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PAPER

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Safety evaluation of plasma-treated lettuce broth using *in vitro* and *in vivo* toxicity models

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Abstract

Cold atmospheric plasma is a promising new non-thermal technology for improving the microbiological safety and shelf-life of food products, particularly fresh produce and minimally processed fruit and vegetables. Limited research has been conducted on the safety of plasma-treated foods for human or animal consumption. This study focuses on basic safety studies by investigating lettuce broth treated with a di-electric barrier discharge plasma device as a fresh produce model in terms of *in vitro* cytotoxic and mutagenic effects on mammalian cells and its *in vivo* toxicity on *Galleria mellonella* larvae. Low cytotoxic effects were detected *in vitro* and mutagenic events were likely to be spontaneous mutations. However, a strong response of *G. mellonella* larvae to injection with plasma-treated lettuce broth was observed for 5-min-treated broth, with larvae survival of less than 10%. No significant effects on quality attributes such as colour were detected and only low concentrations of peroxide were generated in the broth. This study highlights the need for more detailed investigations into the impact of plasma treatment on food components and the subsequent *in vitro* and *in vivo* effects to ensure safe implementation of plasma technology for the processing of food products.

Keywords: cold atmospheric plasma, toxicity, food model

(Some figures may appear in colour only in the online journal)

1. Introduction

Investigation into the application of cold plasma treatment in the food sector has mainly focused on the inactivation of undesirable microorganisms and the effect on the quality of fresh produce in addition to extension of shelf-life leading to enhanced sustainability [1]. Currently there are very few data and little knowledge available about potential cytotoxic and mutagenic effects associated with plasma treatment of food products. While there have been studies conducted on disinfection efficacy and short-term cytotoxicity, improvement of the shelf-life of fresh produce and maintenance of aesthetic appeal, there have been few long-term studies into the safety of consuming cold plasma-treated produce or its direct biological application. Cold plasma treatment can bring about chemical transformations in biologically relevant solutions, such as the production of hydrogen peroxide (H_2O_2) [2] and the modification of proteins, lipids and carbohydrates [3, 4]. Unsaturated lipids, in particular, are prone to peroxidation initiated when plasma-reactive species abstract hydrogen ions from the lipid molecule; this can result in the formation of toxic by-products such as malonedialdehyde or 4-hydroxynonenal [5]. The oxidation of fatty acids in food ingredients by plasma treatment has been documented for a range of food products [6]. The formation of potential toxins or mutagens needs to be investigated to ensure safe as well as efficacious development of this novel

technology for applications in the food industry. A study of constant exposure for several weeks of a mammalian cell model to plasma-activated fetal bovine serum (FBS) as a model for a complex biological fluid did show an increased mutagenic potential [7]. Studies employing shorter exposure times to treated liquids, however, detected no increase in mutations [8]. Many plasma discharges generate nitrite in the micro- to millimolar range in the treated solution. The content of nitrite in plasma-activated water has been specifically utilized in the production of emulsion-type sausages as an alternative to curing with sodium nitrite [9]. These sausages showed comparable shelf-life at lower residual nitrite levels, suggesting that plasma functionalization of foods might represent an alternative to the use of chemical additives. When the sausages were subsequently assessed for immunotoxicity in a mouse feeding model no inflammatory response was detected and no increased mutagenic potential was found using the Ames bacterial mutagenicity test [10]; the authors concluded that plasma-treated water could be employed as a potential replacement for nitrite. The oral toxicity of plasma-treated edible films, investigated in rats, showed neither acute nor subacute toxicity, nor any impact on haematological or biochemical parameters or changes to internal organs [11]. However, overall, little assessment of the oral toxicity or general *in vivo* toxicity of plasma-treated food products and liquids has been performed to date.

Assessing the potential toxicity of plasma-treated produce is essential before general application of plasma to food products. *In vitro* toxicity tests can aid in elucidating intrinsic plasma–cell interactions, but metabolism of plasma-generated compounds, the possibility of absorption, biotransformation and distribution are not simulated in cell culture systems. It has been suggested that a robust assessment of toxicity in mammals could be made by measuring the toxicity in cell culture and in the larvae of the greater wax moth, *Galleria mellonella* [12]. Cell culture systems have been reported to overestimate the toxicity of substances and *G. mellonella* has been a reliable predictor for low-toxicity chemicals [12]. The larvae of *G. mellonella* could therefore offer a rapid and inexpensive method for screening plasma-treated products and could reduce the use of mammals in toxicity studies.

Iceberg lettuce was utilized as a plasma-treated food model in this study due to its association with food-borne pathogens which have been shown to be effectively reduced using cold plasma treatment [13]. Lettuce has an average water content of 96% and the presence of phenolics and different vitamins such as A, C, E, B1, B2 and B3 offer free radical scavenging potential. Plasma-treated lettuce broth was assessed for its cytotoxic and mutagenic potential in an *in vitro* mammalian cell model as well as for its short-term toxic effects in an *in vivo* larva model.

2. Materials and methods

2.1. Plasma system

A dielectric barrier discharge (DBD) system (DIT-120) with a maximum voltage output of 120 kV rms at 50 Hz, which is described and characterized in [14], was used. In this system, two 15 cm diameter aluminium disc electrodes are separated by a polypropylene container with wall thickness of 1.2 mm. The reactive species are generated in the air contained in the rigid polypropylene container (310 mm $\times$ 230 mm $\times$ 22 mm) that acts simultaneously as the dielectric barrier and the sample holder between the two aluminium electrodes. For maximum retention of the generated species, the sample holder was sealed in a high-barrier polypropylene bag (Cryovac, Dublin, Ireland). The time for which the plasma-treated samples remained in the sealed polypropylene bag, and were therefore further exposed to plasma-generated species, is referred to as the post-treatment storage time (PTST). A voltage of 80 kV rms and 24 h PTST was applied to plasma-treated liquid samples as this corresponded to the voltage required for microbial inactivation in liquid used in previous studies. The main chemical species produced by this DBD system were characterized by Moiseev *et al* [14] using optical absorption spectroscopy (OAS). The system was found to generate predominantly ozone at up to 5000 ppm at 70 kV rms, and this was confirmed using Gastec gas detection tubes. However, ozone concentrations were dependent on the relative humidity (RH) and decreased with increasing RH. After 24 h of PTST, ozone concentrations inside the sealed package had decreased to undetectable levels. Important amounts of NOx were also generated, particularly NO_2 and N_2O_5. Direct measurements of the reactive species generated during the treatment of the actual lettuce broth, however, were not possible due to the set-up required for the OAS measurements.

2.2. Lettuce broth model

Lettuce broth was prepared in a similar way to that described by Ziuzina *et al*, with minor modifications [15]. Iceberg lettuce was chopped, placed into stomacher bags (BA6041, Seward Ltd, UK) and homogenized in the stomacher (model BA6020, Seward Ltd) for 20 min. The lettuce juice was centrifuged (10 000 rpm for 10 min at 4 °C) twice to remove coarse particles. The supernatant was passed through paper filters (Whatman, Sigma-Aldrich, Arklow, Ireland), vacuum filtered through 0.45 μm membrane filters (Millipore, Cork, Ireland), and finally sterilized through 0.2 μm syringe filters (Millipore). The lettuce broth was separated into Petri dishes in 15 ml aliquots. Petri dishes without lids were placed in polypropylene sample holders and sealed in airtight packaging before undergoing plasma treatment for 1, 5 and 10 min at 80 kV. Samples underwent a post-treatment storage for 24 h at room temperature before undergoing filter sterilization through 0.2 μm syringe filters (Millipore). After the allocated post-treatment storage time, samples were taken for pH and peroxide analysis. Samples were stored at 4°C for the duration of the experiments.

2.3. Mammalian cytotoxicity assay

The Chinese hamster ovary cell line CHO-K1 was used for studies of cytotoxicity and mutagenicity. Cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12

(Sigma-Aldrich D6421) cell culture medium with 2 mM L-glutamine and 10% FBS at 37 °C and 5% CO_2 in a humidified incubator and passaged every 3–4 d through trypsinization. Cell concentrations and viability were assessed using trypan blue counting. For cytotoxicity assays, cells were seeded at 2.5×10^4 cells ml^{-1} in DMEM/F12 + 10% FBS supplemented with up to 10% of lettuce broth (plasma-treated or untreated control) in 96-well plates. Mammalian cell growth was assessed via crystal violet staining after 2–3 d in culture. Adherent cells were fixed with 70% methanol (Sigma-Aldrich) for 1 min. Once the methanol solution was removed the cells were stained with 0.2% crystal violet solution (Sigma-Aldrich) for 10 min. The wells were washed thoroughly with water and air dried. Adherent crystal violet was dissolved using 10% acetic acid (Sigma-Aldrich) and the absorbance was measured at 600 nm using a microplate reader (Biotek, Swindon, UK). The results were represented as a percentage of the control cells.

2.4. Mammalian mutagenicity assay

The hypoxanthine phosphorybosyl transferase (HPRT) mutation assay was used to evaluate the potential of plasma-treated solutions to induce mutations at the *hgprt* locus of Chinese hamster ovary (CHO-K1) cells. CHO-K1 cells have one functional copy of the gene that codes for the HPRT enzyme at the *hgprt* locus. The HPRT enzyme is involved in DNA synthesis. The toxic nucleoside analogue 6-thioguanine (6-TG) acts as the selective agent. Cells with a normal functioning *hgprt* are unable to grow in the presence of the toxic 6-TG but mutant cells survive and form colonies. Cells that are able to grow and form colonies in the presence of 6-TG are assumed to be mutant cells arising from spontaneous mutation or from an induced mutation at the *hgprt* locus.

To assess the long-term effects of plasma-treated biomolecules on mammalian cells, CHO-K1 cells were cultured in T25 flasks or six-well plates in DMEM/F12 medium with 10% FBS and 10% of plasma-treated lettuce broth. Cells were passaged every 3–4 d through trypsinization and reseeded at 2.5×10^4 cells ml^{-1} into fresh six-well plates over a time course of 40 d. Once a week, at the time of reseeding, cells were also plated at 1×10^4 cells ml^{-1} in round dishes (60 mm diameter) containing DMEM/F12 with 10% FBS and 10 μg ml^{-1} 6-TG as a selective agent for the HPRT mutation assay. Colony formation was evaluated after incubation for 10–14 d at 37 °C and 5% CO_2. Plates were scored as HPRT+ or HPRT− depending on the presence or absence of colonies. Colony numbers did not indicate a reliable assessment of mutation frequency due to the tendency of cells to detach from colonies and reattach forming new colonies in other areas of the plate. At the start of the 40 d culture period, the control plates were negative for colony formation and ethyl methanesulfonate (EMS) (Sigma-Aldrich) was used as a positive control to induce colony formation. Cultures were performed as three independent replicates for plasma-treated lettuce broth and the 6-TG plates were set up in triplicate from each of these replicates

2.5. In vivo *model* G. mellonella.

Sixth-instar *G. mellonella* larvae were obtained commercially from Live Foods Direct (Sheffield, UK; https://www.livefoodsdirect.co.uk/) and stored at 15 °C prior to use. Dead larvae or those with dark spots showing signs of melanization were discarded. Three groups of 10 randomly selected larvae, each weighing 0.2–0.3 g, were selected for each test condition.

2.5.1. Injection of G. mellonella *with test solutions.* Each larva was injected with 20 μl of lettuce broth using a 0.3 ml Terumo® Myjector® U-100 insulin syringe (Fisher Scientific, Dublin, Ireland) through the base of the last left proleg. Larvae were incubated at 30 °C for 24 h in Petri dishes containing wood shavings as a source of nutrition. Larvae were examined visually for viability and percentage survival was noted. Larvae were considered dead if they were not moving, failed to reorient themselves if placed on their backs or failed to respond to stimuli.

2.5.2. Microscopic assessment of G. mellonella *haemocytes.* After 24 h incubation at 30 °C, haemolymph was drained from five larvae in each group by piercing the anterior region and draining into chilled 1.5 ml microfuge tubes, which were kept on ice to prevent melanization of the haemolymph. All samples were diluted by adding 100 μl of haemolymph to 900 μl of ice-cold phosphate-buffered saline, and for each extract concentration haemocytes were enumerated microscopically using a haemocytometer and compared with haemolymph samples of control larvae which were injected with 20 μl of sterile water.

2.6. Peroxide quantification using potassium iodide

The quantification of peroxide was modified from a method used by Loreto and Velikova, 2001 [16] for measurement of H_2O_2 in plant tissues. To determine the generation of peroxides under different plasma process parameters, the spectrophotometric measurement of the oxidation of iodide (I^-) to yellow iodine was performed using a microplate reader (Biotek) and compared with a calibration curve using dilutions of H_2O_2. A linear function was obtained for concentrations <0.005% H_2O_2. Samples of 50 μl were incubated with 50 μl of a 10 mM phosphate buffer solution (pH 7) and 100 μl 1 M potassium iodide (KI) (Sigma-Aldrich) at room temperature for 20°min and the absorbance was read at 390 nm.

2.7. Nitrite and nitrate measurements

Nitrite analysis was performed using the Griess reagent and nitrate concentrations were determined spectrophotometrically with dimethylphenol.

2.8. Statistical analysis

Statistical analysis was performed using Prism software (GraphPad). Comparisons between two groups were analysed

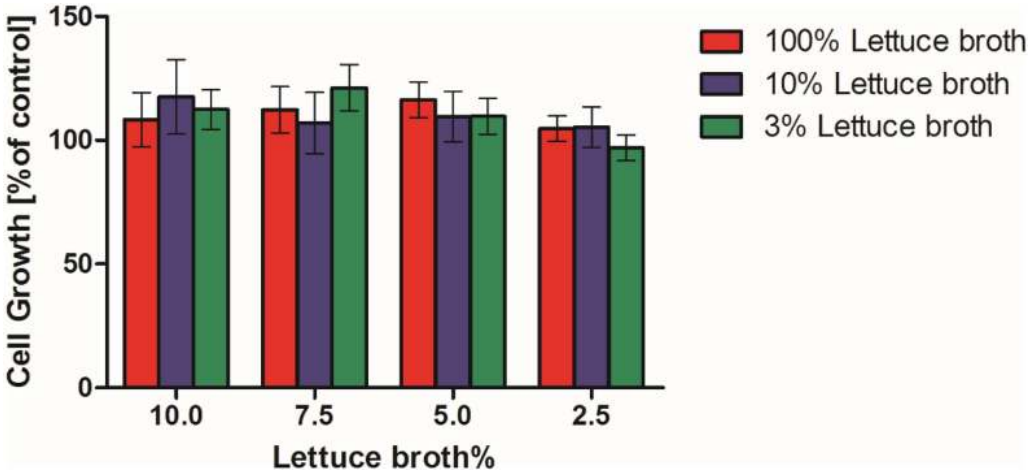


Figure 1. Cell growth with different percentages of lettuce broth cultured with CHO-K1 cells at 2.5%–10% v/v.

by Student's *t*-test and comparisons between more than two groups were analysed by one-way analysis of variance (ANOVA). ANOVA and Tukey´s post hoc-test were used to calculate the statistical significance of the samples referring to the negative untreated control.

3. Results

3.1. Cytotoxic effects of plasma-treated lettuce broth

Lettuce is a leafy vegetable containing antioxidants such as polyphenols and vitamins and has been used in studies of plasma-treated fresh produce with regard to the extension of shelf-life and inactivation of food-borne pathogens [15, 17, 18]. Lettuce broth was developed as a food model to assess the biological impact of plasma-treated fresh produce *in vitro* and *in vivo*.

Cell growth of CHO-K1 cells was assessed using DMEM/F12 supplemented with prepared lettuce broth at different dilutions in water (3%, 10%, 100%) and volume percentages. The cell growth of CHO cells was unaffected when the cell culture medium was supplemented with up to 10% v/v of undiluted non-treated lettuce broth, showing that the lettuce broth itself did not adversely affect the cells (figure 1).

Lettuce broth was prepared and filter sterilized before being treated with plasma for up to 5 min and stored at room temperature in the plasma-treated package for 24 h to ensure maximum retention of plasma-generated species. The lettuce broth was filter sterilized again and used to supplement the DMEM/F12 at 10% v/v. Cell growth in the presence of 10% v/v lettuce broth plasma treated for 1 min decreased to 74% compared with the untreated control (figure 2). CHO cell growth remained stable after the initial decrease with cell growth of 68% compared with the untreated control when supplemented with 10% v/v of lettuce broth subjected to an extended plasma treatment time of 10 min (figure 2).

3.2. Mutagenic potential of plasma-treated lettuce broth

The long-term effect of plasma-treated lettuce broth was evaluated using the HPRT assay in CHO-K1 cells over a

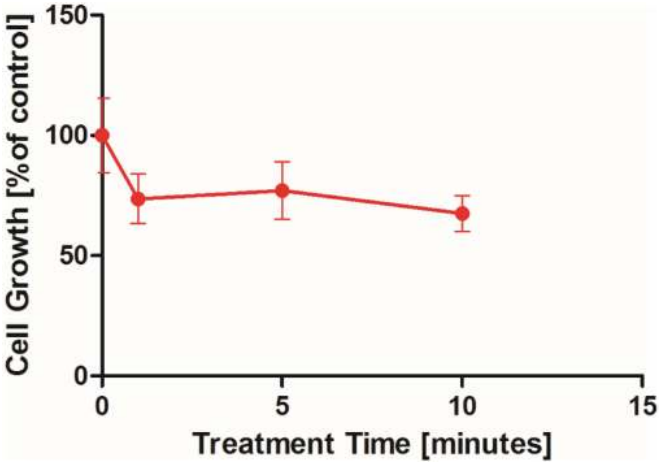


Figure 2. Cell growth of CHO-K1 cells cultured with plasma-treated lettuce broth at 10% v/v.

period of 40 d in culture. Cells were continuously exposed to culture medium freshly supplemented with lettuce broth at every passaging (every 3–4 d), thereby presenting an assessment of long-term exposure. No mutagenic events were noted for 20 d of culture in the presence of lettuce broth. On day 27 of culture, two out of three cultures in one triplicate for the lettuce broth treated with plasma for 1 min were positive for colony growth in the HPRT assay (table 1). On day 27 of culture there were also three out of three cultures in one triplicate positive for colony growth in the lettuce broth treated with plasma for 10 min. On day 34 of culture, one out of three in one triplicate lettuce broth treated for 5 min was positive for HPRT colony growth. These positive cultures did not reappear in the subsequent subcultures, indicating the possibility of spontaneous mutations.

3.3. In vivo safety assessment of plasma-treated food model

The next evaluation for plasma-treated lettuce broth was the *in vivo* model system using the larvae of *G. mellonella* exposed to lettuce broth via injection. *Galleria mellonella* larvae have been used to assess the toxicity of different compounds, and

Table 1. Rate of HPRT-positive colonies in CHO-K1 cells cultured with 10% v/v of plasma-treated lettuce broth. (−) indicates no colonies were observed; (+) indicates colonies were observed.

		Days in culture						
Lettuce broth		0	6	13	20	27	34	40
Control (untreated)	A	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
	B	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
	C	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
1 min	A	nd	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
	B	nd	−/−/−	−/−/−	−/−/−	−/+/+	−/−/−	−/−/−
	C	nd	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
5 min	A	nd	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
	B	nd	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
	C	nd	−/−/−	−/−/−	−/−/−	−/−/−	−/−/ +	−/−/−
10 min	A	nd	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
	B	nd	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
	C	nd	−/−/−	−/−/−	−/−/−	+/+/+	−/−/−	−/−/−

Figure 3. *Galleria mellonella* larvae before treatment with plasma-treated lettuce broth.

offer an inexpensive method to rapidly screen multiple compounds that does not require ethical approval [19].

Figure 3 shows healthy *G. mellonella* larvae prior to the introduction of plasma-treated lettuce broth. Following incubation for 24 h after intra-haemocoelic injection, larval survival was assessed under previously described conditions (figure 4). In the control group that was injected with untreated lettuce broth, three larvae were dead, giving 90% survival (figure 5). There was 100% survival within the group that received lettuce broth treated with plasma for 1 min. In the group that received lettuce broth that was treated with plasma for 5 min, survival was less than 7% and the group that received lettuce broth treated for 10 min showed 50% survival (figure 5). Melanization, which leads to the larvae changing from cream to brown/black, is a common component of the insect immune response occurring as a result of stress or infection [19], and is clearly visible in dead larvae in figure 4. The haemocyte density remained the same among the surviving larvae injected with plasma-treated lettuce broth and the untreated control (figure 6), suggesting that the administration of plasma-treated lettuce broth did not cause adverse stress to the surviving *G. mellonella* larvae and did not elicit an immune response

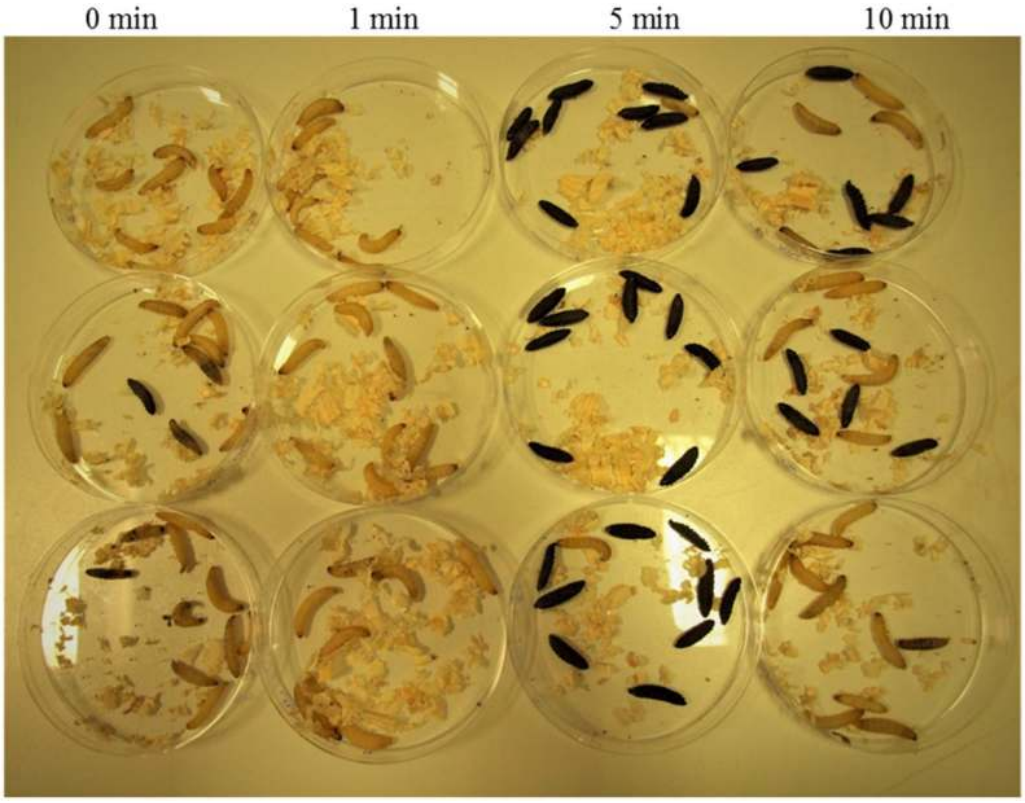


Figure 4. *Galleria mellonella* larvae 24 h after treatment with plasma-treated lettuce broth, showing (from left to right) larvae injected with lettuce broth treated with plasma for 0, 1, 5 and 10 min, respectively.

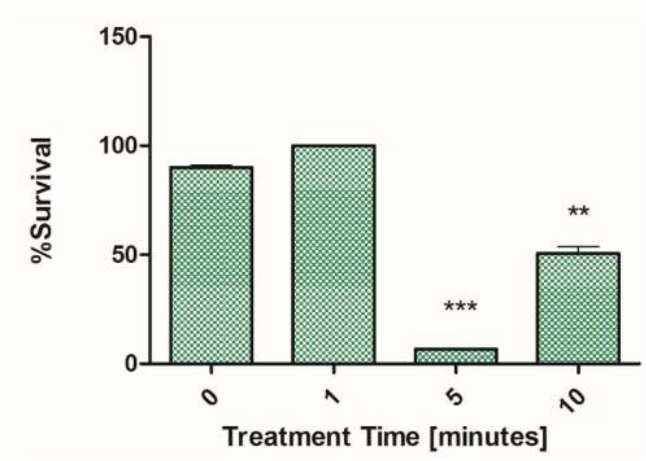


Figure 5. Survival of *G. mellonella* administered with 20 μl of plasma-treated lettuce broth (***$p < 0.001$; **$p < 0.01$ compared with controls injected with untreated lettuce broth).

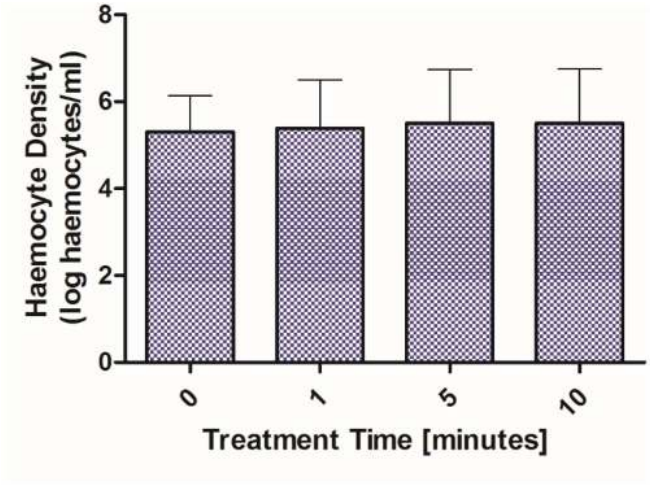


Figure 6. Effect of plasma-treated lettuce broth on the production of haemocytes by *G. mellonella.* Results represent haemocyte counts obtained from combining haemolymph of surviving larvae of each test group and averaging (no significance observed).

in these larvae. The haemocytes of dead larvae could not be analysed, but could offer insights into the induction of stress-related proteins.

3.4. Reactive species quantification in a plasma-treated lettuce broth

Quantification of peroxide levels in plasma-treated liquids presents a rapid technique to assess plasma reactivity and possible biological effects [7]. The liquid environment in contact with cold plasma and the gas used to produce plasma influences the generated plasma chemistry [20]. Although lettuce contains over 90% water, and there is a time-dependent increase in peroxide generation, comparatively low levels were generated, with just over 60 μmol l^{-1} measured in lettuce broth subjected to 10 min of plasma treatment (figure 7).

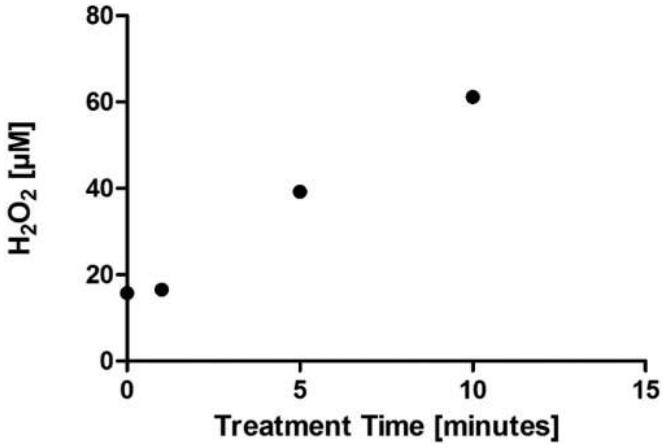


Figure 7. Quantification of peroxide in plasma-treated lettuce broth.

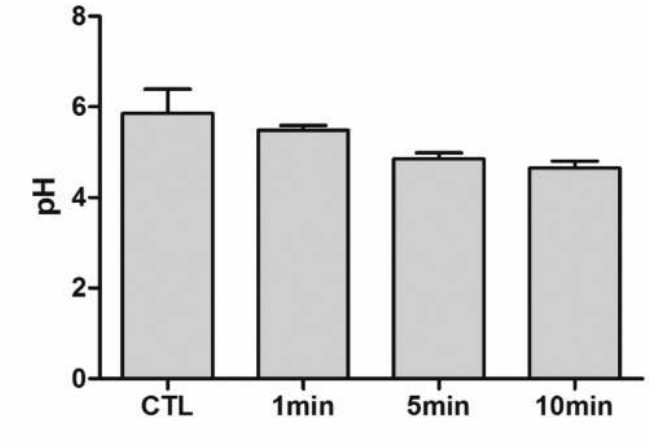


Figure 9. Measurement of pH in plasma-treated lettuce broth.

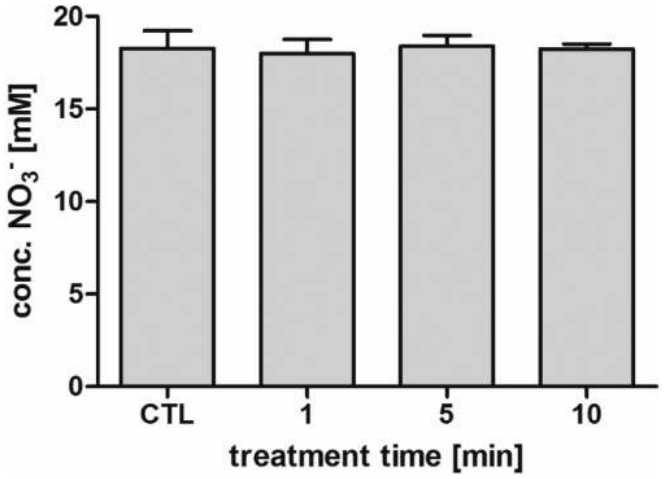


Figure 8. Quantification of nitrate in plasma-treated lettuce broth using the dimethylphenol method.

Less than 20 μM of nitrite was detected in any of the lettuce broth samples, and there was no increase of nitrite with plasma treatment. This is in line with previous findings for this plasma system, where no detectable amounts of nitrite were generated in water during plasma treatment [21, 22].

The nitrate content was analysed using dimethylphenol, and approximately 18 mM or 1.5 g l^{-1} of nitrate was found in the lettuce broth. Concentrations did not change significantly with plasma treatment (figure 8).

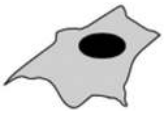
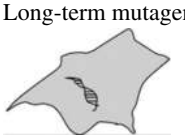
The acidification of solutions through plasma treatment is apparent by a decrease in pH, as can be seen in figure 9. However, a certain buffering capacity of the lettuce broth is evidenced by the much lower decrease in pH compared with treatment of water in the same set-up, where values decreased to below 3 [22].

4. Discussion

There are reports of chemical modification to food induced by plasma treatment, including oxidation of sugars to organic acids, alterations of amino acids and loss of structure in proteins [23]. Assessing the potential toxicity of plasma-treated produce is essential before the general application of plasma to food products. *In vitro* toxicity tests can aid in elucidating intrinsic plasma–cell interactions, but the metabolism of plasma-generated compounds and the possibility of absorption, biotransformation and distribution are not simulated in cell culture systems. Three different toxicity models were therefore employed in this study: the short-term *in vitro* mammalian cytotoxicity model, the long-term *in vitro* genotoxicity model, both using CHO-K1 cells, and the acute *in vivo* toxicity model using *G. mellonella* larvae, which showed very different results (table 2).

The results of short-term mammalian cytotoxicity assays presented here showed stable cell growth at 80% of the untreated control in the presence of lettuce broth treated with plasma for up to 10 min, indicating no acute toxicity of the broth on the cells *in vitro*. This is in contrast to the effects of plasma-activated water and plasma-activated FBS treated with the same plasma device and set-up which in other studies resulted in reduction of cell growth by nearly 90% in the same mammalian cell line [7, 24]. Cytotoxicity of those liquids was found to be mainly mediated by the concentrations of H_2O_2 in agreement with findings by others. There was a plasma treatment time-dependent increase in peroxide measurement of lettuce broth, but after 10 min treatment it was less than 80 μmol l^{-1}, significantly less than concentrations found in plasma-treated water which resulted in reduced cell growth in a previous study [24]. Over a culture period of 40 d, three separate incidents of positive colonies in the HPRT mutagenicity assay occurred for each plasma treatment time. However, these positive colonies did not reappear in subsequent subcultures, indicating spontaneous mutations that may not be directly attributable to the plasma-treated lettuce broth. When plasma-treated lettuce broth was administered to *G. mellonella* larvae there was a clear difference in survival rates. Of the larvae that were administered lettuce broth treated with plasma for 5 min, fewer than 10% survived after 24 h, compared with 100% with lettuce broth treated for 1 min and 50% with lettuce

Table 2. Models and parameters used to assess the toxicity of plasma-treated lettuce broth.

	In vitro		*In vivo*
	Short-term cytotoxicity study 	Long-term mutagenicity study 	Short-term insect toxicity study 
Test subject organism	Chinese hamster ovary cell line (CHO-K1)	Chinese hamster ovary cell line (CHO-K1)	*G. mellonella* larvae
Test subject sample size	2.5×10^4 cells ml^{-1} in 96-well plate; in triplicate	2.5×10^4 cells ml^{-1} in 6-well plates; in triplicate	10 larvae per condition; in triplicate
Test material	Plasma-treated lettuce broth (1, 5, 10 min)		
Application of test material	10 μl in 100 μl cell culture medium	200 μl in 2 ml cell culture medium	20 μl intra-haemocell injection
Exposure duration	3 d	6–40 d	1 d
Effect	Reduction of cell growth by 20% in all plasma-treated samples, no dependence on treatment time	Spontaneous mutations at single time points	Acute toxicity of 5 min treated broth = 90%; 10 min treated broth = 50%

broth treated for 10 min. Some chemicals may not be directly toxic to a cell but can be converted into other toxins through cell metabolism, especially via the cytochrome P450 enzymes which have been suggested to be similar in insects and mammals [25] but could cause differences in response between *in vitro* and *in vivo* models. The mode of administration of the plasma-treated solution differed between the *in vitro* cell-based assays and the *in vivo* insect model. Lettuce broth was introduced to the extracellular environment by supplementation of the cell culture medium at 10% (v/v), resulting in dilution and buffering by the cell culture medium as well as potential interactions with medium components. Administration to the larvae was performed through direct injection of the undiluted broth into the proleg, whereby the broth is diluted into the insect's haemolymph and interactions with haemolymph components are possible. Plasma-treated lettuce broth contains increased concentrations of H_2O_2 and reduced pH compared with the untreated control. However, control experiments using pure H_2O_2 showed the LD50 (lethal dose) to be 675 μM, far in excess of the 60 μM generated in the lettuce broth (results submitted for publication). Studies by Suay-García and co-workers [26] have shown *G. mellonella* larvae to be unaffected by injection of 10%, 20% and 30% acetic acid and HCl at 0.5 M, 1 M and 2.5 M, whereas the pH of the treated lettuce broth did not fall below 4.5 and was therefore only mildly acidic. Therefore neither reduced pH nor increased H_2O_2 are believed to be primary causes of larval death. Maguire *et al* investigated the effects of food preservatives on *G. mellonella* and determined the LD50 for potassium and sodium nitrite to be 0.09–0.10 M (2×10^{-6} mol per/larva) and for sodium nitrate and potassium nitrate 0.66 M and 0.89 M (1.3 and 1.8 $\times$ 10^{-5} mol per larva), respectively [27–29]. Nitrate concentrations determined in the lettuce broth samples were around 0.018 M in all samples (3.6×10^{-7} mol applied per larva) and therefore almost 50-fold lower than the LD50 values. The nitrate content of leafy greens such as iceberg lettuce varies with variety, location and season, with average concentrations around 700–900 mg kg^{-1} of produce [30]. Nitrate concentrations generated by the same plasma device in water were on average around 0.5 mM after 5 min and 1 mM after 10 min of treatment (50 and 100 mg l^{-1}) [22] and therefore would affect total nitrate no more than the overall variance between lettuce samples. Reactive species concentrations characterized for this plasma system set-up therefore cannot explain the distinct difference in viability of larvae exposed to lettuce broth treated for 5 versus 10 min. Although the group that was administered with the 5 min plasma-treated lettuce broth did not tolerate it, the surviving larvae did not exhibit elevated haemocyte densities, which are indicators of a cellular immune response [31]. Potassium nitrate administered at toxic concentrations in the work by Maguire resulted in increased numbers of circulating haemocytes [29]. In the present study no such haemocyte stimulation could be observed, suggesting that an immune response was not triggered prior to cell death or that only certain larvae responded to the lettuce broth with a stress response ultimately leading to death while others were unaffected. The results observed here suggest that toxic intermediates are generated at 5 min plasma treatment, which are themselves further degraded by extending the treatment time to 10 min, resulting in a recovery of viability. These results are in line with previous work by Boehm *et al* that described elevated mutagenicity in FBS treated with plasma for 5 min [7] compared with 10 min treatment. The observed mutagenic potential was attributed

to the possibility of chemical modifications to plasma-treated FBS and the generation of H_2O_2. The peroxide measurements in plasma-treated lettuce broth, however, were comparatively lower. The generation of degradation products more toxic to cells was also reported for antibiotics treated with DBD plasma, with increased antimicrobial activity being observed after the plasma exposure [32]. Other studies have shown various peaks of appearance of by-products, whose intensity increased with time and which again disappeared with further increase in plasma doses [33]. We therefore propose that the plasma treatment initially decomposed certain components of the lettuce broth to intermediates, which may be toxic in contrast to their parent compound, when metabolized, for example by the P450 system, or were more reactive and therefore damaging to the organism. Further plasma treatment then caused further degradation of these intermediates and could eventually lead to complete mineralization of the compound. *Galleria mellonella* larvae accumulate a large fat body, which may constitute 50% of the fresh weight of the insect [34] and serves as an energy reserve to support its metamorphosis and to provide energy for the newly emerged adult [35]. Unsaturated lipids are particularly prone to modification by reactive species, resulting in chain-reaction lipid peroxidation of double bonds. It is therefore possible that the high fat content of the larvae makes these more susceptible to reactive intermediates.

A study examining the effect of plasma treatment on selected flavonoids and phenolic acids due to their antioxidant capabilities used lettuce as a model because of its well-elaborated chemical composition [36]. This study showed a time-dependent degradation of selected antioxidant molecules due to the scavenging of plasma-generated radicals. However, another study reported no effect on the physicochemical properties of plasma-treated lettuce samples [18]. Studies have reported plasma-induced modifications to biomolecules including lipids [37], proteins [3] and amino acids [38, 39]. Plasma-treated products should therefore be examined to assess physical or biochemical modifications of their components and subsequent effects when administered to a biological system. With a water content of 95% in lettuce, potential targets for plasma-induced modifications such as lipids and proteins are at relatively low concentrations (around 1% w/v) in lettuce broth, and the potency of the hypothetical toxic intermediate must be sufficiently strong. Further detailed chemical analyses of the broth after different treatment times are required and separation/fractionation methods could then be employed in an attempt to isolate and identify the toxic components. A model system with controllable composition and biomolecule concentrations may provide clearer insights into the pathways of plasma-induced modifications and their safety implications. The plasma device and the mechanisms and chemistry mediated by a particular set-up are specific to that set-up. Therefore a comparison using model systems is warranted using a range of tunable cold plasma devices.

There is little information on the interaction of plasma-generated species with secondary metabolites in the food matrix [36]. It has been suggested that the generation of electrons, ions, photons of different wavelengths and chemically reactive species in plasma-treated foodstuffs may open reaction pathways different from otherwise endothermic or kinetically hindered reactions, possibly leading to the generation of new intermediates or secondary reaction products [36]. These potential interactions must be elucidated and biologically assessed before cold plasma is established as a mild and safe alternative to conventional food processing technologies.

5. Conclusion

The *in vivo* toxicity observed in plasma-treated lettuce broth after 5 min plasma treatment is in stark contrast to the *in vitro* tests. Further analysis is required on possible secondary reaction products generated by plasma treatment which possess adverse effects that may be further degraded by extending plasma treatment. The discrepancies observed in the biological effects of plasma-treated produce also indicate the importance of assessing plasma-treated products both in isolation and in the context of a biological system and highlight the need for more detailed and relevant exposure studies and standardized assessment procedures.

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