Grower Summary

FV 386

Use of gaseous ozone to prevent microbial post-harvest spoilage and reduce pesticide residue levels

Final 2014

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Project Number:	FV 386
Project Title:	Use of gaseous ozone to prevent microbial post-harvest spoilage and reduce pesticide residue levels
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Report:	Final report 2014
Publication Date:	18 February 2015
Previous report/(s):	Annual report, September 2013
Start Date:	1 October 2011
End Date:	30 September 2014
Project Cost:	£83,150

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.

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	**-**	**-**	**-**	**-**	**-**

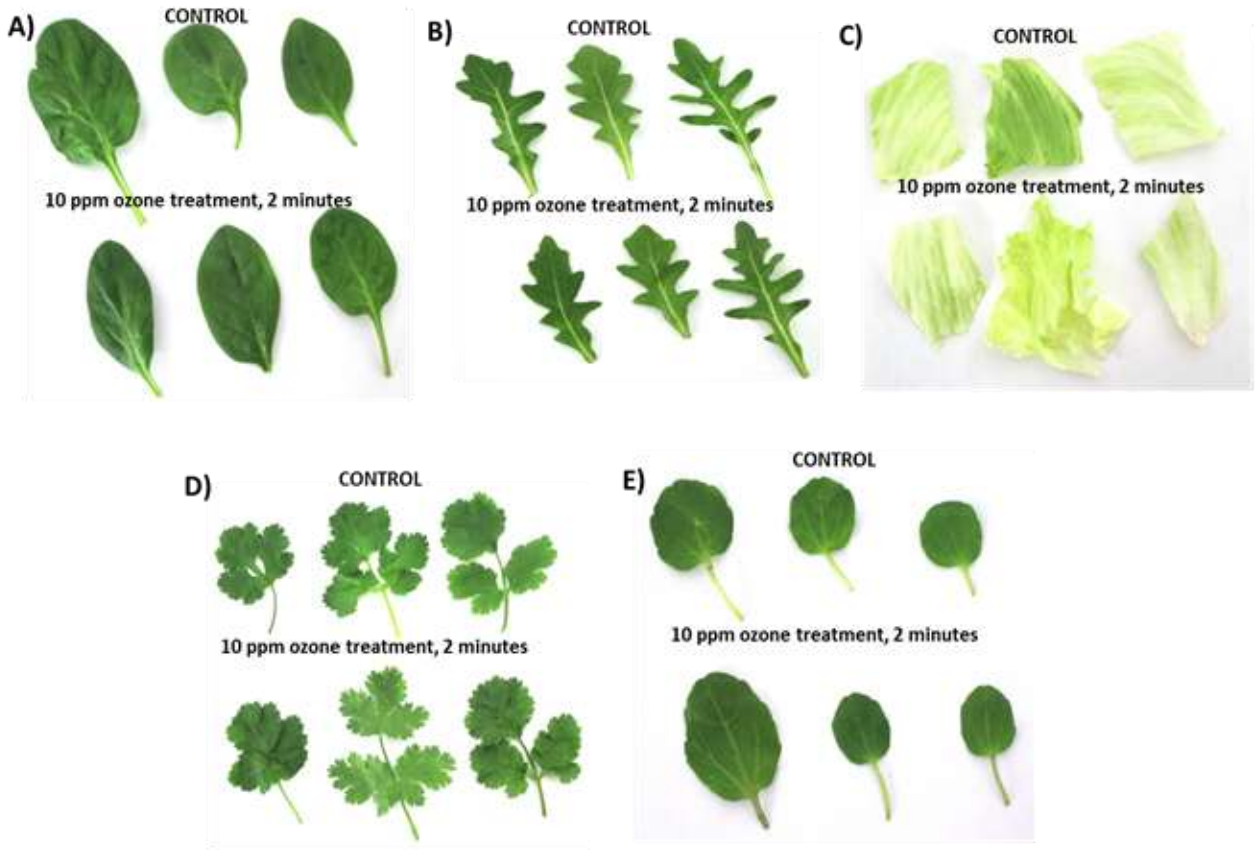

Figure 1: Impact of 10 ppm ozone exposure levels for 2 minutes on visual quality of A) spinach, B) rocket, C) lettuce, D) coriander and E) watercress.

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.

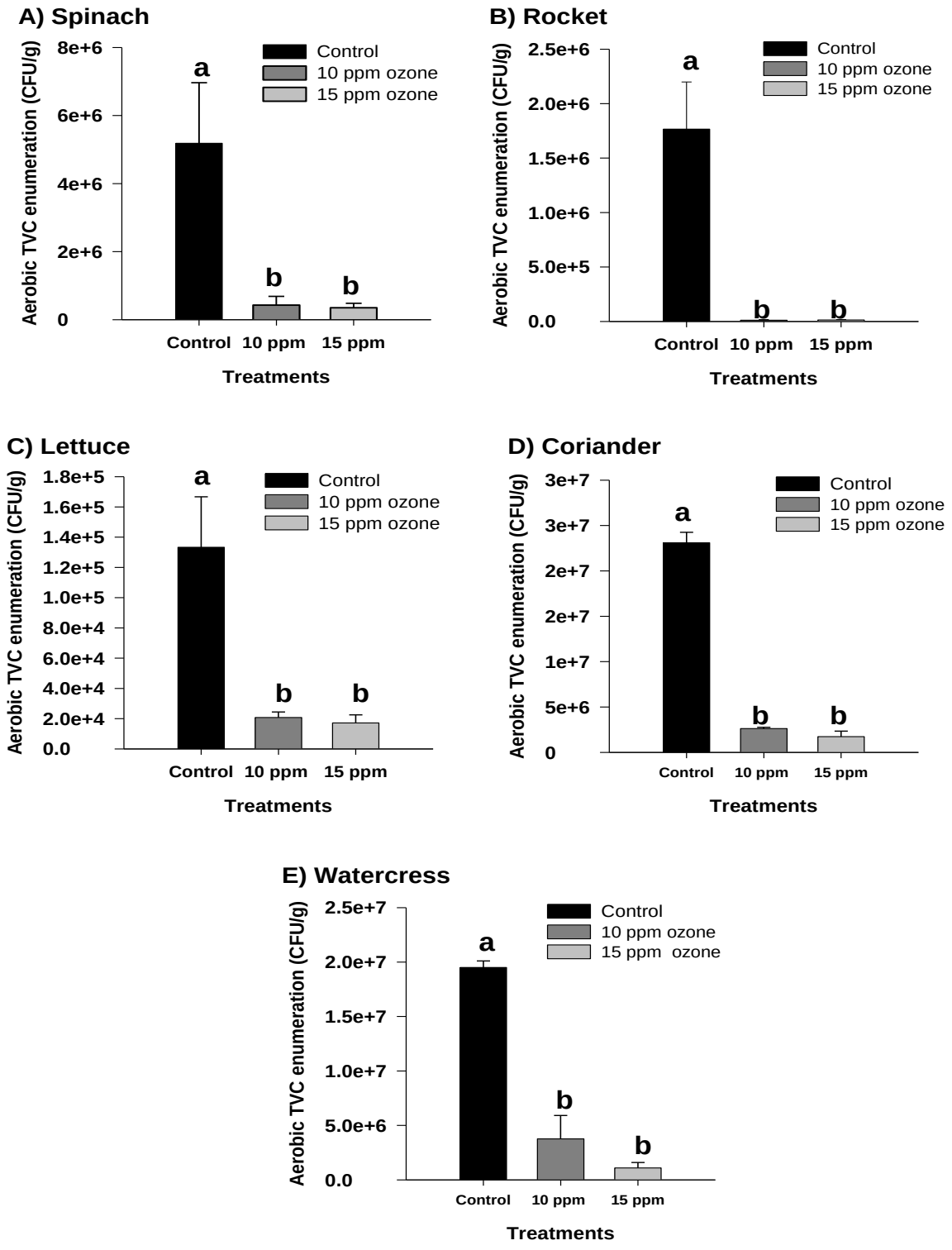

Figure 2: Impacts of high ozone treatment on microbial flora present on surface of A) spinach, B) Rocket, C) lettuce, D) coriander and E) watercress. Produce were either exposed to 15 ppm ozone concentration (grey bar) for 2 minutes, 10 ppm ozone concentration (dark grey bar) for 30 sec or 'clean' air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

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.

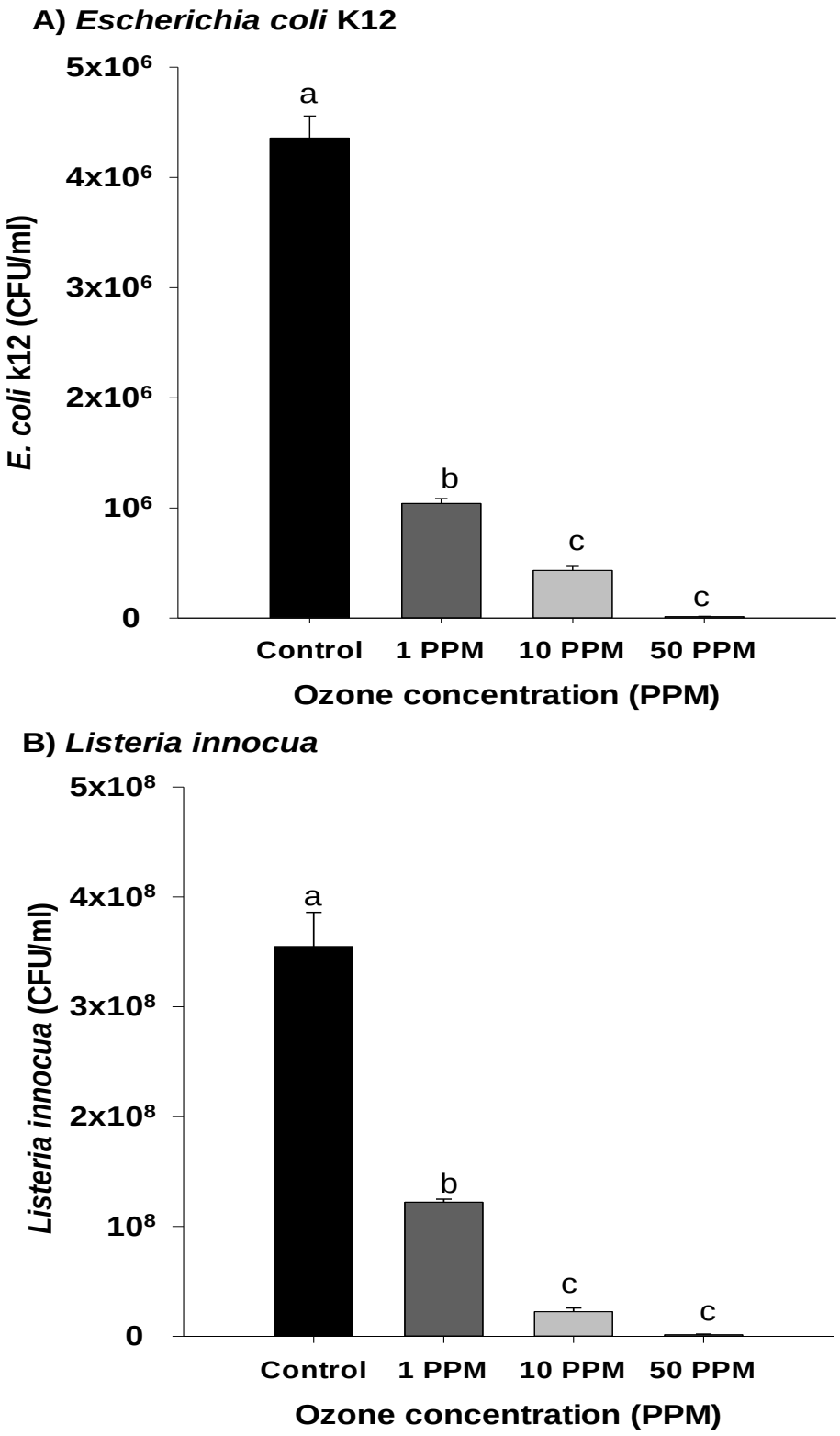

Figure 6: Impacts of ozone treatment on A) *E. coli* K12 and B) *L. innocua* (CFU/ml) grown on agar plates. The treatment chamber was ventilated with 1, 10 or 50 ppm ozone for 10 mins. Controls were exposed to 'clean air'. Values represent the mean (Standard Error) of measurements made on three independent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

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.

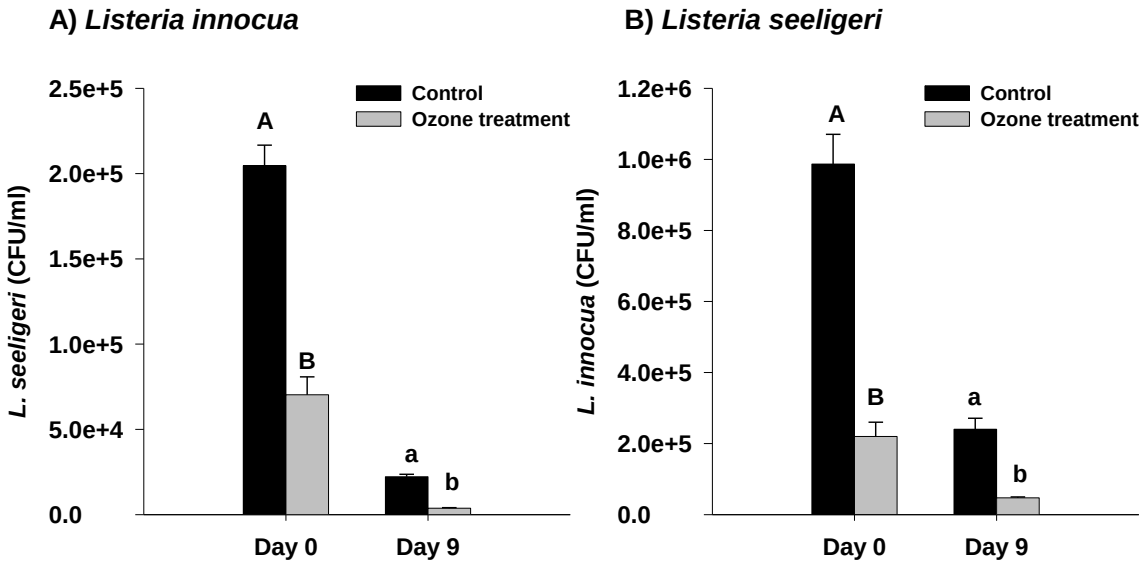

Figure 7: Impacts of ozone-enrichment on *L. innocua* and *L. seeligeri* inoculated onto the surface of spinach leaves. Leaves were either treated with 1 ppm ozone concentration (grey bar) or untreated (black bar) for 10 minute. Colonies were enumerated either directly after the treatments i.e. day 0 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 ($P < 0.05$).

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.

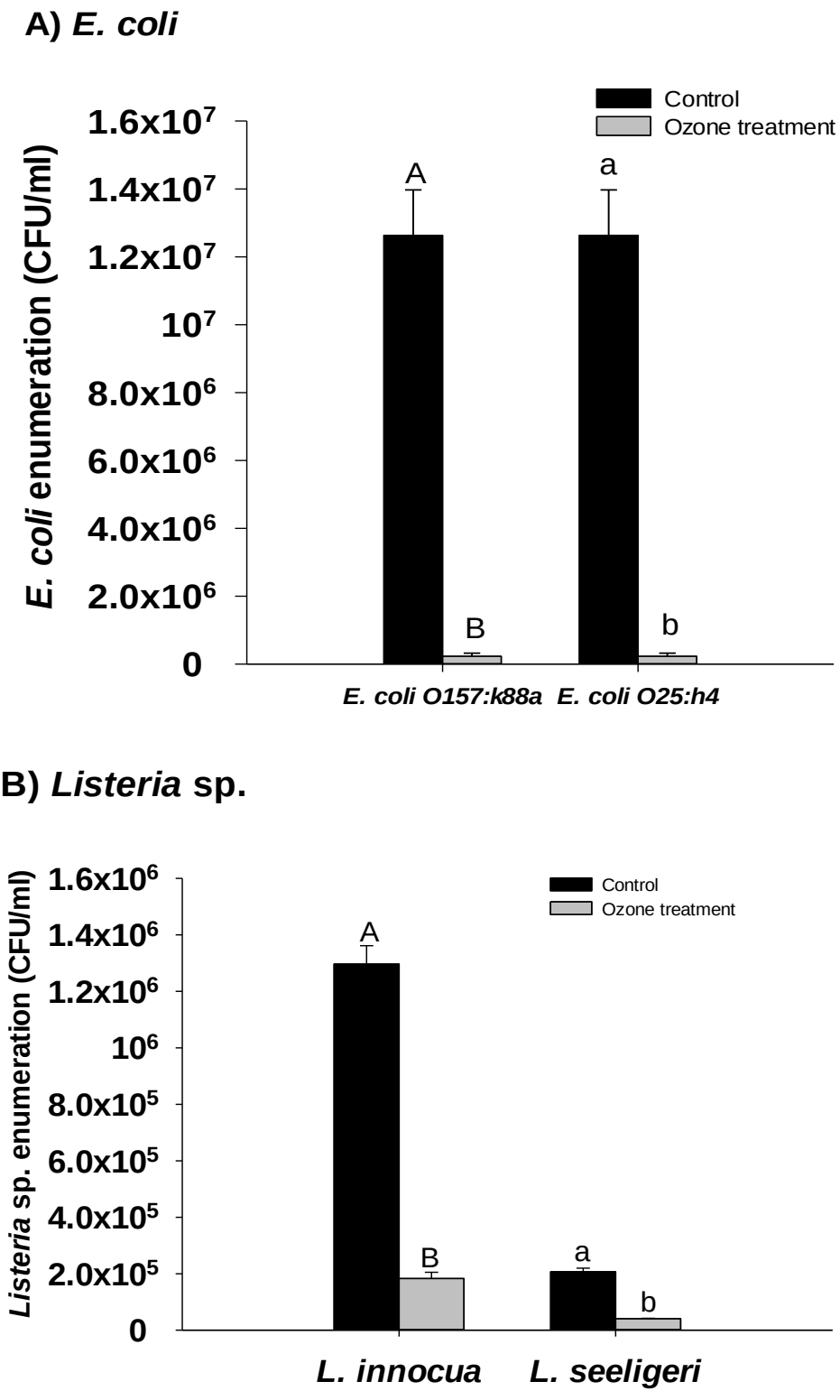

Figure 8: Impacts of increased levels of ozone exposure on two strains of *E.coli* and *Listeria* inoculated onto the surface of spinach leaves. Leaves were either treated with 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minutes. Values represent means (Standard Error) of measurements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

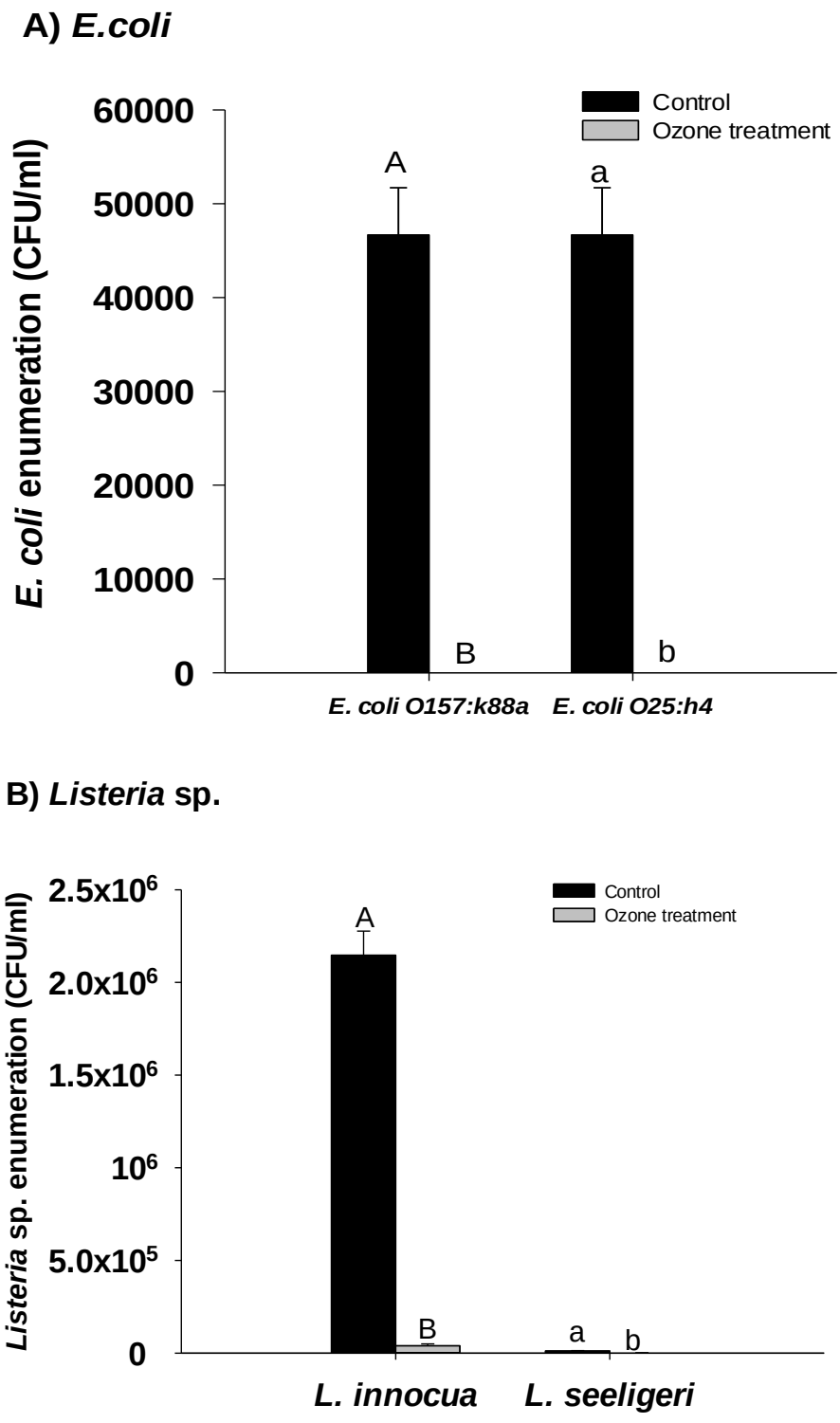

Figure 9: Impacts of ozone treatment on two strains of *E.coli* and *Listeria* inoculated onto the surface of spinach leaves. Leaves were either treated with 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minute. Colonies were enumerated after 9 days storage. Values represent means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

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.

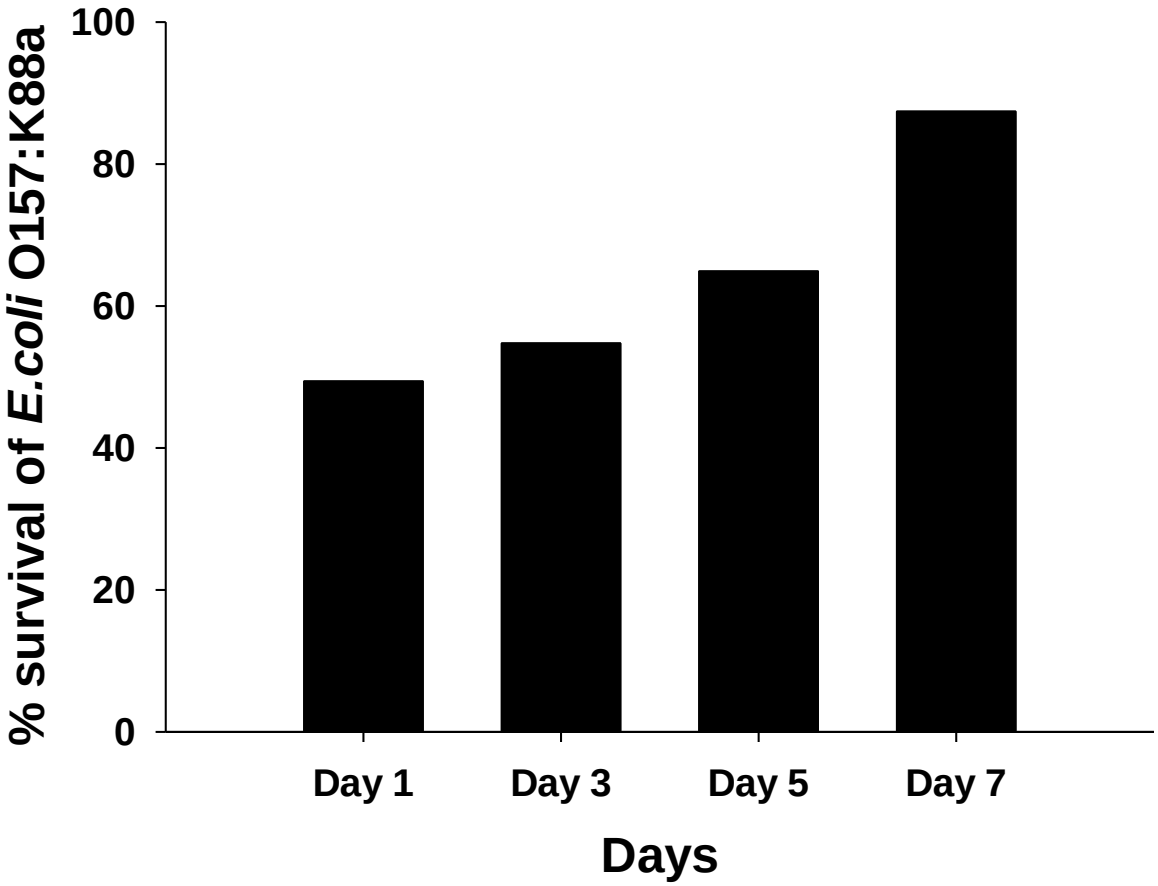

Figure 10: Survival of cells obtained from different colony ages of *E.coli* O157:K88a exposed to 10 ppm ozone concentration for 2 minutes. After ozone exposure, the culture plates were maintained at 37°C for 7 days. Values represent means of measurements made on three independent plates per treatment.

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.

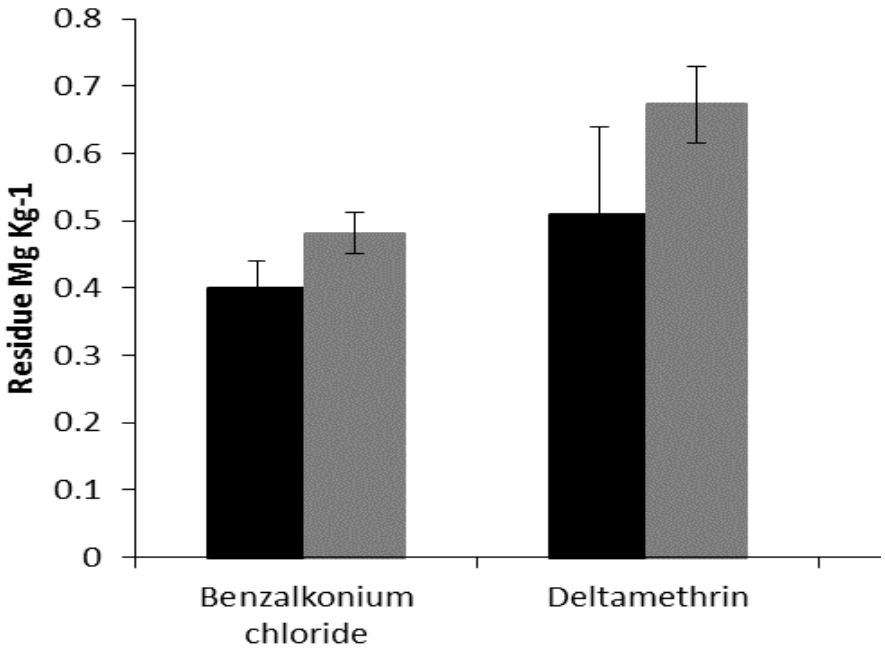

Figure 11: Impacts of ozone treatment on leaves treated with pesticide. Pesticide treated leaves were either exposed to 10 ppm ozone concentration (grey bar) or untreated (black bar) for 2 minute. Values represent means (Standard Error) of measurements made on three independent spinach leaves per treatment.

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.