Cold Atmospheric Gas Plasma Disinfection of Chicken Meat and Chicken Skin contaminated with *Listeria innocua*

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ABSTRACT

Gas plasmas generated at atmospheric pressure and ambient temperatures offer a possible decontamination method for poultry products. The efficacy of cold atmospheric gas plasmas for decontaminating chicken skin and muscle inoculated with *Listeria innocua* was examined. Optimization of operating conditions for maximal bacterial inactivation was first achieved using membrane filters on which *L. innocua* had been deposited. Higher values of AC voltage, excitation frequency and the presence of oxygen in the carrier gas resulted in the greatest inactivation efficiency, and this was confirmed with further studies on chicken muscle and skin. Under optimal conditions, a 10 s treatment gave > 3 log reductions of *L. innocua* on membrane filters, an 8 min. treatment gave 1 log reduction on skin, and a 4 min. treatment gave > 3 log reductions on muscle. These results show that the efficacy of gas plasma treatment is greatly affected by surface topography. Scanning electron microscopy (SEM) images of chicken muscle and skin revealed surface features wherein bacteria could effectively be protected from the chemical species generated within the gas plasma. The developments in gas plasma technology necessary for its commercial application to foods are discussed.

Keywords: cold atmospheric gas plasma, decontamination, chicken skin, chicken muscle, *Listeria innocua*,

1. Introduction

Poultry carcasses are commonly contaminated with enteric pathogens such as *Salmonella, Campylobacter* and *Listeria monocytogenes* (Jacobsreitsma et al., 1994; Murphy et al., 2004), the possibility of cross-contamination of poultry carcasses post slaughter is high. Decontamination of poultry carcasses is therefore desirable. Various decontamination technologies have been proposed including the use of various chemical agents such as alkali (Rodriguez De Ledesma et al., 1996), physical methods such as steam treatment (James et al., 2007) and biological control with bacteriophages (Carvalho et al., 2010), but only treatment with water supplemented with chlorine or a chlorinating agent is used commercially. The effects of such decontaminating treatments are limited (Oyarzaball, 2005; Russel and Axtell, 2005). There is, therefore, need for an effective technology that can be operated within commercial constraints.

The role of gas plasmas in microbial inactivation has been studied since the mid 1990s, but their potential for food decontamination purposes has only recently been recognised. Gas plasmas are usually generated by means of an external electric field; when the voltage applied to a gas exceeds a certain threshold value the gas will become ionized. Gas plasmas comprise mixtures of electrons, ions, atomic species, free radicals and UV photons, all of which have the capability of inactivating micro-organisms (Perni *et al.*, 2007). In order to decontaminate foods without bringing about undesired changes, gas plasmas should ideally be operated at or near room temperature. Until quite recently this could only be achieved under vacuum, which is inconvenient and expensive. Recent advances in plasma source technology allow the generation of plasmas having both spatial and temporal stability at ambient temperatures and atmospheric pressure using relatively simple and inexpensive equipment (Kogelschatz, 2002). Cold atmospheric plasmas have been reported to be very effective against a wide range of microorganisms, including biofilm-formers and bacterial spores (Montie *et al.*, 2000; Deng *et*

68 *al.*, 2005; Vleugels *et al.*, 2005). Although most of the previous work on bacterial inactivation
69 has been conducted with bacteria deposited on the surface of abiotic materials such as
70 membrane filters and glass slides, data on the disinfection of foodstuffs is steadily
71 accumulating. To give examples, Perni et al., (2008b) examined the inactivation of a number
72 of micro-organisms on mango and melon, whilst Kim et al., (2011) reported on the
73 disinfection of bacon.
74 In the present work, the effectiveness of cold atmospheric plasmas for inactivating *Listeria*
75 *innocua*, a non- pathogenic surrogate for *Listeria monocytogenes*, (Perni et al., 2006) on the
76 surface of both membrane filters and chicken breast (skin and muscle) was examined. A range
77 of plasma operating conditions were first investigated in order to arrive at those that were
78 optimum for the inactivation of *L. innocua* deposited on the surface of membrane filters.
79 Those proving to be the most effective conditions were then applied to chicken skin and then
80 further modified for maximum efficacy. Following this, chicken muscle was treated under
81 these optimised conditions.
82
83 **2. Materials and Methods**
84 *2.1 Microorganisms and Cultivation*
85 *Listeria innocua* (ATCC 33090) was stored on cryobeads at -20 ºC. Cells were activated by
86 transferring one of these beads into 100 mL Tryptone Soy Broth (TSB; Oxoid Ltd.,
87 Basingstoke, Hants, UK) in 500 mL Erlenmeyer flasks and incubating overnight at 30 °C and
88 140 rpm. This culture was streaked onto Tryptic Soya Agar plates (TSA; Oxoid Ltd.,)
89 incubated for 24 h at 30 °C. Working cultures were kept at 4 ºC on TSA slopes, prepared
90 from single colonies of the TSA plates, and subcultured every two weeks.

Inocula for deposition on the surfaces of membrane filters and chicken tissue were prepared by transferring single colonies from TSA slopes to 500 mL Erlenmeyer flasks, each containing 100 mL of TSB. After 24 h incubation at 30 °C and 140 rpm, a loopful of cells from these cultures was used to inoculate 100 mL of fresh TSB in 500 mL Erlenmeyer flasks, incubated as before. Cultivation under these conditions yielded cell populations at stationary phase of growth with a concentration of approximately 5.0 x 10^9 CFU mL^{-1}. This second subculture, diluted to 5.0 x 10^7 CFU mL^{-1} was used to surface inoculate membrane filters, chicken skin and muscle. Additionally, the same culture diluted approximately to 5.0 x 10^8 CFU mL^{-1}, was used to load sterile filter papers of 20 mm diameter (No. 1, Whatman, Fisher Scientific, Loughborough, UK) by immersion in the dilute culture for 15 min. These bacteria-laden filters were used to inoculate samples of chicken muscle as described below.

2.2 Preparation of membrane filters

For ease of manipulation, 0.20 μm pore size Whatman polycarbonate membrane filters of 25 mm diameter (Fisher Scientific, Loughborough, UK) were placed on agar plates (Technical Agar No. 3, Oxoid Ltd.,). Bacterial cell suspension (100 μL) was then carefully deposited onto the centre of the sterile membrane filters, and allowed to dry for 60 min. at room temperature in a laminar flow cabinet. All experiments were conducted in triplicate with three independent cultures.

2.3 Preparation of chicken skin and muscle samples

Fresh, raw, boneless chicken breasts with the skin attached were purchased at a local supermarket and transported immediately to the laboratory, where they were kept in a refrigerator overnight at 4°C before use. The entire skin was removed in one piece from the underlying muscle, using a sterile scalpel and forceps, and placed in Petri dishes. Skin tissue was of two types; rough, relatively thick skin and smoother thinner skin. A slice of

115 approximately 2 mm thickness was cut from the subcutaneous muscle and stored in Petri
116 dishes. Discs of tissue were punched from both muscle and skin pieces using a sterile cork
117 borer of 20 mm diameter. The resulting discs were placed singly in Petri dishes.
118 Bacterial suspension (100 µL) was deposited onto the centres of the discs of skin and muscle,
119 taking care not to allow the suspension to overflow the edges. After inoculation the tissue
120 samples were kept at room temperature in a laminar flow cabinet for 60 min. to allow
121 attachment. Additionally, bacteria-laden membrane filters (prepared as described above) were
122 pressed lightly onto a dry, sterile filter paper for a few seconds to remove the excess liquid,
123 placed onto prepared chicken samples for 10 min., then carefully removed with forceps. All
124 experiments were conducted in triplicate with three independent cultures.
125 *2.4 Cold atmospheric plasma apparatus*
126 The cold atmospheric plasma system comprised a 1.5 mm inner diameter ceramic tube
127 confining a helium-oxygen flow; a concentric, ring-shaped, 1 cm wide copper electrode
128 wrapped around the ceramic tube and connected to a high-voltage power supply; and a disc
129 electrode (ground electrode) placed 0.5 to 1.5 cm downstream of the nozzle of the ceramic
130 tube (Figure 1). The powered electrode was energized with a purpose-built, high-voltage, AC
131 power supply with a peak voltage of 6.5 to 16 kV, and a variable excitation frequency
132 between 23 and 38.5 kHz. Helium (99.99 % purity) at a fixed flow rate of 5 L $min.^{-1}$ and
133 oxygen at a flow rate of up to 100 mL $min.^{-1}$ were fed through the hollow ceramic tube for
134 ionization. The ionized gas was flushed out of the electrode unit and into ambient air toward a
135 point 0.5 to 1.5 cm downstream where the samples were placed connected to the ground
136 electrode. In ambient air, the gas plasma appeared as a pale purple plume, termed a cold
137 atmospheric plasma pen (CAP-Pen).
138 *2.5 Microbial inactivation procedure and cell recovery*

The CAP-Pen diameter of 3 mm was much smaller than the bacterial deposition area (approx.1 cm in diameter), therefore plasma treatment was effected by keeping the plasma nozzle fixed and moving the sample beneath it in a zig-zag path by hand so that the entire surface was exposed to the plume. The duration of CAP-Pen treatment varied from 10 s to 8 min. These total treatment times were about 10 fold greater than the time that any part of the treated area was exposed to the plasma. Therefore it should be noted the D values quoted are in reality *apparent* D values. Following CAP-Pen treatment, cells were recovered from membrane filters by transferring them to sterile Universal bottles containing 10 mL of Ringers solution (Oxoid Ltd.) and agitating for 30 s with a vortex mixer. Chicken samples were transferred to stomacher bags containing 10 mL of sterile Ringers solution and surviving cells were recovered by treating for 30 s in the Stomacher 400 (Seward Ltd., Thetford, Norfolk, UK) at intermediate speed. Aliquots (100 µL) of cell suspension or serial dilutions in Ringers solution were spread onto agar plates. Total bacterial counts were determined on TSA plates incubated at 30 ºC for 24 h. *L. innocua* was enumerated on PALCAM *Listeria* selective agar base (Oxoid Ltd) , supplemented with PALCAM *Listeria* selective supplement (Oxoid Ltd.). The plates were incubated at 30 °C for 48 h. All counts were performed in triplicate, and all the results reported here as CFU per square centimetre are mean values.

2.6 Mathematical Modelling of Bacterial Inactivation

The Baranyi inactivation model as modified by Xiong et al., (1989) was applied to all of the data presented here. This was done according to the form of the inactivation curve, and either applied to the entire data (in experiments I, II, V-VII) or, where more than one phase existed, to individual phases (in experiments III and IV). D values, the times required for one log-reduction of the microbial population, were calculated from the corresponding inactivation rate constants (k) i.e. $D = 2.303/k$.

163 *2.7 Scanning electron microscopy (SEM)*

164 Skin and muscle samples were prepared for SEM by chemically fixing them in 0.025 M
165 phosphate buffer (pH 6.8) containing 3 % (v v^{-1}) glutaraldehyde for 1 h. Samples were then
166 washed twice for 15 min. in the same phosphate buffer prior to dehydration in a series of
167 increasing ethanol concentrations (20, 40, 60, 80 and 100 % v v^{-1} ethanol in water; 15 min. in
168 each solution). Then, samples were transferred from 100 % ethanol to 100 % acetone in a
169 similar way (30, 50, 70, 90 and 100 % v v^{-1} acetone in ethanol; 10 min. in each solution) and
170 dried by critical point-drying with liquid CO_2 (Balzers CPD 030, BAL-TEC AG, Fürstentum,
171 Liechtenstein). Membrane filters were also subjected to chemical fixation, but they were first
172 dehydrated by incubation at 60 ºC overnight. Following this, all the samples were mounted on
173 aluminium stubs with double-sided carbon sticky-tape, sputtered with gold in a vacuum
174 evaporator (Balzers SCD 004, BAL-TEC AG, Fürstentum, Liechtenstein), and visualized
175 using a JEOL-6100 SEM microscope (JEOL Ltd., Tokyo, Japan).

176 *2.8 Statistical analysis*

177 Comparisons of experimental data, either in the form of log concentrations or log reductions,
178 were evaluated by means of analysis of variance (ANOVA) tests. The multiple Range Test
179 was used to distinguish means which differed significantly from each other. Standardized
180 skewness and standardized kurtosis were used to assess if data sets were normally distributed.
181 These analyses were performed using STATGRAPHICS PLUS for Windows 3.0® Package
182 (Statistical Graphics, Washington, USA). Test statistics were regarded as significant when p
183 was < 0.05.

184

185 **3. Results and Discussion**

186 *3.1 Inactivation on membrane filters*

187 Plasma inactivation of *L. innocua* deposited on membrane filters at a range of experimental
188 conditions is shown in Figure 2 and Table 1. The temperature of samples undergoing
189 treatment never exceeded 30° C. Increasing the peak voltage, whilst maintaining the gap
190 distance at 1.5 cm, had relatively little effect. However, decreasing the gap distance to 1.0 cm
191 resulted in a biphasic inactivation curve in contrast to the monophasic curves obtained for
192 experiments I and II. A substantial reduction in D value for the first phase of inactivation was
193 followed by a second phase that had a D value almost one order of magnitude greater. This
194 pattern of inactivation was maintained as the voltage was further increased to 7.0 kV with a
195 reduction in D value for both phases of the inactivation curve. Further increasing the voltage
196 to 8.0 kV resulted in a return to monophasic inactivation kinetics, and a reduction in the D
197 value to 4.5 s. Increasing the voltage still further necessitated a decrease in frequency to 23.0
198 kHz in order to maintain stable plasma operation. under these conditions oxygen was supplied
199 to the CAP. This yielded a D value of 3.4 s. at a peak voltage of 9.0 kV and 3.1 s. at 11.0 kV.
200 Table 2 shows that *L. innocua* was inactivated to below the detection limit (1×10^3 CFU cm^{-2})
201 after only 10s treatment in experiments V, VI and VII. Inactivation curves displaying
202 biphasic (or even multiphasic) behaviour have previously been interpreted as being indicative
203 of the occurrence of inactivation by different plasma species (Montie *et al.*, 2000; Moisan *et*
204 *al.*, 2001; Kong et al., 2009; Kong et al., 2005).
205 Perni *et al.* (2008b) reported that 1.5 s of plasma exposure were sufficient to inactivate *L.*
206 *monocytogenes* on membrane filters ($\approx 4 \times 10^6$ CFU/cm^2) to below the detection limit,
207 although their treatment conditions (30 kHz, 8 kV and 25 mL oxygen $min.^{-1}$) were not
208 identical to those employed here. Plasma exposure time to achieve no detectable cell counts
209 was of the same order for both species, although the slightly higher value obtained here

indicates that *L. innocua* may be marginally more resistant to plasma treatment than is *L. monocytogenes*.

When oxygen was added to the helium flow (experiments VI and VII), peak voltage and excitation frequency had to be re-set to achieve stable plasma operation. Molecular gases have higher breakdown voltages than atomic gases, and therefore the applied voltage had to be increased when oxygen was mixed with helium. For the CAP-pen used here it was necessary to increase the voltage to 9 kV at an excitation frequency of 38.5 kHz. However, this was found to result in considerable overheating in, and subsequent failure of, the transformer used in the power supply. To prevent overheating the excitation frequency was reduced to 23 kHz and this resulted in stable operation. The key parameter for comparing the lethality of plasmas is the number densities of the relevant biocidal plasma species. These were taken to be oxygen atoms since they had previously been identified as a key biocidal species in atmospheric He-O_2 plasmas (Deng et al.,2007a). The optical emission intensities at 777 nm and 845 nm (not shown here) were taken as an indirect indicator of atomic oxygen concentrations. Using this criterion, conditions in experiment IV and V were found to be similar to those in VI and VII, respectively. Moreover, previous studies have shown that plasma characteristics do not change in any significant way in the frequency range of 20 to 50kHz (Deng and Kong, 2004).

Although the D values were of the same order of magnitude, log reductions at 10 s in experiments VI and VII were significantly different to the inactivation achieved in the absence of oxygen (experiment V). Therefore, carrier gas composition played a decisive role in plasma inactivation as would be expected (Deng *et al.*, 2007b; Perni *et al.*, 2007).

3.2 Inactivation on skin from chicken breast

Viable counts recovered from chicken skin before inoculation were of the order of 4.0 x 10^5 CFU cm^2, but did not include listeriae. Viable counts after CAP-Pen decontamination of inoculated skin under various experimental conditions are shown in Table 2. The plasma operating parameters that were most effective with membrane filters were applied to the treatment of chicken skin. However, the dielectric properties of membranes and chicken skin are different, and this necessitated adjustment of the operating conditions in order to achieve stable operation. In particular, different peak voltages, excitation frequencies, carrier gas and plume length (experiments IV to VIII) were tested within the plasma stability range, in addition to studying the effect of cell attachment (experiment IX) as well as skin type (experiment III).

Reductions in viable counts were considerably below those achieved using membranes, and the D values were correspondingly higher. In experiments I to III, skin samples were treated for both 4 and 8 min., but no significant reductions in bacterial numbers with treatment times of up to 4 min. were achieved. Therefore in subsequent experiments treatment was for 8 min. only.

Experiments I and II were conducted under conditions previously identified as representing the best conditions for inactivation on membrane filters (V and VII, respectively). After 4 min., the conditions in experiment II resulted in significantly increased log reductions of the total microflora, but not of *L. innocua*. However, after 8 min. of exposure, there were significant decreases in numbers of both the total microflora and *L. innocua*. Curiously, higher levels of inactivation were achieved in the absence of oxygen in the carrier gas (experiment I), which contrasts with the results obtained using membrane filters. When experiments I and II are compared to experiments conducted with membrane filters under the

same conditions (V and VII, respectively), significantly higher ($p < 0.05$) D values (approx. 200 and 600 fold higher, respectively) and log reductions (4- and 15- fold higher, respectively) were achieved with membrane filters with much shorter contact times. Thus chicken skin exerts a protective effect on microorganisms present at the surface.

The effect of skin type i.e. thick rough skin versus thin and relatively smooth skin, on plasma effectiveness was revealed as negligible from experiments II and III, since no significant differences ($p > 0.05$) were found in log reductions at any of the treatment times.

High excitation frequencies have been reported to expand the plasma stability range and enable more abundant reactive plasma species, such as oxygen atoms and hydroxyl radicals to be generated, and this has been correlated with increased anti-microbial efficiency of plasmas (Walsh and Kong, 2006). The importance of this parameter, at constant peak voltage, is evident by comparing experiments II and IV, since significantly higher reductions ($p < 0.05$)were achieved at the highest than at the lower frequencies for both total and listerial populations.

Increasing the oxygen flow rate whilst maintaining all other parameters constant resulted in a significant reduction in D values, as is evident by comparing the results for experiments V and VI. The D value for the latter experiment was not significantly different to that obtained in experiment I. However, when the oxygen flow was set at 100 mL $min.^{-1}$ (experiment VII), more effective inactivation was achieved than in experiment VI. To try to improve plasma effectiveness, the distance between the plasma source and the chicken skin (the 'gap' in Table 2) was decreased to 0.5 cm (experiment VIII). Significantly lower log reductions ($p < 0.05$) were achieved for *L. innocua* and total microflora as compared to experiment VII, although reductions were comparable to those in experiments I, and IV to VI. However, D values for total counts and *L. innocua* in experiment VIII were not significantly different ($p > 0.05$) from

282 the corresponding values in experiment VII. Cell migration into skin tissue during attachment
283 may have occurred and this could well have affected plasma effectiveness, since cells could
284 be transported beyond the reach of plasma species as Perni *et al.* (2008b) demonstrated for the
285 plasma treatment of fresh cut fruit surfaces. To confirm this supposition, samples were treated
286 immediately after deposition onto chicken skin (experiment IX) under the same conditions as
287 in experiment VII. D values for total and listerial counts were not significantly different ($p > 0.05$) between experiments VII and IX. For these same experiments, no significant differences
288 0.05) between experiments VII and IX. For these same experiments, no significant differences
289 were found between log reductions of *L. innocua*, whereas values for the total microflora
290 were significantly lower ($p < 0.05$), although they were of the same order as for experiments
291 I, IV to VI and VIII.
292 Plasma conditions in experiment VII resulted in the most effective inactivation of *L. innocua*
293 on chicken breast skin with a reduction of just under 1 log and a D value of about 9 min.
294

295 *3.3 Inactivation on chicken breast muscle*

296 Chicken skin acts as a physical barrier to microbial migration into the inner tissues, but
297 severed blood vessels or skin cut during skinning, boning and portioning may contaminate the
298 underlying tissue (Avens *et al.*, 2002). Microbiological analysis of uninoculated muscle
299 revealed the absence of listeriae and total counts of only 2.5×10^3 CFU cm^{-2}. Previous
300 experiments conducted with plasma membranes and chicken skin allowed identification of
301 plasma operating conditions that are more effective for inactivation. These were applied to the
302 treatment of chicken muscle (Table 3).
303 In experiment I, no significant differences ($p > 0.05$) were observed after 8 min. of exposure,
304 when compared to the initial concentration, whereas significant reduction in numbers was
305 achieved in experiment II. However, microbial response to plasma treatment was markedly

306 different in experiment III. The survival curve (not shown) displayed biphasic behaviour, with
307 a turning point at 10 s of CAP-Pen exposure, although significant differences were not found
308 between both D values in the overall comparison. Log reductions in experiments I, II and III
309 at 8 min. of plasma exposure, were significantly different ($p < 0.05$), with a much higher
310 inactivation having been achieved in experiment III than in I and II (55-fold and 8-fold
311 higher, respectively). Such a markedly different cell response to plasma treatment may be a
312 reflection of differences in the inoculation procedure (direct deposition in experiment I and II
313 *versus* filter contact in III), since plasma effectiveness has been reported to depend on the cell
314 exposure method (Kong et al., 2005; Kong et al., 2009).
315 Significantly lower log reductions and higher D values were achieved with chicken muscle in
316 experiment I (in the absence of oxygen), when compared to the same conditions on membrane
317 filters (experiment V, Table 1) and skin (experiment I, Table 2). However, when compared
318 with experiment II and the corresponding treatment on chicken skin (experiment IV), no
319 significant differences were found. Therefore, the presence of oxygen in the carrier gas played
320 a more decisive role on muscle than on skin, since inactivation in experiment II was seven-
321 fold higher than in experiment I, whereas similar inactivation was achieved between skin
322 experiments I and IV. Finally, log reductions in muscle experiment III were significantly
323 higher than the values obtained for the corresponding skin experiment (VII).
324 Although the level of inactivation of *L. innocua* achieved on chicken muscle was lower than
325 that on membrane filters, the highest level achieved, approximately 3.3 log reductions, would
326 undoubtedly be of commercial interest.
327 *3.4 Plasma effectiveness and surface topography*
328 Whereas all experiments were conducted under broadly similar conditions, the effectiveness
329 of plasma treatment decreased in the transition from membrane filters to chicken muscle, and

330 from that to chicken skin. This is strongly suggestive that surface topography plays a
331 significant role in inactivation using gas plasmas. An average of 5.2 x 10^6 cells were
332 deposited on each membrane, and from theoretical considerations based on the dimensions of
333 *L. innocua* cells (assumed to be cylinders with dimensions of 1 μm x 0.5 μm) and the
334 membrane surface area (1 cm^2) stacking would start to occur only as the cells exceeded a
335 concentration in excess of 2 x 10^7 cells. Therefore, we can be reasonably certain that all cells
336 on the surface of the membrane were equally exposed to the gaseous plasma species.
337
338 An SEM image of skin removed from chicken breast is shown in Figure 3a, and reveals a
339 highly irregular topography. It is significant that SEMs taken after inoculation of the skin with
340 *L. innocua* did not reveal the presence of an abundance of bacteria at the surface. In Figure 3b
341 for example, only a small cluster of cells is visible slightly to the right of the centre of the
342 image. It is possible that bacteria which were deposited at the surface were drawn through
343 capillary action into feather follicles and other surface irregularities. Kim *et al.* (1996)
344 reported that bacteria could migrate from the surface to depths up to about 140 μm.
345 Microorganisms at these distances from the surface have previously been reported to be
346 largely unaffected by either thermal or chemical treatments. Moreover, the highest levels of
347 inactivation achieved by these methods was typically no more than 1 log-reduction (Thomas
348 and McMeekin, 1980), the maximum value reported in this work.
349 The surface of the chicken muscle immediately following the two different inoculation
350 procedures employed here is shown in Figures 4a and 4b, the former was obtained after direct
351 deposition, and the latter following filter contact. Figure 4a clearly reveals the presence of
352 fissures. As rigor develops, muscle fibres have been reported to undergo radial shrinkage, and
353 pull away from surrounding connective tissue (Frank, 2001). The resulting fissures, which

354 could provide a route for bacterial penetration, may be enlarged in the presence of excess
355 water. Direct deposition causes the tissue to take up water and swell and this could cause
356 bacteria to be drawn into the tissues with the water and become entrapped between swollen
357 fibres. It has been reported that bacteria may be carried to depths of up to 25 μm by this
358 means (Thomas and McMeekin, 1987; Auty et al., 2005). However, after inoculation by filter
359 contact, a dense fibre network covering the muscle surface was observed (Figure 4b), and this
360 possibly prevented cell migration through these channels. Figures 4c and 4d reveal bacteria
361 concentrated around irregularities at the surface of chicken muscle. Some of these cells would
362 presumably still be vulnerable to plasma species, but others might be protected by cells
363 situated above them.
364
365 *3.6 Prospects for employing gas plasmas for the decontamination of chicken tissue*
366 This work has demonstrated that cold atmospheric gas plasmas have the potential to
367 decontaminate chicken muscle. The results obtained with chicken skin are comparable to
368 those obtained by other techniques that fall under the category of minimal processing and are
369 testament to the difficulties in decontaminating this type of tissue owing to its topography.
370 The plasma device employed was a laboratory model and treatment of chicken on a
371 commercial scale would require the issue of scale-up to be addressed. Advances in this
372 direction are already being made; Cao et al., (2010) have described the construction of a so-
373 called '2-D plasma brush' that comprises multiple plasma plumes. Treatment times with such
374 a device would be significantly reduced over those quoted above, and further scale-up would
375 result in a concomitant decrease in the times necessary to bring about the requisite level of
376 surface decontamination in industrial settings.

The chemical species formed during plasma treatment are highly reactive, and as a result, very short-lived. Therefore treatment with gas plasmas should not result in the formation of compounds that could be viewed as residues. However, it remains necessary to assess the effects of plasma treatment on the sensory and nutritional properties of the foods undergoing treatment in a systematic and exhaustive manner. Such studies were beyond the scope of the present work, although treatment as described above did not result in any obvious changes to the appearance of the chicken muscle or skin.

Lastly, it would be important to take into account consumer perception of any novel decontamination technology and before commercialisation of plasma treatment in the food industry could become a reality, this would need to be addressed.

ACKNOWLEDGEMENTS

This work was financially supported by FICYT (Foundation for Scientific and Technical Research, Asturias, Spain) and MEC (Ministry of Education and Science). The authors would like to thank the Scanning Electron Microscopy service of the University of Oviedo for the assistance provided.

REFERENCES

Auty, M., G. Duffy, D. O´beirne, A. McGovern, E. Gleeson, and K. Jordan. 2005. In situ localization of *Escherichia coli* 0157:H7 in food by confocal scanning laser microscopy. J. Food Prot. 68**,**482-486.

Avens, J. S., S. N. Albright, A. S. Morton, B. E. Prewitt, P. A. Kendall, and J. N. Sofos. 2002. Destruction of microorganisms on chicken carcasses by steam and boiling water immersion. Food Con. 13, 445-450.

401 Cao, Z., Nie, Q., Bayliss, D.L., Walsh, J.L., Ren, C.S., Wang, D. Z., and Kong, M.G. 2010.
402 Spatially extended atmospheric plasma arrays. Plasma Sources Sci. Technol. 19**,** 025003.
403 Carvalho, CM; Gannon, BW; Halfhide, DE, Santos, S.B., Hayes, C.M., Roe, J.M., Azeredo, J.
404 2010. The in vivo efficacy of two administration routes of a phage cocktail to reduce numbers
405 of *Campylobacter coli* and *Campylobacter jejuni* in chickens. BMC Microbiol. 10, Article
406 Number: 232.
407 Deng, X. T., and Kong, M.G. 2004. Frequency range for stable generation of atmospheric
408 glow discharges in He and N_2. IEEE Trans. Plasma Sci. 32, 1709-1715.
409 Deng, X. T., Shi, J. J., and Kong, M.G. 2007a. Protein inactivation by atmospheric pressure
410 glow discharges: capability and mechanisms. J. Appl. Phys. 101, 074701.
411 Deng, S., R. Ruan, C. K. Mok, G. Huang, X. Lin, and P. Chen. 2007b. Inactivation of
412 *Escherichia coli* on almonds using non-thermal plasma. J. Food Sci. 72, M62-M66.
413 Deng, X. T., J. J. Shi, G. Shama, and M. G. Kong. 2005. Effects of microbial loading and
414 sporulation temperature on atmospheric plasma inactivation of *Bacillus subtilis* spores. Appl.
415 Phys. Lett. 87,153901.
416 Frank, J. F. 2001. Microbial attachment to food and food contact surfaces. Adv. Food Nutr.
417 Res. 43, 319-370.
418 Jacobsreitsma, W.F., Bolder, N.M., Mulder, R.W.A.W. 1994. Cecal Carriage of
419 *Campylobacter* and *Salmonella* in Dutch Broiler Flocks at Slaughter - A One-Year Study
420 Poultry Sci. 73, 1260-1266.
421 James, C., James, S.J., Hannay, N., Purnell, G., Barbedo-Pinto, C., Yaman, H., Araujo, M.,
422 Kim, B., Yun, H., Jung S., Jung, Y., Jung, H., Choe, W., and Jo, C. 2011. Effect of
423 atmospheric pressure plasma on inactivation of pathogens inoculated onto bacon using two
424 different gas compositions. Food Microbiol. 28, 9-13.

425 Kim, K. Y., J. F. Frank, and S. E. Craven. 1996. Three-dimensional visualization of
426 *Salmonella typhimurium* to poultry skin using confocal scanning laser microscopy. Lett. Appl.
427 Microbiol. 22, 280-282.
428 Kogelschatz, U. 2002. Filamentary, patterned, and diffuse barrier discharges. IEEE Trans.
429 Plasma Sci. 30,1400-1408.
430 Kong, M.G., Kroensen, G. M. W., Morfill, G. E., Nosenko, T., Shimizu, T., van Dijk, J., and
431 Zimmermann, J. L. 2009. Plasma medicine – an introductory review. New J. Phys. 11,
432 115012.
433 Moisan, M., J. Barbeau, S. Moreau, J. Pelletier, M. Tabrizian, and L'H. Yahia. 2001. Low-
434 temperature sterilization using gas plasmas: a review of the experiments and an analysis of the
435 inactivation mechanisms. Int. J. Pharm. 226, 1-21.
436 Montie, T. C., K. Kelly-Wintenberg, and J. R. Roth. 2000. An overview of research using the
437 one atmosphere uniform glow discharge plasma (OAUGDP) for sterilization of surfaces and
438 materials. IEEE Trans. Plasma Sci. 28, 41-50.
439 Murphy, R.Y., Osaili, T., Duncan, L.K., and Marcy, J. A. 2004. Thermal inactivation of
440 *Salmonella* and *Listeria monocytogenes* in ground chicken thigh/leg meat and skin. Poultry
441 Sci. 83, 1218-1225.
442 Oyarzabal, O.A. 2005. Reduction of Campylobacter spp. by commercial antimicrobials
443 applied during the processing of broiler chickens: A review from the United States
444 perspective. J. Food Prot. 68**,** 1752-1760**.**
445 Perni, S., Jordan, S.J., Andrew, P.W., and Shama, G. 2006. Biofilm development by *Listeria*
446 *innocua* in turbulent flow regimes. Food Con. 17, 875-883.
447 Perni, S., G. Shama, J. L. Hobman, P. A. Lund, C. J. Kershaw, G. A. Hidalgo-Arroyo, C. W.
448 Penn, X. T. Deng, J. L. Walsh, and M. G. Kong. 2007. Probing bactericidal mechanisms

449 induced by cold atmospheric plasmas with *Escherichia coli* mutants. Appl. Phys. Lett. 90,
450 073902.
451 Perni, S., D. W. Liu, G. Shama, and M. G. Kong. 2008a. Cold atmospheric plasma
452 decontamination of the pericarps of fruit. J. Food Protect. 71, 302-308.
453 Perni, S., G. Shama, and M. G. Kong. 2008b. Cold atmospheric plasma disinfection of cut
454 fruit surfaces contaminated with migrating microorganisms. J. Food Protect. 71,1619-1625.
455 Rodriguez De Ledesma, A.M., Riemann, H.P., Farver, T. B. 1996. Short-time treatment with
456 alkali and/or hot water to remove common pathogenic and spoilage bacteria from chicken
457 wing skin. J. Food Prot. 59, 746-750.
458 Russell, S.M., Axtell, S.P. 2005. Monochloramine versus sodium hypochlorite as
459 antimicrobial agents for reducing populations of bacteria on broiler chicken carcasses. J. Food
460 Prot. 68**,** 758-763.
461 Thomas, C. J., and T. A. McMeekin. 1980. Contamination of broiler carcass skin during
462 commercial processing procedures: an electron microscopic study. Appl. Env. Microb. 40,
463 133-144.
464 Thomas, C. J., R. D. O´Rourke, and T. A. McMeekin. 1987. Bacterial penetration of chicken
465 breast muscle. Food Microbiol. 4, 87-95.
466 Vleugels, M., G. Shama, X. T. Deng, E. Greenacre, T. Brocklehurst, and M. G. Kong. 2005.
467 Atmospheric plasma inactivation of biofilm-forming bacteria for food safety control. IEEE
468 Trans. Plasma Sci. 33, 824-828.
469 Walsh, J. L., and M. G. Kong. 2006. Sharp bursts of high-flux reactive species in sub-
470 microsecond pulsed atmospheric pressure glow discharges. Appl. Phys. Lett. 89, 231503.

471 Xiong, R., G. Xie, A. S. Edmondson, R. H. Linton, and M. A. Sheard. 1999. Comparison of
472 the Baranyi model with the modified Gompertz equation for modeling thermal inactivation of
473 *Listeria monocytogenes* Scott A. Food Microbiol. 16, 269-279.
474

475 **FIGURE CAPTIONS**
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478 Figure 1. Schematic of the cold atmospheric plasma pen (CAP-Pen) apparatus

479 Figure 2. Plasma inactivation of *Listeria innocua* deposited on the surface of membrane
480 filters: ●-experiment I; ▲-experiment II; ▲-experiment III; ◆-experiment IV; ■-experiment
481 V; ◆-experiment VI; ■-experiment VII (see experimental conditions in Table 1). Dashed
482 lines correspond to concentrations below the detection limit.Figure 3. Scanning electron
483 micrographs. a) non-inoculated skin sample (x1000 magnification), b) cluster of bacteria
484 present on inoculated skin (x1000 magnification).

485 Figure 4. Scanning electron micrographs a) muscle sample newly inoculated by aliquot
486 deposition (x2500 magnification), b) muscle sample newly inoculated by filter contact (x2000
487 magnification) and C) and D) muscle sample inoculated by filter contact after 24 h of
488 incubation at 25 ºC (x 2000 and x 6000 magnification, respectively).

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FIGURE 1

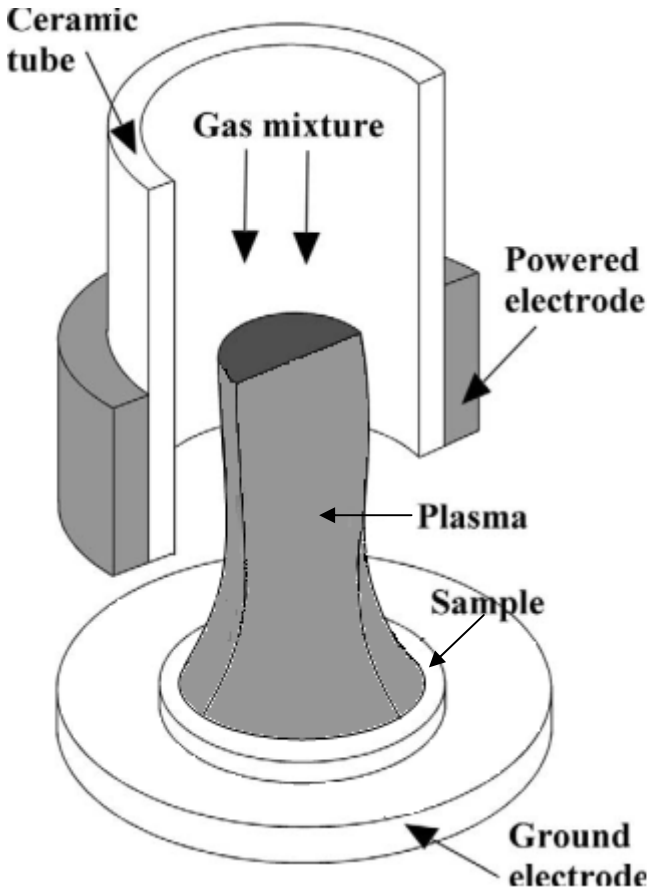

Ceramic tube
Gas mixture
Powered electrode
Plasma
Sample
Ground electrode

FIGURE 2

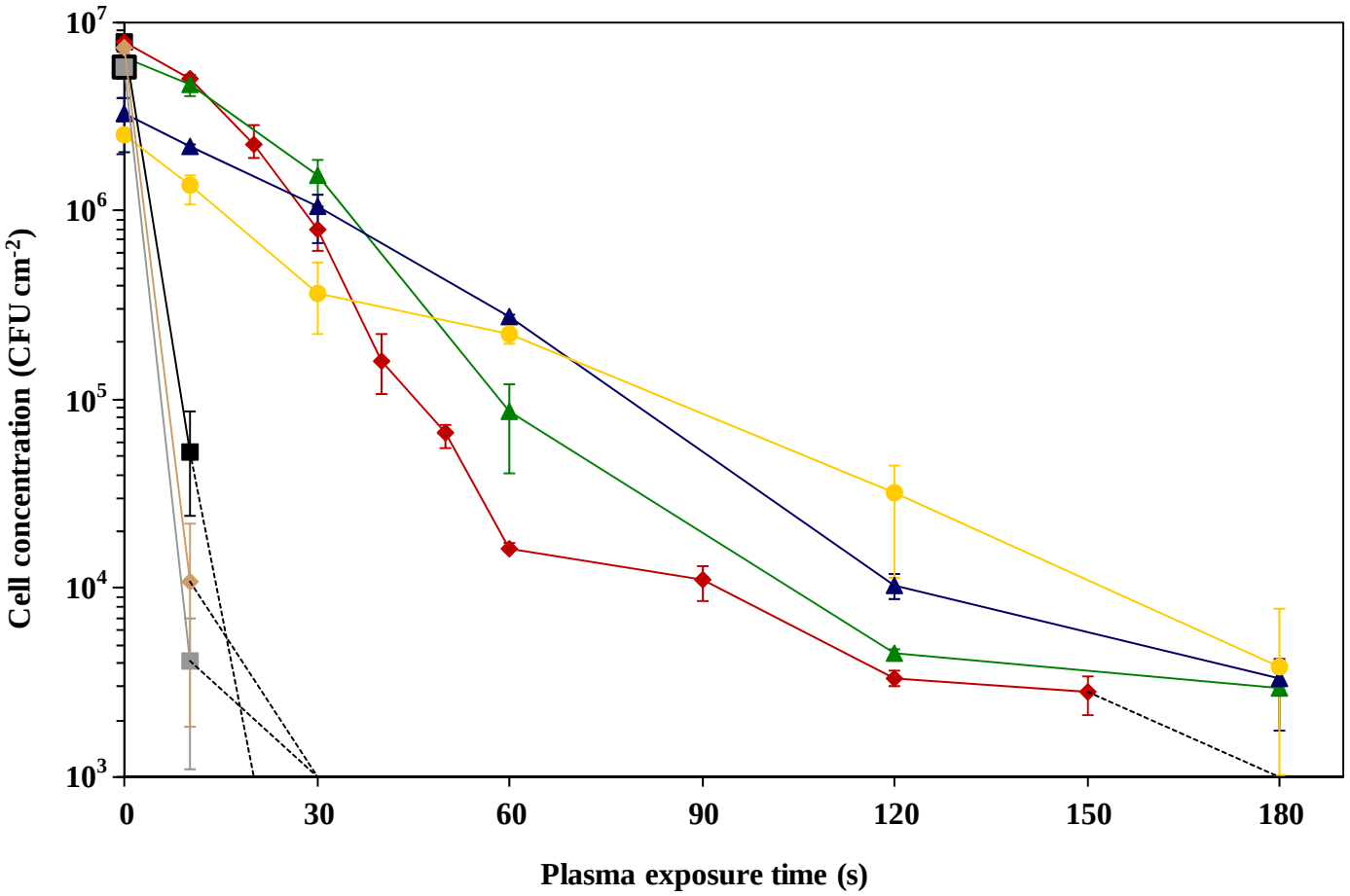

Cell concentration (CFU cm-2)
10^7
10^6
10^5
10^4
10^3
0
30
60
90
120
150
180
Plasma exposure time (s)

TABLE 1. Inactivation of *Listeria innocua* deposited on membrane filters

Exp.[1]	CAP-Pen Treatment Conditions					Log-reductions [2, 3] (log CFU/cm^2)					D-values [4] (s)
	Gap (cm)	Voltage (kV)	Frequency (kHz)	He (L/min)	O_2 (mL/min)	10 s	30 s	1 min	2 min	3 min	
$I_{(a)}$	1.5	6.0	38.5	5.0	0.0	$0.27 \pm 0.07^{a}_{A}$	$0.86 \pm 0.17^{b}_{A}$	$1.05 \pm 0.03^{b}_{A}$	$1.96 \pm 0.31^{c}_{A}$	$2.95 \pm 0.40^{d}_{A}$	60.7 ± 7.8^{a}
$II_{(a)}$	1.5	6.5	38.5	5.0	0.0	$0.16 \pm 0.14^{a}_{A}$	$0.49 \pm 0.01^{b}_{B}$	$1.05 \pm 0.15^{c}_{A}$	$2.48 \pm 0.10^{d}_{B}$	$3.00 \pm 0.06^{e}_{A}$	$56.0 \pm 0.8^{a,b}$
$III_{(b, c)}$	1.0	6.5	38.5	5.0	0.0	$0.15 \pm 0.04^{a}_{A}$	$0.63 \pm 0.12^{b}_{B}$	$1.92 \pm 0.23^{c}_{B}$	$3.15 \pm 0.01^{d}_{C}$	$3.34 \pm 0.01^{d}_{A}$	$37.0 \pm 1.4^{b,c}$ $^{*}322.1 \pm 35.1^{d}$
$IV_{(b)}$	1.0	7.0	38.5	5.0	0.0	$0.19 \pm 0.01^{a}_{A}$	$1.01 \pm 0.10^{b}_{A}$	$2.68 \pm 0.01^{c}_{C}$	$3.37 \pm 0.03^{d}_{C}$	ND	$24.2 \pm 0.8^{c,e}$ $^{*}108.5 \pm 10.2^{f}$
$V_{(b)}$	1.0	8.0	38.5	5.0	0.0	$2.23 \pm 0.23_{B}$	ND	ND	ND	ND	4.5 ± 0.5^{e}
$VI_{(b, c)}$	1.0	9.0	23.0	5.0	25.0	$3.02 \pm 0.51_{C}$	ND	ND	ND	ND	3.4 ± 0.6^{e}
$VII_{(c)}$	1.0	11.0	23.0	5.0	25.0	$3.26 \pm 0.37_{C}$	ND	ND	ND	ND	3.1 ± 0.3^{e}

ND: not detectable (detection limit, 1 x 10^3 CFU/cm^2)
[1] Initial viable counts of experiments lacking common letters are significantly different ($P \leq 0.05$).
[2] Within the same row (same experiment), log-reductions bearing different superscripts are significantly different ($P \leq 0.05$).
[3] Within the same column (same exposure time), log-reductions bearing different subscripts are significantly different ($P \leq 0.05$).
[4] D-values bearing different superscripts are significantly different ($P \leq 0.05$).
*D-values of the second phase (biphasic survival curves)

TABLE 2. Inactivation of *Listeria innocua* and total microflora on the skin of chicken breasts

Exp.	CAP-Pen Treatment Conditions[+]					Log-reductions (log CFU/cm^2)[1,2,3]				D-values[4,5] (min.)
						4 min exposure		8 min exposure		
	Gap (cm)	Voltage (kV)	Frequency (kHz)	O_2 (mL/min)	Skin type	Total	*Listeria*	Total	*Listeria*	($\frac{Listeria}{Total}$)
I	1.0	8.0	38.5	0.0	Rough	$_{(a)}0.16 \pm 0.05^{a}{}_{A}$	$_{(a)}0.14 \pm 0.14^{a}{}_{A}$	$_{(b)}0.49 \pm 0.09^{a}{}_{A}$	$_{(b)}0.56 \pm 0.12^{a}{}_{A,D}$	$16.9 \pm 4.3^{a,d}{}_{A}$ $18.2 \pm 3.5^{a}{}_{A}$
II	1.0	11.0	23.0	25.0	Rough	$_{(a)}0.02 \pm 0.04^{a}{}_{B}$	$_{(a)}0.00 \pm 0.11^{a}{}_{A}$	$_{(a)}0.01 \pm 0.21^{a}{}_{B}$	$_{(a)}0.22 \pm 0.18^{A}{}_{B}$	$28.6 \pm 1.9^{b}{}_{B}$ $52.8 \pm 15.3^{b}{}_{A}$
III	1.0	11.0	23.0	25.0	Smooth	$_{(a)}0.05 \pm 0.01^{a}{}_{B}$	$_{(a)}0.05 \pm 0.04^{a}{}_{A}$	$_{(a)}0.01 \pm 0.02^{a}{}_{B}$	$_{(b)}0.18 \pm 0.01^{b}{}_{B}$	$47.7 \pm 2.7^{c}{}_{B}$ $89.2 \pm 18.9^{c}{}_{A}$
IV	1.0	11.0	30.0	25.0	Rough			$0.48 \pm 0.10^{a}{}_{A}$	$0.50 \pm 0.06^{a}{}_{A}$	$16.3 \pm 2.1^{a,d}{}_{A}$ $17.2 \pm 4.0^{a}{}_{A}$
V	1.0	16.0	30.0	25.0	Rough			$0.69 \pm 0.03^{a}{}_{A,C}$	$0.40 \pm 0.17^{b}{}_{A,B}$	$21.8 \pm 7.3^{d}{}_{A}$ $11.7 \pm 0.6^{a}{}_{A}$
VI	1.0	16.0	30.0	50.0	Rough			$0.50 \pm 0.04^{a}{}_{A}$	$0.55 \pm 0.00^{a}{}_{A,D}$	$14.6 \pm 0.1^{a,e}{}_{A}$ $16.1 \pm 1.2^{a}{}_{A}$
VII	1.0	16.0	30.0	100.0	Rough			$0.91 \pm 0.23^{a}{}_{C}$	$0.94 \pm 0.20^{A}{}_{C}$	$8.8 \pm 1.7^{e}{}_{A}$ $9.1 \pm 2.0^{a}{}_{A}$
VIII	0.5	16.0	30.0	100.0	Rough			$0.57 \pm 0.20^{a}{}_{A}$	$0.60 \pm 0.13^{a}{}_{A,D}$	$13.9 \pm 3.3^{a,e}{}_{A}$ $15.1 \pm 4.7^{a}{}_{A}$
IX*	1.0	16.0	30.0	100.0	Rough			$0.77 \pm 0.25^{a}{}_{C,D}$	$0.77 \pm 0.25^{A}{}_{C,D}$	$11.1 \pm 3.9^{a,e}{}_{A}$ $15.7 \pm 1.5^{a}{}_{A}$

[+] Helium flow (5 L/min)

* Samples were not incubated to allow cell attachment, but treated immediately after cell deposition

[1] Within the same column (4 or 8 min exposure) and the same type of count (Total or *Listeria*), log-reductions lacking common letters (subscripts) are significantly different ($P \leq 0.05$).

[2] Within the same row (same experiment) and same treatment (4 or 8 min), log-reductions bearing different superscripts (uppercase letters on the right) are significantly different ($P \leq 0.05$).

[3] Exp. I to III: within the same row (same experiment) and same type of count (Total or *Listeria*), log-reductions bearing different subscripts (brackets on the left) are significantly different ($P \leq 0.05$).

[4] For like comparisons (either *Listeria* or Total), D-values lacking common superscripts are significantly different ($P \leq 0.05$).

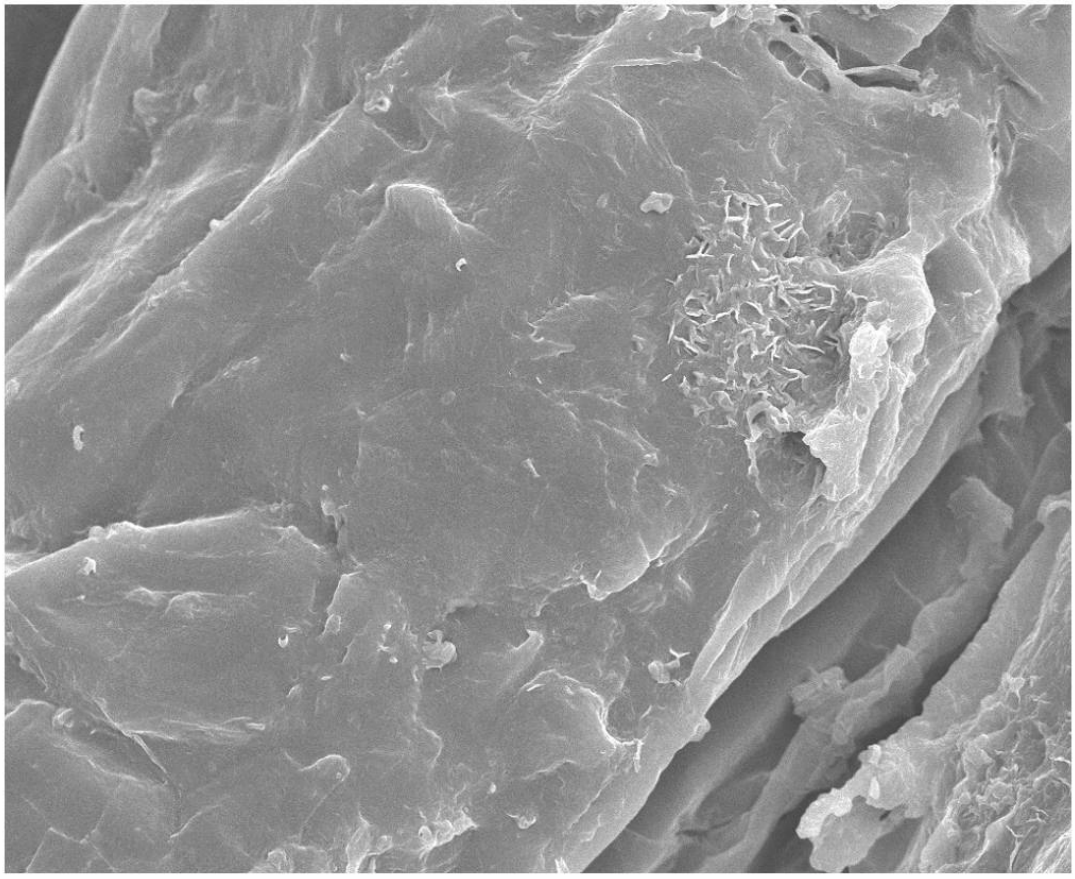
[5] For comparisons between *Listeria* and total counts for the same experiment, D-values lacking common subscripts (capital letters) are significantly different ($P \leq 0.05$).

TABLE 3. Inactivation of *Listeria innocua* on chicken breast muscle

Exp.[1]	CAP-Pen Treatment Conditions						Log-reductions [2, 3] (log CFU/cm^2)						D-values [4] (min)
	Gap (cm)	**Vol.** (kV)	**Freq.** (kHz)	**He** (L min^{-1})	**O_2** (mL min^{-1})	**Inoculum**	**10 s**	**30 s**	**1 min**	**2 min**	**4 min**	**8 min**	
$I_{(a)}$	1.0	8.0	38.5	5.0	0.0	Aliquot	--	--	--	--	--	$0.06\pm0.04_{A}$	162.2 ± 95.8^{a}
$II_{(b)}$	1.0	11.0	30.0	5.0	25.0	Aliquot	--	--	--	--	--	$0.41\pm0.02_{B}$	19.5 ± 0.8^{b}
$III_{(a)}$	1.0	16.0	30.0	5.0	100.0	Filter	1.25 ± 0.26^{a}	1.58 ± 0.34^{a}	$1.61\pm0.03^{a,b}$	$2.17\pm0.56^{b,c}$	2.71 ± 0.54^{c}	$3.30\pm0.14^{d}_{C}$	0.1 ± 0.0^{b} $*3.4 \pm 0.3^{b}$

[1] Initial viable counts of experiments lacking common letters are significantly different ($P \leq 0.05$).
[2] Within the same row, log-reductions bearing different superscripts are significantly different ($P \leq 0.05$).
[3] Within the same column, log-reductions bearing different subscripts are significantly different ($P \leq 0.05$).
[4] D-values bearing different superscripts are significantly different ($P \leq 0.05$)
*D-values of the second phase (dual-slope survival curves)

A)



B)

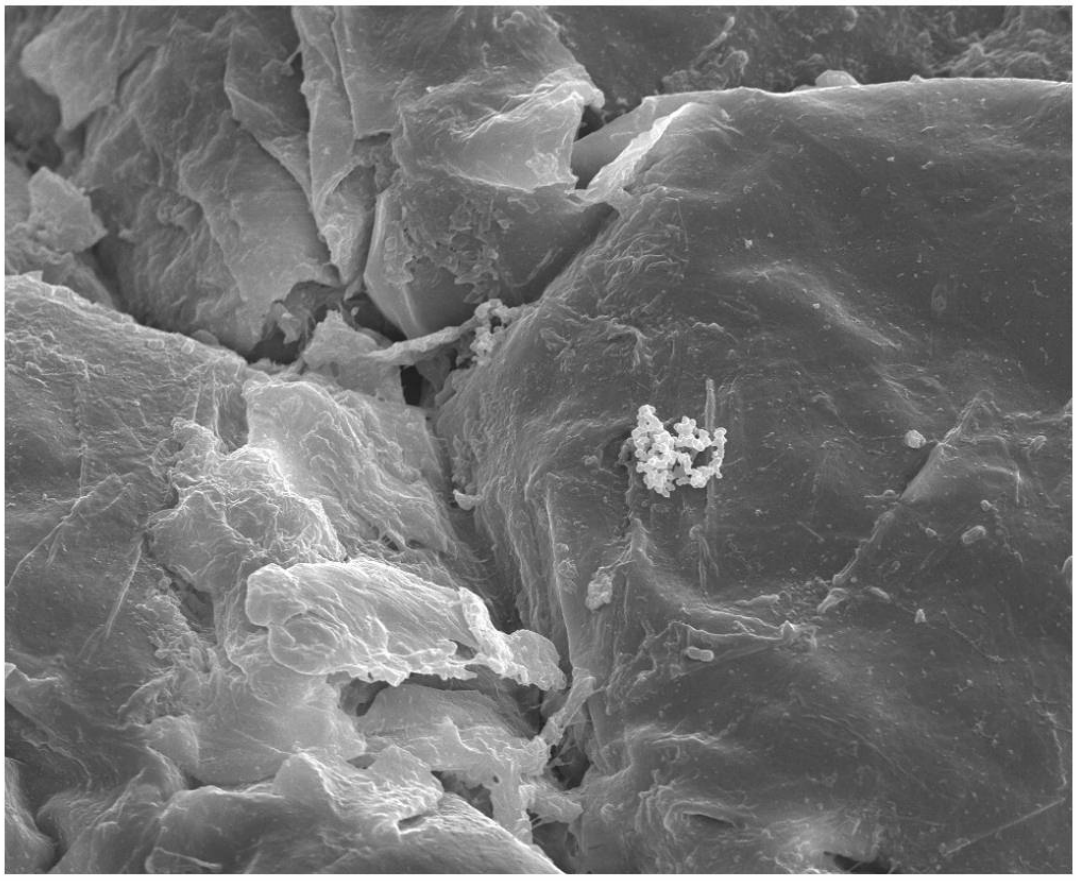


FIGURE 4

A)

B)

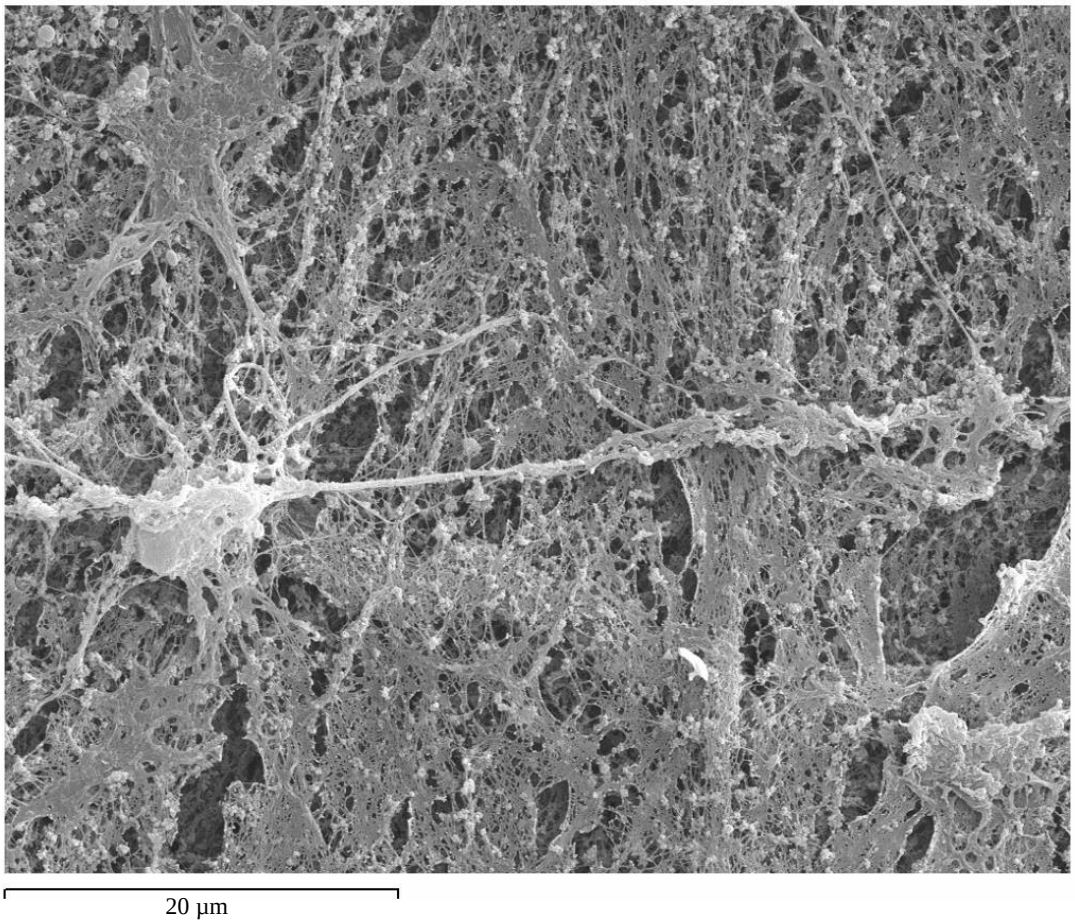
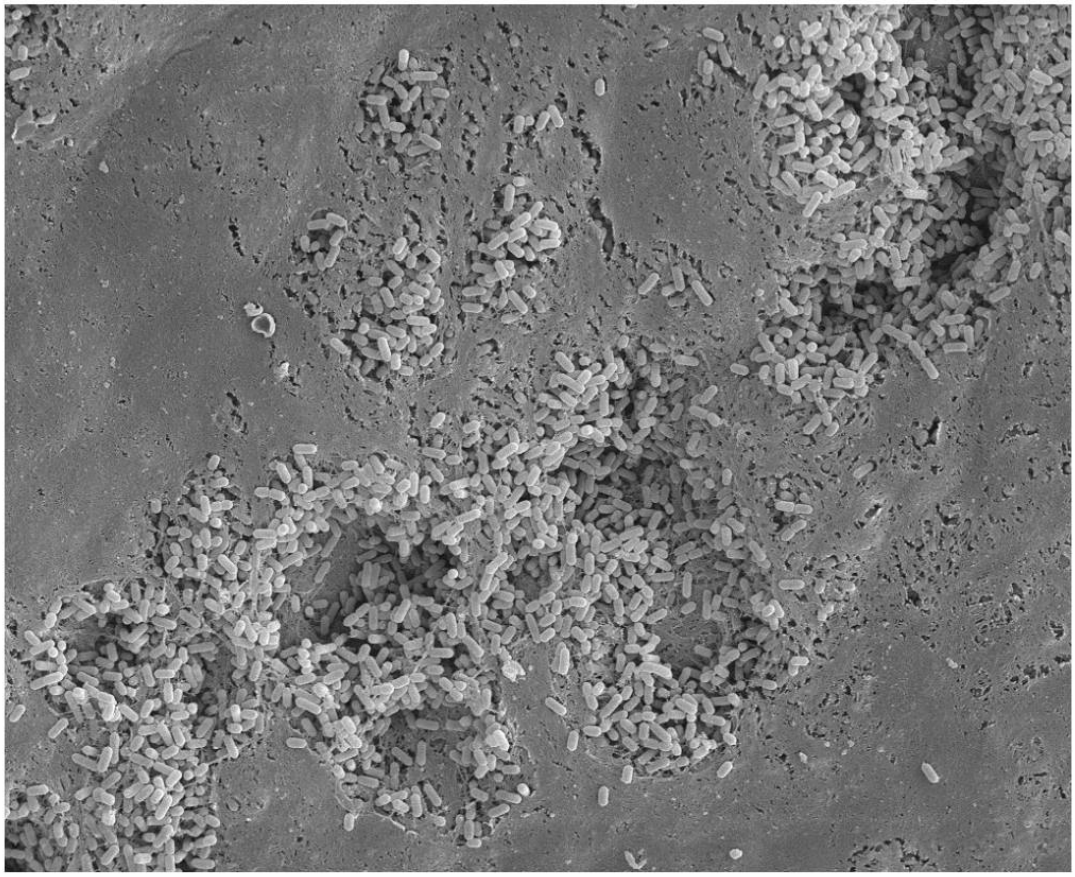


FIGURE 4

C)



D)