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Microbiological Quality Enhancement of Minimally-Processed Enoki Mushrooms Using Ozone and Organic Acids

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Abstract This study examined the effects of ozone exposure alone (1, 3, and 5 ppm) as well as in combination with 1% acetic acid, citric acid, or lactic acid on the growth of indigenous microorganisms in enoki mushrooms. Populations of mesophilic bacteria, yeasts and molds in enoki mushrooms appeared to be decreased by stepwise increases in concentration (1 to 5 ppm) or exposure time (0.5 to 5 min) to ozone. Compared to untreated (control) enoki mushrooms, there were reductions of 1.03 to 2.61 $\log_{10}$ CFU/g in mesophilic bacteria and of 1.21 to 2.75 $\log_{10}$ CFU/g in yeasts and molds in all ozone- treated enoki mushrooms. Combination of 3 ppm ozone and 1% citric acid ($p<0.05$) synergistically brought about significant reductions in both mesophilic bacteria (3.52 $\log_{10}$ CFU/g) and fungi (yeasts and molds) (2.77 $\log_{10}$ CFU/g) from enoki mushrooms. The results of this study show that low concentrations of ozone inhibit indigenous microflora populations in enoki mushrooms. Combination treatments of 3 ppm ozone with 1% citric acid showed greater antimicrobial effectiveness than either 3 ppm ozone or 1% citric acid alone.

Keywords: ozone, acetic acid, citric acid, lactic acid, indigenous microflora

Introduction

In recent years, the consumption of, and demand for, minimally processed fresh (MPF) vegetables as a ready-to-eat (RTE) food has been increasing continuously. However, the risk of contamination from spoilage or pathogenic microorganisms in MPF vegetables has increased because these vegetables usually do not undergo cooking to eliminate these microorganisms before consumption. *Erwinia*, *Psedomonas*, and lactic acid bacteria are the predominant food-borne spoilage organisms related to the degradation of pectin in raw vegetables (1-4). *Escherichia coil* O157:H7 (5-7), *Listeria monocytogenes* (8-11), *Shigella* (12), *Salmonella* (13), and hepatitis A virus (14) are the main sources of food-borne disease outbreaks associated with the consumption of contaminated MPF vegetables. In Korea, mushrooms have been reported to be the main MPF vegetable acting as a vehicle of transmission for food- borne outbreaks.

Therefore, various kinds of antimicrobial treatments such as rinsing with water, chlorine, organic acids, irradiation, and ozone have been used to reduce the number of microorganisms in MPF foods (15,16). Among these antimicrobial treatments, organic acids such as acetic, lactic, citric, and propionic acids are generally recognized as safe (GRAS) disinfectants in MPF vegetables (17-20). Ozone has also been widely used as a disinfectant in foods including MPF vegetables since it was approved as a GRAS substance by a US expert in 1997 (21). Ozone at low concentrations can reduce the populations of a variety of organisms: bacteria, yeasts and molds, parasites, and viruses (22).

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The objective of the current study was to investigate the effects of ozone, alone and in combination with different organic acids, on the growth of indigenous microorganisms in minimally- processed enoki mushrooms.

Materials and Methods

Mushrooms Enoki mushrooms (*Flammulina velutipes* sinh) were purchased from the local supermarket. Wilted enoki mushrooms were removed and discarded. The enoki mushrooms were stored at 5 after purchase and were used for experiments within 48 hr. The mushrooms were cut into pieces using a sterile knife (22).

Ozone generation and measurement Ozone was produced on site by an electrochemical process using an ozone generator (GW-1000, Youlchon, Korea) as shown in Figure 1. For preparation of aqueous ozone, concentration was monitored continuously using a Dissolved Ozone Monitor (Model A15/64, ATI Inc., USA). This particular model was an on-line monitoring system designed for the continuous measurement of ozone gas in solution.

Antimicrobial treatments Two experimental settings were used to determine the effects of ozone alone (first experiment), as well in combination with organic acids (second experiment), on the growth of indigenous microorganisms from enoki mushrooms. A total of 13 treatment solutions were used in the first experiment and 11 were used in the second experiment. Treatments were prepared by mixing 95 mL tap water containing 1, 3, or 5 ppm ozone with appropriate amounts of organic acid (1% acetic acid, 1% citric acid, or 1% lactic acid)(Fisher Scientific Co., Norcrss, GA, USA). In the first experiment, some mushrooms were washed with tap water alone, while the other 12 treatments consisted of immersing the

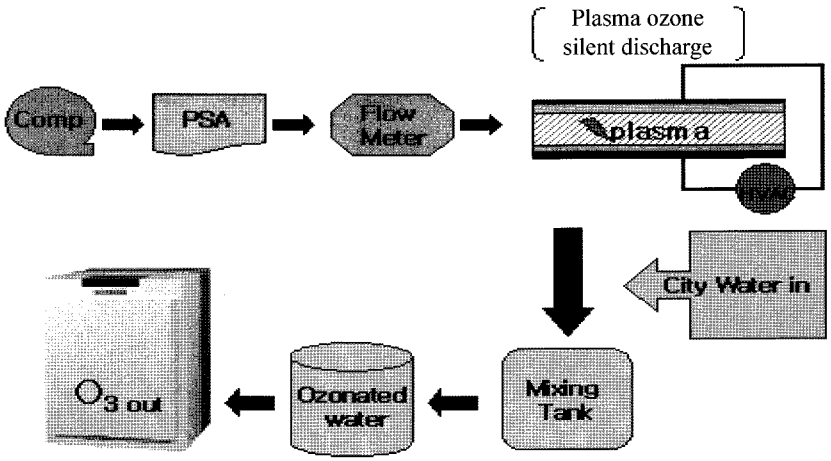

Fig. 1. Conceptural design of plasma ozone generator.

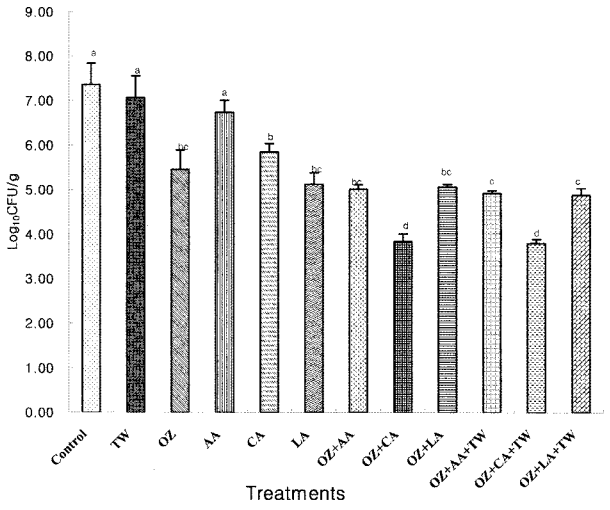

Fig. 2. Single and combined effects of ozone with organic acids on mesophilic bacteria in enoki mushrooms. The data presents means with standard errors (two samples/treatment).

Table 1. Effects of ozone treatment on mesophilic bacteria in enoki mushrooms

Treatment	Concentration (ppm)	Exposure time (min)	Mesophilic bacteria (log_{10} CFU/g)	Reduction[1] (log_{10} CFU/g)
Control[2]			6.39±0.55	-
TW[3]			5.99±0.89	0.40
OZ[4]	1	0.5	5.36±0.11	1.03
		1.5	5.28±0.14	1.11
		3	4.74±0.10	1.65
		5	4.13±0.11	2.26
	3	0.5	4.89±0.18	1.50
		1.5	4.66±0.17	1.73
		3	4.19±0.17	2.20
		5	3.78±0.14	2.61
	5	0.5	4.33±0.34	2.06
		1.5	4.24±0.24	2.15
		3	4.18±0.14	2.21
		5	3.88±0.65	2.51

[1]Reduction from numbers of mesophilic bacteria in untreated enoki mushrooms (control).
[2]Control: untreated enoki mushrooms.
[3]TW: enoki mushrooms washed in tap water.
[4]OZ: ozone-treated enoki mushrooms.

mushrooms in ozone solutions of various strength (1, 3, or 5 parts per million water) for different periods of time (30 seconds, 1.5 minutes, 3 minutes, and 5 minutes). Control mushrooms were not washed at all (Tables 1 and 2). Treatments in the second experiment consisted of washing mushrooms with tap water, ozone alone, organic acids alone, or combinations of 3- ppm ozone solutions with 1% solutions of organic acids. Of the latter, some ozone/ organic acid treatments were followed by washing with tap water, while others were not. In both experiments, untreated enoki mushrooms was used as controls. Enoki mushrooms (10 g) were placed in a plastic basket and submerged in each treatment solution for the required time in the first experiment and for 5 min in the second experiment. Then each treated sample was naturally drained at room temperature. After draining, all treated samples in the first experiment and the samples of treatments (9), (10), and (11) in the second experiment were washed in tap water (Figure 3).

Microbiological analysis Treated samples (10 g) of enoki mushrooms taken from sterile plastic barrier bags were mixed with peptone water (1:10, w/w) in a stomacher bag and homogenized for 2 min in a stomacher (Elmex SH-II M, Tokyo, Japan). Serial dilutions of the samples were made with sterile peptone water. The mesophilic bacteria were plated in duplicate on plate count agar (PCA)(Difco, Detroit, MI, USA) and incubated at 37 °C for 24 hours. Yeasts and molds were also plated in duplicate on potato dextrose agar (PDA)(Difco, Detroit, MI, USA) and incubated 25°C for 48 hr. The numbers of mesophilic bacteria, yeasts and molds were determined by colony enumeration and expressed as log_{10}CFU/g.

Statistical analysis The values of log_{10}CFU/g were analyzed by least square mean separations of the ANOVA procedure in the SAS statistical analysis software program,

Table 2. Effects of ozone treatment on yeasts and molds populations in enoki mushrooms

Treatment	Concentration (ppm)	Exposure time (min)	Yeast and mold (log_{10} CFU/g)	Reduction[1] (log_{10} CFU/g)
Control[2]			6.10±0.14	-
TW[3]			5.81±0.10	0.29
OZ[4]	1	0.5	4.89±0.18	1.21
		1.5	4.56±0.26	1.54
		3	4.15±0.49	1.95
		5	4.10±0.25	2.00
	3	0.5	4.81±0.27	1.29
		1.5	4.52±0.47	1.58
		3	4.20±0.31	1.90
		5	4.04±0.25	2.06
	5	0.5	4.50±0.41	1.60
		1.5	4.01±0.17	2.09
		3	3.85±0.21	2.25
		5	3.35±0.35	2.75

[1]Reductions from numbers of yeasts and molds in untreated enoki mushrooms (control).
[2]Control: untreated enoki mushrooms.
[3]TW: enoki mushrooms washed in tap water.
[4]OZ: ozone- treated enoki mushrooms.

version 6.11 (23). All statistical analyses were considered significantly different at the $p<0.05$ level.

Results and Discussion

Effects of OZ concentrations and exposure time in enoki mushroom RTE vegetables contaminated by 10^5-10^7 CFU/g of total indigenous microflora are often dipped in antimicrobial solutions containing water, 100 ppm chlorine, 1% citric acid, or 1% lactic acid (24). Relatively low concentrations of ozone are also used to inactivate contaminant microflora on diverse food products. Therefore, we examined the effects of different concentrations of ozone for various periods of exposure on the indigenous microorganisms in minimally-processed enoki mushrooms. The number of indigenous mesophilic bacteria in enoki mushroom treated in this manner is shown in Table 1. Mesophilic bacteria counts were decreased by stepwise increases in ozone concentration (1 to 5 ppm) or by stepwise increases in duration of exposure to ozone (0.5 to 5 min) in each aqueous ozone solution. However, reductions in mesophilic bacteria were not maximized even when mushrooms were exposed to the highest concentration (5 ppm) of ozone for the longest time (5 min). Mesophilic bacteria counts were 2.5- to 6.5-fold lower in mushrooms treated with ozone than in those washed with tap water. The number of mesophilic bacteria in enoki mushrooms treated with ozone were decreased by 1.03 to 2.61 log_{10}CFU/g from bacteria levels seen in untreated mushrooms. Similar reduction levels were noted by Khadre *et al.* (25) who reported that aqueous ozone can decrease bacterial populations in fruit and vegetables. They also reported that gram-positive bacteria were reduced in 0.12 to 3.8 mg/mL aqueous solutions of ozone by a factor of 1 to 7 log_{10}CFU/mL, and gram-positive

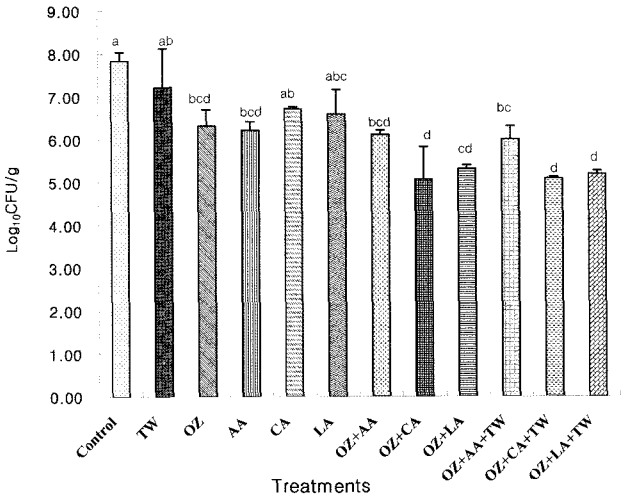

Fig. 3. Single and combined effects of ozone with organic acids on yeasts and molds growth in enoki mushrooms. The data presents means with standard errors (two samples/treatment).

bacteria were reduced by 0.5 to 6.5 log_{10}CFU/ml in 0.004 to 6.5 mg/mL aqueous ozone solutions (25). According to Kim *et al.* (22), treatment of lettuce for 3 minutes with bubbling ozone (1.3 mM) decreased mesophilic bacteria by 1.4 log_{10}CFU/g and psychrotrophic bacteria by 1.8 log_{10}CFU/g . Koseki *et al.* (26) also reported that exposure of lettuce to 5 ppm ozone for 10 minutes decreased aerobic organism counts by 1.5 log_{10}CFU/g. However, washing apples in ozone for more than 5 minutes was considered impractical for the food industry (27). Disinfection of salad leaves using chlorine or organic acids does not usually reduce microbial contamination by more than 2-3 log cycles (17, 28). Adams *et al.* (17) reported that normal washing of lettuce in tap water removed an average of 92.4% of the leaf microflora.

The number of indigenous yeasts and molds in enoki mushrooms exposed to different ozone concentrations for different periods of time are shown in Table 2. Numbers of yeasts and molds among treatments were decreased by stepwise increases (1 to 5 ppm) in ozone concentration or stepwise increases (0.5 to 5 min) in length of exposure time to each aqueous ozone solution. The yeast and mold counts were 4- to 9.5- fold lower in mushrooms treated with ozone than in those simply washed with tap water. The numbers of yeasts and molds in enoki mushrooms treated with ozone were lower than those in untreated enoki mushrooms by 1.21 to 2.75 log_{10}CFU/g. In general, the response of these fungi to ozone showed a similar pattern to that observed with mesophilic bacteria.

Ewell (29) noted that 0.6 to 1.5 ppm ozone was able to inhibit mold growth on eggs and ozone levels of 2.5 to 3 ppm could inactivate the growth of mold on beef. Farooq and Akhlaque (30) reported that 0.23 to 0.26 mg/L of ozone inhibited growth of *Candida parapsilosis* by a log factor of 2.7. Kawamura *et al.* (31) reported that 0.02 to 1.0 mg/L of ozone inactivated *C. tropicalis* by a log factor of 2.0.

Effects of treatment with ozone, organic acids, and combinations of ozone with organic acids Organic

acids are used as GRAS disinfectants to reduce microorganism levels in a variety of foods. Citric acid can inhibit bacterial growth in hard-boiled eggs (32). Lactic acid is a strong disinfectant against the growth of spoilage bacteria in meat (33). In addition, Sanz *et al.* (34) recently reported that 0.02% citric acid and 0.2% acetic acid were more effective than tap water in removing *L. monocytogenes* and *E. coli* O157:H7 from minimally- processed artichokes.

Ozone retains an antimicrobial property in many different foods. However, it is not known if the indigenous microflora of enoki mushrooms would be reduced more by combinations of ozone with organic acids than by ozone or organic acids alone. Therefore, we compared the antimicrobial effect of ozone alone with that of ozone combined with an organic acid on the indigenous microorganisms in minimally- processed enoki mushrooms.

The number of indigenous mesophilic bacteria in enoki mushrooms treated with ozone, organic acids, and combinations of OZ with organic acids isshown in Figure 2. The treatments significantly ($p<0.05$) affected the number of mesophilic bacteria in enoki mushrooms. However, no significant differences in mesophilic bacteria counts were observed between mushrooms rinsed with tap water, those treated with 1% acetic acid, and untreated enoki mushrooms. The mesophilic bacteria counts were also not ($p>0.05$) significantly lower in enoki mushrooms treated with combinations of 3 ppm ozone with 1% acetic acid or of 3 ppm ozone with 1% lactic acid. Subsequent washing with tap water did not significantly affect the results of combined treatments of 3 ppm ozone with 1% acetic acid or 3 ppm ozone with 1% lactic acid. In contrast to these observations, the mesophilic bacteria counts were significantly ($p<0.05$) lower in enoki mushrooms treated with a combination of 3 ppm ozone with 1% citric acid, even when mushrooms were subsequently washed with tap water. The bacterial counts resulting from these ozone-citric acid treatments were lower than those seen from any other single or combination treatment(s). Therefore, minor synergistic effects on mesophilic bacteria were exhibited in combination treatments of 3 ppm ozone with 1% citric acid (3.52 $\log_{10}$CFU/g reductions) when compared to treatments using either 3 ppm ozone (1.90 $\log_{10}$CFU/g reductions) or 1% citric acid alone (1.51 $\log_{10}$CFU/g reductions)(data not shown).

The number of indigenous yeasts and molds in enoki mushrooms treated with ozone, organic acids, and combinations of ozone with organic acids is shown in Figure 3. The treatments significantly ($p<0.05$) affected the numbers of yeasts and molds in enoki mushrooms. Yeast and mold counts were significantly ($p<0.05$) lower in the enoki mushrooms treated with combinations of 3 ppm ozone with 1% citric acid, 3 ppm ozone with 1% citric acid with subsequent washing with tap water or 3 ppm ozone with 1% lactic acid with subsequent rinsing with tap water. Combined ozone/lactic acid treatment (followed by washing with tap water) and all ozone/citric acid treatments (with or without subsequent washing) showed more antifungal effects than treatment with either1% citric acid or 1% lactic acid alone. Therefore, slight synergistic effects on reduction of yeast and mold counts were exhibited in these combined ozone/citric acid (2.77 $\log_{10}$CFU/g reductions [unwashed]) (2.75 $\log_{10}$CFU/g [washed with tap water]) or ozone/lactic acid treatments (2.64 $\log_{10}$CFU/g [washed]) that were greater those of ozone (1.52 $\log_{10}$CFU/g), citric acid(1.13 $\log_{10}$CFU/g), or lactic acid alone (1.25 $\log_{10}$CFU/g)(data not shown).

The antimicrobial properties of ozone are associated with its hydroxyl radical reacting with intracellular enzymes, nucleic material, or components of cell envelopes, spore coats, or viral capsides (25). The antimicrobial properties of organic acids are associated with the depression of pH to below the minimum growth range for microorganisms and with metabolic inhibition by undissociated molecules (35).

High concentrations of ozone may be efficient in eliminating microorganisms in food components, but these ozone levels may alter food sensory attributes and adversely affect acceptability of food products. Therefore, without changing sensory attributes, treatment with ozone at relatively low concentrations and for short periods of time can be used as a conventional method for reducing indigenous microflora on the surfaces of fruits and vegetables.

The results in the current study provide additional evidence that low concentrations of OZ inhibit indigenous microflora populations in enoki mushrooms. In addition, treatments using a combination of 3 ppm ozone with 1% citric acid enhanced antimicrobial effectiveness to a greater degree than either 3 ppm ozone or 1% citric acid alone. Future experiments are needed to examine the effects of ozone, both alone and in combination with organic acids, on parameters of physical and sensory quality in minimally- processed enoki mushrooms.

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