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The impact of food model system structure on the inactivation of *Listeria innocua* by cold atmospheric plasma and nisin combined treatments

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ABSTRACT

Novel processing methods such as cold atmospheric plasma (CAP) and natural antimicrobials like nisin, are of interest to replace traditional food decontamination approaches as, due to their mild nature, they can maintain desirable food characteristics, i.e., taste, texture, and nutritional content. However, the microbial growth characteristics (planktonic growth/surface colonies) and/or the food structure itself (liquid/solid surface) can impact the inactivation efficacy of these novel processing methods. More specifically, cells grown as colonies on a solid(like) surface experience a completely different growth environment to cells grown planktonically in liquid, and thus could display a different response to novel processing treatments through stress adaptation and/or cross protection mechanisms. The order in which combined treatments are applied could also impact their efficacy, especially if the mechanisms of action are complementary.

This work presents a fundamental study on the efficacy of CAP and nisin, alone and combined, as affected by food system structure. More specifically, *Listeria innocua* was grown planktonically (liquid broth) or on a viscoelastic Xanthan gum gel system (1.5% w/v) and treated with CAP, nisin, or a combination of the two. Both the inactivation system, i.e., liquid versus solid(like) surface and the growth characteristics, i.e., planktonic versus colony growth, were shown to impact the treatment efficacy. The combination of nisin and CAP was more effective than individual treatments, but only when nisin was applied before the CAP treatment.

This study provides insight into the environmental stress response/adaptation of *L. innocua* grown on structured systems in response to natural antimicrobials and novel processing technologies, and is a step towards the faster delivery of these food decontamination methods from the bench to the food industry.

1. Introduction

Novel non-thermal technologies such as cold atmospheric plasma (CAP), ultrasound, and high pressure processing (HPP) have gained significant interest among researchers and the food industry over recent years (Deng et al., 2006; Han et al., 2016a; Smet et al., 2017). These technologies are milder than traditional methods (pasteurisation, ultra-high temperature treatments) and thus may be able to meet the increasing consumer demand for minimally processed foods and ready-to-eat produce, e.g. pre-packaged sandwiches, cheeses, cooked meats, fresh fruit and vegetables, which retain their organoleptic properties and nutritional content (Shah et al., 2019; Troy et al., 2016).

CAP in particular shows great promise as a decontamination method. It can be used to inactivate microorganisms on surfaces, packaging, and processing equipment as well as on solid(like) food products or in liquid foods, with its diffuse nature (as an ionized gas) increasing the efficacy of microbial inactivation in pores or crevices that are difficult to reach using traditional methods (Karam et al., 2016; Millan-Sango et al., 2015; Min et al., 2016; Patange et al., 2018). Furthermore, its non-thermal nature, and its application at atmospheric pressure, mean that CAP can be applied to delicate and heat-sensitive foods (De Geyter and Morent, 2012). Also, it requires a low input power, and all chemical species return to their neutral ground state when the electrical supply is stopped (Coutinho et al., 2018; Smet et al., 2017).

CAP is generated by applying a high voltage to a gas stream at room temperature and atmospheric pressure, causing the gas molecules to

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become ionized resulting in the formation of ultraviolet (UV) photons, chemical species such as free radicals, reactive oxygen species (ROS), reactive nitrogen species (RNS), and charged particles including electrons and ions (Millan-Sango et al., 2015; Min et al., 2016; Smet et al., 2017). Cell damage/inactivation by CAP is suggested to occur through (i) permeabilisation of the cell membrane or wall leakage of cellular components by free radicals (e.g. OH, NO) and/or charged particles, (ii) critical damage of intracellular proteins by ROS or RNS, and (iii) direct DNA damage by UV photons (Laroussi, 2002; Surowsky et al., 2015). These species have been reported to be capable of inactivating microorganisms individually, however their bactericidal efficiency and inactivation mechanisms have not yet been fully quantified in the context of CAP (Bourke et al., 2017; Deng et al., 2006; Smet et al., 2017; Ziuzina et al., 2015).

CAP also has the potential to be combined with other treatments, such as natural antimicrobials, to act as a hurdle for microbial growth and thus further enhance the inactivation of food-related bacteria such as *Listeria* (for example, Barba et al., 2017; Bleoancă et al., 2016; Liao et al., 2018; Millan-Sango et al., 2016; Muñoz et al., 2012; Ross et al., 2003). Natural antimicrobial compounds such as essential oils (e.g. carvacrol, thyme, tea tree, oregano), chlorine dioxide, fumaric acid, and bacteriocins produced by lactic acid bacteria (e.g. subtilosin, pediocin and nisin) have been shown to act individually against food-related pathogenic bacteria (see as examples Ale et al., 2015; Bhatti et al., 2004; Costello et al., 2018, 2019; de Sousa Guedes and de Souza, 2018; Gutierrez and Bourke, 2009; Mariam et al., 2014; Zapico et al., 1998).

Nisin is produced by some species of *Lactococcus lactis* and generally recognised as safe by the Food and Drug Administration (FDA), and is the only natural antimicrobial approved by the European Union for use as a preservative in selected foods (Gharsallaoui et al., 2016). Nisin is currently used as a preservative to prevent the growth of microorganisms, particularly in dairy products and acidic foods, but is of increasing interest as a novel non-thermal inactivation method (Abee et al., 1994; Collins et al., 2010; NicAogain and O'Byrne, 2016). Nisin acts by binding to Lipid II, a precursor to cell wall peptidoglycan present in the cell membrane. Cell membrane synthesis is catalysed by a penicillin-binding protein (PBP) which uses Lipid II as the substrate, thus binding to Lipid II is understood to prevent cell membrane synthesis and hence cause pore formation, resulting in the release of intracellular contents and a collapse of the proton motive force (Abee et al., 1994; Zhou et al., 2014). Gram negative bacteria are usually resistant to nisin as the additional outer membrane prevents nisin from reaching the cell membrane (Gharsallaoui et al., 2016; Wang et al., 2018).

CAP is not yet used in food applications, however research into CAP inactivation of a wide range of food-related pathogens has begun to be reported in recent years: in liquid systems such as ultra-heat treated (UHT) and raw milk, orange juice, and waste water (Gurol et al., 2012; Patange et al., 2018; Shi et al., 2011), on the surface of real food products e.g. cheese, fish, tomatoes, ready-to-eat meats, lettuce (Albertos et al., 2017; Lis et al., 2018; Millan-Sango et al., 2015; Min et al., 2016, 2017, 2018; Patange et al., 2019; Wan et al., 2019), and on abiotic surfaces such as glass, polymers such as polyethylene, and stainless steel (De Geyter and Morent, 2012; Puligundla et al., 2016).

To date, the combination of CAP treatment with nisin has been restricted to the development of novel packaging methods (Hu et al., 2018; Karam et al., 2016; Lu et al., 2018), or the inactivation of food-related pathogens on the surfaces of food products such as apples and beef (Stratakos and Grant, 2018; Ukuku et al., 2019). However the use of specific real food products to investigate the microbial response to novel processing technologies such as CAP or nisin treatment is limited in applicability: food products vary significantly in structure and composition, which can affect CAP inactivation both through the growth type (colony/planktonic) and by means of the support system on which the cells are inactivated (liquid/solid surface) (Baka et al., 2014; Kowalik and Lobacz, 2015; Smet et al., 2018; Surowsky et al., 2015). More specifically, cells grown in/on a solid system grow as colonies and experience a completely different environment as compared to (liquid) planktonic growth. Diffusional limitations of oxygen, nutrients and nisin, as well as the accumulation of (acidic) metabolic products around the colony, could affect the efficacy of nisin (Aspridou et al., 2014; Noriega et al., 2013; Velliou et al., 2011a, 2011b). As such, the use of a food model system in inactivation studies allows for the extrapolation of results to food systems of similar rheological and structural characteristics, without the drawbacks associated with batch-to-batch variation and structural differences of different food products (Baka et al., 2016). While food model solid(like) systems (generally comprising gelatin or agar as the gelling agent) have been introduced recently to model the inactivation of different growth types, i.e., planktonic/surface colonies/immersed colonies (Han et al., 2016c; Smet et al., 2016, 2018, 2019; Wan et al., 2019), a systematic fundamental study on the efficacy of CAP treatment in combination with nisin, against food-related bacteria grown in/on systems of controlled rheological complexity/composition, is currently lacking.

Furthermore, while both CAP and nisin treatments are promising to replace traditional methods and thus maintain food characteristics, their mild nature (alone or combined) may instead represent a mild, sublethal stress which could lead to microbial stress adaptation and post-treatment survival (Davidson and Harrison, 2002; Poole, 2012; Zimmermann et al., 2012). This is especially concerning for pathogenic bacteria commonly associated with ready-to-eat food products such as *Listeria*, as the mechanisms of resistance to nisin can lend resistance to antibiotics with a similar mode of action, meaning that typical treatments for listeriosis (for example) may not be as effective (Martínez and Rodríguez, 2005; Zhou et al., 2014). In particular, reports of antimicrobial resistance (AMR) in *Listeria* species isolated from the environment have become more prevalent in recent years, which is of huge concern due to the high mortality rates associated with listeriosis (Charpentier and Courvalin, 1999; Escolar et al., 2017; Gómez et al., 2014; Granier et al., 2011; Wang et al., 2013). Thus, it is important to identify and minimise the potential for AMR and/or stress adaptation in *Listeria* species when investigating and developing novel processing technologies.

This work aims to identify the combined inactivation effect of CAP and nisin on *L. innocua* grown planktonically or on the surface of a viscoelastic food model system of controlled rheological complexity and composition. Furthermore, the effect of the inactivation carrier, i.e., inactivation in/on liquid or solid(like) surface food models, is also studied.

2. Materials and methods

2.1. Preparation of food model systems

For the liquid system, Tryptic Soy Broth (Oxoid Ltd., UK) supplemented with 0.6% Yeast Extract (TSBYE) (Oxoid Ltd., UK) was used. For the solid(like) Xanthan gum (XG) system, a modified method of Velliou et al. (2013) was used. Briefly, XG (Xantural® 75; CP Kelco, UK) was added to TSBYE at a concentration of 1.5% *w/v* and mechanically stirred for at least 5 min until fully homogenised (Omni Mixer Homogenizer, Omni International Inc., USA). The homogenised mixture was centrifuged at 4000 ×*g* in 50 mL Falcon tubes (Corning Inc.®, USA) for at least 30 min to remove entrapped air bubbles (Megafuge 16R, ThermoFisher, USA). After autoclaving at 121 °C for 15 min, the medium was centrifuged again to remove additional air bubbles (Costello et al., 2018, 2019). Xanthan gum was selected as the gelling agent as it is stable at a wide range of temperatures and is widely used in the food industry as a thickener and stabiliser in products such as sauces, dressings and bakery products (Das et al., 2015; Kang and Pettitt, 1993). 1.5% XG was selected as it is the lowest concentration at which a solid(like) surface system is possible. For surface growth or inactivation on the 1.5% XG system, 3 mL of the viscoelastic medium was transferred by a pipette for viscous media (MICROMAN® E, Gilson Ltd., USA) onto a 50 mm petri

plate to form a disc of 3 cm diameter.

2.2. Inoculum preparation and growth

Stock cultures of *Listeria innocua* ATCC 33090, a model for the foodborne pathogen L. *monocytogenes*, were stored at −80 °C in TSBYE (Oxoid Ltd., UK), supplemented with 15% glycerol. A loopful of thawed culture was inoculated in 15 mL TSBYE for 9.5 h at 37 °C. A volume of 20 μL was subsequently transferred to fresh 15 mL TSBYE and cultured for 15 h at 37 °C until early stationary phase was reached (10^9 CFU/mL).

Samples treated with CAP were grown (i) planktonically in TSBYE or (ii) inoculated onto a 1.5% XG disc for an initial concentration of 10^3 CFU/mL on the disc for surface colony growth. Discs were prepared as described in Section 2.1. Samples were incubated at 37 °C for 15 h until early stationary phase (Costello et al., 2018). A temperature of 37 °C was selected as the optimum temperature for *Listeria* growth (Ryser and Marth, 2007), and also so that the only stressing factors are the CAP and nisin treatments. Identifying the potential for stress adaptation and AMR development under optimum conditions is a crucial first step in understanding adaptation under non-optimal conditions. Stationary phase cells were studied as they are the most resistant to inactivation treatments (Navarro Llorens et al., 2010).

2.3. CAP experimental set-up

A dielectric barrier discharge (DBD) reactor was used for CAP inactivation; a diagram of the DBD set-up and image of the reactor vessel is displayed in Fig. 1. The discharge was generated between two electrodes of 5.5 cm diameter which were covered by a dielectric barrier layer (7.5 cm diameter) (Fig. 1a), fixed inside the reactor vessel (22.5 cm × 13.5 cm × 10 cm) (Fig. 1b). The gap between electrodes was set at 0.8 cm. The plasma was generated in a gas mixture comprising 4 L/min helium (purity 99.996%, Praxair, USA) with 40 mL/min oxygen (purity ≥99.995%, Air Liquide, France), i.e., 1% oxygen, 99% helium, with a gas residence time inside the reactor vessel of approximately 45 s (Smet et al., 2019). The gases were mixed together before entering the reactor vessel.

For all systems under study, samples were placed between the electrodes inside the DBD reactor vessel, and the system was flushed with the gas mixture for 4 min to ensure a homogeneous gas mixture before plasma generation commenced (Govaert et al., 2018). Samples were held in a 50 mm petri plate, with the lid removed for treatment. The plasma power supply transforms the low voltage direct current input (17.88 V) to a high voltage alternating current (AC) signal, with a peak-to-peak voltage of approximately 7 kV and dissipated plasma power of approximately 7 W. The frequency was set at 15 kHz. Samples were treated at room temperature (approximately 20 °C) for 0–30 min, not including the 4 min flush through of gases, then removed and processed immediately. It is noted that the temperature increase following a CAP treatment of 30 min was identified as 2 °C only (Smet et al., 2019), which is not sufficiently high to cause thermal stress. As such, any inactivation observed is due to CAP effects, not temperature.

Additional control samples, which were not treated with CAP, were placed in the reactor vessel then flushed with the gas mixture for 4 min + treatment time. No effect of drying from the gas flow was observed as compared to samples which were not flushed with the gases; it is noted that liquid samples (1 mL total volume) did demonstrate evaporation but only during plasma generation (the largest volume was 100 μL, for 30 min treatment).

2.4. CAP inactivation experiments

Four conditions were investigated, as indicated in Table 1, with different combinations of growth type (planktonic/surface colonies) and inactivation support (liquid/solid(like) surface), to mimic the cells' physiological state along with the surrounding (food) structural environment for different 'real-life' scenarios (Table 1). Samples were prepared as described in Section 2.2. before treatment with CAP, nisin, or a combination of both. This concentration was selected to still be able to

Table 1
Different combinations of growth type and inactivation carrier, with real food processing examples for each combination.

Growth type	Inactivation carrier	Food processing example
Planktonic (TSBYE)	Liquid (TSBYE)	Microorganisms which have grown in a liquid food product, or in water which is added to a liquid food product to dilute it, that is then treated with CAP.
Planktonic (TSBYE)	Solid(like) surface (1.5% XG)	Microorganisms grown in water used to wash a solid food product (e.g. ready-to-eat lettuce) before CAP treatment.
Solid(like) surface (1.5% XG)	Liquid (TSBYE)	Microorganisms grown on the surface of a food product (e.g. apple) that are then processed into a liquid food product (e.g. apple juice) before CAP treatment.
Solid(like) surface (1.5% XG)	Solid(like) surface (1.5% XG)	Microorganisms grown on one solid product that are transferred to another before CAP treatment, such as transfer from processing surfaces to solid food, or transfer from a contaminated solid food to a non-contaminated food during handling before CAP processing.

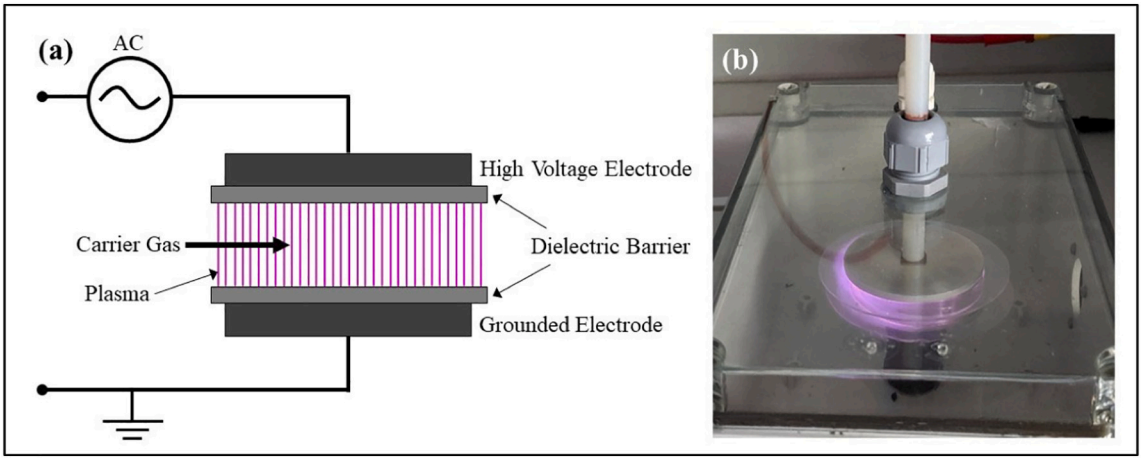

Fig. 1. a diagram indicating the experimental configuration of the reactor vessel internals and an image of the reactor vessel during plasma generation. Carrier gas enters through the blue tube and exits the reactor vessel through vents in the vessel (into the fume hood) after a residence time of approximately 45 s.

observe sufficient reduction in the kinetics following CAP treatment.

For planktonic growth systems inactivated in a liquid carrier, *L. innocua* was cultured and grown to stationary phase in TSBYE as previously described. The culture was diluted in TSBYE to a final cell density of 10^6 CFU/mL, and 1 mL of this cell suspension was transferred to a 50 mm diameter petri dish.

For planktonic growth systems inactivated on a solid carrier, *L. innocua* was cultured and grown to stationary phase in TSBYE as previously described in Section 2.2. The culture was diluted in TSBYE and 50 μL of inoculum was spread on the surface of a 1.5% XG disc for a final concentration of 10^6 CFU/mL.

For surface colony growth inactivated in a liquid carrier, a 1.5% XG disc was inoculated with *L. innocua* at a concentration of 10^3 CFU/mL and grown to early stationary phase (15 h at 37 °C). The entire disc was transferred to a stomacher bag (BA6040, Seward, UK), an appropriate volume of TSBYE added for a 1:10 dilution, and the contents homogenised for 1 min (IUL0470 Basic Masticator, Led Techno, Belgium). Serial dilutions were subsequently performed in TSBYE until a cell density of 10^6 CFU/mL was achieved. 1 mL of this was transferred to a 50 mm diameter petri dish for CAP treatment.

For surface colony growth inactivated on a solid carrier, a 1.5% XG disc was inoculated with *L. innocua* at a concentration of 10^3 CFU/mL and grown to early stationary phase (15 h at 37 °C). The disc was serially diluted as described above, and 50 μL of this cell suspension was spread on the surface of a new 1.5% XG disc for a final concentration of 10^6 CFU/mL.

2.5. Combined inactivation experiments: CAP and nisin

For combined treatment with CAP and nisin, the order of the treatments was varied (as indicated in the experimental schematic in Fig. 2). A concentration of 35 IU/mL of nisin was selected in order to observe the combined effects of CAP and nisin: when applied alone, 35 IU/mL nisin resulted in approximately 1-log reduction, leaving room for further reduction in population while still being able to accurately conduct serial dilutions and plate counts (data not shown).

For nisin treatment of solid samples, 50 μL of an appropriate concentration of nisin was dropped onto the surface of the inoculated XG disc before or after CAP treatment, and gently spread across the surface with a spreader for a final concentration of 35 IU/mL. The sample was incubated at room temperature for 30 min before either CAP treatment (0–30 min) or sample processing in the same manner as for CAP treatment only.

For nisin treatment of liquid samples before CAP, 50 μL of nisin solution was added to 950 μL of *L. innocua* at 10^6 CFU/mL (for a total volume of 1 mL and concentration of 35 IU/mL) in an Eppendorf tube, and vortexed well. The sample was incubated at room temperature for 30 min before CAP treatment (0–30 min) then sample processing in the same manner as for CAP treatment only. For nisin treatment of liquid samples after CAP, the CAP-treated sample was first pipetted into an Eppendorf tube then the same method followed for nisin treatment before sample processing.

2.6. Sample processing

After treatment of cells inactivated in a liquid carrier (1 mL TSBYE), where necessary (due to sample evaporation at higher treatment times as noted in Section 2.3) an appropriate volume of PBS (determined by the difference in sample weight before and after CAP treatment) was added to the sample for a final volume of 1 mL. This was to ensure serial dilutions were performed correctly, as the cells would be more concentrated following evaporation of liquid. This was also conducted before nisin treatment to ensure a consistent concentration across samples. The sample was re-suspended well and transferred to a sterile Eppendorf tube, from which serial dilutions were prepared using TSBYE. For each sample, 1–4 dilutions were plated in duplicate using the drop plate method onto TSAYE plates, i.e., three drops of 20 μL per dilution, for 1–4 dilutions per replicate (Herigstad et al., 2001).

For cells treated on a solid surface carrier (3 mL 1.5% XG disc), the entire disc was removed to a stomacher bag, an appropriate volume of PBS was added to form an initial 1:10 dilution, and the contents homogenised for 1 min. Serial dilutions were subsequently performed in TSBYE and samples were plated as described above for liquid carrier samples.

2.7. Statistical analysis

Independent experiments were performed at least twice, with a minimum of two samples for each treatment time for each individual experiment. The statistical difference/indifference between means of logarithmically transformed viable counts was evaluated with an analysis of variance (significance $p < 0.05$). These analyses were performed using Microsoft Excel (2016).

3. Results and discussion

3.1. Inactivation of L. innocua by CAP

Fig. 3 displays the inactivation kinetics of *L. innocua* following CAP treatment under the four different conditions investigated, i.e., planktonic or surface growth, followed by inactivation in a liquid or solid (like) surface carrier (Table 1).

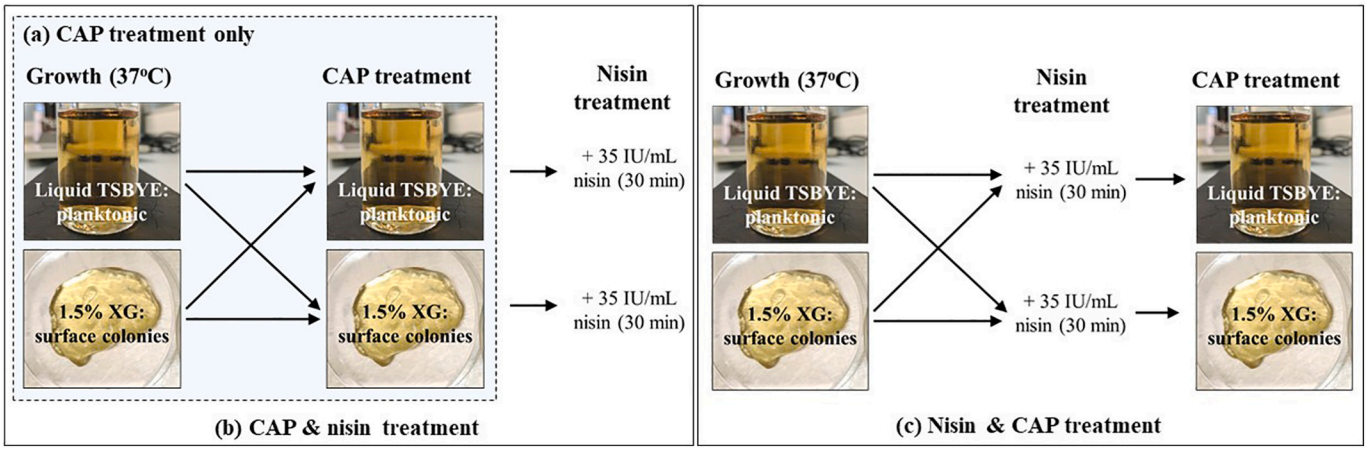

Fig. 2. Schematic for inactivation experiments. (a) schematic for CAP treatment only, (b) schematic for CAP treatment followed by nisin treatment, and (c) schematic for nisin treatment followed by CAP treatment.

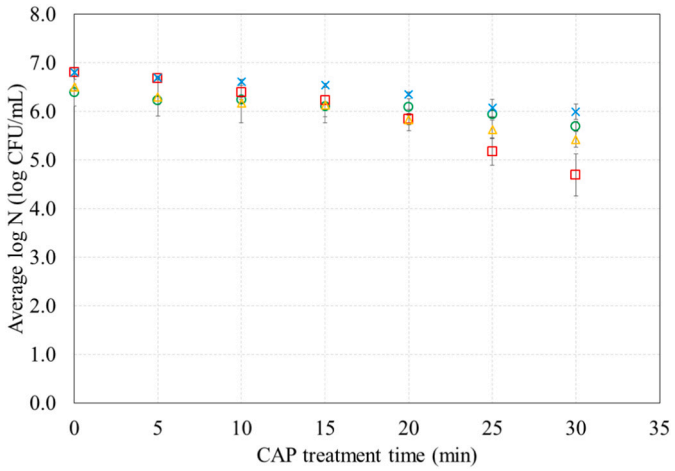

Fig. 3. Inactivation kinetics of *Listeria* following CAP treatment for 0–30 min. (○) planktonic growth → liquid carrier, (□) planktonic growth → solid surface carrier, (×) surface growth → liquid carrier, (△) surface growth → solid surface carrier. Error bars indicate the standard deviation.

3.1.1. *Effect of inactivation carrier (liquid versus solid surface)*

Cells inactivated in the liquid carrier, whether grown planktonically or as surface colonies, are observed to be more resistant to CAP treatment than when inactivated on the 1.5% XG solid(like) surface, suggesting that the liquid system lends a protective effect against CAP treatment (Fig. 3). This observation is in accordance with previous studies on CAP inactivation. Millan-Sango et al., (2015) used CAP (air DBD, up to 90s treatment time) to inactivate planktonically-grown *E. coli* in (i) a broth system or (ii) on the surface of lettuce leaves. They observed a greater log reduction for the cells on the lettuce surface than in the broth system. Similarly, Smet et al. (2016, 2017, 2018, 2019) show in several studies that L. *monocytogenes* and *S.* Typhimurium are inactivated to a greater extent by CAP on the surface of a viscoelastic model system comprising 5% gelatin than in a liquid carrier (TSB). These studies were performed using the same CAP set-up and similar operating conditions as the present work. Other studies report a similar trend (Stoffels et al., 2008; Surowsky et al., 2015).

As discussed in Section 1: Introduction, cell damage/inactivation by CAP is suggested to occur through cell membrane permeabilisation, critical damage of intracellular proteins, and direct DNA damage (Laroussi, 2002; Surowsky et al., 2015). Reactive species, i.e., free radicals e.g. OH, NO, can be absorbed onto the cell surface and form volatile compounds, provoking damage of the cell wall (Coutinho et al., 2018; Millan-Sango et al., 2015). ROS and RNS can react with membrane lipids to form unsaturated fatty acid peroxides, causing the leakage of macromolecules from the cell. ROS can also cause DNA and protein damage through oxidation (Bourke et al., 2017; Han et al., 2016b; Stoffels et al., 2008). Charged particles can cause cell membrane rupture due to electrostatic tension arising from intense electric fields and high intracellular electric charge. Furthermore, charged particles can cause lesions in the cell membrane through which toxic reactive species can diffuse and cell leakage can occur (Coutinho et al., 2018; Lu et al., 2008; Smet et al., 2019; Surowsky et al., 2015). UV photons can induce the formation of thymine dimers in DNA, inhibiting replication and causing the destruction of genetic material. Photodesorption of atoms and molecules from the cell can also result in the breakage of chemical bonds in cells, potentially leading to the formation of volatile substances e.g. CO, CHx (Coutinho et al., 2018; Hoffmann et al., 2013; Ziuzina and Misra, 2016).

On a solid(like) surface, the above CAP agents can easily reach and inactivate cells (Coutinho et al., 2018). However, in a liquid system, diffusional limitations of chemical species may result in reduced CAP effectiveness. More specifically, fewer CAP species may reach the cells, or their efficacy could be impacted: short-lived charged particles may not retain their activity following diffusion, free radicals may react with components in the liquid system before reaching the cells, and the efficacy of UV photons may be affected by the medium (Smet et al., 2016; Stoffels et al., 2008; Surowsky et al., 2015). Overall, this can explain the reduced effects of CAP in the liquid carrier.

3.1.2. *Effect of growth type (planktonic versus surface colonies)*

Planktonically grown cells inactivated on the 1.5% XG solid(like) surface system are shown to be the most susceptible to CAP treatment, with an approximately 2-log reduction in cell count after 30 min of treatment (Fig. 3). In comparison, cells grown as surface colonies and inactivated on the 1.5% XG solid(like) surface system display a 1-log reduction only (Fig. 3).

This observation is supported by previous studies. Smet et al. (2017, 2019) observed that L. *monocytogenes* and *S.* Typhimurium grown planktonically were more susceptible to CAP treatment, when inactivated on a solid(like) surface (5% gelatin), than when grown as surface colonies. Furthermore, resistance of surface colonies (as compared to planktonic cells) to stresses other than CAP has been demonstrated. For example, Valdramidis et al. (2008) show that *L. innocua* as surface attached cells on Teflon® were more heat resistant than planktonic cells in liquid medium. Aryani et al. (2016) observed that *L. monocytogenes* and *Lactobacillus plantarum* on the surface of ham slices were more resistant to thermal inactivation (65 °C or 60 °C for each species respectively) than cells in liquid broth. Further studies also demonstrate resistance of surface colonies to heat treatment and osmotic stress (Noriega et al., 2013; Smet et al., 2015; Velliou et al., 2013; Verheyen et al., 2019; Wang et al., 2017).

As discussed in Section 1: Introduction, cells grown on the surface of a solid(like) system experience a different environment as compared to planktonic growth in a liquid system. More specifically, on a solid surface, microorganisms evolve as colonies and due to diffusional limitations of oxygen and nutrients, as well as the accumulation of (acidic) metabolic products around the colony, they may experience a self-induced (acid) stress which can lead to stress adaptation, affecting their response to CAP treatment (Aspridou et al., 2014; Costello et al., 2018, 2019; Skandamis and Jeanson, 2015; Velliou et al., 2010, 2012, 2013; Yousef and Juneja, 2003).

3.2. *Inactivation of L. innocua by CAP and nisin combined treatments*

Fig. 4 presents the inactivation kinetics of *L. innocua* following the combination of CAP and nisin treatments under the conditions identified in Table 1. The order in which nisin and CAP were applied was varied, as indicated previously.

3.2.1. *Effect of growth type (planktonic versus surface colonies)*

For cells grown planktonically in TSBYE, enhanced inactivation of *L. innocua* is achieved when nisin is added to the system before CAP treatment (nearly 2-log reduction) for both inactivation carriers, i.e., liquid and solid(like) surface, as compared to the treatments applied individually (Fig. 4 a, c). No additional effect of nisin is observed when added after CAP treatment for both liquid and solid(like) inactivation carriers (Fig. 4 a, c).

Ukuku et al., (2019) observed, for L. *monocytogenes* grown planktonically and spread on an apple surface, an enhanced inactivation for the combination of CAP treatment (air plasma jet, up to 40 s treatment time) followed by treatment with nisin solution (1.5-log reduction), as compared to a 0.4-log reduction for each treatment applied individually. Similarly, enhanced antimicrobial effects were also observed for the combination of CAP with essential oils pre-treatment on planktonically-grown *E. coli* and *Salmonella* inactivated on lettuce and eggshell surfaces (nitrogen DBD, up to 3 min) (Cui et al., 2016a, 2016b), with Vitamin C pre-treatment on bacterial biofilms (air plasma jet, up to 60 min) (Helgadóttir et al., 2017), and with lactic acid or sodium dodecyl sulfate pre-treatment on planktonically-grown L. *monocytogenes* inoculated on red

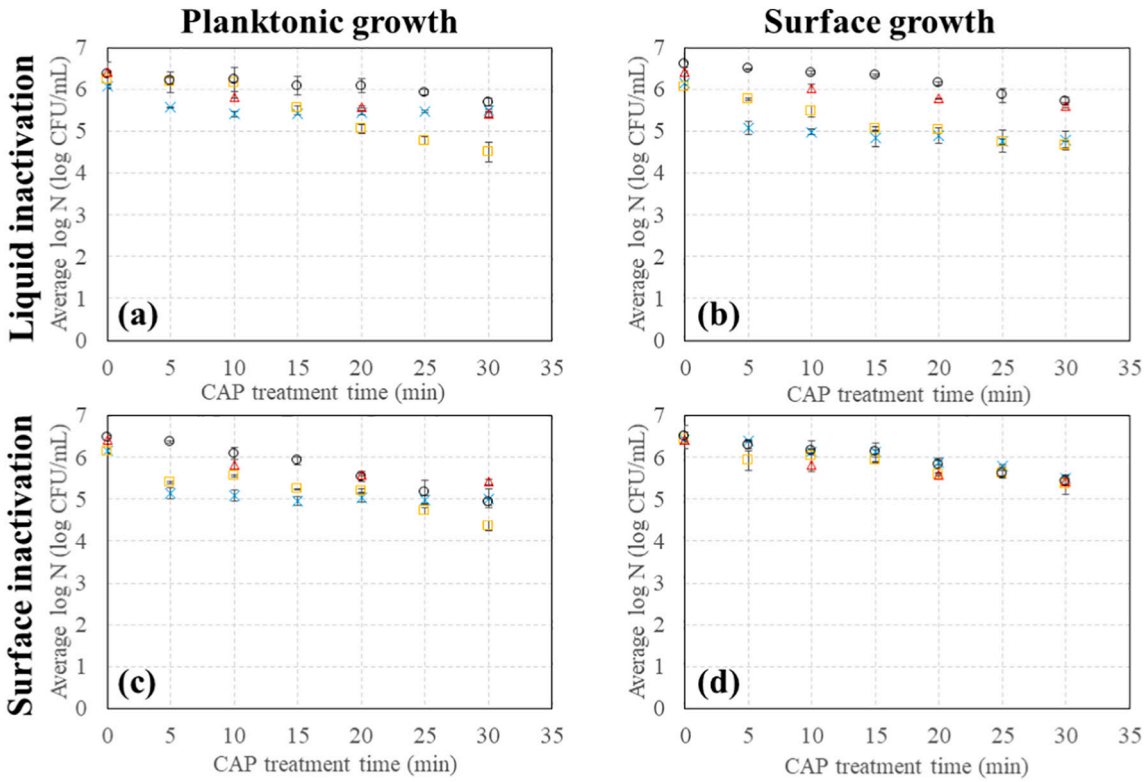

Fig. 4. Inactivation kinetics of *Listeria* following combinations of CAP and nisin treatments for (a) planktonic growth → liquid carrier inactivation, (b) surface growth → liquid carrier inactivation, (c) planktonic growth → solid surface carrier inactivation, (d) surface growth → solid surface carrier inactivation. In each graph, (×) CAP treatment (0–30 min) followed by 30 min nisin, (□) 30 min nisin followed by CAP treatment (0–30 min), (○) CAP treatment only (0–30 min), (△) nisin treatment only (0–30 min). Error bars indicate the standard deviation.

chicory leaves (air DBD, 15 min) (Trevisani et al., 2017). However, none of these studies investigated the impact of the order in which combined treatments were applied, or the effect of structure on the efficacy of inactivation. Furthermore, to the authors' best knowledge, there are no studies on the combined effects of CAP and nisin (or other natural antimicrobials) in liquid carrier systems with which to compare the current work. It is noted that CAP and nisin have also been investigated for the development of novel packaging methods using low-density polyethylene (LDPE) (Hu et al., 2018; Karam et al., 2016; Lu et al., 2018). However, for each of these studies, CAP was used solely for surface functionalisation of polylactic acid to facilitate the attachment of nisin to the surface and not as an inactivation treatment.

The combination of nisin and CAP clearly results in an enhanced antimicrobial effect, but only when nisin is applied first. When combining inactivation technologies, it is often beneficial to combine techniques which target different elements of the cell (Berdejo et al., 2019; Ross et al., 2003). As discussed in Section 1: Introduction, nisin is known to form pores in the cell membrane (Zhou et al., 2014), while CAP acts on microorganisms both internally and externally through various mechanisms (Hoffmann et al., 2013; Stoffels et al., 2008). Previous emission spectrum analysis by the group indicates that highly reactive excited and ground state species including O, OH, H and nitrogen species are produced from the CAP set-up used in the present work (Smet et al., 2018). As discussed in Section 3.1.1., these species can cause damage to the cell membrane, DNA, and proteins, with the potential for complete cell membrane rupture (Coutinho et al., 2018; Laroussi, 2002; Surowsky et al., 2015). The application of nisin and subsequent pore formation could enable CAP species to enter the cell more easily and hence act on cellular components, i.e., critical damage of intracellular proteins by reactive oxygen or nitrogen species (ROS, RNS), direct DNA damage, and the inhibition of DNA replication, thus reducing the surviving population further than when applied invidually or in reverse order (Coutinho et al., 2018; Han et al., 2016b; Ziuzina and Misra, 2016). Furthermore, many CAP species are short-lived and return quickly to their ground state when CAP treatment is ended (Coutinho et al., 2018; Smet et al., 2017), which can explain why pore formation as a result of nisin treatment does not have an additional effect when applied after CAP.

Cells grown as colonies on the solid(like) 1.5% XG surface system are observed to be more resistant to the CAP and nisin combined treatment, showing a 1.5-log reduction (Fig. 4b, d) as compared to a 2-log reduction for cells grown planktonically (Fig. 4a, c), regardless of the order in which treatments are applied. Furthermore, an enhanced effect of the combined treatment is observed only for inactivation in the liquid carrier (Fig. 4b), with no significant differences observed between systems inactivated on the solid(like) surface (1-log reduction in all cases) (Fig. 4d).

As discussed in Section 1: Introduction, cells that are grown on the surface of a solid(like) system can demonstrate increased stress adaptation as a result of the stresses experienced during growth, i.e., oxygen and nutrient diffusional limitations, and the accumulation of (acidic) metabolic products around the colony, thus explaining the observed resistance to the combined treatment of CAP and nisin. Furthermore, our previous work has shown that cells grown as colonies on a surface system are less responsive to nisin than in a liquid system, which is attributed to diffusional limitations across the surface and through the colony which restrict the action of nisin on cells (Costello et al., 2018, 2019). It is noted that in the present study, cells grown as colonies are diluted and redistributed evenly on the surface system as individual cells rather than colonies and as such there will be very little protective effect against nisin from other cells, as there would in a colony. However, diffusional limitations will nevertheless affect the action of nisin on the cells, as the effect of each nisin molecule will be restricted to the area immediately surrounding it (as a result of the solid surface). As such, the

reduced impact of nisin and CAP (in any order) for cells inactivated on the solid(like) surface is attributed to diffusional limitations. Similar observations on the effect of growth type (planktonic/surface colony) are reported in the literature, but only for CAP treatment alone, as discussed previously in Section 3.1.1. To the authors' best knowledge, this is the first report on the influence of growth type on the combined efficacy of CAP and nisin inactivation.

3.2.2. Effect of inactivation carrier (liquid versus solid surface)

The inactivation system, i.e., liquid or solid(like) surface, was observed to affect the level of enhanced antimicrobial activity for the combined treatments: overall, a reduced efficacy of combined nisin and CAP treatments is observed when cells are inactivated on the 1.5% XG solid(like) surface carrier as compared to the liquid carrier (Fig. 4). More specifically, for inactivation of planktonic cells in the liquid carrier, an additional reduction of approximately 1-log is observed for treatment with nisin followed by CAP as compared to individual treatments, but only in this order (Fig. 4a). Cells grown as colonies inactivated in the liquid carrier demonstrate a further reduction of approximately 1-log when combined treatments are applied, regardless of the treatment order (Fig. 4b). For inactivation on the solid(like) 1.5% XG surface system, the additional effect of the combined treatments is only 0.5-log greater than the individual treatments, and only for cells grown planktonically – no differences are observed between treatments for cells grown as colonies, as noted and discussed in Section 3.1.2 (Fig. 4c,d).

Our previous work has shown that nisin is more effective in a liquid system than on a solid surface, as it will be evenly dispersed throughout the system and have easy access to all cells (Costello et al., 2019). On a solid surface, diffusional limitations are likely to restrict the action of nisin, i.e., the number of cells nisin can interact with will be limited. This explains the reduced effect of nisin on the solid(like) surface as compared to the liquid inactivation system. To the author's best knowledge, this is the first report on the influence of the inactivation carrier structure on the combined efficacy of CAP and nisin inactivation.

As discussed in Section 3.1.1, in the liquid system CAP species must diffuse through the liquid to reach the cell surface, which may cause a reduction in activity and thus a reduced effect of CAP treatment alone, as observed.

4. Conclusions

This work presents a fundamental study on the efficacy of treatment with cold atmospheric plasma (CAP) and nisin against *Listeria innocua*, grown planktonically or as surface colonies, in/on viscoelastic food model systems of controlled rheological complexity/composition.

The impact of the growth type and the inactivation carrier on the combined treatment of CAP with nisin is reported for the first time. More specifically, cells grown as surface colonies are more resistant to CAP and nisin inactivation treatments than those grown as planktonic cells, and a greater impact of CAP and nisin treatments, alone and combined, is observed for cells inactivated on the solid(like) surface carrier. Furthermore, the order in which treatments are applied is shown to have a significant effect on the efficiency of inactivation for planktonically grown cells.

This study provides a further step towards the optimisation of cold atmospheric plasma and nisin treatments for microbial inactivation in/on liquid and solid(like) food products. Furthermore, these combined treatments have the potential for use in foods that already contain nisin as a preservative, and for the microbiological safety of heat-sensitive foods. Future work will investigate the efficacy of these combined treatments on more complex food model systems to identify the impact of surface microstructure, topography, and composition. Techniques such as flow cytometry, confocal microscopy and scanning electron microscopy will be used to identify the effect of CAP and nisin treatments on a sub-population/cellular level.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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