

The use of gaseous ozone and gas packaging to control populations of *Salmonella infantis* and *Pseudomonas aeruginosa* on the skin of chicken portions

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Received 11 October 2003; received in revised form 28 April 2004; accepted 29 April 2004

Abstract

Chilled breasts of chicken were inoculated with *Salmonella infantis* or *Pseudomonas aeruginosa* and then given one of the following treatments: (i) exposure to gaseous ozone (>2000 ppm for up to 30 min); (ii) storage under 70% CO₂:30% N₂; and (iii) exposure to gaseous ozone (>2000 ppm for 15 min) followed by storage under 70% CO₂:30% N₂; all storage at 7 °C. Gaseous ozone reduced the counts of salmonellae by 97% and pseudomonads by 95%, but indigenous coliforms were unaffected. Under the modified atmosphere, the cell count of *S. infantis* was reduced by 72% following initial exposure and then stabilised, coliforms grew, but *Ps. aeruginosa* behaved like *S. infantis*—initial reduction (58%) followed by stability. Exposure to gaseous ozone followed by gas packaging allowed survival of *S. infantis*, *Ps. aeruginosa* and coliforms over 9 days at 7 °C, but there was no evidence of any sensory deterioration. It is proposed that the latter treatment could, in a modified form perhaps, be used to reduce the contamination of chicken carcasses with salmonellae and improve their shelf-life.

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Keywords: *Salmonella infantis*; *Pseudomonas aeruginosa*; Modified Atmosphere Packaging (MAP); Ozone; Poultry

1. Introduction

There have been numerous reports of viable salmonellae being present on the carcasses of chickens (Wilson, 2002) and, although this situation is a major concern for poultry processors and retailers alike, it has proved extremely difficult and expensive to raise chickens free of salmonellae (Corry, James, James, & Hinton, 1995). Equally important is the fact that *Pseudomonas* spp. (including the human pathogens, *Ps. aeruginosa*, and *Ps. fluorescens* that are rarely implicated in food-borne disease outbreaks) have been linked with the spoilage of fresh poultry (Allen, Russell, & Fletcher, 1997; Ellis & Goodacre, 2001; Kelley, Pancorbo, Merka, & Barnhart, 1998), and contamination with pseudomonads could be one of the causes of the shelf-life problems that plague the industry.

The application of strict hygiene regimes during the production and processing of chickens can reduce the incidence of salmonellae on carcasses (Al-Haddad & Robinson, 2003; Bolder, 1997), but the essential use of water during defeathering and to wash the carcasses after evisceration provides ideal conditions for the spread of both salmonellae and pseudomonads.

The use of final wash waters containing lactic or ethanoic acid (Yang, Li, & Slavik, 1998) or sparged with ozone to give a concentration of 6.0–8.0 mg l⁻¹ (Rice, Graham, & Sopher, 2001) does lead to a reduction in the total bacterial load on chicken carcasses, but overall the impact of either treatment tends to be limited. Surface treatment with gamma-radiation can be effective as a means of destroying the surface microflora (Katta, Rao, Sunki, & Chawan, 1991) as can antibiotic dips, but both treatments are prohibited in many countries. For carcasses that are frozen, a prolonged period at –20 °C appears to reduce the populations of Gram-negative species.

Gas packaging as applied to some vegetables and meats (Narasimha Rao & Sachindra, 2002) could

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provide one option for controlling the surface contaminants of chilled carcasses, but the effect is usually to prevent excessive growth of genera like *Pseudomonas* or *Salmonella* rather than their eradication. As food standards tend to stipulate a 'low tolerance' of *Salmonella* spp. in poultry (Gulf Standard, 1998), what is needed is a post-chilling process that will eliminate salmonellae from a chicken carcass in a manner that poses no risk to the consumer. If the same process would reduce populations of pseudomonads as well, an extension of shelf-life could be an additional benefit.

One technique that might achieve these aims is the exposure of the chilled carcasses to gaseous ozone for, according to one hypothesis, ozone is active against the glycoproteins or glycolipids of the bacterial cell surface membrane; rapid bacterial death is often attributed to changes in cell permeability followed by cell lysis (Moore, Griffith, & Peters, 2000). In addition, ozone may act on the sulfhydryl groups of certain enzymes, resulting in disruption of normal cellular activities (Komanapalli & Lau, 1996). It is possible, therefore, that molecules of ozone in air above a portion of chicken could impact directly onto contaminant bacterial cells with lethal consequences, and hence the aims of this work were to

- (i) inoculate the skin of pieces of chilled chicken breast with *Salmonella infantis* or *Pseudomonas aeruginosa*, and then expose them to high (>2000 ppm) concentrations of gaseous ozone for varying periods of time; and
- (ii) attempt to recover the test organisms immediately after exposure to ozone, and after storage in air or a modified atmosphere of CO₂ and N₂ (Jiménez et al., 1997; Sawaya, Elnawawy, Aburuwaida, Khalafawi, & Dashti, 1995) for 1, 2, 3, 5, 7 and 9 days.

2. Materials and methods

2.1. Inoculation procedure

Pure cultures of *S. infantis*—isolated from a chicken carcass—and *Ps. aeruginosa* (NCIMB 10421) were grown separately on Plate count agar (PCA) (Oxoid Code No: CM 463; Unipath Ltd., Basingstoke, Hants), and one or two colonies from a plate were suspended in saline (2 ml, 8.5 g l⁻¹) to give a density of 0.5 MacFarland Units—roughly 1.5 × 10⁸ colony-forming units (cfu) ml⁻¹—using the Densimat (bioMérieux, 1997). This suspension was then diluted in Maximum Recovery Diluent (9.0 ml, Oxoid Code No: CM 733) to give a working suspension of 1.5 × 10⁴ cfu ml⁻¹.

Pieces of chicken breast were purchased from the chill cabinet of a local supermarket and stored at 4 °C for a maximum of 16 h. Two pieces were then placed on a

sterile china plate and the skin-sides marked with duplicate squares using a standard wire template (10 cm²). Each square was inoculated with an aliquot (0.1 ml) of the suspension of *S. infantis* to give counts of approximately 1500 cfu per 10 cm²; similar pieces were inoculated with *Ps. aeruginosa*. Each plate was then covered with aluminium foil prior to treatment of the chicken pieces with ozone. Duplicate plates of PCA were inoculated at the same time in order to confirm the count in the inoculum.

No attempt was made to establish the natural microfloras on the skins of the chicken pieces, because different batches of chicken breast had to be purchased for each trial. Obviously this adventitious flora could have affected the survival of the test organisms but, as the species composition could well have varied from batch to batch, it was decided that it was a variable that could not be controlled.

3. System of ozonation

Ozonation of the chicken pieces took place in a Perspex box (30 × 30 cm² × 45 cm in height) with an open base and, 15 cm from the base, a sliding shelf separated the lower section of the box. Ozone was fed via a central entry port into the upper chamber from a generator (Model AQ2, Ozone Industries Ltd., Farnborough, Hant), and the concentration in the upper chamber was monitored continuously with a meter (Model 3600/313 E, Orbisphere Laboratories, Geneva, Switzerland). To avoid burning/drying the skin of the chicken, the ozonised air was passed through a flask of water before reaching the box.

In operation, pure oxygen was fed via a flow meter into the ozone generator, and the flow-rate of the oxygen was adjusted to provide the concentration of ozone required; a standard graph of oxygen flow-rate/ozone concentration provided by Ozone Industries gave an initial indication of the desired flow-rate. The whole system was installed in a fume cupboard, and the experiment was completed at room temperature (20 °C).

3.1. Experimental procedure

During the experimental work, ozone was fed into the upper chamber until the concentration reached ~3000 ppm, and then a plate containing two pieces of chicken was placed in the lower chamber. The sliding shelf was then withdrawn, and the surfaces of the chicken skin were exposed to the ozone for 1, 3, 5, 10, 20 or 30 min; two pieces of inoculated chicken exposed to air provided a control (0 min). At the end of each time interval, the sliding shelf was inserted back into place, and the plate removed as quickly as possible and exposed to the normal atmosphere. After treatment for the specified

time interval, each plate of chicken pieces was covered with foil and taken to the laboratory for examination.

Although the flow of ozone into the chamber remained constant throughout the experiment, a dilution effect caused by opening the sliding shelf and some losses of ozone, e.g. leakage from the base of the chamber of ozone and absorption by the flesh of the chicken, were inevitable during the treatment. It was noted, however, that the concentration of ozone in the upper chamber adjacent to the meter never fell below 1500 ppm even with the shelf withdrawn and the chicken pieces in situ.

Each square from each piece of chicken breast that had been inoculated with either *S. infantis* or *Ps. aeruginosa* was swabbed twice using separate cotton swabs (Bibby Sterilin Ltd., Stone, UK) moistened with MRD. The tips of both swabs were then placed in MRD (9.0 ml) and vigorously shaken on a Whirlmixer (Fisher Scientific Ltd., Loughborough, UK). Aliquots (0.1 ml) were then spread onto the surfaces of duplicate plates of Xylose lysine desoxycholate (XLD) agar (Oxoid Code No: CM 496) to recover *S. infantis* (24–48 h at 37 °C) or *Pseudomonas* C–N agar (Oxoid Code No: CM 559+SR 102) for *Ps. aeruginosa* (24–48 hours at 37 °C).

XLD was selected because: (i) it allowed easy differentiation between *S. infantis* (black, irregular colonies), any *Salmonella* spp. from the chicken itself (likely to be black regular colonies) and coliforms (yellow colonies); and (ii) a preliminary trial employing PCA as the control medium (data not shown) indicated that this strain of *S. infantis* was not inhibited by XLD. A similar trial comparing the growth of *Ps. aeruginosa* (NCIMB 10421) on PCA and *Pseudomonas* C–N agar suggested that the latter medium did not restrict the number of colonies being recovered. These trials did not confirm, of course, that cells of the species would grow on their respective selective media after possible sub-lethal physiological injury from ozone, and hence some of the recorded reduction in cell counts in Tables 1 and 2 could

Table 1

Mean counts of *S. infantis* and *Ps. aeruginosa* (cfu per 10 cm² of chicken skin) following inoculation of chicken breasts and exposure to ozone for the time intervals shown; the average concentration of ozone was >2000 ppm

Time under ozone (min)	<i>S. infantis</i>	<i>Ps. aeruginosa</i>	Coliforms
Inoculum applied	1560	940	NK
0	950	575	850
1	350	550	700
3	175	250	725
5	125	200	475
10	100	125	650
20	75	100	500
30	50	50	700

NK = not known.

All the figures are means of two trials.

Table 2

Mean counts of *S. infantis* and coliform (cfu per 10 cm² of chicken skin) following inoculation of chicken breast and exposure to ozone for 15 min; the average concentration of ozone was >2000 ppm

Days in Storage	<i>S. infantis</i>	<i>Ps. aeruginosa</i>	Coliforms
Inoculum applied	1510	940	NK
0	88	13	38
1	43	0	55
2	95	13	100
3	125	13	575
5	100	0	450
7	63	13	835
9	38	13	2710

NK = not known.

All the pieces of chicken were then placed in plastic trays and gas-packed (70% CO₂:30% N₂) before storage at 7 °C. All the figures are means of two trials.

result from a combination of treatment and inhibition by the selective media.

After recording the counts of typical colonies of the organisms (Bridson, 1998), the results were calculated as overall means (cfu per 10 cm²). Although one common pseudomonad on chickens, *Ps. fluorescens*, will not grow at 37 °C, it is possible that other species from the chicken were counted as *Ps. aeruginosa*. However, as it would have been wasteful of time and resources to check every colony—in any event, all the colonies appeared similar—it was decided to record all colonies as the inoculated species.

Sensory observations of odour and colour were recorded before and after treatment with ozone. The entire trial was repeated twice.

4. Gas packaging

The preparation of the inoculum and the inoculation of the chicken breast were completed exactly as described above, except that the two inoculated pieces of chicken breast were placed on polystyrene trays (176 mm × 134 mm × 42 mm) (Cryovac Europe Sealed Air Ltd, Knowsley Business Park, Prescot, Merseyside, UK).

4.1. Experimental procedure

After inoculation, the polystyrene trays were placed individually into multi-layer gas barrier bags (Type BB4L, Cryovac Europe Sealed Air Ltd.) with the following properties: 59 µm thick; O₂ and CO₂ permeability of 30 and 150 cm³ m⁻² d⁻¹, respectively, at 23 °C and 0% RH; and moisture vapour transmission rate of 20 g m⁻² d⁻¹ at 38 °C and 90% RH. Air was removed from each bag in turn, and the bags were then filled with a mixture of CO₂:N₂ (70:30). Filling was completed using a vacuum-packaging machine (MULTIVAC Ltd.,

Swindon, UK), which also heat-sealed the bags after injection of the gas. Following packaging, the samples were kept at 7 °C to simulate poor refrigeration and, at 1, 2, 3, 5, 7 and 9 days of storage, the inoculated squares were examined for the presence of either *S. infantis* or *Ps. aeruginosa*; a 'zero time' sample was swabbed within one hour of packaging. At each time interval and prior to opening, the composition of the atmosphere in the head-space of the packs was analysed for CO₂ using a CO₂ Meter (Servomex Ltd, Crowborough, UK). A tentative assessment of colour and odour was completed ahead of the microbiological examination.

A control group (air-packed) was inoculated with either *S. infantis* or *Ps. aeruginosa* in same manner but, in this case, the chicken breasts were tray-packaged with an over-wrap of cling film (ASDA Stores Ltd., Leeds, UK). After packaging, the samples were stored at 7 °C and sampling carried out at the same intervals. The entire trial was repeated twice.

5. Use of gaseous ozone and map

The chicken breasts inoculated with *S. infantis* or *Ps. aeruginosa* were exposed to gaseous ozone for 15 minutes to reduce the level of contamination, and then stored under a modified atmosphere of 70% CO₂:30% N₂ as recommended by Sawaya et al. (1995).

The experimental procedures, except that the chicken portions were transferred from the china plates to polystyrene trays after ozonation, were identical to those described earlier. Following packaging, the samples were kept at 7 °C and examined at 0, 1, 2, 3, 5, 7 and 9 days of storage for the presence of *S. infantis* and *Ps. aeruginosa*. The entire trial was repeated twice.

6. Results and discussion

6.1. Ozone treatment

The results of the treatment with gaseous ozone are shown in Table 1, and it is clear that, for both *S. infantis* and *Ps. aeruginosa*, the recovery by swabbing from the unexposed control (0 min) was around 60%. As the recovery procedure was, as far as possible, standardised, it was assumed that any further decline in counts following exposure to ozone could, in the main, be attributed to the treatment.

The numbers of both *S. infantis* and *Ps. aeruginosa* decreased during exposure to ozone in a time-dependent manner, but ozone did not, over the maximum period of the treatment (30 min), achieve total eradication of *S. infantis* or *Ps. aeruginosa*, and appeared to have no impact at all on the indigenous coliforms. This contrast in behaviour may have arisen because the coliforms were

attached to, or had penetrated into pores in, the skin, so that the ozone was neutralised by organic matter before it could be effective; by contrast, the inoculated pseudomonads and salmonellae were more likely to be superficial contaminants. Furthermore, although ozone inactivates numerous types of bacteria, including Gram-negative and Gram-positive types and spore-formers, their sensitivity varies enormously and depends on many factors (Thanomsut et al., 2002).

This problem of accessibility to the ozone is clearly critical because, while the reduction in viable count of *Ps. aeruginosa* of 95% might help to delay spoilage at <4 °C and the decrease in *S. infantis* (97%) should reduce the risk of cross-contamination, these results may only apply to freshly deposited cells. Whether a longer exposure, say 60 min, would have reduced to counts of *Ps. aeruginosa* or *S. infantis* even further was not examined because, in a commercial situation, exposing chicken breasts to 3000 ppm of ozone for 60 min would be both expensive and, probably, impractical. On a more positive note, the ozone treatment did not cause any changes in the colour of the skin or flesh, even though the skin did appear slightly dry after 30 min.

6.2. Gas packaging

Sawaya et al. (1995) and Jiménez et al. (1997) recommended a gas mixture of 70% CO₂:30% N₂ for the storage of refrigerated chicken carcasses, and suggested that it delayed microbial growth and extended the shelf-life of the carcasses up to 21 days. Whether this span could have been achieved in this study is not clear but, at day 9, it was noted that the skin was quite dry and the overall appearance of the breasts was still acceptable. No off-odours developed during storage, and the records of CO₂ levels (data not shown) indicated that the composition of the atmospheres remained stable during storage of all the samples. The means counts for coliforms, *S. infantis* and *Ps. aeruginosa* are shown in Figs. 1 and 2.

What is noticeable immediately is the dramatic fall in viable count of *Ps. aeruginosa* after exposure of the chicken breasts to the gas mixture for around one hour at ambient temperature, for the population had fallen from 950 to 300 cfu per 10 cm². A comparison with the mean figure for 'zero' time in air (980 cfu per 10 cm²) suggests that it is the high concentration of CO₂ that has been inhibitory, and that, perhaps, carbonic acid formed on the surface of the chicken has been diffusing into the bacterial cells and acidifying the cytoplasm, so that around 70% of the cells had died/could be recovered. In the remaining cells, a stress-induced homeostasis mechanism may well have begun to operate in time to prevent death and, once operational, the mechanism was able to protect the cells during the further exposure for 9 days (Dixon & Kell, 1989). Although the cell counts remained

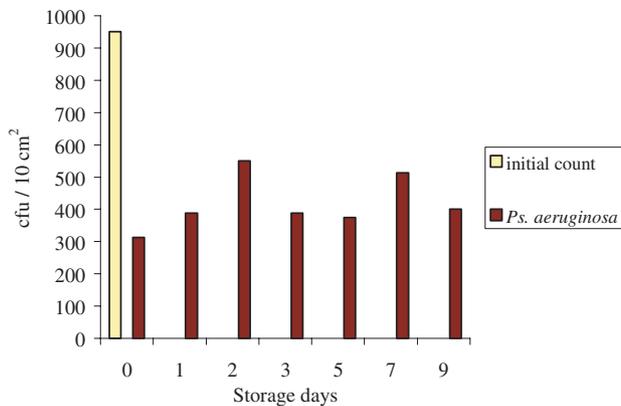


Fig. 1. Mean counts of *Ps. aeruginosa* (cfu per 10 cm² of chicken skin) following inoculation of the chicken breast and storage at 7 °C under a gas mixture of 70% CO₂ and 30% N₂; all the figures are the means of two trials.

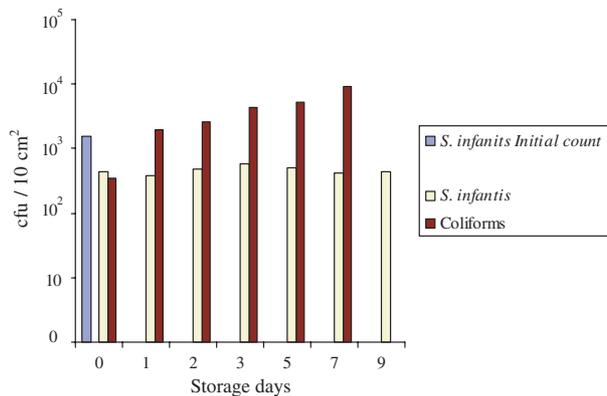


Fig. 2. Mean counts of *S. infantis* and coliforms (cfu per 10 cm² of chicken skin) following inoculation of the chicken breast and storage at 7 °C under a gas mixture of 70% CO₂ and 30% N₂; all the figures are the means of two trials.

broadly stable for the duration of storage, it was noted that colour and odour did as well.

This same stability is evident in Fig. 2, for the combination of low temperature and high CO₂ has produced an inhibition of growth of *S. infantis* that lasted for 9 days, and gave a food that appeared organoleptically acceptable. Nevertheless, coliforms grew to give counts that are over the acceptable limit for foods (1000 cfu g⁻¹) (Canadian Food Inspection Agency, 1999), and this conflict between favourable appearance and the presence of human pathogens is not unusual with gas packaged foods (Farber, 1991; Narasimha Rao & Sachindra, 2002; Phillips, 1996).

However, the results, as well as those from the available literature, confirm that the use of modified atmosphere packaging (MAP) using 70% CO₂:30% N₂ can extend the shelf-life of chilled chicken breasts. Thus, in the air-packed (control) samples, the increases in bacterial counts over 48 h at 7 °C were: coliforms from 125 to 2.56 × 10⁴ cfu per 10 cm², *S. infantis* from 940 to

2200 cfu per 10 cm² and *Ps. aeruginosa* from 980 to 2200 cfu per 10 cm². These changes, together with possible increases in other non-identified populations, led to a short shelf-life; the off-odour was so strong by day 2 that further microbial counts were abandoned.

However, some survival of organisms like *S. infantis* and *Ps. aeruginosa* could not be avoided under MAP, so that the study was extended to examine a combination treatment involving a prior decontamination procedure with ozone followed by MAP.

6.3. Ozonation and gas packaging

The results for the microbial counts for this latter trial are shown in Table 2 and, given the size of the initial inocula, it is clear that exposure to gaseous ozone has produced a dramatic decline in numbers—the ‘zero’ time sample was examined immediately after exposure to ozone for 15 min. The figures for *S. infantis* compare well with those in Table 1, but the rapid decline of *Ps. aeruginosa* could not be explained. What was equally unexpected was that gas packaging would have little further impact, because it was anticipated that some of the cells remaining at time ‘zero’ would be sub-lethally damaged by the ozone and die over time under the adverse levels of CO₂. However, the data in Table 2 suggest that the counts of both *Ps. aeruginosa* and *S. infantis* have varied very little, and it may be that any stress induced by the ozone triggered a homeostasis mechanism that nullified the effect of CO₂ (Farber, 1991; Phillips, 1996).

What was equally noticeable was the absence of any off-odours even at 9 days, and it may be that, in this instance, the ozone treatment had an impact on coliforms and coliform behaviour as well. Thus, the mean count recorded at time ‘zero’ was 38 cfu per 10 cm² of skin compared with 125 cfu per 10 cm² of skin in the air-packaged control sample mentioned earlier and, while the latter rose to 2560 cfu per 10 cm² at 2 days, the coliforms on the ozone-treated/MAP sample took 9 days to achieve a similar count. In other words, exposure to gaseous ozone and gas packaging could produce a dramatic benefit in terms of shelf-life of fresh chicken pieces, especially as the sensory aspects remained acceptable throughout the storage period.

7. Conclusion

The data presented in this paper suggest that, while ozone is bactericidal against *Ps. aeruginosa* and *S. infantis*, the eradication of the organisms from a natural substrate like chicken skin presents problems. Thus, not only does the organic matter absorb ozone and reduce the active concentration, but the bacterial cells appear to become embedded in folds or pores in the skin and

protected from direct contact with the gas. Whether or not a prolonged exposure of chicken portions to a much lower concentration of ozone would be more effective remains an open question, but any system that might reduce the incidence of *Salmonella* spp. on poultry deserves serious consideration.

Acknowledgements

The authors are extremely grateful to Ozone Industries Ltd, Farnborough, Hampshire (UK) for the loan of the ozone generator and meter, for without their generosity the project would not have been possible. The support of the Ministry of Higher Education, Abu Dhabi (UAE) is also acknowledged with gratitude.

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