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| **Project title:** | Use of gaseous ozone to prevent microbial post-harvest spoilage and reduce pesticide residue levels |
| **Project number:** | Project FV 386 |
| **Project leader:** | Ian Singleton and Shreya Wani, Newcastle University |
| **Report:** | Final report (Year 3), December 2014 |
| **Previous report:** | Annual report, September 2013 |
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| **Date project commenced:** | 1st October 2011 |
| **Date project completed (or expected completion date):** | 30th September 2014 |

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## *AUTHENTICATION*

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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## *CONTENTS*



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## *GROWER SUMMARY*

### Headline

- Ozone treatment regimes were optimized to achieve approximately 90% reduction of bacteria on leafy produce surfaces without causing physical damage e.g. 10 ppm for 2 minutes.
- Food pathogens (*E. coli* and *Listeria* sp.) are sensitive to ozone treatment.

### Background & Summary

Fresh leafy produce is rendered unmarketable after harvest by microbes. The increasing pesticide resistance problem and consumer demands for residue-free produce has led to the research and promotion of alternative produce treatment practices such as the use of ozone to reduce microbial loads and curb spoilage of crops in storage and/or transit.

Previous work has demonstrated that long-term exposure to low atmospheric concentrations of ozone can be effective in some crops (e.g. kiwi, avocado, berries, etc.) in significantly reducing mould proliferation but less work has been done on leafy produce. The initial aim of this work was to determine ozone exposure levels that did not damage produce but which reduced microbial loads. Different produce types had different abilities to resist ozone damage e.g. coriander and rocket were resistant to ozone (10 ppm for 10 minutes) while spinach, watercress and lettuce were more sensitive (1 ppm for 10 minutes). However, all ozone exposures used reduced bacterial loads by at least one order of magnitude. Confocal microscopy was used to visualise microbes on plant cell surfaces before and after ozone treatment. Direct observation (live/dead cell staining) of cells after ozone exposure showed that some cells were still alive; this included cells in small micro-colonies and cells present as individuals on the leaf surface. These visual observations demonstrated the heterogeneity in ozone resistance of leaf surface bacteria. In order to investigate this further it was hypothesized (Finkel, 2006) that cell age and stress (cold) may be responsible for the variation in ozone resistance. Interestingly both older cells and cold stressed cells of *Pseudomonas* sp. (isolated from coriander) showed higher ozone resistance than control cells. Subsequent gene expression analysis of old and cold stressed cells (using RNA-Seq technology) showed significant changes in genes related to stress resistance compared to controls. In particular, it was observed that in aged cells, about 90% of genes expressed mapped to one gene (a non-coding RNA that is part of RNase P). This gene interacts with cellular mRNA transcripts and may be involved in controlling expression of other genes.

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In parallel, work on the use of ozone to kill bacterial food pathogens on leafy produce was carried out. Results showed that 10 ppm ozone treatment for 2 minutes gave at least a 1 order of magnitude reduction in *E.coli* and *Listeria* spp. on spinach and that the pathogens did not re-grow after treatment (over a 9 day storage period). Overall it can be concluded that ozone treatment is a potential alternative method to reduce microbial spoilage and food pathogen contamination of leafy produce and is worth exploring on a pilot-scale in an industrial setting.

## Exploration of higher ozone exposure levels to treat leafy produce without causing visual damage

This section of work aimed to develop a shorter produce ozone exposure period so that the technology could be applied to other stages of the fresh produce processing chain e.g. vacuum cooling, where shorter treatments are needed.

No visual ozone damage was observed when leafy produce was exposed to higher concentrations such as 10, 15 and 20 ppm ozone for short durations (Table 1). Ozone treated produce visually looked as fresh as untreated produce (control) after 7 days of storage (Figure 1). Ozone injury/visible damage were observed on all produce when exposed to 25 ppm ozone concentration.

**Table 1:** The maximum ozone exposure levels that can be applied on the targeted produce without causing visible damage

| | **Duration of the exposure of targeted leafy produce** | | | | |
|---|---|---|---|---|---|
| | **Spinach** | **Rocket** | **Watercress** | **Lettuce** | **Coriander** |
| **10 ppm** | **2 min** | **2 min** | **2 min** | **2 min** | **2 min** |
| **15 ppm** | **45 sec** | **45 sec** | **30 sec** | **30 sec** | **30 sec** |
| **20 ppm** | **30 sec** | **30 sec** | **15 sec** | **15 sec** | **30 sec** |
| **25 ppm** | **-** | **-** | **-** | **-** | **-** |

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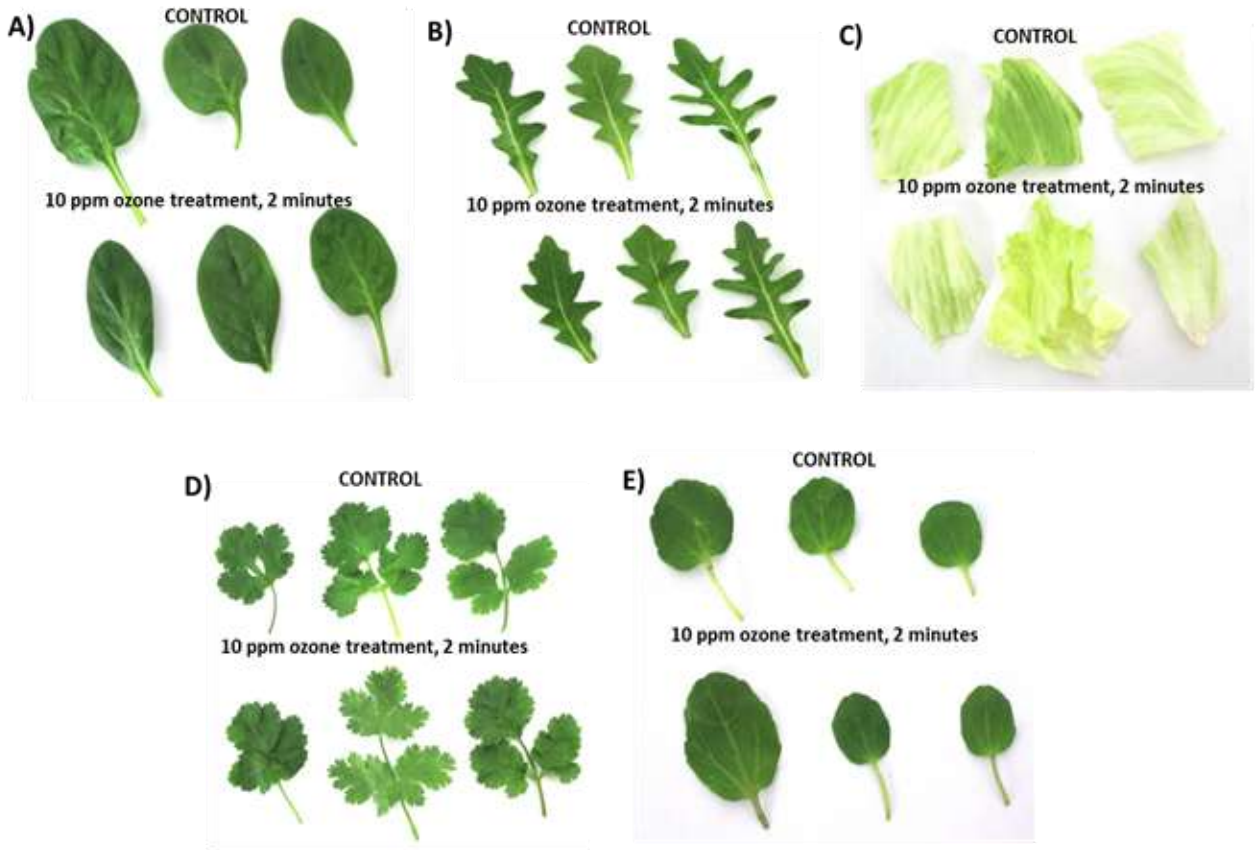


**Figure 1:** Impact of 10 ppm ozone exposure levels for 2 minutes on visual quality of A) spinach, B) rocket, C) lettuce, D) coriander and E) watercress.

## Impact of the higher ozone exposure levels to reduce microbial load present on the surface of leafy produce (*in vivo*)

Having demonstrated that ozone exposure levels up to 20ppm did not damage produce the next aim of the work was to assess if the highest safe levels (with shorter duration of exposure) were able to reduce microbial counts on produce surfaces.The impact of high ozone concentration on microbes present on the surface of the spinach, rocket, lettuce, coriander and watercress leaves is shown in Figure 2. The number of colonies (CFU/g) showed an order of magnitude CFU reduction of aerobic bacteria present on the surface of the produce when subjected to 10 ppm and 15 ppm ozone treatment as compared to untreated control produce. There was no significant reduction in the number of colonies on all leafy produce treated with 10 ppm ozone treatment and that treated with15 ppm ozone.

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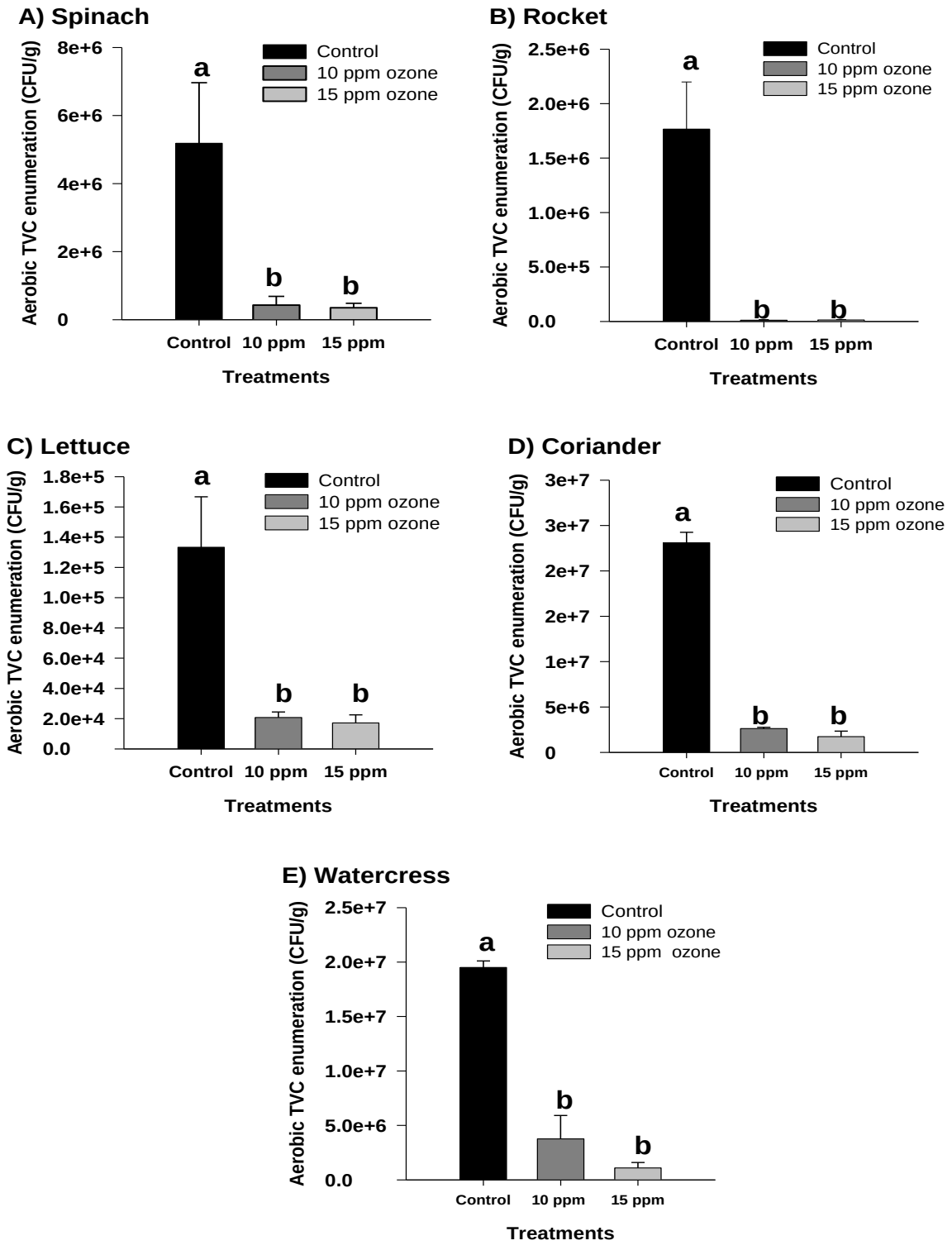


**Figure 2:** Impacts of high ozone treatment on microbial flora present on surface of A) spinach, B) Rocket, C) lettuce, D) coriander and E) watercress. Produce were either exposed to 15 ppm ozone concentration (grey bar) for 2 minutes, 10 ppm ozone concentration ( dark grey bar) for 30 sec or 'clean' air (black bar).Values represent means (±Standard Error) of measurements made on three independent plates per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

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## Gene expression in cold stressed and aged bacteria by RNA sequencing

The aim of this work was to determine the potential genetic mechanisms by which bacteria are able to resist ozone treatment. Understanding such mechanisms may aid the development of future novel produce treatment options. This was in itself a very academic exercise (this project is a PhD Studentship) therefore i*f you wish to read through the materials and methods and results obtained then either read through the full report or alternatively go to Appendix 1.*

## Effect of ozone exposure on *E. coli* and *Listeria* sp*. in vitro*

Colony numbers (CFU) of *E.coli* K12 and *L. innocua in vitro* were significantly reduced ($P < 0.05$) by all ozone treatments (Figure 6), even at the lowest level used (1 ppm for 10 mins). Less than 1-log reduction was achieved when exposed to 1 ppm for 10 mins but more than 1-log reduction was achieved when both the strains of food pathogens were treated with ozone concentrations of 10 ppm and 50 ppm. This implies that ozone concentrations of 10 ppm and 50 ppm reduced counts significantly more compared to 1ppm ozone. However, there was no significant difference in colony counts between 10 ppm and 50 ppm ozone concentration treatment in both strains of food pathogens.

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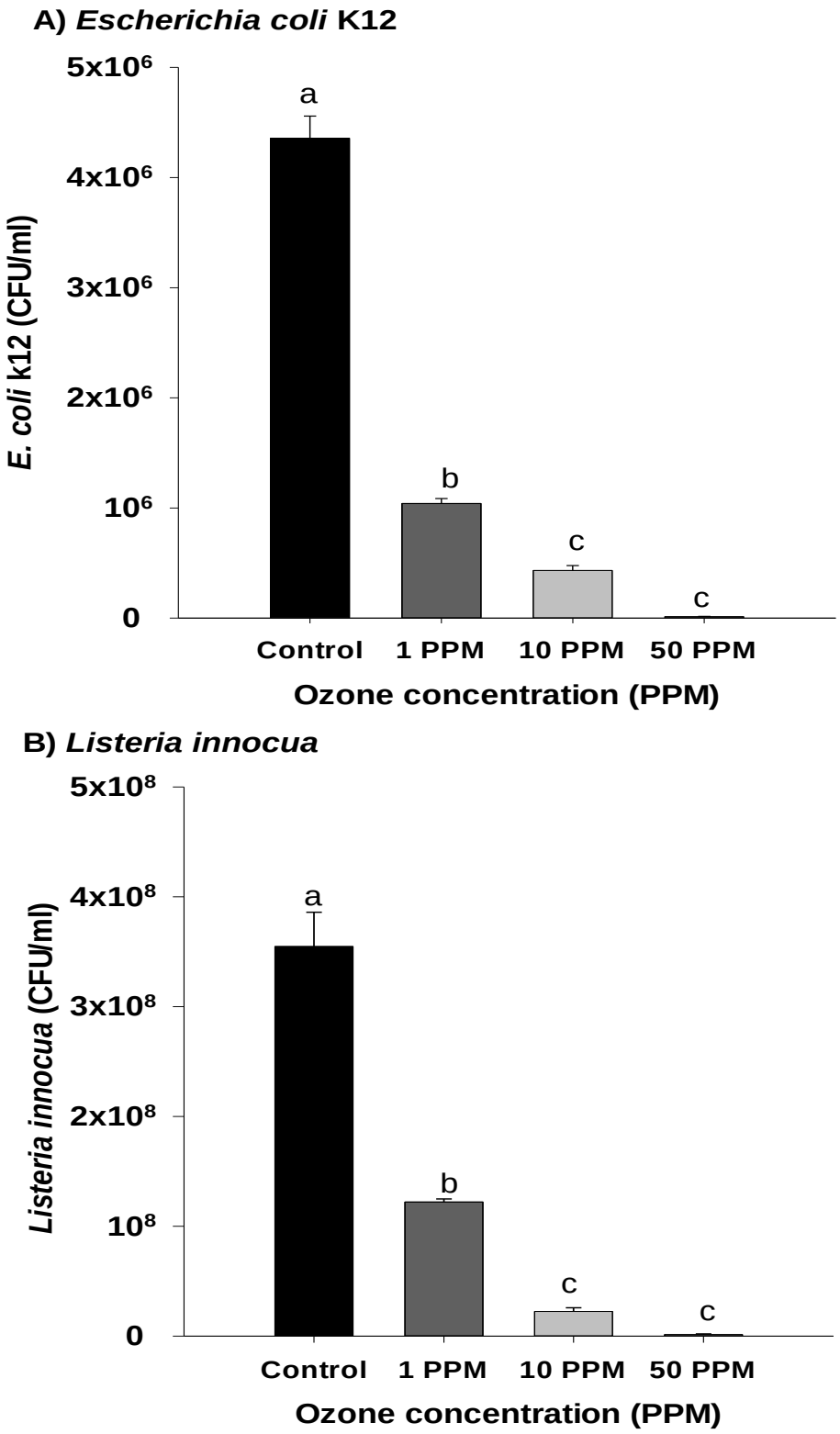


**Figure 6:** Impacts of ozone treatment on A) *E. coli* K12 and B) *L. innocua* (CFU/ml) grown on agar plates. The treatment chamber was ventilated with 1, 10 or 50 ppm ozone for 10 mins. Controls were exposed to 'clean air'. Values represent the mean (Standard Error) of measurements made on three independent plates per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

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## Impact of ozone treatment on *Listeria innocua* and *L. seeligeri* inoculated onto spinach leaves

Colony numbers (CFU) of *L. innocua* and *L. seeligeri* obtained directly from ozone exposed leaves (1ppm) i.e. day 0 were significantly reduced ($P < 0.05$) compared to non-ozone exposed controls (Figure 7). A similar trend was also observed when ozone treated leaves were stored for 9 days (Figure 7). No *Listeria* colonies were isolated from non-inoculated spinach leaves.

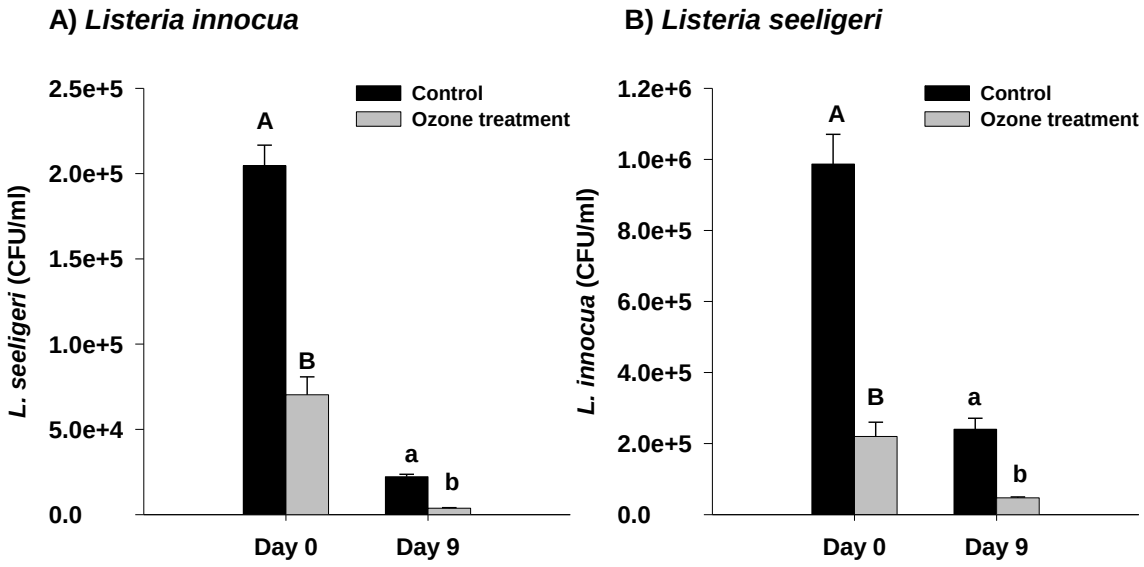


**Figure 7:** Impacts of ozone-enrichment on *L. innocua* and *L. seeligeri* inoculated onto the surface of spinach leaves. Leaves were either treated with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 10 minute. Colonies were enumerated either directly after the treatments i.e. day 0 or after 9 days storage. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are significantly different ($P < 0.05$).

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## Effect of higher ozone treatment on *E.coli* and *Listeria* sp. inoculated onto spinach leaf surface

Results of spinach artificially contaminated with two strains of *E.coli* (*E.coli* O157:K88a and *E.coli* O25:h4) and *Listeria* (*L. innocua* and *L. seeligeri*) treated with 10 ppm of ozone concentration for 10 minutes are shown in Figure 8. For *E.coli* O157:K88a and *E.coli* O25:h4, ozone treatment significantly ($P < 0.05$) reduced counts by 1-log compared with the untreated control (Figure 8A). Ozone had less than 1-log effect on *L. innocua* and *L. seeligeri* (Figure 8B). Overall this treatment on both the strains of food pathogen showed greater reductions than that observed at lower ozone levels.

To investigate the after effects of the ozone treatment on pathogen growth, artificially contaminated spinach was stored at 7°C for 9 days. Figure 9 shows populations of both *E.coli* (*E.coli* O157:K88a and *E.coli* O25:h4) and *Listeria* sp. (*L. innocua* and *L. seeligeri*) after 9 day storage did not regrow as a significant reduction in number of colonies was observed as compared with the untreated control.

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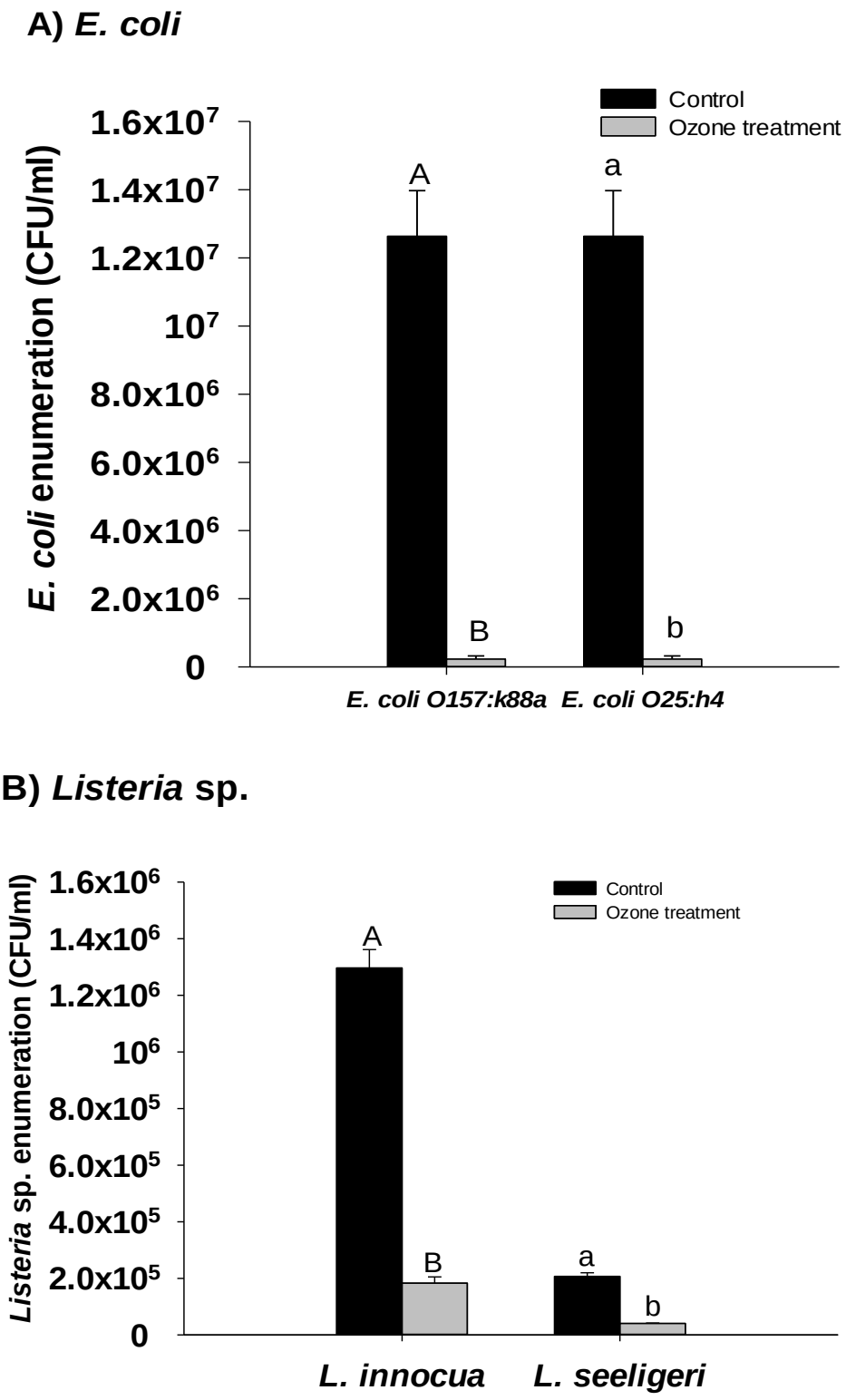


**Figure 8:** Impacts of increased levels of ozone exposure on two strains of *E.coli* and *Listeria* inoculated onto the surface of spinach leaves. Leaves were either treated with 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minutes. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are significantly different ($P < 0.05$).

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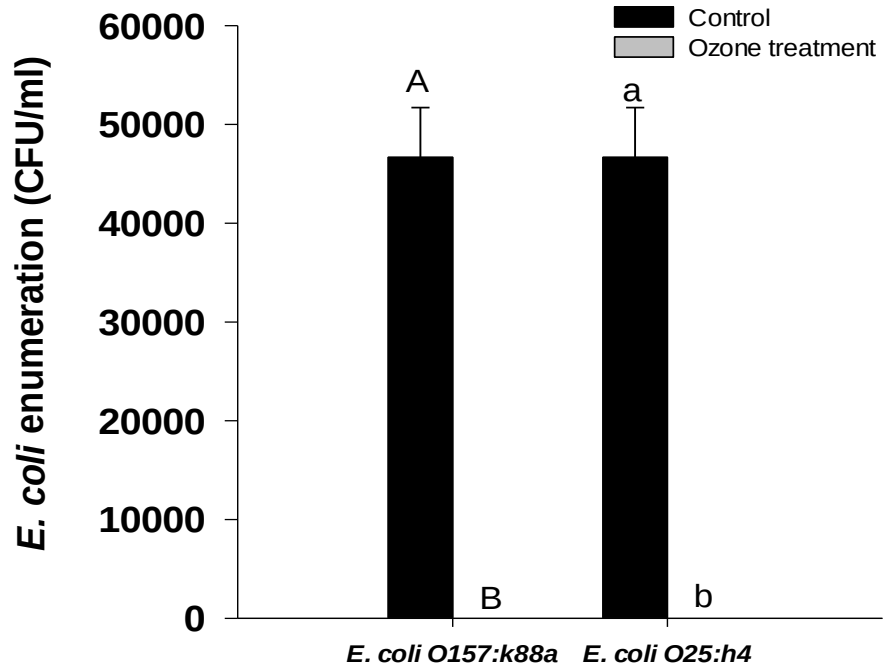
**A) *E.coli***




**B) *Listeria* sp.**

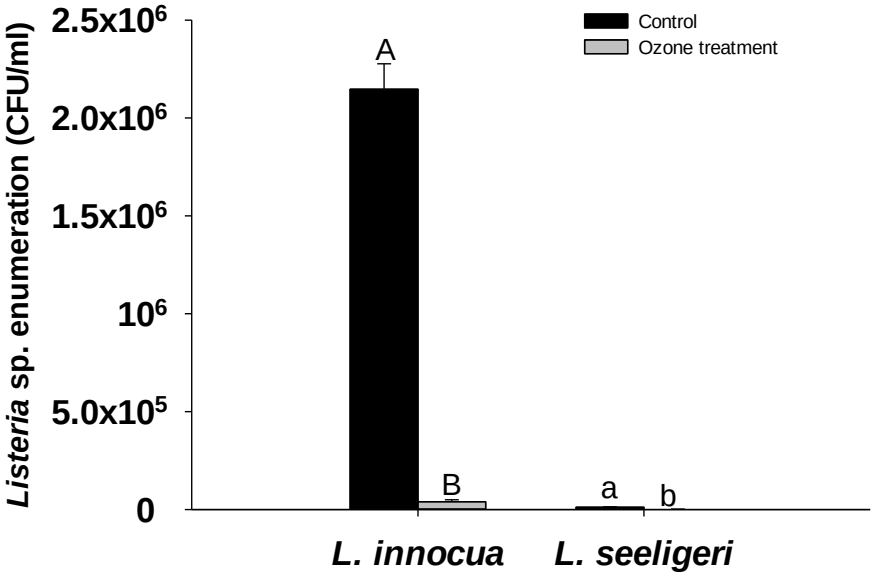



**Figure 9:** Impacts of ozone treatment on two strains of *E.coli* and *Listeria* inoculated onto the surface of spinach leaves. Leaves were either treated with 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minute. Colonies were enumerated after 9 days storage. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are significantly different ($P < 0.05$).

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### Effect of age on ozone resistance of *E. coli* O157:k88a *in vitro*

*E.coli* cultures of increasing age were exposed to ozone (10ppm for 2 minutes) (*in vitro*) and results demonstrated a clear increase in ozone resistance of *E. coli* O157:k88a with increasing colony age. For example, survival of *E. coli* O157:k88a was observed to be greater (approximately 15%) after 5 days of growth compared to 1 day old cultures. An further increase in the level of survival was observed at day 7 (Figure 10) suggesting that cells in older bacterial colonies are more ozone resistant than cells from younger colonies.

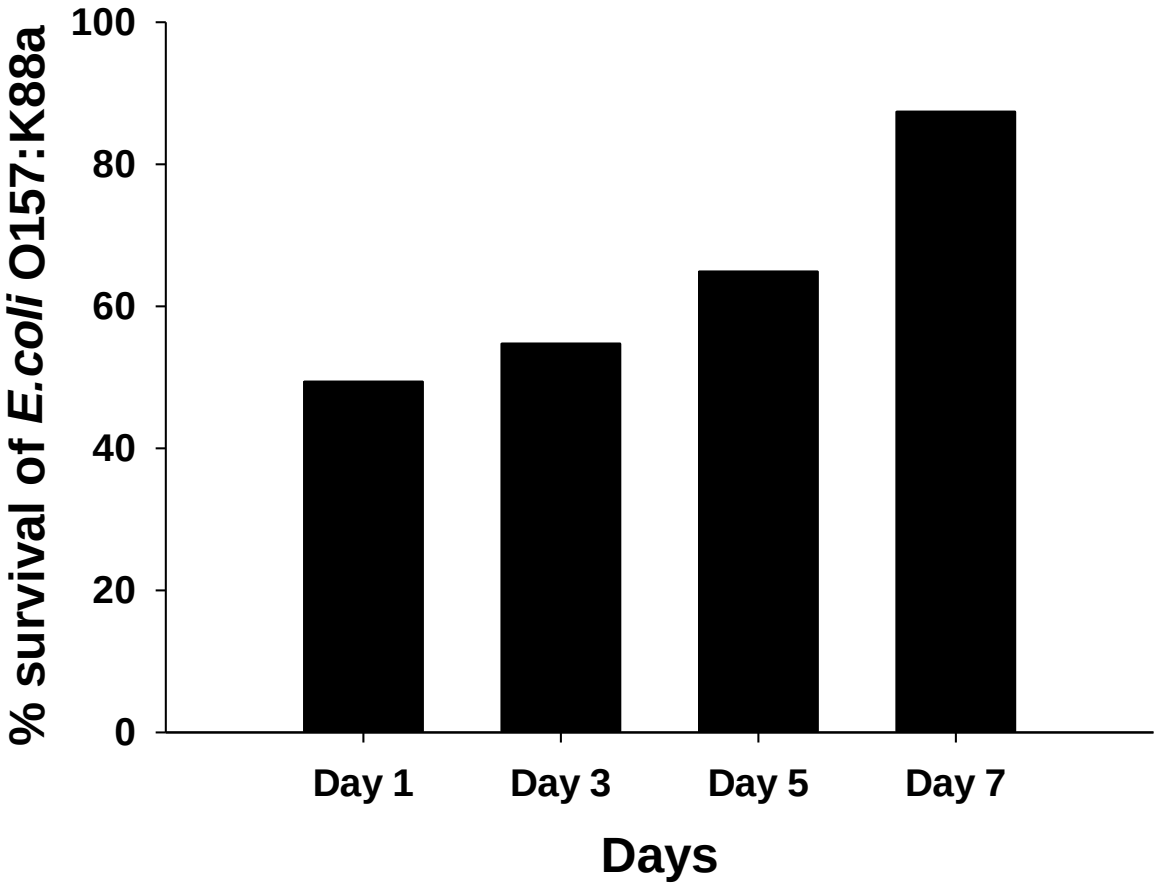


**Figure 10:** Survival of cells obtained from different colony ages of *E.coli* O157:K88a exposed to 10 ppm ozone concentration for 2 minutes. After ozone exposure, the culture plates were maintained at 37°C for 7 days. Values represent means of measurements made on three independent plates per treatment.

### Effects of ozone treatment on leaves treated with pesticides

Ozone treatment had no significant effect on any pesticide residue levels found on spinach leaf surfaces. The cationic surfactant Benzalkonium chloride was not displayed on the list of actives of the foliar pesticide used but was observed in chromatography analysis. This could be possibly introduced through post-harvest cleaning or washing processes.

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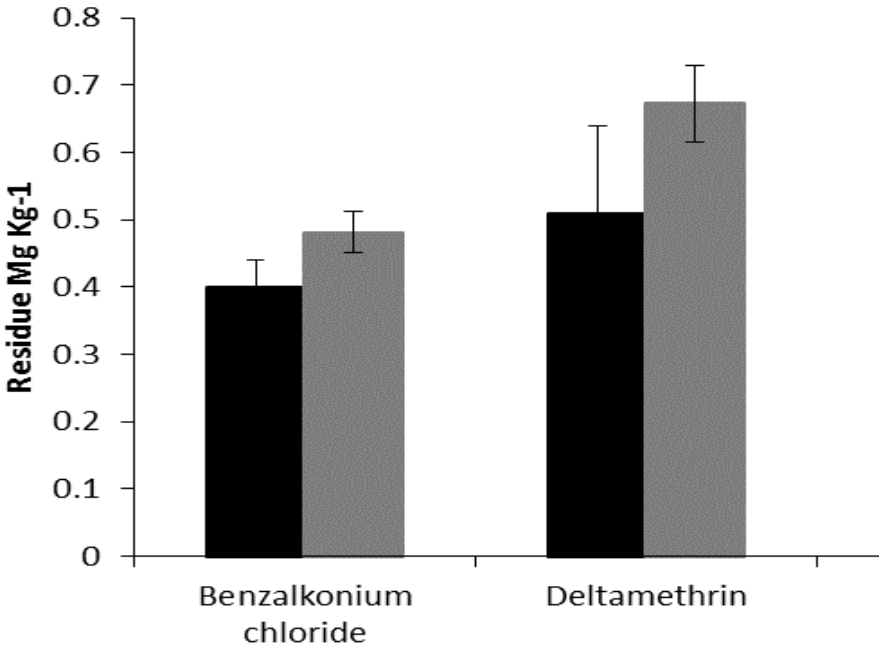


**Figure 11:** Impacts of ozone treatment on leaves treated with pesticide. Pesticide treated leaves were either exposed to 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minute. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment.

## Financial Benefits & Action Points

No financial benefit can be derived from the laboratory work but the student has shown that there is potential for scaling up ozone treatment technologies for the industry. The use of ozone would lead to reduced residues in produce and potentially less microbial deterioration of produce would would benefit the industry immensely. Discussion with industry representatives has taken place on up-scaling the work and to determine the most suitable place to introduce ozone while processing fresh produce. It would appear that the vacuum cooling process may be the best place to trial ozone application on a pilot scale as during vacuum cooling maximum air (and hence ozone) exposure of produce would occur. Also the vacuum cooler is an enclosed treatment process meaning ozone could safely be introduced at this stage with minimal risk of worker exposure to high ozone levels. More funding and time would be required for such commercial trials.

The novel work on gene expression by spoilage microbes could lead to the exploration of new treatment options because if we have a better understanding of how microbes are able to resist treatments then it may be possible to apply simple external treatments to alter microbial gene expression (i.e. turn off resistance mechanisms) and hence reduce microbial produce loads even further.

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## *SCIENCE SECTION*

### Introduction

The increasing popularity of fresh produce as a food source is due to its accepted importance as a source of vitamins, fibre and nutrients to humans (Olaimat and Holley, 2012). Production of leafy produce has grown rapidly in the past few decades due to increases in year-round consumption and rising demand for worldwide distribution (Little and Gillespie, 2008). Research has proved that a low fat and high fibre diet which includes abundant consumption of fruits and vegetables are protective against illness such as cancers and cardiovascular diseases (Mercanoglu Taban and Halkman, 2011). Increased consumption of vegetables is predominantly encouraged by the trend of healthier lifestyle and the 5 A DAY – live well concept promoted by the Government and independent health authorities (Little and Gillespie, 2008).

Fresh leafy produce receives minimum processing and is generally consumed raw (Naito and Takahara, 2006). The microbial flora is assumed to be limited to the surface of the healthy produce whereas the internal tissue remains sterile (Naito and Takahara, 2006). Spoilage of produce is caused by numerous bacterial species by either breaking the protective cover of the leaf or by entering the plant tissue through wounds (Tournas, 2005). It appears that microbial contamination can arise at any stage from production to consumer handling and this contamination can occur from animal, environmental, human sources or by simple multiplication of surface biofilms to create slime and off odours (Olaimat and Holley, 2012). The microbial communities found on leafy produce are commonly dominated by bacteria from the *Enterobacteriaceae* and *Pseudomonadaceae* families and the total microbial population ranges from $10^5$ – $10^7$ CFU/cm$^2$ (Ragaert *et al.*, 2007). Generally the microbial populations present do not affect the leaf in the field but after harvesting and storage certain bacteria such as *Pseudomonas* spp., *Bacillus spp*. and *Erwinia* spp. can begin to cause soft rots of the produce (P. Saranraj, 2012). The microbial proteolytic and pectinolytic activities that cause soft rot can be carried out by these microbes at storage temperatures as low as 0.2°C (P. Saranraj, 2012).

In addition to produce spoilage, microbes present on leafy produce have also been implicated in human disease. For example, leafy produce may be contaminated with food-borne pathogens, such as enterohaemorrhagic *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* (Abadias *et al.*, 2008). During processing, leafy produce are washed with either chlorinated water or spring water to reduce the microbial load. However, these approaches have their limitations and hence, there is a major commercial interest in optimising a potential treatment to reduce losses of leafy salad by microbial spoilage.

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Ozone is well known for its strong oxidizing capacity and has been recognized for over a century as a powerful antimicrobial agent, reacting with organic substances approximately 3,000 times quicker than chlorine (Singh *et al*., 2002). In 1997, the United States Food and Drug Administration (US-FDA) in union with an expert panel granted ozone as GRAS (Generally Recognised as Safe) status (Graham *et al*., 1997) and later, in 2003, it received formal approval from the US-FDA as a 'direct contact food sanitizing agent' (Karaca and Velioglu, 2007). Previous work has demonstrated that long-term exposure to low atmospheric concentrations of ozone can be effective in some crops (e.g. asparagus, kiwi, avocado, berries, etc.) in significantly reducing mould proliferation but less work has been done on leafy produce. One of the major advantages of ozone treatment is the fact the gas leaves no detectable residues in/on treated products as ozone rapidly decomposes into oxygen (Guzel-Seydim *et al*., 2004).

## Summary of results from year 2:

The aim of this work in Yr 2 were to determine ozone exposure levels that did not damage produce, but reduced microbial loads significantly. Different produce types had different abilities to resist ozone damage e.g. coriander and rocket were resistant to ozone (10 ppm for 10 minutes) while spinach, watercress and lettuce were more sensitive (1 ppm for 10 minutes). However, all ozone exposures used reduced bacterial loads by at least one order of magnitude. Confocal microscopy was used to visualize microbes on plant cell surfaces before and after ozone treatment. Direct observation (live/dead cell staining) of cells after ozone exposure showed that some cells were still alive; this included cells in small micro-colonies and cells present as individuals on the leaf surface. These visual observations clearly demonstrated the heterogeneity in ozone resistance of leaf surface bacteria. In order to investigate this further it was hypothesized (Finkel, 2006) that cell age and stress (e.g. cold) may be responsible for the variation in ozone resistance seen. Interestingly both older cells and cold stressed cells of *Pseudomonas* sp. (isolated from coriander) showed higher ozone resistance than control cells. In parallel, work on the use of ozone to kill inoculated bacterial food pathogens on leafy produce was carried out. Results showed that 1 ppm ozone treatment for 1 minute gave at least a 1 order of magnitude reduction in *E.coli* on spinach.

A meeting with industry representatives at the end of Year 2 identified several objectives to take forward into year 3.

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**Year 3 Objectives:**

a) To optimize higher ozone concentration treatments to reduce microbial loads on leafy produce (this required development of a modified ozone fumigation system) – Previous results focused on lower ozone levels for longer periods and industrial representatives were interested in developing a shorter treatment using higher ozone concentration

b) To study bacterial (*Pseudomonas* sp.) gene expression in response to increasing cell age and cold stress by using RNA-Seq to develop a better understanding of ozone resistance mechanisms

c) To determine the impacts of ozone treatment on artificially inoculated food pathogens (*E.coli* and *Listeria* spp.) on spinach leaves

d) To investigate the effects of ozone treatment on leaves treated with pesticides

## Materials and methods

### Modified ozone fumigation system – delivery of high ozone concentrations for short time durations (seconds)

A modified ozone fumigation system was engineered to improve application of ozone to produce surfaces and also to reduce the time required to build up the desired ozone concentrations needed for produce treatment. The aim was to develop a system allowing application of higher ozone concentrations for shorter durations to improve bacterial kill without damaging the produce. The system was housed in a fume hood and constructed from 20 $cm^2$ x 20 $cm^2$ Perspex. Produce was placed on a steel mesh in a 2 cm deep tray within the box and produce was then exposed to ozone once the desired concentration was achieved (as shown in Figure 12). Ozone was added with 1 inlet pipe generated by electric discharge from oxygen, with the introduction of ozone controlled manually. The ozone concentration was recorded by a photometric analyzer (model 450, manufactured by Advanced Pollution Instrumentation Inc.). The ozone monitor employed in these studies was serviced weekly and calibrated routinely.

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**Figure 12:** Modified ozone fumigation system

### Exploration of higher ozone exposure levels to treat leafy produce without causing visual damage

This experiment focused on optimizing (high) ozone concentration for shorter duration on organic baby spinach, Iceberg lettuce, wild rocket, coriander and watercress without causing visible damage/deterioration. To determine the impact on visual quality of the produce, leaves were exposed to 10, 15, 20 or 25 ppm ozone or 'clean air' (controls) for varying periods of time ranging from 30 seconds to 2 minutes. Following exposure to ozone, produce was packed in a sealed plastic bag and maintained at 4°C in dark conditions. Ozone injury was assessed visually by comparing ozone exposed produce with control (non-ozone exposed) produce every alternate day for 7 days.

### Use of the highest ozone exposure levels to reduce microbial load present on the surface of leafy produce (*in vivo*)

Once the highest ozone exposure levels that didn't cause produce damage were determined (from section above) the same concentrations were used to examine reduction of microbial load on the surface of the leafy produce. Organic baby spinach, Iceberg lettuce, wild rocket, coriander and watercress were treated with either 10 ppm ozone for 2 minutes, 15 ppm ozone for 30 seconds or untreated in the ozone treatment box as mentioned in section above. After treatment, total viable counts (CFU/g) were enumerated using standard plate count agar (PCA) in petri-dishes and incubated at 30°C for 3 days after serial dilution in minimum recovery diluent (MRD).

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## Gene expression in aged and cold stressed bacteria by RNA sequencing

Results from previous years had revealed that older and cold stressed cells of *Pseudomonas* sp. (isolated from coriander) showed higher ozone resistance than control cells. This suggested that common crop growth and storage conditions may enhance bacterial resistance to treatments applied to reduce their numbers. In order to examine the mechanisms of ozone resistance, gene expression in stressed bacteria and control cells were compared using RNA-Seq techniques. To examine responses of cold stressed cultures, a colony of *Pseudomonas* sp. isolated from coriander was inoculated in 9ml of sterile MRD (Minimum Recovery Diluent) and serial diluted to standardized concentration $10^{-4}$ cells per mL. This inoculum (100 µl) was spread onto sterile CFC plates and maintained at 4°C (mimicking cold storage conditions) for 7 days. For examining aged culture responses, the plates were incubated at 25°C for 10 days. Control cultures were incubated at 25°C for 48 h. The inoculum was prepared by swabbing colonies of bacteria from the CFC plates into a 3-fold buffer/PBS solution (150 ml 1xPBS + 300ml Nutrient broth) using a sterile loop. Cold conditions were maintained throughout for cold stressed cultures. The inoculum was then homogenized using a bead beater. RNase-away treated glass beads 2mm in diameter were added to the samples and shaken at 30 Hz (hertz) for 30 seconds.

### RNA extraction

Three biological replicates were used for all RNA-Seq experiments from each culture type. The total RNA from the bacterial cells was extracted using ISOLATE RNA kit (Bioline). All procedures were carried out using the manufacturer's protocol and guidelines. The integrity and quality of the total RNA was determined by a NanoDrop 1000 spectrophotometer and Agilent's 2100 Bioanalyzer using the Agilent RNA 6000 Nano kit.

### Construction of RNAseq libraries enriched for bacterial mRNA

RNAseq libraries were constructed using the TruSeq Stranded RNA Sample Preparation Kit (Illumina San Diego, CA). Briefly, total RNA was recovered from the RNAStable columns using the manufacturer's protocol. RNA was DNAsed and then quantitated by Qubit (Life Technologies, Grand Island, NY) and checked for integrity on a 1% eGel (Life Technologies). Ribosomal RNA was removed from 1ug of total RNA using the Ribo-Zero™ Magnetic Bacteria kit (Illumina, CA). First-strand synthesis was synthesized with a random hexamer and SuperScript II (Life Technologies). Double stranded DNA was blunt-ended, 3'-end A-tailed and ligated to indexed adaptors. The adaptor-ligated double-stranded cDNA was amplified by PCR for 12 cycles with the Kapa HiFi polymerase (Kapa Biosystems, Woburn, MA). The final libraries were quantified on Qubit and the average size determined on an Agilent bioanalyzer DNA7500 DNA chip (Agilent Technologies, Wilmington, DE) and

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diluted to 10nM final concentration. The 10nM dilution was further quantified by qPCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc. CA), which results in high accuracy and consistent pooling of barcoded libraries and maximization of the number of clusters in the flowcell.

**Sequencing on an Illumina HiSeq2000**

The pooled libraries were loaded onto one lane of an 8-lane flowcell for cluster formation on the cBOT and then sequenced on an Illumina HiSeq2000 from one end of the molecules for a total read length of 100 nucleotides (nt) from that end. The typical output from a lane with version 3 sequencing reagents and Casava1.8.2 is 150 to 200 million reads; 192M reads were generated from this run. The run generated .bcl files which were converted into demultiplexed compressed fastq files using Casava 1.8.2 (Illumina, CA). A secondary pipeline decompressed the fastq files, generated plots with quality scores using FastX Tool Kit, removed perfect matches to reads that contain only adaptor and generated a report with the number of reads per sample/library. Demultiplexed fastq files were .tgz compressed and posted to a password-secured FTP site.

**RNA-Seq alignment**

Raw FASTQ data was subjected to a head crop of 1 base due to a fair number of low-quality bases at positions 2-3, then quality-trimmed from both 3' and 5' ends using the program Trimmomatic v 0.30 (Lohse *et al*., 2012), using a minimal phred33 quality score of 20 and a minimal length of 15. Sequences were then aligned using Novoalign v3.00.05, Novocraft using the default parameters for single-end reads and the *Pseudomonas* sp. GM60 genome (Genbank: AKJI00000000.1) from NCBI as the reference genome. The raw read counts were tabulated for each sample using the GFF gene model file from NCBI and htseq-count, from HTSeq v0.6.1 using parameters -m intersection-nonempty -s reverse -t gene -i Name.

The raw read counts were input into R v3.1.1 (R Core Team, 2013) for data pre-processing and statistical analysis using packages from Bioconductor (Carey *et al*., 2004) as indicated below. 2207 genes out of 5943 did not have at least 1 count per million mapped reads in at least 2 samples and were filtered out. The remaining 3736 genes were analyzed for differential expression using edgeR v3.6.8 (Robinson and Smyth, 2010). The raw count values were used in a negative binomial model (Robinson and Smyth, 2007) that accounted for the total library size for each sample and an extra TMM normalization factor (Robinson and Oshlack, 2010) for any biases due to changes in total RNA composition of the samples, along with qCML tagwise dispersion estimates. Pairwise comparisons of cold stressed (4C)

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vs. Control (C) and aged (D10) vs. C were calculated using exact tests, and a False Discovery Rate correction (Hochberg and Benjamini, 1995) was done separately for each comparison. All clustering of samples was done using normalized individual sample expression levels obtained from edgeR's cpm function, which adjusts for the total number of reads and the extra TMM normalization factor, plus adds a proportional constant (0.25 average) to avoid zero values, then transforms to the log2 scale.

## Assessing the impact of ozone treatment on food pathogens: *E. coli* and *L. innocua in vitro*

*E.coli K12* and *L. innocua* were obtained from a culture collection maintained by Geneius Laboratories Ltd. (44 Colbourne Crescent, Nelson Park, Cramlington, NE23 1WB). These cultures were sub-cultured on Nutrient agar (NA) and Agar *Listeria* according to Ottaviani and Agosti (ALOA) agar plates respectively by spread plating. A single colony was isolated from each culture plate after incubation at 37°C for 24 h and 30°C for 48 h respectively and transferred to MRD. A standardized concentration $10^4$ cells per mL of each culture was spread (100 µL) onto sterile NA and ALOA agar plates respectively. These plates were then either exposed to 1 ppm, 10 ppm, 50 ppm ozone concentration or charcoal filtered 'clean air' (controls) for 10 minutes. After treatment, the NA and ALOA agar plates were incubated at 37°C for 24 h and 30°C for 48 h respectively. The number of colonies produced on control plates (non-ozone exposed) were compared to the numbers found on ozone-treated plates based on three replicate observations.

## Impact of ozone treatment on *Listeria innocua* and *L. seeligeri* inoculated onto spinach leaves

*Listeria innocua* and *L. seeligeri* were obtained from a culture collection maintained by Geneius Laboratories Ltd. Cultures were stored at 4°C on ALOA agar plates. Spinach leaves were then aseptically cut into discs measuring 1.13 $cm^2$ using sterile cork borer. A suspension of *Listeria* sp. ($10^7$ – $10^8$ CFU/ml MRD) was applied directly to the leaf disc in 300 µl aliquots and the inoculated leaves were maintained at 7°C to mimic produce storage conditions for 2 hours to allow attachment of *Listeria* sp. to the leaf surface. Inoculated leaves were either exposed to 1 ppm ozone or 'clean air' for 10 minutes. To determine the survival and growth of *Listeria* sp. during storage, the treated and untreated inoculated leaves were maintained at 7°C for 9 days. The number of colonies remaining (control and ozone exposed) on day 0 and day 9 was determined by vigorously shaking the leaf disc in MRD for 2 minutes after 1 h incubation at room temperature and then serially diluting in diluent followed by standard spread technique on ALOA agar plates. Plates were incubated at 30°C for 48 h, and colonies were counted.

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## Impact of higher/increased ozone concentrations on 2 strains of *E.coli* and *Listeria* inoculated onto spinach leaves

This experiment used the highest ozone exposure levels that didn't cause produce damage to try and achieve higher reductions in pathogenic bacteria on the surface of baby spinach leaves. Two strains of *E.coli* (*E.coli* O157:K88a and *E.coli* O25:h4) and *Listeria* (*L. innocua* and *L. seeligeri*) were inoculated onto spinach leaves as described in previous sections. Inoculated leaves were either treated with 10 ppm ozone concentration or charcoal filtered 'clean air' for 2 minutes. The number of *E. coli* and *Listeria* sp. remaining (control and ozone exposed) was determined as described above. To determine the impact of highest ozone exposure levels on the survival and growth of *E.coli* (*E.coli* O157:K88a and *E.coli* O25:h4) and *Listeria* (*L. innocua* and *L. seeligeri*) during storage, the inoculated leaves were treated as mentioned in previous section. After the treatment, inoculated and control leaves were maintained at 7°C for 9 days. The number of colonies remaining (control and ozone exposed) on day 9 was determined as mentioned in previous section.

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## Age effects on ozone resistance of *E.coli in vitro*

To determine whether cell age affected the ozone resistance of the bacteria, a colony of *E. coli* O157:K88a obtained from a culture collection maintained by Geneius Laboratories Ltd. was sub-cultured onto NA plates and incubated at 37°C for 7 days. A single colony was isolated on the 1st, 3rd, 5th and 7th day of the incubation and transferred to MRD. A standardized concentration 104 cells per mL of each cell age was spread (100 µL) onto sterile NA plates and these plates were then exposed to either 10 ppm ozone concentration or charcoal filtered 'clean air' for 2 minutes. Colony counts were carried out after incubating NA plates at 37°C for 24 hr.

## To investigate the effects of ozone treatment on leaves treated with pesticides

This experiment focused on investigating the effects of ozone treatment on spinach leaves treated with pesticides. Packets of spinach were purchased from a local retailer and then individual leaves were placed on paper towel with adaxial leaf surface facing upwards. The samples (15 g) were treated with Bayer household insecticide foliar product with the main active ingredient of 0.0075g/l Deltamethrin. Deltamethrin was chosen after discussion with industry representatives as a typical surface applied pesticide. Systemic pesticides were not considered as they are taken up by plants and unlikely to be exposed to ozone during treatment. In order to allow attachment of foliar applicant, the treated samples were maintained at room temperature for 24 hrs. These samples were either treated with 10 ppm ozone or charcoal filtered 'clean- air' for 2 minutes. The ozone treated and control samples were further analyzed by Geneius Laboratories Ltd. (UKAS accredited technique) for pesticide residue recovery using analytical chromatography techniques.

## Statistical analysis

Data were analysed using SPSS (IBM SPSS Statistics 19 64Bit) and graphs were produced using Microsoft Office Excel 2010 and SigmaPlot 11.0. Normal distribution was tested using Normality test and significant differences between mean values were verified using LSD ($P < 0.05$) following one-way ANOVA.

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## Results

### Exploration of higher ozone exposure levels to treat leafy produce without causing visual damage

No visual ozone damage was observed when leafy produce was exposed to concentrations such as 10, 15 and 20 ppm ozone for shorter durations (Table 4). Ozone treated produce looked as fresh as the untreated produce (control) after 7 days (Figure 13). Ozone injury/visible damage were observed on all produce when exposed to 25 ppm ozone concentration.

**Table 4:** The maximum ozone exposure levels that can be applied on the targeted produce without causing visible damage

| | **Duration of the exposure of targeted leafy produce** | | | | |
|---|---|---|---|---|---|
| | **Spinach** | **Rocket** | **Watercress** | **Lettuce** | **Coriander** |
| **10 ppm** | **2 min** | **2 min** | **2 min** | **2 min** | **2 min** |
| **15 ppm** | **45 sec** | **45 sec** | **30 sec** | **30 sec** | **30 sec** |
| **20 ppm** | **30 sec** | **30 sec** | **15 sec** | **15 sec** | **30 sec** |
| **25 ppm** | **-** | **-** | **-** | **-** | **-** |

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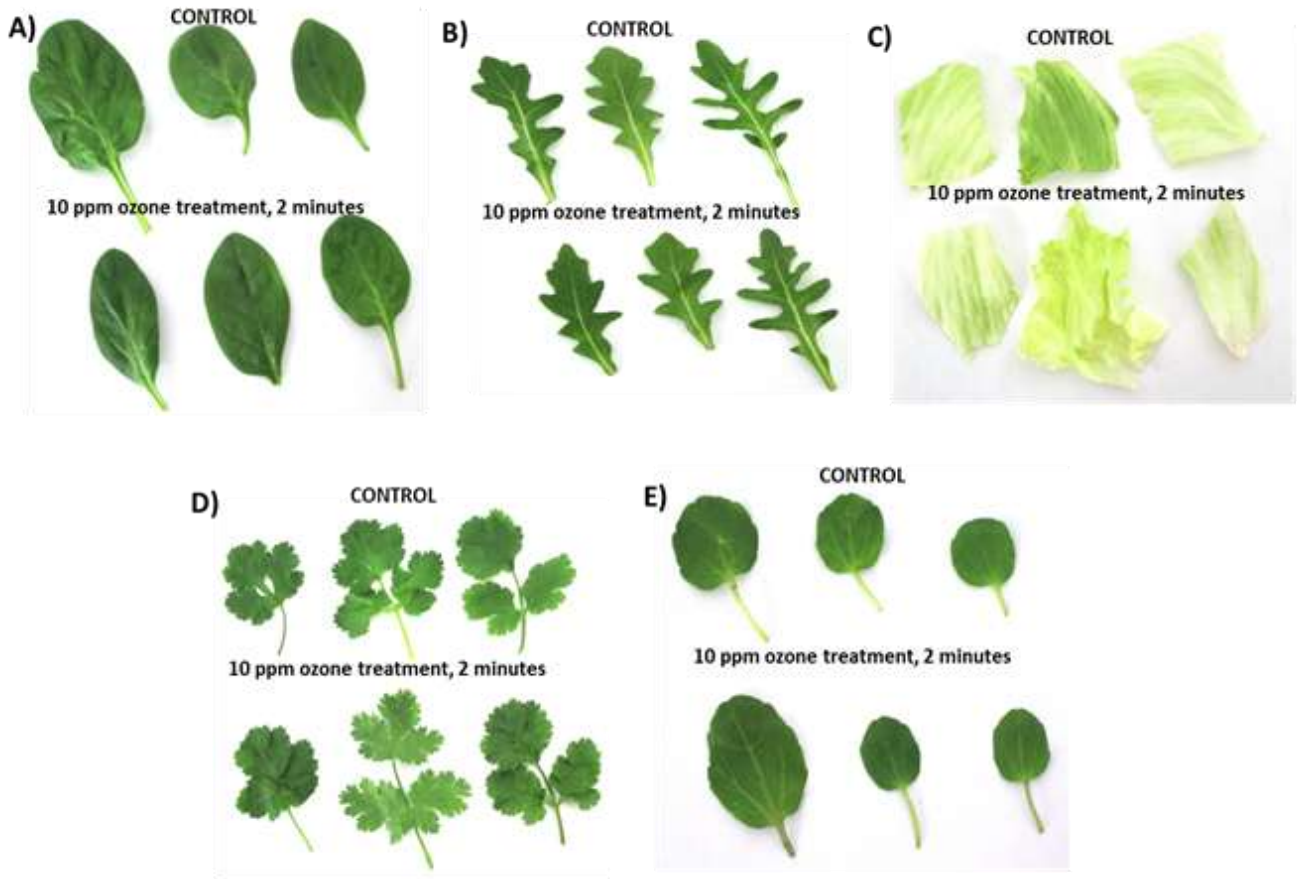


**Figure 13:** Impact of 10 ppm ozone exposure levels for 2 minutes on visual quality of A) spinach, B) rocket, C) lettuce, D) coriander and E) watercress.

## Impact of the higher ozone exposure levels to reduce microbial load present on the surface of leafy produce (*in vivo*)

The impact of high ozone concentration treatments on microbes present on the surface of the spinach, rocket, lettuce, coriander and watercress leaves is shown in Figure 14. The number of colonies (CFU/g) showed 1 log CFU reduction of aerobic bacteria present on the surface of the produce when subjected to 10 ppm and 15 ppm ozone treatment as compared to untreated control produce. However, there was no significant reduction in the number of colonies when treated with 10 ppm ozone treatment as compared to 15 ppm ozone treatment. This was observed on all targeted leafy produce.

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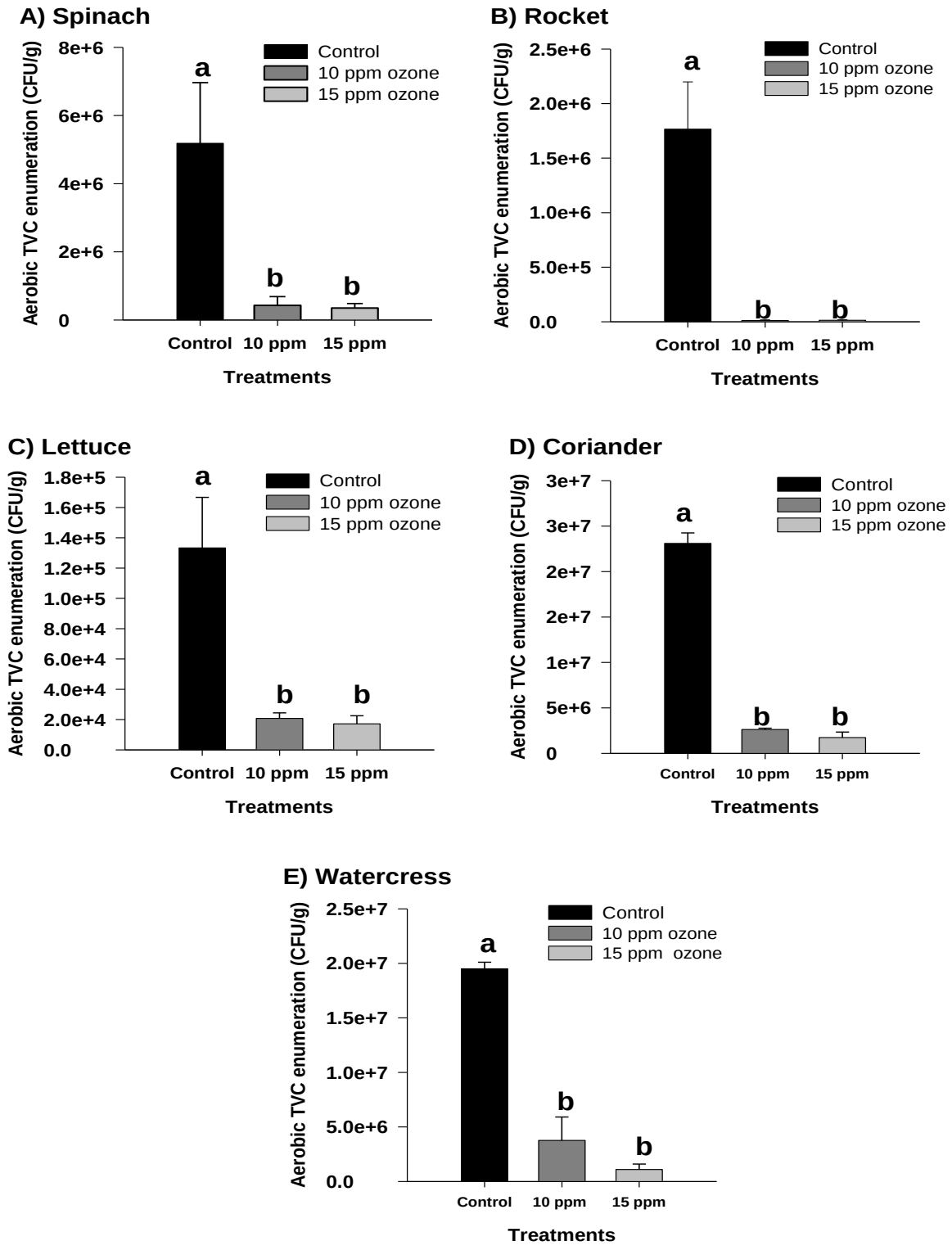


**Figure 14:** Impacts of high ozone treatment on microbial flora present on surface of A) spinach, B) Rocket, C) lettuce, D) coriander and E) watercress. Produce were either exposed to 15 ppm ozone concentration (grey bar) for 2 minutes, 10 ppm ozone concentration ( dark grey bar) for 30 sec or 'clean' air (black bar).Values represent means (Standard Error) of measurements made on three independent plates per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

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## Gene expression in cold stressed and aged bacteria by RNA sequencing

The *Pseudomonas* sp. cold stressed/ aged/ control RNA samples were used for Illumina Genome Analyzer deep sequencing. There were nine samples in total, with each condition having three replicates. The raw sequence output generated 192 million reads, each with a length of 100nt. Those reads mapping to the reference genome (*Pseudomonas* sp. GM60) were first categorized into three classes (Table 5). Uniquely mapped reads are those that map to only one position in the genome, and gapped alignment are those that have a (limited) mismatch as compared to the reference genome. Unmapped reads are those that do not (share sufficient sequence similarity to) map to any position in the reference genome.

**Table 5:** Number of reads sequenced and mapped

4C, 10D and C stand for cold stressed, aged and control samples respectively. Numbers I, II and III indicate the three replicates..

| Sample | Read Sequences | Unique Alignment | Gapped Alignment | Unmapped reads |
|---|---|---|---|---|
| 4C_I | 25,748,666 | 17,053,980 | 2,922,953 | 8,688,761 |
| 4C_II | 21,949,529 | 15,677,275 | 2,848,392 | 6,267,351 |
| 4C_III | 21,107,733 | 15,729,041 | 3,018,542 | 5,374,059 |
| C_I | 19,333,104 | 14,605,701 | 2,389,820 | 4,724,414 |
| C_II | 19,842,086 | 15,181,454 | 2,724,031 | 4,657,447 |
| C_III | 21,722,592 | 16,535,963 | 2,435,825 | 5,183,392 |
| D10_I | 20,758,340 | 14,542,190 | 1,213,218 | 6,215,488 |
| D10_II | 19,940,059 | 14,311,523 | 1,169,298 | 5,627,645 |
| D10_III | 21,465,952 | 16,539,000 | 1,122,241 | 4,925,913 |

The assembled transcripts were then classified into two main categories (Table 6): aligned transcripts and unmapped transcipts.

**Table 6:** Classification of transcripts

1 million reads aligned to *Pseudomonas* sp. GM60

| Sample | Aligned (%) | Unmapped (%) |
|---|---|---|
| 4C_I | 66.16 | 33.09 |
| 4C_II | 71.33 | 27.27 |
| 4C_III | 74.40 | 24.33 |
| C_I | 75.51 | 22.86 |
| C_II | 76.54 | 21.79 |
| C_III | 76.07 | 22.10 |
| D10_I | 70.70 | 15.71 |
| D10_II | 72.26 | 17.20 |
| D10_III | 77.37 | 16.23 |

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After the alignment of the sequence and statistical analysis, the genes differentially expressed between aged cultures (D10) and control (C) as well as between cold stressed cultures (4C) and control were identified. The two pairwise comparisons yielded approximately 500 up-regulated genes and approximately 500 down-regulated genes in each of the treatments. Overall, the 3 replicates in each sample were similar to each other. At the level of the treatments, cold stressed (4C) and control (C) samples were more comparable to each other than the aged (D10) sample (Figure 15).

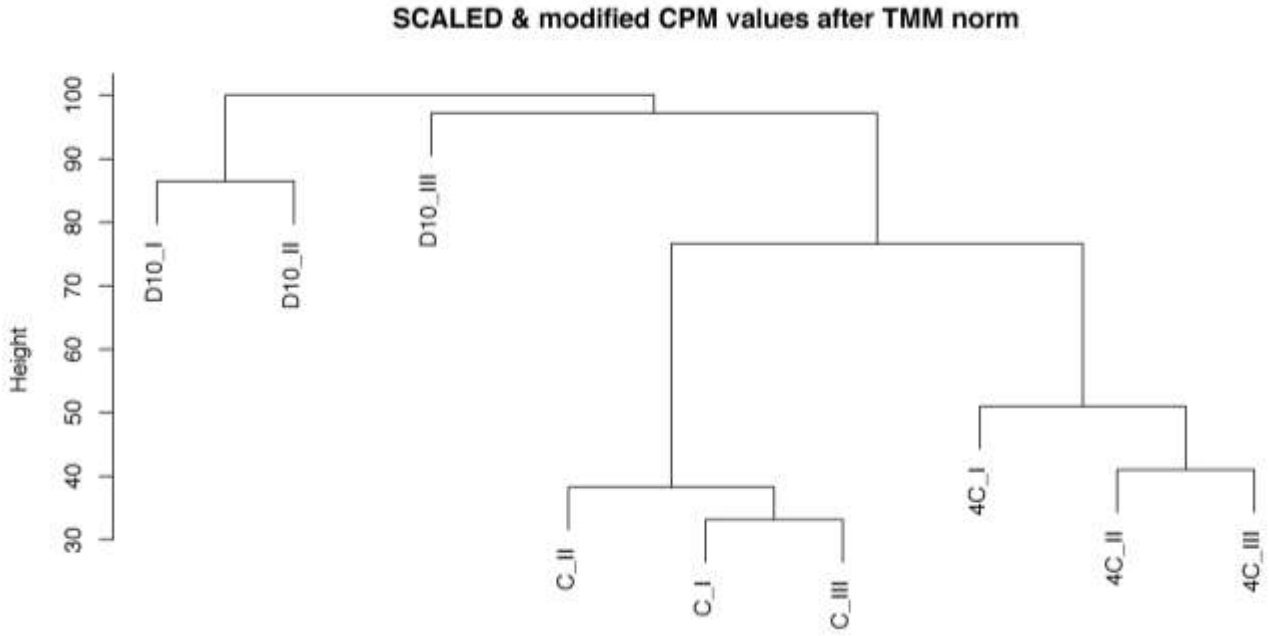


**Figure 15:** Overview of gene expression in all three bacterial samples. 4C, 10D and C stand for cold stressed, aged and control samples respectively. Numbers I, II and III indicate the three biological replicates

The analysis of differentially expressed genes (DEGs) revealed that very few genes in aged (D10) samples were responsible for the observed differences as compared to cold stressed (4C) and control (C) samples. Figure 16 illustrates that approximately 98% (Y-axis) of the reads were assigned to <5% of the genes (x-axis) in aged (D10) samples. In addition, >90% of the reads map to one gene in all three aged (D10) samples, subsequently characterised to be a non-coding RNA which is a component of RNaseP.

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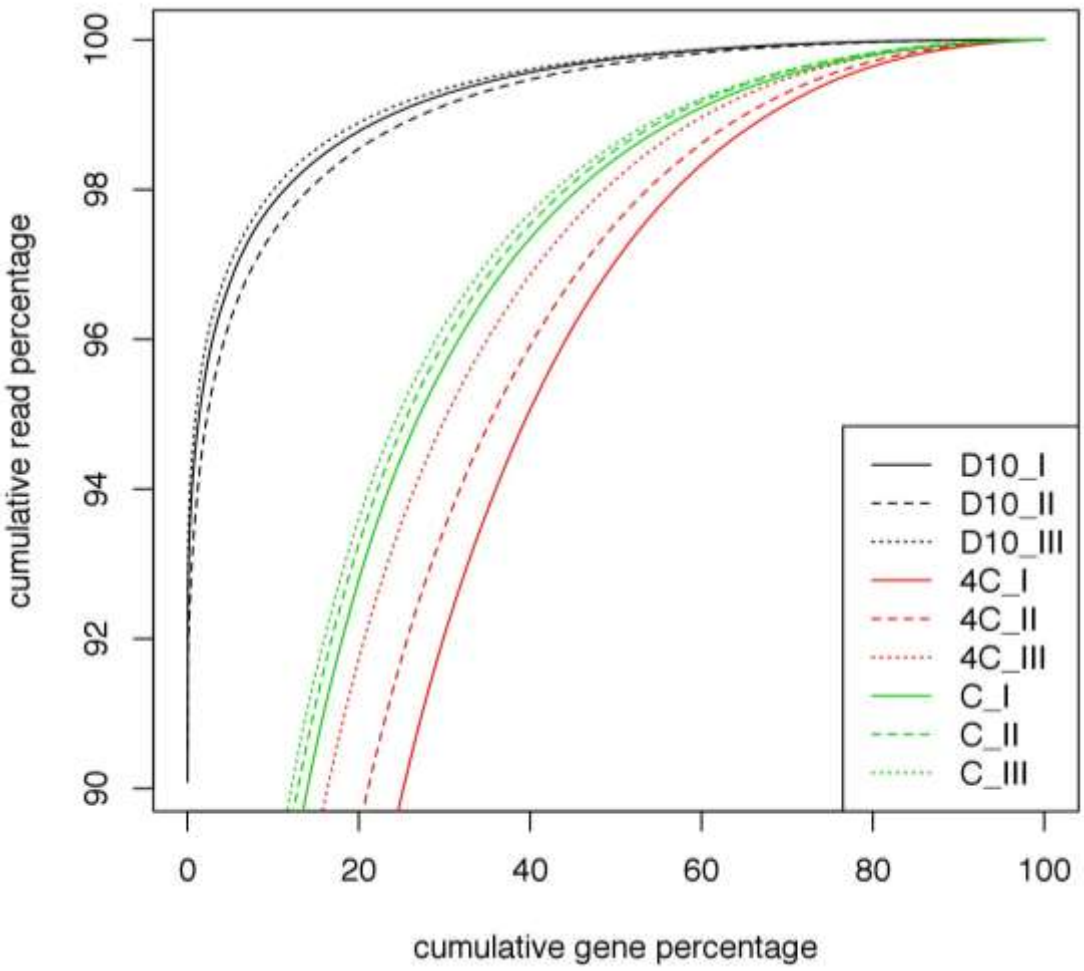


**Figure 16:** Cumulative gene percentage graph of all three samples

The heatmap shows some complex patterns of expression, with two distinct groups (clusters) of genes in each pairwise comparison (Figures 17A & 17B). Within these clusters, control (C) and cold stressed (4C) samples show limited variation in expression, while expression in aged (D10) samples was observed to be more variable.

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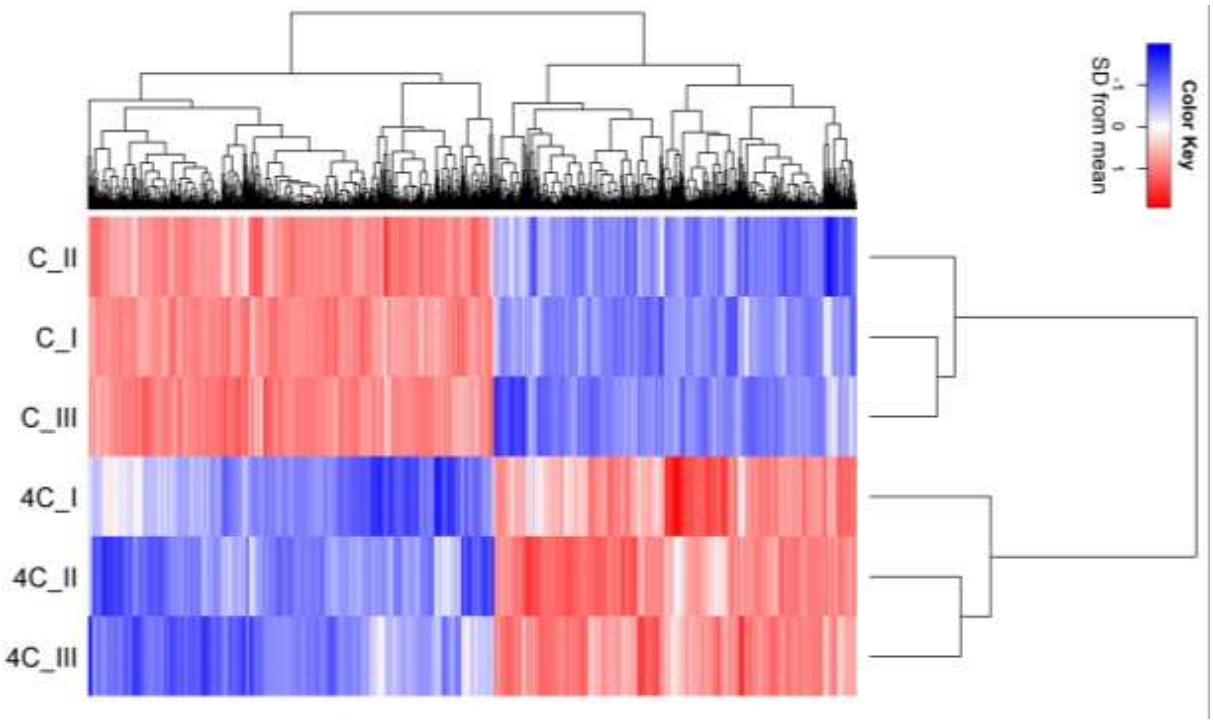
A)




B)

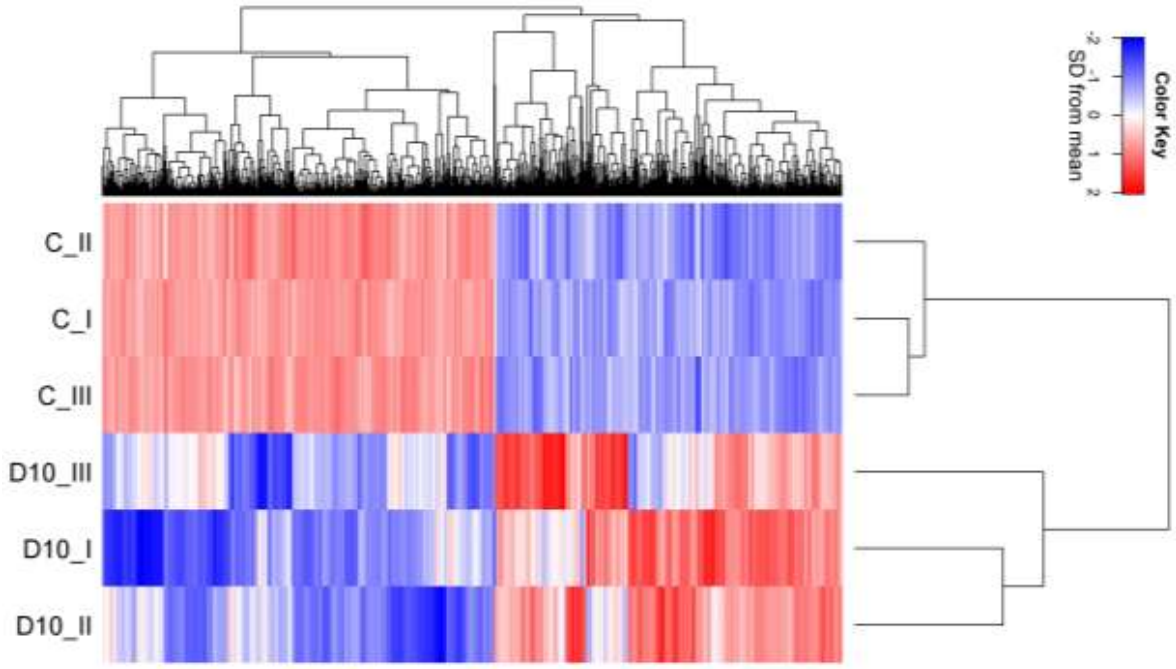



**Figure 17:** Heat maps showing the different genes present in A) control bacterial cells (C) v/s cells under cold stress (4C) and B) control cells v/s aged cells (10D). Red denotes a relative increase in gene expression (upregulation) and blue denotes a relative decrease in gene expression (down regulation).

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Preliminary analysis of gene expression in aged and cold stressed cells compared to control cells revealed significant differences that probably account for the increased ozone resistance seen in aged and cold stressed cells. Further understanding of these resistance mechanisms may ultimately result in novel anti-microbial treatments for fresh produce.

### Effect of ozone exposure on *E. coli* and *Listeria* sp*. in vitro*

Colony numbers (CFU) of *E.coli* K12 and *L. innocua in vitro* were significantly reduced ($P < 0.05$) by all ozone treatments (Figure 18), even at the lowest level used (1 ppm for 10 mins). Less than 1-log reduction was achieved when exposed to 1 ppm for 10 mins but more than 1-log reduction was achieved when both the strains of food pathogens were treated with ozone concentrations of 10 ppm and 50 ppm. This implies that ozone concentrations of 10 ppm and 50 ppm reduced counts significantly more compared to 1 ppm ozone. However, there was no significant difference in colony counts between 10 ppm and 50 ppm ozone concentration treatment in both strains of food pathogens.

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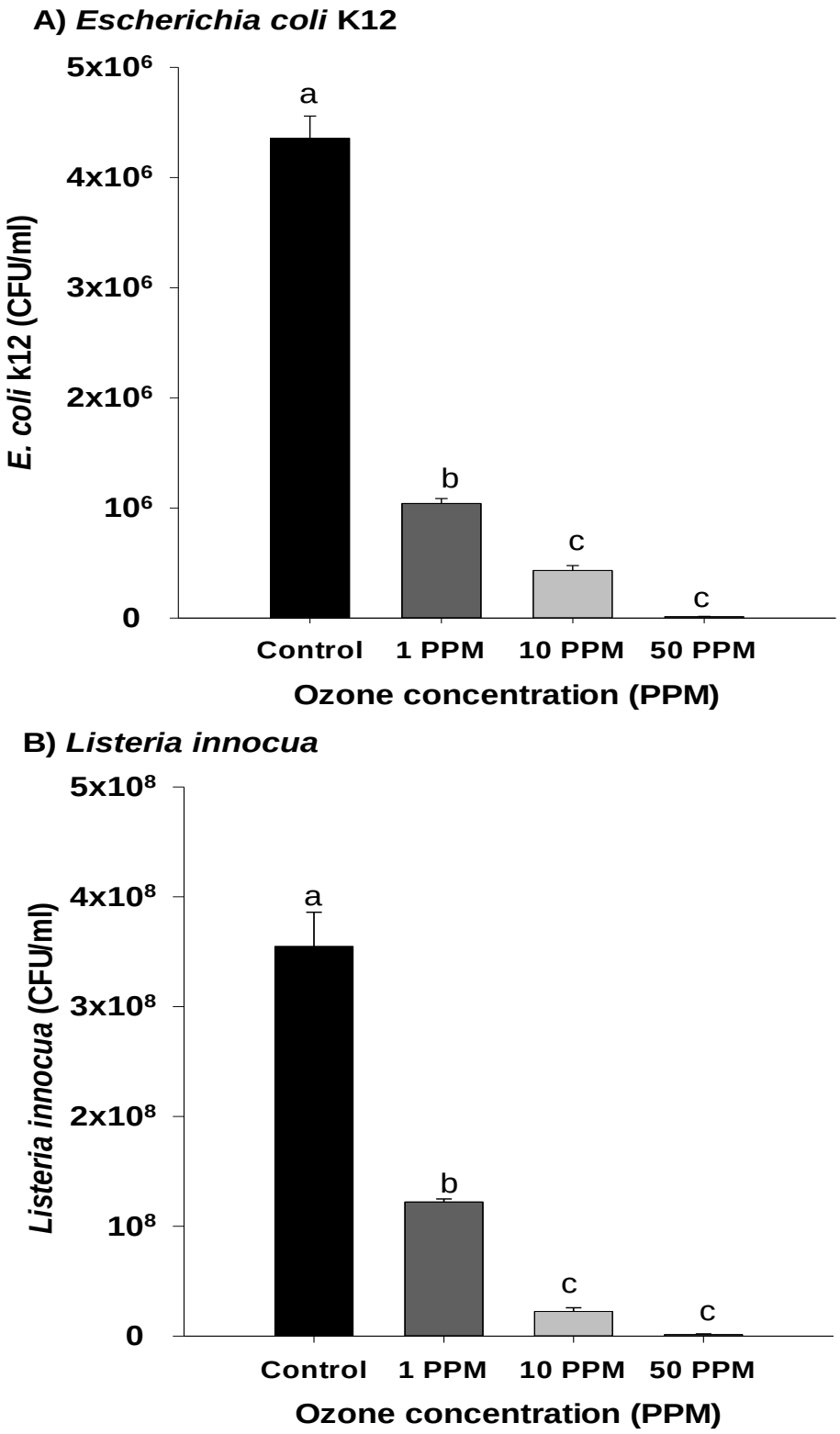


**Figure 18:** Impacts of ozone treatment on A) *E. coli* K12 and B) *L. innocua* (CFU/ml) grown on agar plates. The treatment chamber was ventilated with 1, 10 or 50 ppm ozone for 10 mins. Controls were exposed to 'clean air'. Values represent the mean (Standard Error) of measurements made on three independent plates per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

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## Impact of ozone treatment on *Listeria innocua* and *L. seeligeri* inoculated onto spinach leaves

Colony numbers (CFU) of *L. innocua* and *L. seeligeri* obtained directly from ozone exposed leaves i.e. day 0 were significantly reduced ($P < 0.05$) compared to non-ozone exposed controls (Figure 19). Similar trend was also observed when ozone treated leaves were stored for 9 days (Figure 19). No *Listeria* colonies were isolated from non-inoculated spinach leaves.

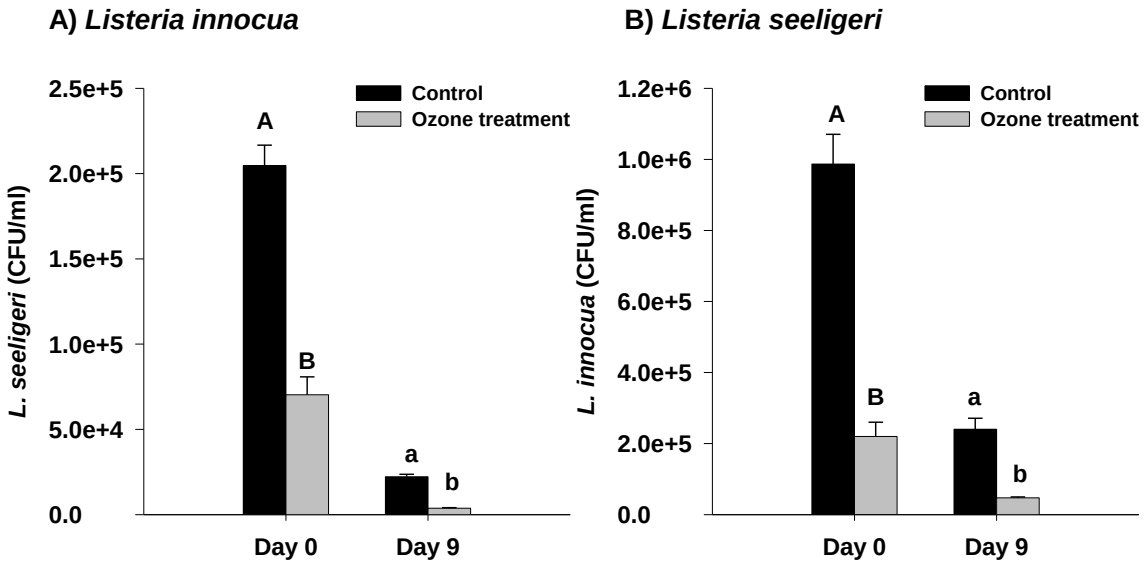


**Figure 19:** Impacts of ozone-enrichment on *L. innocua* and *L. seeligeri* inoculated onto the surface of spinach leaves. Leaves were either treated with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 10 minute. Colonies were enumerated either directly after the treatments i.e. day 0 or after 9 days storage. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are significantly different ($P < 0.05$).

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## Effect of higher ozone treatment on *E.coli* and *Listeria* sp. inoculated onto spinach leaf surface

Results of spinach artificially contaminated with two strains of *E.coli* (*E.coli* O157:K88a and *E.coli* O25:h4) and *Listeria* (*L. innocua* and *L. seeligeri*) treated with 10 ppm of ozone concentration for 10 minutes are shown in Figure 20. For *E.coli* O157:K88a and *E.coli* O25:h4, ozone treatment significantly ($P < 0.05$) reduced counts by 1-log compared with the untreated control (Figure 20A). Ozone had less than 1-log effect on *L. innocua* and *L. seeligeri* (Figure 20B). Also the results obtained from this treatment i.e. 10 ppm for 2 min weren't significantly effective against bacterial reduction as compared to previous ozone treatment i.e. 1 ppm for 10 min.

To investigate the after effects of the ozone treatment on pathogen growth, artificially contaminated spinach was stored at 7°C for 9 days. Figure 21 shows populations of both *E.coli* (*E.coli* O157:K88a and *E.coli* O25:h4) and *Listeria* sp. (*L. innocua* and *L. seeligeri*) after 9 day storage did not regrow as a significant reduction in number of colonies was observed as compared with the untreated control.

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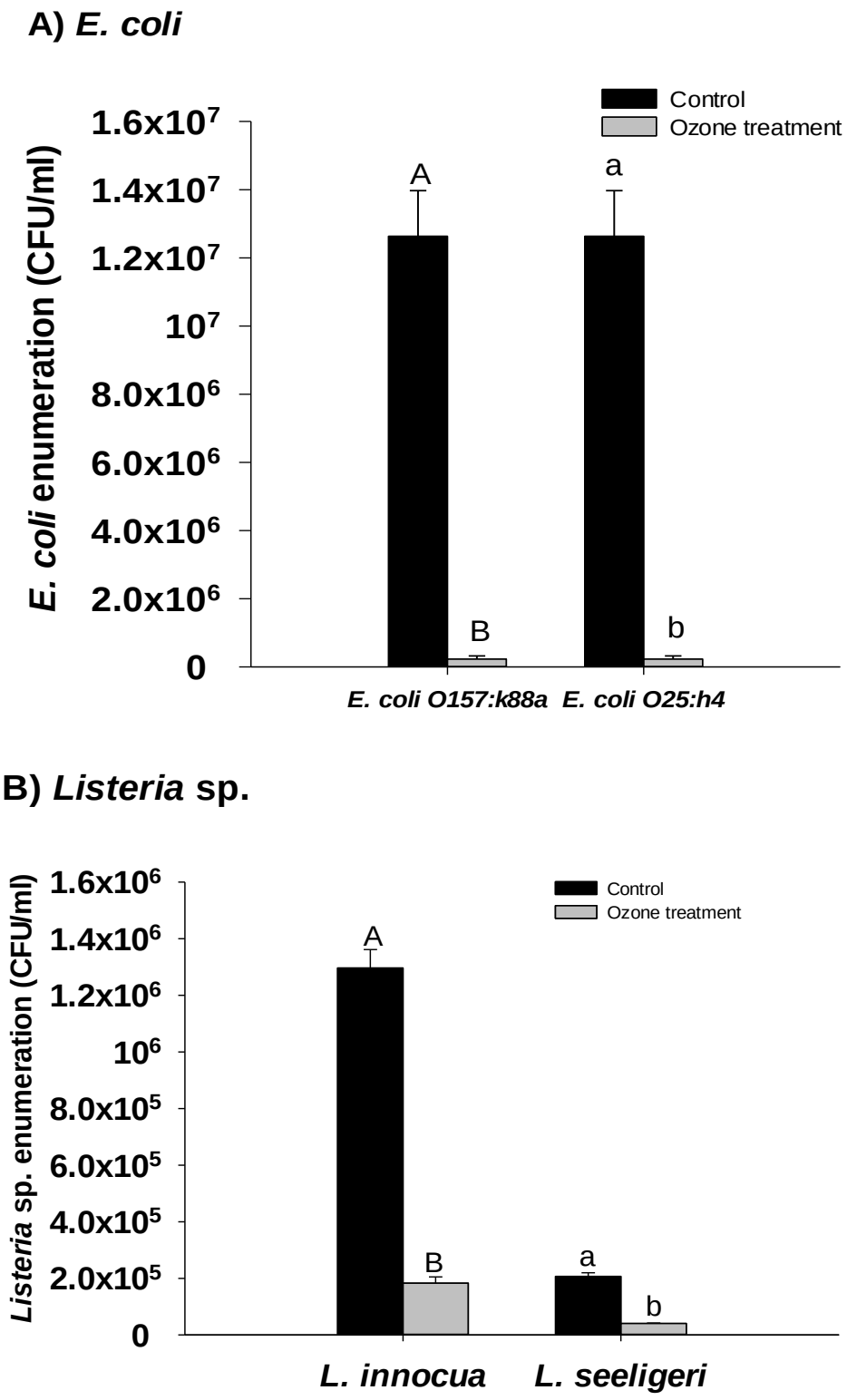


**Figure 20:** Impacts of increased levels of ozone exposure on two strains of *E.coli* and *Listeria* inoculated onto the surface of spinach leaves. Leaves were either treated with 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minutes. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are significantly different ($P < 0.05$).

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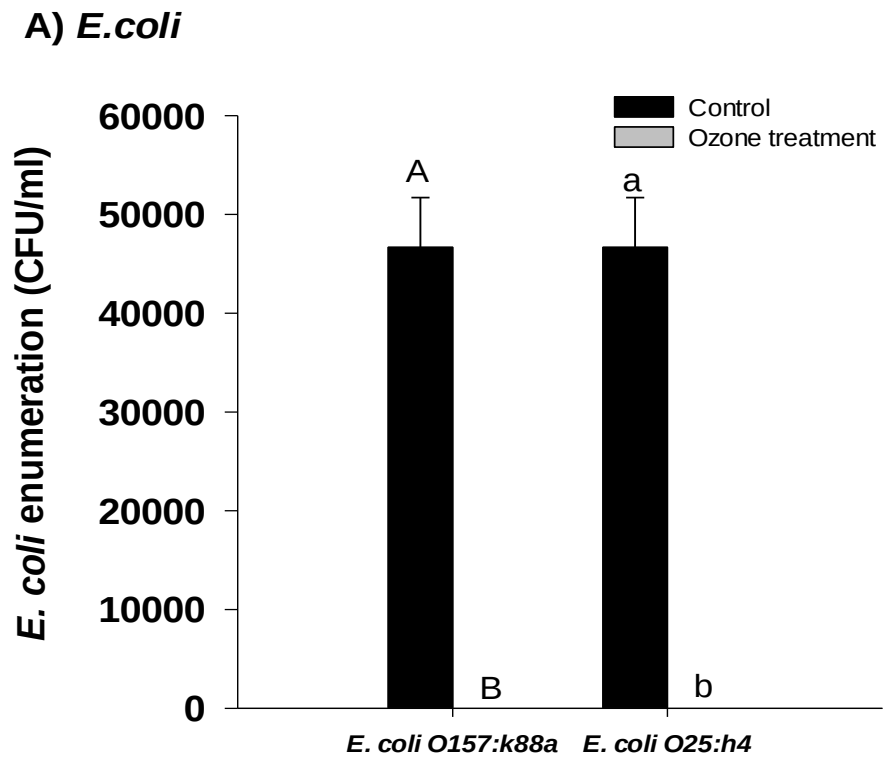


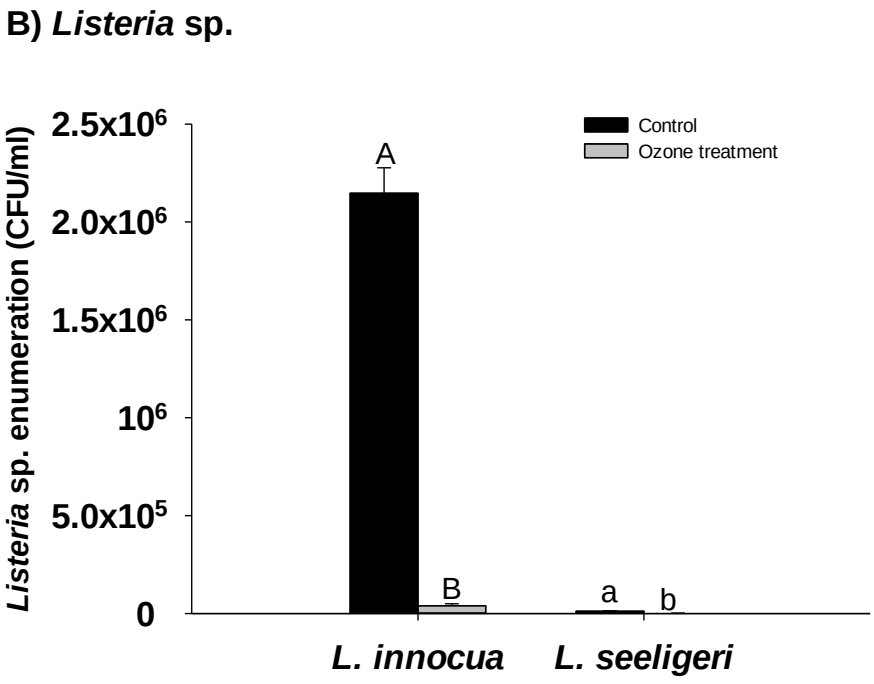


**Figure 21:** Impacts of ozone-enrichment on two strains of *E.coli* and *Listeria* inoculated onto the surface of spinach leaves. Leaves were either treated with 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minute. Colonies were enumerated after 9 days storage. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment. Bars with different letters are significantly different ($P < 0.05$).

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### Effect of age on ozone resistance of *E. coli* O157:k88a *in vitro*

*E.coli* cells of increasing age were exposed to ozone (*in vitro*) and results demonstrated a clear increase in ozone resistance of *E. coli* O157:k88a with increasing colony age. For example, survival of *E. coli* O157:k88a was observed to be greater (approximately 15%) after 5 days of growth compared to day 1 time point. An further increase in the level of survival was observed at day 7 (Figure 22) suggesting that cells in older bacterial colonies are more ozone resistant than cells from younger colonies.

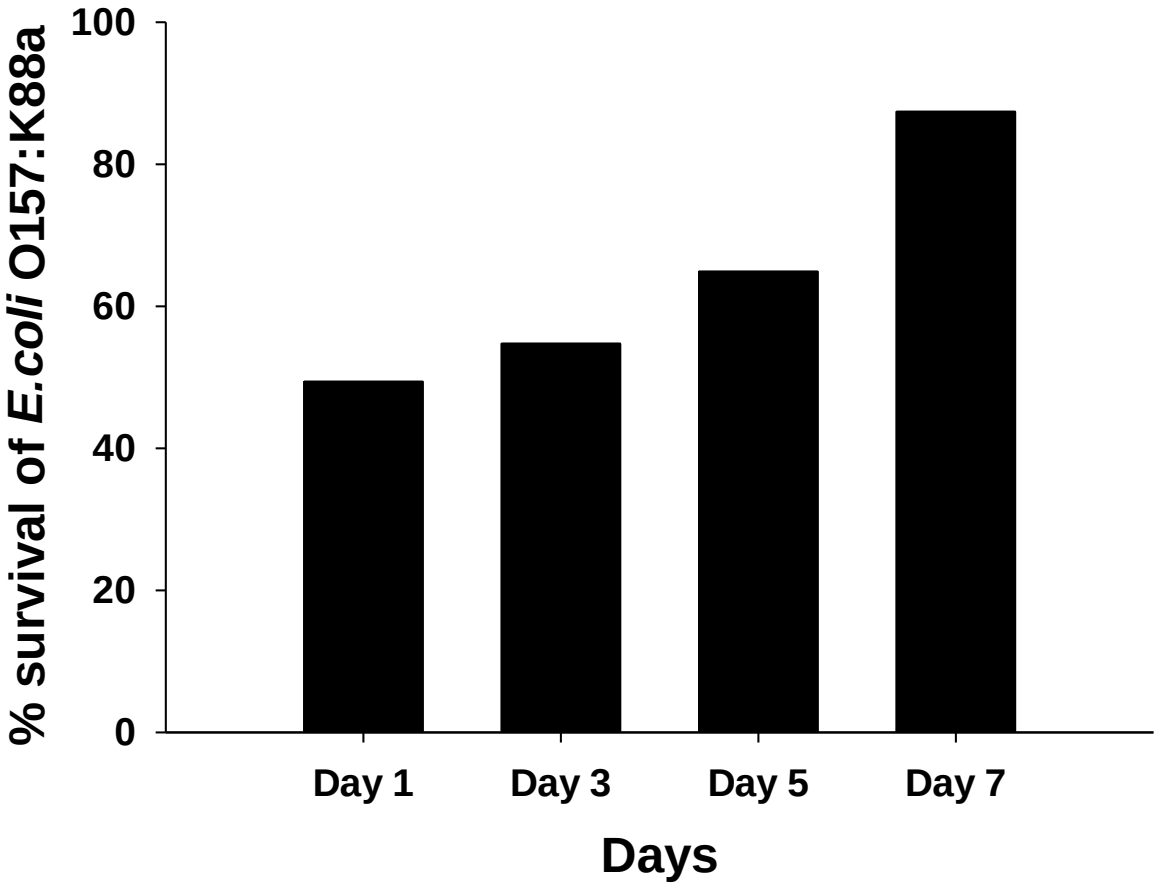


**Figure 22:** Survival of cells obtained from different colony ages of *E.coli* O157:K88a exposed to 10 ppm ozone concentration for 2 minutes. After ozone exposure, the culture plates were maintained at 37°C for 7 days. Values represent means (Standard Error) of measurements made on three independent plates per treatment.

### Effects of ozone treatment on leaves treated with pesticides

Ozone treatment had no significant effect on any pesticide residue levels found on spinach leaf surfaces. The cationic surfactant Benzalkonium chloride was not displayed on the list of actives of the foliar applicant used but was observed in chromatography analysis. This could be possibly introduced through post-harvest cleaning or washing processes.

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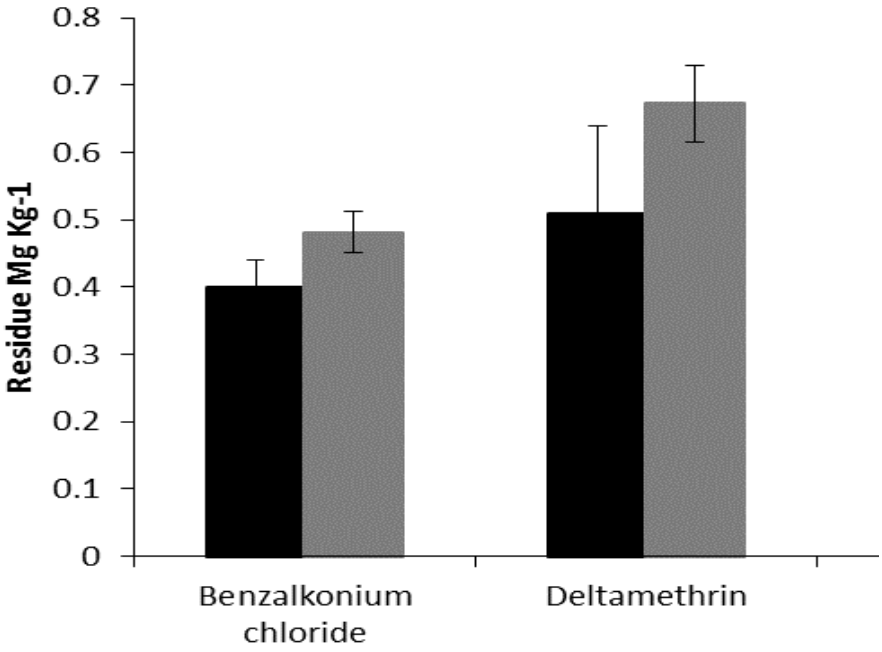


**Figure 23:** Impacts of ozone treatment on leaves treated with pesticide. Pesticide treated leaves were either exposed to 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minute. Values represent means (±Standard Error) of measurements made on three independent spinach leaves per treatment.

## Discussion

**Exploration of higher ozone exposure levels to treat leafy produce without causing visual damage:**

No visual damage was observed on leafy produce when exposed to ozone exposure concentrations such as 10, 15 and 20 ppm for shorter duration of exposure but different produce types had different ozone tolerance levels and changes in overall visual quality were observed after the maximum ozone exposure limit (20 ppm). This could be due to the fact that ozone, being a powerful oxidising agent, acts on the tissue of the targeted produce promoting enzymatic activity and the action of ozone could vary depending upon the enzymatic composition, pigments and other compounds that affect the colour of the targeted produce (Bermúdez-Aguirre and Barbosa-Cánovas, 2013). All leafy produce visually appeared to be as fresh as the untreated control produce when exposed to its maximum ozone concentration (see Figure 13). However, ozone concentrations higher than 20 ppm for 30 sec exposure time damaged leafy produce.

**The impact of higher ozone exposure levels to reduce microbial load present on the surface of leafy produce (*in vivo*):**

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A significant reduction of microbial count was observed on the surface of the targeted leafy salads when treated with high ozone concentration for shorter duration of exposure (10ppm for 2 minutes) using the modified ozone fumigation system. However, 10% of surface bacteria survived ozone treatment. This may be due to exopolysaccharides (EPS) which are responsible for protecting bacterial cells against stress (Monier and Lindow, 2003). Therefore, the 'naked' cells of identical physiological state may not have retained adequate EPS to confer protection against ozone treatment. This indicates metabolic differences between the cells present on the surface of leaves. Overall the results indicate that although the vast majority of bacteria are killed by ozone there are a number of cells that survive and this could be due to a combination of physical protection in a micro-colony and increased stress resistance in certain cells due to environmental conditions and cell age.

**Gene expression in stressed bacteria by RNA sequencing**

During transcriptome sequencing using Illumina technology, a total of 192 million raw sequencing reads with a length of 100nt were generated. Compared with other transcriptomes in *Pseudomonas* sp., (e.g. the *P. aeruginosa* transcriptome consisted of 84.1 million reads, each with a length of 36nt), it was observed that this study produced a much better transcriptome assembly (Dotsch *et al*., 2012). The analysis of the treated *Pseudomonas* sp. culture led to approximately 72% of the assembled reads matched to known transcripts i.e. *Pseudomonas* GM 60 as predicted by genome sequencing and annotation (Table 5 & 6). The remaining transcripts comprise of approximately 28 % of unmapped reads. This suggests that there might be new gene sequences present in our isolate (Xia *et al*., 2013).

To obtain a general view of differentially expressed gene (DEG) expression patterns, pairwise comparison of expression was conducted. It showed that genes were differentially expressed between the aged/stress treatments and the control. In particular, it was observed that in aged cells, about 90% of genes expressed mapped to one gene i.e. a non-coding RNA that is part of RNase P. This gene interacts with cellular mRNA transcripts and may be involved in controlling various levels of gene expression in physiology and development (Mattick and Makunin, 2006). Overall, approximately 500 DEGs were up-regulated and approximately 500 DEGs were down-regulated. The observed values for DEGs are higher to those in *P. aeruginosa* study based on RNA-seq used to evaluate gene expression between mature *P. aeruginosa* and planktonic culture, in which 227 up-regulated and 46 down-regulated were observed (Dotsch *et al*., 2012). Also, the heatmaps showed clear expression patterns, showing that genes were expressed differentially among the different treatments.

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The initial analysis undertaken is promising and current work to look at more detailed gene expression variation is being undertaken. This analysis includes:

a) Pathway analysis, e.g. determining which cell pathways are the most affected by age and cold stress e.g. cellular component, molecular function and biological process

b) Detailed analysis of individual genes showing the highest expression changes

It is hypothesized that a more detailed understanding of bacterial ozone resistance mechanisms may lead to future novel anti-microbial treatments.

**Effect of ozone treatment on *E.coli* and *L. innocua in vitro*:**

The antimicrobial effects of gaseous ozone on *E.coli* K12 and *L. innocua in vitro* are presented in Figure 18. The data obtained in this study have shown that the treatment resulted in significant reduction in *E.coli* K12 and *L. innocua*. Similar results were observed by (Alwi *et al*., 2014) on *E.coli* O157, *L. monocytogenes*, *Salmonella Typhimurium in vitro* when treated with 0.1, 0.3, 0.5 and 1.0 ppm ozone concentration for exposure times of 0.5, 3, 6 and 24h.

The *in vitro* assay on both pathogens showed the effectiveness of gaseous ozone increased with increasing ozone concentration from 1 to 10 ppm but that the effectiveness of ozone exposure did not significantly increase above this level. This is possibly due to live bacterial cells being physically protected by other cells on the surface of the agar plates – thus interfering with the oxidation action of ozone treatment (Alwi *et al*., 2014). Alternatively some cells may have an intrinsic resistance to ozone exposure perhaps due to their age and exposure to stress (see below – Effect of cell age on ozone resistance). Fan and colleagues (2007) reported that the maximum death rate of *L. innocua* cells was observed in less than 2 h and the death rate reached a plateau after 4 h when treated with gaseous ozone *in vitro*.

**Impact of ozone treatment on *L. innocua* and *L. seeligeri* onto spinach leaves:**

In this work, *L. innocua* and *L. seeligeri* were used as microbial surrogate of *L. monocytogenes* as they are known to be useful indicators of contamination and have also demonstrated similar behaviour to *L. monocytogenes* on fresh vegetables (Alwi *et al*., 2014). Results from spinach artificially contaminated with *L. innocua* and *L. seeligeri* treated with 1 ppm ozone for an exposure time of 10 min showed 1-log reduction in colony count compared with the untreated control. Karaca and his colleague (2014) reported *L. innocua* reduction of 1.14 $\log_{10}$ cfu/g on parsley when treated with high ozone concentration of 950 ppm for 20 min. Similar results have been shown by previous research on mushrooms, alfalfa sprouts, alfalfa seeds and lettuce (Yuk *et al*., 2007). The growth of *L. innocua* and *L. seeligeri* on spinach remained significantly reduced after day 9 storage. This is possibly due to the

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interactions between the natural background microflora of spinach and *L. innocua* which can affect its growth and survival (O'Beirne, 1998). O'Berine and his colleague (1998) reported that Lactic acid bacteria and mixed population of natural microflora isolated from shredded lettuce reduced *L. innocua* growth in model media. Rodgers and colleagues (2004) demonstrated complete inactivation of *L. monocytogenes* on lettuce during 9 days storage when treated with 3 ppm ozone for 3 min.

**Impact of high ozone treatment on *E.coli* and *Listeria* sp. inoculated onto spinach leaves:**

Increasing ozone exposure levels i.e. 10 ppm for 2 min on (*E.coli* O157:K88a and *E.coli* O25:h4) and *Listeria* sp. (*L. innocua* and *L. seeligeri*) onto spinach resulted in 1 log and less than 1 log reduction respectively. Awli and his colleague (2014) achieved reduction of 2.89 and 3.06 $\log_{10}$ for *E.coli* O157 and *L. monocytogenes* respectively on bell pepper when exposed to 9 ppm ozone for 6 h. Their work met the standards for an antimicrobial agent by attaining a minimum of 2 $\log_{10}$ reduction (Alwi *et al*., 2014). Similar reductions were observed from application of 5 ppm ozone for 3 min on whole tomato (Bermúdez-Aguirre and Barbosa-Cánovas, 2013). When results from this work (on leafy produce) are compared to other hardy produce, it appears that ozone treatment was less successful. This is most probably due to the delicate nature of leafy produce which limits the use of increased ozone concentration and exposure time.

Ozone inactivates bacterial cells by the progressive oxidation of important cellular constituents (Karaca, 2014). Suggestions for the principal target of ozonation include the bacterial cell surface. Bacterial cell death was observed as a consequence of a ruptured cell membrane and as a result disintegration of cell wall to function as a barrier (Fan *et al*., 2007; Alwi 2014; Karaca 2014). *E.coli*, Gram-negative bacteria is more susceptible to ozone treatment since it has thin peptidoglycan lamella which is covered by an outer membrane made of polysaccharides and lipoproteins (Zuma *et al*., 2009). In contrast, some studies claimed that Gram-negative bacteria were more resistant to ozone treatment as compared to Gram-positive bacteria (Vaz-Velho, 2006). Results from this study show that ozone treatment was effective in both *E. coli* and *Listeria* sp. inactivation but *Listeria* sp. were slightly more resistant. These results are in line with Yuk and colleagues (2007) who showed that *E.coli* O157:H7 to be more sensitive than *Listeria monocytogenes*.

After high ozone treatment i.e. 10 ppm for 2 min *E.coli* O157:K88a and *E.coli* O25:h4 and *Listeria* sp. (*L. innocua* and *L. seeligeri*) did not show significant regrowth on produce

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(spinach) This is an important observation as it means the ozone treatment is effective in keeping pathogen levels low during storage and transport.

**Effect of cell age on ozone resistance of *E. coli in vitro*:**

The work showed that older cells (5-7 days old) of *E.coli* O157:k88a were more ozone resistant than younger cells (3 days old). This is possibly because the older *E. coli* cells might to be in their long-term stationary phase (fifth phase of bacterial growth cycle which survives on the nutrient released by the dead population of bacteria). These older cells can survive external stress unlike the younger cells (probably in first or second phase of bacterial growth cycle) and can remain viable for months or even years once they enter long-term stationary phase (Navarro Llorens *et al*., 2010). This stationary phase is dominated by the accumulation of the sigma factor RpoS (Hengge-Aronis, 2002). The entire cellular physiology of *E.coli* is influenced by RpoS which directly or indirectly affects the expression of 10% of the *E.coli* genes. These genes are involved in morphological variations within the cell and responsible for increasing resistance during numerous stress conditions e.g. oxidative stress, osmotic stress, heat shock, etc. (Navarro Llorens *et al*., 2010). Further work would be needed to compare the ozone resistance mechanisms of *Pseudomonas* with *E. coli*.

**Effects of ozone treatment on leaves treated with pesticides:**

The results of this study indicated that 10 ppm ozone treatment for 2 minutes was not effective in degrading benzalkonium chloride and deltamethrin. This could be due to numerous factors as removal efficiency of pesticides highly depends on the ozone levels, temperature and exposure time (Wu *et al*., 2007). Residual reduction by any treatment also varies with the nature of pesticide molecule (Bajwa and Sandhu, 2014). Similar results were observed on boscalid and iprodione residues by Karaca and his colleagues (2012) when exposed to 0.3 µl/L gaseous ozone for 36 days in storage. In contrast, Gabler and colleagues (2010) showed that residues of fenhexamid, cuprodinil, pyrimethanil and pyraclostrobin were reduced by 68, 75, 83 and 100% respectively when treated with 10,000µL/L ozone for 60 minutes.

Further investigation will be required to test different ozone-level and exposure times required to achieve optimum pesticide removal

## Conclusions

- Ozone treatment regimes were optimized to treat leafy produce without causing physical damage e.g. 10 ppm for 2 minutes

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- Optimized ozone treatment achieved about 90% reduction of bacteria on leafy produce surfaces
- Cells on a leaf surface appear to survive ozone treatment by a combination of physical protection (micro-colony) and physiological adaptation
- Cold stress and increasing age of the leaf surface bacteria (*Pseudomonas* sp.) enhances ozone resistance
- Difference in gene expression of cold stress and aged bacteria (*Pseudomonas* sp.) was observed by RNA sequencing
- Food pathogens like *E. coli* and *Listeria* spp. are sensitive to ozone treatment

## Knowledge and Technology Transfer

Second prize in the Post-graduate Research (PGR) Poster Conference 2014, Newcastle University

Talk in 'British Leafy Salad Association' (BLSA) annual meeting, October 2014

'Best poster' award in the HDC Studentship Conference 2014, York

Awarded 'Institute for Sustainability Responsive Mode 2014' grant by Institute for Sustainability, Newcastle University

Awarded PGR Innovation Fund by Newcastle University

'Best speaker' award in the Post-graduate Research (PGR) Conference 2013, Newcastle University

Second prize in the NIRes poster competition 2012, Newcastle University

An article of my work was published in 'Horticulture Week' magazine in November 2012, in 'HDC News' magazine in March 2013 and in 'Field vegetables' review 2013 magazine in September 2013

Presented posters in PGR Poster Conference 2014, HDC Studentship Conference 2014, FoodMicro Conference 2014, North East Postgraduate (NEPG) Conference 2013, Society of General Microbiology (SGM) Conference 2013, 'British Leafy Salad Association' (BLSA) Conference 2012 and NIRes poster competition 2012

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# Appendices

## Appendix 1 – Moved from the Grower Summary: Gene expression in cold stressed and aged bacteria by RNA sequencing

The ultimate aim of this work is to determine the potential mechanisms by which bacteria are able to resist ozone treatment. Better understanding of these mechanisms may allow future novel produce treatments to be developed and such treatments may be applicable in a wide range of commercial situations.

The *Pseudomonas* sp. cold stressed/ aged/ control RNA samples were used for Illumina Genome Analyzer deep sequencing. There were nine samples in total, with each condition having three biological replicates. The raw sequence output generated 192 million reads, each with a length of 100nucleotide (nt). Those reads mapping to the reference genome (*Pseudomonas* sp. GM60) were first categorized into three classes (Table 2). Uniquely mapped reads are those that map to only one position in the genome, and gapped alignment are those that have a (limited) mismatch as compared to the reference genome. Unmapped reads are those that do not (share sufficient sequence similarity to) map to any position in the reference genome.

**Table 2:** Number of reads sequenced and mapped

4C, 10D and C stand for cold stressed, aged and control samples respectively. Numbers I, II and III indicate the three biological replicates.

| Sample | Read Sequences | Unique Alignment | Gapped Alignment | Unmapped reads |
|---|---|---|---|---|
| 4C_I | 25,748,666 | 17,053,980 | 2,922,953 | 8,688,761 |
| 4C_II | 21,949,529 | 15,677,275 | 2,848,392 | 6,267,351 |
| 4C_III | 21,107,733 | 15,729,041 | 3,018,542 | 5,374,059 |
| C_I | 19,333,104 | 14,605,701 | 2,389,820 | 4,724,414 |
| C_II | 19,842,086 | 15,181,454 | 2,724,031 | 4,657,447 |
| C_III | 21,722,592 | 16,535,963 | 2,435,825 | 5,183,392 |
| D10_I | 20,758,340 | 14,542,190 | 1,213,218 | 6,215,488 |
| D10_II | 19,940,059 | 14,311,523 | 1,169,298 | 5,627,645 |
| D10_III | 21,465,952 | 16,539,000 | 1,122,241 | 4,925,913 |

The assembled transcripts were then classified into two main categories (Table 3): aligned transcripts and unmapped transcipts.

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**Table 3:** Classification of transcripts

1 million reads aligned to *Pseudomonas* sp. GM60

| Sample | Aligned (%) | Unmapped (%) |
|---|---|---|
| 4C_I | 66.16 | 33.09 |
| 4C_II | 71.33 | 27.27 |
| 4C_III | 74.40 | 24.33 |
| C_I | 75.51 | 22.86 |
| C_II | 76.54 | 21.79 |
| C_III | 76.07 | 22.10 |
| D10_I | 70.70 | 15.71 |
| D10_II | 72.26 | 17.20 |
| D10_III | 77.37 | 16.23 |

After the alignment of the sequence and statistical analysis, the genes differentially expressed between aged cultures (D10) and control (C) as well as between cold stressed cultures (4C) and control were identified. The two pairwise comparisons yielded approximately 500 up-regulated genes and approximately 500 down-regulated genes in each of the treatments. Overall, the 3 biological replicates in each sample were similar to each other. At the level of the treatments, cold stressed (4C) and control (C) samples were more comparable to each other than the aged (D10) sample (Figure 3).

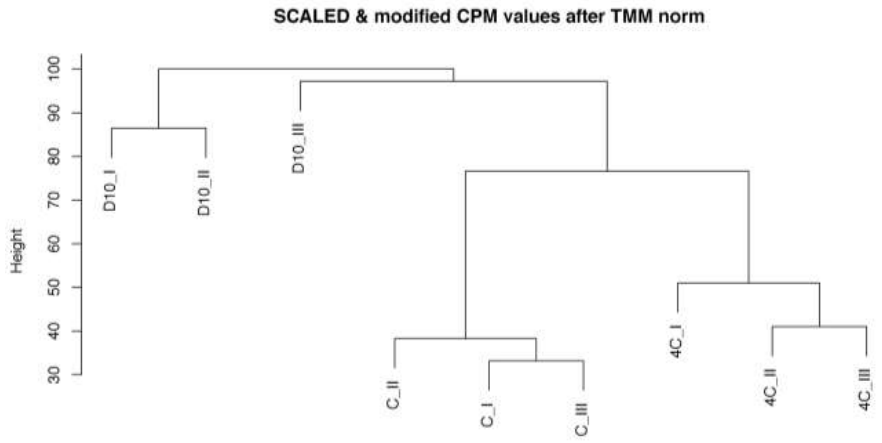


**Figure 3:** Overview of gene expression in all three bacterial samples. 4C, 10D and C stand for cold stressed, aged and control samples respectively. Numbers I, II and III indicate the three biological replicates.

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The analysis of differentially expressed genes (DEGs) revealed that very few genes in aged (D10) samples were responsible for the observed differences as compared to cold stressed (4C) and control (C) samples. Figure 4 illustrates that approximately 98% (Y-axis) of the reads were assigned to <5% of the genes (x-axis) in aged (D10) samples. In addition, >90% of the reads map to one gene in all three aged (D10) samples, subsequently characterised to be a non-coding RNA which is a component of RNaseP.

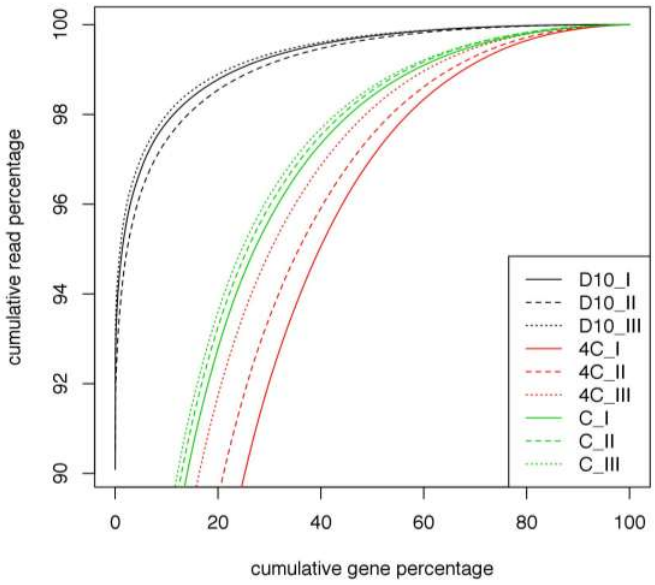


**Figure 4:** Cumulative gene percentage graph of all three samples

The heatmap shows some complex patterns of expression, with two distinct groups (clusters) of genes in each pairwise comparison (Figure 5A & 5B). Within these clusters, contol and cold stressed samples show limited variation in expression, while expression in aged samples was observed to be more variable.

A)

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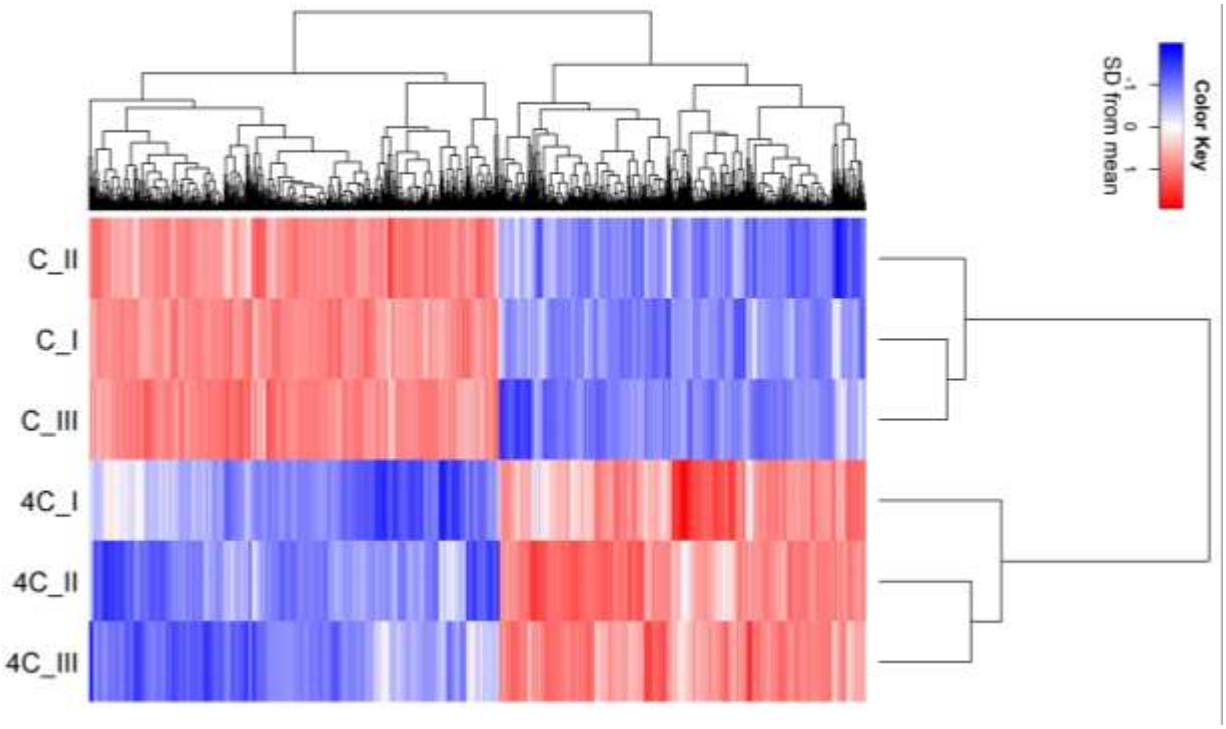


B)

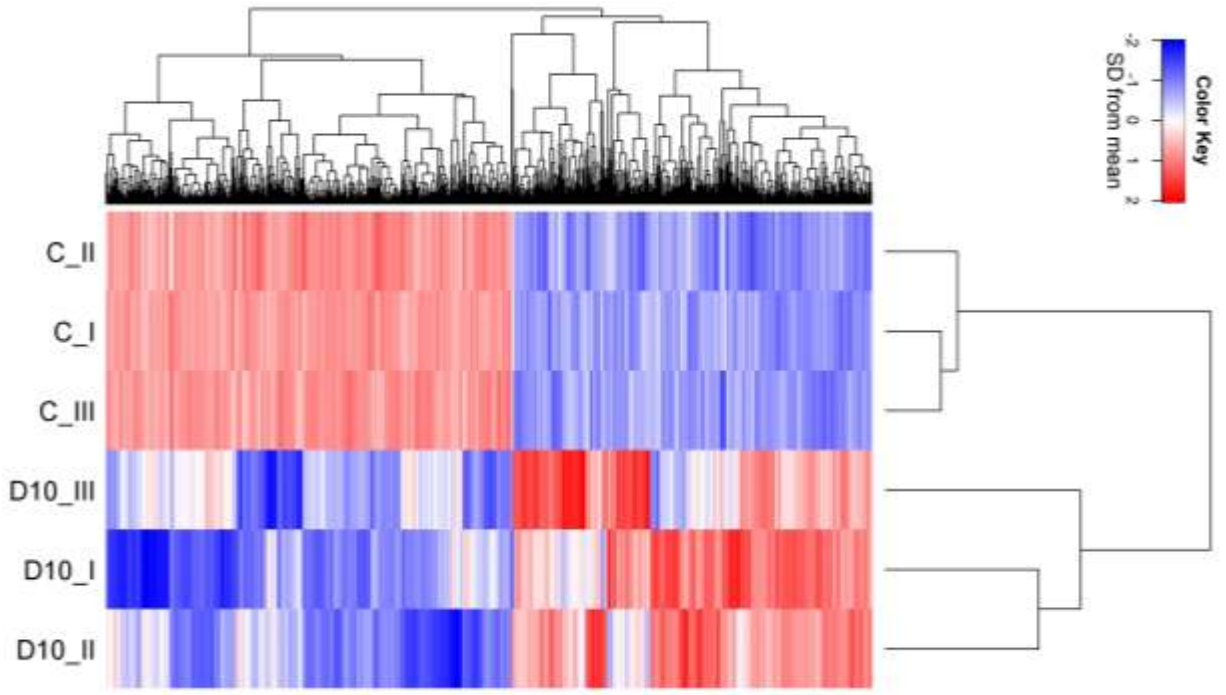


**Figure 5**: Heat maps showing the different genes present in A) control bacterial cells (C) v/s cells under cold stress (4C) and B) control cells v/s aged cells (10D). Red denotes a relative increase in gene expression (upregulation) and blue denotes a relative decrease in gene expression (down regulation).

Preliminary analysis of gene expression in aged and cold stressed cells compared to control cells revealed significant differences that probably account for the increased ozone

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resistance seen in aged and cold stressed cells. Further understanding of these resistance mechanisms may ultimately result in novel anti-microbial treatments for fresh produce.

## Appendix 2 - Raw data for impact of the highest ozone exposure levels to reduce microbial load present on the surface of leafy produce (*in vivo*)

| Target produce | Treatment | Replicate I (CFU/g) | Replicate II (CFU/g) | Replicate III (CFU/g) |
|---|---|---|---|---|
| Spinach | Control | 3090000 | 8730000 | 3730000 |
| | 10 ppm $O_3$, 2 mins | 291000 | 927000 | 63600 |
| | 15 ppm $O_3$, 2 mins | 600000 | 155000 | 300000 |
| Rocket | Control | 2090000 | 909000 | 2300000 |
| | 10 ppm $O_3$, 2 mins | 3000 | 22700 | 7570 |
| | 15 ppm $O_3$, 2 mins | 19100 | 5320 | 15200 |
| Lettuce | Control | 200000 | 100000 | 100000 |
| | 10 ppm $O_3$, 2 mins | 27500 | 14700 | 20000 |
| | 15 ppm $O_3$, 2 mins | 14000 | 10000 | 27600 |
| Coriander | Control | 25100000 | 24000000 | 20200000 |
| | 10 ppm $O_3$, 2 mins | 2400000 | 3000000 | 2450000 |
| | 15 ppm $O_3$, 2 mins | 1900000 | 3000000 | 300000 |
| Watercress | Control | 18300000 | 20000000 | 20200000 |
| | 10 ppm $O_3$, 2 mins | 1000000 | 8000000 | 2290000 |
| | 15 ppm $O_3$, 2 mins | 1000000 | 2000000 | 300000 |

## Appendix 3: Raw data for effect of ozone exposure on *E. coli* and *Listeria* sp*. in vitro*

| | E.coli | | |
|---|---|---|---|
| | Replicate I | Replicate II | Replicate III |
| Control | 4610000 | 3960000 | 4500000 |
| 1 PPM | 1130000 | 1010000 | 980000 |
| 10 PPM | 430000 | 510000 | 360000 |
| 50 PPM | 10000 | 20000 | 10000 |

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| | Listeria sp. | | |
|---|---|---|---|
| | **Replicate I** | **Replicate II** | **Replicate III** |
| **Control** | 394000000 | 377000000 | 293000000 |
| **1 PPM** | 122000000 | 117000000 | 127000000 |
| **10 PPM** | 28000000 | 23000000 | 16000000 |
| **50 PPM** | 0 | 2000000 | 2000000 |

**Appendix 4: Raw data for impact of ozone treatment on *L. innocua* and *L. seeligeri* onto spinach leaves**

| | | Day 0 | | | Day 9 | | |
|---|---|---|---|---|---|---|---|
| | | **Replicate I** | **Replicate II** | **Replicate III** | **Replicate I** | **Replicate II** | **Replicate III** |
| ***Listeria innocua* (cfu/ml)** | **No Ozone treatment** | 1420000 | 1270000 | 1200000 | 2070000 | 1970000 | 2400000 |
| | **10 ppm ozone treatment** | 200000 | 210000 | 140000 | 50000 | 20000 | 50000 |
| ***L. seeligeri* (cfu/ml)** | **No Ozone treatment** | 20400 | 187000 | 230000 | 9600 | 14700 | 11600 |
| | **10 ppm ozone treatment** | 38000 | 44000 | 40000 | 1600 | 2700 | 1500 |

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**Appendix 5: Raw data for effect of higher ozone treatment on *E.coli* and *Listeria* sp. inoculated onto spinach leaf surface**

| **Strain count cfu/ml** | **Treatment** | Day 0 | | | **Day 9** | | |
|---|---|---|---|---|---|---|---|
| | | Replicate I | Replicate II | Replicate III | Replicate I | Replicate II | Replicate III |
| ***E.coli* 0157:K 88a** | **No ozone treatment** | 15300000 | 11100000 | 11500000 | 37000 | 54000 | 49000 |
| | **10 ppm Ozone treatment** | 200000 | 100000 | 400000 | 0 | 0 | 0 |
| ***E.coli* O25:h4** | **No ozone treatment** | 16900000 | 14300000 | 15600000 | 18000 | 10000 | 6000 |
| | **10 ppm Ozone treatment** | 0 | 500000 | 1000000 | 0 | 0 | 0 |
| ***L. innocua*** | **No ozone treatment** | 1420000 | 1270000 | 1200000 | 2070000 | 1970000 | 2400000 |
| | **10 ppm Ozone treatment** | 200000 | 210000 | 140000 | 50000 | 20000 | 50000 |
| ***L. seeligeri*** | **No ozone treatment** | 204000 | 187000 | 230000 | 9600 | 14700 | 11600 |
| | **10 ppm Ozone treatment** | 38000 | 44000 | 40000 | 1600 | 2700 | 1500 |

**Appendix 6: Raw data for effect of age on ozone resistance of *E. coli* O157:k88a *in vitro***

| | **Control I** | **Control II** | **Control III** | **Ozone I** | **Ozone II** | **Ozone III** |
|---|---|---|---|---|---|---|
| **Day 1** | 129000000 | 142000000 | 122000000 | 50000000 | 68000000 | 76000000 |
| **Day 3** | 185000000 | 151000000 | 256000000 | 105000000 | 117000000 | 102000000 |
| **Day 5** | 310000000 | 330000000 | 300000000 | 210000000 | 220000000 | 180000000 |
| **Day 7** | 142000000 | 93000000 | 122000000 | 137000000 | 100000000 | 75000000 |

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