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Authors(s)	Heslin, Caitlin; Boehm, Daniela; Gilmore, Brendan F.; Megaw, Julianne; Bourke, Paula
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Safety evaluation of plasma-treated lettuce broth using in vitro and in vivo toxicity models

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

Caitlin Heslin[1], Daniela Boehm[1,*], Brendan F. Gilmore[2], Julianne Megaw[2], and Paula Bourke[1,3,4]

[1] School of Food Science and Environmental Health, Technological University Dublin, Dublin, Ireland
[2] School of Pharmacy, Queens University Belfast, Belfast, Northern Ireland, United Kingdom
[3] School of Biological Sciences, Queens University Belfast, Belfast, Northern Ireland, United Kingdom
[3] Plasma Research Group, School of Biosystems and Food Engineering, University College Dublin, Dublin 4, Ireland

*corresponding author: daniela.boehm@tudublin.ie

Abstract

Cold atmospheric plasma is a promising new non-thermal technology for improving 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 focusses 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 *Galleria* larvae to injection with plasma-treated lettuce broth was observed for 5-min treated broth, with less than 10% larvae survival. 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 on the impact of plasma treatment on food components and the subsequent *in vitro* and *in vivo* effects to ensure a safe implementation of plasma technology for processing of food products.

Keywords: cold atmospheric plasma, toxicity, food model

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 quality of fresh produce in addition to shelf life extension leading to enhanced sustainability [1]. Currently there is very scarce data and knowledge available on potential cytotoxic and mutagenic effects associated with plasma treatment of food products. While there have been studies conducted on the disinfection efficacy and short-term cytotoxicity, improvement of shelf life of fresh produce and maintenance of aesthetic appeal, there have been few long-term studies into the safety considerations on the consumption of 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 [2], 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 and which can result in the formation of toxic by-products such as malonedialdehyde or 4-hydroxynonenal [5]. The oxidation of fatty acids of 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 of several weeks of a mammalian cell model to plasma activated foetal bovine serum as a model for a complex biological fluid did show an increased mutagenic potential [7]. Studies employing shorter exposure times of treated liquids, however, detected no increase in mutations [8]. Many plasma discharges generate nitrite in the micro - millimolar range in the treated solution. The content of nitrite in plasma activated water (PAW) has been specifically utilized in the production of emulsion-type sausages as an alternative curing agent to sodium nitrite [9]. These sausages showed comparable shelf-life at lower residual nitrite levels, suggesting that plasma

functionalizing 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] and the authors concluded that plasma treated water could be employed as a potential nitrite replacer. The oral toxicity of plasma-treated edible films investigated in rats showed neither acute nor sub-acute toxicity, nor impacts 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 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 inexpensive method to screen 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% 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 short-term toxic effects in an *in vivo* larvae 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 was used which has been described and characterised [14]. In this system, two 15 cm diameter aluminium disk 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 x 230 x 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 duration that the plasma treated samples remained in the sealed polypropylene bag and therefore further exposed to plasma generated species is referred to as post-treatment storage time (PTST). A voltage of 80 kV rms and 24h PTST was applied to plasma-treated liquid samples as it corresponded with 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 5000ppm at 70kV rms, which was confirmed using Gastec gas detection tubes. However, O_3 concentrations were dependant on the relative humidity (RH) and decreased with increasing RH. After 24h 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 similar to the method described by Ziuzina *et al.*, with minor modifications [15]. Iceberg lettuce was chopped, placed into stomacher bags (BA6041, Seward

LTD, UK) and homogenised in the stomacher (Model: BA6020, Seward LTD, UK) for 20 minutes. The lettuce juice was centrifuged (10,000 rpm for 10 minutes 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, Cork, Ireland). 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 minutes at 80 kV. Samples underwent a post-treatment storage time of 24 hours at room temperature before undergoing filter sterilization through 0.2 µm syringe filters (Millipore, Cork, Ireland). After the allocated post-treatment storage time, samples were taken for pH and peroxide analysis and 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 cytotoxicity and mutagenicity studies. Cells were grown 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 days through trypsinization. Cell concentrations and viability were assessed using trypan blue counting. For cytotoxicity assays, cells were seeded at $2.5x10^4$ cells/ml 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 days in culture. Adherent cells were fixed with 70% methanol (Sigma-Aldrich, Arklow, Ireland) for one minute. Once the methanol solution was removed the cells were stained with 0.2% crystal violet solution (Sigma-Aldrich, Arklow, Ireland) for ten minutes. The wells were washed thoroughly with water and air dried. Adherent crystal violet was dissolved using 10% acetic acid (Sigma-Aldrich, Arklow, Ireland) and the absorbance was

measured at 600nm using a microplate reader (Biotek, Swindon, UK). The results were represented as a percentage of the control cells.

2.4 Mammalian mutagenicity assay

The 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 while 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 6-well plates in DMEM/F12 medium with 10% FBS and 10% of plasma treated lettuce broth. Cells were passaged every 3-4 days through trypsinization and reseeded at 2.5×10^4 cells/ml into fresh 6-well plates over a time course of 40 days. Once a week at the time of reseeding, cells were also plated at 1×10^4 cells/ml in round dishes (60mm diameter) containing DMEM/F12 with 10% FBS and 10µg/ml 6-TG as a selective agent for the HPRT mutation assay. Colony formation was evaluated after 10-14 days incubation 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-day culture period, the control plates were negative for colony formation and ethyl methanesulfonate (EMS) (Sigma-Aldrich, Arklow, Ireland) was used as a positive control to induce colony formation. Cultures were

performed as 3 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 *Galleria mellonella*

Sixth-instar *Galleria mellonella* larvae were obtained commercially from livefoodsdirect.co.uk and stored at 15°C prior to use. Dead larvae or those with dark spots showing signs of melanisation were discarded. Three groups of ten 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 in petri dishes containing wood shavings as a source of nutrition, at 30°C for 24 hours. Larvae were examined visually for viability and percentage survival was noted. Larvae were considered dead if they were unmoving, failing 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 hours 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 melanisation of the haemolymph. All samples were diluted by adding 100 µl haemolymph to 900 µl ice-cold PBS and for each extract concentration, haemocytes were enumerated microscopically using a haemocytometer, and compared to haemolymph samples of control larvae which were injected with 20 µl of sterile H_2O.

2.6 Peroxide Quantification using Potassium Iodide

The quantification of peroxide was modified from a method used in Loreto and Velikova, 2001 [16] for H_2O_2 measurement 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, Swindon, UK) and compared to calibration curve using dilutions of H_2O_2. A linear function was obtained for concentrations <0.005% H_2O_2. 50 µl sample were incubated with 50 µl of a 10 mM phosphate buffer solution (pH 7) and 100 µl 1M potassium iodide (KI) (Sigma-Aldrich, Arklow, Ireland) at room temperature for twenty minutes 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 (DMP).

2.8 Statistical analysis

Statistical analysis was performed using Prism software (GraphPad). Comparisons between two groups were analysed by Student's t test and comparisons between more than two groups were analysed by ANOVA. One-way analysis of variances (ANOVA) and Tukey´s post hoc-test were used to calculate 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 examining plasma-treated fresh produce investigating 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 supplementing the cell culture media 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 sterilised before being treated with plasma for up to 5 minutes and stored at room temperature in the plasma treated package for 24 hours to ensure maximum retention of plasma-generated species. The lettuce broth was filter sterilised 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 minute decreased to 74% compared to the untreated control, Figure 2. CHO cell growth remained stable after the initial decrease with a cell growth of 68% compared to the untreated control when supplemented with 10% v/v of the lettuce broth subjected to an extended plasma treatment time of 10 minutes, Figure 2.

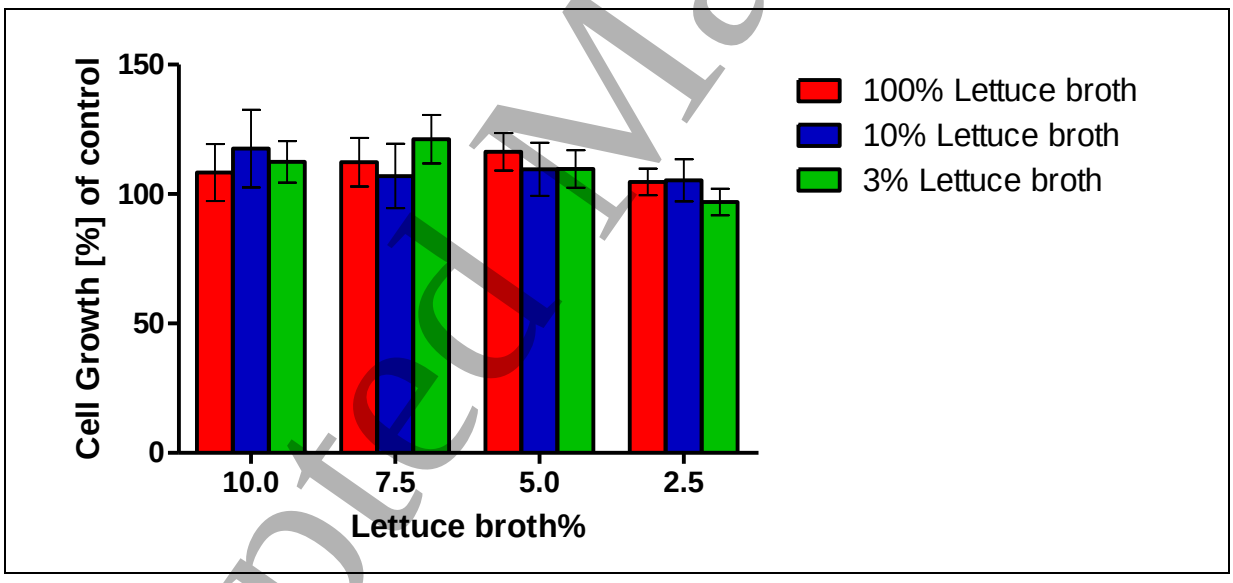

Figure 1: Cell growth with different percentages of lettuce broth cultured with CHO-K1 cells at 2.5-10% v/v.

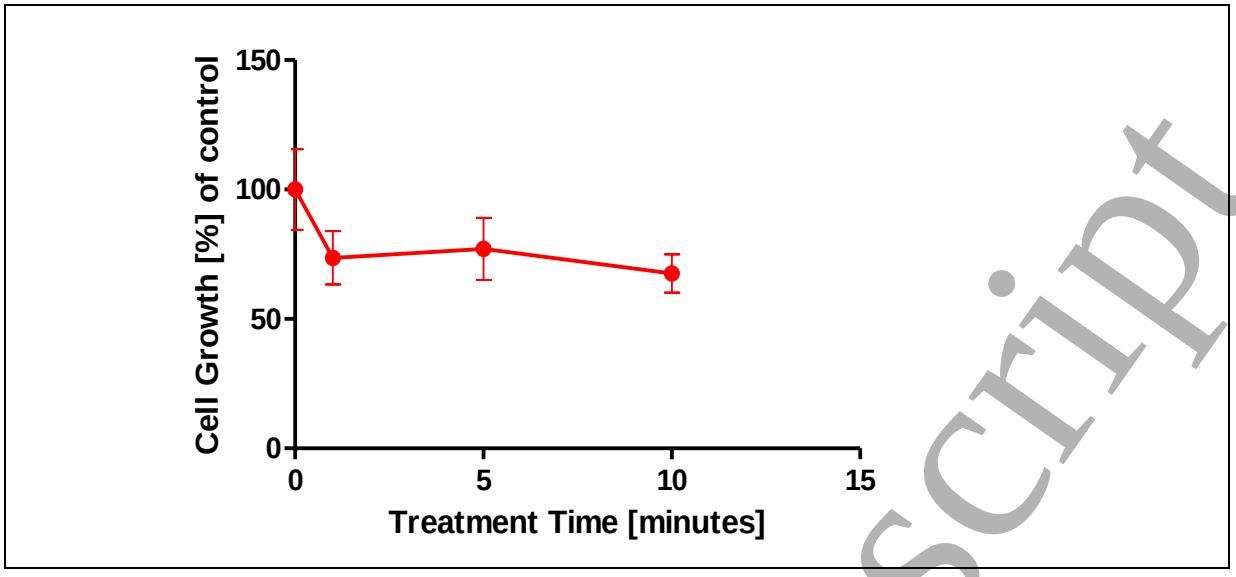

Figure 2: Cell growth of CHO-K1 cells cultured with plasma treated lettuce broth at 10% v/v.

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 period of 40 days in culture. Cells were continuously exposed to culture medium freshly supplemented with lettuce broth at every passaging (every 3-4 days), thereby presenting an assessment of long-term exposure. No mutagenic events were noted for 20 days of culture in the presence of lettuce broth. On Day 27 of culture, 2 out of 3 cultures in one triplicate for the lettuce broth treated with plasma for 1 minute were positive for colony growth in the HPRT assay, Table 1. On Day 27 of culture, there were also 3 out of 3 cultures in one triplicate positive for colony growth in the lettuce broth treated with plasma for 10 minutes. On Day 34 of culture, 1 out of 3 in one triplicate lettuce broth treated for 5 minutes was positive for HPRT colony growth. These positive cultures did not reappear in the subsequent subcultures indicating the possibility of spontaneous mutations.

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

Lettuce Broth		Days in Culture						
		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	-/-/-	-/-/-	-/-/-	+/+/+	-/-/-	-/-/-

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 the Greater Wax Moth, *Galleria mellonella*, exposed to lettuce broth via injection. The larvae of *G. mellonella* have been used to assess the toxicity of different compounds and offer an inexpensive, ethical approval-free method to rapidly screen multiple compounds [19]. Figure 3 shows healthy larvae of *G. mellonella* prior to the introduction of plasma treated lettuce broth. After 24 hours incubation post intra-haemocoelic injection, larvae survival was assessed under previously described conditions, Figure 4. In the control group that was injected with untreated lettuce broth, 3 larvae were dead leaving a 90% survival, Figure 5. There was 100% survival within the group that received lettuce broth treated with plasma for 1 minute. In

the group that received lettuce broth that was treated with plasma for 5 minutes, there was less than 7% survival and the group that received lettuce broth treated for 10 minutes had a 50% survival, Figure 5. Melanisation, 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* and did not elicit an immune response in these larvae. The haemocytes of dead larvae could not be analysed but could offer insights into the induction of stress related proteins.

Figure 3: G. mellonella before treatment with plasma treated lettuce broth.

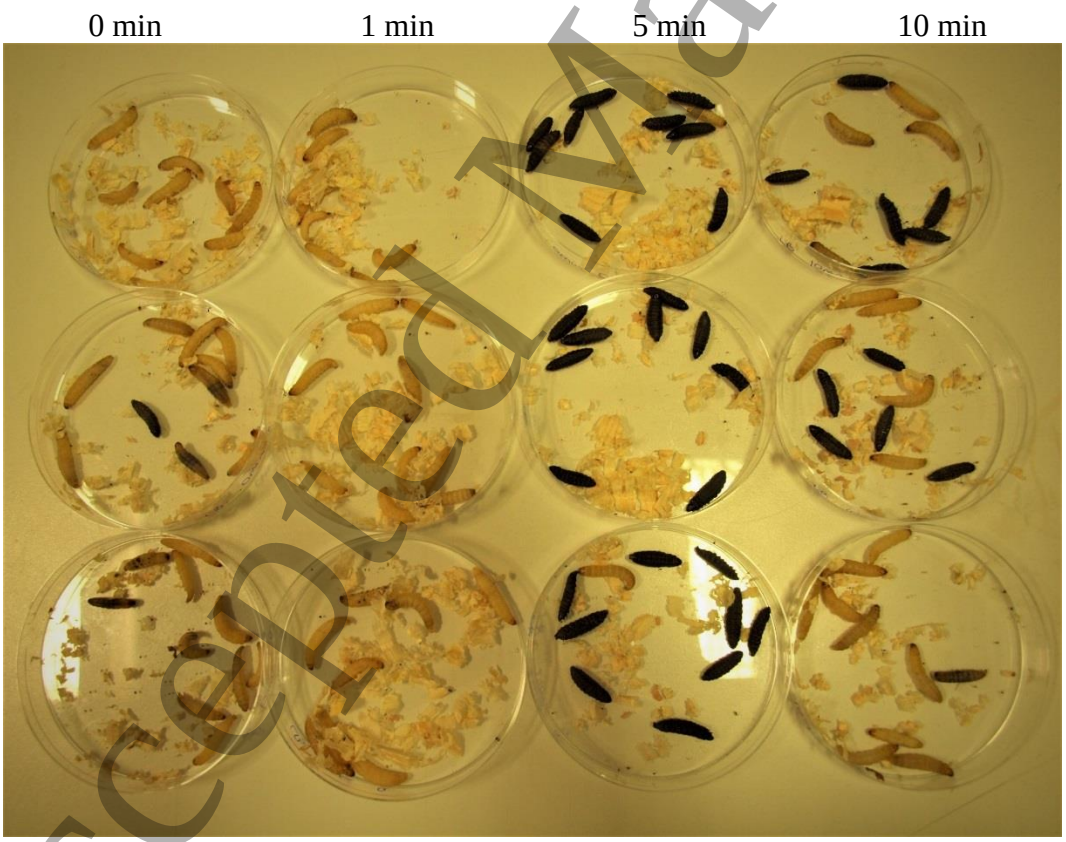

Figure 4: G. mellonella 24 hours after treatment with plasma treated lettuce broth, showing from left to right larvae injected with lettuce broth treated with plasma for 0, 1, 5 and 10 min, respectively.

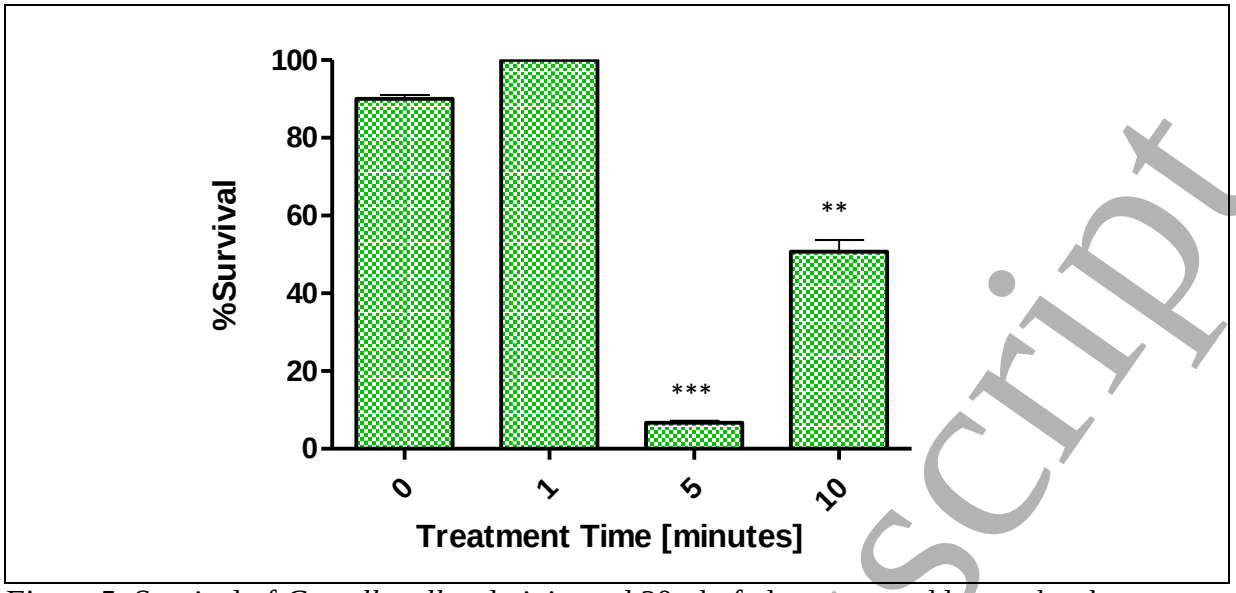

Figure 5: Survival of *G. mellonella* administered 20 µl of plasma treated lettuce broth (***p<0.001; **p<0.01 compared to controls injected with untreated lettuce broth).

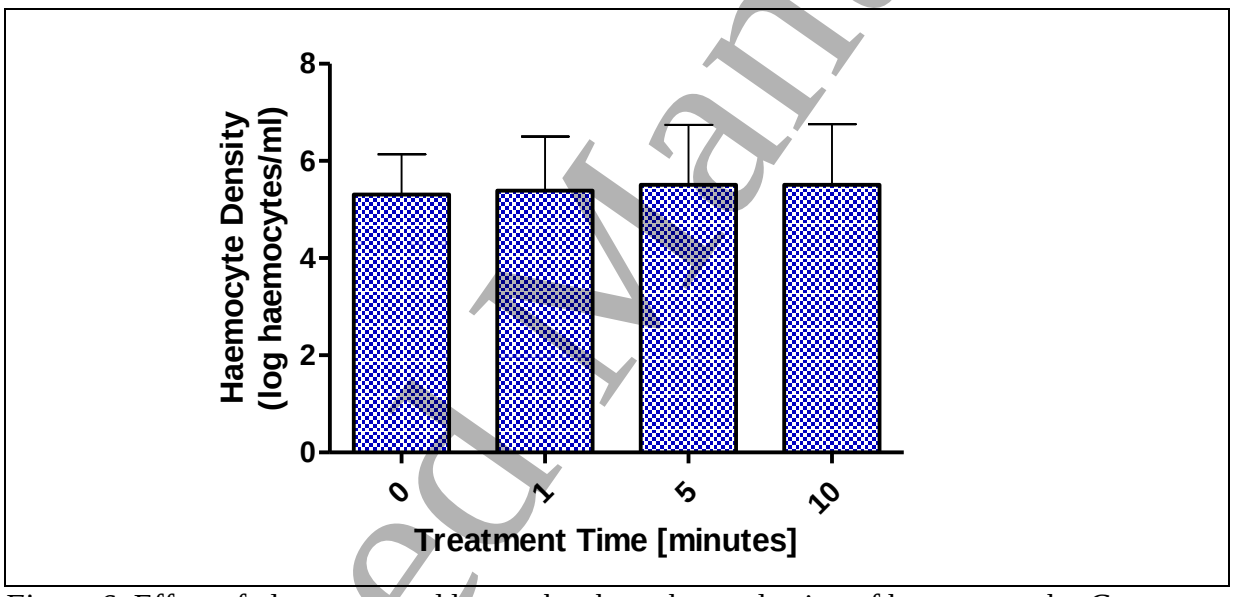

Figure 6: Effect of plasma-treated lettuce broth on the production of haemocytes by *G. mellonella*. Results represent haemocyte counts obtained from combining haemolymph of surviving larvae of each test group and averaged, no significance observed.

3.4 Reactive species quantification in a plasma treated lettuce broth

Quantifying 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 measured in lettuce broth subjected to ten minutes of plasma treatment, Figure 7.

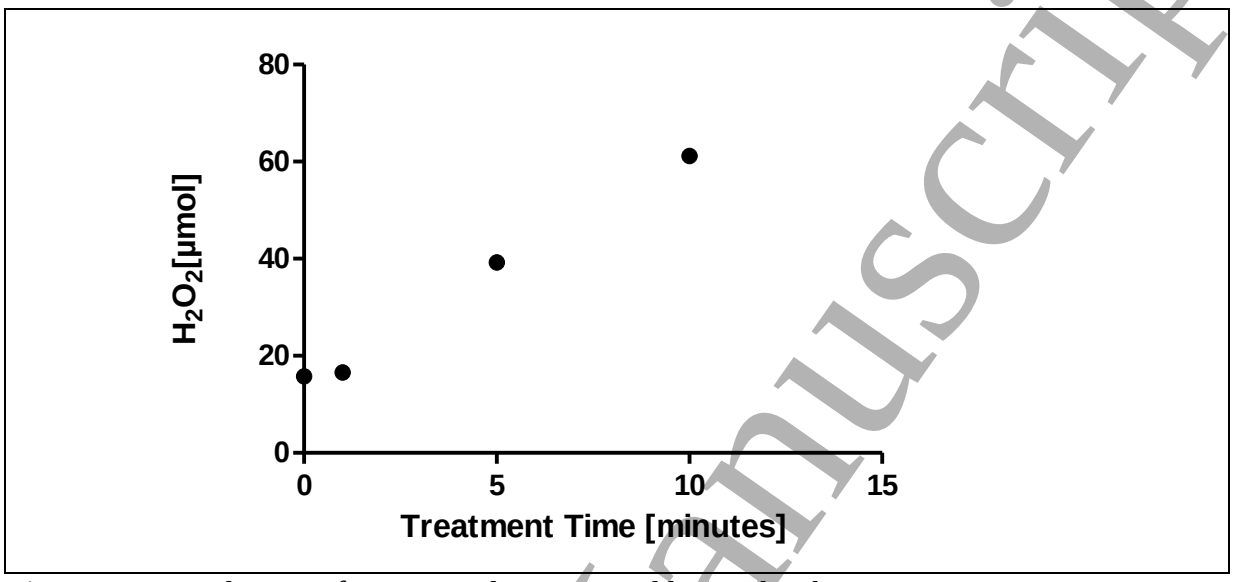

Figure 7: Peroxide quantification in plasma treated lettuce broth.

Less than 20µM of nitrite were 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 showed approximately 18mM or 1.5g/l of nitrate in the lettuce broth. Concentrations did not change significantly with plasma treatment.

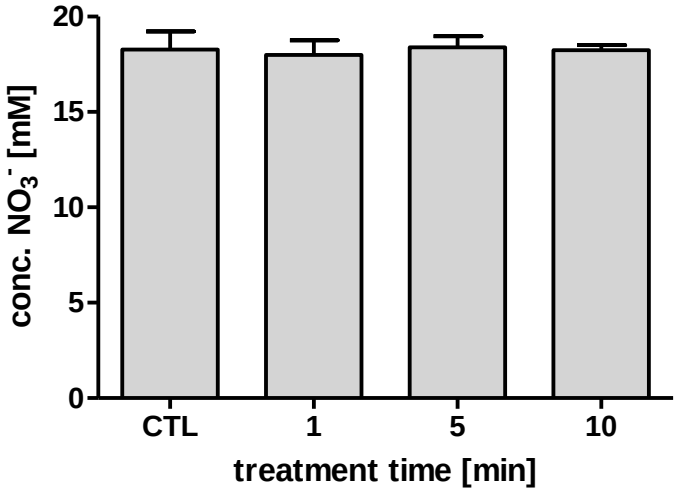

Figure 8: Nitrate quantification in plasma treated lettuce broth using dimethylphenol method.

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 by the lettuce broth is evidenced by the much lower decrease in pH compared to treatment of water in the same set-up, where values decreased to below 3 [22].

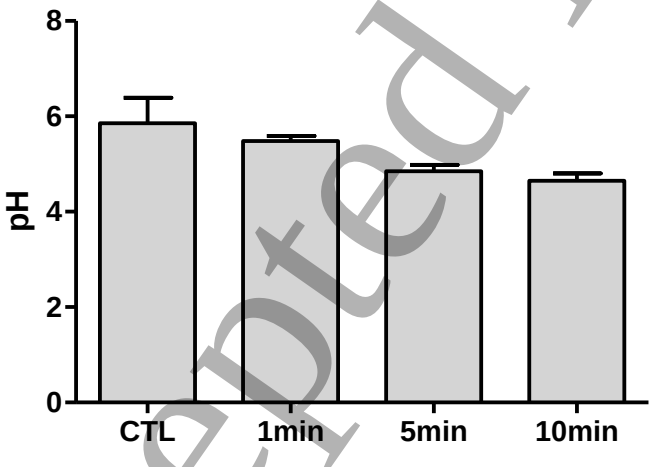

Figure 9: Measurement of pH in plasma treated lettuce broth.

4. Discussion

There are reports of chemical modification to food induced by plasma treatment including oxidation of sugars to organic acids, amino acid alterations and loss of structure in proteins [23]. 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, the metabolism of plasma-generated compounds, 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 *Galleria* larvae, which showed very different results (Table 2).

Table 2: Models and parameters used to assess 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)	*Galleria mellonella* larvae
Test subject sample size	2.5 x 10^4 cells/ml in 96-well plate; in triplicate	2.5×10^4 cells/ml 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	10µl in 100µl cell culture medium	200µl in 2ml cell culture medium	20µl intra-haemocell injection
Exposure duration	3 days	6-40 days	1 day
Effect	reduction of cell growth by 20% in all plasma-treated samples, no treat-ment time dependency	Spontaneous mutations at single time points	Acute toxicity of 5min treated broth: 90% 10 min treated broth: 50%

The results of short-term mammalian cytotoxicity assays presented here showed cell growth stable at 80% of the untreated control in the presence of lettuce broth treated with plasma for up to ten minutes, 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 foetal bovine serum treated with the same plasma device and set-up which resulted in reduction of cell growth by nearly 90% in the same mammalian cell line in other studies [7, 24]. Cytotoxicity of those liquids was found to be mainly mediated by the concentrations of hydrogen peroxide in agreement with findings by others. There was a plasma treatment time-dependent increase in peroxide measurement of lettuce broth but after 10 minute treatment it was less than 80 µmol/l, 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 days, 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 attributed to the plasma treated lettuce broth. When plasma treated lettuce broth was administered to the larvae of *G. mellonella*, there was a clear difference in survival rates. Of the larvae that were administered lettuce broth treated with plasma for 5 minutes, <10% survived after 24 hours, compared to 100% for lettuce broth treated for 1 minute and 50% survival for lettuce broth treated for 10 minutes. Some chemicals may not be directly toxic to a cell but can be converted into other toxins through the 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 to the untreated control. However, control experiments using pure H_2O_2 showed the (lethal dose) LD50 to be 675µM, 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 *Galleria* larvae to be unaffected by injection of acetic acid of 10, 20, 30% and HCl at 0.5, 1 and 2.5M, 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 larvae 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 M - 0.10 M ($2x10^{-6}$ mol/larvae) and those of sodium nitrate 0.66 M and potassium nitrate 0.89 M (1.3 and $1.8x10^{-5}$ mol/larvae) [27-29]. Nitrate concentrations determined in the lettuce broth samples were around 0.018M in all samples, ($3.6\ x10^{-7}$ mol applied per larvae) and therefore almost 50-fold lower than the LD50 values. Nitrate content of leafy greens such as iceberg lettuce varies with variety, location and season with average concentrations around 700-900mg/kg of produce [30]. Nitrate concentrations generated by the same plasma device in water were on average around 0.5mM after 5 min and 1mM after 10 min of treatment (50 and 100mg/l) [22] and therefore would impact 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 5 versus 10min treated lettuce broth. Although the group that was administered the 5 minute 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 the generation of toxic intermediates 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 describe elevated mutagenicity in FBS treated with plasma for 5 minutes [7] compared to 10 min treatment. The observed mutagenic potential was attributed to the possibility of chemical modifications to plasma treated FBS and the generation of hydrogen peroxide. 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 where increased antimicrobial activity was observed after the plasma exposure [32]. Other studies have shown various peaks of by-products to appear, 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, e.g. 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. *G. 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 due to 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 to 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 its 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 biomolecules concentrations may provide clearer insights to the pathways of plasma induced modifications and the 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, different wave length photons and chemically reactive species in plasma-treated food stuffs may open reaction pathways different to 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

the establishment of cold plasma 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 minutes 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 context with a biological system and highlight a need for more detailed and relevant exposure studies and standardized assessment procedures.

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