Type of the Paper (Original Research Paper)

Investigation of potential reasons for bacterial survival on 'ready-to-eat' leafy produce during exposure to gaseous ozone

Shreya Wani, Jeremy Barnes * and Ian Singleton*

School of Biology, Newcastle University, Newcastle upon Tyne, NE1 7RU;

E-Mail: shrenrique@gmail.com (S.W)

***** Authors to whom correspondence should be addressed;

E-Mail: jerry.barnes@ncl.ac.uk (J.B); ian.singleton@ncl.ac.uk (I.S)

Tel.: +44 (0) 191 243 0871; +44 (0) 191 208 3043

Received: / Accepted: / Published:

Abstract: Fresh leafy produce, such as lettuce and coriander, are subject to post-harvest microbial contamination and decay. Because of increasing pesticide resistance and consumer pressures, alternative residue-free treatments, such as ozone, are being actively explored and encouraged to reduce microbial loads and curb spoilage of crops in storage/transit. However, several researchers have reported that a component of the bacterial population on leaf surfaces is resistant to ozone treatment. To investigate the potential reasons for this bacterial survival, confocal microscopy was used to visualise microbes on leaf 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 colonies as well as individual cells. We

hypothesised that cell (colony) age and prior stress (cold) contributes to, or is responsible for, the ozone resistance observed. Interestingly, cells derived from older agar-grown colonies (7–12-day-old) and cold stressed cells of a *Pseudomonas* sp. (isolated from coriander) showed higher ozone resistance than that of control cells (4-day-old colonies). These findings suggest that a range of factors are responsible for ozone resistance and further work to improve our understanding of the mechanisms of ozone resistance may lead to improved methods to reduce microbial spoilage of fresh produce.

Keywords: fresh produce, ozone resistance, confocal microscopy, *Pseudomonas* sp., spoilage, ozone gas

1.0 Introduction

Over the past decade, there have been increasing concerns about food quality and microbial safety, especially with regard to leafy salads, herbs, seed and vegetables which may be minimally processed and are often consumed raw (Losio *et al.*, 2015). It appears that microbial contamination can occur at any stage from production to consumer handling, and may arise from animal, environmental or human sources or by simple multiplication of surface biofilms to create slime and off odours (Olaimat and Holley, 2012). The microbial flora is assumed, often incorrectly, to be limited to the surface of the healthy produce, whereas the internal tissue remains sterile (Naito and Takahara, 2006). To prevent potential microbial spoilage, ozone has been suggested as an alternative sanitizer because of its strong oxidizing capacity (Goncalves, 2009). It has been used as a key disinfectant to treat municipal and drinking water since the late 19^{th} century, but has lately gained attention in the agrifood sector. The use of ozone is already permitted in many Asian and European countries, and the gas holds Generally Recognised as Safe (GRAS) status in USA and was approved by US-FDA as a 'direct contact food sanitizing agent' in 2001 (Palou *et al.*, 2003). One of the major advantages of ozone treatment is that the gas spontaneously decomposes in to inert products unlike other sanitizers used in the food processing industry (Mahapatra *et al.*, 2005). However, research shows that treatment with ozone does not completely inactivate bacteria on fresh produce (Wei *et al.*, 2007; Srey *et al.*, 2013; Wani *et al.*, 2015). This could be due to a combination of physical protection of cells in micro-colonies and/or increased ozone resistance induced by parallel factors such as refrigeration (Finkel, 2006). Epiphytic bacteria i.e. bacteria present on plant surfaces are exposed to numerous environmental stresses in nature, such as nutrient stress, water stress, variable weather conditions, and exposure to UV radiation (Capozzi *et al.*, 2009). However, bacteria are capable of adapting to, and growing, under stressful conditions (Beattie, 1999) and initiating stress response mechanisms (Capozzi *et al.*, 2009).

In this study, we used confocal scanning laser microscopy (CSLM) to differentiate between live and dead bacteria on the surface of a range of leafy salads and herbs (spinach, rocket, watercress, coriander and lettuce) in the presence and absence of ozone treatment. CSLM allows quick and direct assessment of microbial colonization on leaf surface by producing sharp, in-focus images from three-dimensional specimens (Ferrando and Spiess, 2000). We also tested the hypothesis that prior exposure to stress (cold) and increasing cell age would enhance cell resistance to ozone exposure using a *Pseudomonas* species isolated from coriander. *Pseudomonas* sp. was used as a model in this work as species from this genera are known to be involved in the microbial proteolytic and pectinolytic activities that cause soft rot of fresh produce at storage temperatures as low as 0.2°C (Saranraj, 2012).

2.0 Materials and methods

2.1 Bacterial staining and visualisation for cell viability assessment on leafy produce

Packets of 'ready-to-eat' organic baby spinach, Iceberg lettuce, wild rocket, coriander and watercress were purchased from a local retailer and stored at 4°C until the use-by-date i.e. 'EOL' (end of life). The leaves were then aseptically cut into discs measuring 1.13 cm^2 using a sterile cork borer for visualization of cell viability and enumeration of natural flora bacteria. Cell viability stains (LIVE/DEAD® BacLight™ Viability Kit, Invitrogen/Molecular Probes, Eugene, Oregon, USA) were prepared separately as per the manufacturer's instructions. This protocol utilizes green-fluorescent SYTO®9 stain to label live bacterial cells green, whereas red-fluorescent propidium iodide stains dead cells red. The staining solutions were prepared in Mueller Hinton Broth (MHB) and filter-sterilized using a syringe-mounted membrane filter of 0.2 µm pore size prior to use. The BacLight stains were added directly to the leaf surfaces which were placed onto sterile glass slides in 250 µL aliquots before placing a coverslip on top of the stain. The stained leaf was then incubated in the dark for 30 min before viewing with a Confocal Scanning Laser Microscopy (Leica TCS SP2, Leica Microsystems, GMBH, Heidelberg, Germany). The samples were scanned with a 488 nm Argon laser for the SYTO®9-stained bacteria using emission wavelengths collected at 500–550 nm, whereas a 543 nm Helium/Neon laser was used for the propidium iodide-stained bacteria using emission wavelengths collected at 574–714 nm. The microscope was equipped with either 40× HCX Plan (numerical aperture = 0.85) or 63× oil immersion objective (numerical aperture = 1.32) to image the leaf surfaces.

2.2 Ozone fumigation system and optimization of ozone exposure levels to treat leafy salads

Ozone concentration and exposure time were optimized to treat 'ready-to-eat' organic baby spinach, Iceberg lettuce, wild rocket, coriander and watercress. The ozone fumigation system stainless steel container (35 cm in diameter) placed in a fume hood into which ozone gas was introduced (produced by an electric discharge ozone generator supplied with oxygen using a model SGA01 Pacific Ozone Technology Inc., Brentwood, California, USA). Stainless steel needle valves/gap flow meters were used to manually control the introduction of ozone. Produce was placed in the treatment chamber which was closed using a Pyrex cover (Wani *et al.*, 2015). Targeted produce were exposed to 1, 10, 25, 50 µL L^{-1} ozone or 'charcoal-filtered air' (control) for varying durations (from 1 to 60 min). A photometric analyzer (model 450, manufactured by Advanced Pollution Instrumentation Division, 9480 Carroll Park Drive, San Diego, CA 92121-5201) was used to accurately monitor the ozone concentration in the system. Following targeted ozone exposure, the produce was then placed in a sterile self-seal bag and maintained at 4°C in the dark to mimic commercial storage conditions. The appearance treated produce was assessed visually using a 5-point scale (5 being unaffected and 1 being the worst colour quality)

2.3 Direct enumeration of bacteria on leafy produce after ozone treatment using confocal microscopy

'Ready-to-eat' organic baby spinach, Iceberg lettuce, wild rocket, coriander and watercress were aseptically cut into discs using a sterile cork borer and placed onto sterile glass slides. Produce was treated with either 0 (charcoal-filtered air), 1 (spinach, lettuce and watercress) or 10 (rocket and coriander) µL L^{-1} ozone for 10 min (results obtained from Section 3.2). The leaf surface bacterial staining procedure as described in Section 2.1 was then performed. Images were captured at 40× magnification. Three replicates (leaf discs) of each product per treatment were used for enumeration of viable cells (stained green). Bacteria from 20 microscopic fields were counted on each replicate leaf for each type of fresh produce using

image J software (Selinummi *et al.*, 2005), and results were expressed as average numbers of bacteria per square centimeter (cm^2) of leaf.

2.4 Investigating potential reasons for bacterial survival during ozone treatment

2.4.1 Isolation and identification of *Pseudomonas* sp.

The effect of stress on ozone resistance was determined on a *Pseudomonas* isolate from coriander. Samples (25 g) were stomached in Buffered Peptone Water (BPW) and the total viable count (TVC) determined after growth on Plate Count Agar (PCA) using standard spread plate technique. PCA agar plates were incubated at 30°C for 3 days after serial dilution in minimum recovery diluent (MRD). Discrete colonies of one morphologically dominant microbial type were subsequently re-cultured for microbial identification using 16S rRNA gene sequence. The total DNA from agar grown cells was extracted using a QIAGEN kit and extracted DNA was stored at −20°C. Using the universal prokaryotic primers, (27F) (5′-AGAGTTTGATCMTGGCTCAG-3′) and (1525R) (5′-AAGGAGGTGWTCCARCC-3′), a segment of the bacterial 16S rRNA gene was amplified using a Hybaid PCR Express thermal cycler; PCR cycles were performed at 94°C for 3 min, 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. A total of 30 cycles were performed with a final extension step at 72°C for 5 min. PCR amplification was performed using reaction mixtures (final volume 10 μL) consisting of 2 ng template, buffer incubation mix with 1.5 mM $MgCl_2$, 0.2 mM dNTP (Qiagen), 0.5 mM primer 27F, 0.5 mM primer 1525R, and 2.5 U of DreamTaq proof-reading DNA Polymerase (Fermentas). Amplification of PCR products was confirmed by 1.5% agarose gel electrophoresis with ethidium bromide staining and visualised using a UV transilluminator. PCR products were then purified using Exonuclease 1 & Alkaline phosphatase prior to sequencing (ABI 3730, 96 capillary array sequencer). The sequences were generated using Sanger sequencing and the sequences were assembled by aligning the forward and reverse

sequences using ABI MicroSeq software to form a consensus sequence. This consensus sequence was then compared with sequences in the ABI MicroSeq database as well as with those in the BLAST nucleotide database (NCBI) to allow for genus/species matching. The nucleotide sequence for the isolate employed in this study has been deposited in GenBank (NCBI) under the accession number: KR067481.

2.4.2 Effect of temperature on ozone resistance of *Pseudomonas* sp. *in vitro*

Confocal microscopy images of ozone-treated leaves revealed that two/three cells often survived in micro-colonies surrounded by dead cells. Interestingly, individual survivors were also visible (See Results Section 3.3). This indicated that cells could be physically protected by other cells when present in small colonies but also that some invidual cells appear to display some type of inherent resistance to ozone exposure. To find potential reasons for the ozone resistance observed by individual bacteria, we hypothesised that both previous stress exposure and cell age contribute to ozone resistance.

To determine the effect of prior cold exposure on ozone resistance *in vitro*, a colony of *Pseudomonas* sp. (isolated from coriander) was sub-cultured on to plates and incubated at optimum conditions i.e. 25°C for 48 h (control) and 4°C (test) to mimic produce storage conditions for 7 days. A colony of *Pseudomonas* sp. from each temperature plate was serially diluted to a standard concentration of 10^4 cells per mL (maintaining respective temperature conditions) in MRD and 100 µL of the cell suspension was spread on to Cephaloridin Fucidin Centrimide (CFC) agar plates. Each plate (containing either bacteria grown at 4°C or 25°C) was then treated with either 1 µL L^{-1} ozone concentration or 'clean air' for 10 min. Colony count was determined after incubating all plates at 25°C for 48 h.

2.4.3 Colony age effects on ozone resistance of *Pseudomonas* sp. *in vitro*

To determine whether cell age affected the ozone resistance of bacteria, a colony of the *Pseudomonas* sp. (see Section 2.4.1) was sub-cultured on to CFC plates and incubated at 25°C for up to 12 days. A single colony was isolated on the 2^{nd}, 4^{th}, 7^{th}, 10^{th} and 12^{th} day of incubation and transferred to MRD. A volume of 10^4 cells per mL of each cell age was spread (100 µL) onto sterile CFC plates and these plates were then exposed to either 1 µL L^{-1} ozone or 'clean air' for 10 min (control). Colony count was determined after incubating CFC plates at 25°C for 48 h. The % survival of *Pseudomonas* sp. was calculated by comparing the ozone treated colonies to the control colonies (not ozone treated).

2.5 Statistical analysis

Data were analysed using SPSS (IBM SPSS Statistics 19 64Bit) and graphs were produced using Microsoft Office Excel 2010 and SigmaPlot 12.5. Normal data distribution was tested using Normality test and significant differences between mean values were verified using LSD ($P < 0.05$) following one-way ANOVA.

3.0 Results

3.1 Confocal microscopy: Visualization of bacteria on leaves

Spinach leaves were observed using confocal scanning laser microscopy together with LIVE/DEAD® BacLight™ Viability Kit to determine if the bacteria that survived ozone treatment were typically present in colonies or individual cells. Bacteria were attached mainly to the leaf epidermal cell margins, observed at 20× magnification, scale bar = 47.6 µm (Fig. 1).

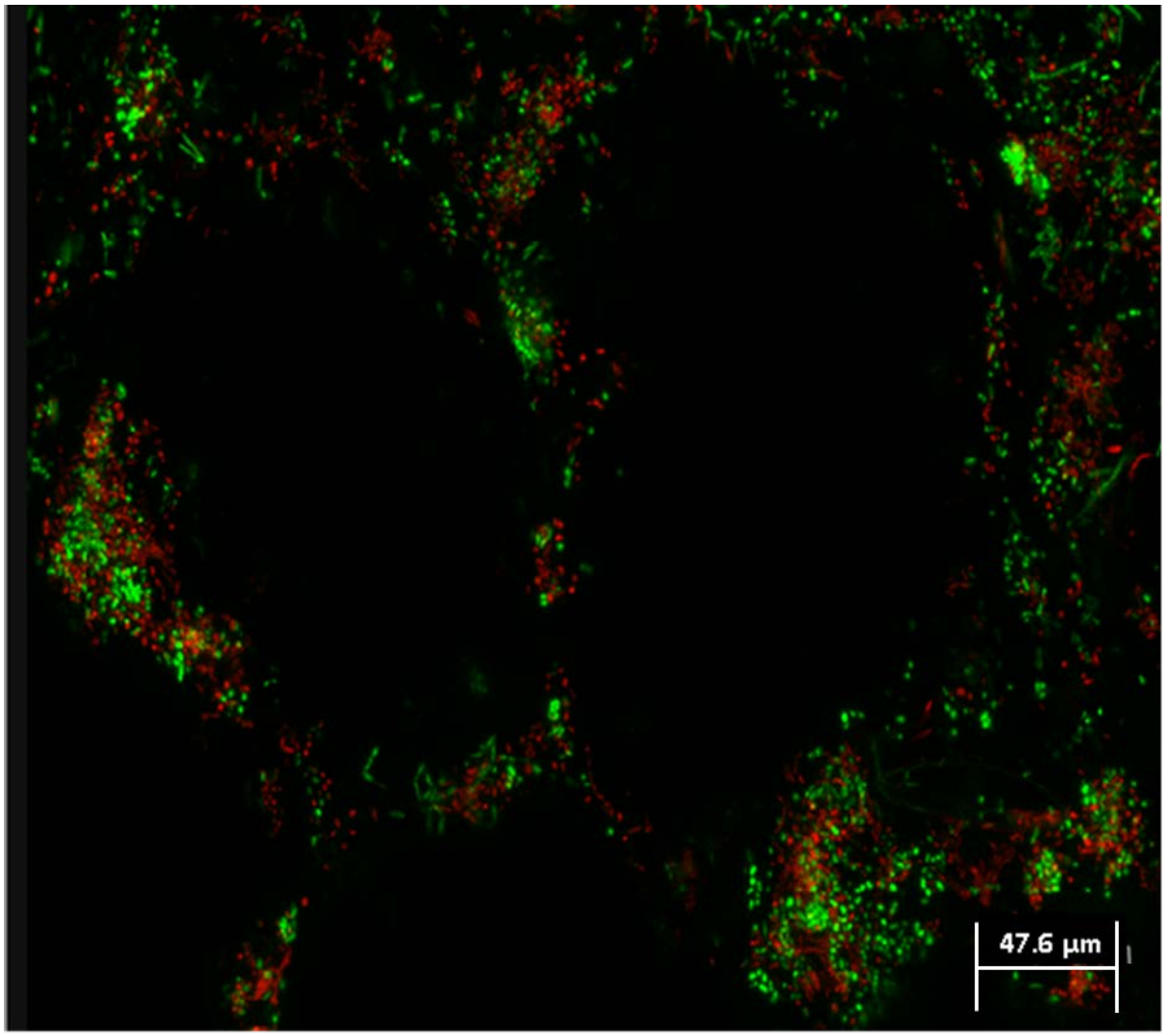

Figure 1: Confocal microscopy image of a control (not exposed to ozone) baby spinach leaf. Bacteria appeared to attach preferentially to the epidermal cell margins. Scale bar = 47.6 µm.

3.2 Optimized ozone exposure levels to treat leafy produce

All treated leafy produce showed varying levels of discoloration, whereas non-exposed controls showed little to no discolouration. Non-exposed controls and all leaves treated with 1 µL L^{-1}ozone for duration time 10 min or less received a value of '5' on the 5-point scoring scale. All produce scored '1 to 4' with the score reciprocally related to ozone * exposure time. Only coriander and rocket scored '5' when exposed to 10 µL L^{-1} ozone for up to 10 min. Table 1 shows maximum ozone exposure levels achievable to score '5' on the 5-point quality scale.

Table 1: Maximum ozone exposure levels of different types of leafy produce (ozone exposure levels that received a value of '5' on the 5-point quality scale)

Target produce	Ozone exposure limit	
	Concentration of ozone exposure (µL L^{-1})	**Duration of ozone exposure (min)**
Baby spinach	**1**	**10**
Watercress	**1**	**10**
Coriander	**10**	**10**
Lettuce	**1**	**10**
Rocket	**10**	**10**

3.3 Direct enumeration of bacteria on leafy produce after ozone treatment using confocal microscopy

Bacterial viability on non-ozone exposed control leaves was nearly 90% (Fig. 2A & 2B), whereas only 10% of bacteria on ozone-treated leaf surfaces appeared viable. On control leaves, large aggregations of live cells stained green are visible (see Fig. 2A indicated by the blue arrow). Micro-colonies and cells in twos/threes, as indicated by the orange arrow (Fig. 2A – spinach leaf as an example), were frequent. Individual dead cells stained red are visible in Fig. 2A (indicated by a white arrow). Similar bacterial aggregates were also observed on watercress, coriander, rocket and lettuce leaf surfaces (results not shown). In Fig 2B, yellow arrows indicate individual bacteria surviving ozone treatment. Similar results were also observed on watercress, coriander, rocket and lettuce leaf surfaces (results not shown). Enumeration of bacterial viability after ozone exposure showed at least 1-log reduction in all targeted produce (Fig. 3).

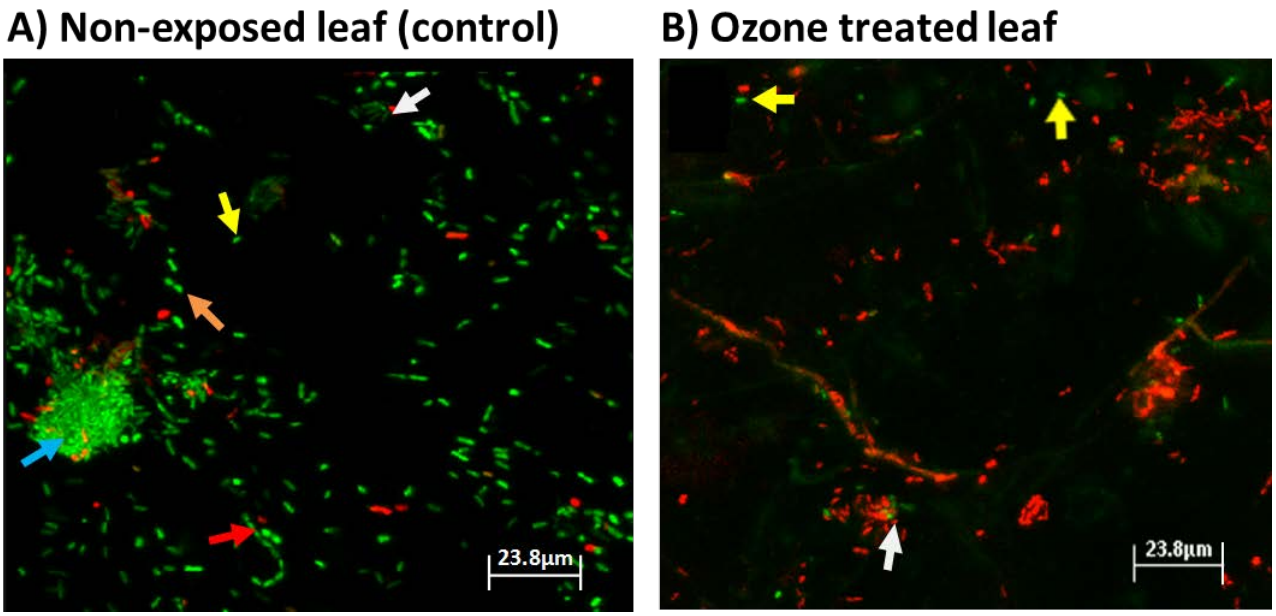

Figure 2: Confocal microscopy image of a baby spinach leaf. Bacteria were stained with green-fluorescent SYTO®9 to label live bacterial cells green and with red-fluorescent propidium iodide to label dead bacterial cells red. Scale bar = 23.8 µm (A) Non-ozone exposed leaf (control). Blue arrow indicates large aggregates of live cells, orange arrow indicates small colonies in two/threes, red arrow indicates bacteria in chains, yellow arrow indicates individual cells present on a leaf surface and white arrow indicates individual dead cell (B) Leaf treated with 1 µL L^{-1} ozone for 10 min. White arrow indicates live cell present in micro-colony of dead cells and yellow arrows indicate individual live cells surviving ozone treatment.

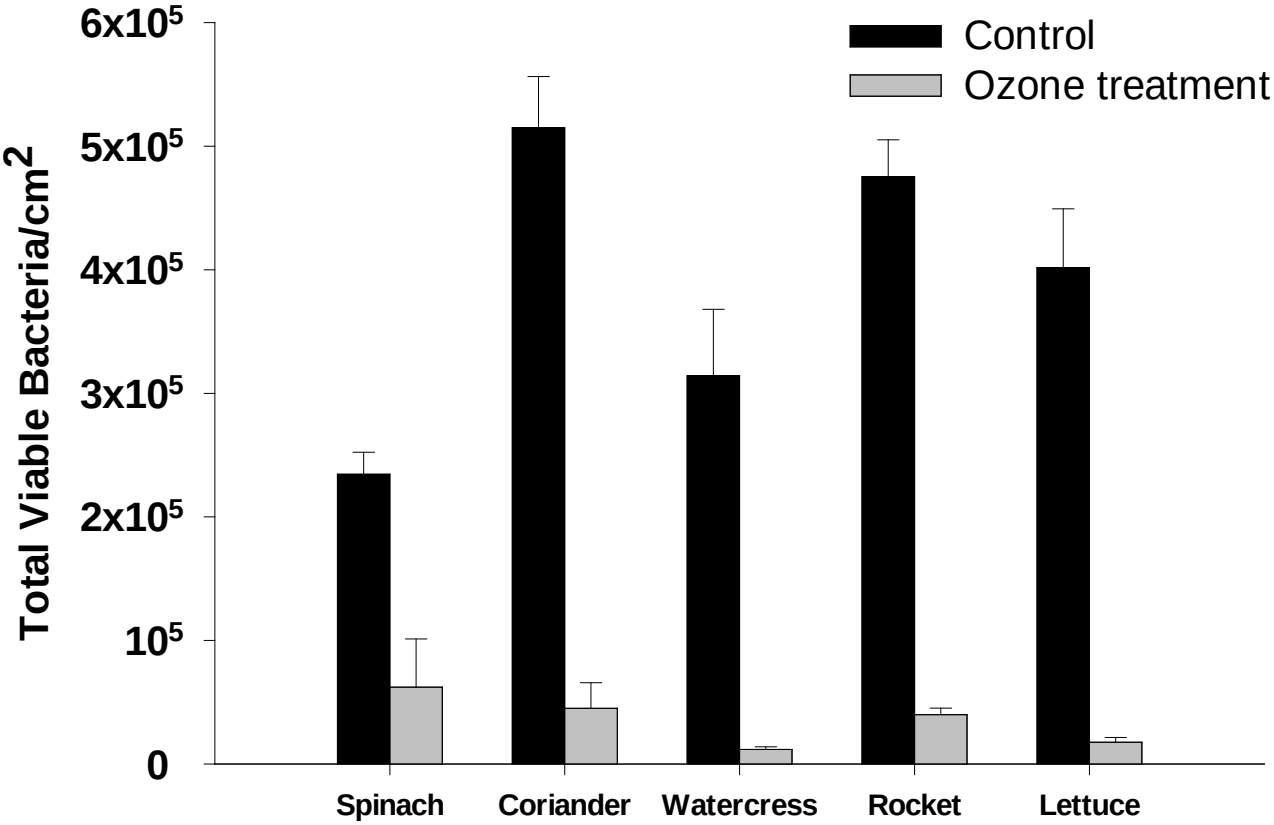

Figure 3: Total viable bacterial counts from the surfaces of spinach, watercress, and lettuce leaves treated with 1 µL L^{-1} ozone and coriander and rocket treated with 10 µL L^{-1} (grey bars) versus leaves not treated with ozone and maintained in 'clean air' for an equivalent period (black bars) for 10 min. Data derived from microscopic counts of SYTO®9/PI stained bacteria on leaves. Values represent means (+/−Standard Error) of measurements made on three separate leaves per treatment.

3.4 Investigating potential reasons for bacterial survival on leaf surfaces after ozone treatment

3.4.1 Effect of temperature on ozone resistance of *Pseudomonas* sp. *in vitro*

Colony numbers (CFU) of *Pseudomonas* sp. grown in optimum conditions (25°C) *in vitro* were significantly ($P < 0.05$) reduced by ozone treatment (Fig. 4). In contrast, colony numbers of *Pseudomonas* sp. maintained in cold conditions (i.e. stored at 4°C) *in vitro* were not

significantly ($P < 0.05$) reduced by ozone treatment (Fig. 4) implying that bacteria submitted to refrigerated conditions show enhanced resistance to ozone.

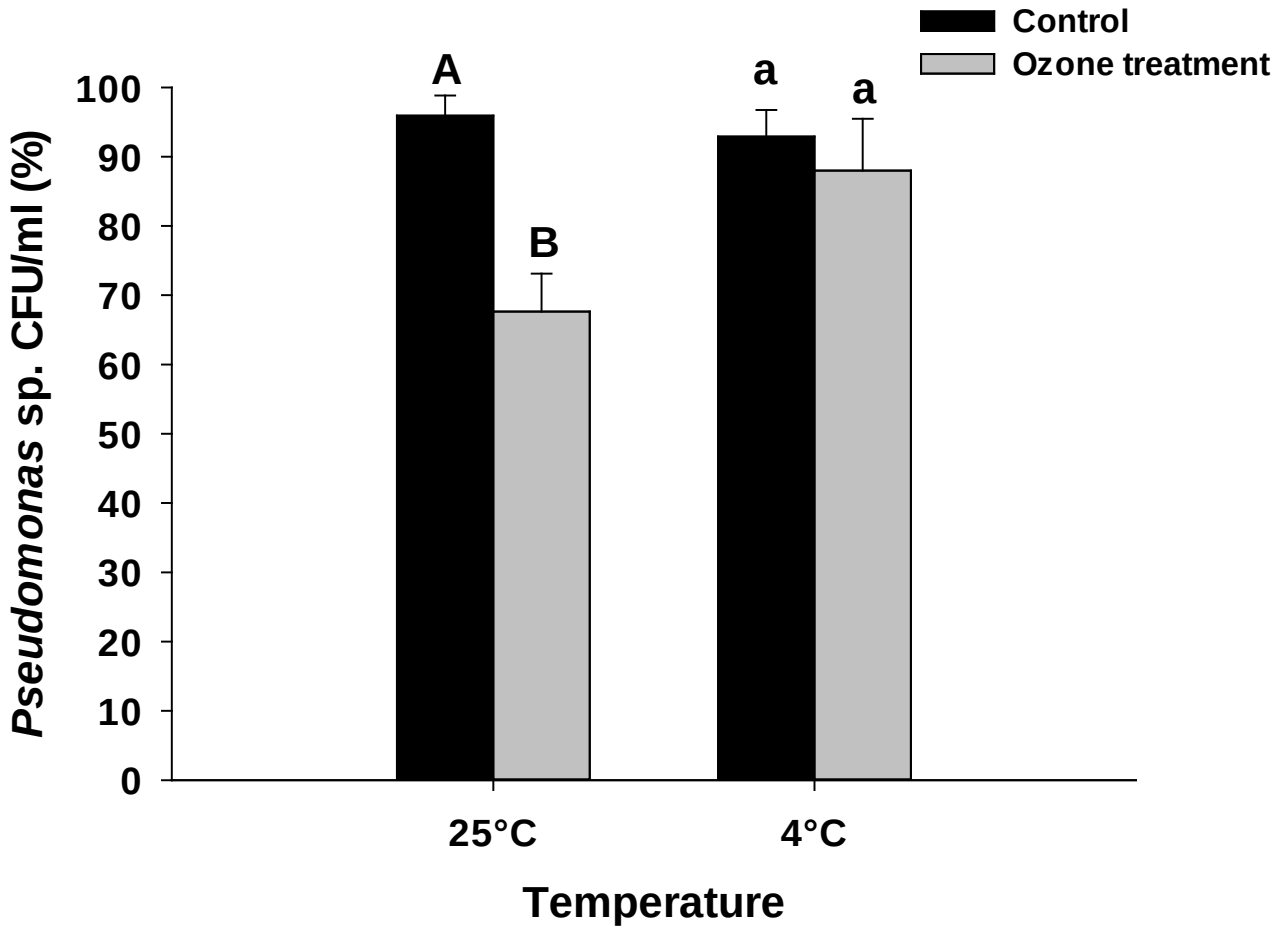

Figure 4: Impacts of ozone-exposure on *Pseudomonas* sp. grown at 25°C and 4°C and exposed to either 1 µL L^{-1} ozone concentration (grey bar) or 'clean' air (black bar) for 10 min. After the treatment plates were incubated at optimum temperature i.e. 25°C for 48 h. Values represent means (±Standard Error) of measurements made on three independent plates per treatment. Bars with different letters are statistically significantly different ($P < 0.05$).

3.4.2 Effect of age on ozone resistance of the leaf surface bacteria *in vitro*

Pseudomonas cells derived from 7, 10 and 12 day old colonies showed approximately 40% greater survival to ozone treatment than those from 2 and 4 day old cells (Fig. 5), suggesting

that cells from older bacterial colonies are more ozone resistant than cells from younger colonies. The increase in survival was statistically significant ($P < 0.05$).

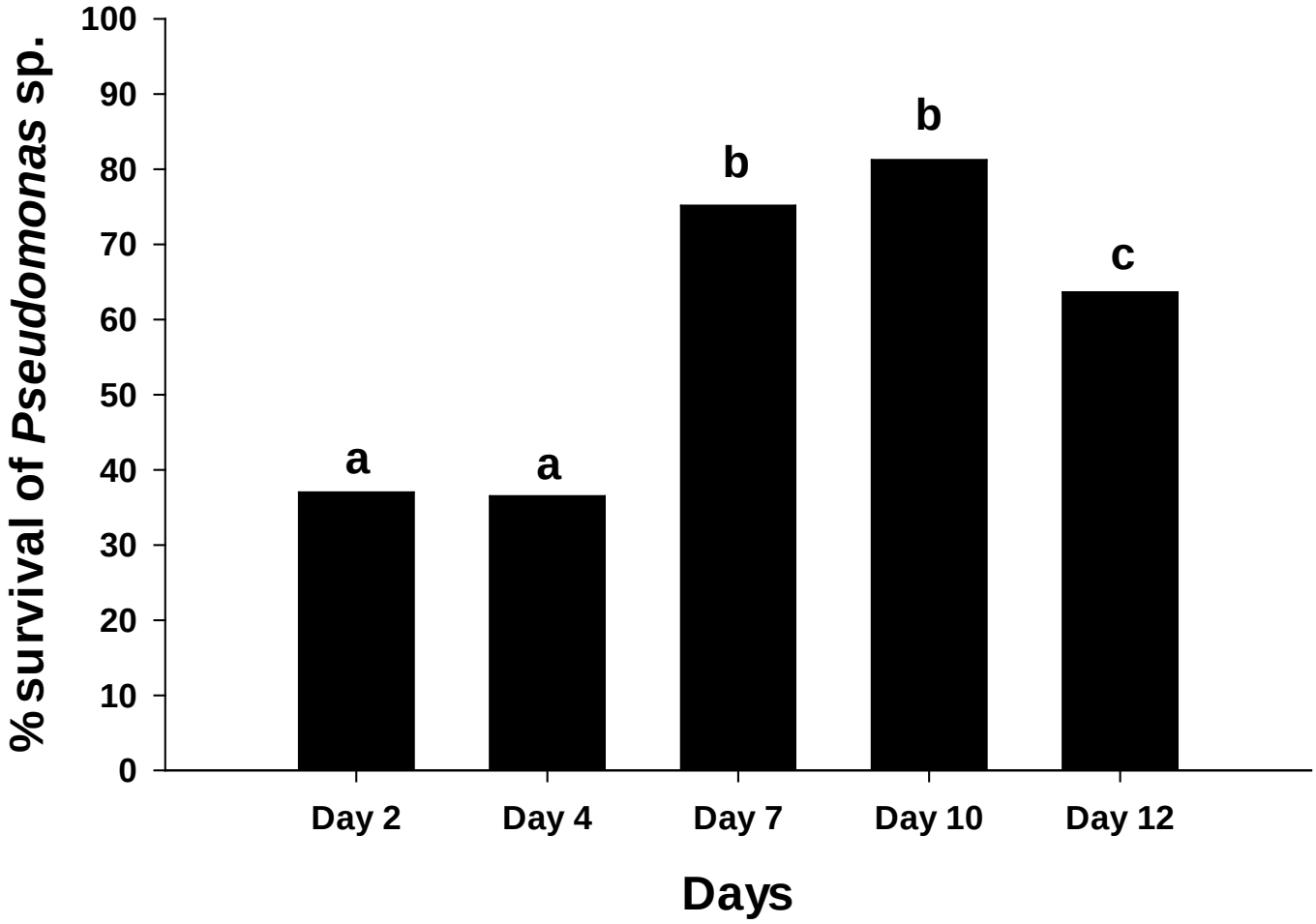

Figure 5: Survival of cells obtained from different colony ages of *Pseudomonas* sp. exposed to 1 µL L^{-1} ozone concentration for 10 min. After ozone exposure, the culture plates were maintained at 25°C for 12 days. Bars with different letters are statistically significantly different ($P < 0.05$).

4.0 Discussion

Confocal microscopy revealed that bacteria were mainly attached to the epidermal plant cell margins, consistent with the report by Romantschuk *et al*. (1996). SYTO®9/PI staining in conjunction with CSLM allowed *in situ* observation of bacteria on untreated leaf surfaces (control) and they appeared to be present as small micro-colonies and as individual cells. Similar observations were obtained by Carmichael *et al.* (1999) who used fluorescein isothiocyanate (FITC) staining together with confocal imaging techniques to observe both clusters and individual bacteria on the surface of lettuce leaves.

The visual appearance and freshness of leafy produce has been the main judging criteria for quality distinction at purchase or consumption (Rico *et al.*, 2007). No visual discolouration was observed when leafy produce was treated with 1 µL L^{-1} gaseous ozone but higher levels, e.g. 10 µL L^{-1} for 10 min, caused discolouration to spinach, watercress and lettuce. Similar results were previously observed on fresh produce like lettuce, spinach, rocket leaves when treated with different ozone concentrations (Alexopoulos *et al.*, 2013). Only coriander and rocket leaves retained freshness when exposed to 10 µL L^{-1} ozone treatment. This may be related to the difference in the physiology of the produce e.g. stomatal conductance (Jin-Gab Kim, 1998; Alexopoulos *et al.*, 2013). Discolouration was observed when high ozone dosages were applied. Bacterial colonization varies between leaves and largely depends on the properties of the leaf surface e.g. leaf surface morphology, hydrophobicity, waxiness, leaf surface chemistry etc (Golberg *et al.*, 2011). Confocal microscopy revealed no obvious link between physical surface morphology and bacterial colonization.

The data presented here indicate that although ozone treatment significantly reduced bacterial viable counts on the leaf surface, approximately 10% of the bacterial flora exhibited resistance to the ozone treatment employed. Confocal images of ozone treated leaves revealed that

two/three live cells survived in micro-colonies (surrounded by dead cells). Micro-colonies and biofilms are formed on leaf surfaces due to bacterial attachment and production of exopolymeric substances (Mah, 2001). This motivates microbial cells to stimulate activities unachievable alone or outside of micro-colonies. The possible effect of micro-colonies or biofilms on microbial biology and ecology are protection from UV, desiccation and predation, and biofilms potentially allow genetic exchange, gene transfer and synergistic interaction between cells (Morris and Monier, 2003). Biofilms allow microbes to remain in close contact and communicate by quorum sensing, and thus, combat anti-microbial treatments as a community (Jahid and Ha, 2012). The survival of bacteria to ozone exposure could also be due to the presence of a small sub-population of persister cells. These cells are invulnerable cells that neither grow nor die, which may enter a highly-protected state exhibiting intense resistance, and develop more commonly in micro-colonies or biofilm (Van Houdt and Michiels, 2010; Bridier *et al.*, 2011). Therefore cells in micro-colonies/biofilms on leaf surfaces may resist ozone treatment by both physical protection (i.e. surrounding cells are killed but the cells in the centre of a colony are physically protected) or by the biofilm bacteria having inherent enhanced resistance mechanisms.

Interestingly, some individual cells on the leaf surface also survived ozone treatment suggesting that they also have inherent resistance mechanisms. We hypothesised that the survival of the individual bacteria on the leaf surface after ozone exposure is due to ageing or prior exposure to cold (Johnson, 2008; Wani *et al.*, 2015).

During growth in the field, the bacteria present on the surface of leaves are continually subjected to changes in temperature, nutrient availability and osmotic pressure (Lindow, 1995). In addition, to prevent microbial spoilage and contamination by pathogens, cumulative mild processing steps are employed during the production of fresh produce increasing chances of

312 additional stress and potentially developing hardy bacteria that are able to resist any further
313 applied treatments such as ozone exposure (Capozzi *et al.*, 2009).

314 A number of stresses have been shown to induce such 'cross protection', and in this study, cold
315 stress was used as a model to determine if prior stress exposure enhanced the ozone resistance
316 of a typical leaf surface bacterium. Our results suggest that pre-exposure of bacteria
317 (*Pseudomonas* sp.) to cold stress enhanced ozone resistance *in vitro*. Survival of these bacteria
318 in stressed conditions is a combination of cell responses designed to minimise the lethal effects
319 or repair damage (Jozefczuk *et al.*, 2010). When repairing damage, the presence of cold shock
320 proteins in bacteria overcomes growth-limiting effects by either altering redox status or
321 increasing stability of RNA and DNA secondary structures (Reva *et al.*, 2006). Cold shock
322 acclimation proteins are produced in high abundance during low temperature and have been
323 identified in *Pseudomonas* sp. (Reva *et al.*, 2006). Our results indicate that such stress-related
324 temperature responses may also help bacteria to survive subsequent ozone exposure.

325 We also hypothesised that cell age is a factor contributing to the ozone resistance of individual
326 leaf surface bacteria (Wani *et al.*, 2015). Fresh produce typically takes weeks to grow and any
327 cells present on the leaf surface could easily have been present and persisting for a prolonged
328 period. Our results clearly demonstrated that cells derived from older colonies were more
329 resistant to ozone than cells from younger colonies and this observation is strengthened by
330 previous work showing that older biofilm cells of *Pseudomonas aeruginosa* were more
331 resistant to biocides than younger cells (Bridier et al., 2011) and that the older cells had an
332 increased expression of RpoS genes.

333 Therefore, further understanding of the molecular basis of ozone resistance of leaf surface
334 bacteria is required. A detailed understanding of the resistance mechanisms involved may help
335 to develop novel methods to control the contamination of fresh produce.

5.0 Conclusions

This work focused on visualising microbes on leaf surfaces after ozone treatment by using confocal scanning microscopy and investigating potential reasons for ozone resistance in leaf surface bacteria. Confocal microscopy demonstrated that bacterial cells able to survive ozone exposure occurred both in micro-colonies and as individuals on the leaf surface. This suggested that bacterial ozone resistance was likely due to a number of factors e.g. physical protection in small colonies and inherent resistance of individual cells. Subsequent results suggested that increasing cell (colony) age and prior exposure to cold stress of a typical leaf surface bacterium (*Pseudomonas* sp.) enhances ozone resistance *in vitro*. Therefore, further investigation on understanding the mechanisms of ozone resistance in aged and cold stressed cells of *Pseudomonas* sp. is required, and this may lead to methods that can overcome resistance. Such applications could deliver immense potential benefits for commercial use.

Acknowledgements

This work was supported by AHDB/Horticulture Development Company (HDC); project number FV 386 via award of a PhD studentship to Shreya Wani. The authors would like to thank Dr Matthew Peake for technical support; Dr Trevor Booth (Bio-Imaging services, William Leech Bldg, Newcastle University Medical School, NE2 4HH, UK) for assisting with CSLM; Alan Craig for maintaining and calibrating the ozone fumigation equipment; and Jagpreet K. Maker for editing the manuscript.

Author Contributions

The work presented is a part of a PhD project funded by the UK-ADHB/HDC (project number FV 386) and was also supported financially by Newcastle University Institute for Sustainability; the data were managed, designed, collected and analyzed by Shreya Wani, who

took the lead on writing the manuscript. Ian Singleton and Jeremy Barnes supervised the work, assisted in experimental design/ideas, data interpretation, manuscript writing and won the grant award.

Conflicts of Interest

The authors declare no conflict of interest.

References

Alexopoulos, A., Plessas, S., Ceciu, S., Lazar, V., Mantzourani, I., Voidarou, C., Stavropoulou, E., Bezirtzoglou, E., 2013. Evaluation of ozone efficacy on the reduction of microbial population of fresh cut lettuce (*Lactuca sativa*) and green bell pepper (*Capsicum annuum*). Food Control. 30, 491–496.

Beattie, G., A., Lindow, S., E., 1999. Bacterial colonization of leaves: A spectrum of strategies. Phytopathology. 89, 353–359.

Bridier, A., Briandet, R., Thomas, V., Dubois-Brissonnet, F., 2011. Resistance of bacterial biofilms to disinfectants: a review. Biofouling. 27, 1017–1032.

Capozzi, V., Fiocco, D., Amodio, M.L., Gallone, A., Spano, G., 2009. Bacterial stressors in minimally processed food. Int. J. Mol. Sci. 10, 3076–3105.

Ferrando, M., Spiess, W.E.L., 2000. Review: Confocal scanning laser microscopy. A powerful tool in food science Revision: Microscopia laser confocal de barrido. Una potente herramienta en la ciencia de los alimentos', Food Sci. Technol. Int. 6, 267–284.

Golberg, D., Kroupitski, Y., Belausov, E., Pinto, R., Sela, S., 2011. *Salmonella Typhimurium* internalization is variable in leafy vegetables and fresh herbs. Int. J. Food. Microbiol. 145, 250–257.

Goncalves, A.A., 2009. Ozone - an Emerging Technology for the Seafood Industry. Braz. Arch. Biol. Tech. 52, 1527–1539.

Jahid, I.K., Ha, S.-D., 2012. A review of microbial biofilms of produce: Future challenge to food safety. Food Sci. Biotechnol. 21, 299–316.

Kim, J-G., Yousef, AE.,Chism. G.W., 1998. Use of ozone to inactivate microorganisms on lettuce. J. Food Saf. 19, 17–34.

Johnson, L.R., 2008. Microcolony and biofilm formation as a survival strategy for bacteria. J. Theor. Biol. 251, 24–34.

Jozefczuk, S., Klie, S., Catchpole, G., Szymanski, J., Cuadros-Inostroza, A., Steinhauser, D., Selbig, J., Willmitzer, L., 2010. Metabolomic and transcriptomic stress response of *Escherichia coli*. Mol. Syst. Biol. 6, 364.

Beattie, G.A., Lindow, S.E., 1995. The secret life of foliar bacterial pathogens on leaves. Annu. Rev. Phytopathol. 33, 145–172.

Losio, M.N., Pavoni, E., Bilei, S., Bertasi, B., Bove, D., Capuano, F., Farneti, S., Blasi, G., Comin, D., Cardamone, C., Decastelli, L., Delibato, E., De Santis, P., Di Pasquale, S., Gattuso, A., Goffredo, E., Fadda, A., Pisanu, M., De Medici, D., 2015. Microbiological survey of raw and Ready-To-Eat leafy green vegetables marketed in Italy. Int. J. Food Microbiol. 210, 88–91.

Mah, T.F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9, 34–39.

Mahapatra, A.K., Muthukumarappan, K., Julson, J.L., 2005. Applications of ozone, bacteriocins and irradiation in food processing: a review. Crit. Rev. Food Sci. Nutr. 45, 447–461.

401 Morris, C.E., Monier, J.M., 2003. The ecological significance of biofilm formation by plant-associated
402 bacteria. Annu. Rev. Phytopathol. 41, 429–453.
403 Naito, S., Takahara, H., 2006. Ozone Contribution in Food Industry in Japan. Ozone Sci. Eng. 28, 425–
404 429.
405 Olaimat, A.N., Holley, R.A., 2012. Factors influencing the microbial safety of fresh produce: a review.
406 Food Microbiol. 32, 1–19.
407 Ölmez, H., Akbas, M.Y., 2009. Optimization of ozone treatment of fresh-cut green leaf lettuce. J.
408 Food Eng. 90, 487–494.
409 Palou, L.S., Smilanick, J.L., Crisosto, C.H., Mansour, M., Plaza, P., 2003. Ozone gas penetration and
410 control of the sporulation of *Penicillium digitatum* and *Penicillium italicum* within commercial
411 packages of oranges during cold storage. Crop Protect. 22, 1131–1134.
412 Reva, O.N., Weinel, C., Weinel, M., Bohm, K., Stjepandic, D., Hoheisel, J.D., Tummler, B., 2006.
413 Functional genomics of stress response in *Pseudomonas putida* KT2440. J. Bacteriol. 188, 4079–
414 4092.
415 Rico, D., Martín-Diana, A.B., Barat, J.M., Barry-Ryan, C., 2007. Extending and measuring the quality of
416 fresh-cut fruit and vegetables: a review. Trends Food Sci. Technol. 18, 373–386.
417 Saranraj, P., Stella, D., Reetha, D., 2012. Microbial spoilage of vegetables and its control measures: a
418 review. Int. J. Nat. Prod. Sci. 2, 1–12.
419 Selinummi, J., Seppälä, J., Yli-Harja, O., Puhakka, J., 2005. Software for quantification of labeled
420 bacteria from digital microscope images by automated image analysis. BioTechniques. 39, 859–863.
421 Srey, S., Jahid, I.K., Ha, S.-D., 2013. Biofilm formation in food industries: A food safety concern. Food
422 Control. 31, 572–585.
423 Van Houdt, R., Michiels, C.W., 2010. Biofilm formation and the food industry, a focus on the bacterial
424 outer surface. J. Appl. Microbiol. 109, 1117–1131.
425 Wani, S., Maker, J., Thompson, J., Barnes, J., Singleton, I., 2015. Effect of Ozone Treatment on
426 Inactivation of *Escherichia coli* and *Listeria* sp. on Spinach. Agriculture. 5, 155–169.
427 Wei, K., Zhou, H., Zhou, T., Gong, J., 2007. Comparison of Aqueous Ozone and Chlorine as Sanitizers
428 in the Food Processing Industry: Impact on Fresh Agricultural Produce Quality. Ozone Sci. Eng. 29,
429 113–120.

430