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ORIGINAL ARTICLE

# Inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* in apple juice at different pH levels by gaseous ozone treatment

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**Keywords**
apple juice, foodborne pathogen, inactivation, ozone, pH.

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## Abstract

**Aims:** We investigated the effect of ozone treatment of apple juice at different pH levels for inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes*.

**Methods and Results:** Apple juice (pH 3·0, 4·0 and 5·0) inoculated with the three pathogens were treated with gaseous ozone (3·0 l $min^{-1}$ flow rate and 2·0–3·0 g $m^{-3}$) for up to 4 min. Ozone treatment (4 min) of pH 3·0 apple juice resulted in >5·36 log CFU $ml^{-1}$ reduction of *E. coli* O157:H7. Ozone treatment of pH 4·0 and 5·0 apple juice for 4 min reduced this pathogen by 5·12 log CFU $ml^{-1}$ and 1·86 log CFU $ml^{-1}$ respectively. The combination of low pH and ozone showed a great antimicrobial effect in apple juice. *Salm.* Typhimurium and *L. monocytogenes* showed a reduction trend similar to *E. coli* O157:H7. There were no significant changes of colour values when apple juice was treated with ozone, except for *b* values. Among all ozone treated samples, the browning index was lower than that of nontreated samples and there were no significant differences in total phenolic contents.

**Conclusions:** In conclusion, ozone treatment of low pH apple juice was significantly effective in inactivation of foodborne pathogens while maintaining acceptable apple juice quality.

**Significance and Impact of the Study:** The antimicrobial effect of ozone treatment on foodborne pathogens in apple juice can be reinforced by lowering the pH of apple juice.

## Introduction

Enterohaemorrhagic *Escherichia coli* O157:H7 causes haemorrhagic colitis, and can lead to haemolytic uraemic syndrome which produces symptoms such as haemolytic anaemia, thrombocytopenia and renal injury (Rangel *et al.* 2005; Reiss *et al.* 2006). *Salmonella enterica* serovar Typhimurium causes nontyphoidal salmonellosis and typically presents itself as a self-limiting gastroenteritis (Boyle *et al.* 2007). *Listeria monocytogenes* is generally known as a pathogen which causes bacteremia, meningoencephalitis. But, unfortunately, gastroenteritis due to *L. monocytogenes* has been reported (Ooi and Lorber 2005). Scallan *et al.* (2011) estimated the annual number of foodborne illnesses caused by 31 pathogens in the United States. Of these, there are over 3·6 million foodborne illnesses caused by pathogenic bacteria annually. Over 60 thousand of these cases are caused by Shiga toxin-producing *E. coli* (STEC) O157. About a million other cases are caused by *Salmonella* spp. and over one thousand cases are caused by *L. monocytogenes*. In the case of *L. monocytogenes*, the total number of illnesses is much fewer than for STEC O-157 or *Salmonella* spp., but the death rate is 15·9%, which is much higher than that of STEC O-157 or *Salmonella* spp.

Apple juice is a rich source of antioxidants that may defend humans from free radical damage (Lee *et al.* 2003). Antioxidants in apple juice, such as flavonoids, can reduce coronary heart disease and cancer (Hertog *et al.* 1993). Apple juice consumption is continuously increasing because of its health benefits, as well its taste and flavour (USDA, 2013). Generally, apple juice is believed to be free from the presence of foodborne pathogens because it has low pH in the range of 3·3–4·1 (Mattick and Moyer 1983). But, unfortunately, there have been several outbreaks caused by apple juice (Sivapalasingam *et al.* 2004; Rangel *et al.* 2005). In 1996, in the USA, there was a large outbreak caused by *E. coli* O157:H7 in unpasteurized apple juice which resulted in 25 hospitalizations and one death (Cody *et al.* 1999). Between 1995 and 2005, in the USA, there were five incidents of juice-associated outbreaks caused by *Salmonella*. Due to these outbreaks, 710 people contracted illnesses (Vojdani *et al.* 2008). There have been no juice-related outbreaks associated with *L. monocytogenes*, but it was identified as a potential pathogen which threatens juice safety in the final ruling published in the Federal Register (Gabriel and Nakano 2009).

Today, pasteurization of juice by conventional heating is undesirable because juice quality deterioration occurs during thermal treatment. During high temperature-short time pasteurization (HTST), loss of flavouring agents occurred (Aguilar-Rosas *et al.* 2007). Therefore, many researchers are focusing on novel methods for nonthermal pasteurization of juices. Ozone is one of the most popular nonthermal pasteurization methods. In 2001, the USFDA approved gaseous and aqueous ozone treatment as an antimicrobial agent for food (Khadre *et al.* 2001). After this approval several studies which confirmed the microbial inactivation effect of ozone on juices were reported. Ozone showed great antimicrobial effect on orange juice, apple cider (Williams *et al.* 2004) and apple juice (Choi *et al.* 2012).

The effect of ozone is affected by pH. Patil *et al.* (2010) reported that the $t_{5d}$ of *E. coli* ATCC 25922 and NCTC 12900 (nontoxigenic strains) in pH 5·0 apple juice was much higher than in pH 3·0 juice. But there has been no study investigating the antimicrobial effect of ozone on foodborne pathogens in apple juice of different pH levels. Therefore, this study was undertaken to evaluate the antimicrobial effect of ozone on *E. coli* O157:H7, *Salm.* Typhimurium and *L. monocytogenes* in apple juice at different pH levels. To ascertain quality changes due to ozone treatment, colour values, nonenzymatic browning indices and total phenolic contents were investigated.

## Materials and methods

### Bacterial strains and inoculum preparation

Three strains of *Escherichia coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *Salmonella* Typhimurium (ATCC 19585, ATCC 43971, ATCC 700408) and *Listeria monocytogenes* (ATCC 19114, ATCC 19115, ATCC 15313) were obtained from the bacterial culture collection of Seoul National University (Seoul, Korea). Stock cultures were prepared by growing strains at 37°C in Tryptic Soy Broth (TSB; Difco, BD, Sparks, MD) for 24 h, combining 0·7 ml of the stationary phase cultures with 0·3 ml of sterile 50% glycerol, and storing at −80°C in vials. Bacteria were streaked onto Tryptic Soy Agar (TSA; Difco, BD), incubated at 37°C for 24 h and stored at 4°C before use. Each strain of *E. coli* O157:H7, *Salm.* Typhimurium, and *L. monocytogenes* was cultured in 5 ml TSB at 37°C for 24 h and centrifuged at 4000 ***g*** for 20 min at 4°C. Cell pellets were washed three times with sterile 0·2% peptone (Bacto, Sparks, MD) water and resuspended. After this, the final cell concentration was approx. 8–9 log CFU $ml^{-1}$. To inoculate apple juice, three strains of each bacterium were equally combined to construct a mixed culture cocktail.

### Sample preparation and inoculation

Nonfrozen apple juice concentrate (72 °Brix) was purchased at a local market (Chung-Book, Korea) and diluted with sterile distilled water to 18 °Brix, because apple juice usually has a soluble solids content ranging from 10 to 22 °Brix (Eisele and Drake 2005). Soluble solids content was measured with a digital refractometer (Atago Co., Ltd., Tokyo, Japan). Initial pH of 18 °Brix apple juice was 2·51. The pH of samples was adjusted to 3·0, 4·0 and 5·0 with 40 g $l^{-1}$ (1 mol $l^{-1}$) sodium hydroxide. During juice preparation, pH was measured with a pH meter (Mettler-Toledo, Greifensee, Switzerland). Then, 200 ml of each apple juice sample was dispensed into a 500 ml bottle along with 0·1 ml of antifoam B Emulsion (Sigma-Aldrich, St. Louis, MO, USA) to prevent the overflow of juice. After that, 0·1 ml of mixed culture cocktail (*E. coli* O157:H7, *Salm.* Typhimurium and *L. monocytogenes)* was inoculated into the apple juice immediately before ozone treatment. The final cell concentration was approx. 5–6 log CFU $ml^{-1}$.

### Ozone treatment

The apparatus used in this study was the same as the one used in our previous investigation (Fig. 1) (Sung *et al.*

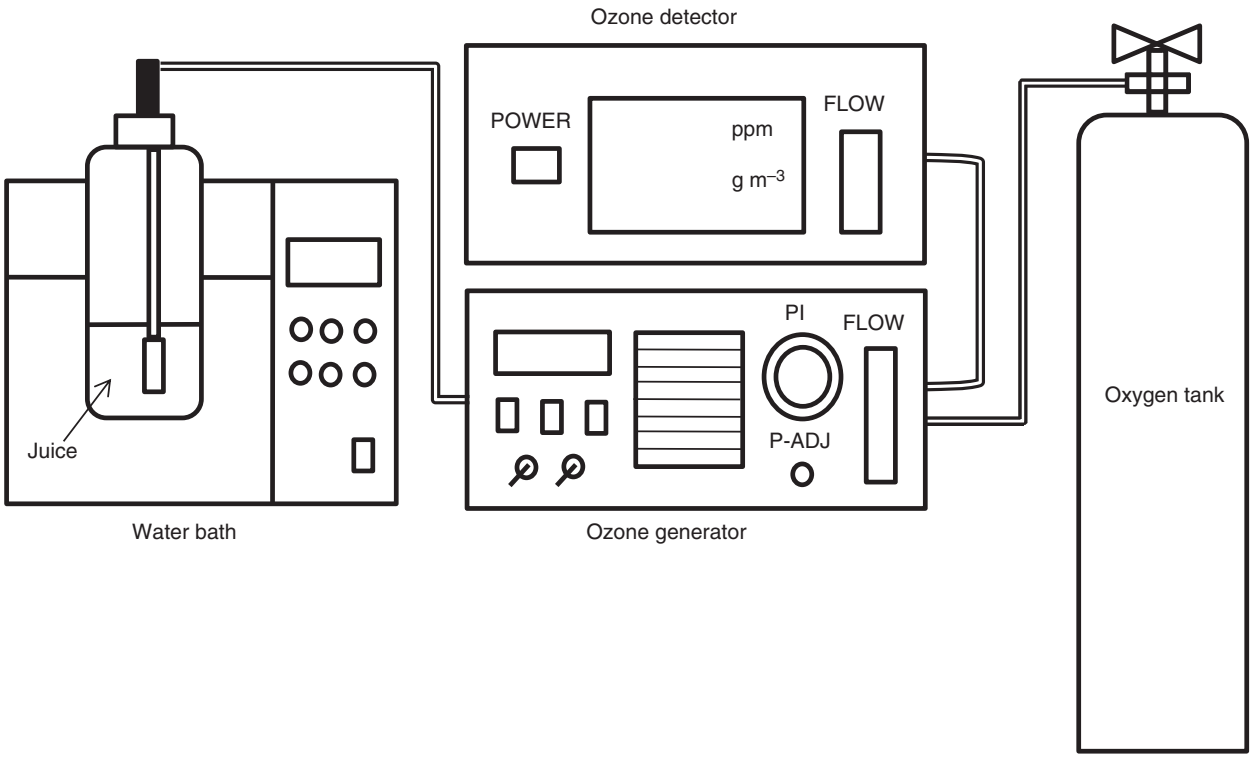


**Figure 1** Schematic diagram of the ozone treatment apparatus at Seoul National University (Seoul, Republic of Korea) (Sung *et al.* 2014).

2014; Song *et al.* 2015). Gaseous ozone was produced from pure oxygen with an ozone generator (Ozonetech Co., Ltd., Daejeon, Korea) at generation rates of 2·0–3·0 g $m^{-3}$ and a flow rate of 3·0 l $min^{-1}$. An ozone detector (Okitrotec, Tokyo, Japan) was used to continuously monitor the concentration of ozone gas. Pumped ozone was delivered through a tube and sparged directly into the sample with a porous tube. The apple juice samples were treated for 1, 2, 3 and 4 min in a shaking water bath at a mixing rate of 150 rev $min^{-1}$ to achieve uniform distribution. During ozone treatment the temperature was held at 22°C.

### Microbiological analysis

After ozone treatment, 1 ml sample aliquots were transferred into test tubes containing 9 ml of D/E neutralizing broth (Difco, Becton Dickinson, Sparks, MD). Each test tube was homogenized with a vortex mixer (VM-10, Daihan Scientific Co., Ltd., Wonju, Korea). The samples were then 10-fold serially diluted with 0·2% sterile peptone water and 0·1 ml of diluted samples was spread-plated onto selective media. Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco) and Oxford Agar Base with antimicrobial supplement (OAB; MB Cell) were used as selective media to enumerate *E. coli* O157:H7, *Salm.* Typhimurium, and *L. monocytogenes* respectively. Where low populations of surviving cells were anticipated, 0·25 ml aliquots of the original homogenate were pipetted onto four plates of each respective medium and spread-plated. All plates were incubated at 37°C for 24 h before enumeration.

### Colour measurement

Colour values of *L*, *a* and *b* were used to evaluate the colour changes of apple juice at the three pH levels after ozone treatment. Colour values were measured by using a Minolta colorimeter (model CR400; Minolta Co., Osaka, Japan). Nonozone treated apple juice was used as the control. Two millilitre of sample was placed in the bottom half of the measurement device. The measuring head of the colorimeter was located on top of the measurement device. Colour values for the control and treatments were measured after storage at 4°C for 0, 1, 3, 5 and 7 days. The parameters *L*, *a*, and *b* values are indicators of lightness, redness and yellowness respectively. All measurements were performed in triplicate.

### Nonenzymatic browning index measurement

The nonenzymatic browning index was determined by a modification of the method described by Caminiti *et al.* (2012). Five ml of sample was mixed with 5 ml of 95%

ethanol, centrifuged for 10 min at 8000 ***g*** and absorbance of the supernatant was measured by a spectrophotometer (Spectramax M2e; Molecular Devices, Sunnyvale, CA) at 420 nm. Nonenzymatic browning is a valuable quality indicator as it is an important chemical reaction causing quality and colour changes during storage of citrus products such as apple juice (Bharate and Bharate 2014). The nonenzymatic browning index for the controls and treatments was measured after storage at 4°C for 0, 1, 3, 5 and 7 days. All measurements were performed in triplicate.

### Total phenolic content measurement

Total phenolic content was determined by colorimetric assay based on the procedure of Singleton and Rossi (1965) and modified by Torlak (2014). Briefly, 1 ml of a 10 fold diluted with distilled water juice sample was mixed with 5 ml of 0·2 N Folin–Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO). After maintaining the mixture at room temperature (22°C) for 3 min, 4 ml of 7·5% sodium carbonate (Samchun Pure Chemicals Co., Ltd., Pyeongtaek, Korea) solution was added to the mixture and then shaken. After storage for 2 h at room temperature in the dark, absorbance was measured at 765 nm against a blank sample using a spectrophotometer (Spectramax M2e; Molecular Devices, Sunnyvale, CA). The results were expressed as gallic acid (Sigma-Aldrich) equivalents (mg GAE $l^{-1}$).

### Statistical analysis

All experiments were duplicate-plated and replicated three times. All data were analysed with one-way ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC) and Duncan's multiple range test to determine if there were significant differences ($P < 0{\cdot}05$) in mean values of pathogen reductions, Hunter colour values, browning indices and total phenolic contents. Microbial counts were transformed to $\log_{10}$ values for analysis.

## Results

Initial populations of *E. coli* O157:H7, *Salm.* Typhimurium and *L. monocytogenes* in inoculated apple juice were approx. $10^5$–$10^6$ CFU $ml^{-1}$ and the detection limit was 1·0 log CFU $ml^{-1}$. Ozone treatment exhibited a great effect in reducing *E. coli* O157:H7, *Salm*. Typhimurium and *L. monocytogenes* in apple juice of varying pH. Figure 2–4 represent the populations of surviving *E. coli* O157:H7, *Salm*. Typhimurium and *L. monocytogenes,* respectively, at each of three different pH levels (3·0, 4·0 and 5·0) of apple juice after treatment.

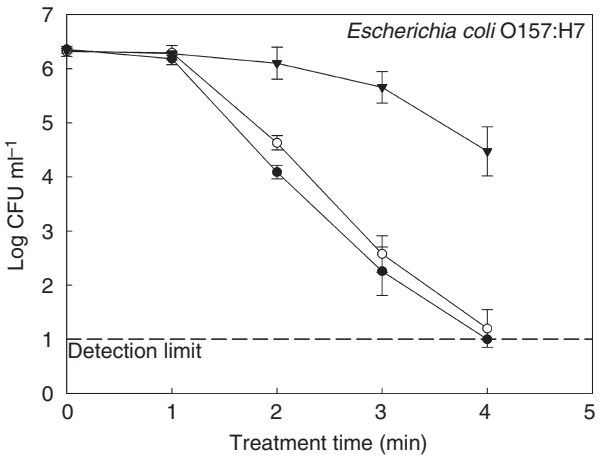


**Figure 2** Inactivation of *Escherichia coli* O157:H7 in apple juice at different pH levels treated with gaseous ozone treatment. ● pH 3·0 apple juice; ○ pH 4·0 apple juice; ▼pH 5·0 apple juice.

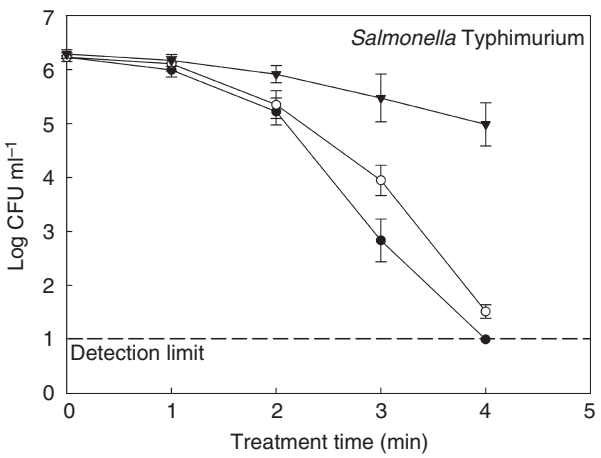


**Figure 3** Inactivation of *Salmonella* Typhimurium in apple juice at different pH levels treated with gaseous ozone treatment. ● pH 3·0 apple juice; ○ pH 4·0 apple juice; ▼ pH 5·0 apple juice.

Figure 2 shows the bactericidal effect of ozone against *E. coli* O157:H7 in apple juice of pH 3·0, 4·0, and 5·0. The levels of surviving pathogens were reduced in all treated apple juice samples as treatment time increased from 0 min to 4 min. When pH 3·0 apple juice was treated with ozone (1, 2, 3 and 4 min), populations of *E. coli* O157:H7 were decreased by 0·17, 1·97, 4·10 and >5·36 log CFU $ml^{-1}$, respectively. Ozone treatment for 1, 2, 3 and 4 min inactivated *E. coli* O157:H7 by 0·02, 1·69, 3·74 and 5·12 log CFU $ml^{-1}$ in pH 4·0 apple juice, respectively. In the case of pH 5·0 apple juice, ozone treatment for 1, 2, 3 and 4 min reduced *E. coli* O157:H7 by 0·05, 0·23, 0·68 and 1·86 log CFU $ml^{-1}$ respectively.

The reduction of *Salm.* Typhimurium in pH 3·0, 4·0 and 5·0 apple juice following ozone treatment is shown

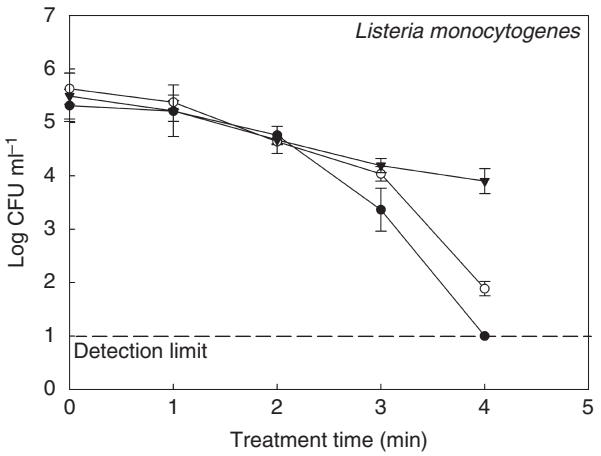


**Figure 4** Inactivation of *Listeria monocytogenes* in apple juice at different pH levels treated with gaseous ozone treatment. ● pH 3·0 apple juice; ○ pH 4·0 apple juice; ▼ pH 5·0 apple juice.

in Fig. 3. The reduction trend was similar to that of *E. coli* O157:H7. Levels of surviving populations were decreased by 0·24, 1·01, 3·42 and >5·23 log CFU ml$^{-1}$ in pH 3·0 apple juice after 1, 2, 3 and 4 min ozone treatment, respectively. In pH 4·0 apple juice treated with ozone for 1, 2, 3 and 4 min, *Salm.* Typhimurium was decreased by 0·12, 0·88, 2·28 and 4·71 log CFU ml$^{-1}$ respectively. In pH 5·0 apple juice, ozone treatment for 1, 2, 3 and 4 min inactivated 0·12, 0·37, 0·81 and 1·30 log CFU ml$^{-1}$ of this pathogen respectively.

Figure 4 shows the reduction of *L. monocytogenes* by ozone treatment. It was not different from reduction trends of *E. coli* O157:H7 and *Salm.* Typhimurium. Ozone treatment for 1, 2, 3 and 4 min reduced this pathogen by 0·10, 0·55, 1·95 and >4·17 log CFU ml$^{-1}$ in pH 3·0 apple juice respectively. In pH 4·0 apple juice treated with ozone for 1, 2, 3 and 4 min, *L. monocytogenes* was decreased by 0·25, 0·99, 1·59 and 3·75 log CFU ml$^{-1}$ respectively. In the case of pH 5·0 apple juice, ozone treatment for 1, 2, 3 and 4 min reduced *L. monocytogenes* by 0·27, 0·82, 1·30 and 1·59 log CFU ml$^{-1}$ respectively.

When we treated apple juice for 1 min there were no significant differences in reduction between any of the pathogens. In pH 3·0 apple juice, *E. coli* O157:H7 was reduced faster than the other two pathogens, but there were no significant differences between *E. coli* O157:H7 and *Salm.* Typhimurium when apple juice was treated with ozone for 3 or 4 min. *L. monocytogenes* populations decreased slower than the other two pathogens but this pathogen was reduced to under the detection limit after 4 min ozone treatment. This trend was also observed in the case of pH 4·0 apple juice. But, in pH 5·0 apple juice, there were no significant differences in the rate of reduction between any of the three pathogens, except that samples treated with ozone for 2 min showed greater reduction of *L. monocytogenes*.

The Hunter colour values of apple juice treated with ozone for 4 min and nontreated apple juice are shown in Table 1. In all ozone treated apple juice samples of pH

**Table 1** The Hunter's colour *L* (lightness), *a* (redness) and *b* (yellowness) values* of gaseous ozone treated (4 min) and nontreated apple juices at different pH levels during storage at 4°C

| Parameter | Treatment | Day 0 | Day 1 | Day 3 | Day 5 | Day 7 |
|---|---|---|---|---|---|---|
| *L* | pH 3 | 24·47 ± 0·16 A | 24·60 ± 0·23 A | 24·47 ± 0·28 A | 24·52 ± 0·07 A | 24·41 ± 0·26 A |
| | pH 3, ozone | 24·64 ± 0·33 A | 24·66 ± 0·27 A | 24·56 ± 0·15 A | 24·62 ± 0·12 A | 24·67 ± 0·12 A |
| | pH 4 | 24·49 ± 0·08 A | 24·59 ± 0·05 A | 24·47 ± 0·30 A | 24·55 ± 0·06 A | 24·52 ± 0·24 A |
| | pH 4, ozone | 24·71 ± 0·24 A | 24·81 ± 0·15 A | 24·54 ± 0·18 A | 24·76 ± 0·21 A | 24·60 ± 0·17 A |
| | pH 5 | 24·85 ± 0·24 A | 24·61 ± 0·12 A | 24·54 ± 0·07 A | 24·66 ± 0·20 A | 24·52 ± 0·28 A |
| | pH 5, ozone | 24·80 ± 0·27 A | 24·84 ± 0·08 A | 24·75 ± 0·09 A | 24·75 ± 0·10 A | 24·83 ± 0·24 A |
| *a* | pH 3 | 0·34 ± 0·02 A | 0·34 ± 0·00 A | 0·35 ± 0·01 A | 0·33 ± 0·01 A | 0·34 ± 0·03 A |
| | pH 3, ozone | 0·36 ± 0·01 A | 0·34 ± 0·01 A | 0·37 ± 0·05 A | 0·34 ± 0·04 A | 0·34 ± 0·01 A |
| | pH 4 | 0·31 ± 0·05 A | 0·35 ± 0·03 A | 0·35 ± 0·01 A | 0·34 ± 0·04 A | 0·34 ± 0·02 A |
| | pH 4, ozone | 0·32 ± 0·03 A | 0·33 ± 0·00 A | 0·35 ± 0·03 A | 0·31 ± 0·01 A | 0·33 ± 0·02 A |
| | pH 5 | 0·32 ± 0·04 A | 0·35 ± 0·04 A | 0·34 ± 0·01 A | 0·31 ± 0·04 A | 0·31 ± 0·03 A |
| | pH 5, ozone | 0·35 ± 0·04 A | 0·34 ± 0·01 A | 0·34 ± 0·05 A | 0·31 ± 0·03 A | 0·32 ± 0·02 A |
| *b* | pH 3 | 4·56 ± 0·01 A | 4·49 ± 0·07 A | 4·50 ± 0·06 A | 4·58 ± 0·11 A | 4·57 ± 0·11 A |
| | pH 3, ozone | 4·34 ± 0·08 B | 4·28 ± 0·04 B | 4·32 ± 0·11 B | 4·38 ± 0·07 B | 4·32 ± 0·05 B |
| | pH 4 | 4·52 ± 0·02 A | 4·48 ± 0·06 A | 4·45 ± 0·14 A | 4·54 ± 0·09 A | 4·52 ± 0·08 A |
| | pH 4, ozone | 4·40 ± 0·09 B | 4·35 ± 0·05 B | 4·35 ± 0·05 A | 4·38 ± 0·03 B | 4·42 ± 0·03 A |
| | pH 5 | 4·57 ± 0·05 A | 4·52 ± 0·07 A | 4·51 ± 0·05 A | 4·56 ± 0·11 A | 4·58 ± 0·07 A |
| | pH 5, ozone | 4·33 ± 0·03 B | 4·28 ± 0·09 B | 4·29 ± 0·04 B | 4·31 ± 0·02 B | 4·30 ± 0·01 B |

*Mean values ± standard deviation. Means followed by the same letter within a column per parameter and pH are not significantly different. There were no significant differences within a row ($P < 0·05$).

3·0, 4·0, and 5·0, *L* and *a* values were not significantly different ($P > 0·05$) from those of the control. But *b* values of ozone treated samples were significantly lower than those of nontreated samples. During storage, no significant changes were observed.

Browning indices of ozone treated and nontreated apple juice are shown in Table 2. Ozone treated apple juice showed significantly lower browning indices than nontreated apple juice. During storage, no significant changes of nontreated samples were observed, except in pH 5·0 apple juice. Ozone and nonozone treated pH 5·0 apple juice showed a lower browning index after storage.

Total phenolic contents of apple juice treated and nontreated with ozone for 4 min are shown in Table 3. In all ozone treated apple juice samples of different pH, total phenolic contents were not significantly different ($P > 0·05$) from those of the control.

**Table 3** Total phenolic content* of gaseous ozone treated (4 min) and nontreated apple juices at different pH levels

| | Total phenolic content (mg GAE $l^{-1}$) | |
|---|---|---|
| pH | Nontreated sample | Ozone treated sample |
| 3 | 144·1 ± 7·8 | 141·9 ± 11·9 |
| 4 | 148·6 ± 9·7 | 139·8 ± 1·7 |
| 5 | 140·7 ± 8·7 | 140·7 ± 9·5 |

*Mean values ± standard deviation. Mean values in same column and row did not differ significantly ($P < 0·05$).

## Discussion

Traditionally, apple juice has been pasteurized by conventional heating. HTST pasteurization is the most common pasteurization system used by the juice industry which uses a temperature range of 76·6–87·7°C and a holding time range of 20–30 s. But as we mentioned previously, HTST pasteurization leads to quality deterioration of apple juice (Aguilar-Rosas *et al.* 2007). Therefore, many researchers have focused on nonthermal treatments to inactivate micro-organisms in apple juice, such as pulsed light treatment (Sauer and Moraru 2009) and ultraviolet light (Caminiti *et al.* 2012).

Ozone is an allotropic form of oxygen which has great oxidation potential and can act as a powerful antimicrobial agent (Guzel-Seydim *et al.* 2004). It has an oxidation potential of 2·07 V in alkaline solution which is higher than that of chlorine (1·36 V) and also does not produce any harmful byproducts, such as trihalomethanes (THMs) which can be produced by chlorine (Glaze 1987; Sung *et al.* 2014). Excess ozone rapidly decomposes into diatomic oxygen ($O_2$) (Khadre *et al.* 2001). Ozone reduces micro-organisms by attacking various cellular elements such as lipoprotein and lipopolysaccharide layers in Gram-negative bacteria and double bonds of unsaturated lipids in the cell envelope, resulting in cell lysis (Scott and Lesher 1963; Murray *et al.* 1965).

Patil *et al.* (2010) reported on the inactivation effect of nonpathogenic *E. coli* by ozone on apple juice at different pH levels. The time required for 5 log reduction of *E. coli* increased as pH increased from 3·0 to 5·0. This supports the results of this study which show that ozone treatment of low pH apple juice shows great antimicrobial activity. When pathogens are inoculated into pH 3·0 apple juice and held for 4 min, populations of pathogens were not significantly changed (data not shown). But ozone treatment for 4 min reduced populations of the three pathogens to under the detection limit in pH 3·0 apple juice, whereas in pH 5·0 juice, pathogens were reduced by 1·30–1·86 log CFU $ml^{-1}$. Low pH and ozone treatment showed a synergistic antimicrobial effect. These results can be explained in two aspects. First, ozone reacts in two ways with organic compounds: direct reaction of the ozone molecule with organic compounds or decomposition of the ozone molecule to produce radicals which can then react with these compounds (Staehelin and Hoigné 1985). Ozone is more stable at low than at high pH (Khadre *et al.* 2001), which means direct reaction with ozone molecules accounts for the majority of pathogen inactivation in low pH apple juice. Finch *et al.* (1992) and Labatiuk *et al.* (1994) also reported that direct reaction with molecular ozone is more effective for inactivation of micro-organisms than reaction with radicals

**Table 2** Browning indices* of gaseous ozone treated (4 min) and nontreated apple juices at different pH levels during storage at 4°C

| | Day 0 | Day 1 | Day 3 | Day 5 | Day 7 |
|---|---|---|---|---|---|
| pH 3 nontreated | 0·274 ± 0·016 Aa | 0·283 ± 0·022 Aa | 0·287 ± 0·010 Aa | 0·284 ± 0·003 Aa | 0·285 ± 0·028 Aa |
| pH 3 ozone treated | 0·213 ± 0·011 Ba | 0·224 ± 0·015 Ba | 0·229 ± 0·009 Ba | 0·237 ± 0·008 Ba | 0·230 ± 0·021 Ba |
| pH 4 nontreated | 0·243 ± 0·011 Aa | 0·241 ± 0·015 Aa | 0·235 ± 0·008 Aa | 0·232 ± 0·003 Aa | 0·232 ± 0·012 Aa |
| pH 4 ozone treated | 0·206 ± 0·008 Ba | 0·195 ± 0·012 Ba | 0·192 ± 0·005 Ba | 0·196 ± 0·003 Ba | 0·192 ± 0·007 Ba |
| pH 5 nontreated | 0·258 ± 0·008 Aa | 0·226 ± 0·006 Ab | 0·236 ± 0·005 Ab | 0·230 ± 0·002 Ab | 0·229 ± 0·006 Ab |
| pH 5 ozone treated | 0·222 ± 0·003 Ba | 0·195 ± 0·008 Bb | 0·199 ± 0·004 Bb | 0·194 ± 0·005 Bb | 0·191 ± 0·002 Bb |

*Mean values ± standard deviation. Means followed by the same uppercase letters within a column per pH and by the same lowercase letters within a row are not significantly different ($P < 0·05$).

arising from decomposition of ozone molecules. Second, the combination effect of ozone with low pH. Low pH produces antimicrobial and growth inhibition effects. These effects occur due to disruption of the outer membrane of Gram-negative micro-organisms (Paul Ross *et al.* 2002). Alakomi *et al.* (2000) reported that NPN (1-*N*-phenylnaphthylamine) uptake by *E. coli* and *Salmonella enterica* serovar Typhimurium at low pH (pH 3·6 and 4·0 adjusted with lactic or hydrochloric acid) was much larger than that at neutral pH (pH 7·2). Uptake of NPN through the bacterial membrane is an indicator of damage in the outer membrane of Gram-negative bacteria. Low pH disrupts the outer membrane of gram negative bacteria resulting in its permeabilization which allows passage of ozone through the cell membrane and enhances the antimicrobial effect of ozone. In the case of Gram-positive bacteria, weak acids produce a potential antimicrobial effect through permeabilization of the cell membrane and diffusion of undissociated forms of weak acids through the cell membrane. Weak acids then dissociate and release protons which result in lowering the cytoplasmic pH. By contrast, cytoplasm of cells with intact membranes has higher pH than that of general growth media (Cotter and Hill 2003). Malic acid, a weak organic acid, is the predominant acid found in apple juice. When we treated Gram-positive bacteria in apple juice with ozone, the cell membrane was permeabilized due to ozone damage which enhanced entry of malic acid into the cytoplasm resulting in an enhanced antimicrobial effect.

During oxidative treatment involving reactive oxygen species including ozone, injury of foodborne pathogens can occur (Stephens *et al.* 2000; Thanomsub *et al.* 2002). Injured cells cannot form colonies on selective agar because selective agents such as antibiotics, dyes, or bile salts interfere with growth of injured micro-organisms (Prentice and Clegg, 1974). For these reasons, the inactivation effect of gaseous ozone treatment on *E. coli* O157:H7, *Salm.* Typhimurium and *L. monocytogenes* could be overestimated. But there are some studies which show that during storage following control interventions, injured pathogens in fruit juice were reduced continuously. Jordan *et al.* (2001) reported that high hydrostatic pressure of 500 MPa for 5 min achieved *E. coli* O157 C9490 reductions of about 1–2 log in orange juice. But following storage of pressure-treated orange juice at 4°C for 24 h and at 25°C for 3 h, *E. coli* O157 C9490 was reduced by 4·4 and >7 log CFU $ml^{-1}$, respectively. *L. monocytogenes* showed a similar trend to that of *E. coli* O157. Also, García *et al.* (2005) reported that during storage at 4°C after pulsed electric field treatment, *E. coli* O157:H7 in apple juice reduced consistently. They stated that pulsed electric field treatment of *E. coli* O157:H7 in apple juice produced injured cells which became more sensitive to subsequent storage under refrigeration. Ozone can also injure pathogens, but injured cells are not able to adapt to the storage environment of apple juice (low pH and low temperature).

Ozone can be used as a decolorizing agent because it has a great oxidation potential. Ozone breaks double bonds between carbon (-C=C-), nitrogen (-N=N-) and heterocyclic, aromatic rings in foods (Strickland and Perkins 1995). Torres *et al.* (2011) reported that ozone treatment (4·8% w/w) for 10 min in apple juice resulted in increased *L* and *b* values and decreased *a* value. In our study, there were no significant changes of *L* and *a* values. But the *b* value of apple juice was decreased after ozone treatment for 4 min. There are studies which confirmed that ozone treatment (3–4 min) decreased the *b* value of strawberry juice, orange juice and grape juice (Tiwari *et al.* 2008, 2009a,b). However, in this study, changes in *b* value were very small and those could not be detected visually. During 7 days of storage at 4°C, no significant changes were observed, which means that colour of apple juice was affected by ozone during treatment but not affected after treatment.

Browning is one of the main quality deteriorations of citrus juices, and can occur during thermal treatment and storage. Toribio and Lozano (1986) reported that conventional heating increased browning of apple juice of varying solids content. Leizerson and Shimoni (2005) reported that ohmic heating induced nonenzymatic browning of orange juice during storage at 4°C. In the case of apple juice, nonenzymatic browning by the Maillard reaction is the main cause of browning (Toribio and Lozano 1984). The Maillard reaction takes place between reducing sugars and amino acids and ultimately yields melanoidin which is manifested by brown colour (Baltes 1982; Toribio and Lozano 1984). In this study, we can confirm that ozone reduced the browning index in apple juice and did not stimulate browning during storage. During storage, there was no significant difference observed except in pH 5·0 apple juice which showed a reduced browning index. Reducing browning indices were also observed during other nonthermal treatments of apple juice. Caminiti *et al.* (2012) reported that during UV treatment browning indices were reduced, and a reduced browning index during gamma irradiation treatment was also reported (Fan and Thayer 2002).

Total phenolic content is one of the main factors responsible for health benefit of consuming apple juice (Kahle *et al.* 2005). But several researchers reported that during ozone treatment of apple juice total phenolic content was reduced. Torlak (2014) reported that ozone treatment (2·8 and 5·3 mg $l^{-1}$) of apple juice at 4 and

22°C for 40 min produced significant reductions of total phenolic content. Torres *et al.* (2011) reported that total phenolic content of fresh apple juice was 63·8 mg GAE $ml^{-1}$ but that of ozone treated (4·8% for 10 min) apple juice was 32·1 mg GAE $ml^{-1}$. In our study, there were no significant differences of total phenolic contents between ozone treated and nontreated samples. Changes in total phenolic content of apple juice during ozone treatment can be affected by various control parameters such as ozone concentration and treatment time. It is noteworthy that in this study, treatment time was shorter and ozone concentration was lower than that of the cited investigations.

Our research showed that gaseous ozone can be used as a pasteurization intervention for apple juice at different pH levels. Ozone treatment for 4 min resulted in 1·30–1·86 log reductions of three pathogens in pH 5·0 apple juice and reduced levels of three pathogens to under the detection limit in pH 3·0 apple juice. During ozone treatment, *b* values were decreased but could not be detected with the unaided eye. Ozone treatment reduced browning of apple juice and did not result in browning during storage at 4°C. Also, ozone treatment of apple juice of different pH levels did not affect the total phenolic content. In this study, we found there were large significant differences in surviving pathogen populations between pH 3·0 and 5·0 apple juice, but no large differences between pH 3·0 and 4·0 apple juice. Therefore, further investigations are needed to confirm the exact pH which shows the synergistic effect with ozone and also elucidates the mechanism of the combination effect of ozone and low pH.

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## Conflict of Interest

No conflict of interests declared.

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