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Short communication

Synergistic effect of carvacrol and ohmic heating for inactivation of *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*, and MS-2 bacteriophage in salsa

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ABSTRACT

Foodborne outbreaks have still been reported worldwide, and acid resistant foodborne pathogens are biological hazards in salsa. We investigated the combination effect of carvacrol and ohmic heating for inactivation of foodborne pathogens in salsa. Salsa samples were subjected to carvacrol, ohmic heating, and the combination treatment to identify any synergistic effect. Quality aspects of salsa such as color and lycopene content were also observed after each treatment. The synergistic bactericidal effect of combination treatment was observed for *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes*. A synergistic virucidal effect of combination treatment was also observed for MS-2 bacteriophage, which is a surrogate for human norovirus. Moreover, L* and a* values were improved by combination treatment compared to ohmic heating. Therefore, the combination treatment of carvacrol and ohmic heating could be used effectively to process salsa without incurring quality degradation.

1. Introduction

Foodborne outbreaks have still been reported worldwide even though food technology is continuously being developed to help ensure food safety. The U. S. Centers for Disease Control and Prevention (CDC) reported that one out of six people get sick, 128,000 are hospitalized, and 3000 die of foodborne illness in the United States each year (CDC, 2010). Moreover, new challenges such as demographic changes, climate changes, globalization of trade in food, and consumer's preferences for minimally processed foods have arisen (Doyle et al., 2015). Most microorganisms cannot survive at low pH an extended time, and acidic food has been considered as biologically safe (Song, Sung, & Kang, 2015). However, not only have many researchers reported that foodborne pathogens such as *E. coli* O157:H7, *L. monocytogenes*, and norovirus can survive at low pH for a long time, but also several outbreaks have been reported involving acidic foods such as juices and salsa (Lee, Kim, & Kang, 2015; Raghubeer, Dunne, Farkas, & Ting, 2000; Vimont, Fliss, & Jean, 2015). Therefore, it is necessary to ensure microbiological safety in acidic foods while maintaining quality aspects such as color, nutrient content, and flavor.

Carvacrol is a major component of certain essential oils, and possesses potential as a natural agent for food preservation by means of its antimicrobial properties (Helander et al., 1998; Lambert, Skandamis, Coote, & Nychas, 2001; Solórzano-Santos & Miranda-Novales, 2012). The possible antimicrobial mechanism of carvacrol is to increase permeability of the cytoplasmic membrane or cause oxidative damage to DNA in bacteria, whereas it targets capsids and subsequent RNA in viruses (Chueca, Pagán, & García-Gonzalo, 2014; Gilling, Kitajima, Torrey, & Bright, 2014; Ultee, Kets, & Smid, 1999). Resistance of microorganisms increase in the food matrix, and thus a high concentration of carvacrol is needed to ensure microbiological safety in food. Because such a high concentration of carvacrol results in undesirable flavor, the concentration should be reduced. Several research have recently reported

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that combining carvacrol with other treatments such as medium chain fatty acids, mild heat, and pulsed electric fields could reduce the concentration of carvacrol needed (Ait-Ouazzou, Espina, García-Gonzalo, & Pagán, 2013; Kim & Rhee, 2016).

Ohmic heating facilitates uniform and rapid heating inside of food by means of electric current through food components (Ramaswamy, Marcotte, Sastry, & Abdelrahim, 2014). Quality aspects of food could be improved by ohmic heating compared to conventional heating (Leizerson & Shimoni, 2005). In particular, solids and liquids are heated simultaneously with ohmic heating, and solid-liquid food such as salsa could be processed effectively (Lee, Ryu, & Kang, 2013). Nevertheless, there still exist limitations. First, the heating rates of solids and liquids would differ significantly if the electrical conductivity of solids and liquids are different (Ye, Ruan, Chen, & Doona, 2004). Secondly, quality aspects of solid-liquid food could be degraded due to severe heat damage. To overcome these limitations, combination treatments of ohmic heating with other technologies or chemicals have been investigated recently (Choi, Lee, Kim, & Jun 2015; Moreno et al., 2016). However, to the best of our knowledge, the combination treatment of carvacrol and ohmic heating has not been reported.

In the present study, we investigated the combination effect of carvacrol and ohmic heating in salsa. First, a synergistic bactericidal effect of combination treatment was identified for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. Secondly, we identified a synergistic virucidal effect of combination treatment for MS-2 bacteriophage. Finally, quality aspects including color and lycopene content were compared between ohmic and combination treated salsa. Even though widespread outbreaks caused by human norovirus have been reported worldwide, it is still not possible to cultivate human norovirus in vitro (Moore, Goulter, & Jaykus, 2015). Feline calicivirus (FCV), murine norovirus (MNV), and MS-2 bacteriophage are used as representative human enteric virus surrogates. MS-2 bacteriophage was used as a surrogate for human norovirus in the present study.

2. Materials and methods

2.1. Bacterial cultures and cell suspension

Three strains each of *E. coli* O157:H7 (ATCC 35150 (American Type Culture Collection, Rockville, MD), ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), and *L. monocytogenes* (ATCC 19111, ATCC 19115, ATCC 15313) were obtained from the bacteria culture collection of Seoul National University (Seoul, Korea). Stock and working cultures were prepared according to a previously described method (Kim & Kang, 2015a). A single colony cultivated from frozen stocks on tryptic soy agar (TSA; Difco, Becton, Dickinson, Sparks, MD) was inoculated into 5 ml of tryptic soy broth (TSB; Difco, Becton, Dickinson, Sparks, MD), incubated at 37 °C for 24 h, collected by centrifugation at 4000 × g for 20 min at 4 °C, and washed three times with 0.2% peptone water (PW; Bacto, Becton, Dickinson, Sparks, MD). The final pellets were resuspended in 0.2% PW, corresponding to approximately 10^8–10^9 CFU/ml. Afterwards, suspended pellets of the three pathogens were combined to comprise a mixed culture cocktail containing approximately equal numbers of cells of each strain of *E. coli* O157:H7 (10^7 CFU/ml), *S.* Typhimurium (10^7 CFU/ml), and *L. monocytogenes* (10^6 CFU/ml).

2.2. Sample preparation and inoculation

Pasteurized salsa (pH 3.7) was purchased at a local grocery store (Seoul, South Korea). The salsa contained no chemical preservatives and included tomatoes, jalapeño peppers, onions, garlic, and distilled vinegar. Twenty five g of salsa were put into the ohmic heating chamber at room temperature (22 ± 1 °C). A mixed culture cocktail (0.2 ml) was inoculated into 25 g of each prepared salsa sample before treatment.

2.3. Carvacrol, ohmic heating, and combination treatment

Inoculated salsa samples were treated with carvacrol, ohmic heating, and carvacrol + ohmic (combination) treatment. Purified carvacrol (98%) was purchased from Sigma-Aldrich (St. Louis, MO). Final concentration of carvacrol in salsa was adjusted to 1.3 mM (0.2 mg/g) which showed less than 0.2 log reduction for *E. coli* O157:H7 (Ait-Ouazzou et al., 2013). Ohmic heating was carried out in a previously described apparatus (Kim & Kang, 2015b). Salsa without or with carvacrol was subjected to pulsed ohmic heating (0.05 duty ratio, 60 Hz) with fixed electric field strength of 12.1 V_{rms}/cm. Addition of carvacrol did not significantly ($p > 0.05$) influence the temperature history (Fig. 1). Samples were taken at regular intervals, and populations of surviving microorganisms were enumerated.

2.4. Bacterial enumeration

For microbial enumeration, each treated 25 g sample was immediately transferred into a sterile stomacher bag (Labplas, Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of sterile 0.2% peptone water and homogenized for 2 min using a stomacher (Easy Mix; AES Chemunex, Rennes, France). After homogenization, 1 ml samples were 10-fold serially diluted with 9 ml of sterile 0.2% peptone water and 0.1 ml of stomached or diluted samples were spread plated onto each selective or non-selective medium. Sorbitol MacConkey (SMAC) agar (Difco), xylose lysine deoxycholate (XLD) agar (Difco), and Oxford agar base (OAB; Difco) with antimicrobial supplement (Bacto Oxford antimicrobial supplement; Difco) were used as selective media for enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. All plates were incubated at 37 °C for 24–48 h before counting colonies characteristic of the pathogens. The overlay method (OV), developed by (Hartman, Hartman, & Lanz, 1975), and verified by (Lee & Kang, 2001) was used to recover sub-lethally injured cells of *S.* Typhimurium and *L. monocytogenes*. After cells were resuscitated on tryptic soy agar (TSA; Difco) at 37 °C for 2 h, plates were overlaid with 7–8 ml of XLD and OAB for *S.* Typhimurium (XLD-OV) and

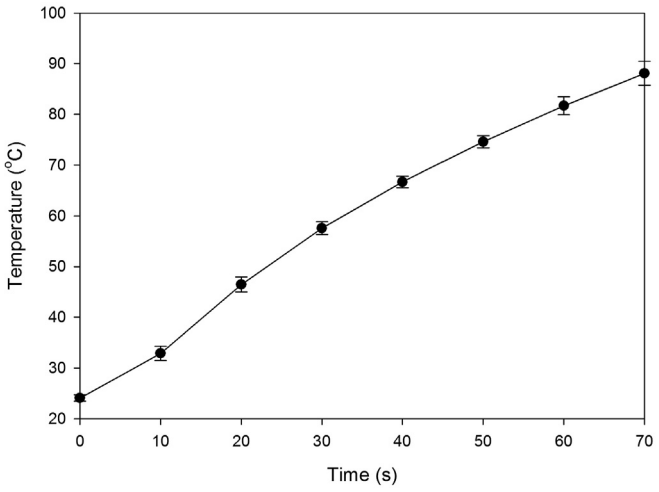

Fig. 1. Temperature history of salsa subjected to ohmic heating without carvacrol. The temperature history was not significantly influenced by addition of 1.3 mM carvacrol.

L. monocytogenes (OAB-OV), respectively. The plates were further incubated for 22–46 h at 37 °C before colonies were counted. Phenol red agar base with 1% sorbitol (SPRAB; Difco) was used to recover injured cells of *E. coli* O157:H7. After incubation at 37 °C for 24 h, typical white colonies characteristic of *E. coli* O157:H7 were enumerated. Randomly selected isolates from SPRAB plates were subjected to serological confirmation as *E. coli* O157:H7 (RIM, *E. coli* O157:H7 latex agglutination test; Remel, Lenexa, KS), because SPRAB is not typically used as aselective agar for enumerating *E. coli* O157:H7.

2.5. Inactivation and enumeration of MS-2 bacteriophage

Stock cultures of MS-2 bacteriophage ATCC 15597 were prepared by a previously described method (Cho, Cates, & Kim, 2011). Treatment conditions for MS-2 bacteriophage were not different from that for bacterial pathogens except treatment time. Each treated sample was immediately transferred into a sterile stomacher bag and homogenized with a stomacher as described previously. For enumeration, the soft-agar overlay (double-agar layer) plaque assay method was used (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009). Tryptic soy agar (TSA; Difco) was used for the bottom agar layer and Lactose Broth (LB; Difco) with 7 g/L agar was used as the top agar medium.

2.6. Color, lycopene measurement

The color and lycopene content of untreated (control) and treated samples were measured. Treatment times for ohmic heating (75 s) and combination treatment (50 s) were chosen from preliminary experiments to ensure 5 log reductions of all three bacterial pathogens. All treated samples were cooled immediately in a crushed ice water mixture. Color values were measured with a Minolta colorimeter (model CR400; Minolta Co., Osaka, Japan). The values for L^*, a^*, and b^* were measured to evaluate color changes of salsa. The parameter L^* is a measure of lightness, a^* is an indicator of redness, and b^* is a measure of yellowness. Lycopene content in salsa was measured according to the previously described method (Lee et al., 2013).

2.7. Statistical analysis

All experiments were duplicate-plated and replicated three times. All data were analyzed by the analysis of variance procedure of the Statistical Analysis System (SAS Institute, Cary, NC) and mean values were separated using Duncan's multiple-range test. Significant differences in the processing treatments were determined at a significance level of $p = 0.05$.

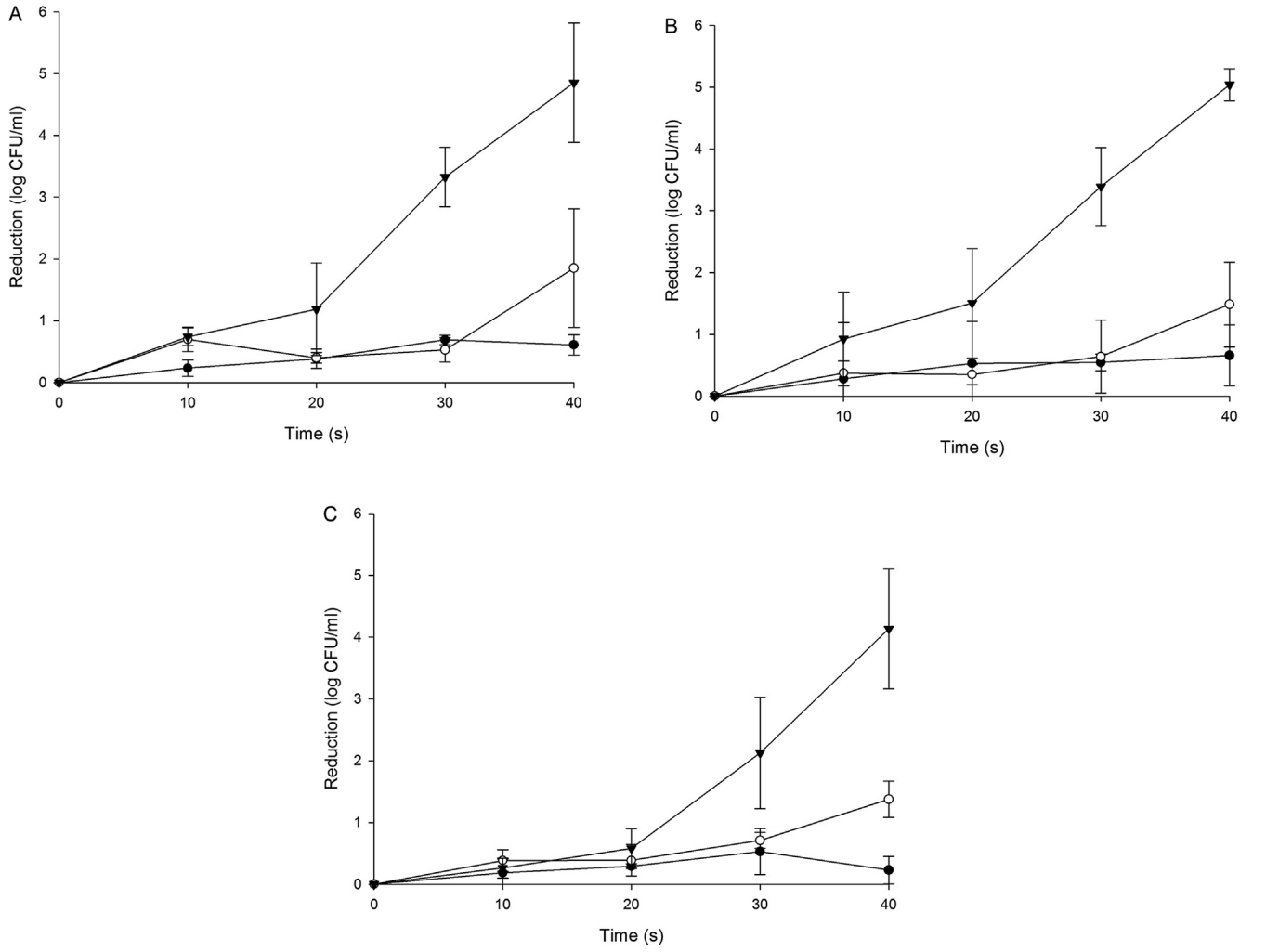

Fig. 2. Reduction (log CFU/g) of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) in salsa subjected to 1.3 mM carvacrol (●), 12.1 V_{rms}/cm ohmic heating (○), and combination treatment (▼). Selective media were used to enumerate populations of bacterial cells. Detection limit was 1.0 log CFU/g.

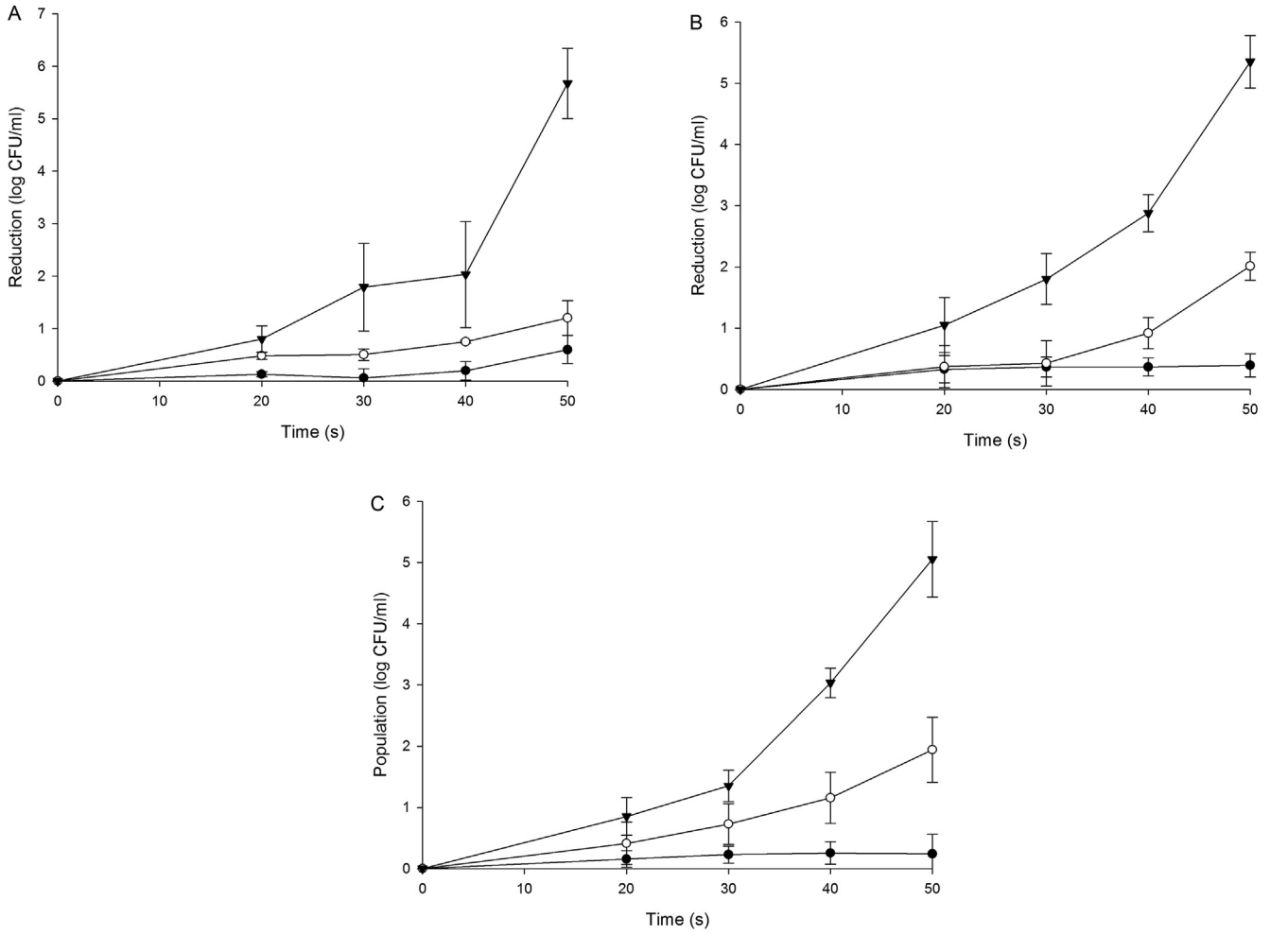

Fig. 3. Reduction (log CFU/g) of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) in salsa subjected to 1.3 mM carvacrol (●), 12.1 V_{rms}/cm ohmic heating (○), and combination treatment (▼). Resuscitation media were used to recover sub-lethally injured bacterial cells. Detection limit was 1.0 log CFU/g.

3. Results

3.1. Synergistic bactericidal effect of carvacrol and ohmic heating

The combination treatment of carvacrol and ohmic heating exhibited a synergistic effect for inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on both selective and resuscitation media (Fig. 2 and Fig. 3). The synergistic effect was observed after 30 and 40s treatment on selective and resuscitation media, respectively. When enumerated on resuscitation media, all three pathogens were inactivated by less than 0.6 and 2.1 log after 50 s treatment with carvacrol and ohmic heating, respectively. On the other hand, all three pathogens were inactivated by over 5.0 log following 50 s combination treatment. The synergistic effect was enhanced with increasing treatment time for all three pathogenic bacteria.

3.2. Synergistic virucidal effect of carvacrol and ohmic heating

The combination treatment of carvacrol and ohmic heating exhibited a synergistic effect for inactivation of MS-2 bacteriophage (Table 1). The synergistic effect was observed after 100 s treatment. Reduction (log PFU/g) of MS-2 bacteriophage after 100 s combination treatment was 6.20 whereas reductions by carvacrol and ohmic heating were 0.16 and 4.23, respectively. On the other hand, reductions (log PFU/g) of MS-2 bacteriophage were not significantly different ($p > 0.05$) between ohmic heating and combination treatment up through 60–90 s.

3.3. Effect of combination treatment on product quality

Quality aspects (L* and a*) of salsa were improved by combination treatment compared to ohmic heating (Table 2). Quality aspects (L*, a*, b*, and lycopene content) of combination treated samples were not significantly different from those of untreated samples ($p > 0.05$). On the other hand, a* value of ohmic treated

Table 1
Reduction (log PFU/g) of MS-2 bacteriophage in salsa subjected to 1.3 mM carvacrol, 12.1 V_{rms}/cm ohmic heating, and combination treatment[a,b].

Time (s)	Carvacrol	Ohmic heating	Combination
60	0.10 ± 0.14 Aa	1.4 ± 0.23 Ba	1.7 ± 0.11 Ba
80	0.11 ± 0.14 Aa	2.8 ± 0.33 Bb	2.7 ± 0.59 Bab
90	0.03 ± 0.07 Aa	3.3 ± 0.67 Bbc	3.2 ± 0.45 Bb
100	0.16 ± 0.11 Aa	4.2 ± 0.73 Bc	6.2 ± 0.77 Cc

Mean values ± standard deviation.
[a] Values in the same row followed by the same uppercase letter are not significantly different ($p > 0.05$).
[b] Values in the same column followed by the same lowercase letter are not significantly different ($p > 0.05$).

Table 2
Quality aspects of salsa untreated and treated with 12.1 Vrms/cm ohmic heating or combination treatment with 1.3 mM carvacrol[a,b].

Treatment	Color			Lycopene content (mg/kg tissue)
	L*	a*	b*	
Untreated	30.38 ± 1.55 AB	8.93 ± 2.37 A	12.97 ± 3.94 A	78.29 ± 4.94 A
Combination	31.62 ± 1.13 A	7.48 ± 0.67 A	10.13 ± 1.03 A	78.19 ± 3.84 A
Ohmic heating	29.21 ± 0.37 B	5.81 ± 0.29 B	9.69 ± 2.08 A	81.52 ± 4.01 A

Mean values ± standard deviation.
[a] Values in the same column followed by the same uppercase letter are not significantly different ($p > 0.05$).
[b] Treatment times for ohmic heating and combination treatment were 75 s and 50 s, respectively.

samples were significantly different from untreated samples ($p < 0.05$).

4. Discussion

This research identified the effect of combining carvacrol and ohmic heating on pathogen inactivation. The synergistic bactericidal effect of ohmic heating and carvacrol was observed in the present study. Because the bactericidal effect of ohmic heating is mainly due to thermal inactivation (Kim & Kang, 2015c), the primary synergistic mechanism would be the combination effect of carvacrol with heat as reported by previous researchers (Ait-Ouazzou et al., 2013; Juneja & Friedman, 2008). However, there have also been research reporting non-thermal bactericidal effects of ohmic heating (Park & Kang, 2013; Somavat, Mohamed, Chung, Yousef, & Sastry, 2012). Electroporation of the bacterial cell membrane was pointed out as a prime inactivation mechanism of the non-thermal effect of ohmic heating (Park & Kang, 2013). Because carvacrol is also known to permeabilize bacterial cell membranes (Ultee et al., 1999), the non-thermal effect of ohmic heating and carvacrol may work together to destroy bacterial cell membranes. Previous research also indicated that the combination treatment of carvacrol with other non-thermal treatments targeting cell membranes showed a synergistic bactericidal effect. Ait-Ouazzou et al. (2013) reported the synergistic effect of pulsed electric field (PEF) and carvacrol for inactivation of *E. coli* O157:H7 in various juices. Kim and Rhee (2016) also reported a synergistic bactericidal effect of medium chain fatty acids (MCFA) and essential oils. Therefore, the primary target of synergistic bactericidal effect in the present study would be cell membrane destruction.

Individual treatment of carvacrol was not sufficient to inactivate MS-2 bacteriophage in the present study. Previous studies investigating the virucidal effect of essential oil also showed only marginal effect. Su and D'Souza (2011) reported only 1.13–1.60 log reductions of MS-2 bacteriophage after 2 h treatment of grape seed extract. Gilling et al. (2014) also reported only 1.95 log reduction of MNV following 1 h treatment with 0.25% carvacrol. Compared to these individual treatments, the combination of carvacrol and ohmic heating presented a significant synergistic effect (6.2 log reduction) for inactivation of MS-2 bacteriophage with significantly reduced treatment time (100 s) in the present study. The virucidal mechanism of essential oil is known to target capsids and subsequent RNA (Gilling et al., 2014), but no research have been reported investigating the virucidal effect of ohmic heating. Therefore, further study is needed to identify the mechanism of the synergistic virucidal effect of ohmic heating and carvacrol.

Because the temperature history of salsa was not significantly influenced ($p > 0.05$) by addition of carvacrol in the present study, heat damage of salsa is dependent on treatment time. Seventy-five seconds was required to inactivate all three pathogens by > 5 log reduction with ohmic heating whereas only 50 s was needed for the combination treatment. Temperature increase of the combined treatment was reduced by ca. 20 °C compared to individual ohmic heating treatment. Severe heat damage by ohmic heating would result in a significant difference ($p < 0.05$) of a* compared to untreated salsa whereas heat damage by the combination treatment was not significant ($p > 0.05$). Sung and Kang (2014) also reported that quality of salsa could be significantly degraded by severe heat damage. Therefore, the combination treatment could be used as alternative technology to improve the quality of salsa instead of individual thermal treatment.

In conclusion, combination treatment of carvacrol and ohmic heating showed the synergistic bactericidal and virucidal effect in salsa. Moreover, quality aspects of salsa were improved by combination treatment compared to ohmic heating. Therefore, combination treatment could be used effectively to process salsa with reduced treatment time. For further study, comparing the combination treatment of ohmic heating with other essential oil compounds is required to identify the most effective combination treatment.

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