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ORIGINAL PAPER

Synergistic Effects of Combined Chlorine and Vitamin B_1 on the Reduction of Murine Norovirus-1 on the Oyster (*Crassostrea gigas*) Surface

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Abstract
This study investigated the synergistic effects of combined chlorine (200, 500, 700, and 1000 ppm) and vitamin B_1 (1000, 2000, and 3000 ppm) on the murine norovirus-1 (MNV-1), a human norovirus (NoV) surrogate, on oyster surface. Vitamin B_1 slightly reduced MNV-1 (0.04–0.3 log-reduction), whereas chlorine significantly reduced MNV-1 (0.4–1.0 log-reduction). The combined chlorine and vitamin B_1 resulted in a 0.52–1.97 log-reduction of MNV-1. The synergistic reduction in the MNV titer was not dependent on the concentrations of chlorine and vitamin B_1, and it ranged between 0.08 and 1.03 $\log_{10}$ PFU/mL. The largest synergistic reduction observed was for the combined 700 ppm chlorine and 1000 ppm vitamin B_1. The pH and mechanical texture of the oysters were not significantly changed by the combined 0–1000 ppm chlorine and 3000 ppm vitamin B_1. The overall sensory acceptability were significantly ($P < 0.05$) reduced in oysters treated with 1000 ppm chlorine and 3000 ppm vitamin B_1 than in those treated with 0–700 ppm chlorine and 3000 ppm vitamin B_1. This study suggests that the combined 700 ppm chlorine and 3000 ppm vitamin B_1 could potentially be used to reduce NoV on oyster surface without causing concomitant changes in the mechanical texture, pH, or sensory qualities of the oysters.

Keywords MNV-1 · Chlorine · Vitamin B_1 · Synergistic effects · Oyster

Introduction

Human noroviruses (NoVs) belonging to the family *Caliciviridae* and the genus *Norovirus* comprise a group of positive-sense, single-stranded, polyadenylated RNA viruses. NoVs are considered as the most frequent and common foodborne viruses responsible for sporadic and epidemic gastroenteritis outbreaks worldwide (Siebenga et al. 2009). NoVs exhibit a high particle stability and infectivity, and there is no specific or effective therapy against NoV infection (Anonymous 2018). Therefore, NoVs are a major public health problem worldwide. NoVs cannot proliferate outside of their hosts, which hinders studies on NoV control measures. Therefore, murine norovirus (MNV) has recently emerged as a model for studies on NoVs owing to its similar genetic organization and identical fecal-oral transmission route (Kingsley 2013; Wobus et al. 2004).

NoVs cause frequent and large foodborne outbreaks that impose substantial economic losses (Gibson and Ricke 2012). NoV outbreaks have often been associated with the consumption of contaminated raw oysters in all age groups worldwide (Wang and Deng 2012). Previous studies have suggested that NoV can persist in oyster tissues for weeks and cannot be effectively removed during commercial depuration (Hewitt and Greening 2004; Provost et al. 2011; Ueki et al. 2007). Cooking by heating is the most effective tool to eliminate NoVs in shellfish including oysters; however, oysters are often consumed raw and fresh raw oysters are most preferably consumed in many regions including Europe, the United States of America, Korea, and Japan. Therefore, the seafood industry needs an effective non-thermal process that

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is milder than commercial sterilization because of the high customer demand for minimally processed foods to maintain the nutritional and quality aspects of the food product.

Several single-agent disinfection and sanitization techniques have been proposed in the last decade to reduce the contamination of foods by pathogenic microorganisms. Physical sanitization and disinfection methods, such as heating (Browne and Dowds 2001) and electron beam irradiation (Sarrias et al. 2003), and chemical sanitization and disinfectant methods using chlorine, hydrogen peroxide, ethanol, and ozone (Chang et al. 2004; Wisniewsky et al. 2000) have been used to reduce the pathogenic bacterial contamination of foods. Sodium hypochlorite is the most effective chlorine compound used as a disinfectant. The bactericidal efficacy of chlorine is based on the penetration of the chemical and its oxidative action on essential enzymes in the bacterial cells (Kumar and Anand 1998; Lomander et al. 2004). Kim et al. (2005) showed that vitamin B_1 possessed antimicrobial activities against *Escherichia coli* and *Staphylococcus aureus*. Especially, Lee et al. (2010) demonstrated that treatment of rice with a sanitizer produced a greater reduction in the abundance of *Bacillus cereus* than water treatment, while a combined treatment with sanitizer and vitamin B_1 produced an even greater reduction in the abundance of *B. cereus*.

Many studies have been performed to evaluate the inactivation efficiency or synergy of combinations of chemical treatments and physical sanitization techniques such as ethanol, hydrogen peroxide, sodium hypochlorite, ozone, ultraviolet processes, and γ-irradiation compared with the same treatments applied individually. Studies of combined disinfection treatments are common in the literature (Chawla et al. 2006; Kanatt et al. 2006). Kim et al. (2011) reported that a combined disinfection treatment with chlorine and vitamin B_1 followed by γ-irradiation synergistically reduced the microbial populations in oysters and short-necked clams. Our previous study identified the combined treatment of 100 ppm chlorine and 1000 ppm vitamin B_1 as an optimum hurdle technology against *Aeromonas hydrophila* in squid (Park et al. 2012).

There is a need to further investigate the potential synergistic effects of combined treatment with chlorine and vitamin B_1 on the reduction of foodborne virus titers, especially during the washing of food. The present study therefore aimed to assess the effects of chlorine (0–1000 ppm) and vitamin B_1 (0–3000 ppm), and their combinations at different doses, on the reduction of the titers of MNV-1 as a NoV surrogate on oyster surface and evaluate the overall quality (pH, texture, and sensory attributes) of the treated oysters.

Materials and Methods

Virus Cell Culture

MNV-1 (a surrogate for NoV) was maintained in murine RAW 264.7 cells. Cells were cultured in Dulbecco's minimum essential medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 44 mM sodium bicarbonate (Sigma-Aldrich), and 1% antibiotic–antimycotic (penicillin/streptomycin; Gibco) and were seeded into 75-cm^2 culture flasks for incubation at 37 °C in a humidified incubator containing 5% CO_2. The cells were subcultured every 2 or 3 days.

Preparation of Viral Suspension

When the RAW 264.7 cell monolayers achieved 90% confluency in a 150-cm^2 tissue culture flask, the growth medium was aspirated and the monolayers were washed with phosphate-buffered saline (pH 7.4; Oxoid, Basingstoke, Hampshire, England). A 200-µL aliquot of the MNV-1 inoculum was added to the flasks, which were incubated for 30 min to allow virus adsorption. Then, 15 mL of the maintenance medium (DMEM + 2% FBS + 44 mM sodium bicarbonate + 1% antibiotic–antimycotic) was transferred to the flasks containing cells and MNV-1 and incubated at 37 °C in a 5% CO_2 atmosphere for 3 days. If cytopathic effects were observed in over 90% of cells, the virus-infected flasks were frozen and thawed three times. The viruses were released by cell lysis. The above contents were centrifuged at 1500×*g* for 10 min to remove cell debris, and the supernatant was subsequently harvested. The viruses were stored at −70 °C until further use.

Preparation of Fresh Oyster and Virus Inoculation

Fresh oysters sold as meat without the shell were purchased from a local market (Ansung, Korea). The oysters were of a medium market size, with a meat weight of approximately 7–8 g per oyster. Water present on the surface of each oyster was absorbed using paper wipes (Kimtech, Yuhan-Kimberly, Daejeon, Korea). A 200-µL aliquot of MNV-1 [6–7 $\log_{10}$ plaque-forming units (PFU)/mL] was spot inoculated onto the meat surface and gills of each whole oyster in a sterilized petri-dish. To allow the virus to attach to the surfaces and gills of the samples, the samples were placed in a 4 °C refrigerator for 1 h.

Single and Combined Chlorine and Vitamin B_1 Treatments

For the single treatments with chlorine, 4.5% chlorine (free chlorine, Shimadzu Co., Kyoto, Japan) was diluted with sterile distilled water to yield 35 mL solutions of 0, 200,

500, 700, or 1000 ppm of chlorine. Vitamin B_1 (Shinko Sci. Osaka, Japan) dissolved in 30% ethanol to a concentration of 0, 1000, 2000, or 3000 ppm was used and then immediately added to the sanitizer solution.

The inoculated samples were immersed in a single treatment of chlorine or vitamin B_1, or a combined treatment of chlorine and vitamin B_1, at room temperature for 5 min with mild agitation. For the combined treatments, the chlorine treatment (200, 500, 700, or 1000 ppm) was first conducted as a primary disinfectant, and the vitamin B_1 treatment (1000, 2000, or 3000 ppm) followed immediately as a secondary disinfectant, as described by Koivunen and Heinonen-Tanski (2005).

Synergistic Effects of Chlorine and Vitamin B_1

The MNV-1 inactivation efficacies of the combined treatments were compared with those of the individual treatments to assess any synergistic effects resulting from combined treatment with chlorine and vitamin B_1. The procedure described by Koivunen and Heinonen-Tanski (2005) was used for the combined disinfectant treatments. The combined disinfection experiments were carried out by first applying chlorine as a primary disinfectant and then applying vitamin B_1 as a secondary disinfectant. The disinfection efficacy after each treatment was determined by measuring the extent of microbial reduction. The synergistic effect values of the combined chlorine–vitamin B_1 treatments were calculated using the following equation:

$$\text{Synergistic effect value} = A - (B + C),$$

where A is the reduction in MNV-1 titer after combined chlorine and vitamin B_1 treatment, B is the reduction after chlorine treatment alone, and C is the reduction after vitamin B_1 treatment alone.

Sample Processing for Virus Recovery and Virus Titration

MNV-1 recovery from the samples was performed as described previously, with slight modifications (Son et al. 2014). Following the single and combined treatments, each contaminated sample was placed into a 50-mL centrifuge tube and 10 mL of phosphate-buffered saline (PBS) was added. The samples were subjected to thorough vortexing and shaking (300 rpm, 1 h), followed by centrifugation (10,000×*g*, 1 h, 4 °C). Supernatants were sequentially filtered using sterile 5-, 1.2-, 0.8-, and 0.45-mm filters. Each eluted viral suspension was then subjected to tenfold serial dilutions in DMEM. The virus infection titers were determined using a plaque assay. MNV-1 titration was performed as described previously, with minor modifications as suggested by Wobus et al. (2004) and Bidawid et al. (2000). Briefly, confluent RAW 264.7 cells were seeded into a 12-well plate and incubated at 37 °C with 5% CO_2 for 36 h to reach 90% confluence. Viral suspensions eluted from the samples were serially diluted in the maintenance medium (DMEM + 2% FBS + 44 mM sodium bicarbonate + 1% antibiotic–antimycotic). Serially diluted viral suspensions (100-µL aliquots) were used to inoculate the cells. After shaking the plates for 10 min (FMS2, FINEPCR, Gunpo, Korea), they were incubated at 37 °C in the presence of 5% CO_2. One hour later, 2× type II agarose (Sigma-Aldrich) supplemented with 2× DMEM was added to the inoculated cells at a volume of 1 mL per well. The plates were left at room temperature for 20 min and then incubated at 37 °C in 5% CO_2 for 2–3 days to culture MNV-1. Next, the cells were fixed with 2 mL of 3.7% formaldehyde for 4 h. Then, the formaldehyde was discarded and the agarose overlays were removed carefully with tap water. The fixed cells were stained with 0.1% (w/v) crystal violet solution (Invitrogen, Carlsbad, CA, USA) for 20 min to visualize the plaques. Viral titers were calculated as the number of PFU/mL.

Physical Quality Measurement: Texture

The center of each treated sample was compressed twice to reach 25% of its original thickness using a TA-XT Express texture analyzer with an SMSP/35 probe (Stable Micro Systems Ltd., Surrey, UK). The probe was moved over the sample as follows: pre-test speed of 3.0 mm/s, test speed of 0.5 mm/s, and post-test speed of 5.5 mm/s, with a trigger force of 5.0 g. Texture data were analyzed using Exponent Lite Express software (version 4.0.8.0) and the following parameters were recorded: hardness (g/cm^2), cohesiveness, and springiness. Samples were maintained at 4 °C after treatment and then texture analysis was performed.

Chemical Quality Measurement: pH

Each treated sample was homogenized at a 1:10 dilution (weight/volume) in sterile distilled water. The pH of each sample was measured using a pH meter (Thermo Electron Corporation, Waltham, MA, USA).

Sensory Quality Measurement: Seven-Point Hedonic Scale Test

Twenty untrained panelists who studied in the Graduate School of Food Science and Technology at Chung-Ang University, Korea, evaluated the sensory qualities of the treated samples. The samples were evaluated for color, flavor, texture, appearance, and overall acceptability by using the hedonic scale. Each sensory quality was assessed using the seven-point hedonic scale as follows: '1' for extreme dislike, not acceptable; '2' for much dislike; '3' for dislike; '4'

for neither liked nor disliked, lower limit of the acceptable range; '5' for like; '6' for much like; and '7' for extreme like, essentially free from any defect, original quality preserved. A rating higher than 5 indicated greater acceptability of the food product. Prior to sample evaluation, untrained panelists participated in orientation sessions to familiarize themselves with the attributes of the hedonic scale and the related quality assessment. The panelists independently evaluated the samples in identical environments.

Statistical Analysis

Data for MNV-1 titers expressed as logarithmic functions, log10 reduction, pH, texture, and sensory measurement were analyzed by the least significance difference method using the Pdiff option of the generalized linear model procedure in the SAS statistical software program (SAS version 9.1; SAS Institute, Cary, NC, USA). Model and parameter adequacy were considered significant at $P < 0.05$, unless otherwise noted. The means represent the average of three replicate samples, and they were considered significantly different at $P < 0.05$.

Results

Synergistic Reduction of MNV-1 by Combined Chlorine and Vitamin B_1 on Oyster Surface

To determine the viricidal effects of combined chlorine and vitamin B_1 against MNV-1 on oyster surface, the reduction of MNV-1 titers by different concentrations of chlorine and vitamin B_1 was determined (Table 1). After treatment with 200, 500, 700, and 1000 ppm chlorine alone, the MNV-1 titers were significantly ($P < 0.05$) reduced by 0.40, 0.41, 0.50, and 1.0 $\log_{10}$ PFU/mL, respectively. The MNV-1 titers were also significantly ($P < 0.05$) reduced by 0.04, 0.20, and 0.30 $\log_{10}$ PFU/g after treatment with 1000, 2000, and 3000 ppm vitamin B_1 alone, respectively. These findings indicated that the reductions in MNV-1 titers were primarily dependent on the chlorine concentration rather than the vitamin B_1 concentration. Reductions in MNV-1 titers of 1–2 $\log_{10}$ were observed following combined treatments with 200 ppm chlorine and 3000 ppm vitamin B_1; 500 ppm chlorine and 2000 ppm vitamin B_1; 500 ppm chlorine and 3000 ppm vitamin B_1; 700 ppm chlorine and 2000 vitamin B_1; 700 ppm chlorine and 3000 ppm vitamin B_1; 1000 ppm chlorine and 1000 ppm vitamin B_1; 1000 ppm chlorine and 2000 ppm vitamin B_1; and 1000 ppm chlorine and 3000 ppm vitamin B_1. The maximum reduction of MNV-1 titer detected was 1.97 $\log_{10}$ PFU/mL ($P < 0.05$) after the combined treatment with 1000 ppm chlorine and 3000 ppm vitamin B_1.

Table 2 shows the synergistic effects of chlorine and vitamin B_1 on the reduction of MNV-1 titers on the oysters. No antagonistic reduction effects of chlorine and vitamin B_1 against MNV-1 were observed for all the combined treatments, while synergistic reduction effects of chlorine and vitamin B_1 against MNV-1 were observed for all the combined treatments. Specifically, the mean synergistic reduction values for the treatments of oysters were 0.19 [calculated as $(0.08 + 0.28 + 0.23 + 0.15) \div 4$], 0.38 [calculated as $(0.34 + 0.48 + 0.49 + 0.19) \div 4$], and 0.78 [calculated as $(0.58 + 0.83 + 1.03 + 0.67) \div 4$] $\log_{10}$ PFU/mL after the combined treatments with 1000, 2000, and 3000 ppm vitamin B_1, respectively, with any concentration of chlorine. That means that for any combined treatment with chlorine, the mean synergistic reduction value showed an approximate twofold increase for each 1000-ppm increase in the vitamin B_1 concentration. These findings suggest that vitamin B_1 functions only as a synergizer, rather than as a main disinfectant in the combined treatments. The largest synergistic reduction value was 1.03 $\log_{10}$ PFU/mL ($P < 0.05$) after the combined treatment with 700 ppm chlorine and 3000 ppm vitamin B_1. The synergistic reduction effects against MNV-1 were not dependent on the concentration of chlorine or vitamin B_1 unlike the effect on the reduction of MNV-1 titers. The findings suggest that the combined treatment of 700 ppm chlorine and 3000 ppm vitamin B_1 is an ideal choice for reducing the titers of MNV-1 on oyster surface.

Table 1 Reduction of MNV-1 on oyster surface after the treatment of chlorine and vitamin B_1 alone and chlorine/vitamin B_1 mixture

Vitamin B_1 (ppm)	Mean ± (SD) reduction value ($\log_{10}$PFU/ml) Chlorine (ppm)				
	0	200	500	700	1000
0	–	$0.40 \pm 0.05^{C,d}$	$0.41 \pm 0.02^{C,d}$	$0.50 \pm 0.04^{B,d}$	$1.00 \pm 0.01^{A,d}$
1000	$0.04 \pm 0.04^{D,b}$	$0.52 \pm 0.02^{C,c}$	$0.73 \pm 0.08^{B,c}$	$0.77 \pm 0.02^{B,c}$	$1.19 \pm 0.08^{A,c}$
2000	$0.20 \pm 0.07^{D,a}$	$0.94 \pm 0.04^{C,b}$	$1.09 \pm 0.08^{B,b}$	$1.19 \pm 0.15^{AB,b}$	$1.39 \pm 0.05^{A,b}$
3000	$0.30 \pm 0.02^{E,a}$	$1.28 \pm 0.04^{D,a}$	$1.54 \pm 0.03^{C,a}$	$1.83 \pm 0.01^{B,a}$	$1.97 \pm 0.03^{A,a}$

SD standard deviation, *PFU* plaque-forming units

A–E Means in the same row are significantly ($P < 0.05$) different by Duncan's multiple range test

a–d Means in the same column are significantly ($P < 0.05$) different by Duncan's multiple range test

Texture, pH, and Sensory Analysis of Oysters Treated with Combined Chlorine and Vitamin B_1

To determine the effects of combined treatments with chlorine (200–1000 ppm) and vitamin B_1 (maximum dose; 3000 ppm) on the texture of oyster surface, the changes in the texture were analyzed (Table 3). No significant differences ($P > 0.05$) in hardness, cohesiveness, and springiness were observed between samples treated with 3000 ppm vitamin B_1 alone and those treated with 3000 ppm vitamin B_1 in combination with chlorine at any concentration. Moreover, no differences in pH were observed between the oysters treated with the combination of 200–1000 ppm chlorine and 3000 ppm vitamin B_1 and the oysters treated with 3000 ppm vitamin B_1 alone (Table 3).

The combined treatment of chlorine (200–1000 ppm) and vitamin B_1 (maximum dose; 3000 ppm) on the sensory qualities (color, flavor, texture, and appearance) and the overall acceptability of the oysters were evaluated by untrained panelists (results shown in Table 4). No differences in color, texture, and appearance were observed between the oysters treated with the combination of 200–1000 ppm chlorine and 3000 ppm vitamin B_1 and the oysters treated with 3000 ppm vitamin B_1 alone. However, the flavor values were significantly ($P < 0.05$) reduced after treatment with 1000 ppm chlorine and 3000 ppm vitamin B_1 compared with those recorded after the treatment with 0–200 ppm chlorine and 3000 ppm vitamin B_1. This may be related to the observation that the overall acceptability values were significantly ($P < 0.05$) reduced in oysters treated with 1000 ppm

Table 2 Synergistic effects of chlorine/vitamin B_1 mixture on the reduction of MNV-1 titers on oyster surface

Vitamin B_1 (ppm)	Mean ± (SD) synergistic value of reduction value (log_{10}PFU/ml) Chlorine (ppm)			
	200	500	700	1000
1000	$0.08 \pm 0.04^{C,c}$	$0.28 \pm 0.08^{A,c}$	$0.23 \pm 0.12^{AB,c}$	$0.15 \pm 0.08^{B,b}$
2000	$0.34 \pm 0.21^{AB,ab}$	$0.48 \pm 0.08^{A,b}$	$0.49 \pm 0.02^{A,b}$	$0.19 \pm 0.08^{B,b}$
3000	$0.58 \pm 0.04^{C,a}$	$0.83 \pm 0.03^{B,a}$	$1.03 \pm 0.04^{A,a}$	$0.67 \pm 0.06^{C,a}$

SD standard deviation, *PFU* plaque-forming units

A–C Means in the same row are significantly ($P < 0.05$) different by Duncan's multiple range test

a–c Means in the same column are significantly ($P < 0.05$) different by Duncan's multiple range test

[a]Synergistic effects indicated as + = (reduction achieved with the chlorine treatment and the vitamin B_1 treatment) − (reduction achieved by the chlorine + vitamin B_1 treatment)

Table 3 Comparison of texture and pH change on oyster surface after chlorine and vitamin B_1 3000 ppm mixture

Texture/pH	Vitamin B_1 3000 ppm Chlorine (ppm)				
	0	200	500	700	1000
Hardness (g/cm^2)	85.95 ± 10.11	92.85 ± 12.42	84.24 ± 13.29	88.96 ± 11.25	90.12 ± 10.49
Cohesiveness	0.68 ± 0.03	0.65 ± 0.05	0.62 ± 0.02	0.66 ± 0.03	0.65 ± 0.05
Springiness	0.87 ± 0.03	0.89 ± 0.04	0.84 ± 0.02	0.87 ± 0.03	0.86 ± 0.03
pH	6.38 ± 0.11	6.28 ± 0.00	6.46 ± 0.12	6.31 ± 0.05	6.37 ± 0.03

Table 4 Comparison of sensory change in oyster after chlorine and vitamin B_1 3000 ppm mixture

Sensory	Vitamin B_1 3000 ppm Chlorine (ppm)				
	0	200	500	700	1000
Color	6.58 ± 0.52	6.58 ± 0.79	6.50 ± 0.67	6.33 ± 0.78	6.50 ± 0.87
Flavor	6.50 ± 0.67^{a}	6.33 ± 0.78^{a}	6.17 ± 0.72^{ab}	6.17 ± 0.72^{ab}	5.54 ± 0.52^{b}
Texture	6.25 ± 0.75	6.17 ± 0.83	6.08 ± 0.90	6.12 ± 0.99	6.22 ± 0.83
Appearance	6.33 ± 0.78	5.92 ± 0.90	6.25 ± 0.75	6.08 ± 0.67	5.75 ± 0.87
Overall acceptability	6.58 ± 0.52^{a}	6.25 ± 0.62^{a}	6.25 ± 0.74^{a}	6.08 ± 0.29^{a}	5.42 ± 0.67^{b}

SD standard deviation

a, b Means in the same row are significantly ($P < 0.05$) different by Duncan's multiple range test

chlorine and 3000 ppm vitamin B_1 than in those treated with 0–700 ppm chlorine and 3000 ppm vitamin B_1.

Discussion

Oysters are referred to as "the milk of the sea" because they are high in protein, amino acid, minerals, and taurine and have a pleasing taste. Oysters are easy to digest for the absorption of their nutrients because of their soft consistency and are consumed as a fresh raw food in most countries. In addition, oysters are processed to produce diverse food products such as fermented and non-fermented oyster "*Jeotgal*" (salted seafood product), oyster "*Kimchi*" (salted, seasoned, and fermented vegetables), and oyster "*Jeon*" (a kind of pancake) in Korea. Most of the oysters distributed in the commercial markets of Korea are cultured and shucked.

Moon et al. (2011) reported that in the retail markets of Korea, noroviral RNA sequences were detected in 14.1% of oysters, with 12.2% of the sequences corresponding to genogroup I (GI) and 1.9% to genogroup II (GII). Shin et al. (2014) also reported that NoV GI and NoV GII sequences were detected in 24% (8.97×10^2–2.24×10^2 copies/g) and 29% (3.05×10^2–7.47×10^1 copies/g), respectively, of oysters collected from Tongyeong, Korea. Recently, the Korean Ministry of Oceans and Fisheries (MOF 2018) recommended that consumers should consume heated and cooked oysters because NoV was isolated from oysters (*Crassostrea gigas*) cultured in the coastal area of Kyungnam province in Korea. Furthermore, the oysters are often distributed without packaging at traditional local markets during the Korean winter. Because of potentially unsanitary conditions (open or no packaging, no heating, high moisture content, and cross-contamination from hand to oyster), there are microbial safety concerns regarding unpackaged oysters including the potential for NoV contamination. Owing to the frequent problem of NoV contamination, the safety of fresh oysters is currently considered an important social issue in Korea.

There has been increasing interest worldwide in employing hurdle approaches to enhance the shelf life of perishable foods such as seafood and ensure the microbiological safety of such products during food production and processing. Specifically, there have been some studies on the effects of combined treatments with chlorine and vitamin B_1 for reducing the abundance of pathogenic bacteria on food products. However, no studies have been reported on the effects of combined treatments with chlorine and vitamin B_1 for reducing the titers of foodborne viruses including NoV in food products. Chlorine is a disinfectant that is also widely used as the primary chemical sanitization method in the food industry. Chlorine can be converted into hypochlorous acid (HOCl) (unionized form), which is a strong bactericidal agent, through hydrolysis ($\text{Chlorine} + H_2O \rightarrow HOCl + NaOH^-$). HOCl is a weak acid and dissociates to the hypochlorite ion (^-OCl) and a proton (H^+) depending on the solution pH (Fukuzaki 2006). Hirneisen et al. (2010) reported that chlorine could denature the capsid proteins and RNA of NoV. Specifically, Park and Sobsey (2011) noted that the titer of MNV-1 on stainless steel showed 1.5 and 1.3 $\log_{10}$ reductions after treatment with 5000 and 2500 ppm chlorine, respectively. Their results correspond somewhat with those of the current study, in which a 1.0 $\log_{10}$ reduction of MNV-1 titer was observed in oysters treated with 1000 ppm chlorine alone. Vitamin B_1 is typically used as a synergistic bactericidal agent for the treatment of noodles in Korea. The antibacterial effects of vitamin B_1 may originate from its structure and components such as sodium lauryl sulfate and a thiazole ring. Sodium lauryl sulfate can damage biological membranes and inhibit the proliferation of microorganisms by disrupting the conformation of protein molecules (Rykke et al. 1990). In addition, the quaternary amine group in the thiazole ring of vitamin B_1 can perturb the lipid bilayer membranes that constitute the cytoplasm membrane (Thorsteinsson et al. 2003). Lee et al. (2010) suggested that vitamin B_1 could also be used as an additive to reduce the amount of disinfectant required for reducing the abundance of *B. cereus* in rice via a synergistic effect. Kim et al. (2011) noted that treatment with vitamin B_1 at a concentration of 1000 ppm resulted in a 1.0 log-reduction in the abundance of *Vibrio vulnificus*. Furthermore, Srey et al. (2014) suggested that the combined treatment of 200 ppm chlorine and 1000 ppm vitamin B_1 could have practical applications in removing *Listeria monocytogenes* biofilms from surfaces in the food industry. In the study by Park et al. (2012), the synergistic reduction values for combined treatment with 100 ppm chlorine and 1000 ppm vitamin B_1 against *A. hydrophila* in squid were 1.35 $\log_{10}$ CFU/g. This value was much higher than that (0.08 $\log_{10}$ CFU/g) of the oysters treated with 200 ppm chlorine and 1000 ppm vitamin B_1 for the reduction of MNV-1 titers in the current study. Vitamin B_1 has antiviral properties. Thiamin was shown to be a potent inhibitor of human immune deficiency virus (HIV) replication (Shoji et al. 1994) and an effective treatment for chronic hepatitis B virus infection (Wallace and Weeks 2001). The mechanism underlying the vitamin B1 antivirus effects has not been fully elucidated; the disulfide bridge of thiamine disulfide was essential for anti-HIV activity because thiamine alone, the reduced form of thiamine disulfide, neither inhibited HIV-1 production nor HIV-Tat (transactivator of transcription, a regulator protein of HIV-1) activity (Shoji et al. 1994). It is possible that thiamine disulfide can cause the blockage of adsorption of HIV-1 on its target cells by disulfide exchange and thiol-disulfide interchange (Shoji et al. 1994). In general, foodborne viruses including NoV seem to be more resistant than foodborne bacteria to antimicrobial agents, although

differences in the experimental conditions and food matrices tested may also contribute to the variations among studies.

In the absence of a cell culture method for NoVs, some cultivable surrogates have been widely studied; MNV-1, feline calicivirus (FCV), bacteriophage MS2, and (or) tulane virus have been widely used as a surrogate of human norovirus due to the absence of a cell culture method for NoVs (Bae and Schwab 2008; Cannon et al. 2006; Cromeans et al. 2014; Hirneisen et al. 2010; Hirneisen and Kniel 2013; Kitajima et al. 2010; Sinclair et al. 2012). Hirneisen and Kniel (2013) noted that one surrogate alone cannot fully mimic the characteristics of human norovirus stability in different treatments. They also suggest that several surrogates should be considered in the evaluation of disinfection treatments because the many different genogroups and genotypes of NoVs have different environmental responses. However, MNV-1 is generally accepted as a best surrogate for NoVs (Cannon et al. 2006; Cromeans et al. 2014; Kitajima et al. 2010; Wobus et al. 2006). Especially, Kitajima et al. (2010) reported that there was no significance in the overall viral RNA reduction rate between human norovirus and MNV-1, indicating similar persistence against free chlorine disinfection. Based on these previous studies, we may speculate that the viricidal effects of combined chlorine and vitamin B_1 against NoVs could be similar to MNV-1.

pH is an important factor for assessing oyster freshness, and fresh oysters should have a pH between 6.29 and 6.59 (Kim et al. 2014). Texture is also an important factor for the consumer acceptability of raw molluscan shellfish foods including oysters. Therefore, texture and pH were used as indirect indicators of the sensory attributes of oysters. The results for pH (6.31–6.46) and texture parameters were not changed by the combined treatment of 0–1000 ppm chlorine and 3000 ppm vitamin B_1. According to the study by Lee and Ha (2008), the sensory properties of cooked rice treated with 300 ppm chlorine and 0–1000 ppm vitamin B_1 did not differ significantly from those of water-treated cooked rice. Their observations were somewhat similar to the results of the sensory analysis in the present study. However, some panelists in the current study mentioned that there were some off-odors and/or chemical-like odors for the oysters treated with concentrations of over 1000 ppm chlorine, regardless of the concentration of vitamin B_1 (data not shown). This finding is likely to be due to the odor attributes of chlorine, while vitamin B_1 has no smell or taste.

Conclusion

The current results provide additional evidence that all combinations of chlorine (200–1000 ppm) and vitamin B_1 (1000–3000 ppm) resulted in synergistic reductions in the titers of MNV-1 (as a NoV surrogate) on oyster surface (synergistic reduction values of 0.08–1.03). Vitamin B_1 (100–1000 ppm) did not greatly reduce MNV-1 titers when applied alone, but it contributed to minimizing the concentration of chlorine required for the reduction of MNV-1 titers in the combined treatments. Our findings indicate that the combined treatment of 700 ppm chlorine and 1000 ppm vitamin B_1 could serve as an optimum treatment for the reduction of NoV titers during the processing of oyster surface to enhance microbiological safety without producing any physical, chemical, and sensorial changes in the food qualities of raw oysters.

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