Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Inactivation of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in apple juices with different soluble solids content by combining ozone treatment with mild heat

W.-J. Song[1,2,*], H.-J. Sung[1,2,*] and D.-H. Kang[1,2]

1 Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Center for Food and Bioconvergence, and Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, Korea
2 Institutes of Green Bio Science & Technology, Seoul National University, Pyeongchang-gun, Gangwon-do, Korea

Keywords
apple juice, foodborne pathogens, heat treatment, inactivation, ozone.

Correspondence
Dong-Hyun Kang, Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Seoul National University, Kwanak-ro 1, Kwanak-gu, Seoul 151-742, Korea.
E-mail: Kang7820@snu.ac.kr

*These two authors contributed equally to this article.

2014/0870: received 17 June 2014, revised 16 September 2014 and accepted 16 October 2014

doi:10.1111/jam.12671

Abstract

Aims: This study evaluated the combined effects of ozone and heat treatment to inactivate *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in three types of apple juice of different soluble solids content.

Methods and Results: Three types of apple juice (18, 36, 72 °Brix) inoculated with pathogens were subjected to ozone (3·0 l min^{-1} flow rate and 2·0–3·0 g m^{-3} concentration) and heat treatment (25, 45, 50 and 55°C) simultaneously for 20, 40 and 60 s. Initial populations of pathogens in inoculated apple juice were approximately 10^5–10^6 CFU ml^{-1}. Heat treatment alone (25, 45, 50 and 55°C) for 1 min reduced populations of *E. coli* O157:H7 by 0 to 4·75 log CFU ml^{-1} in three types of apple juice. The combination of ozone and heat treatment for 1 min at 25 and 45°C reduced *E. coli* O157:H7 by 0·93–3·87 log CFU ml^{-1} and below the detection limit (>1 log CFU ml^{-1}) at 50 and 55°C. A similar tendency was observed for *S.* Typhimurium. In several instances, results showed a synergistic effect of ozone and heat treatment. Colour values were not changed during ozone and heat treatment.

Conclusion: These results show that the combination of ozone and heat treatment can be used as a potential inactivation intervention for *E. coli* O157:H7 and *S.* Typhimurium in apple juice.

Significance and Impact of the Study: The combination of ozone treatment and mild heat can be used as an alternative intervention for pasteurization of varying soluble solids content apple juice in food industries.

Introduction

Humankind has enjoyed apples since ancient times. Not only because of their taste, sweetness and texture, but also for their health benefits, as shown in phrase 'an apple a day keeps the doctor away'. Because apples have antioxidant activity and a cholesterol lowering effect, apple consumption can reduce the risk of cancer, heart disease, asthma and type II diabetes. Also, they have the positive effects of increasing lung function and weight loss (Boyer and Liu 2004). These healthful benefits also operate when apple juice is consumed. Apple juice contains antioxidants, iron, vitamin C and low levels of sodium, cholesterol and fat (Patil *et al.* 2010). It is also reported that apple juice has beneficial effects on degenerative diseases and protective effects against cardiovascular diseases and cancer (Torres *et al.* 2011). Also, apple juice consumption has increased continuously for more than 30 years (USDA 2012).

However, several outbreaks have been caused by pathogens in fruit juices. In 1996, there was a large outbreak of *Escherichia coli* O157:H7 traced to unpasteurized commercial apple juice, resulting in 56 illnesses, 25 hospitalizations, 14 people developing the haemolytic uremic syndrome and one death (Cody *et al.* 1999). From 1995 to 2005, there were 21 outbreaks associated with juice products; ten of these were caused by apple juice (Vojdani *et al.* 2008). Furthermore, *Salmonella* showing

increased heat and acid resistance was isolated from unpasteurized juice (Sharma *et al.* 2005).

For storage and transportation, juice is usually condensed to a high solids content. Juice concentrates have low pH, which is an extreme challenge for pathogen survival. However, pathogens can contaminate juices during extraction and postprocessing (Enache *et al.* 2006). There was one study which confirmed the survival of pathogens in juice of high solids content (Oyarzábal *et al.* 2003). Because of juice concentrate's low pH, pathogens can adapt to an acidic environment. Acid-adapted pathogens also exhibit enhanced heat resistance and increased D-value. Thus, it is extremely hard to kill pathogens in juice concentrate by thermal treatment (Mazzotta 2001; Enache *et al.* 2006). Therefore, an improved method is needed to kill pathogens in high solids content juices.

Ozone is a triatomic allotrope of oxygen with blue colour and has great oxidation potential (Patil *et al.* 2009). Because of this oxidation potential, ozone attacks numerous components of micro-organisms. Scott and Lesher (1963) reported that ozone attacked the double bonds of lipids in the cell membrane, so cell leakage or cell lysis occurred. Also, ozone inactivates enzymes by the oxidation of sulfhydryl groups produce DNA lesions and inhibits transcriptional ability (Chang 1971; Mura and Chung 1990). In 2001, the gaseous and aqueous phases of ozone were approved by the FDA as a direct food additive for the treatment, storage and processing of foods (Khadre *et al.* 2001). This approval led many food researchers to apply ozone to fruit juices to inactivate pathogens (Williams *et al.* 2004; Patil *et al.* 2009; Choi *et al.* 2012).

The recent concept of hurdle technology for inactivating pathogens in apple juice needs to be investigated. Williams *et al.* (2004) reported that ozone treatment of apple juice with mild heat (50°C) reduced foodborne pathogens more rapidly than ozone treatment alone. Sung *et al.* (2014) reported that the combination of mild heat and ozone treatment had a great antimicrobial effect on pasteurized apple juice. But, to date, there has been no study exploring the bactericidal effect of the combination of ozone and heat treatment on juices of different °Brix levels. Therefore, this study evaluates the bactericidal effect of the combination of ozone and heat treatment on three concentrations of apple juice (18, 36 and 72 °Brix). To confirm quality changes occurring during the combination treatment, colour values were measured.

Materials and methods

Preparation of bacterial strains and inoculums

Strains of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890) and *Salmonella* Typhimurium (ATCC 19585, ATCC 43971, ATCC 700408) were obtained from the bacterial culture collection of Seoul National University (Seoul, Korea). Stock cultures were maintained by combining 0·7 ml of 24 h cultures in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and 0·3 ml of 50% glycerol and storing at −80°C. Working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37°C for 24 h, and stored at 4°C before use. Each strain of *E. coli* O157:H7 and *S.* Typhimurium was incubated in 5 ml of TSB at 37°C for 24 h, harvested by centrifugation at 4000 ***g*** at 4°C for 20 min and washed twice with sterile 0·2% peptone (Bacto, Sparks, MD) water. The final pellets, corresponding to approximately 10^8–10^9 CFU ml^{-1}, were obtained through resuspension in sterile 0·2% peptone water. The cell concentration was determined by plating aseptically on TSA and incubating at 37°C for 24 h. Mixed culture cocktails were prepared by blending equal volumes of each test strain.

Sample preparation and inoculation

Apple juice (72 °Brix) was purchased at a local supermarket (Chung-Book, Korea) and stored at 4°C before use. Thirty-six and 18 °Brix apple juices were prepared by diluting 72 °Brix apple juice with sterile distilled water. Soluble solids content was measured by a digital refractometer (Atago Co., Ltd., Tokyo, Japan). Two hundred ml of apple juice was dispensed into a 500-ml bottle and 0·2 ml of antifoam B Emulsion (Sigma-Aldrich, St. Louis, MO, USA) was added to apple juice to prevent excessive foaming. Then, 0·1 ml of mixed culture cocktail (*E. coli* O157:H7 and *S.* Typhimurium) was inoculated into apple juice when the preset temperature (25, 45, 50 and 55°C) in the shaking water bath stabilized. Apple juice was shaken vigorously by hand for 10 s for equilibration of inoculum in apple juice. The final cell concentration in apple juice was approximately 10^5–10^6 CFU ml^{-1}.

Ozone and heat treatment

The apparatus used in this study was the same as the one used in our previous study (Fig. 1) (Sung *et al.* 2014). An ozone generator (Ozonetech Co., Ltd., Daejeon, Korea) was used to produce gaseous ozone from ambient air at generation rates of 2·0–3·0 g m^{-3} and a flow rate of 3·0 l min^{-1}. The concentration of ozone was continuously monitored with an ozone monitor (Okitrotec, Tokyo, Japan). Ozone was directly injected into 500 ml bottles containing juice samples through a delivery tube and a perforated tube for sparging. When target temperatures (25, 45, 50 and 55°C) were attained in a shaking water bath, 0·1 ml of mixed culture cocktail was inoculated into apple juice and heat treatments were performed. Juice

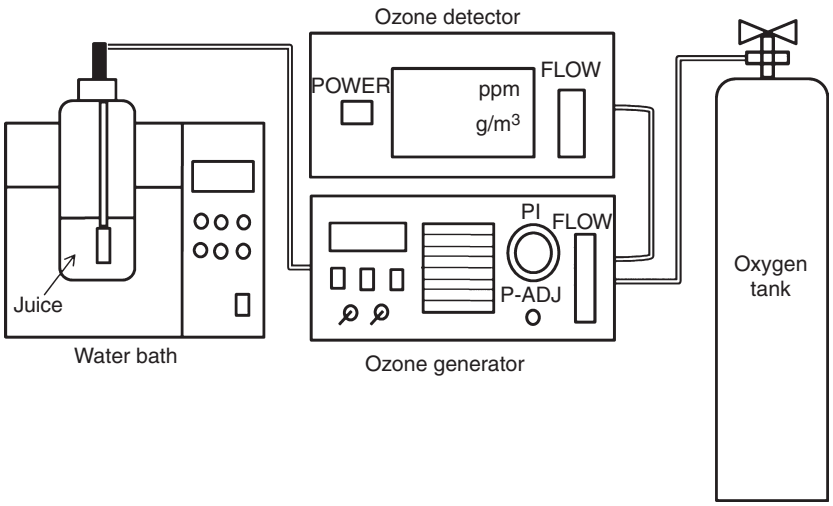

Figure 1 Schematic diagram of the ozone treatment apparatus at Seoul National University (Seoul, Republic of Korea) (Sung *et al.* 2014).

samples treated with heat alone were designated as the heat treatment group. Apple juice samples treated at 25°C without or with ozone were designated as controls for confirming the effect of heat or ozone at 25°C, respectively. Treatment times were 20, 40 or 60 s for all samples. Juice samples were treated by mixing at 150 rev min^{-1} in a shaking water bath during the entire treatment time for even distribution of inoculum and dispersal of ozone. All experiments were performed in a fume hood. An ozone decomposer was used for decomposition of excess ozone.

Bacteriological analysis

After treatment, sample aliquots (1 ml) were transferred into test tubes containing 9 ml of D/E neutralizing broth (Difco, Becton Dickinson) and homogenized using a vortex mixer (VM-10, Daihan Scientific Co., Ltd., Wonju, Korea) for bacteriological analysis of pathogens. Samples were 10-fold serially diluted with 9 ml of sterile 0·2% peptone, and 0·1 ml of samples was spread-plated onto selective media. Sorbitol MacConkey agar (SMAC; Difco) and xylose lysine desoxycholate agar (XLD; Difco) were used as selective media for enumeration of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Where low populations of surviving cells were anticipated, 1 ml aliquots of the original homogenate in D/E neutralizing broth were equally distributed onto four plates of each selective medium and spread-plated. All plates were incubated at 37°C for 24 h and colonies enumerated. The detection limit was 1·0 log CFU ml^{-1}.

Colour measurement

Colour of apple juice was measured using a Minolta colorimeter (model CR400; Minolta Co., Osaka, Japan). The *L*-, *a*- and *b*- values which indicate lightness, redness and yellowness, respectively, were measured to investigate the colour changes of apple juice after each heat and/or ozone treatment. Untreated apple juice was used as the control. Before measurement, treated juice was cooled to about 15°C by dipping the bottle in crushed ice. Two ml of sample was poured into the bottom half of the measurement equipment. The measuring head of the colorimeter was placed on top of the measurement equipment. All measurements were conducted in triplicate.

Statistical analysis

All experiments were duplicate-plated and replicated three times. All data were analysed with one-way ANOVA using Statistical Analysis System (SAS Institute, Cary, NC) and Duncan's multiple range test to investigate whether there were significant differences ($P < 0·05$) in mean values of micro-organism populations and colours. Microbial counts were transformed to $\log_{10}$ values for analysis. One log was used for calculations where populations were reduced to under the detection limit.

Results

Initial populations of *E. coli* O157:H7 and *S.* Typhimurium in inoculated apple juice were approximately 10^5–10^6 CFU ml^{-1}, and the detection limit was 1·0 log CFU ml^{-1}. The combination of ozone and heat treatment exhibited a great effect in reducing *E. coli* O157:H7 and *S.* Typhimurium in apple juice. Figure 2 through 7 represent the populations of surviving *E. coli* O157:H7 and *S.* Typhimurium in each of three different concentrations (18, 36, 72 °Brix) of apple juice after treatments, respectively.

Figures 2–4 show the bactericidal effect of the combination treatment of ozone and heat against *E. coli* O157:H7 in apple juice of 18, 36, 72 °Brix. The levels of surviving pathogens were reduced in all treated apple juice samples as treatment temperature increased from 25 to 55°C. When 18 °Brix apple juice was treated with heat alone (45, 50 and 55°C) for 1 min, populations of *E. coli* O157:H7 were decreased by 1·34, 2·36 and 4·75 log CFU ml^{-1}, respectively. The combined treatment of heat with ozone for 1 min inactivated *E. coli* O157:H7 by 1·65 and 3·87 log CFU ml^{-1} at 25 and 45°C, respectively. In the case of the combination treatment at 50 and 55°C, surviving populations were reduced to below the detection limit (1·0 log CFU ml^{-1}). In 36 °Brix apple juice, pathogen populations were reduced by 1·56, 2·58 and 3·40 log CFU ml^{-1} after heat treatment for 1 min at 45, 50 and 55°C, respectively. Populations were reduced by 0·93 and 2·49 log CFU ml^{-1}, respectively, when ozone and heat (25 and 45°C) were simultaneously applied to apple juice for 1 min. Pathogens were reduced to below the detection limit after combination treatments at 50 and 55°C for 1 min. The surviving populations of *E. coli* O157:H7 in 72 °Brix apple juice were reduced by 1·66, 4·18 and 4·38 log CFU ml^{-1} at 45, 50 and 55°C, respectively. Regarding the combination treatment for 1 min, populations were decreased by 1·13 and 2·49 log CFU ml^{-1} at 25 and 45°C, respectively, and to below the detection limit at 50 and 55°C.

The reduction of *S.* Typhimurium in 18, 36 and 72 °Brix apple juice after the combination treatment of ozone and heat is shown in Figs 5–7. The reduction trend was similar to that of *E. coli* O157:H7. Levels of surviving populations were decreased by 1·40, 3·95 and >5·05 log CFU ml^{-1} in 18 °Brix apple juice after 1 min heat treatment at 45, 50 and 55°C, respectively. In apple juice treated with ozone for 1 min, *S.* Typhimurium was decreased by 1·38 and 2·64 log CFU ml^{-1} at 25 and 45°C, respectively. After ozone treatment at 50 and 55°C, populations were reduced to below the detection limit. In 36 °Brix apple juice, heat treatment of 45, 50 and 55°C for 1 min inactivated 1·60, 3·03 and 3·35 log CFU ml^{-1} of this pathogen, respectively. The combined treatment of heat with ozone for 1 min inactivated 0·74 and 2·83 log CFU ml^{-1} of *S.* Typhimurium at 25 and 45°C, respectively. Populations were reduced to below the detection limit at 50 and 55°C. Surviving populations of *S.* Typhimurium in 72 °Brix apple juice were reduced by 2·09, 4·11 and 3·81 log CFU ml^{-1} after heat treatment at 45, 50 and 55°C for 1 min, respectively. In the case of the combination treatment for 1 min, populations were decreased by 1·17 and 2·80 log CFU ml^{-1} at 25 and 45°C and to below the detection limit at 50 and 55°C.

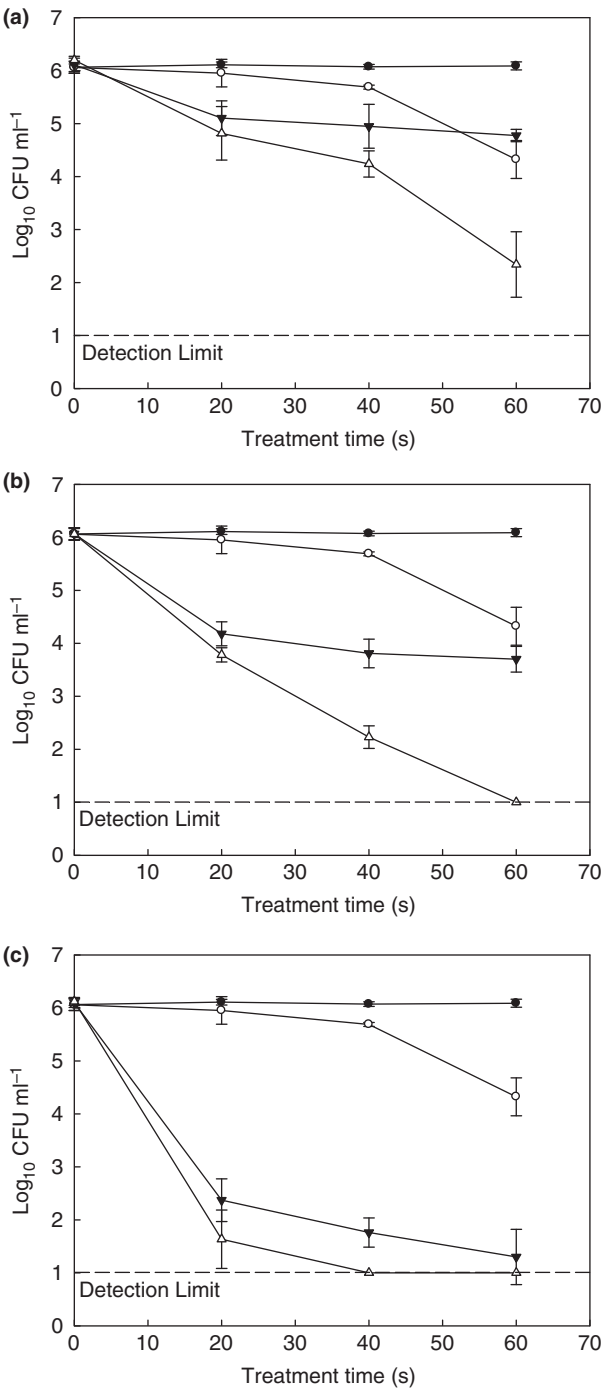

Figure 2 Inactivation of *Escherichia coli* O157:H7 in 18 °Brix apple juice treated with the combination of ozone and heat. (a) ● 25°C; ○ 25°C + ozone; ▼ 45°C; △ 45°C + ozone, (b) ● 25°C; ○ 25°C + ozone; ▼ 50°C; △ 50°C + ozone, (c) ● 25°C; ○ 25°C + ozone; ▼ 55°C; △ 55°C + ozone.

The Hunter colour values of apple juice of 18, 36 and 72 °Brix treated with heat alone or with the combination of ozone and heat are shown in

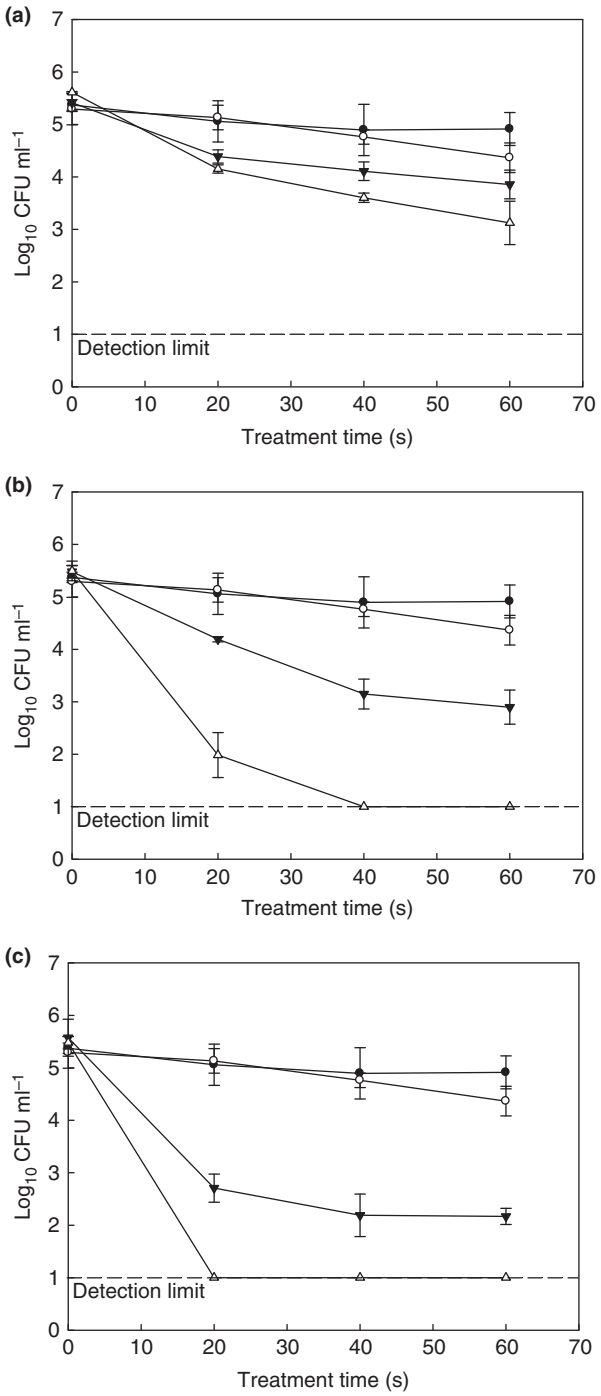

Figure 3 Inactivation of *Escherichia coli* O157:H7 in 36 °Brix apple juice treated with the combination of ozone and heat. (a) ● 25°C; ○ 25°C + ozone; ▼ 45°C; △ 45°C + ozone, (b) ● 25°C; ○ 25°C + ozone; ▼ 50°C; △ 50°C + ozone, (c) ● 25°C; ○ 25°C + ozone; ▼ 55°C; △ 55°C + ozone.

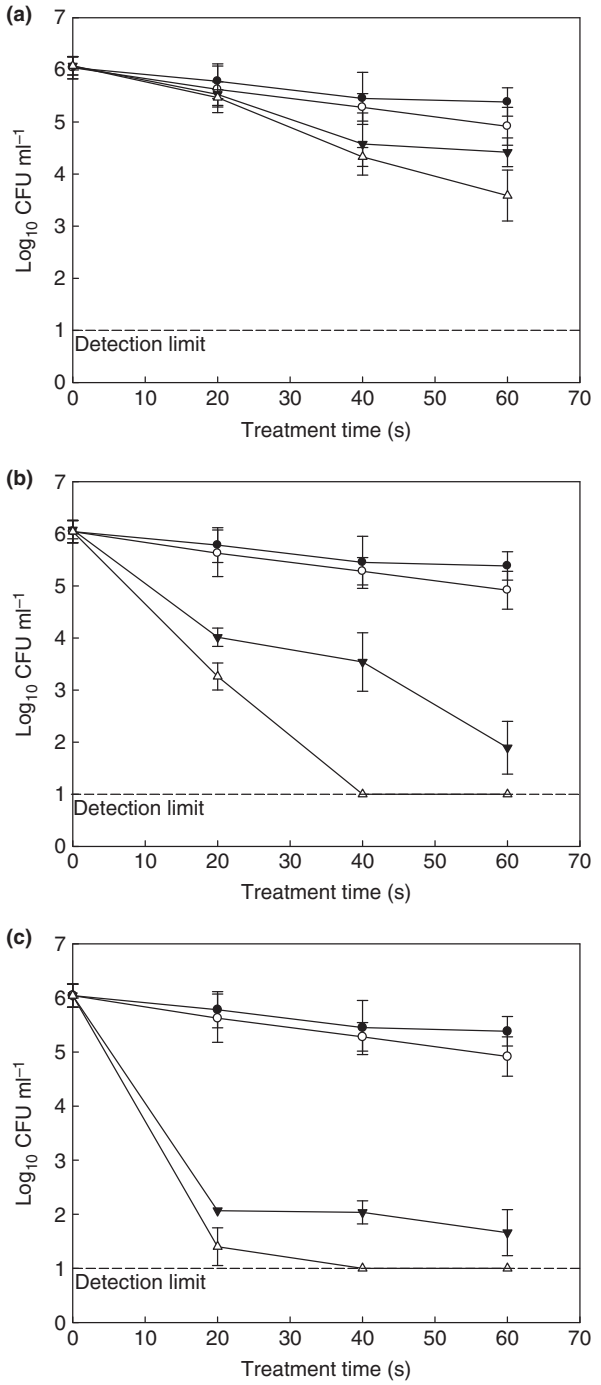

Figure 4 Inactivation of *Escherichia coli* O157:H7 in 72 °Brix apple juice treated with the combination of ozone and heat. (a) ● 25°C; ○ 25°C + ozone; ▼ 45°C; △ 45°C + ozone, (b) ● 25°C; ○ 25°C + ozone; ▼ 50°C; △ 50°C + ozone, (c) ● 25°C; ○ 25°C + ozone; ▼ 55°C; △ 55°C + ozone.

Tables 1–3. In all treated apple juice samples of 18, 36 and 72 °Brix, *L*-, *a*- and *b*- values were not significantly different ($P > 0{\cdot}05$) from those of the control. The combination treatment of both heat and ozone did not significantly affect the colour value of apple juice.

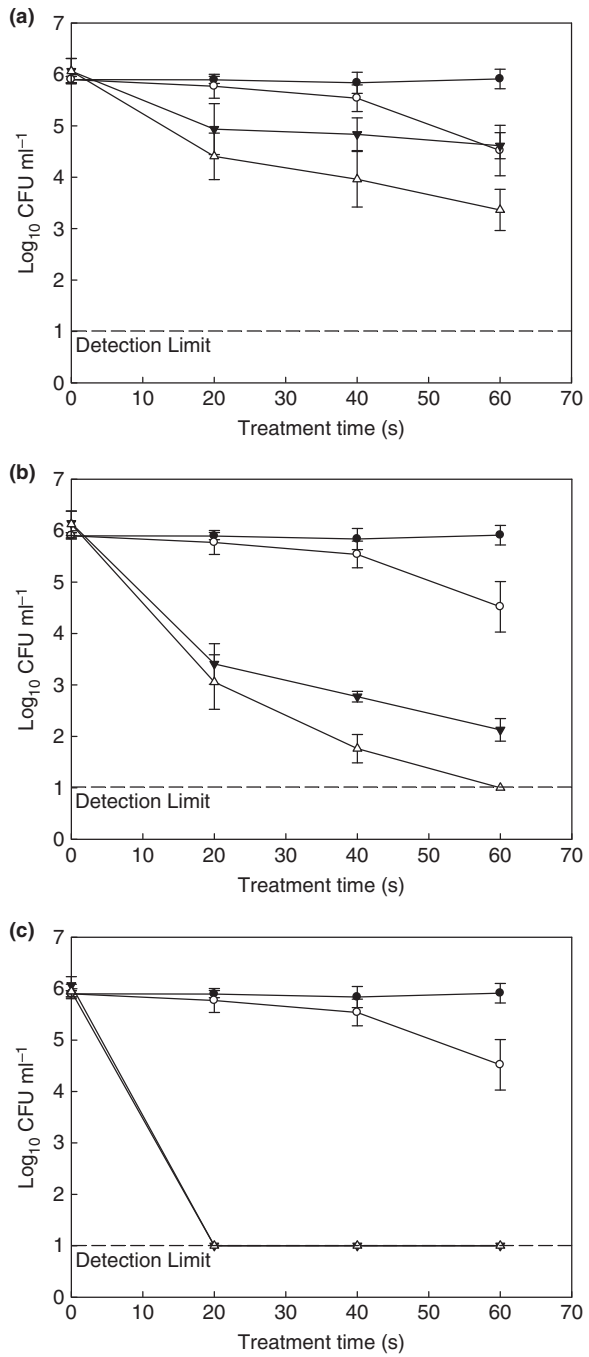

Figure 5 Inactivation of *Salmonella* Typhimurium in 18 °Brix apple juice treated with the combination of ozone and heat. (a) ● 25°C; ○ 25°C + ozone; ▼ 45°C; △ 45°C + ozone, (b) ● 25°C; ○ 25°C + ozone; ▼ 50°C; △ 50°C + ozone, (c) ● 25°C; ○ 25°C + ozone; ▼ 55°C; △ 55°C + ozone.

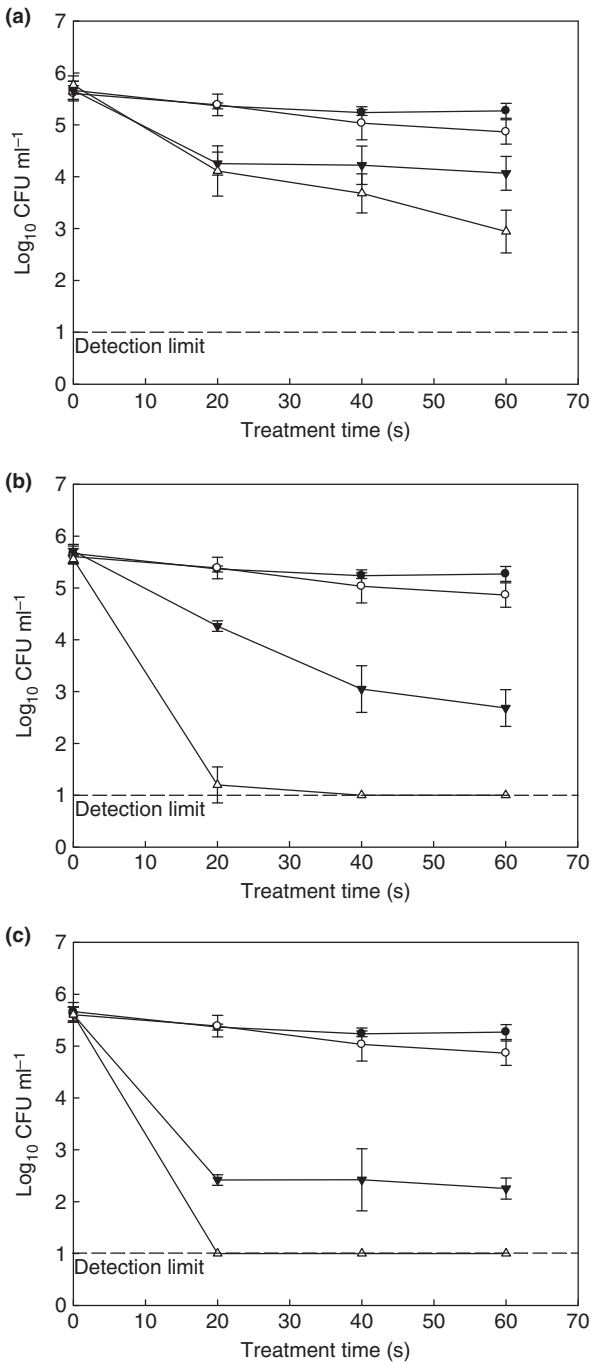

Figure 6 Inactivation of *Salmonella* Typhimurium in 36 °Brix apple juice treated with the combination of ozone and heat. (a) ● 25°C; ○ 25°C + ozone; ▼ 45°C; △ 45°C + ozone, (b) ● 25°C; ○ 25°C + ozone; ▼ 50°C; △ 50°C + ozone, (c) ● 25°C; ○ 25°C + ozone; ▼ 55°C; △ 55°C + ozone.

Discussion

Apple juice is of low pH (3·1–4·4), and thus formerly was considered to be hostile to the survival of foodborne pathogens (Mattick and Moyer 1983). But several studies have confirmed numerous outbreaks associated with the consumption of apple juice (Goverd *et al.* 1979; Steele

Table 1 Hunter's colour *L* (lightness), *a* (redness) and *b* (yellowness) values of 18 °Brix apple juice treated simultaneously with gaseous ozone and mild heat

	Parameter*		
Treatment	*L*	*a*	*b*
Control	25·02 ± 0·24	0·27 ± 0·06	3·89 ± 0·61
25°C	25·35 ± 0·36	0·30 ± 0·04	4·11 ± 0·60
25°C, ozone	25·39 ± 0·07	0·28 ± 0·06	4·10 ± 0·33
45°C	25·08 ± 0·01	0·27 ± 0·02	3·96 ± 0·43
45°C, ozone	25·24 ± 0·08	0·30 ± 0·07	3·40 ± 0·24
50°C	25·20 ± 0·32	0·38 ± 0·03	3·22 ± 0·40
50°C, ozone	25·44 ± 0·08	0·31 ± 0·04	3·46 ± 0·76
55°C	25·19 ± 0·10	0·34 ± 0·02	3·09 ± 1·12
55°C, ozone	25·05 ± 0·34	0·44 ± 0·24	3·37 ± 0·73

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

Table 2 Hunter's colour *L* (lightness), *a* (redness) and *b* (yellowness) values of 36 °Brix apple juice treated simultaneously with gaseous ozone and mild heat

	Parameter*		
Treatment	*L*	*a*	*b*
Control	25·81 ± 0·32	0·29 ± 0·09	4·36 ± 0·30
25°C	25·57 ± 0·24	0·29 ± 0·07	4·61 ± 0·15
25°C, ozone	25·53 ± 0·22	0·31 ± 0·13	4·55 ± 0·31
45°C	25·41 ± 0·21	0·24 ± 0·11	4·67 ± 0·22
45°C, ozone	25·65 ± 0·19	0·29 ± 0·12	4·43 ± 0·57
50°C	25·63 ± 0·46	0·28 ± 0·06	4·76 ± 0·09
50°C, ozone	25·26 ± 0·15	0·25 ± 0·06	4·77 ± 0·16
55°C	25·42 ± 0·11	0·29 ± 0·02	4·57 ± 0·05
55°C, ozone	25·25 ± 0·45	0·32 ± 0·05	4·24 ± 0·24

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

Table 3 Hunter's colour *L* (lightness), *a* (redness) and *b* (yellowness) values of 72 °Brix apple juice treated simultaneously with gaseous ozone and mild heat

	Parameter*		
Treatment	*L*	*a*	*b*
Control	27·52 ± 0·14	0·30 ± 0·04	4·06 ± 0·06
25°C	27·65 ± 0·41	0·27 ± 0·03	4·01 ± 0·08
25°C, ozone	27·93 ± 0·88	0·25 ± 0·05	4·20 ± 0·16
45°C	28·11 ± 0·90	0·30 ± 0·10	4·15 ± 0·19
45°C, ozone	28·24 ± 1·26	0·34 ± 0·16	3·91 ± 0·11
50°C	27·90 ± 0·72	0·34 ± 0·06	3·60 ± 0·78
50°C, ozone	27·69 ± 1·24	0·28 ± 0·16	3·90 ± 0·47
55°C	27·81 ± 0·82	0·28 ± 0·07	3·73 ± 0·32
55°C, ozone	27·38 ± 0·77	0·25 ± 0·08	3·87 ± 0·50

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

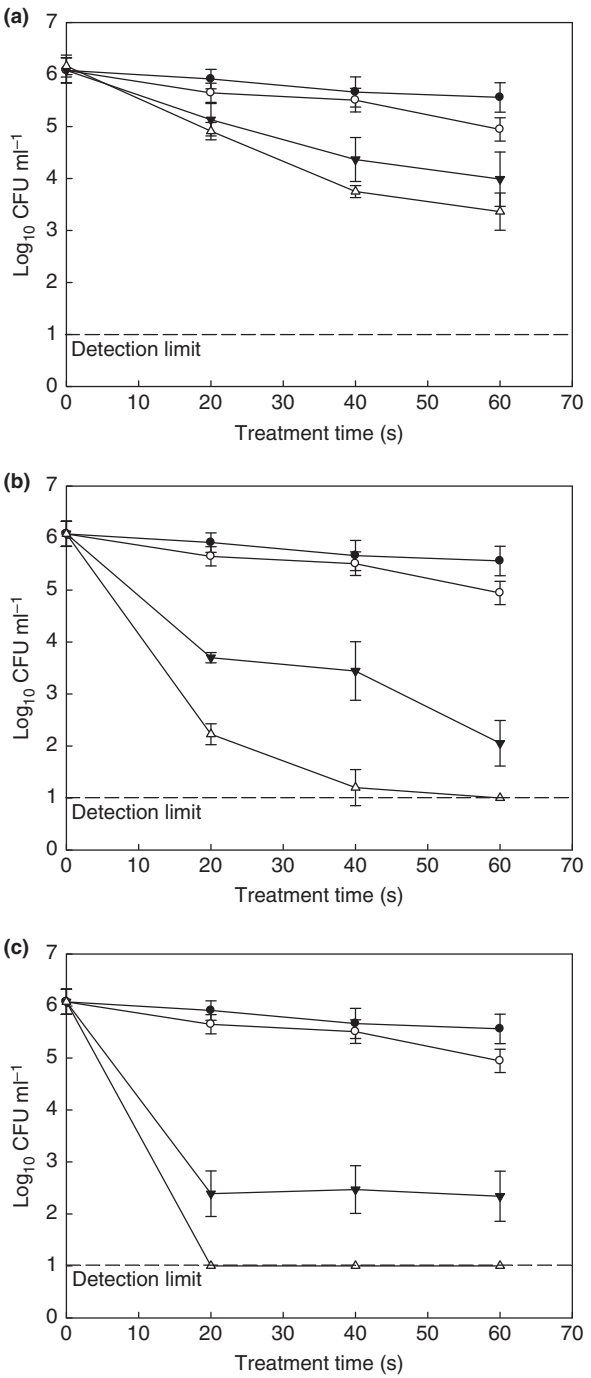

Figure 7 Inactivation of *Salmonella* Typhimurium in 72 °Brix apple juice treated with the combination of ozone and heat. (a) ● 25°C; ○ 25°C + ozone; ▼ 45°C; △ 45°C + ozone, (b) ● 25°C; ○ 25°C + ozone; ▼ 50°C; △ 50°C + ozone, (c) ● 25°C; ○ 25°C + ozone; ▼ 55°C; △ 55°C + ozone.

et al. 1982; Parish 1997; Sivapalasingam *et al.* 2004; Rangel *et al.* 2005; Vojdani *et al.* 2008). Conventional heating is the usual intervention performed for pasteurization of

apple juice. But thermal treatment leads to quality deterioration of apple juice. Natural flavouring compounds found in apple juice are destroyed by conventional heat treatment. During high-temperature short-time (HTST) treatment more than 50% of acetic acid, hexanal, ethyl acetate, ethyl butyrate, methyl butyrate and 1-hexanal are lost. Also, 36% of butyl hexanoate and 22% of hexyl acetate are lost to evaporation (Aguilar-Rosas *et al.* 2007). Moreover, during HTST treatment, turbidity and lightness of cloudy apple juice are increased (Krapfenbauer *et al.* 2006).

Because conventional heating has many disadvantages, numerous other nonthermal treatments have been evaluated for inactivation of pathogens in apple juices. Yuste and Fung (2004) used nisin and cinnamon to rapidly reduce *S.* Typhimurium and *E. coli* O157:H7 in apple juice. Buchanan *et al.* (1998) used gamma irradiation for inactivating *E. coli* O157:H7 in apple juice and reported that the D-value of apple juice is in the range of 0·26–0·35 kGy. Also, dense phase carbon dioxide (Liao *et al.* 2007), high hydrostatic pressure (Teo *et al.* 2001), ultraviolet (Gachovska *et al.* 2008; Keyser *et al.* 2008) and pulsed electric fields (Evrendilek *et al.* 1999; Gachovska *et al.* 2008) were also tested to control pathogens in apple juice.

Ozone is one of the most popular nonthermal treatments to reduce pathogens in foods. There are studies which confirm the antimicrobial effect and influence of ozone on quality in juice. Patil *et al.* (2009) reported the bactericidal effect of ozone treatment on model orange juice. There was a 6 log CFU ml^{-1} reduction of *E. coli* during 60 s ozone treatment with 0·12 l min^{-1} flow rate and 0·075–0·078 mg ml^{-1} of ozone concentration. Steenstrup and Floros (2004) reported the effect of ozone (860 ppm) and low temperature on inactivation of *E. coli* O157:H7 in apple cider. D-values of *E. coli* O157:H7 ranged from 0·6 to 1·5 min at 20 and 5°C. Anthocyanin and ascorbic acid degradation of strawberry juice by ozone treatment was also reported (Tiwari *et al.* 2009c).

In this study, it was tested the effect of ozone treatment with a mild heat for inactivation of pathogens in apple juice. The combination of ozone and heat treatment showed great efficacy for reducing pathogens in apple juice with different soluble solids content. Williams *et al.* (2004) reported inactivation of *E. coli* O157:H7 and *Salmonella* in orange juice by applying ozone at various treatment temperatures. Mild heat treatment combined with ozone showed a much better bactericidal effect than ozone treatment alone. To reduce pathogen levels to below the detection limit, more than 240 min was required for *E. coli* O157:H7 and *Salmonella* at 4°C but at 50°C, 45 min for *E. coli* O157:H7 and 15 min for *Salmonella* was necessary. This tendency was also observed in our study. When juices were treated with ozone alone, 0·74 to 1·73 log CFU ml^{-1} reductions occurred after 1 min. But ozone treatment combined with heat at 50 or 55°C reduced pathogens to under the detection limit after <1 min.

Choi *et al.* (2012) reported on the efficacy of ozone to inactivate pathogens in apple juice of different levels of soluble solids (°Brix). When 18 and 36 °Brix apple juice was treated with ozone, *E. coli* O157:H7 was reduced to under the detection limit after 10 min. *S.* Typhimurium and *Listeria monocytogenes* also showed a similar tendency. But in the case of 72 °Brix apple juice concentrate, ozone produced 0·41–1·73 log reductions of all 3 pathogens during 10 min of treatment. This indicates that ozone treatment alone is not suitable for high solids content juice pasteurization. However, in this study, using the combination of ozone and heat treatment, we were able to reduce pathogens to under the detection limit after 1 min treatment in 72 °Brix apple juice. The combination of ozone and heat treatment can be used as a pasteurization intervention for high solids content juices.

The results of this study demonstrated that ozone combined with heat shows great antimicrobial effect when applied to apple juice of different solids content. Especially in some cases, we obtained a greater effect than expected. Tables 4 and 5 show the comparison of the sum of reductions and the combination effect on *E. coli* O157:H7 and *S.* Typhimurium. The combined treatment showed a synergistic effect on *E. coli* O157:H7 at 45 and 50°C in 18 °Brix apple juice and at 50°C in 36 °Brix apple juice, respectively. In the case of *S.* Typhimurium, the combined treatment showed a synergistic effect at 50°C in 36 °Brix apple juice. The combined treatment did not show any synergistic effect on *E. coli* O157:H7 and *S.* Typhimurium in 72 °Brix apple juice.

During heat treatment alone, large pathogen reductions occurred. The reason was due to the low pH of apple juice. The pH values of apple juice were 2·35 (72 °Brix), 2·39 (36 °Brix) and 2·51 (18 °Brix). When pathogens were inoculated into 18, 36 and 72 °Brix apple juice and held for 1 min, populations of pathogens were not significantly changed (data not shown). In low pH foods, pathogens are easily reduced by heat treatment if they are not adapted to an acidic environment. Beuchat *et al.* (1986) reported that D-values of *L. monocytogenes* Scott A at 50°C was over 60 min in pH 5·6 cabbage juice and 25·0 min in pH 4·6 cabbage juice. Because of the combination of low pH and heat treatment, pathogens experienced shock and died. Eighteen °Brix apple juice underwent a larger reduction of pathogens following heat treatment than did 36 and 72 °Brix juice. This is due to water activity. Mattick *et al.* (2000), reported that *Salmonella* shows higher heat resistance in TSB at a_w 0·91 than TSB at a_w 0·95. Heat treatment alone of 36 and 72 °Brix

Table 4 Comparison of reductions of *Escherichia coli* O157:H7 in apple juice by heat or ozone alone and the combined treatment

°Brix	Treatment temperature (°C)	Heat	Ozone (at 25°C)	Sum	Combination
18	45	1·34 ± 0·12	1·65 ± 0·38	2·99 ± 0·38B	3·87 ± 0·52A
	50	2·36 ± 0·14	1·65 ± 0·38	4·02 ± 0·30B	5·07 ± 0·12A
	55	4·75 ± 0·52	1·65 ± 0·38	6·40 ± 0·22A	5·12 ± 0·08B
36	45	1·56 ± 0·69	0·93 ± 0·44	2·49 ± 0·35A	2·49 ± 0·43A
	50	2·58 ± 0·39	0·93 ± 0·44	3·51 ± 0·20B	4·49 ± 0·19A
	55	3·40 ± 0·49	0·93 ± 0·44	4·33 ± 0·34A	4·48 ± 0·12A
72	45	1·66 ± 0·39	1·13 ± 0·55	2·78 ± 0·26A	2·49 ± 0·20A
	50	4·18 ± 0·23	1·13 ± 0·55	5·31 ± 0·12A	5·04 ± 0·21A
	55	4·38 ± 0·13	1·13 ± 0·55	5·51 ± 0·17A	5·04 ± 0·21B

Mean values ± standard deviation. Means with the same upper-case letter in the same row are not significantly different ($P > 0·05$).

Table 5 Comparison of reductions of *Salmonella* Typhimurium in apple juice by heat or ozone alone and the combined treatment

°Brix	Treatment temperature (°C)	Heat	Ozone (at 25°C)	Sum	Combination
18	45	1·40 ± 0·11	1·38 ± 0·45	2·78 ± 0·34A	2·64 ± 0·25A
	50	3·95 ± 0·20	1·38 ± 0·45	5·33 ± 0·33A	5·03 ± 0·20A
	55	5·05 ± 0·15	1·38 ± 0·45	6·44 ± 0·30A	4·95 ± 0·14B
36	45	1·60 ± 0·47	0·74 ± 0·35	2·34 ± 0·23A	2·83 ± 0·59A
	50	3·03 ± 0·27	0·74 ± 0·35	3·77 ± 0·43B	4·56 ± 0·08A
	55	3·35 ± 0·29	0·74 ± 0·35	4·10 ± 0·40A	4·61 ± 0·15A
72	45	2·09 ± 0·31	1·17 ± 0·46	3·26 ± 0·29A	2·80 ± 0·46A
	50	4·11 ± 0·41	1·17 ± 0·46	5·28 ± 0·45A	5·08 ± 0·24A
	55	3·81 ± 0·41	1·17 ± 0·46	4·98 ± 0·30A	5·08 ± 0·24A

Mean values ± standard deviation. Means with the same upper-case letter in the same row are not significantly different ($P > 0·05$).

apple juices also showed large reductions. This is due to the inoculation method. When we inoculated pathogens into apple juice, we used a mixed cocktail culture suspended in sterile 0·2% peptone water. Due to the high water activity of the surrounding environment immediately after inoculation, pathogens were readily killed by heat and low pH. Actually, in the majority of cases, most pathogen reductions resulting from heat treatment happened within 20 s of treatment, which included the shaking time required for equilibration of inoculum and juice. After 20 s, pathogens showed increased heat resistance in most cases.

Generally, food scientists used to believe that juice was safe from foodborne pathogens due to the low pH of juice. But, unfortunately, there are several studies which confirm the survival of pathogens in juice (Ryu and Beuchat 1998; Oyarzábal *et al.* 2003). Pathogens can survival in acidic fruit juice because of acid adaptation. *Escherichia coli* O157:H7 and *S.* Typhimurium cells can develop adaptive mechanisms by undergoing genetic and physiologic changes that allow the cells to stay viable in acidic juice (Foster and Hall 1990; Lin *et al.* 1996). Acid adaption of pathogens shows cross-protection against thermal treatment (Ryu and Beuchat 1998). In actuality, the presence of pathogens in acidic juice triggers the acid adaptation phenomenon, which can produce a different response to treatment. In this study, we did not confirm the effect of acid adaptation of pathogens on combined treatment of heat and ozone. But Gabriel (2012) reported that the effect of physicochemical combination on decimal reduction times showed that acid-adapted *E. coli* O157:H7 had no cross-protection effect against thermal treatment in liquid medium (pH 3·0, 55 °Brix at 55°C). In this respect, we can postulate that acid-adapted pathogens in apple juice with high soluble solids content do not show any cross-protection effect.

Ozone has a high oxidation potential, so it can be used as a decolorizing agent. Ozone oxidizes or breaks -C=C- bonds, the -N=N- bonds, and heterocyclic and aromatic rings (Strickland and Perkins 1995). These lead to colour changes of food during ozone treatment. During ozone treatment, colour changes of blackberry juice (Tiwari *et al.* 2009a), grape juice (Tiwari *et al.* 2009b), apple juice (Torres *et al.* 2011) and orange juice (Tiwari *et al.* 2008) were reported. However, in this study, no significant

changes of *L*-, *a*- and *b*- values were observed in apple juice. Colour changes of fruit juices by ozone treatment can be affected by parameters such as treatment time, concentration of ozone and flow rate of ozone. Particularly, in this study, treatment time was shorter than that of other investigations.

In conclusion, this study validates the possibility of the combination of ozone and heat treatment as a novel technology for inactivating foodborne pathogens in apple juice of different solids content (°Brix). We found the combination effect and, in some cases the synergistic effect, when apple juice was treated with ozone and heat. Also, following treatment, there were no significant differences in colour values. The combination of ozone and heat treatment could be applied by the apple juice industry to control *E. coli* O157:H7 and *S.* Typhimurium. However, further investigations taking place in an industrial environment under varying conditions need to be performed to confirm the feasibility of this combination treatment.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the ministry of Education, Science and Technology (NRF-2012R1A1A2003838). This research was also supported by the Public Welfare & Safety research program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF-2012M3A 2A1051679).

Conflict of Interest

No conflict of interests declared.

References

Aguilar-Rosas, S.F., Ballinas-Casarrubias, M.L., Nevarez-Moorillon, G.V., Martin-Belloso, O. and Ortega-Rivas, E. (2007) Thermal and pulsed electric fields pasteurization of apple juice: effect on physicochemical properties and flavour compounds. *J Food Eng* **83**, 41–46.

Beuchat, L.R., Brackett, R.E., Hao, D.Y.Y. and Conner, D.E. (1986) Growth and thermal inactivation of *Listeria monocytogenes* in cabbage and cabbage juice. *Can J Microbiol* **32**, 791–795.

Boyer, J. and Liu, R.H. (2004) Apple phytochemicals and their health benefits. *Nutr J* **3**, 5.

Buchanan, R.L., Edelson, S.G., Snipes, K. and Boyd, G. (1998) Inactivation of *Escherichia coli* O157:H7 in apple juice by irradiation. *Appl Environ Microbiol* **64**, 4533–4535.

Chang, S.L. (1971) Modern concept of disinfection. *J Sanit Eng Div* **97**, 689–707.

Choi, M.R., Liu, Q., Lee, S.Y., Jin, J.H., Ryu, S. and Kang, D.H. (2012) Inactivation of *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes* in apple juice with gaseous ozone. *Food Microbiol* **32**, 191–195.

Cody, S.H., Glynn, M.K., Farrar, J.A., Cairns, K.L., Griffin, P.M., Kobayashi, J., Fyfe, M., Hoffman, R. *et al.* (1999) An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. *Ann Intern Med* **130**, 202–209.

Enache, E., Chen, Y., Awuah, G., Economides, A. and Scott, V.N. (2006) Thermal resistance parameters for pathogens in white grape juice concentrate. *J Food Prot* **69**, 564–569.

Evrendilek, G.A., Zhang, Q.H. and Richter, E.R. (1999) Inactivation of *Escherichia coli* O157:H7 and *Escherichia coli* 8739 in apple juice by pulsed electric fields. *J Food Prot* **62**, 793–796.

Foster, J.W. and Hall, H.K. (1990) Adaptive acidification tolerance response of *Salmonella typhimurium*. *J Bacteriol* **172**, 771–778.

Gabriel, A.A. (2012) Influences of heating temperatures, pH, and soluble solids on the decimal reduction times of acid-adapted and non-adapted *Escherichia coli* O157:H7 (HCIPH96055) in a defined liquid heating medium. *Int J Food Microbiol* **160**, 50–57.

Gachovska, T.K., Kumar, S., Thippareddi, H., Subbiah, J. and Williams, F. (2008) Ultraviolet and pulsed electric field treatments have additive effect on inactivation of *E. coli* in apple juice. *J Food Sci* **73**, M412–M417.

Goverd, K.A., Beech, F.W., Hobbs, R.P. and Shannon, R. (1979) The occurrence and survival of coliforms and salmonellas in apple juice and cider. *J Appl Bacteriol* **46**, 521–530.

Keyser, M., Műller, I.A., Cilliers, F.P., Nel, W. and Gouws, P.A. (2008) Ultraviolet radiation as a non-thermal treatment for the inactivation of microorganisms in fruit juice. *Innov Food Sci Emerg Technol* **9**, 348–354.

Khadre, M.A., Yousef, A.E. and Kim, J.G. (2001) Microbiological aspects of ozone applications in food: a review. *J Food Sci* **66**, 1242–1252.

Krapfenbauer, G., Kinner, M., Gössinger, M., Schönlechner, R. and Berghofer, E. (2006) Effect of thermal treatment on the quality of cloudy apple juice. *J Agric Food Chem* **54**, 5453–5460.

Liao, H., Hu, X., Liao, X., Chen, F. and Wu, J. (2007) Inactivation of *Escherichia coli* inoculated into cloudy apple juice exposed to dense phase carbon dioxide. *Int J Food Microbiol* **118**, 126–131.

Lin, J., Smith, M.P., Chapin, K.C., Baik, H.S., Bennett, G.N. and Foster, J.W. (1996) Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol* **62**, 3094–3100.

Mattick, L.R. and Moyer, J.C. (1983) Composition of apple juice. *J Assoc Off Anal Chem* **66**, 1251–1255.

Mattick, K.L., Jørgensen, F., Legan, J.D., Lappin-Scott, H.M. and Humphrey, T.J. (2000) Habituation of *Salmonella* spp. at reduced water activity and its effect on heat tolerance. *Appl Environ Microbiol* **66**, 4921–4925.

Mazzotta, A.S. (2001) Thermal inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juices. *J Food Prot* **64**, 315–320.

Mura, C. and Chung, Y.S. (1990) In vitro transcription assay of ozonated T7 phage DNA. *Environ Mol Mutagen* **16**, 44–47.

Oyarzábal, O.A., Nogueira, M.C.L. and Gombas, D.E. (2003) Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* in juice concentrates. *J Food Prot* **66**, 1595–1598.

Parish, M.E. (1997) Public health and nonpasteurized fruit juices. *Crit Rev Microbiol* **23**, 109–119.

Patil, S., Bourke, P., Frias, J.M., Tiwari, B.K. and Cullen, P.J. (2009) Inactivation of *Escherichia coli* in orange juice using ozone. *Innov Food Sci Emerg Technol* **10**, 551–557.

Patil, S., Torres, B., Tiwari, B.K., Wijngaard, H.H., Bourke, P., O' Donnell, C.P. and Valdramidis, V.P. (2010) Safety and quality assessment during the ozonation of cloudy apple juice. *J Food Sci* **75**, M437–M443.

Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M. and Swerdlow, D.L. (2005) Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerg Infect Dis* **11**, 603–609.

Ryu, J.H. and Beuchat, L.R. (1998) Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int J Food Microbiol* **45**, 185–193.

Scott, D.B.M. and Lesher, E.C. (1963) Effect of ozone on survival and permeability of *Escherichia coli*. *J Bacteriol* **85**, 567–576.

Sharma, M., Adler, B.B., Harrison, M.D. and Beuchat, L.R. (2005) Thermal tolerance of acid-adapted and unadapted *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* in cantaloupe juice and watermelon juice. *Lett Appl Microbiol* **41**, 448–453.

Sivapalasingam, S., Friedman, C.R., Cohen, L. and Tauxe, R.V. (2004) Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J Food Prot* **67**, 2342–2353.

Steele, B.T., Murphy, N., Arbus, G.S. and Rance, C.P. (1982) An outbreak of hemolytic uremic syndrome associated with ingestion of fresh apple juice. *J Pediatr* **101**, 963–965.

Steenstrup, L.D. and Floros, J.D. (2004) Inactivation of *Escherichia coli* O157:H7 in apple cider by ozone at various temperatures and concentrations. *J Food Process Preserv* **28**, 103–116.

Strickland, A.F. and Perkins, W.S. (1995) Decolorization of continuous dyeing wastewater by ozonation. *Text Chem Color* **27**, 11–15.

Sung, H.J., Song, W.J., Kim, K.P., Ryu, S. and Kang, D.H. (2014) Combination effect of ozone and heat treatment for the inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in apple juice. *Int J Food Microbiol* **171**, 147–153.

Teo, A.Y., Ravishankar, S. and Sizer, C.E. (2001) Effect of low-temperature, high-pressure treatment on the survival of *Escherichia coli* O157:H7 and *Salmonella* in unpasteurized fruit juices. *J Food Prot* **64**, 1122–1127.

Tiwari, B.K., Muthukumarappan, K., O'Donnell, C.P. and Cullen, P.J. (2008) Kinetics of freshly squeezed orange juice quality changes during ozone processing. *J Agric Food Chem* **56**, 6416–6422.

Tiwari, B.K., O'Donnell, C.P., Muthukumarappan, K. and Cullen, P.J. (2009a) Anthocyanin and colour degradation in ozone treated blackberry juice. *Innov Food Sci Emerg Technol* **10**, 70–75.

Tiwari, B.K., O'Donnell, C.P., Patras, A., Brunton, N. and Cullen, P.J. (2009b) Anthocyanins and color degradation in ozonated grape juice. *Food Chem Toxicol* **47**, 2824–2829.

Tiwari, B.K., O'Donnell, C.P., Patras, A., Brunton, N. and Cullen, P.J. (2009c) Effect of ozone processing on anthocyanins and ascorbic acid degradation of strawberry juice. *Food Chem* **113**, 1119–1126.

Torres, B., Tiwari, B.K., Patras, A., Wijngaard, H.H., Brunton, N., Cullen, P.J. and O' Donnell, C.P. (2011) Effect of ozone processing on the colour, rheological properties and phenolic content of apple juice. *Food Chem* **124**, 721–726.

USDA. U. S. Apple Statistics table 18, Apple juice and cider: Supply and Utilization in the United States, 1980/81 to date. Data set released May, 2012. Available at http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1825 (last access on February 2014).

Vojdani, J.D., Beuchat, L.R. and Tauxe, R.V. (2008) Juice-associated outbreaks of human illness in the United States, 1995 through 2005. *J Food Prot* **71**, 356–364.

Williams, R.C., Sumner, S.S. and Golden, D.A. (2004) Survival of *Escherichia coli* O157:H7 and *Salmonella* in apple cider and orange juice as affected by ozone and treatment temperature. *J Food Prot* **67**, 2381–2386.

Yuste, J. and Fung, D.Y.C. (2004) Inactivation of *Salmonella* Typhimurium and *Escherichia coli* O157:H7 in apple juice by a combination of nisin and cinnamon. *J Food Prot* **67**, 371–377.