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Short communication

Effects of oxygen-depleted atmospheres on survival and growth of *Listeria monocytogenes* on fresh-cut Iceberg lettuce stored at mild abuse commercial temperatures

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ABSTRACT

The effects of oxygen-depleted atmospheres, 0.25% O_2 + 12% CO_2 (balance N_2) and 2% O_2 + 6% CO_2 (balance N_2), on growth of *Listeria monocytogenes* on fresh-cut Iceberg lettuce were determined. The study was carried out at mild abuse temperatures using controlled atmosphere chambers. During storage at a constant temperature of 7 °C, growth was enhanced at the lower oxygen level of 0.25% O_2 by Day 10. Over 17 days of storage at temperatures designed to mimic mild abuse commercial conditions, there were again significantly higher counts under 0.25% O_2 from Day 10 onwards. These were 0.9 and 0.7 log cycles higher on Days 14 and 17, respectively. When a model lettuce agar medium was used to eliminate possible interactions with competing flora the direct effects of the atmosphere enhancing the growth of *L. monocytogenes* was also observed. It is concluded that use of very O_2-depleted atmospheres for control of enzymatic browning of fresh-cut Iceberg lettuce may introduce a potential hazard under some commercial conditions. There is a need for greater vigilance and possibly additional measures to ensure consumer safety.

1. Introduction

Increasing consumption of fresh produce has been parallelled by an increase in produce-linked food poisoning outbreaks. Fresh-cut vegetables, notably lettuce and leafy greens, have been implicated in a significant number of food-borne disease outbreaks in the USA since 1995 (Beuchat, 2006). To prevent contamination and growth by human pathogens, safety assurance of these products depends on Good Agricultural Practices, Good Manufacturing Practices, and on the environmental conditions prevailing between production and consumption, notably temperature and atmosphere. The benefits of low temperatures and modest shelf-lives for control of pathogenic bacteria are well documented. However, the practical implications of interactions between modified atmospheres (MAs) and storage temperatures/times on survival and growth of some pathogens are less well understood. On the one hand, there appear to be no effects of MAs on growth of *Escherichia coli* O157:H7 (Diaz and Hotchkiss, 1996; Delaquis et al., 2007; Abadias et al., 2012; Posada-Izquierdo et al., 2013), and enhanced effects of MAs on growth of *Salmonella* species on leafy greens have only been reported at quite high abuse temperatures of 15 or 25 °C (Oliveira et al., 2010; Sant'ana et al., 2013). By contrast, there are significant effects of MAs on enhanced survival and growth of *Listeria monocytogenes* at even modest abuse temperatures of 8 °C (Francis and O'Beirne, 1997, 1998a). However, in these previous publications, oxygen depletion was not as low as those oxygen concentrations found in commercial packages of fresh-cut produce.

Commercial modified atmospheres for fresh-cut products are generally in the range of 0.25–3% O_2 + 3–12% CO_2, balance N_2. The benefits of MAP arise from a general reduction in the rates of metabolic processes and the retardation of senescence. A key consideration for some minimally processed products, for example fresh-cut lettuce, is sufficient oxygen depletion to ensure good control of enzymatic browning while at the same time avoiding off-odours and flavours due to anaerobiosis. Martínez-Sánchez et al. (2011) have shown that for fresh-cut Romaine lettuce, the optimum oxygen atmosphere is between 0.2 and 0.5%. Atmospheres

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above 0.5% O_2 resulted in increased enzymatic browning, while atmospheres below 0.2% resulted in off-odours and flavours. Smyth et al. (1998) have produced similar data for Iceberg lettuce. As a result, an intended oxygen level in this range, approximately 0.25%, is targeted in commercial practice. Since O_2 depleted atmospheres may exert selective pressures favouring *Listeria* growth at mild abuse temperatures, this study examined whether such very low O_2 levels (0.25 and 2%) represented a hazard under commercial conditions.

Based on data for temperatures experienced by fresh-cut products during distribution (Nunes et al., 2009), retail (Jacxsens et al., 2002; Tromp et al., 2010) and domestic refrigeration (Marklinder et al., 2004) and on the practical experience of the current authors, a mild abuse temperature profile was used in this study. It consisted of two days at 4 °C (storage by processor), followed by two days at 8 °C (distribution), followed by subsequent storage at 7 °C (retail display/domestic refrigerator).

2. Material and methods

2.1. Preparation of inoculum

Three strains of *L. monocytogenes* were used: CECT 940, CECT 4032 and CECT 5947. Cultures were rehydrated in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, United Kingdom) and consecutively subcultured twice in 10 mL of BHI at 37 °C for 24 h. After the second incubation, cultures were mixed and equal volumes of cell suspensions were combined to give approximately equal populations of each culture. The pool was centrifuged (2000 × *g*, 15 min, 4 °C), resuspended and diluted in phosphate buffered saline (PBS) (Oxoid) to the desired concentration of approximately 10^4 cfu/mL. Final concentrations of the inoculum solutions were confirmed by spread plating on Oxford agar (Scharlau, Sentmenat, Spain).

2.2. Preparation and inoculation of fresh-cut lettuce

Iceberg lettuce (cultivar Yukaipa) was subjected to a standard fresh-cut process, including anti-microbial dipping in aqueous solution containing 100 mg/L free chlorine for 3 min followed by rinsing in tap water and spin drying. Thirty gram samples were placed in unsealed perforated low density polyethylene (LDPE) bags (200 mm × 100 mm) with 20 × 5 mm perforations per bag. The function of the bags was to prevent surface drying, while the holes enabled the samples to be stored in the desired atmosphere. In order to achieve an inoculation close to 1000 cells/g lettuce, two mL (approx 10^4 cfu/mL) were spot inoculated on to the 30 g of fresh-cut lettuce in each bag. The bags were heat-sealed and carefully placed in the controlled atmospheres chambers, explained below, at random.

2.3. Controlled atmosphere (CA) chambers

Inoculated lettuce was stored in 1.5 L glass jars connected to a flow-through board system providing air or different controlled atmospheres at a constant flow rate of 40 mL/min (Martínez-Sánchez et al., 2006). Jars were arranged in rows, each with five jars in series, and the required gases passed through the jars. Each jar had a gas-tight rubber bung, perforated by two metal tubes through which the gases entered and left. Appropriate lengths of silicone tubing joined the jars in series, and brought the entering gas to the bottom of each jar. Atmospheres of 0.25% O_2 + 12% CO_2, balance N_2 or 2% O_2 + 6% CO_2, balance N_2, or air were used. These atmospheres were humidified by bubbling through a preliminary chamber containing 100 mL of water. After passing through the jars, the gases passed though a micropore filter and were vented outside the cold room. The composition of the gas mixes (O_2 and CO_2) was monitored daily with an O_2 analyser (CG-1000, Ametek, Thermox Instruments Co., Pittsburgh, PA, USA) and a CO_2 analyser (Via 510, Horiba Instruments Co., Irvine, CA, USA) to ensure that target gas levels were maintained. The jars were placed in a cold room equilibrated to the temperature(s) required. Three replicates were used for each atmosphere and for each sampling date. The last jar in each row was removed and counts of *Listeria* determined.

2.4. Preparation and inoculation of lettuce agar model medium

A solid lettuce agar model medium was prepared using surface washings from shredded Iceberg lettuce as described previously (Francis and O'Beirne, 1998a). Three 60 g batches of shredded lettuce were gently shaken in 250 mL sterile distilled water. This extract was filtered aseptically using a 0.45 μm (Sartorius Microsart Filter, Goettingen, Germany) followed by a 0.22 μm micropore filters (Fisher, Pittsburgh, USA). The solid medium was prepared by heating the aseptic extract to 50 °C and mixing two parts with one part 0.3 M potassium phosphate buffer (pH 7.2) containing 3% agar (Scharlau), giving a final concentration of 1% agar. Aliquots of 9 mL were pipetted into 55 mm diameter petri dishes and allowed to solidify.

Diluted cultures of *L. monocytogenes* (50 μm, containing approx 10^4 cfu/mL) were surface spread on to the model medium. Inoculated packs were placed in triplicate CA chambers. The gas mixtures and flow rate described above were used.

2.5. Microbiological analyses

2.5.1. Fresh-cut lettuce

On sampling days, the contents of each experimental bag (~30 g) were transferred to a stomacher bag followed by nine times their weight of PBS, and homogenised for 1 min at high speed (Smasher, AES, Bruz, France). Serial dilutions of homogenised samples were made in PBS and were surface spread on Oxford agar. Colonies were counted after incubation at 37 °C for 24 h and reported as log cfu/g sample.

2.5.2. Vegetable agar medium

The entire agar in each plate was aseptically transferred to a stomacher bag followed by four times its weight of Fraser broth (Merck, Darmstadt, Germany) to give a fivefold dilution, and homogenised at high speed for 1 min as described above. Serial dilutions of homogenised samples were made in PBS and were surface spread on Oxford agar. Plates and stomacher bags were incubated at 37 °C for 24 h. Thereafter, the number of colonies was counted and reported as log cfu/plate. Where present but below the limit of detection (log 0.7 cfu/plate), *Listeria* was confirmed by loop transfer from Fraser broth, streaking on Oxford agar and incubation at aforementioned conditions.

2.6. Experiments

Three experiments were carried out to determine whether very O_2-depleted atmospheres enhanced the survival and growth of *L. monocytogenes*.

1. The effects of 0.25% O_2 + 12% CO_2, balance N_2 versus 2% O_2 + 6% CO_2, balance N_2, were determined on fresh-cut iceberg lettuce over 10 days at 7 °C.
2. The effects of 0.25% O_2 + 12% CO_2, balance N_2; 2% O_2 + 6% CO_2, balance N_2; or air were determined on fresh-cut iceberg lettuce over 17 days at temperatures designed to mimic mild abuse

commercial temperatures. These were two days at 4 °C (storage by processor), two days at 8 °C (distribution), and thirteen days at 7 °C (retail display/domestic refrigerator).

3. The effects of 0.25% O_2 + 12% CO_2, balance N_2; 2% O_2 + 6% CO_2, balance N_2; or air were determined on a model lettuce agar medium using the same temperatures and times as described above in the 2nd experiment. The objective was to investigate the effects of atmosphere on growth on a sterile substrate, eliminating possible interactions with competing flora.

2.7. Statistical methods

All experiments were carried out in triplicate. Reported counts are means of three values with standard deviations. Means were compared by Kruskall–Wallis test followed by Mann–Whitney test at $p < 0.05$ (SPSS Statistics 21).

3. Results

3.1. Effects of O_2-depleted atmospheres at 7 °C

The effects of very O_2-depleted atmospheres (0.25% O_2) on growth of *L. monocytogenes* on fresh-cut iceberg lettuce were compared with those of a less O_2-depleted atmosphere of 2% O_2 at 7 °C. The pathogen grew from 10^4 cells per gram (Day 0) to between 10^5 and 10^6 cfu by Day 7 and to over 10^6 cfu by Day 10. There were no significant effects of oxygen level on counts up to Day 7 (Fig. 1). However, counts were 0.5 log cycles higher under 0.25% O_2 on Day 10.

3.2. Effects of O_2-depleted atmospheres at mild abuse commercial temperatures

Based on the data in Fig. 1, the effects of very O_2-depleted atmospheres on growth of *L. monocytogenes* were determined under storage temperatures considered close to mild abuse commercial temperatures. The experiment produced similar results, though growth was slower initially due to the lower initial temperatures used. The *Listeria* strains survived in air at these temperatures, but there was little change in the counts observed, remaining close to 10^4 cfu per gram. Growth in 2% O_2 was more rapid than in air, reaching between 10^5 and 10^6 cfu by Day 10 and over 10^7 and 10^6 cfu on Days 14 and 17, respectively. Interestingly, growth was faster in the more O_2-depleted atmosphere of 0.25%. There were no significant differences between 0.25% O_2 and 2% O_2 to Day 7, but significantly higher counts under 0.25% O_2 from Day 10 onwards, increasing to approximately 0.7 log cycles higher on Days 14 and 17.

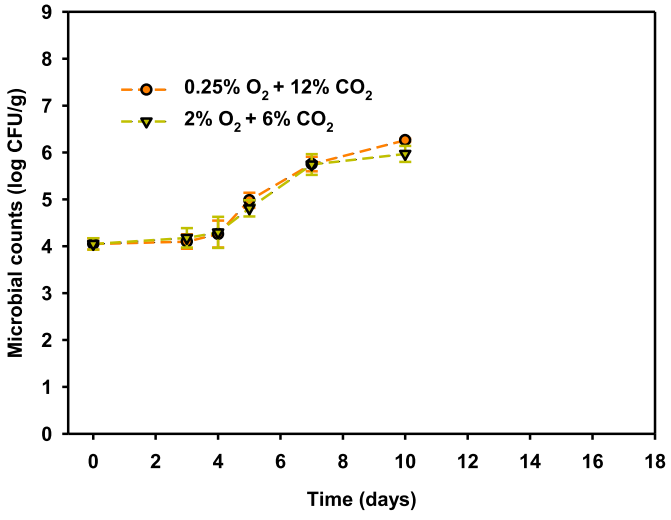

Fig. 1. Effects of atmosphere on survival and growth of *Listeria monocytogenes* (mean log cfu/g) on fresh-cut Iceberg lettuce stored at 7 °C for 10 days.

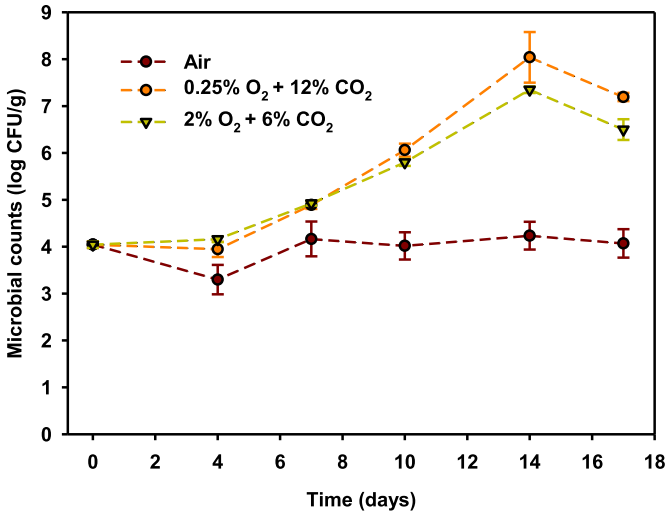

Fig. 2. Effects of atmosphere on survival and growth of *Listeria monocytogenes* (mean log cfu/g) on fresh-cut Iceberg lettuce stored for 17 days at commercial temperatures of 4 °C for two days (processor), 8 °C for two days (distribution) and 7 °C for thirteen days (retail and domestic refrigeration).

3.3. Effects of O_2-depleted atmospheres in lettuce agar medium

Inoculation levels were between 10^2 and 10^3 cells per plate at Day 0 (Fig. 3). Counts fell rapidly in air under this culture regime to below the detection limit by Day 4. By contrast, *Listeria* grew well under both of the low oxygen atmospheres, reaching around 10^4 cfu by Day 4, 10^7 by Day 10 and close to 10^8 by Day 14. Up to this point there were no significant differences between the two low oxygen atmospheres. By Day 17, however, counts under the very O_2-depleted atmosphere of 0.25% were 0.38 log cycles higher than under 2% O_2.

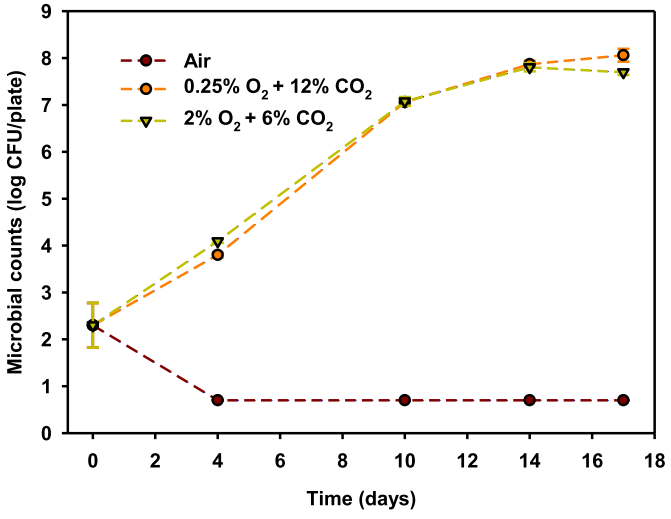

Fig. 3. Effects of atmosphere on survival and growth of *Listeria monocytogenes* (mean log cfu/plate) on Iceberg lettuce agar medium stored for 17 days at commercial temperatures of 4 °C for two days (processor), 8 °C for two days (distribution) and 7 °C for thirteen days (retail and domestic refrigeration).

4. Discussion

The very O_2-depleted atmospheres (0.25%) used to control enzymatic browning enhanced *Listeria* growth in fresh-cut Iceberg lettuce stored at mild abuse temperatures that can occur commercially. The enhanced growth was in addition to the already enhancing effects of less depleted low O_2 atmospheres compared to storage in air. These results are consistent with previous reports that mild temperature abuse permits more rapid growth of psychotropic pathogens (Berrang et al., 1989; Carlin and Peck, 1996; Farber et al., 1998; Francis and O'Beirne, 1998a; Conway et al., 2000; Rodriguez et al., 2000). The differences in CO_2 levels are unlikely to have made much contribution as significant inhibitory effects of CO_2 on bacteria generally require levels >20% (Wolfe, 1980) and *L. monocytogenes* itself is little affected by CO_2 in this range (Francis and O'Beirne, 1998a).

Enhancement of the growth of *L. monocytogenes* by O_2-depleted atmospheres appears to be due, in part, to inhibition by these atmospheres of some natural competitors within the indigenous lettuce leaf flora (Francis and O'Beirne, 1998 a,b). However, direct effects of the atmospheres on *L. monocytogenes* itself under these conditions (as seen in Fig. 3 after extended storage), and direct effects on the levels of natural anti-listerial chemicals in lettuce may also be involved. Delaquis et al. (2006) have demonstrated that restricting some of the chemical changes taking place during enzymatic browning, as would occur in O_2-depleted atmospheres, may facilitate better *L. monocytogenes* growth. Greater understanding is needed of the effects of low O_2 atmospheres (0.25%) on specific competitors, and on natural anti-listerial compounds in lettuce.

The implications of these data are that very O_2-depleted atmospheres introduce a potential hazard, where products may experience mild abuse temperatures, and extended storage lives. As can be seen in Fig. 2, these effects were only present from Day 10 onwards. However, the temperatures and times selected in this study are by no means unrealistic “worst case scenarios”.

Safety assurance of fresh-cut produce occurs during growing and harvesting, processing, packaging and storage. Safe atmospheres are relevant to packaging and storage decisions and controls. The issues include choice of safe intended target atmospheres, optimum package-product compatibility, storage at safe low temperatures, and avoidance of excessive storage lives. Based on the data presented, consideration needs to be given to avoiding excessively O_2-depleted atmospheres where possible. More research and technical focus is needed on development and commercial exploitation of cost-effective alternative controls of enzymatic browning. These include argon enriched low-oxygen atmospheres, high-oxygen atmospheres (O'Beirne et al., 2011) and mild thermal treatments (Loaiza-Velarde et al., 1997) among others.

Ensuring good temperature control is also essential to maintaining target atmospheres, and low storage temperatures (3/4 °C) minimise the microbial risks from the use of excessively O_2-depleted atmospheres. Temperature is controlled best during processing and short-term storage at the factory, where products remain under the manufacturer's control. Greater attention is required to ensure that low temperatures are maintained during distribution by use of suitably designed vehicles and optimal vehicle loading practices. Similarly, more effective temperature control and chill cabinet loading practices are needed at retail level.

5. Conclusions

As fresh-cut products are unpasteurised, every effort must be made to minimise potential hazards such as those introduced by the use of O_2-depleted atmospheres. These are controllable by greater attention to temperature control, alternative control measures for enzymatic browning, and greater attention to product-package compatibility. There is continuing need for vigilance, and additional anti-microbial hurdles are desirable. Of all the options for ensuring safety, maintenance of low temperature throughout this food chain, and use of modest shelf lives are likely to be the most effective.

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