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Short communication

Inactivation of *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes* in apple juice with gaseous ozone

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ABSTRACT

This research was initiated to assess the efficacy of gaseous ozone for inactivation *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes* in apple juice. Juice samples with solids content of 18, 36, and 72 °Brix inoculated with a culture cocktail of three foodborne pathogens were treated with gaseous ozone at a flow rate of 3.0 L/min and an ozone generation rate of 0.10, 0.90, 3.51, and 5.57 g/h for 0.5, 1, 5, and 10 min, respectively. The inactivation kinetics of gaseous ozone on foodborne pathogens conformed to the Weibull model. The time required to achieve a 5 log reduction (t_{5d}) was estimated using the parameters of the Weibull model. The t_{5d} increased with increasing solids content of apple juice. The ozone generation rate did not impart a significant effect ($p > 0.05$) on t_{5d}. Gaseous ozone is effective at inactivating foodborne pathogens in apple juice but the efficacy is dependent on the solids content of the juice sample.

1. Introduction

Apple juice is consumed because of its health-related nutritional value and sensory attributes. Apple juice has a pH range of 3.1–4.4, which was historically considered a barrier against contamination and survival of foodborne pathogens (Mattick and Moyer, 1983). However, acid stress resistance of foodborne pathogen has led to outbreaks in traditional pressed juice products. An outbreak of *Escherichia coli* O157:H7 infections was linked to apple cider in 1991, and *E. coli* O157:H7 can survive for several days in acidic pH between 3.6 and 4.0 (Vojdani et al., 2008). Moreover, acid stressed *Salmonella* isolated from unpasteurized juice products exhibited increased heat and acid resistance (Sharma et al., 2005).

Outbreaks of foodborne pathogen infections associated with consumption of juice products led to new regulations and application of sterilization methods in juice processing to improve safety of these products. The final juice hazards analysis and critical control point (HACCP) regulation required that pasteurization processing used to prevent biological contamination must achieve at least a 5 log reduction of pathogens of concern to public health (U.S. FDA, 2001). Traditional pasteurization processing involving heating juices at a temperature below 100 °C for several seconds results in loss of organoleptic compounds in juice products. Moreover, traditional thermal pasteurization is not suitable for small juice producers from an economic perspective. Non thermal methods such as UV radiation (Forney et al., 2004) and ozone treatment (Patil et al., 2010) are utilized as alternative pasteurization methods.

Ozone is a triatomic molecule that is considered to be an allotropic modification of oxygen. Ozone causes cell damage to foodborne pathogens in the unsaturated lipids in the microbial cell envelope, lipopolysaccharides layer of Gram negative bacteria, intracellular enzymes, and genetic materials (Kim et al., 2003). When applied in food processing, the bacterial inactivation effect of ozone is affected by water activity (Kuprianoff, 1953), pH (Patil et al., 2010), and antioxidant compounds (Restaino et al., 1995) in the food matrix. However, the effect of apple juice solids content on pathogen inactivation efficacy of gaseous ozone has not been reported.

The objectives of this study were to 1) investigate how the solids content of apple juice affects gaseous ozone treatment efficacy; 2) quantitatively describe the inactivation kinetics of foodborne pathogens in apple juice treated by gaseous ozone.

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2. Material and methods

2.1. Bacteria strains and preparation of inoculum

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889 and ATCC 43890), *Salmonella typhimurium* (ATCC 19585, ATCC 43971 and DT 104) and *Listeria monocytogenes* (ATCC 7644, ATCC 19114 and ATCC 19115) were obtained from the School of Food Science Culture Collection at Washington State University (Pullman, WA, USA). Each strain of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* was cultured in 5 ml Tryptic Soy Broth (TSB, Becton Dickinson, US) at 37 °C for 24 h, harvested by centrifugation at 4000×*g* for 20 min at 4 °C and washed three times with buffered peptone water (Difco, US). The final cell concentration in the buffer was approximately 8–9 log CFU/ml. After that, the three strains of each foodborne pathogen were combined to construct a nine-strain mixed culture cocktail. This multi-pathogen culture cocktail was used for subsequent experiments.

2.2. Preparation of apple juice

Apple juice was purchased from a Chung-Book Nonghyup processing factory (Chung-Book, Korea), and stored at 4 °C. Single-strength apple juice had a solids content of 72 °Brix and pH value of 3.8. Apple juice was diluted with sterile distilled water to 36 and 18 °Brix. Then 100 μl of the pathogen cocktail was inoculated into each 200 ml apple juice sample.

2.3. Ozone treatment

Gaseous ozone was produced by an activated oxygen generator (Ozonetech Co., Ltd, Korea) at generation rates of 0.10, 0.90, 3.51, and 5.57 g/h from ambient air at a flow rate of 3.0 L/min. Ozonated air was pumped directly into the juice through a delivery tube and sparged through a perforated tube into a 1-L bottle. To prevent excess foaming, 0.1 ml sterile anti-foaming agent (Antifoam B emulsion, Sigma Aldrich, Ireland Ltd.) was added before each ozone treatment. During ozone treatment, juice samples were stirred at a medium rate using a stir plate (MS-100, Universal Scientific co., Ltd, Korea) with a magnetic spin bar to ensure even dispersal of ozone. The apple juice samples were treated for 0.5, 1, 5 and 10 min. Juice samples not subjected to ozone treatment were designated as the control group. All experiments were conducted in a fume hood.

2.4. Bacteria enumeration

After 0.5, 1, 5 and 10 min of ozone treatment, sample aliquots were aseptically removed and placed in test tubes containing 9 ml D/E neutralizing broth (Difco) and homogenized for 2 min using a vortex (VM-10, Daihan Scientific co., Ltd, Korea). After homogenization, samples were 10-fold serially diluted with 9 ml sterile buffered peptone water and 0.1 ml of diluent was spread-plated onto selective media. Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco) and Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Bacto Oxford Antimicrobic Supplement, Difco) were used as selective media to enumerate *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes*, respectively. All plates were incubated at 37 °C for 24–48 h, and colonies typical of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* were counted.

2.5. Inactivation kinetics

The survival curves of food pathogens in apple juice demonstrated a non-linear characteristic. GInaFit tool was employed to analyze the microbial inactivation data (Geeraerd et al., 2005). The Weibull model was able to describe foodborne pathogens inactivation kinetics.

$$\log_{10}(N) = \log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \quad (1)$$

N (CFU/ml) is the survival population of the microorganism, N_0 is the initial microorganism population, t (min) is the treatment time, δ (min) is the time required for the first decimal reduction and p is the parameter related to the scale and shape of the inactivation curve. If $p < 1$, the Weibull model presents as an upward concave curve and downward if $p > 1$ (Van Boekal, 2002).

The numerical estimates of δ and p were used to calculate a time required for 5 log reduction to occur (t_{5d}, U.S. FDA, 2001). In this equation, x is equal to 5.

$$t_{xd} = \delta \times (x)^{1/p} \quad (2)$$

The fitting ability of the Weibull model was evaluated by calculating root mean squared error (RMSE) (Lyman and Longnecker, 2008) by the following equation:

$$\mathrm{RMSE} = \sqrt{\sum_{i=1}^{nt} \frac{\left(y_{expi} - y_{pre}\right)^2}{n_t - n_p}} \quad (3)$$

Where y_{expi} refers to the experimental observation, y_{pre} refers to the model predictions, n_t is the number of data, n_p is the number of parameters.

2.6. Statistical analysis

All experiments were repeated three times with duplicate samples and viable plate counts from three replications were converted to units of log CFU/ml. Data were analyzed by the ANOVA procedure of Minitab (Version 15, Minitab Inc., PA, USA) for a completely randomized design. Means were separated and compared using the *T*-test procedure of Minitab at $p < 0.05$ level.

3. Results and discussion

3.1. Effect of ozone on inactivation foodborne pathogens in apple juice

The t_{5d} values of three foodborne pathogens treated with four selected ozone generation rates are shown in Table 1. Generally, t_{5d} of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* in apple juice samples of 18, 36 and 72 °Brix decreased as the ozone generation rates increased. However, this trend was not significant ($p > 0.05$). After ozone treatment at four selected generation rates, the populations of three foodborne pathogens were not detected with the current agar-based detection method which has a detection limit of 1 log CFU/ml (Figs. 1–3, data of *S. typhimurium*, and *L. monocytogenes* were not shown) in 18 and 36 °Brix apple juice samples. The population reduction of three foodborne pathogens in 72 °Brix apple juice was in a range of 0.41 ± 0.02 to 1.73 ± 0.10 log CFU/ml after ozone treatment.

Compared to the ozone generation rate, efficacy of gaseous ozone on the inactivation of foodborne pathogens is more dependent on residual ozone in the medium. Residual ozone refers to the detectable ozone concentration in apple juice after it has been applied (Kim et al., 2003). Decomposition of ozone is related to pH of the medium, temperature, and presence of ozone-consuming

Table 1
The effect of ozone generation rate on the t_{5d} values for *E. coli* O157:H7, *S. typhymurium* and *L. monocytogenes* strains treated in apple juice with solids content of 18, 36, and 72 °Brix.

Solids content (°Brix)	Ozone generation rate (g/h)	*E. coli* O157:H7		*S. typhimurium*		*L. monocytogenes*	
		t_{5d}	RMSE	t_{5d}	RMSE	t_{5d}	RMSE
18	0.10	4.82 ± 0.40A[a]a[b]	0.89 ± 0.03	5.70 ± 0.63Ba	0.78 ± 0.01	5.70 ± 0.21Cb	0.83 ± 0.06
	0.90	4.88 ± 0.41Ac	0.92 ± 0.10	5.56 ± 1.38Bc	1.38 ± 0.83	6.02 ± 0.49Cd	1.15 ± 0.20
	3.51	5.18 ± 0.95Ae	1.11 ± 0.24	6.11 ± 1.33Be	0.81 ± 0.14	5.49 ± 0.11De	0.98 ± 0.07
	5.57	3.76 ± 0.59Af	1.54 ± 0.08	6.61 ± 1.27Bf	1.15 ± 0.31	4.55 ± 0.38Df	1.22 ± 0.26
36	0.10	7.56 ± 0.35Eg	0.85 ± 0.11	7.12 ± 0.40Fg	0.51 ± 0.14	9.21 ± 1.84Hg	0.74 ± 0.23
	0.90	6.19 ± 1.44Eh	0.95 ± 0.14	7.26 ± 0.27Fh	0.49 ± 0.02	8.06 ± 1.05Hh	0.32 ± 0.02
	3.51	5.79 ± 2.32Ei	0.79 ± 0.22	6.06 ± 1.02Fi	0.80 ± 0.18	5.49 ± 1.36Hi	1.02 ± 0.24
	5.57	5.83 ± 0.91Ej	0.84 ± 0.19	4.70 ± 0.11Gj	0.86 ± 0.04	4.96 ± 0.42Ij	0.79 ± 0.33
72	0.10	1377.56 ± 1390.17Jk	0.17 ± 0.02	2.03e17 ± 2.06e17Kk	0.08 ± 0.11	32.24e4 ± 54.61e4Lk	0.17 ± 0.22
	0.90	976.35 ± 1607.27Jl	0.16 ± 0.06	57.95e6 ± 85.28e6Kl	0.19 ± 0.19	2246.83 ± 1967.04Ll	0.43 ± 0.29
	3.51	33.07e7 ± 56.7e7Jm	0.15 ± 0.11	44.56e11 ± 77.18e9Km	0.13 ± 0.17	1615.81 ± 1231.66Lm	0.22 ± 0.09
	5.57	24.70e3 ± 34.89e3Jn	0.33 ± 0.15	56.43 ± 66.1Kn	0.14 ± 0.08	596.98 ± 693.28Ln	0.40 ± 0.10

[a] Within each solid content level, values within one column followed by different upper case letters indicate significantly different ($p < 0.05$).
[b] Values within a row followed by different lower case letters indicate significantly different ($p < 0.05$).

materials. In this study, we focused on the effect of ozone-consuming materials in apple juice on the inactivation efficacy of gaseous ozone. Ozone-consuming materials present in apple juice reduced the amount of ozone applied and decreased the amount available to inactivate target pathogens. These ozone-consuming materials contributing to ozone chelation in apple juice are anti-oxidant compounds such as polyphenols, phenolic acids, flavo-noids, and vitamin C (Leontowicz et al., 2002). Moreover, the metal ions present in apple juice also consume effective molecular ozone and byproducts. Hague reported that in apple juice samples, the predominant metals are Rb, Mn, Cr, Zn and Cu with a total measured metal ion concentration of 1339.87 ± 10.84 ppb (Hague et al., 2008). Therefore, the actual effective ozone dose was over-estimated according to the applied dose. Although t_{5d} has a tendency towards reduction with an increasing rate of ozone generation, this trend is not inevitable due to the complex components of apple juice.

3.2. Inactivation kinetics of foodborne pathogen by ozone gas

The inactivation kinetics of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* were analyzed by 10 model types. The Weibull model provided the best fit for the data among the tested models (Table 1), especially for data obtained from high solids content juice samples with lower RMSE value.

With respect to population reduction and the overall pattern of survival curves, no significant differences ($p > 0.05$) were observed among the three tested foodborne pathogens in apple juice with the same solids content treated by ozone (Figs. 1–3). Restaino et al. (1995) also reported that *S. typhimurium*, *E. coli*, *Pseudomonas*

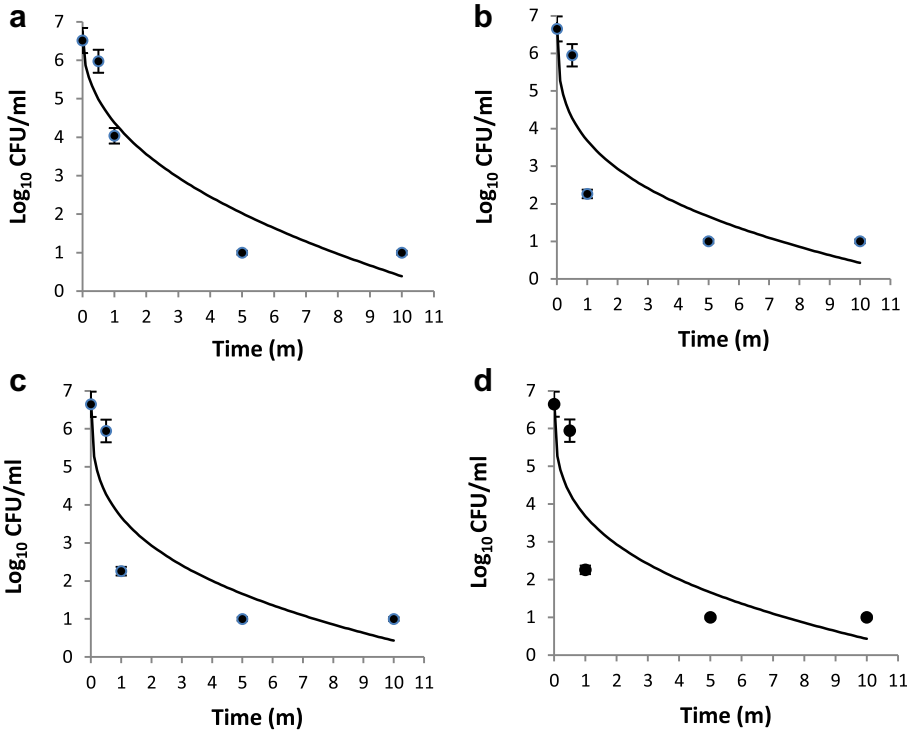

Fig. 1. Ozone gas inactivation of *E. coli* O157:H7 in apple juice (18 °Brix) at room temperature (22 ± 2 °C). Error bars indicate 95% confidence intervals. Ozone generation rate: (a) 0.10 g/h; (b) 0.90 g/h; (c) 3.51 g/h; (d) 5.57 g/h.

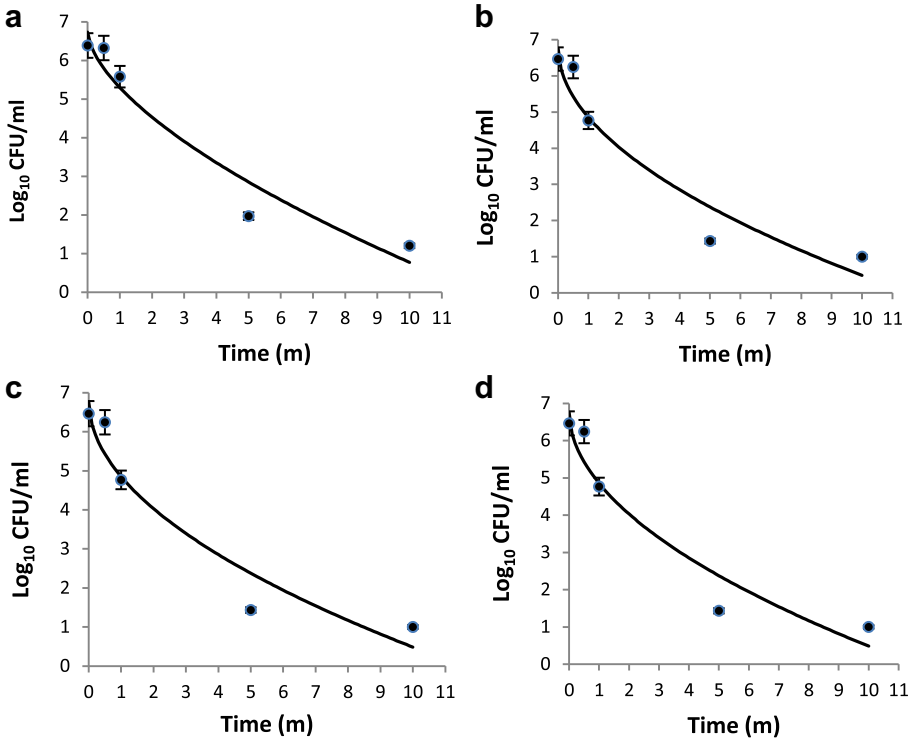

Fig. 2. Ozone gas inactivation of *E. coli* O157:H7 in apple juice (36 °Brix) at room temperature (22 ± 2 °C). Error bars indicate 95% confidence intervals. Ozone generation rate: 0.10 g/h; (b) 0.90 g/h; (c) 3.51 g/h; (d) 5.57 g/h.

aeruginosa and *Yersinia enterocolitica* were not significantly different in death rate and death pattern when treated with ozonated deionized water with an ozone concentration of 0.188 mg/L. However, *L. monocytogenes* demonstrated a significantly greater death rate ($p < 0.05$) compared to *Staphylococcus aureus* and *Enterococcus faecalis* (Restaino et al., 1995).

In this study, the survival curves were concave downward ($p > 1$) which indicates the cells became increasingly damaged

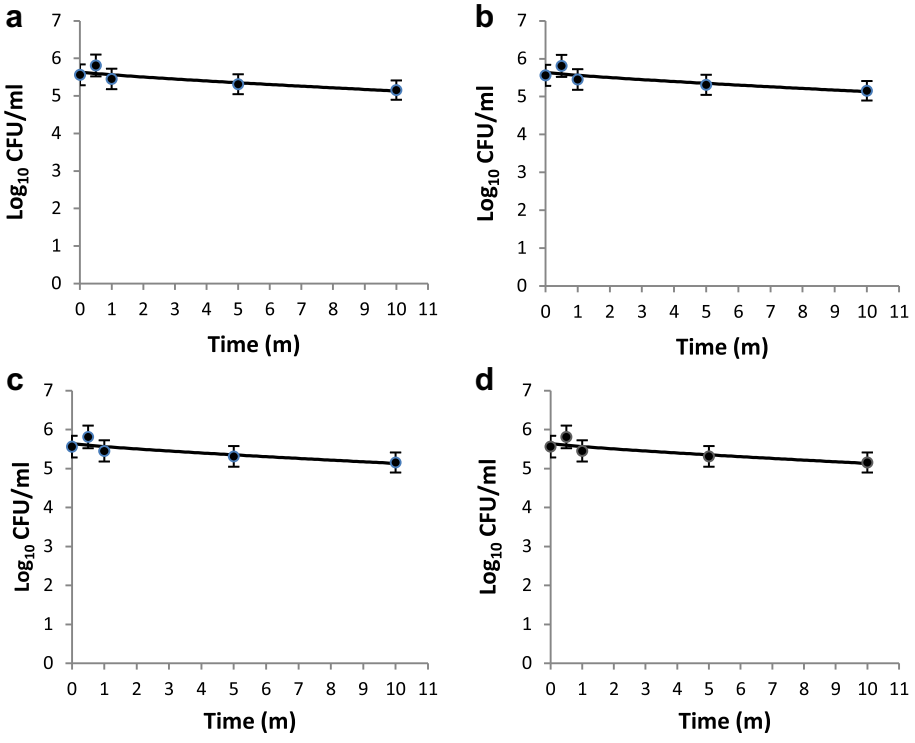

Fig. 3. Ozone gas inactivation of *E. coli* O157:H7 in apple juice (72 °Brix) at room temperature (22 ± 2 °C). Error bars indicate 95% confidence intervals. Ozone generation rate: 0.10 g/h; (b) 0.90 g/h; (c) 3.51 g/h; (d) 5.57 g/h.

(Figs. 1–3). Therefore, these sensitive microorganisms were possibly injured and dying as a result of ozone treatment. However, the pattern of survival curves in this study was different from that reported by Patil et al. (2010), which showed an upward concave curve. The upward concavity ($p < 1$) of survival curves means the cells remaining have the ability to adapt to the applied stress (Van Boekel, 2002). This difference may result from the status of tested strains. According to Fisher et al. (2000), mid-exponential and late stationary phase cells of *L. monocytogenes* are more sensitive to ozonated water than early stationary phase cells. They also found sensitivity to ozonated water varied among tested *L. monocytogenes* strains. Catalase and superoxide dismutase produced by *L. monocytogenes* cells played an important role in protecting cells from ozone attack (Fisher et al., 2000).

The parameters of the Weibull model were calculated to estimate the time duration required to achieve a 5 log reduction. These parameters may be dependent on external conditions such as temperature, pressure, pH, and presence of preservative. Patil et al. (2010) reported that the pH values of apple juice imparted a significant effect on Weibull model parameters, and thus on calculation of t_{5d} of ozone gas inactivation of *E. coli*. However, some researchers have argued that the shape factor (p) only represents the inactivation kinetic patterns and is independent of external factors. According to Fernandez et al. (2002), the effect of pH and temperature on the thermal resistant ability of *Bacillus cereus* spores can be ignored. However, in this study, the solids content of juice samples did affect the parameters significantly ($p > 0.05$).

4. Conclusion

This work demonstrated that gaseous ozone can be used to inactivate *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* in apple juice directly. The inactivation kinetics were described by the Weibull model and the t_{5d} values can be estimated. The efficacy of ozone gas was significantly influenced by the solid contents of the juice sample. *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* did not demonstrate significant differences in death rate.

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