FINAL REPORT

Microplasma Disinfection of Meat

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1.0   EXECUTIVE SUMMARY

Quality and reliability of meat products is highly important to the consumer. Consequently, prevention and control of bacterial contamination is vital to the reputation and commercial viability of meat processors.

Recent technological innovation has allowed the economic electrical generation of microplasmas in gases, which then acquire a short-lived ability to kill micro-organisms. It was the aim of this work to investigate the new opportunity presented by microplasma treatments of atmospheric gases. Such treatments have the advantage, over chemical treatments, that no chemical residue will be left.

As a response to the above motivations, inhibition of the growth of micro-organisms using air that had been passed through a microplasma apparatus was investigated. In preliminary work, yeast (*Saccharomyces cerevisiae*) was used as a test organism. When air or oxygen-enriched air were used as feed gas to the microplasma apparatus, the product gas stream was found to inhibit yeast growth. No inhibition was found when the inert gas argon was used as feed gas, suggesting that ozone produced from oxygen in the air was responsible for the effect. Accordingly, measurements of the ozone level were made. It was found that a high degree of inactivation of yeast, exposed as a thin layer on agar medium, was observed if an ozone concentration of 25 parts per million (ppm) was applied for 10 minutes or longer. In some cases only 2.5 or 5 minutes exposure was enough. However, thicker layers of yeast were not effectively inactivated.

Work was extended to bacteria found on retail meat samples by the use of selective diagnostic agar plates. These enabled the presumptive identification of four different families of pathogenic bacteria: *Campylobacter*, coliforms (including *E. coli*) *Salmonella*, and *Staphylococcus*. In addition, a more generally-sensitive plate was used (detecting not only *E. coli* but also *Enterococcus/Enterobacter*). The effects of ozone on these specific organisms was followed by the use of these selective plates for both treated and control cultures. As with yeast, an ozone concentration of at least 25 ppm was found to be necessary for appreciable inactivation of these bacteria. 24-30 plates, for each species, were tested at 25-38 ppm of ozone. Most exposures were 8 minutes long. Growth was assessed by counting of bacterial colonies.

- Coliforms, as well as *Enterobacter*/*Enterococcus,* were found to be reliably inactivated on agar plates by ozone concentrations between 25 ppm and 38 ppm. There were many instances where growth was inhibited by 40 times or more.

- *Campylobacter* species and *Staphylococcus* species showed inconsistent or only moderate inactivation by ozone levels between 25 ppm and 38 ppm. At least one plate of each showed no effect, but at least one showed 40 times reduction.
- *Salmonella* species proved to be the most resistant, with no instance of growth reduction of 40 times or more by 25-38 ppm of ozone.

It seems likely that a further increase in ozone concentration would be useful, and should be investigated. This is especially relevant if insensitive organisms such as *Salmonella* are to be controlled.

An estimate was made of the cost of up-scaling the microplasma-based treatment to suit a meat processing facility. Data on meat plant size and cost obtained from published studies was combined

with project data on ozone sensitivity, and additional published data on the sensitivity of different organisms, to estimate the amount of ozone required. Equipment manufacturer's data and prices, as well as data on ozone lifetimes was then been used to estimate the ozone generation costs and the cycle time. Finally an estimate of metering and control costs was made. It was found that the costs were dominated by the depreciation on the building, including the air-lock mechanism required for reasons of safety. Projected operating costs were dominated by the labour costs incurred in loading/unloading the transport mechanism required to transit the airlock.

There have been well-publicised and - to the meat industry - very costly outbreaks of STEC (Shiga-toxin producing *E. coli*) in Canada and the USA, as well as consequent sensitivity about the STEC content of meat imported into the USA. An extension of the present work to STEC was also considered here. It was concluded that this is desirable, but that work with STEC will involve a high standard of laboratory containment.

2.0 INTRODUCTION

This project was designed to assess the feasibility of using gases treated with an electrical micro-discharge to reduce the levels of bacterial contamination of meat. The micro-discharge produces a plasma which can exist for short periods at room temperature and pressure. Depending on the feed gas used, then ozone or other reactive species (free radicals) are produced. This technique appears promising, because even relatively-resistant, dormant forms of bacteria (spores) were inactivated [1,2] by the activated gases produced in such discharges. In air or oxygen, one of the main products of the micro-discharge is ozone.

The scope of the project included acquisition and assembly of suitable equipment, and validation of the operation of that equipment. This enabled the development of experimental protocols by testing the effect of the activated gases on harmless organisms (yeast and lactobacillus were chosen). The concentrations of ozone that could be produced, and which were effective in inactivation of yeast, were measured. Following this, tests on several mildly-pathogenic types of bacteria could be carried out.

Once the efficacy of the micro-discharge process had been demonstrated, the applicability to industry needed to be assessed. Accordingly, the capital and running costs of a scaled-up plant were estimated, based on published data and costings for meat-processing facilities.

This research was able to operate at ozone concentrations up to 38 parts per million (ppm). This is far in excess of the 0.04 to 0.1 ppm reported to be lethal to bacteria (Stockinger, 1959, quoted by Scott and Leshner [3]). Whilst the concentrations used here proved very effective at inhibiting the growth of yeast and of some species of the bacteria, they were inadequate for some other bacteria.

3.0 PROJECT OBJECTIVES

The Project Objectives were defined by the scope of 6 Milestones:

1. New purchase, assembly and functioning of micro-discharge equipment: the report for this Milestone 1 was submitted 30th November 2016.

2. Demonstration of the generation, by micro-discharge, of active species as a function of different feed gases (e.g. ozone from pure oxygen compared to ozone from air). The report for this Milestone 2 was submitted 17th February 2017.

3. Quantitate the degree of inactivation of non-pathogenic microbes such as yeast or lactobacillus by active species produced from two different gases (report submitted 17th May 2017).

4. Estimate the operating costs of a scaled-up plant based on the power consumption and capital depreciation, to be informed by the performance of the lab-scale equipment (report submitted 26th June 2017).

5. Quantitate the degree of inactivation of PC-2 grade pathogenic microbes (*E. coli, Staph. aureus* etc) by active species produced from two different gases (report submitted 4th September 2017).

6. Compile a review of results with a case for extension to STEC organisms (report submitted 7th November 2017).

4.0   METHODOLOGY

4.1   Assembly and functioning of micro-discharge equipment

Micro-discharge apparatus (Model Pure-2002) was purchased from EP Purification Inc (Illinois, USA), and ozone measurement (series 200) and safety monitoring (Model SM70) meters from Aeroqual Limited (Auckland, NZ). As quantities of ozone were generated, and this can be toxic to the operator above a low threshold, both measurement and thresholded alarm devices were required.

Exposure of yeast and bacteria to micro-plasma product gas was carried out in a fume-extraction hood. Therefore all the required equipment was assembled in a laboratory, previously used for chemical synthesis, which contains such a hood.

The micro-plasma apparatus requires a source of **dry** gas: air, oxygen or argon. Dry gases were obtained from certified, highly compressed gas cylinders (20,000 kPa). The necessary valves and pressure-reduction regulators were obtained locally.

4.2 Demonstration of active species as a function of different feed gases

The milestone required the running of the micro-plasma equipment, and measurement of the ozone output. This was done for both dry air and oxygen-enriched feed gases.

A gas handling, metering and dilution panel was constructed from stock items to enable the measurement of higher ozone concentrations than were measurable directly. This permitted quantitation of the ozone output. In addition to dry air from a cylinder, oxygen-enriched air was generated using an "oxygen-generator" hired from a medical supplies company. Using this apparatus, the operation of the micro-discharge apparatus in both air and oxygen-enriched air was verified.

As ozone is toxic to personnel at above 0.1 ppm in 8-hour exposure, or above 0.3 ppm in short-term exposure [4], the fixed ozone monitor/alarm (Aeroqual SM70, 0 - 0.15 ppm) mounted close to the apparatus was set to a threshold of 0.1 ppm, and the hand-held ozone monitor (Aeroqual Series 200) was also used whenever an ozone generator was running.

The results indicate that the ozone generator, when supplied with dry air as a feed gas, generated ozone at a concentration of approximately 2000 ppm. When supplied with the output of the oxygen concentrator, it generated ozone at concentration of approximately 4500 ppm. The ozone increase obtained in this way ranged between 110% and 140% in different experiments. See Appendices 1 & 2 for details.

4.3 Quantitation of inactivation of non-pathogenic microbes

For this milestone, the output of the micro-plasma equipment was applied to non-pathogenic microbes. For these, microbes used in fermentation of foods were used: yeast, *Saccharomyces cerevisiae*, and a bacterium cultivated from commercial yoghurt. Two different feed gases, air and argon were used.

The gas from the micro-plasma apparatus was diluted with air and used to treat agar plates seeded (Appendix 3) with cultures of yeast or lactobacillus. Treatment was carried out for specified times whilst monitoring the ozone concentration in the treatment chamber. The plates were sealed and incubated at 30˚C for 3-7 days and compared with non-exposed controls. It could then be observed how many colonies could grow compared to the non-exposed microbes.

All media were pre-sterilised by steam autoclave, and plastic consumables (Petri dishes, inoculation loops etc) were obtained gamma-sterilised.

Culture of the non-pathogenic microbes:
1. Bakers' yeast (*Saccharomyces cerevisiae*) was seeded into liquid YEP medium (Yeast Extract/Peptone) and shaken at 30˚C;
2. commercial yoghurt was seeded into a medium appropriate for lactobacillus (MRS broth) and shaken at 30˚C until a dense suspension was obtained.

The liquid cultures were inoculated by a 3-way streak procedure onto YM (Yeast Morphology) agar or MRS agar respectively in small open Petri plates. This procedure results in a two-stage thinning of the culture such that dense streaks of culture as well as isolated single colonies of the organisms can normally be produced (see Appendix 1). The Petri plates were then exposed for defined times to the activated gases in question. Air or argon were used as feed gases to the micro-discharge apparatus.

The gas from the micro-discharge apparatus was fed into a vertical cylinder (19cm internal diameter and 35 cm tall) where it was entrained and mixed with ascending, circulating air. The airflow was driven around the cylinder by an electrical fan, and forced to ascend by the draught within a fume cupboard. In the case of air feedstock, the mixing ratio was adjusted to give pre-determined concentrations (approx. 10 or 26 ppm) of ozone. The same mixing ratios were used for argon feedstock. Open Petri plates containing appropriate agar medium inoculated with the micro-organism of interest were exposed to the circulating gas for defined times.

Exposed plates were sealed immediately after exposure and incubated for 3 – 7 days at 30˚C.

The plates were then examined for growth of the two different organisms, and compared with control plates (prepared at the same time but not exposed). Examples are shown in Appendix 4.

4.4 Estimate the operating costs of a scaled-up plant

Based on literature and own data on the ozone concentration required to kill micro-organisms, as well as upon two published studies of the costs of meat plants from the USA, the operating cost of a scaled-up plant was estimated: see Appendix 5.

The approach was to estimate the add-on cost to an already-costed plant to take advantage of the new

technology.

The cost and specific building needs of ozone-generating and application equipment were covered, as were estimates of safety and regulating equipment. In a first instance, a batch-operated system was envisaged. This was done because it requires less investment: if successful a continuous, and likely more expensive, system could be envisaged at a later date.

4.5 Quantitation of inactivation of pathogenic microbes

(i) Experimental design

Retail-packaged meat samples (chicken, beef or lamb) from local outlets were purchased at least 3 days before the "best by" date. Sterile foam bud swabs ("PUR-blue", World Bioproducts) were used to wipe the surface of each meat sample and then to inoculate species-selective agar plates. These plates not only inhibited the growth of most families of bacteria, but were also "chromogenic" in that they contained dye pre-cursors such that different species of bacteria gave differently-coloured colonies. Control plates were incubated under appropriate conditions for 2-3 days in order to reveal what organisms had been present on the samples. Experimental plates were exposed to ozone before being incubated and examined in parallel with the controls.

Five different types of agar plates were used, four of these were specific enough to give at least presumptive indication of the presence of:

1. *Campylobacter jejuni* or *Campylobacter coli*.

2. Coliforms, specifically *Escherichia coli* (other coliforms, e.g. *Klebsiella*, are also detected but can be distinguished from E. coli).

3. *Salmonella* (no distinction possible between *S. typhimurium*, *S. salford* or *S. IIIb*)

4. *Staphylococcus aureus* or *Staphylococcus saprophyticus* (separately identifiable).

5. A further chromogenic, but not growth-selective, agar was used to give an indication of the presence of other bacteria, even if not within one of the above pathogenic classifications.

(ii) Experimental method

All media were obtained sterile, and plastic consumables (sample swabs, inoculation loops etc) were obtained gamma-sterilised.

Surface samples were obtained from the meat pieces (chicken, beef or lamb from retail outlets), and then inoculated on to one of a series of selective agar medium Petri plates. As described in Paragraph 4.3 and Appendix 3, the inoculum was spread out ("streaking out") in two directions from the inoculation site to give a dilution effect. However, for the present Milestone two distinct inocula were used per plate, which was accordingly delimited along one diameter. The photographs in the Appendices, e.g. the control plates in Appendix 6 (e.g. Figure A6.2), show the results of this after growth of bacterial colonies. The Petri plates were then exposed for defined times to the activated gases in question. Air or argon were used as feed gases to the micro-discharge apparatus.

The gas from the micro-discharge apparatus was fed into a vertical cylinder where it was entrained and mixed with ascending, circulating air. In the case of air feedstock, the mixing ratio was adjusted to give pre-determined concentrations (approx. 25 or 35 ppm) of ozone. The same mixing ratios were used for argon feedstock. Open Petri plates containing appropriate agar medium inoculated with the micro-organism of interest were exposed to the circulating gas for defined times.

Exposed plates were sealed immediately after exposure and incubated for 2 days at 37°C. Most plates were incubated aerobically. However, plates selective for the oxygen-intolerant *Campylobacter* had to be incubated in a micro-aerobic atmosphere. This was produced in a container of 1 litre volume by allowing a small candle to burn until oxygen was sufficiently reduced. After incubation, the plates were examined for growth in the two different regions, and compared with control plates (prepared at the same time but not exposed to ozone). Examples are shown in Appendix 6.

Meat pieces were sampled on the day of purchase and then on further days up to the "best by date". Between experiments, they were stored at 6°C in a sterile container.

4.6 Compilation of a case for extension to STEC organisms

This milestone was fulfilled by reviewing the nature and risks posed by STEC. The following headings were used:

(i) Background information on STEC bacteria

STEC (Shiga-toxin producing *E. coli*) organisms such as serotype O157:H7 and other variants [5] such as O26, O45, O103, O111, O121, and O145 were introduced. The ability of the Shiga toxins to cause severe and bloody diarrhea, and sometimes death, was pointed out [6-8]. The tendency of cattle to act as hosts for the STEC *E. coli* O157:H7, and the resulting shedding of this organism in the faeces, was mentioned. Several aspects of STEC outbreaks were highlighted:

1. Risks to the meat industry: the consequences of notable outbreaks of STEC organisms in meat products were reviewed. The risk is greatest with ground meat products: outbreaks have occurred not only with beef [9] but also pork [10]. The consequent sensitivity and imposition of strict procedures and penalties by markets such as the USA was pointed out [11]. The history of significant financial penalties in civil cases following STEC outbreaks in N. America was also mentioned [12,13].

2. Non-meat-related outbreaks: the many outbreaks of illness due to STEC where vegetables rather than meat were the cause, was mentioned [14]. In most cases these seem to involve one of the six non-O157 STECs.

3. Detection method for STEC O157. The standard microbiological methods [15] for detecting *E. coli* O157:H7, were mentioned, as was the disadvantage of the prolonged time required. The recent introduction of quicker techniques such as DNA extraction followed by PCR, as well as the on-going development of fluorescent and antibody detection methods were also brought in.

(ii) Relevance of this project's results

The results presented here give some indications as to the possible sensitivity of STEC to ozone.

1. It was demonstrated that air processed in the microplasma apparatus was capable, due its ozone content, of inactivating thin layers of both *E. coli* and other coliforms on agar. At ozone concentrations of 28 ppm or greater, a high degree of inactivation (10 times or even 50 times or more in the case of coliforms) was often observed.

2. In the case of *Salmonella* species, even when ozone concentrations of 38 ppm were employed, only a poor degree of inactivation was observed. In the case of *Campylobacter* and *Staphylococcus* species, inconsistent growth suppression was achieved.

3. It seems likely that STECs, being strains of *E. coli*, will also be sensitive to ozone treatment. However, some STEC are more resistant to certain environmental challenges (e.g. acidic pH) than non-STEC strains, so there must remain some doubt on this point.

Therefore, if ozone is to be used to control bacterial contamination of meat, then tests of its efficacy against STECs and particularly *E. coli* O157:H7 would be advisable.

5.0   PROJECT OUTCOMES

5.1 Inactivation of yeast

Tests on yeast were done as envisaged. This is an easy organism to culture and work with, and a useful preliminary to working with bacteria.

Yeast from a laboratory culture was available: streaking of this liquid on agar plates gave, if unexposed to ozone, dense colonies after 2-3 days of incubation (see Appendix 3).

It was found that exposure to ozone prior to incubation inhibited the growth of yeast in a concentration- and time-dependent manner:

1. After exposure to less than 12 ppm, for up to 45 minutes, there was some inactivation of yeast, but this was poorly reproducible (Figures A4.1 and A4.2).

2. After exposure to more than 25 ppm, for more than 10 minutes, there was definite inactivation of yeast by these higher ozone concentrations (25-37 ppm), see Figures A4.3 and A4.4. The agar plates showed very definite reduction in the number and density of colonies after 10, 20 and especially after 30 minutes exposure.

3. After exposure to 25-37 ppm, for a series of shorter times designed to tie down the time required, it was found (Figures A4.5 to A4.8) that moderate inactivation (colonies reduced in the secondary streak, much reduced in the tertiary streak) was observed after 2.5, and 5 minutes exposure. Very great inactivation (colonies much reduced in the secondary and

tertiary streaks, moderate reduction in the primary streak) was observed after 7.5, 10, 12.5 and 15 minutes exposure.

It can be concluded that an exposure period of 7.5 minutes was sufficient for thinly-spread inoculations, but that thicker ones required either much longer times or higher ozone concentrations.

5.2 Inactivation of bacteria

The results presented in Appendices 6-8 were collated according to the degree of inactivation achieved with a given organism. The many different concentrations of ozone that were used were put into two groups of lower and higher concentrations. As also with yeast, it was noticed that there was a substantial increase in the inhibitory effect at ozone concentrations > 25 ppm, which was therefore made the dividing line between the two groups.

Table 5.2.1. The results of multiple tests of the efficacy of two concentrations of ozone at preventing bacterial growth of five classes of bacteria. The data includes all durations of treatment.

Colony count reduction	Campylobacter		Coliforms		Enterobacter/ Enterococcus		Salmonella		Staphyloccus	
	21-25 ppm O_3	28-39 ppm O_3	21-25 ppm O_3	28-39 ppm O_3	21-25 ppm O_3	28-39 ppm O_3	21-25 ppm O_3	28-39 ppm O_3	21-25 ppm O_3	28-39 ppm O_3
0		1					4			1
1.5-9.9x	4	7	4			3	5	14	4	7
>1.5-9.9x	1	5		2		2	1	11	2	12
10-40x		6		7	4	13		4	1	2
>10-40x			2	1						4
> 40x		5		19	2	15			1	

Table 5.2.2. The data of Table 5.1 in which the 21-25 ppm data have been normalised against the 28-39 ppm data by multiplying them by the ratio of the numbers of experimental runs exposed within the two concentration ranges. The normalization enables a comparison of the effects of the two concentration ranges to be made.

Colony count reduction	Campylobacter		Coliforms		Enterobacter/ Enterococcus		Salmonella		Staphyloccus	
	21-25 ppm O_3	28-39 ppm O_3	21-25 ppm O_3	28-39 ppm O_3	21-25 ppm O_3	28-39 ppm O_3	21-25 ppm O_3	28-39 ppm O_3	21-25 ppm O_3	28-39 ppm O_3
0		1					11.6			1
1.5-9.9x	19.2	7	19.3			3	14.5	14	13	7
>1.5-9.9x	4.8	5		2		2	2.9	11	6.5	12
10-40x		6		7	22.7	13		4	13.3	2
>10-40x			9.7	1						4
> 40x		5		19	11.3	15			3.3	

The extent of the effects can be ranked as follows:

1. Severely affected. Coliforms (i.e. *E. coli* and related species), and also *Enterobacter* or *Enterococcus* were most affected. For coliforms, the lower concentration range of 21-25 ppm gave more than 10x inhibition in 2 out of 6 cultures, whereas use of 28-39 ppm gave more than 40x inhibition in 19 out of 28 cases. For *Enterobacter* or *Enterococcus*, the lower concentration range of 21-25 ppm gave more than 40x inhibition in 2 out of 6 cultures, whereas use of 28-39 ppm gave more than 40x inhibition in 15 out of 33 cases. All cultures of these species showed at least some effect by all concentrations of ozone tested.

2. Moderate, or variable, effects. Both *Campylobacter* and *Staphylococcus* showed mostly moderate inhibition, but also in a few cases a wide variation in sensitivity: at least one culture showed no effect, and at least one culture more than 40x inhibition.

3. Little or no effect. The species that was least affected by ozone was *Salmonella*. After treatment with 21-25 ppm ozone, 4 cultures from 10 appeared completely unaffected, the other 6 showed less than 90% inhibition. At the higher ozone concentrations (28-39 ppm), all cultures were somewhat affected, but only 4 cultures from 29 showed inhibition in the 10-40x range, and none more than 40x inhibition.

Some indication of the effect of the duration of exposure to ozone was seen. Appendix 7 summarizes the tabulated data presented in Appendix 8. For most work a standard exposure times of 8 or 10 minutes and a concentration of above 25 ppm were used. It was seen that:

1. The 8 or 10 minute standard exposure was effective against coliforms, but 5 minutes exposure was insufficient. There was some evidence that 16 or 32 minutes was still better.

2. Whilst the standard exposure was not very effective against *Staphylococcus* or *Salmonella*, there is some evidence that 16 or 32 minute exposures were more effective, particularly against *Salmonella.*

6.0   DISCUSSION

6.1 Sensitivity of yeast

A high degree of inactivation of yeast films applied to agar was obtained when:

1. The ozone concentration was greater than 25 ppm (up to 37 ppm was used).

2. The ozone was applied for 7.5 minutes or longer.

   Only a moderate degree of inactivation was observed at 8-11 ppm, even after prolonged exposure to ozone (up. to 45 minutes). Likewise, exposure to the higher concentrations for only 2.5 or 5 minutes gave only moderate inactivation. It was not possible, at least at these ozone concentrations, to eliminate all growth of the yeast: the persistent growth seemed to correlate with areas of heavy inoculation.

6.2 Sensitivity of bacteria

A high degree of inactivation of bacteria was obtained for two species, if the ozone concentration was 25-37 ppm. One species was only slightly or moderately affected by these concentrations, whist another two species showed wide variation in sensitivity. Concentrations below 25 ppm showed only a moderate degree of inactivation, even for the sensitive species.

As indicated in the Introduction, this contrasts with the statement that "The concentration of ozone which kills bacteria has been variously reported to be 0.04 to 0.1 ppm" (Stockinger, quoted in Scott and Lesher 1963). It may be that variations in treatment conditions (dry versus wet, for example) may account for the large difference.

With regard to the differences found here between bacterial species, it is of interest that industry publications suggest that the bacterial cell wall is the primary site of attack by ozone [16], even stating in one case "Disinfection by ozone is a direct result of bacterial cell wall disintegration, also known as lysis" [17]. There have been academic papers supporting this view [3, 18]. On this basis, it might be expected that bacteria that possess a cell wall, i.e. "Gram-positive" species, might be less sensitive than those that have only a double membrane ("Gram-negative").

However, the present results do not support a critical role for the cell-wall: both the most-sensitive (*E. coli*) and least-sensitive strains (*Salmonella*) are Gram-negatives. By contrast, the Gram-positive Staphylococcus showed a widely varied, although mostly moderate, sensitivity.

The lack of correlation with the presence or absence of a cell wall is consistent with more recent ozonisation work using several strains of *E. coli* [19]. This showed that cell lysis was not the major mechanism of inactivation, even if some slight membrane damage was observed after ozone treatment. Instead the oxidation of DNA was implicated, as was the normally protective effect of (anti-) oxidative and stress genes.

This might suggest that the differences in the ozone resistance of bacterial strains could be due to differences in activities of their oxidative stress genes. In any case, if marked reduction in the numbers of resistant bacteria such as *Salmonella* is required, then the effect of higher ozone concentrations than 38 ppm should be investigated. Just how much higher is unclear from present data, although it has been reported that even 80,000 ppm in air gave a ten times reduction in *Salmonella enteritidis* on broiler carcasses (Ramirez et al., 1994: quoted by Kim et al [20]).

As with yeast, less effective inhibition was observed when the microbial film was thick, suggesting that the ozone was unable to penetrate beyond the surface layers.

6.3 Practicality of scale-up

Consideration of the costs of add-on buildings and equipment for an ozone-treatment plant capable of handling 25-32 head of beef per week resulted in estimates of the depreciation of AU $ 20,176 p.a. for the first 5 years, and of operating costs of AU$ 14,592 p.a. (Appendix 5).

The ozone level foreseen was 50 ppm, with a reserve of nine times this to account for tissue uptake.

Hence this should be an effective plant, but the acceptability of the costs involves knowledge of market

conditions which is beyond the scope of this report.

7.0   CONCLUSIONS/RECOMMENDATIONS

Ozone treatment at 25-38 ppm for 8 or 10 minutes is an effective method to inhibit the growth of yeast and bacteria such as *E. coli* by 40 times or more. This was achieved for the majority of samples, when these were presented as thin films on agar plates. For some other bacteria (*Campylobacter* and *Staphylococcus*), the inhibitory effect was less at roughly 10 times for the majority of samples, but with the risk of a small number being unaffected. For Salmonella, an even smaller degree of inhibition was achieved on average. However, there are indications that 16 or 32 minute treatments are more effective against both *Staphylococcus* and *Salmonella.*

Thus higher concentrations and treatment times of 16 to 32 minutes will be required to achieve a high degree of inhibition of *Campylobacter* and *Staphylococcus*, and especially of *Salmonella*. Higher concentrations, or possibly prolonged treatment times, are likely to help where a thick microbial layer must be inactivated.

In addition, if certainty that suppression of the growth of STEC organisms is achievable is required, then a project for that purpose would be necessary. Due to the hazardous nature of these organisms, this would involve a PC3 class laboratory.

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9.0 APPENDICES

9.1   Appendix 1: Measurements, using a gas dilution set-up, of ozone production

Preliminary Run #1: air input to ozoniser

Ozone-rich input to first mixer	10 ml/min
First mixer air diluent input	140 ml/min
Diluted ozone input to second mixer	60 ml/min
Second mixer air diluent input	350 ml/min
Measured ozone concentration at output	19.9 ppm

Calculation: Dilution factor = (10/140) x (60/350) = 1/81.7

Inferred concentration supplied by the ozoniser = 19.9 x 81.7 ppm = 1626 ppm

Run #2: air input to ozoniser

Ozone-rich input to first mixer	4 ml/min
First mixer air diluent input	140 ml/min
Diluted ozone input to second mixer	60 ml/min
Second mixer air diluent input	350 ml/min
Measured ozone concentration at output	9.6 ppm

Calculation: Dilution factor = (4/140) x (60/350) = 1/204

Inferred concentration supplied by the ozoniser = 9.6 x 204 ppm = 1958 ppm

Run #3: oxygen-enriched input to ozoniser

Ozone-rich input to first mixer	4 ml/min
First mixer air diluent input	130 ml/min
Diluted ozone input to second mixer	24 ml/min
Second mixer air diluent input	360 ml/min
Measured ozone concentration at output	9.2 ppm

Calculation: Dilution factor = (4/130) x (24/360) = 1/488

Inferred concentration supplied by the ozoniser = 9.2 x 488 ppm = 4490 ppm.

Runs #2 and #3 indicate that use of the oxygen-enrichment in the feed air gave an increase in ozone

concentration of 130%.

9.2 Appendix 2: Tests of the full output of the ozoniser

The tests in Appendix 1 used only a small fraction of the ozoniser output, which was then diluted in 2 stages before measurement. In case of possible errors in a rather complex set-up, a full-flow test was devised.

The full output of the ozoniser was used to feed a cylindrical treatment chamber (185 mm diameter x 350 mm long) inside a fume extraction hood which had a minimum flow speed of 0.2 m/s. Concentrations in the treatment chamber were measured after 5 minutes operation, to allow a steady state to be established. Even so, some fluctuations due to turbulence seemed unavoidable: this is the disadvantage of this sort of measurement.

Run #1: Down-shift in oxygen feed.

With oxygen-enriched feed air	6.2 ± 0.4 ppm
With ordinary air as feed gas	2.8 ± 0.1 ppm

   Ratio = 2.2 ± 0.2,   i.e. a 120% increase with oxygen-enriched air.

Run #2: Upshift in oxygen feed

With ordinary air as feed gas	3.0 ± 0.1 ppm
With oxygen-enriched feed air	7.1 ± 0.3 ppm

   Ratio = 2.4 ± 0.2,   i.e. a 140% increase with oxygen-enriched air.

Run #3: Upshift in oxygen feed

With ordinary air as feed gas	4.15 ± 0.1 ppm
With oxygen-enriched feed air	8.85 ± 0.6 ppm

   Ratio = 2.1 ± 0.3,   i.e. a 110% increase with oxygen-enriched air.

Runs #1 to #3 indicate that use of oxygen-enrichment in the feed air gave an increase in ozone concentration of 110% – 140%, in agreement with the dilution measurement.

9.3   Appendix 3: the procedure of a 3-way streak on agar

The microbial suspension was transferred to an agar medium's surface using a sterile plastic loop. Streaks of suspension were made in one direction (at the "top" of the Petri dish in Fig. 1). Using a fresh sterile loop, part of the initially deposited film was drawn at right angles (i.e. to the left side in Fig. 1). Then using a third sterile loop, a further set of streaks was drawn at right angles again (not shown in Fig. 1).

Figure A3.1. "Streaking out" a microbial suspension (only two directions of three are shown). This particular agar gel was made unusually soft to show the indentation better.

Figure A3.2. Result, after 5 days incubation, of the above procedure is shown here. Cell growth results in visible colonies from each originally viable cell. The density of the colonies covers a wide "dynamic range" caused by the successive streak operations. The first streak caused a mass of colonies, the second gave rise to strings of merging ones, whilst the third gave individual colonies (which could if necessary be picked out and further cultured).

The picture is hazy because it was taken through the thickness of the agar medium (and the image then flipped horizontally).

9.4   Appendix 4: Results of exposure of yeast to ozone
(i) Yeast exposed at 8-11 ppm for up to 45 minutes

Figures A4.1 and A4.2 below are examples of the poorly reproducible inactivation of yeast by lower ozone concentrations (8 – 11 ppm) applied for 6 minutes or more in air. Incubation of the plates was for 3 days at 30˚C.

These two runs are representative of seven runs carried out with yeast at this ozone concentration.

Ozone concentration 8 - 11 ppm: with the exposures being, clockwise from top left:

Upper panel, 0 minutes, 6 minutes, 12 minutes, 18 minutes exposure. **Progressive inactivation!**

Lower panel, 0 minutes, 15 minutes, 30 minutes, 45 minutes exposure. **Little inactivation.**

Figure A4.1.

Figure A4.2.

(ii) Yeast exposed at > 25 ppm, > 10 minutes

Figures A4.3 and A4.4 below demonstrate definite inactivation of yeast by higher ozone concentrations (> 25 ppm) applied for 10 minutes or more in air.

Ozone concentration 25-37 ppm: with the exposures being, clockwise from top left:

0 minutes, 10 minutes, 20 minutes, 30 minutes.

Incubation of the plates was for 4 days at 30 C.

Very definite reduction in colonies can be observed after 10, 20 and especially 30 minutes exposure.

The large mass of cell growth in the control plate (primary streak) is reduced but not absent from all the exposed plates. There are some cracks visible in the agar, which are of no consequence.

The extent of the reduction is seen better in transmitted light (lower picture: colonies appear dark) than in incident light (upper picture, colonies appear white, but there are interfering highlights).

Figure A4.3.

Figure A4.4.

(iii) Yeast exposed at > 25 ppm, < 16 minutes

Investigation of the inactivation of yeast by shorter exposures to higher ozone concentrations (25-37 ppm) in air, Run A. Other methods were the same as above, except for the exposure times:

Upper panel, clockwise from top left: 0 minutes, 2.5 minutes, 5 minutes and 7.5 minutes exposure.

Lower panel, clockwise from top left: 0 minutes, 10 minutes, 12.5 minutes and 15 minutes exposure.

Moderate inactivation (colonies reduced in the secondary streak, much reduced in the tertiary streak) is observed after 2.5, and 5 minutes exposure.

Very great inactivation (colonies much reduced in the secondary and tertiary streaks, moderate reduction in the primary streak) is observed after 7.5, 10, 12.5 and 15 minutes exposure.
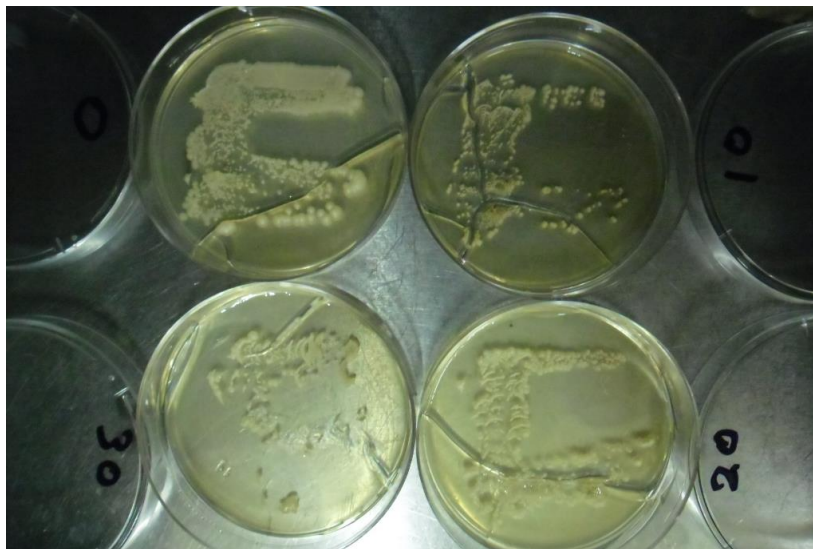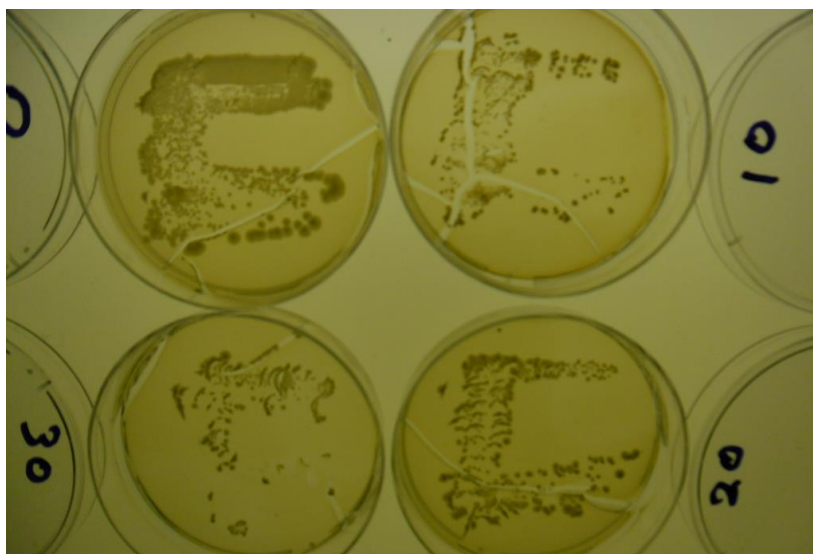
Figure A4.5.
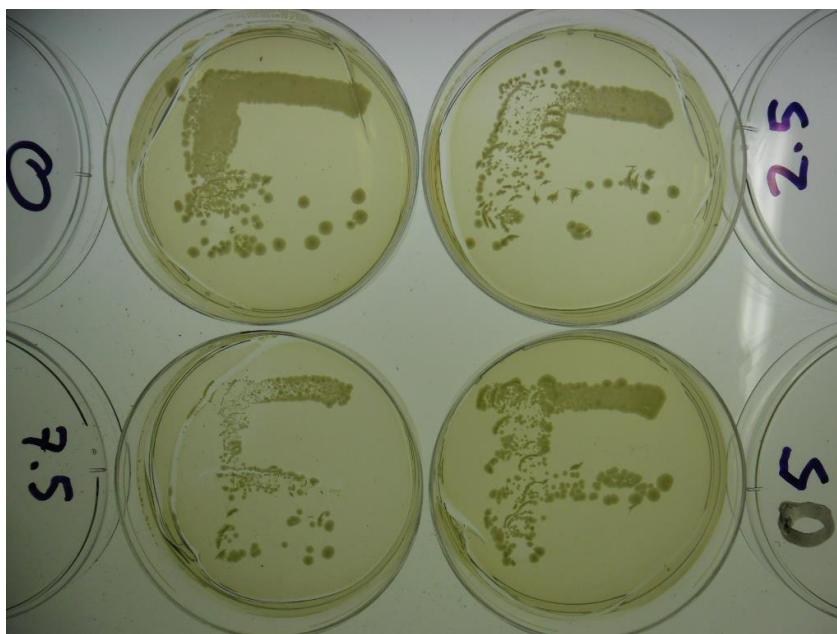
Figure A4.6.
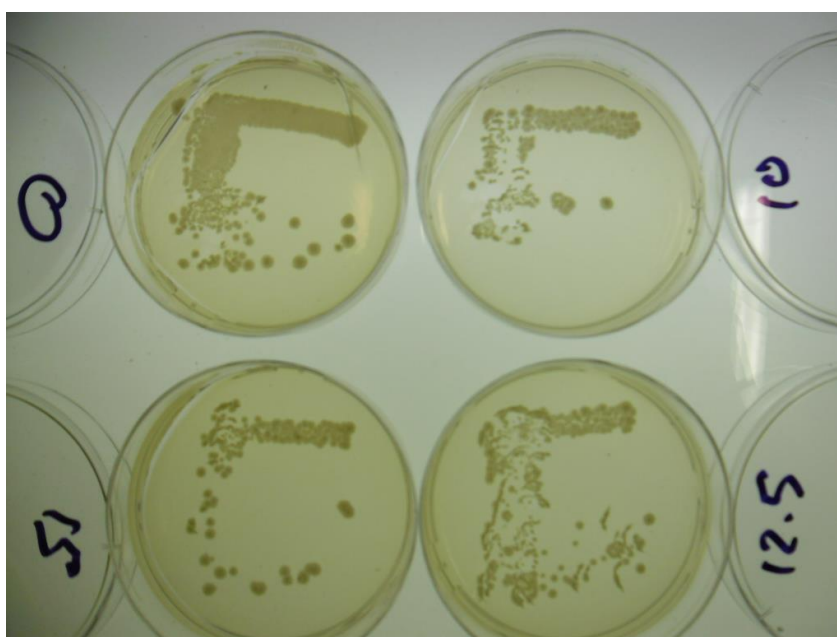
(iv) Repeat of exposure to > 25 ppm

Investigation of the inactivation of yeast by shorter exposures to higher ozone concentrations (25-37 ppm) in air, Run B. Other methods were the same as above, except for the exposure times:

Upper panel, clockwise from top left: 0 minutes, 2.5 minutes, 5 minutes and 7.5 minutes exposure.

Lower panel, clockwise from top left: 0 minutes, 10 minutes, 12.5 minutes and 15 minutes exposure.

Moderate inactivation (colonies reduced in the secondary streak, almost absent from the tertiary streak) is observed after 2.5, 5 and 7.5 minutes exposure.

Very great inactivation (colonies reduced in the primary streak, much reduced in the secondary streak, absent from the tertiary streak) is observed after 10, and 12.5 minutes exposure.
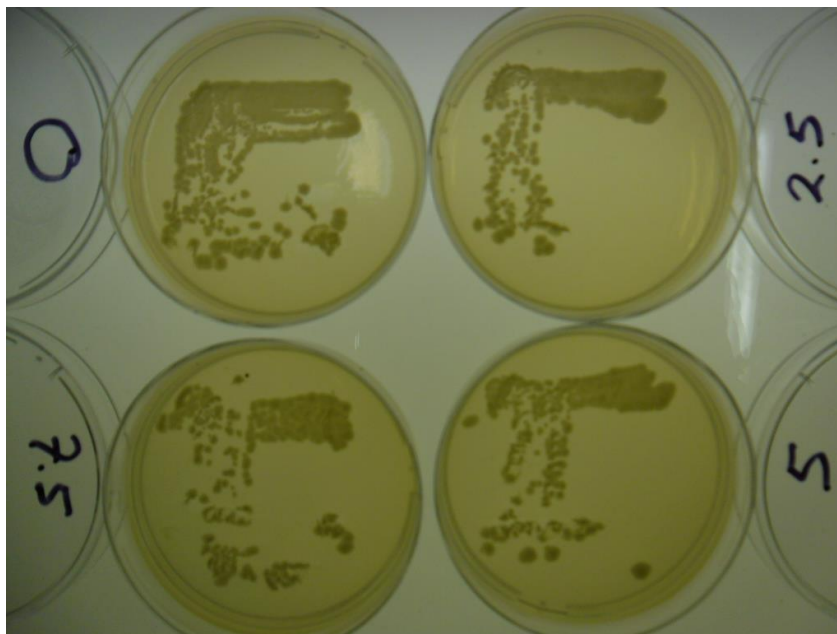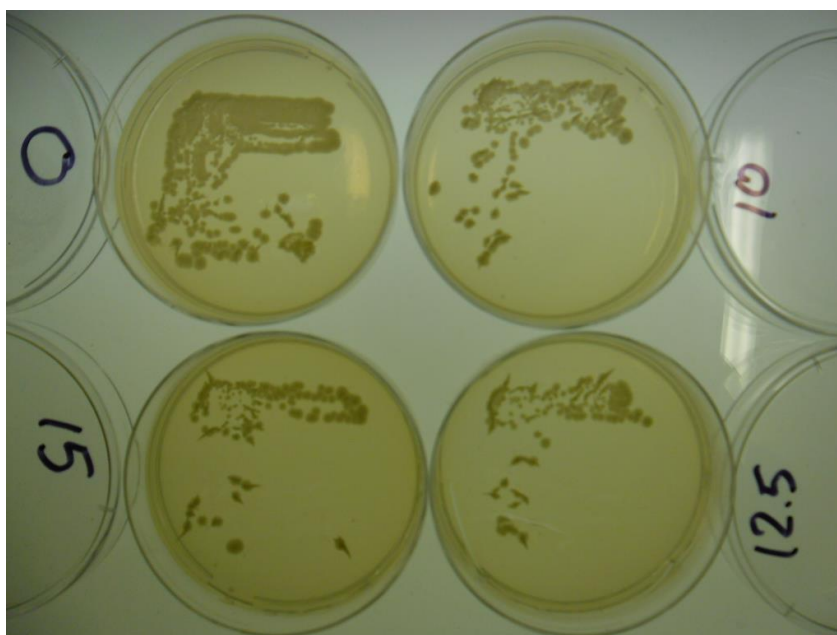
Figure A4.7.

Figure A4.8.

9.5   Appendix 5: Estimation of the operating costs of scale-up

(i) Introduction

The model chosen is that of a meat processing plant with a throughput of 30 beef cattle per week, and uses capital estimates derived from two publicly-available studies from the USA. The approach is to estimate the add-on cost to a new plant to take advantage of the new technology.

The cost and specific needs of ozone-generating and application equipment are covered, as are estimates of safety and regulating equipment. In a first instance, a batch-operated system is envisaged, although if this is successful a continuous, and likely more expensive, system could be envisaged at a later date.

It must be emphasized that these costings are based only on technical data, and not on any commercial information. The viability of implementing such a system will depend on market, profitability and site-specific factors that will need to be factored in before a meat-plant operator makes a go/no-go decision.

(ii) Methodology

The levels of ozone generated by the microplasma apparatus that were capable of inactivating thin layers of certain micro-organisms in a continuous flow system were taken as a starting point for this study. However, literature evidence suggested that, in a batch system, the consumption of ozone by a commercially useful amount of product would require additional amounts of ozone. This information was combined with the specifications on up-rated micro-plasma based ozone generators to derive a conservative cost estimate.

Safety considerations dictated that not only the ozone generation, but also the treatment area and airlocks/interlocks, be costed at this stage. These can only be estimates as the nature of the transport system (hooks on a chain, conveyor belt, or motorized trolley) will affect the type and cost of airlock.

Data on meat plant size and cost has been obtained from published sources to enable an estimate of the cost of a suitable treatment room. This was combined with this study's data on ozone sensitivity, and additional published data on the sensitivity of different organisms, to estimate the amount of ozone required. Equipment manufacturer's data and prices, as well as data on ozone lifetimes has then been used to estimate the ozone generation costs and the cycle time. Finally, an estimate of metering and control costs is made. These costings are combined below.

(iii) Building Cost Estimates

Two models of meat plant were used as the basis of calculations. These are similar, with buildings of 6,200 sq.ft. [A1] and 5,250 sq.ft. (larger of two options in [A2]) respectively. Both models include a "Boning Room" [A1] also known as a "Processing Room" [A2].

In the Generic Meat Plant [A1], this is 412 sq.ft. and processes 25-32 head of beef per week. In the "Large Plant" version of [A2] this is 392 sq. ft. and processes roughly 4,750 lbs of product in 12 hr.

These figures are roughly in accordance, assuming 460 lbs (210 kg) carcass weight [A3] per animal, when it is realised that processing operates only 2-3 days per week, with animal killing occupying the other 2-3 days per week.

It is assumed that ozone-treatment will require a similarly-sized room to the Boning/Processing Room. It will need to be fitted with an air-locked volume, preferably configured as a tunnel. This is made necessary by the toxicity of ozone.

This system is foreseen, at least at first, to operate as a batch system. A continuous system is conceivable, but would incur much increased costs for equipment.

When meat products are carried by some mechanical system through the air-lock, air will have to be blown in at the same time in order to keep ozone from entering the rest of the building. Consequently, ozonised air will need to exit the treatment room and then be vented to atmosphere external to the plant. A fresh charge of ozone must then be supplied for the next batch. For the most general application, the transport system could be a conveyor belt, or a wheeled truck.

The building cost of the room, at the same rates as assumed in [A1] will be US $412 x 150

	= US $ 61,800
Applying 2.5% inflation over 2011 to 2017 this becomes	US $ 71,670
Costs for installation of an air-lock tunnel (rough estimate, 20%)	US $ 14,334
Total buildings etc	US $ 86,004
Using linear depreciation over 20 years, this amounts to	US $ 4,300 p.a.

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Est. cost of a transport mechanism to carry one carcass of 210kg in-out	US $ 20,000
Using linear depreciation over 5 years, this amounts to	US $ 4,000 p.a.

In the simplest conceivable arrangement, the products will be transported out through the same airlock after treatment, whilst more air is blown in.

(iv) Estimation of Ozone Requirements and Costs

There are two factors to be considered, either:

a) the concentration required in a flow system where effectively an infinite amount of ozone is available from further flow

b) the amount (weight) of ozone required in a batch system to satisfy the binding of a certain mass of food-stuff.

As reported previously, the concentration of ozone required to kill organisms such as yeast is 25 ppm. Note that this was applied in a continuous flow system for at least 10 minutes. Applying an engineering margin of 2 suggests that a figure of 50 ppm should be designed for: this translates to 0.06 mg/litre. (The density of air at 15˚C is 1.225 kg/m^3, see the International Standard Atmosphere). However, published work with insect pests [A5] in a batch system, suggests that initial values of 13.9 mg/litre may be required. That large dose was attributed to "passive" absorption by the foodstuff, and the size of such pests may also be a factor. Also, pests buried deep within the corn were unaffected. Insect eradication, and indeed treatment deep within a sample is outside the scope of this project which is aimed at surface micro-organisms. An indication of the amount of ozone required for micro-fungi, on a foodstuff-weight basis, was provided by Wu et al [A6]. It was found that 0.33 mg/g was required for 5 minutes to eradicate fungi in wheat.

The processing room described earlier [A2] has a height of 10 feet and therefore a volume of 412 x 10 cu. ft., or 116.5 m^3. A concentration of 0.06 mg/litre (50 ppm) will require a weight of ozone of 7.0 g.

However, with a batch system, due to expected absorption by the meat involved, then the above figure of 0.33 mg/g of foodstuff would indicate an additional 69.3 g of ozone would be required per carcass (210kg of meat), assuming that this had been prepared as small cuts. The total would then be 76.3 g. This is likely only required if the load is entirely made up of small cuts: less would be required for a whole carcass of this weight.

The cost of producing ozone consists of:

1) the cost of supplying dry air or oxygen at a suitable pressure, and
2) the cost of the ozone generator itself, and
3) cost of ozone metering and control.

It will be assumed that the plant already has the ability to supply compressed air, although a drying unit may be required to ensure longevity of the ozone generator(s).

The units considered here differ in their capability, best characterised as the weight of ozone produced per hour. All units provide essentially the same concentration, but the flow rate rises with the capability. Oxygen feed was not considered due to the high cost of producing it. The microplasma units presently available are:

Existing experimental unit EP-2002 (Option 0)

2-3 g/hour ozone   US $1,300

Requires a dried air feed of 10 lpm @ dewpoint = 10˚C and 120W power.

Up-rated unit from EP-Pure (Option 1)

15g/hour ozone   US $5,000

Requires a dried air feed of 50-60 lpm @ dewpoint = 10˚C and 600W power.

Up-rated unit EP_Pure (Option 2)

25 g/hour ozone   US $ 8,500

Requires a dried air feed of 100 lpm @ dewpoint = 10˚C and 1.2 kW power.

(v) Times required to generate the required amounts of ozone

In accordance with the capabilities of the plant, it is assumed that six beef carcasses per shift (30 per week @ I shift per day) need to be treated. Hence a maximum treatment time of 1.5 hours per carcass is implied.

Alternatively, 2 carcasses could be treated for 3 hours, but the limited lifetime of ozone might be thought to cause a drop in effectiveness in this case. There is some disagreement about this lifetime in the literature, although there is agreement that it is shortened by increase of temperature [A4, A7]. According to Ref. [A4], a half-life of 72 hours is expected in air at 20˚C, but Ref. [A7] found only about 7.5 hours at 24˚C and 87% humidity. Longer half-lives were found at lower humidity (e.g. 25 hours in dry air at 24˚C), which may explain the difference. Vigorous stirring of the air can shorten the lifetime, to less than 1 hour at 24˚C [A7]. However, data from the same source indicates that at 4˚C the half-life would still exceed 3 hours.

If the expected additional ozone consumption of a single batch of 210 kg of cut meat is included, then the requirement is for 76 g of ozone to be supplied in 1.5 hours or less. The existing experimental unit would be insufficient: it would require 3.5 hours even to raise the level of an empty room to the expected required concentration of 0.06 mg/litre.

However this weight of ozone could be generated by using either:

four of the Option 1 generators (4x US $5,000 = US $20,000), or

two of the Option 2 generators (2x US $ 8,500 = US 17,000).

The second option would then result, over a 5-year linear depreciation, in US $ 3,400 p.a.

(vi) Metering and Control

Process control and safety dictate that the airlock to the treatment room can only be opened after the process has run its allotted time and ozone in the treatment room has been flushed out. Therefore, a sequencer and a metering system (with sensors in both the treatment room and the airlock) will be required. The metering of ozone levels during the process will also be necessary for quality assurance.

The cost of a suitable range of ozone meters using optical absorbtion is in the Euro 3,000-4,000 range, depending on sensitivity (BMT Messtechnik Berlin, Dr. Ing. Franz Wallner GmbH). These have a 5-year

service interval. Therefore, a high-sensitivity (for the airlock) and a low-sensitivity (for the treatment room) pair will cost Euro 7,000 (AU $10,300). A high-sensitivity meter based on electrochemistry is available at lower cost in NZ from Aeroqual. However, the sensor for this requires annual replacement.

The process-control electronics, including switching of in/out drive power to the transport system, can be made up locally for an estimated AU$ 10,000.

(vii) Estimation of Cost of a Microplasma-Based Ozone Treatment

Buildings are depreciated over 20 years, equipment over 5 years.

	Capital Cost US $	Depreciation (20 yr) US $	Depreciation (5 yr) US $	Other US $
Building: Room with in/out airlock tunnel (App. 1, 2)	86,004	4,300 p.a.		
Transport mechanism (App. 2)	20,000		4,000 p.a.	
Labour for loading & unloading the belt/wagon/hooks: 1 hour/day				10,000 p.a.
Dried compressed air	(assumed on-hand)			
Ozone Generators (App. 3-5)	17,000		3,400 p.a.	
Power: 2.4 kW, 7.5 h/day 330 days/year, 10c/kWh	included above			594 p.a.
Totals: US $	128,004	4,300 p.a.	7,400 p. a.	10,594 p.a.
or AU $ (@.726 USD)	**176,314**	**5,923 p.a.**	**10,193 p.a**	**14,592 p.a.**
Metering/Control (App. 6)	AU $ 20,300		4,060 p.a.	
Totals: AU $	**196,614**	**5,923 p.a.**	**14,253 p.a.**	**14,592 p.a.**

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Total CapEx AU $ 196,614

Total Depreciation AU $ 20,176 p.a. (first 5 years)

Operating Costs AU$ 14,592 p.a.

(viii) Discussion

The costs are dominated by CapEx for the building, including the air-lock mechanism required. Operating costs are dominated by the labour costs incurred in loading/unloading the transport mechanism required to transit the airlock.

These costs must be considered against the increase in value from the improved food safety of the meat from 30 animals per week.

(ix) Bibliography (for Appendix 9)

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9.6   Appendix 6: Visualisations of bacteria exposed to ozone

(i) On *Campylobacter*-selective plates

For all of chicken, beef and lamb samples low levels of contamination in most fresh samples developed into medium or heavy contamination after 3-5 days storage. After treatment, usually only a moderate degree (10-20 times) of inactivation was observed, although two samples showed complete inactivation after only 5 minutes treatment.

Figure A6.1. Example of results using *Campylobacte*r-selective plates. Effect of exposure to ozone for 0, 8 and 16 minutes on the growth of bacterial colonies from samples swabbed from beef (left half of each plate) and chicken (right half). In this case, bacteria were found on the chicken only, and the colonies become stained brown. Growth was for 2 days at 37˚C in micro-aerobic conditions after ozone treatment.

(ii) On Coliform-selective plates

Medium to heavy contamination was observable for all of chicken, beef and lamb samples after 3-5 days storage. These levels developed from usually low levels, although moderate contamination was seen in one of two fresh lamb samples. After treatment, provided the ozone concentration was 25 ppm or greater, a high degree of inactivation (50 times or more) was observed for both *E. coli* and other coliforms. A single trial at 21 ppm gave only marginal inactivation.

At the higher concentrations, 5-minute exposures were not as effective as 8 or 10 minute exposures. There is some evidence that a further increase in effect occurred when the exposure was increased from 8 minutes to 16 or 32 minutes.
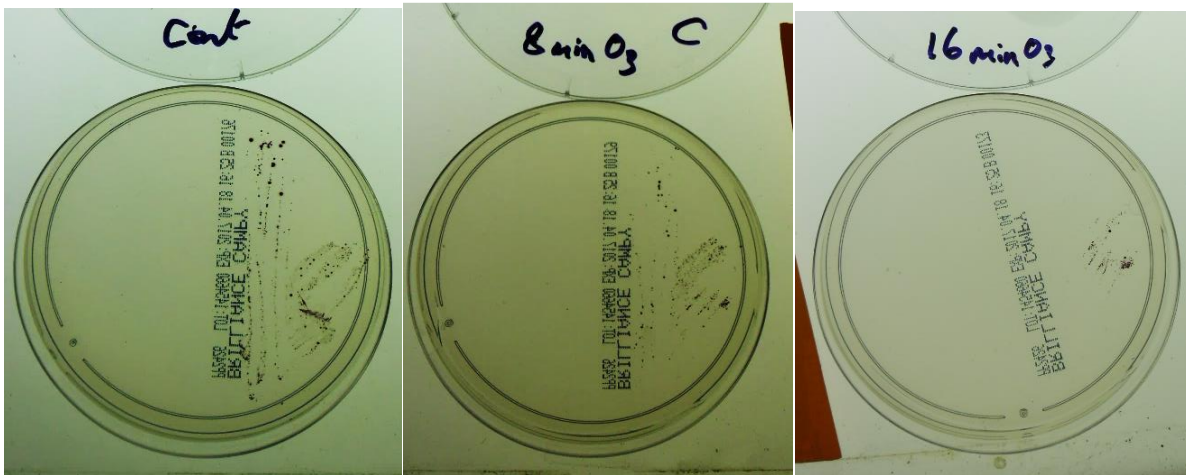

Figure A6.2. Example of results using coliform-selective plates. Effect of exposure to ozone for 0, 5 and 10 minutes on the growth of bacterial colonies from samples swabbed from beef (left half of each plate) and chicken (right half). Bacteria were found on both meat samples in large numbers. *E. coli* colonies become stained purple, other coliforms are pink. Yet other bacteria form white colonies. Growth was for 2 days at 37˚C in aerobic conditions.

(iii) On *Salmonella*-selective plates

Summary: medium to heavy contamination was observable for all of chicken, beef and lamb samples after 3 or 5 days storage. Moderate contamination was seen in some fresh lamb samples. After treatment, provided the ozone concentration was 25 ppm or greater, some degree of inactivation was observed for *Salmonella sp.* The single trial at 21 ppm gave no marginal inactivation. However, even with ozone concentrations of 25 ppm or greater, the degree of inactivation was never very great especially where the bacterial film appeared thicker.

Whilst 16-minute exposures were more effective than 10, 8, or 5-minute exposures, total eradication was only achieved where the original colony count was 25 or less and the ozone concentration was 35 ppm or higher.
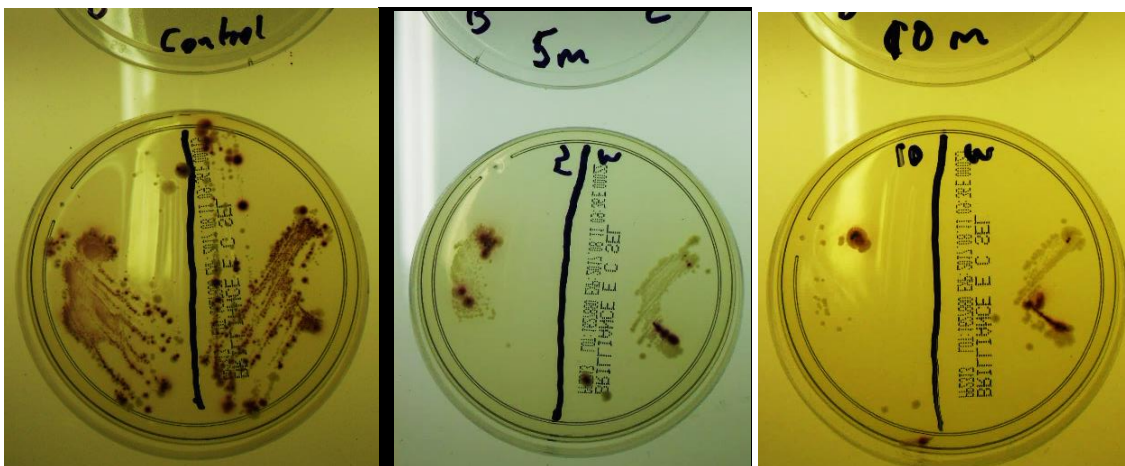

Figure A6.3. Example of results using *Salmonella*-selective plates. Effect of exposure to ozone for 0, 8 and 16 minutes on the growth of bacterial colonies from samples swabbed from beef (left half of each plate) and lamb (right half). Bacteria were found on both meat samples in moderate numbers. *Salmonella* colonies become stained purple, other bacteria are blue or colourless. Growth was for 2 days at 37˚C in aerobic conditions.

(iv) On *Staphylococcus*-selective plates

Summary: light to medium contamination with *Staphylococcus aureus* was observable for all chicken, beef and lamb samples after 3 or 5 days storage. Samples stored for 1 day or less showed no or very light contamination. After treatment, even at an ozone concentration of 21 ppm or greater, a fair degree of inactivation was observed. With ozone concentrations of 25 ppm or greater, the degree of inactivation was good especially for exposures of 16 or 32 minutes.

The ozone treatment was able to achieve a consistent 10 times reduction in *Staphyloccus* colony number. However, no heavily contaminated samples were encountered that would have tested the real capability of ozone against this bacterium. Colonies of *Staphylococcus saprophyticus* were often seen but not counted.
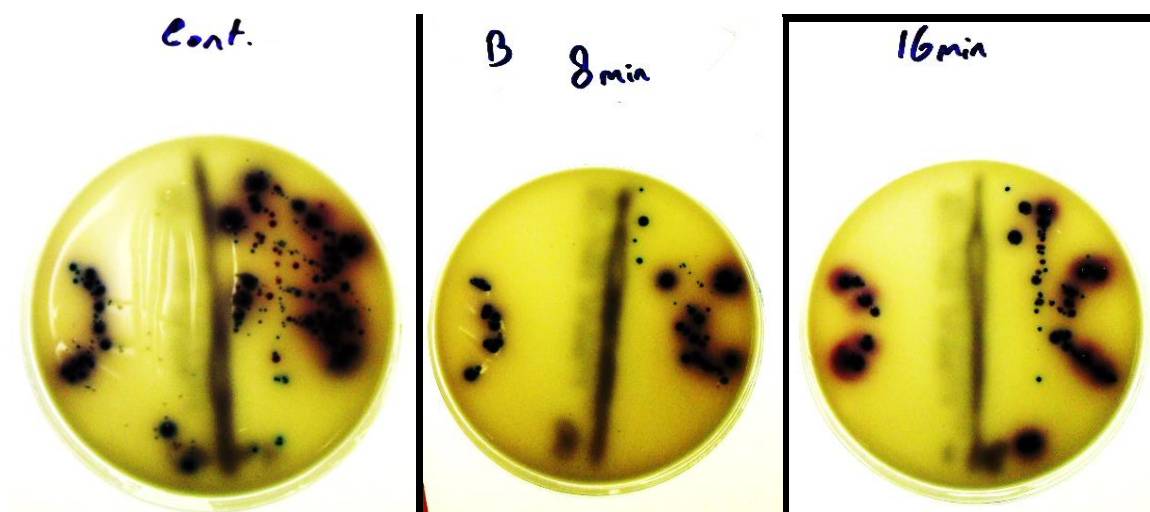

Figure A6.4. Example of results using *Staphylococcus*-selective plates. Effect of exposure to ozone for 0, 5 and 10 minutes on the growth of bacterial colonies from samples swabbed from beef (left half of each plate) and lamb (right half). Bacteria were found on both meat samples in moderate numbers. *Staphylococcus aureus* colonies become stained purple, *Staphylococcus saprophyticus* are blue. Growth was for 2 days at 37˚C in aerobic conditions after ozone treatment.

(v) On non-selective "Orientation" plates

Depending on the bacteria present, these plates can grow a number of different-coloured colonies. Only the data on pink/red (R) and blue (B) colonies is presented here. However, in some cases a very dense blue masks any red. The manufacturer indicates that the red are *E. coli*, whilst the blue are *Enterococcus* or *Enterobacter* (these plates are intended for urinary diagnosis).

In general, these non-selective plates develop very dense, uncountable, areas of colonies. Hence an estimate of 10-30 times inhibition can be made, but may well be an underestimate. However, complete inhibition was rarely achieved.
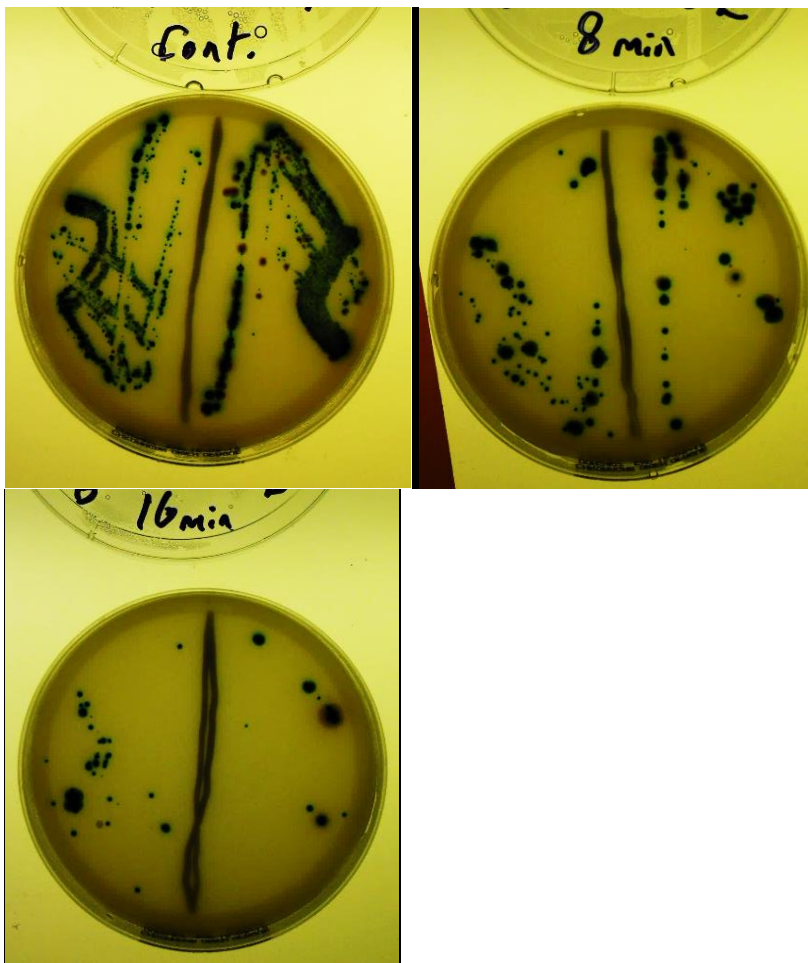
Figure A6.5. Example of results using non-selective plates. Effect of exposure to ozone for 0, 8 and 16 minutes on the growth of bacterial colonies from samples swabbed from beef (left half of each plate) and lamb (right half). Bacteria were found on both meat samples in considerable numbers. Growth was for 2 days at 37˚C in aerobic conditions after ozone treatment.

9.7 Appendix 7: Summaries of the effects according to bacterial species

(i)   *Campylobacter*-selective plates

Most samples from any of chicken, beef or lamb showed low levels of contamination when fresh, which developed into medium or heavy contamination after 3-5 days storage. After treatment of the agar plates, usually only a moderate degree (10-20 times) of inactivation was observed, although two samples showed complete inactivation after only 5 minutes treatment.

(ii) Coliform-selective plates

Medium to heavy contamination was observable for all of chicken, beef and lamb samples after 3-5 days storage. These levels developed from usually low levels, although moderate contamination was seen in one of two fresh lamb samples. After treatment, provided the ozone concentration was 25 ppm or greater, a high degree of inactivation (50 times or more) was observed for both *E. coli* and other coliforms. The single trial at 21 ppm gave only marginal inactivation. At the higher concentrations, 5-minute exposures were not as effective as 8 or 10 minute exposures. There is some evidence that a further increase in effect occurred when the exposure was increased from 8 minutes to 16 or 32 minutes.

(iii) *Salmonella*-selective plates

Medium to heavy contamination was observable for all of chicken, beef and lamb samples after 3 or 5 days storage. Moderate contamination was seen in some fresh lamb samples. After treatment, provided the ozone concentration was 25 ppm or greater, some degree of inactivation was observed for *Salmonella sp.* The single trial at 21 ppm gave no marginal inactivation. However, even with ozone concentrations of 25 ppm or greater, the degree of inactivation was never very great especially where the bacterial film appeared thicker. Whilst 16-minute exposures were more effective than 10, 8, or 5-minute exposures, total eradication was only achieved where the original colony count was 25 or less and the ozone concentration was 35 ppm or higher.

(iv) *Staphylococcus*-selective plates

Light to medium contamination with *Staphylococcus aureus* was observable for all chicken, beef and lamb samples after 3 or 5 days storage. Samples stored for 1 day or less showed no or very light contamination. After treatment, even at an ozone concentration of 21 ppm or greater, a fair degree of inactivation was observed. With ozone concentrations of 25 ppm or greater, the degree of inactivation was good especially for exposures of 16 or 32 minutes. The ozone treatment was able to achieve a consistent 10 times reduction in *Staphylococcus* colony number. However, no heavily contaminated samples were encountered that would have tested the real capability of ozone against this bacterium. Colonies of *Staphylococcus saprophyticus* were often seen but not counted.
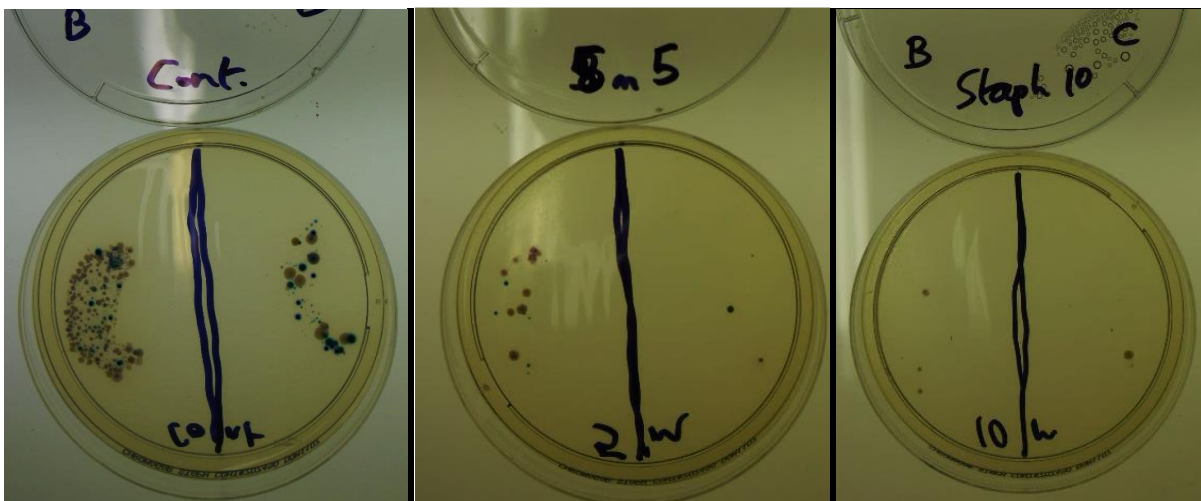
(v) Non-selective "Orientation" plates

Depending on the bacteria present, these plates can grow a number of different-coloured colonies. Only the data on pink/red (R) and blue (B) colonies is presented here. However, in some cases a very dense blue masks any red. The manufacturer indicates that the red are *E. coli*, whilst the blue are *Enterococcus* or *Enterobacter* (these plates are intended for urinary diagnosis). In general, these non-selective plates develop very dense, uncountable, areas of colonies. Hence an estimate of 10-30 times inhibition can be made, but may well be an underestimate. However, complete inhibition was rarely achieved.

9.8   Appendix 8: Tabulated results according to bacterial species

(i) *Campylobacter*-selective plates

In the Table below, the numbers up to 50 are colony counts, beyond that they are indicative estimates:

999 = thick, merged colonies over entire inoculated and streaked area;

666 = merged growth over the inoculated area, also over some of streaked area

333 = broken-up or gapped but otherwise merged colonies

99 = primary inoculation area only populated with colonies, not merged.

Exposure key: ^ = 5 minute exposure, instead of the usual 8 minute;   *= 16 minute exposure;

" = 32 min exposure.

Run ID	Age, days	Chicken, un-exposed	Ozone-treated	Beef, Unexposed	Ozone-treated	Lamb, unexposed	Ozone-treated	Ozone Conc.
-12	1	2	-	5	-	-	-	
-10	3	9	0	0	0	-	-	25
-6	1.5	2	0	1	0	-	-	38
-3	4.5	99	10, 10*	99	15, 20*	-	-	21
00	0	0	0	0	0	-	-	39
01	1	0	0	0	0	-	-	39
04	4	999	50, 20*	0	0	-	-	36
07	0	0	0	0	0	-	-	38
09	2	0	0^	4	0^	-	-	35
11	4	333	0^, 0^^	333	0^, 0^^			37
14	1	-	-	1	0, 0*	1	0, 0*	25
15	2	-	-	2	2,2*	15	1, 1*	30
18	5	-	-	-	-	-	-	28
21	1			999	99*	999	99*	36
23	3			50	1, 1"	99	9, 4"	37
25	5	-	-	-	-	-	-	38
29	3	-	-	-	-	-	-	33

### (ii)	Coliform-selective plates

These plates give separate colours for *E. coli* as opposed to other coliform bacteria. Hence these are keyed as: E= E. coli., f= other coliform.

The numbers up to 50 are colony counts, beyond that they are estimates:
999 = thick, merged colonies over the entire inoculated and streaked area;
666 = merged growth over the inoculated area, also some of the streaked area
333 = broken-up or gapped but otherwise merged colonies
99 = primary inoculation area only populated
Exposures are keyed as: ^ = 5 minutes exposure, instead of 8 minutes; ^^= 10 minutes exposure
*= 16 minutes exposure;   " = 32 minutes exposure.

Run ID	Age, days	Chicken, un-exposed	Ozone-treated	Beef, un-exposed	Ozone-treated	Lamb, Un-exposed	Ozone-treated	Ozone Conc. ppm
-12	1	-	-	-	-	-	-	-
-10	3	-	-	-	-	-	-	25
-6	1.5	-	-	-	-	-	-	38
-3	4.5	13f	4,3*	25f	11,11*	-	-	21
00	0	2E	0	0	0	-	-	39
01	1	20	0	0	0			39
04	4	999 (20%E)	17, 4*	0	0,0*	-	-	36
07	0	30f	0,0	21E	0,0	-	-	38
09	2	50	0^,0^^	99	5^,0^^	-	-	35
11	4	999	99^, 9^^	999	50^, 5^^	-	-	37
14	1	-	-	11	0	35	0	25
15	2	-	-	99E most	1,1*	99 f most	3E, 2f*	30
18	5	-	-	999E most	9E*	999f most	20f*	28
21	1	-	-	50f most	0*	300f most	0*	36
23	3	-	-	99mixed	2,0"	500f most	9,0"	37
25	5	-	-	-	-	999mixed	20f	38
29	3	-	-	-	-	-	-	33

(iii)   *Salmonella*-selective plates

The numbers up to 50 are colony counts, beyond that they are estimates:
999 = thick, merged colonies over entire inoculated and streaked area;
666 = merged growth over the inoculated area, also some of the streaked area
333 = broken-up or gapped but otherwise merged colonies
99 = primary inoculation area only populated.
Exposure key: ^ and ^^ = 5 and 10 minute exposures, instead of the usual 8 or 10 minute;
*= 16 minute exposure.

Run ID	Age, Days	Chicken, un-exposed	Ozone-treated	Beef, Un-exposed	Ozone-treated	Lamb, Un-exposed	Ozone-treated	Ozone Conc. ppm
-12	1					-	-	
-10	3	5	0	9	1	-	-	25
-6	1.5	3	0,2*	6	0,0*	-	-	38
-3	4.5	99	99,99*	99	99,99*	-	-	21
00	0	1	0,0*	0	0,0*	-	-	39
01	1	-	-	-	-	-	-	39
04	4	999	333,99*	0	0,0*	-	-	36
07	0	3	0,0*	1	0,0*	-	-	38
09	2	0	0^,0^^	25	2^,0^^	-	-	35
11	4	999	333^	999	333^			37
14	1	-	-	11	2,1*	20	15,10*	25
15	2	-	-	25	10,10*	99	25,25*	30
18	5	-	-	-	-	999	99*	28
21	1	-	-	1	0,0*	99	30,10*	36
23	3	-	-	-	-			37
25	5	-	-	333	33	999	333,99*	38
29	3	-	-	999	333	999	333	33

(iv) *Staphylococcus*-selective plates

The numbers up to 50 are colony counts, beyond that they are estimates:
999 = thick, merged colonies over entire inoculated and streaked area;
666 = merged growth over the inoculated area, also some of streaked area
333 = broken-up or gapped but otherwise merged colonies
99 = primary inoculation area only populated.
Exposure key: ^ and ^^ = 5 and 10 minute exposures, instead of the usual 8 minute;   *=
16 minute exposure; " 32 minute exposure.

Run ID	Age, days	Chicken, un-exposed	Ozone-treated	Beef, unexposed	Ozone-treated	Lamb, unexposed	Ozone-treated	Ozone Conc. ppm
-12	1	-	-	-	-	-	-	
-10	3	12	4	8	2	-	-	25
-6	1.5	1	0,0*	0	0,0*	-	-	38
-3	4.5	50	4,0*	9	2,1*	-	-	21
00	0	2	0,0*	0	1,1*	-	-	39
01	1	-	-	-	-	-	-	39
04	4	0	0,0*	0	0,0*	-	-	36
07	0	0	1,0*	2	0,0*	-	-	38
09	2	0	0^,0^^	5	0^,0^^	-	-	35
11	4	7	1^,0^^	40	5^,3^^	-	-	37
14	1	-	-	1	0	2	0	25
15	2	-	-	4	2,0*	6	1,0*	30
18	5	-	-	1	1*	99	15*	28
21	1	-	-	0	0*	2	0*	36
23	3	-	-	10	0,0"	10	0,0"	37
25	5	-	-	25	-	25	2,0*	38
29	3	-	-	3	0	25	5	33

(v) Non-selective "Orientation" plates

Data on pink/red (R) and blue (B) colonies could be derived.
The numbers up to 50 are colony counts, beyond that they are estimates:
999 = thick, merged colonies over entire inoculated and streaked area;
666 = merged growth over the inoculated area, also some of streaked area
333 = broken-up or gapped but otherwise merged colonies
99 = primary inoculation area only populated
Exposure key: ^ and ^^ = 5 and 10 minute exposures, instead of the usual 8 minute exposure;
*= 16 minute exposure; " 32 minute exposure.

Red are *E. coli*, whilst the blue are *Enterococcus* or *Enterobacter*

Run ID	Age, days	Chicken, un-exposed		Ozone-treated	Beef, Un-exposed	Ozone-treated	Lamb, Un-exposed	Ozone-treated	Ozone Conc.
-12	1	-		-	-	-	-	-	
-10	3	6R 99B		1R 2B	10R 99B	1R 6B	-	-	25
-6	1.5	0R 50B		0R 0B	1R 99B	0R 14B	-	-	38
-3	4.5	999B		99B	999B	99B	-	-	21
00	0	99B		0	0	0	-	-	39
01	1	-		-	-	-	-	-	39
04	4	999B		99,10*	99B	0,0*			36
07	0	4B 4R		0B,0B* 0R	99B 10R	2B,1B*			38
09	2	99B		1B^, 0^^	333B	50B^, 16B^^			35
11	4	666B		50B^, 25B^^	999B	333B^, 99B^^			37
14	1	-		-	666B	10B	666B 20R	20B 0R	25
15	2	-		-	666B	40B, 20B*	666B 20R	25B, 5B* 10R, 4R*	30
18	5				666B	50B	999B	333B	28
21	1				333B 4R	10B*	999B 15R	5B* 1R*	36
23	3	-		-	999B	25B, 2B"	999B	35B, 5B"	37
25	5	-		-	-	-	999B	99B*	38
29	3				999B	15B*	999B	50B*	33