be attributed to the "quenching" effects between reactive oxygen/nitrogen species formed in packages during extended treatment time [21,22]. At this power level the UV radiation does not play a significant direct role in the inactivation process. In DBD plasma discharges, reactive species generated through various collisional pathway, commonly, used oxygen and nitrogen gas plasma are excellent sources of reactive oxygen-based and nitrogen-based species, such as O, •OH, NO and $NO_2$ [23]. Firstly, reactive species were regarded as the primary factors in killing microorganisms. They caused oxidation of unsaturated fatty acids and produced unsaturated fatty acid peroxide formation, thereby impeding transport of the biochemical by-products across the membrane. Beyond that, proteins and nucleic acids react with reactive species to formation of 2-oxohistidine and 8-hydroxy-2 deoxyguanosine [24]. Besides, some reports pointed out that charged particles play important roles in inactivation. Electrons and ions can cause surface lesions in membranes by direct bombardment damage. Mendis et al. [25] and Laroussi et al. [26] suggested that electrostatic force caused by charge accumulation on the outer surface of the cell membrane could overcome the tensile strength of the membrane and cause its rupture. This phenomenon is more likely to occur on gram-negative bacteria, whose cell membrane is always irregular. These irregularities offer small radii of curvatures that cause localized high outward electrostatic forces which trigger rupture of gram-negative bacteria [23].

**Table 1.** Parameters of the kinetics model for the inactivation of *S. typhimurium* by dielectric barrier discharge non-thermal plasma (DBD-NTP). Cells were inactivated on a solid surface or in a liquid suspension.

| | Population | log $N_0$ (log (CFU/mL))/ log $N_0$ (log (CFU/cm$^2$)) | $K_{max}$ | $t_l$ | log $N_{res}$ (log (CFU/mL))/ log $N_{res}$ (log (CFU/cm$^2$)) | $D$ value [1] | Log Reduction [2] | RMSE [3] | R [2] |
|---|---|---|---|---|---|---|---|---|---|
| NaC l0.9% | Total | $7.91 \pm 0.10^{aAB}$ | $0.26 \pm 0.011^{aB}$ | $73.3 \pm 4.08^{aC}$ | | $81.71 \pm 3.72^{aC}$ | $8.07 \pm 0.10^{aA}$ | 0.4002 | 0.9895 |
| | Uninjured | $7.76 \pm 0.15^{bBC}$ | $0.22 \pm 0.007^{bB}$ | $63.8 \pm 1.08^{bC}$ | | $73.6 \pm 0.78^{bD}$ | $8.09 \pm 0.07^{aA}$ | 0.4319 | 0.9876 |
| NaC l2% | Total | $7.76 \pm 0.15^{aB}$ | $0.34 \pm 0.011^{aA}$ | $50.2 \pm 1.15^{aD}$ | | $56.62 \pm 0.94^{aD}$ | $8.09 \pm 0.07^{aA}$ | 0.6705 | 0.9815 |
| | Uninjured | $7.64 \pm 0.16^{aC}$ | $0.32 \pm 0.015^{aA}$ | $48.2 \pm 1.93^{aD}$ | | $55.02 \pm 1.62^{aE}$ | $8.08 \pm 0.09^{aA}$ | 0.7184 | 0.9783 |
| PBS pH = 6.0 | Total | $7.93 \pm 0.14^{aAB}$ | $0.21 \pm 0.041^{aC}$ | $72.5 \pm 1.42^{aC}$ | | $83.2 \pm 1.63^{aC}$ | $6.57 \pm 1.23^{aB}$ | 0.1618 | 0.9968 |
| | Uninjured | $7.82 \pm 0.15^{aABC}$ | $0.20 \pm 0.031^{aBC}$ | $67.8 \pm 3.31^{aBC}$ | | $78.84 \pm 2.97^{aCD}$ | $6.83 \pm 0.99^{aB}$ | 0.2185 | 0.9948 |
| PBS pH = 7.3 | Total | $7.97 \pm 0.12^{aA}$ | $0.17 \pm 0.025^{aD}$ | $82.5 \pm 4.18^{aB}$ | | $95.25 \pm 5.41^{aB}$ | $4.72 \pm 0.88^{aC}$ | 0.2623 | 0.9863 |
| | Uninjured | $7.88 \pm 0.14^{aAB}$ | $0.16 \pm 0.007^{aDE}$ | $71.2 \pm 2.85^{bB}$ | | $84.34 \pm 2.37^{bC}$ | $5.50 \pm 0.03^{aC}$ | 0.3250 | 0.9842 |
| BSA 1% | Total | $7.97 \pm 0.07^{aA}$ | $0.17 \pm 0.004^{aD}$ | $91.4 \pm 3.47^{aA}$ | | $104.03 \pm 3.48^{aA}$ | $3.93 \pm 0.24^{aCD}$ | 0.2126 | 0.9867 |
| | Uninjured | $7.78 \pm 0.19^{aBC}$ | $0.17 \pm 0.033^{aCD}$ | $81.7 \pm 4.25^{bA}$ | | $94.49 \pm 1.72^{bB}$ | $4.95 \pm 0.74^{aCD}$ | 0.3515 | 0.9761 |
| BSA 12% | Total | $8.02 \pm 0.10^{aA}$ | $0.12 \pm 0.009^{aE}$ | $88.3 \pm 2.56^{aA}$ | | $106.03 \pm 1.73^{aA}$ | $2.87 \pm 0.20^{aE}$ | 0.0620 | 0.9980 |
| | Uninjured | $7.99 \pm 0.08^{aAB}$ | $0.13 \pm 0.020^{aEF}$ | $87.6 \pm 5.81^{aA}$ | | $104.01 \pm 7.28^{aA}$ | $3.24 \pm 0.71^{aE}$ | 0.0961 | 0.9961 |
| Glass | Total | $8.05 \pm 0.07^{bA}$ | $0.09 \pm 0.007^{bF}$ | | $4.91 \pm 0.61^{aA}$ | $9.11 \pm 3.37^{aF}$ | $3.11 \pm 0.58^{aDE}$ | 0.0432 | 0.9991 |
| | Uninjured | $8.04 \pm 0.07^{aA}$ | $0.10 \pm 0.001^{aF}$ | | $4.83 \pm 0.59^{aA}$ | $8.11 \pm 3.35^{aF}$ | $3.20 \pm 0.56^{aE}$ | 0.0665 | 0.9980 |
| Gelatin | Total | $8.06 \pm 0.07^{aA}$ | $0.10 \pm 0.005^{aEF}$ | | $4.52 \pm 0.15^{aA}$ | $23.66 \pm 2.72^{aE}$ | $3.66 \pm 0.02^{bDE}$ | 0.1083 | 0.9964 |
| | Uninjured | $8.06 \pm 0.07^{bA}$ | $0.06 \pm 0.001^{bG}$ | | $3.83 \pm 0.18^{bB}$ | $9.02 \pm 1.68^{bF}$ | $4.23 \pm 0.08^{aD}$ | 0.1629 | 0.9928 |

[1] $D$ value is defined as time needed for the 1 log reduction of initial target microorganism population. [2] Log reduction is calculated from the difference between log $N_0$ to log $N$ ($n$ = 140 s). [3] RMSE stands for the root mean squared error. [a–b] Means without common superscripts in each inactivation media (NaCl, PBS, BSA or solid media) within a parameter are different ($p < 0.05$). [A–G] Means without common superscripts in each population type (total or uninjured population) within a parameter are different ($p < 0.05$).

As shown in Tables 1 and 2, *D* values for *S. typhimurium* and *S. aureus* on the surface of glass slides were 9.14 s and 9.11 s, respectively, and on the surface of gelatin plates were 23.66 s and 25.47 s, respectively. These results indicated that bacteria on the gelling plates were much harder to kill by DBD-NTP than bacteria loaded on glass slides. The differences between glass slides and gelatin plates could be due to the amount of water and chemical composition of medium effect [27]. Dobrynin et al. [27] reported that charged particles generated in plasma catalyzed peroxidation of lipids and polysaccharides on the cell membranes, with reaction rate described above. Bacteria loaded on surface of slides are covered with layer of water molecules bound by van der Waals forces, whereas bacteria loaded on gelatin are covered with thick layer of free water. Charged particles catalyze oxidation and erosion processes of both surface and interior of microorganisms and presence of water and reactive species play a synergistically effect role which contribute to faster inactivation. Cell seems to be less susceptible and slopes of survival curves became flatter at the latter half of inactivation process. Plasma acts only on the surface and charged particle and reactive species cannot penetrate deep into the substrates. It seems plausible that dead bacteria on top layers could form a physical protective layer covering the surviving bacteria. Meanwhile, disruption of membrane integrity could lead to the release of highly oxidative materials (e.g., membrane lipids, free fatty acids, etc.) [28]. The oxidizable materials could serve as a "quenching" phase for the highly reactive, oxidizing species produced by plasma, thereby resulting in a decline in microbial inactivation. Although the nature of this tailing effect is not exactly known, it has been attributed to the existence of a fraction of the population more resistant to plasma [2]. Tailing effect is not exactly known and the prospect warrants further investigation.

#### 3.1.2. Embedding *S. Typhimurium* and *S. Aureus* in Liquid Media

Survival curves of *S. typhimurium* and *S. aureus* in liquid media are shown in Figure 1b–d, and Figure 2b–d. The survival curves exhibited a shoulder region followed by a log-linear phase regardless of liquid medium and bacterial species. The shoulder region has been attributed to the time required by generated germicidal agents in NTP treatments to reach the minimum concentrations on cell membranes for causing damages to bacterial cells [29]. Hence, the differences in shoulder phase length or duration reflect the requirement of limiting levels of germicidal agents to start inactivating bacteria or thresholds for formed germicidal agents to start damaging microbial cells on different media. Once the concentration of germicidal agents passes the threshold, a log-linear phase is triggered, resulting in irreversible damage and lysis of the cells [29].

The kinetics parameters from Tables 1 and 2 for *S. typhimurium* and *S. aureus*, respectively, show that compared with buffer liquid system, a shorter shoulder and a higher maximum specific inactivation rate were found with salt solutions. In addition, no visible colony formation was detected in 2% NaCl solution after samples were treated for as short as 120 s (Figures 1b and 2b) regardless of bacterial species and recovery method. However, it took 140 seconds to finish inactivation in 0.9% NaCl solution. The plasma–liquid interaction likely generated other oxidizing species such as hypochlorous acid (HOCl) [30]. With higher concentration of NaCl in liquids, the more HOCl was produced [31]. However, for the other two media, microbial populations were still countable after 140s' treatments. These differences could be due to the acidification effect of aqueous solution by DBD-NTP. In our present study, it was noted that the pH values of the salt solution descended to 3.7 (data not shown) after NTP treatments, whereas pH of buffer liquid system did not significantly change. Acidification has been hypothesized to be one of the main mechanisms for DBD-NTP treatments to reduce microbial loads in an aqueous solution. Our results are in agreement with what was reported by Zhou et al. [32]. Oehmigen et al. [33] studied the effect of plasma on properties of water and concluded that the NOx formation, including nitrous $HNO_2$ and nitric acid $HNO_3$, is responsible for acidification of aqueous solution. Bacteria are negatively charged colloidal particles. Their isoelectric points are disturbed in the acidic conditions, which leads to the death of microorganism [34]. Further study with NTP [35] showed that addition of nitrate ions in aqueous solution alone was not able to inactivate microorganisms,

but it somehow enhanced antimicrobial effect of nitrate, nitrite, hydrogen peroxide, peroxynitrite, and hydroxyl radicals. In addition, DBD-NTP treatments can also produce hydrogen peroxide in high relative moisture environment. Hydrogen peroxide in acidic conditions is a strong oxidizer and can have a greater impact on the bacterial cell membrane [36]. pH effect on bacterial surviving DBD-NTP treatments was also demonstrated by the inactivation kinetics analyses conducted in this study. *D*-values for *S. typhimurium* and *S. aureus* in pH 7.3 PBS were consistently higher than those in pH 6.0 PBS (Tables 1 and 2).

Peroxynitrous acid enters the bacterial by passive diffusion. Once inside the cell, ONOOH can damage DNA, lipids, and proteins via direct oxidation reactions or by decomposing to OH and $NO_2\bullet$. Peroxynitrite also reacts with $CO_2$ to form a nitrosoperoxycarbonate anion ($ONOOCO_2^-$) followed by decomposing to $NO_2\bullet$ and a carbonate radical ($CO_3\bullet^-$), which also can damage biomolecules [37]. The reaction mentioned above can occur only in acidic conditions. Coinciding with this research, *D*-values of *S. typhimurium* and *S. aureus* planktonic were significantly different between pH = 7.3 PBS and pH = 6.0 PBS. Adverse environmental conditions such as extremes of temperature and pH, osmotic shock, anaerobicity and composition of the growth medium can induce stress responses in *S. typhimurium*, which in turn affects its ability to withstand antimicrobial treatments [38]. Kayes et al. [10] reported that lower pH rendered *Escherichia coli* O157:H7, *Listeria monocytogenes*, *S. aureus*, more susceptible to the plasma. Along with the number of stressful environmental factors increasing, microorganism became more vulnerable when treated by plasma. *D* values of *S. typhimurium* and *S. aureus* planktonic were higher in 12% BSA compared to 1% BSA ($p < 0.05$). Log reduction of *S. typhimurium* and *S. aureus* (total) were significantly different between 1% BSA and 12% BSA ($p < 0.05$). The inactivation rate of the mixed culture cocktail was slower by comparison to the respective mono culture preparations, which may be due to the greater potential for organic-based quenching of the reactive species generated by DBD-NTP treatment [39]. Higher resistance of bacteria at 12% BSA to plasma may be attributed to BSA reacting with active species of plasma which are supposed to disinfect microorganisms. Zhou et al. [30] showed that plasma induced the change of the side chains of the amino acids by hydroxylation, nitration, dehydrogenation and dimerization. Scholtz et al. [33] showed the $He/O_2$ plasma jet was used for destruction of BSA proteins and in the case of incomplete protein removal, the treated BSA suffered considerable degradation. This leads to the assumption that existence of BSA could protect microorganisms from being killed by plasma.

**Table 2.** Parameters of the kinetics model for the inactivation of *S. aureus* by DBD-NTP. Cells were inactivated on a solid surface or in a liquid suspension.

| | Population | log $N_0$ (log (CFU/mL))/ log $N_0$ (log (CFU/cm$^2$)) | $K_{max}$ | $t_l$ | log $N_{res}$ (log (CFU/mL))/ log $N_{res}$ (log (CFU/cm$^2$)) | $D$ value [1] | Log Reduction [2] | RMSE [3] | R [2] |
|---|---|---|---|---|---|---|---|---|---|
| NaCl0.9% | Total | $7.65 \pm 0.1^{aA}$ | $0.26 \pm 0.015^{aB}$ | $74.57 \pm 2.78^{aC}$ | | $82.97 \pm 2.29^{aC}$ | $7.87 \pm 0.12^{aA}$ | 0.3392 | 0.9921 |
| | Uninjured | $7.46 \pm 0.09^{aC}$ | $0.23 \pm 0.013^{bB}$ | $67.27 \pm 3.17^{bB}$ | | $76.87 \pm 2.61^{bCD}$ | $7.86 \pm 0.11^{aA}$ | 0.4218 | 0.9874 |
| NaCl2% | Total | $7.65 \pm 0.12^{aA}$ | $0.31 \pm 0.021^{aA}$ | $64.44 \pm 2.97^{aD}$ | | $71.61 \pm 2.48^{aD}$ | $7.89 \pm 0.14^{aA}$ | 0.4283 | 0.9896 |
| | Uninjured | $7.55 \pm 0.1^{aBC}$ | $0.27 \pm 0.015^{aA}$ | $59.01 \pm 2.86^{aCD}$ | | $67.07 \pm 2.41^{aE}$ | $7.86 \pm 0.11^{aA}$ | 0.4726 | 0.9869 |
| PBS pH=6.0 | Total | $7.82 \pm 0.06^{aA}$ | $0.11 \pm 0.002^{aC}$ | $68.26 \pm 0.81^{aD}$ | | $87.98 \pm 0.74^{aC}$ | $3.57 \pm 0.06^{bB}$ | 0.069 | 0.9985 |
| | Uninjured | $7.78 \pm 0.1^{aA}$ | $0.12 \pm 0.007^{aC}$ | $55.94 \pm 0.82^{bD}$ | | $74.28 \pm 1.31^{bD}$ | $4.46 \pm 0.05^{aB}$ | 0.1464 | 0.9955 |
| PBS pH=7.3 | Total | $7.78 \pm 0.11^{aA}$ | $0.11 \pm 0.013^{aC}$ | $82.47 \pm 5.77^{aB}$ | | $102.28 \pm 5.06^{aB}$ | $2.94 \pm 0.34^{bCD}$ | 0.1012 | 0.9948 |
| | Uninjured | $7.76 \pm 0.1^{aA}$ | $0.12 \pm 0.007^{aC}$ | $62.66 \pm 2.49^{bBC}$ | | $80.54 \pm 1.56^{bC}$ | $4.26 \pm 0.08^{aB}$ | 0.089 | 0.9982 |
| BSA 1% | Total | $7.77 \pm 0.11^{aA}$ | $0.1 \pm 0.004^{bC}$ | $86.22 \pm 1.16^{aAB}$ | | $107.39 \pm 2.05^{aB}$ | $2.59 \pm 0.16^{bD}$ | 0.1176 | 0.9907 |
| | Uninjured | $7.67 \pm 0.12^{aAB}$ | $0.13 \pm 0.009^{aC}$ | $79.66 \pm 4.62^{aA}$ | | $96.62 \pm 4.2^{bB}$ | $3.71 \pm 0.3^{aCD}$ | 0.2096 | 0.9854 |
| BSA 12% | Total | $7.85 \pm 0.14^{aA}$ | $0.05 \pm 0.017^{bD}$ | $88.86 \pm 1.51^{aA}$ | | $134.57 \pm 15.59^{aA}$ | $1.25 \pm 0.43^{bE}$ | 0.0485 | 0.993 |
| | Uninjured | $7.75 \pm 0.1^{aA}$ | $0.1 \pm 0.002^{aC}$ | $83.48 \pm 5.41^{aA}$ | | $104.6 \pm 5.05^{bA}$ | $2.8 \pm 0.24^{aD}$ | 0.1838 | 0.9799 |
| Glass | Total | $7.84 \pm 0.12^{aA}$ | $0.04 \pm 0.011^{aD}$ | | $4.88 \pm 0.09^{aA}$ | $9.14 \pm 2.14^{aF}$ | $2.9 \pm 0.1^{aCD}$ | 0.0464 | 0.9987 |
| | Uninjured | $7.84 \pm 0.09^{aA}$ | $0.06 \pm 0.035^{aD}$ | | $4.95 \pm 0.05^{aA}$ | $7.44 \pm 4.18^{aF}$ | $2.94 \pm 0.14^{aD}$ | 0.1196 | 0.9919 |
| Gelatin | Total | $7.86 \pm 0.08^{aA}$ | $0.06 \pm 0.011^{aD}$ | | $4.7 \pm 0.09^{aB}$ | $25.47 \pm 6.38^{aE}$ | $3.06 \pm 0.06^{aC}$ | 0.0393 | 0.9993 |
| | Uninjured | $7.85 \pm 0.08^{aA}$ | $0.02 \pm 0.003^{bE}$ | | $3.76 \pm 0.7^{aB}$ | $3.44 \pm 0.85^{bF}$ | $3.48 \pm 0.07^{aC}$ | 0.1465 | 0.9901 |

[1] $D$ value is defined as time needed for the 1 log reduction of initial target microorganism population. [2] Log reduction is calculated from the difference between log $N_0$ to log $N$ ($n$ = 140 s). [3] RMSE stands for the root mean squared error. [a–b] Means without common superscripts in each inactivation media (NaCl, PBS, BSA or solid media) within a parameter are different ($p < 0.05$). [A–F] Means without common superscripts in each population type (total or uninjured population) within a parameter are different ($p < 0.05$).

### 3.2. Effects of NTP on Sublethal Injury

Sublethal injury (SI) is a consequence of exposure to a chemical or physical process that damages but does not kill microorganism cells [40]. Bacteria death can be induced by the effect of one or more sublethal treatments on a microorganism. Sublethal injury implies that cell membrane permeability or functional cell components of microorganisms are damaged. The degree of damage can be estimated by the difference in plate counts between non-selective and selective media as a proportion or percentage of the entire population [23]. Plasma interactions with bacteria are quite complex, the dose-dependent effects range from lethal to sublethal [34].

Figures 3 and 4 show the percentage of sublethal injury as a function of the NTP exposure time for *S. typhimurium* and *S. aureus*, respectively. The maximum value and time to reach a maximum peak of sublethal injury varied with medium state, pH, and osmolarity.

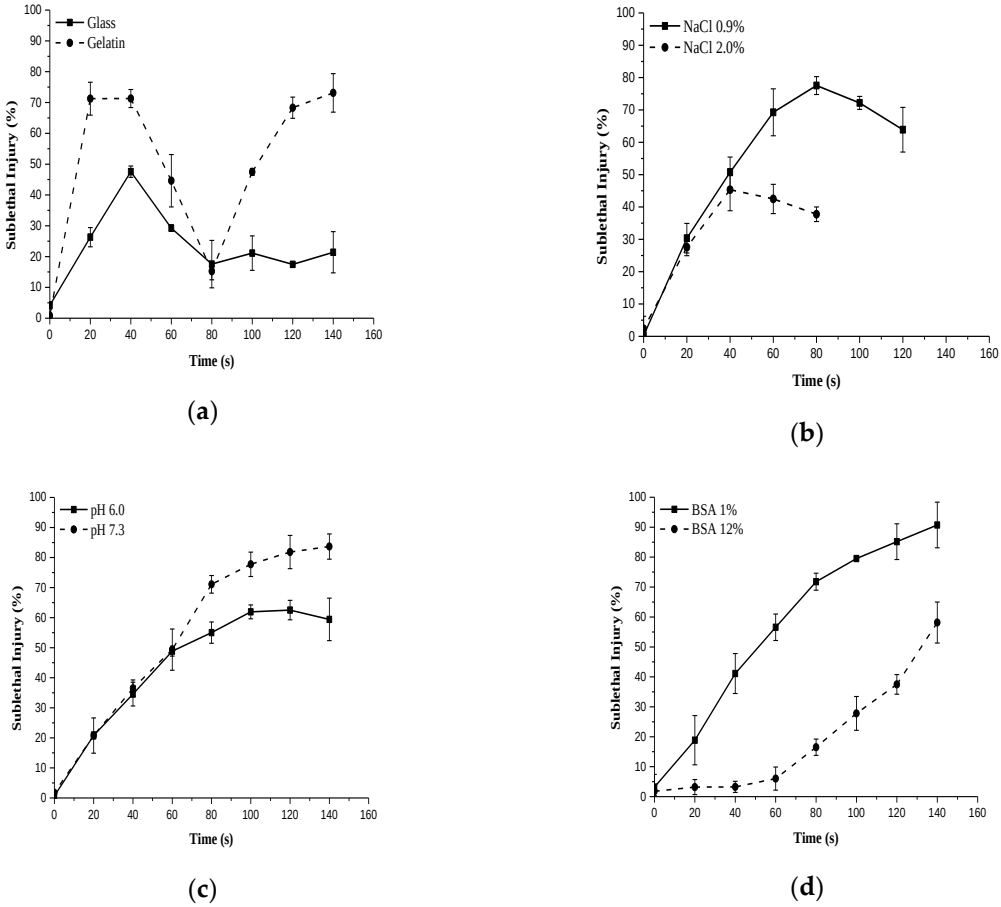


**Figure 3.** Changes in sublethal injury (%) of *S. typhimurium* during DBD-NTP treatment. Cells were inactivated on glass and gelatin surfaces (**a**) and in liquid suspensions of NaCl (**b**), PBS (**c**), and BSA (**d**).

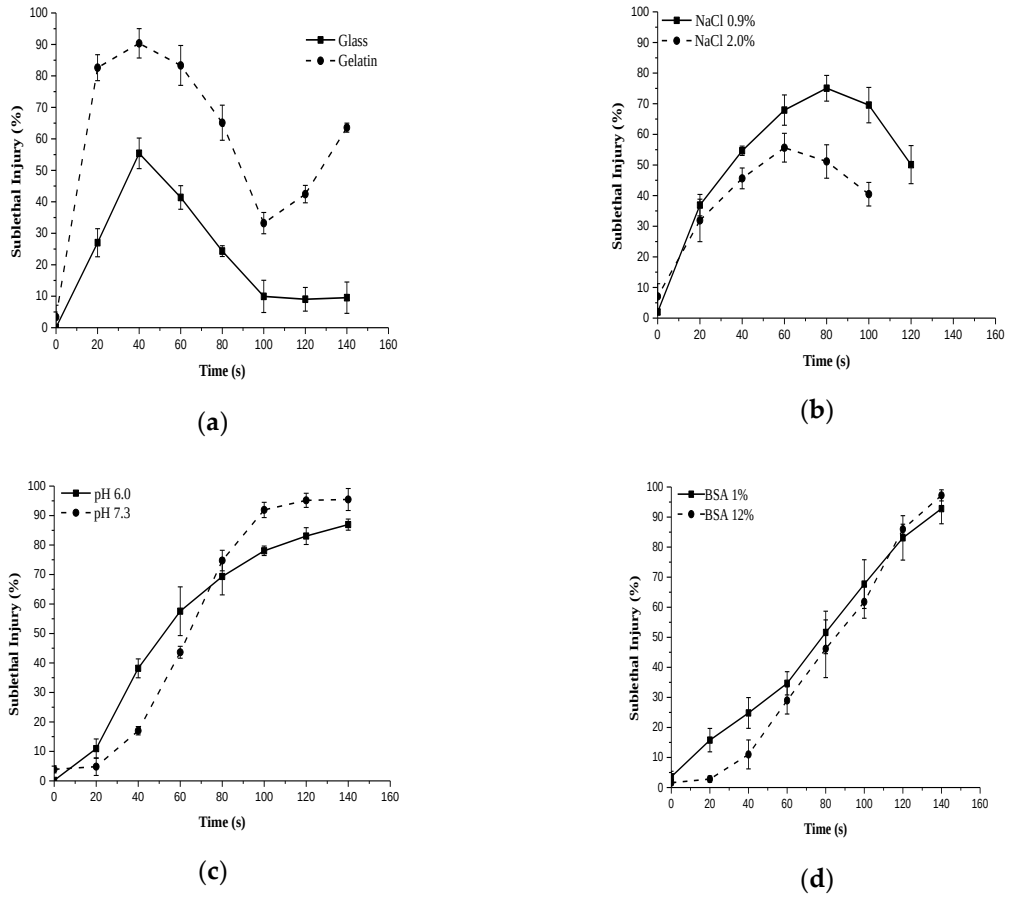


**Figure 4.** Changes in sublethal injury (%) of *S. aureus* during DBD-NTP treatment. Cells were inactivated on glass and gelatin surfaces (**a**) and in liquid suspensions of NaCl (**b**), PBS (**c**), and BSA (**d**).

The maximum value of SI of *S. aureus* was consistently higher than that of *S. typhimurium* during the whole treatment period, both on solid surfaces (glass and gelatin) and in liquid (NaCl and PBS solution) media. This maximum illustrates the phenomenon of injury accumulation finally culminating into cell death [41], and coincides to the start of a new phase in the inactivation kinetics. For example, when *S. typhimurium* was treated in 2.0% NaCl solution, SI increased rapidly with exposure time and reached the maximum value 45.35% at 40 s. Then it decreased progressively. However, in 0.9% NaCl, SI value increased along with the exposure time and reached the maximum value >70% at 80 s (Figure 3b). When *S. typhimurium* was treated in 1 % BSA phosphate buffered solution, sublethal damage increased with increasing exposure time and reached the maximum value 90.74% after 140 s. However, in 12% BSA, it increased to only approximate 55% after 140s' treatment (Figure 3d). SI values for *S. typhimurium* on gelatin plates were consistently higher than those on glass slides regardless of exposure time (Figure 3a). However, there was no difference in SI values between pH 6.0 and 7.3 bacterial suspensions in the first 60 s. After 60 s, SI values in pH 7.3 medium was higher than those in pH 6.0 medium (Figure 3c). Similar results were also noticed with *S. aureus* (Figure 4). Smet et al. [11] found that a maximum SI value was usually detected at optimal conditions or moderately stressing environmental conditions. However, our results indicate that in DBD-NTP treatment, maximum SI values for individual bacteria species could be affected by various factors.

## 4. Conclusions

In conclusion, our data demonstrate that DBD-based NTP can effectively inactivate microbial pathogens both on a solid surface and in liquid suspensions, and inactivation kinetics of *S. typhimurium* and *S. aureus* by DBD-NTP treatments on both media can be well predicted with mathematical models.

However, both the inactivation effects on microbial pathogens and sublethal injury of microbial pathogens by DBD-NTP treatments could be significantly affected by solid materials and properties of liquid suspensions. In application of DBD-NTP for inactivating solid food surface and liquid food products and extending shelf life and safety, solid materials and properties of liquids should be considered for achieving the optimal results.

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