Food Sci. Biotechnol. 25(6): 1801-1804 (2016)
DOI 10.1007/s10068-016-0274-5

Food Science
and
Biotechnology

Research Note

Effects of malic acid or/and grapefruit seed extract for the inactivation of common food pathogens on fresh-cut lettuce

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Received September 6, 2016
Revised October 6, 2016
Accepted October 18, 2016
Published online December 31, 2016

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pISSN 1226-7708
eISSN 2092-6456

Abstract This study investigated the antimicrobial activity of malic acid (MA), grapefruit seed extract (GSE), and combined (MA+GSE) treatment against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on fresh-cut lettuce. The antimicrobial effects of 1% MA and 0.5% GSE alone and in combination (1% MA+0.5% GSE) were tested on artificially inoculated lettuce during storage at 5°C for 14 days. The maximum reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were 4.96, 4.80, and 3.95 log CFU/g observed with MA+GSE during storage for 14 days, respectively. MA+GSE showed the greatest reduction against in *E. coli* O157:H7 and *L. monocytogenes*. These results indicate that the combined treatment was more effective than MA and GSE alone treatment. Therefore, it suggests that MA + GSE could be used as an effective intervention method for improving microbiological safety of fresh-cut lettuce.

Keywords: malic acid, grapefruit seed extract, foodborne pathogens, lettuce, reduction

Introduction

Consumption of ready-to-eat vegetables has been increasing as consumers' demand for safe food that can maintain their health (1-3). These products are usually sealed in packages and stored at low temperatures. They can possess diverse pathogenic microorganisms, such as *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* (1,4-6).

Among foodborne pathogens, *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* are of high public health concern (2,7). These foodborne pathogens have been isolated from lettuce and cause severe cases of foodborne illnesses (1-3,8-11). Various sanitizers such as chlorine dioxide (ClO_2), hydrogen peroxide (H_2O_2), and ozonized water have been used for enhancing microbiological safety of fresh products (1,2). Chlorine-based sanitizers are most widely used in fresh produce industry. But the use of chlorine-based sanitizers is likely to produce toxic chemicals such as trihalomethanes formed with organic matter, considered as carcinogens (12). Therefore, there is a continuous demand from consumers for safe sanitizers such as natural sanitizers.

Organic acids are considered generally-recognized-as-safe (GRAS) products. Several organic acids have been used as sanitizers on vegetables (13-16). Among organic acids, malic acid (MA) has been found to be highly effective against *L. monocytogenes* compared to other organic acids (17). In addition, MA showed more reduction activity against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* than other organic acids (propionic, acetic, lactic, or citric acid) when applied on unwaxed apples at room temperature (22±2°C) (2).

Grapefruit seed extract (GSE) is also known as a natural antimicrobial. It possesses antimicrobial, antiviral, antifungal, and antiparasitic properties (18,19). In addition, GSE contains beneficial products such as catechins, epocatechin-3-O-gallate, epicatechin, and trimeric, dimeric, and tetrameric procyanidins (20). These properties of GSE have been attributed to the antioxidative activity of citrus flavonoids.

In a previous study, Poimenidou *et al*. (21) reported that combined treatment of commercial sanitizers with natural antimicrobials was more effective for pathogen reduction than the application of single treatments. Therefore, combination of natural antimicrobials, such as organic acids and GSE, could be effective in the reduction of foodborne pathogens. Hence, this study was performed to investigate the combined effect of natural antimicrobials (MA and GSE) for inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on fresh-cut lettuce.

Materials and Methods

Bacterial strains Strains of *E. coli* O157:H7 (ATCC 43895, ATCC 35150, and ATCC 43894), *S.* Typhimurium (ATCC 19585, ATCC 6994,

and ATCC 14028), and *L. monocytogenes* (ATCC 7644, ATCC 19111, and ATCC 19115) were obtained from the ATCC (Manassas, VA, USA). Stock cultures in 0.5 mL of tryptic soy broth (TSB; Difco, Flanklin Lakes, NJ, USA) and 0.5 mL of 50% glycerol were stored at 80°C. Before use, all strains were separately grown in TSB (Difco) at 37°C. Then, the cultures were incubated at 37°C for 24 h and stored at 4°C.

Preparation of culture cocktail for artificial inoculation Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was cultured in 10 mL TSB at 37°C for 24 h. All cultures were separately centrifuged at 4,000× *g* for 15 min at 4°C and the supernatants were discarded. Cell pellets were washed twice in 0.85% saline solution (8.5 g/L sodium chloride; Sigma-Aldrich, St. Louis, MO, USA) and resuspended (7). Then, the three strains of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were combined to construct a cocktail with approximately 10^5–10^6 CFU/mL.

Artificial inoculation of fresh-cut lettuce Lettuce was purchased from a local market (Seoul, Korea) and stored prior to the experiment. The lettuce leaves were cut into 3×3 cm pieces using a sterile knife. For inoculation, 0.1 mL of the mixed strain cocktail (*E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*) was added to 25 g of lettuce (7). After inoculation, the lettuces were kept at room temperature in a laminar flow for 20 min to allow attachment (22). This procedure resulted in initial pathogen inoculum levels. The initial inoculum levels of *E. coli* O157:H7 and *S.* Typhimurium were 10^5 CFU/g, and for *L. monocytogenes*, the level was 10^4 CFU/g.

Preparation of sanitizing agents 1% w/v MA (D-L MA, 99%, Sigma-Aldrich), 0.5% w/v GSE (Seoul Food R&D, Seongnam, Korea), and combined treatment (1% MA+0.5% GSE) were used. Inoculated lettuce was dipped for 5 min into 1% w/v MA (Sigma-Aldrich), 0.5% w/v GSE, and its combined treatment prepared using sterile distilled water. From each treated sample, 25 g was aseptically placed in a sterile whirl-pak (19x30 cm; Nasco, Fort Atkinson, WI, USA). Duplicate packed samples were prepared and stored at 5°C for 14 days for periodic enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*.

Microbiological analysis For counting of pathogens, inoculated sliced lettuces were aseptically transferred into a stomacher bag and homogenized with 225 mL sterile 0.85% (wv) saline solution for 2 min using a stomacher (Laboratory Blender Stomacher 400; Seward Ltd., Worthing, UK). The stomached samples were serially diluted and plated onto eosin methylene blue agar (EMB) to enumerate *E. coli* O157:H7, xylose lysine desoxycholate agar (XLD) to count *S.* Typhimurium, and Oxford agar base (OAB) to enumerate *L. monocytogenes* (15,23). All plates were incubated at 37°C for 24–48 h, and then typical colonies were counted.

Statistical analysis All experiments were replicated three times. Reported plate count data are represented as mean±standard deviation. CFU data were transformed to log CFU/g. Data were analyzed by the general linear model of the Statistical Analysis System (version 9.1, SAS Institute, Cary, NC, USA). SAS was used for the analysis of variance (ANOVA; Duncan's multiple range test) to determine the significant effect (p<0.05) of sanitizing agents (i.e., MA and GSE) and their combination (MA+GSE) (7).

Results and Discussion

Table 1 shows the log reductions of *E. coli* O157:H7 artificially inoculated on lettuce and treated with MA, GSE, and MA+GSE. *E. coli* O157:H7 was decreased significantly (p<0.05) with MA, GSE, and MA+GSE treatment during storage for 14 days at 5°C. *E. coli* O157:H7 populations were reduced to 1.02, 0.67, and 0.46 log CFU/g, respectively, in the three treatment conditions. All treatments showed more than 4.4 log reductions. Therefore, it was concluded that MA, GSE, and MA+GSE were effective reduction methods for *E. coli* O157:H7 on lettuce during storage at 5°C.

Table 2 shows the survival of *S.* Typhimurium on lettuce. *S.* Typhimurium was statistically significantly reduced compared with control to 0.68, 0.88, and 0.16 log CFU/g, resulting in more than 4.1 log CFU/g reductions with MA, GSE, and MA+GSE treatments during storage for 14 days at 5°C, respectively.

Table 3 shows the effects of sanitizers against *L. monocytogenes*

Table 1. Effect of MA, GSE, and combined treatment against *E. coli* O157:H7 artificially inoculated on lettuce[1)]

Concentration	Population ($\log_{10}$ CFU/g) by storage time							
	0 Day	1 Day	2 Day	3 Day	5 Day	7 Day	10 Day	14 Day
Water	5.41±0.15 Aa	5.22±0.17 Aa	5.02±0.07 Aa	5.13±0.27 Aa	4.79±0.31 Aa	4.82±0.08 Aa	3.72±0.39 Ba	4.06±0.71 Ba
MA[2)] 1%	5.33±0.18 Aa	3.73±0.34 Bb	3.75±0.6 Bb	3.08±0.54 Bb	2.59±0.14 Bb	2.77±0.19 Bb	2.51±0.31 Bab	1.02±1.77 Cb
GSE[3)] 0.5%	5.30±0.13 Aa	4.67±2.34 Aab	2.15±1.25 Bc	2.34±0.09 Bb	2.08±1.31 Bb	1.79±1.33 Bb	1.51±2.14 Bb	0.67±1.17 Bb
MA+GSE[4)]	5.40±0.17 Aa	1.91±1.44 Bc	1.51±0.91 Bc	0.99±0.86 Bc	1.58±1.39 Bb	1.63±0.33 Bb	1.07±1.2 Bb	0.46±0.55 Bb

Different capital letters indicate significant differences (p<0.05) among storage times for each treatment; different lower case letters show significant differences (p<0.05) among treatments for each storage time.

[1)]Mean±standard deviation obtained in two experiments, one of two experiments in duplicate (n=3).

[2)]MA=1% malic acid.

[3)]GSE=0.5% grapefruit seed extract.

[4)]Combined=1% malic acid+0.5% grapefruit seed extract.

Table 2. Effect of MA, GSE, and combined treatment against *S.* Typhimurium artificially inoculated on lettuce[1)]

Concentration	Population (log_{10} CFU/g) by storage time							
	0 Day	1 Day	2 Day	3 Day	5 Day	7 Day	10 Day	14 Day
Water	4.95±0.26 Aa	4.50±0.06 Ba	3.81±0.16 Ca	3.68±0.32 Ca	3.48±0.27 CDa	3.18±0.28 Da	3.19±0.24 Da	3.35±0.30 CDa
MA[2)] 1%	4.46±0.18 Aab	2.59±0.66 Bbc	2.53±0.43 Bc	2.41±0.52 Bbc	1.25±1.09 Cbc	0.84±0.62 Cb	0.92±0.11 Cb	0.68±0.60 Cb
GSE[3)] 0.5%	4.43±0.03 Ab	3.70±0.44 ABab	3.20±0.27 ABCb	2.87±0.72 BCab	2.08±0.59 CDb	1.76±1.52 CDEab	0.51±0.89 Eb	0.88±0.84 DEb
MA+GSE[4)]	4.87±0.40 Aab	1.80±1.22 Bc	1.95±0.01 Bd	1.68±0.72 BCc	1.30±0.65 CDc	0.10±0.17 Db	0.26±0.24 Db	0.16±0.28 Db

Different capital letters indicate significant differences (p<0.05) among storage times for each treatment; different lower case letters show significant differences (p<0.05) among treatments for each storage time.
[1)]Mean±standard deviation obtained in two experiments, one of two experiments in duplicate (n=3).
[2)]MA=1% malic acid.
[3)]GSE=0.5% grapefruit seed extract.
[4)]MA+GSE=1% malic acid+0.5% grapefruit seed extract.

Table 3. Effect of MA, GSE, and combined treatment against *L. monocytogenes* artificially inoculated on lettuce[1)]

Concentration	Population (log_{10} CFU/g) by storage time							
	0 Day	1 Day	2 Day	3 Day	5 Day	7 Day	10 Day	14 Day
Water	4.21±0.19 Aa	4.04±0.17 Aa	4.01±0.27 Aa	3.78±0.47 ABCa	3.27±0.32 Ca	3.92±0.12 ABa	3.44±0.38 BCa	3.69±0.13 ABCa
MA[2)] 1%	3.86±0.38 Aa	3.27±0.91 ABa	2.22±1.19 BCab	2.42±0.81 ABb	1.89±0.49 BCDb	1.99±1.02 BCDb	0.75±0.65 CDb	0.65±0.91 Db
GSE[3)] 0.5%	3.90±0.08 Aa	3.51±0.08 ABa	2.53±1.36 BCab	1.79±0.16 CDb	1.02±1.05 DEb	1.36±0.21 CDEbc	0.55±0.95 DEb	0.39±0.36 Eb
MA+GSE[4)]	4.07±0.27 Aa	1.62±0.14 Bb	1.03±0.97 Bb	0.77±0.40 Bc	0.76±0.36 Bb	0.72±0.73 Bc	0.68±0.36 Bb	0.25±0.24 Bb

Different capital letters indicate significant differences (p<0.05) among storage times for each treatment; different lower case letters show significant differences (p<0.05) among treatments for each storage time.
[1)]Mean±standard deviation obtained in two experiments, one of two experiments in duplicate (n=3).
[2)]MA=1% malic acid.
[3)]GSE=0.5% grapefruit seed extract.
[4)]MA+GSE=1% malic acid+0.5% grapefruit seed extract.

on inoculated lettuce at 5°C during storage for up to 14 days. *L. monocytogenes* was decreased significantly (p<0.05) with MA, GSE, and MA+GSE treatments for 14 days at 5°C. Populations of *L. monocytogenes* were reduced to 0.65, 0.39, and 0.25 log CFU/g, respectively.

In a previous study, treatment with 1% MA for 5 min resulted in significant decrease in the number of *E. coli* O157:H7 (2.11 log CFU/g), *S.* Typhimurium (1.78 log CFU/g), and *L. monocytogenes* (1.95 log CFU/g) on lettuce (2). Ramos-Villarroel *et al*. (24) indicated that *E. coli* was reduced with 2% MA treatment from 1.73 to 1.82 log CFU/g during 15 days of storage. According to these authors, 1% MA was more effective than propionic acid, 1% acetic acid, 1% lactic acid, and 1% citric acid on lettuce. Sagong *et al*. (15) reported that MA was more effective than lactic acid and citric acid when lettuce-inoculated *E. coli* O157:H7 and *L. monocytogenes* were treated with 1% organic acids for 5 min, while MA was similar to citric acid in reducing *S*. Typhimurium numbers. Several studies have reported that GSE is a natural effective bactericidal extract (19,25). Xu *et al*. (1) reported that 0.1% GSE exhibited reductions of 0.5–0.8 log CFU/g. GSE contains flavonoids, catechins, minerals, tocopherols, and procyanidins. It has been revealed that the extract reduce the cell membrane through the microbial uptake by interrupting enzymatic activities (18,26). Till date, to our knowledge, there are no studies on the combined effect of MA and GSE on *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* in lettuce.

In this study, experiments were performed to compare the effectiveness of MA, GSE, and MA+GSE in reducing the populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on fresh-cut lettuce. Significant differences (p<0.05) in *E. coli* O157:H7 were found between the lettuces treated with MA (3.73 log CFU/g) and MA+GSE (1.91 log CFU/g) on day 1. However, MA+GSE showed a significantly higher reduction (p<0.05) than that shown by MA. Our results demonstrated that the MA+GSE combination greatly improves the reduction effectiveness than MA and GSE alone against *E. coli* O157:H7 on lettuce. After 1 day, MA and MA+GSE showed significantly (p<0.05) different reductions compared with control. However, MA+GSE exhibited more reduction (0.58 log) of *S.* Typhimurium compared to MA on day 2. These results showed that MA+GSE caused significantly higher reductions (p<0.05) than the respective single treatments (MA and GSE). *L. monocytogenes* was greatly reduced to 1.62 log CFU/g by MA+GSE combined treatment but moderately reduced to 3.27 log CFU/g and 3.51 log CFU/g by MA treatment and GSE treatment, respectively. The treatments showed no statistically significant reductions (p>0.05) on day 1 storage, but MA+GSE showed significantly higher reduction efficiency. Hence, it was concluded that the MA+GSE combined treatment is a more effective intervention method for reduction of foodborne pathogens on fresh-cut lettuce stored at 5°C.

All treatments were capable of effectively reducing the foodborne pathogens on fresh-cut lettuce. The reductions were approximately

3.56–4.95 log CFU/g. The combined treatment had more antimicrobial effect than MA and GSE alone treatments on *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on lettuce at day 1 or 2. MA+GSE significantly reduced the number of *E. coli* O157:H7 and *L. monocytogenes* (p<0.05) when compared to MA and GSE on day 1. Regarding *S*. Typhimurium, significant difference was also observed with MA+GSE combined treatment compared with MA and GSE during 1–3 days of storage.

The use of all treatments resulted in similar reductions (4.39, 4.0, and 3.5 log CFU/g) of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* during storage for 14 days, respectively. However, rapid reduction was obtained for *E. coli* O157:H7 and *S*. Typhimurium compared to *L. monocytogenes* with MA treatment. Statistically significant levels of reduction against *E. coli* O157:H7 and *L. monocytogenes* were obtained on storage day 1 with each treatment of MA, GSE, and MA+GSE, and MA+GSE showed the greatest reduction. Noticeable differences in reduction of *S*. Typhimurium were observed for samples treated with MA, GSE, and MA+GSE on day 3.

The antimicrobial effect of MA was greater than that of GSE on day 1 for *E. coli* O157:H7. Eswaranandam *et al*. (17) reported that acids of undissociated molecules enter into the bacterial cell. Among the organic acids, MA and lactic acid have smaller molecules than those of others. The acid that enters into the cell is dissociated into protons and anions. The protons inhibit glycolysis and active transport (27). However, the antimicrobial action of organic acids depends upon several factors, including reduction in pH, ratio of undissociated species of the acid, chain length, and cell physiology and metabolism (28).

Acknowledgments This work was supported by a program of High Value-added Food Technology Development of Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) [grant number 313032-03-3-HD020, 113016-03-3-HD020] and “Development of rapid detection system for foodborne pathogens to strengthen the food safety and to promote the seafoods consumption” funded by the Ministry of Oceans and Fisheries, Korea.

Disclosure The authors declare no conflict of interest.

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