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ARTICLE

Addition of thiamine dilaurylsulfate to reduce the intensity of hydrostatic pressure treatment for microbial safety of Korean *Jogaejeot-muchim* (salted-fermented-seasoned short-neck clam)

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Abstract This study investigated hydrostatic pressure (HP) treatment with the addition of thiamine dilaurylsulfate (TDS) for the microbial safety of the final product (salted-fermented-seasoned short-neck clam) during *Jogaejeot-muchim* manufacturing process. HP treatment (100–600 MPa) was performed at each step (raw, salted-fermented, and salted-fermented-seasoned short-neck clam) of the *Jogaejeot-muchim* manufacturing process. The reduction effect on *Staphylococcus aureus* in the salted-fermented-seasoned short-neck clam step was 1.2 log after 400 MPa treatment, and it was lower than those of raw and salted-fermented short-neck clam steps (4.3 and 4.9 log, respectively) after 400 MPa treatment. To improve the microbial safety of the salted-fermented-seasoned short-neck clam step, TDS (1 g/kg) was added, and 1 log reduction was obtained. In the salted-fermented-seasoned short-neck clam step, HP treatment with TDS addition showed a synergistic effect, and the HP intensity for a 2 log reduction was reduced from 500 to 100 MPa treatment with TDS addition, and a 4 log reduction was achieved by the 300 MPa treatment with addition of TDS. This study concluded that the TDS addition contributed to reducing the intensity of HP treatment required for 99 % inactivation of *S. aureus,* and that combined treatment (300 MPa treatment with TDS) can be used to provide microbial safety assurance in Korean *Jogaejeot-muchim*.

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Keywords Hydrostatic pressure · *Jogaejeot-muchim* manufacturing process · Short-neck clam · *Staphylococcus aureus* · Thiamine dilaurylsulfate

Introduction

Jogaejeot-muchim, a traditional Korean salted-fermented-seasoned (SFS) short-neck clam, is a ready-to-eat, non-thermal product of shellfish brined with salt, fermented, and mixed with seasoning such as garlic, ginger, red pepper, and starch syrup. The demand on a low salt concentration in *Jogaejeot-muchim* has been increasing due to health trends. In low-salted products (<10 % salt con.), maintaining microbial safety is becoming an important issue. There are halo-tolerant bacteria, marine bacteria, and yeast in the raw material of shellfish (Cha et al. 1993). Seasonings such as red pepper, garlic, and ginger are of agricultural origin and therefore are generally highly contaminated by microorganisms (Buckenhuskes and Rendlen 2004). Foodborne pathogens and saprogenic bacteria can cause contamination during food processing and can grow easier than in high-salted products (Ham and Jin 2002). A sanitation process of *Jogaejeot-muchim* is required for microbial safety.

Staphylococcus aureus, a gram-positive bacterium, has high possibility of contamination during processing, such as by worker's hands (Buchanan et al. 1993). Contaminations in several foods, including fish products and salted and fermented shrimp, have been reported (Wieneke et al. 1993; Mok and Song 2000). Normally, gram-positive bacteria are more resistant to environmental stresses than gram-negative bacteria (Cheftel 1995; Patterson et al. 1995; Ananou et al. 2010). Thus, *S. aureus* is a one of the

major pathogens of concern in *Jogaejeot-muchim* due to its resistance.

Non-thermal sterilization technology tends to apply several combination treatments to avoid increases of treatment intensity, extension of duration time, and increase of food damage by a single technology application (Leistner 2000; Lambert and Bidlas 2007). Hydrostatic pressure (HP) treatment, as a non-thermal sterilization technology, is an isostatic system regardless of size or shape of food. Though HP has lots of facility cost (less operational cost), recently through the progress of equipment technology, the cost has been decreased (Sàiz et al. 2008). Since HP treatment is processed in pre-packaged foods, microbial safety of a final product could be assured of avoiding cross-contamination risks by re-packaging food product (Chawla et al. 2011). HP treatment is an appropriate food processing for the Korean *Jogaejeot-muchim*.

The addition of antimicrobials as an ingredient in food is a simple method to combine with other non-thermal sterilization methods, such as HP. Various studies of the combinations of HP treatment with an antimicrobial addition for food safety have been reported in rice pudding, cooked chicken, beverages, and fermented sausages (Corbo et al. 2009; Pina-Pérez et al. 2009; Ananou et al. 2010; Patterson et al. 2011; Pulido et al. 2012). Pina-Pérez et al. (2011) researched HP treatment with the addition of various antimicrobials in beverages, and they reported that additive or synergistic effects were observed depending on the antimicrobial. Some combined effects were antagonistic (Koivunen and Heinonen-Tanski 2005).

Thiamine dilaurylsulfate (TDS) is a nontoxic substance with antimicrobial effects and nutritional benefits as a vitamin B_1 derivative (Zhengyi and Zhengshu 1999). It has been approved for use as a synthetic food additive in Korea and Japan. Commercial antimicrobial agents, such as TDS, can be used in food products as an ingredient (Shin et al. 2008). Therefore, HP treatment with TDS addition was considered an appropriate method for the treatment of Korean *Jogaejeot-muchim* (SFS short-neck clam).

In this study, HP-induced *S. aureus* inactivation was evaluated at each step (raw, salted-fermented (SF), and SFS short-neck clam) of the *Jogaejeot-muchim* manufacturing process. HP treatment can be assigned an additional benefit, avoiding cross contamination, when it was applied on pre-packaged final product. For microbial safety assurance in the final product (SFS short-neck clam, *Jogaejeot-muchim*), addition of TDS was used to reduce the intensity of HP as a method with industrial efficiency.

Materials and methods

Materials

The raw, SF, and SFS short-neck clams (*Tapes philippinarum*) were provided by a seafood company in Korea and were stored at 4 °C. The SF short-neck clam was made by adding salt and fermentation to the raw short-neck clam. The SFS short-neck clam, as the final product, was made by adding mixed crushed garlic and ginger, red pepper, and starch syrup (provided from the seafood industry) to the SF short-neck clam. TDS as a white, crystalloid powder was purchased from Samin Chemical Co. (Korea) and was added at 1 g per 1 kg of SFS short-neck clam.

Measurement of pH, salt concentration, and Aw

The water activity (a_w) of the samples was determined using a a_w measuring apparatus (ms1-a_w, Novasina, Switzerland). The pH of the samples was measured with a pH meter (Corning Inc., USA). For the preparation of the pH measurement, samples (2 g) in 18 mL deionized water (DW) were mixed for 60 s with a homogenizer (Ultra Turrax T18 basic, IKA Works Inc., USA) and filtered through a filter paper (Whatman, No. 4, UK). The salt concentration was measured by the Mohr method (Che 1998). For the preparation of the salt concentration measurement, samples (1 g) were pre-heated on a hot plate, and dry ash was produced in a muffle furnace (at 550 °C, for 5 h). The dry ash was collected in DW (10 mL) and was titrated with silver nitrate in potassium chromate indicator solution.

Preparation of *S. aureus* culture

Staphylococcus aureus (ATCC25923, ATCC12600, and ATCC29213) strains in an ampoule at room temperature were obtained from the Korean Collection for Type Culture, Korea. All strains were maintained at −80 °C in skim milk containing 30 % glycerol. A sterile disposable plastic loop was used to transfer the frozen bacterial cultures to a nutrient agar plate. The cultures were incubated at 37 °C for 24 h, and they were then transferred from the nutrient agar plate to nutrient broth tubes. Each strain was incubated at 37 °C for 15 h and was collected by centrifugation (10,000×*g*, 3 min, RT). Each pellet were re-suspended with PBS and pooled in equal proportions. The pooled suspension (*S. aureus* cocktail, approximately 8–9 log CFU/mL) was used for inoculating samples. *S. aureus* cocktail (1 mL) was used to inoculate 100 g of raw, SF, and SFS short-neck clam, and each ingredient (crushed garlic and ginger, red pepper, starch syrup) (inoculated

level of 7 log CFU/g). Starch syrup was diluted with DW to 25, 50, and 75 % (w/w). *S. aureus* inoculated samples were incubated at 4 °C for 3 h and were then HP treated. During each set of experiments, two non-inoculated samples were taken to determine the initial contamination by *S. aureus* of black colonies with clear halo among staphylococci (not detected).

Hydrostatic pressure treatment

Samples were placed into a sterile polyethylene bag and vacuum-sealed. The bag was placed into an HP unit, which contained a pressure chamber 9 cm in diameter and 30 cm in height with an inner cylinder (8 cm in diameter and 19 cm in height) for loading the sample (Quintus food processor 6, ABB Autoclave System Inc., USA). HP treatment at 100, 200, 300, 400, 500, and 600 MPa was performed at room temperature for 5 min using DW with water glycol fire-resistant hydraulic fluid (Houghto-safe 620-TY, Houghton International Inc., USA) as the pressure medium. The working pressure was increased at a rate of 7 MPa/s, and decompression took place immediately.

Antimicrobial assay by a disk diffusion method

Staphylococcus aureus cocktail (6 log CFU/plate) was spread on Mueller–Hinton agar with a pH of 5.0 or 6.0 and a salt concentration of 4.0 and 7.0 %. After these plates were incubated at 37 °C for 3 h, a filter paper disk (8 mm diameter, Whatman) with 10 μL of TDS (0, 1, 10, and 100 mg/mL, solved in 30 % ethanol) was placed on each plate. After the plates were incubated at 37 °C for 18 h, the diameters of the distinctly clear zones were measured using a digital caliper. The antimicrobial activity of TDS against *S. aureus* was evaluated by measuring the diameter of inhibition zone induced by cell diffusion after incubating at 37 °C for 18 h.

Calculation of the synergistic reduction effects

To estimate any synergistic effect on bacterial inactivation, the reductions of the combined treatment (HP treatment with added TDS) were compared with those of HP treatment or TDS alone. The synergy values of the combined treatment (HP treatment with added TDS) were calculated by the following equation:

$$\begin{aligned}&\text{Synergy value (log CFU/g)}\\&= \text{log reduction by combined treatment}\\&\quad (\text{HP treatment with added TDS})\\&\quad -(\text{log reduction by HP treatment}\\&\quad +\text{log reduction by TDS})\end{aligned}$$

When the synergy value is positive, there is a synergy benefit in the combined treatment, whereas a negative value represents an antagonistic effect. A value of zero means that the efficiency of the combined treatment is the same as the sum of the two individual treatments.

Determination of colony counts after treatment

Changes in the colony counts in *S. aureus* inoculated samples were measured by counting black colonies with clear halo on the Staphylococci-selective Baird-parker agar (Becton, Dickinson and Company, USA). Changes were detected immediately after HP treatment (HP + non-incubation), and colony counts were detected after the HP-treated samples were mixed with ten fold peptone water and incubated for 3 h at 37 °C (HP + incubation).

Determination of Hunter's color value

To quantify the color of samples in terms of Hunter's L* (lightness), a* (redness), and b* (yellowness), each sample was put in a petri dish (diameter 5 cm) and measured using a Minolta CM-700d spectrophotometer(Konica Minolta Optics, Inc., Japan). For each treatment, five measurements along the equatorial area were conducted on at least 3 samples.

Statistical analysis

All experiments were performed in triplicate. The average values and standard deviations were calculated from the data using Excel (Microsoft corp. USA). The data were analyzed via ANOVA using the SAS statistical program (SAS Institute, USA), and the significant differences were compared using Duncan's multiple ranges tests ($P < 0.05$).

Results and discussion

Effect of HP treatment on the inactivation of *S. aureus* in raw, SF, and SFS short-neck clam

In *S. aureus* inoculated raw, SF, and SFS short-neck clam, the changes of *S. aureus* counts were measured twice, immediately after HP treatment (HP + non-incubation) and after HP treatment and 3 h incubation with peptone (HP + incubation) (Fig. 1). Higher intensity of HP induced greater inactivation of *S. aureus*. The reductions of *S. aureus* by 400 MPa were 4.3, 4.9, and 1.2 log CFU/g in *S. aureus* inoculated raw, SF, and SFS short-neck clam, respectively. A reduction of over 2 log CFU/g in raw, SF, and SFS short-neck clam was achieved by 300, 300, and

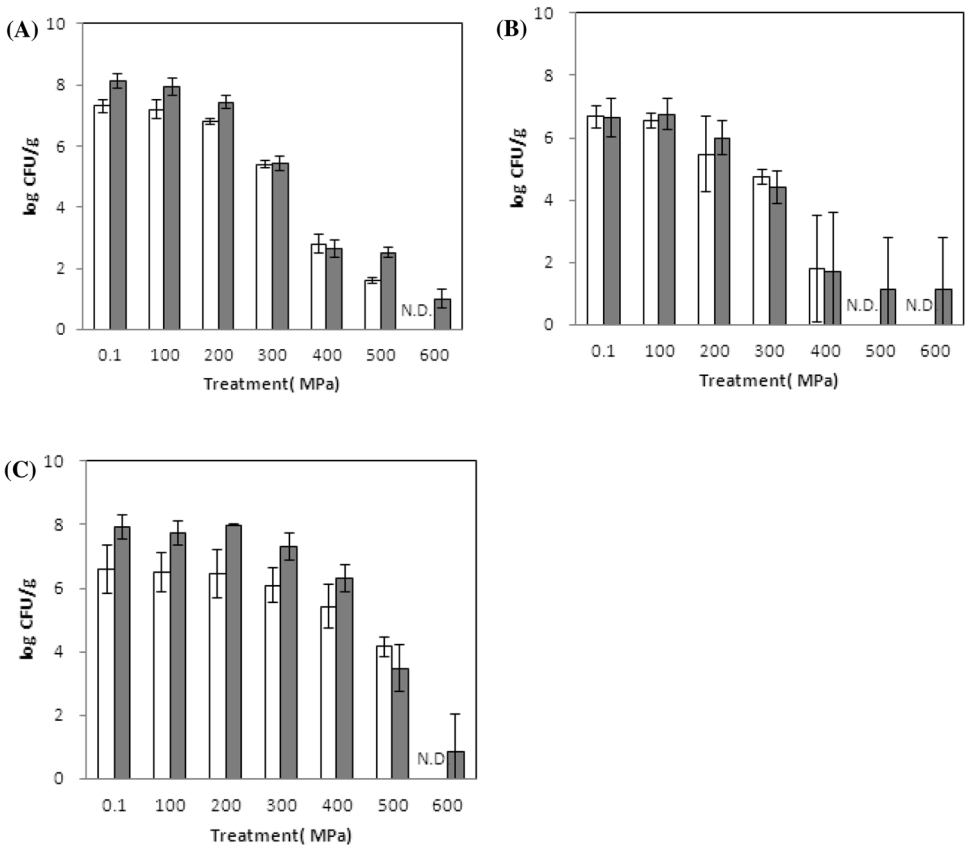

Fig. 1 Changes of the *Staphylococcus aureus* colony counts after hydrostatic pressure (HP) treatment in each step of the *Jogaejeot-muchim* manufacturing process. *White bars* HP + non-incubation: right after HP treatment. *Gray-shaded bar* HP + 3 h incubation: 3 h incubation at 37 °C after HP treatment. (**A**) Raw, (**B**) salted-fermented, and (**C**) salted-fermented-seasoned short-neck clam. *N.D.* 'not detected'

500 MPa treatment, respectively. The reduction of the *S. aureus* counts by HP in SFS short-neck clam was lower than in raw and SF short-neck clam. The *S. aureus* counts were not detected after 600 MPa treatment of raw and SFS short-neck clam or after 500 MPa treatment of SF short-neck calm (2 log CFU/g detection limit).

The *S. aureus* counts after 3 h incubation at 37 °C in HP-treated samples were measured to determine the *S. aureus* count by enrichment of the sample below the detection limit and recovery of injured cells. In most samples, the *S. aureus* counts were increased after 3 h incubation. In the 600 MPa treatment of raw, SF, and SFS short-neck calm, the counts of *S. aureus* were increased over the detection limit after 3 h incubation, which reflects the proliferation of surviving cells or the recovery of injured cells.

Other researchers reported that the 600 MPa-induced reduction was below the detection limit of *S. aureus* inoculum (6–7 log CFU/g) in foods such as rice pudding, milk, and cooked meat (Guan et al. 2006; Patterson et al. 2011; Pulido et al. 2012). However, during storage periods, the colony counts increased to a detectable level (Patterson et al. 2011).

Effect of HP treatment on the inactivation of *S. aureus* in each ingredient for SFS short-neck clam

The inactivation effects of *S. aureus* by HP were determined in each ingredient for SFS short-neck clam: crushed garlic and ginger, red pepper, and starch syrup (Fig. 2). The inoculated *S. aureus* level of the non-treated control was 5.5-6.5 log CFU/g in the ingredients. *S. aureus* in crushed garlic and ginger was reduced below the detection limit by the 300 MPa treatment. In red pepper, *S. aureus* was below the detection limit after 500 MPa treatment. However, starch syrup showed different inactivation effects of *S. aureus* by HP, depending on its concentration (25, 50, and 75 % (w/w)). The a_w values of the 25, 50, 75, and 100 % starch syrup used in this study were 0.95, 0.92, 0.89, and 0.74, respectively. The counts of *S. aureus* in the 25 and 50 % starch syrup were below the detection limit after 400 and 500 MPa treatments, respectively. However, in the 75 % starch syrup, the reduction effect was less than 1 log CFU/g after 400 MPa treatment.

Differences of the ingredient compositions in raw, SF, and SFS short-neck clams affect the HP-resistance of *S. aureus*. Koseki and Yamamoto (2007) reported that high

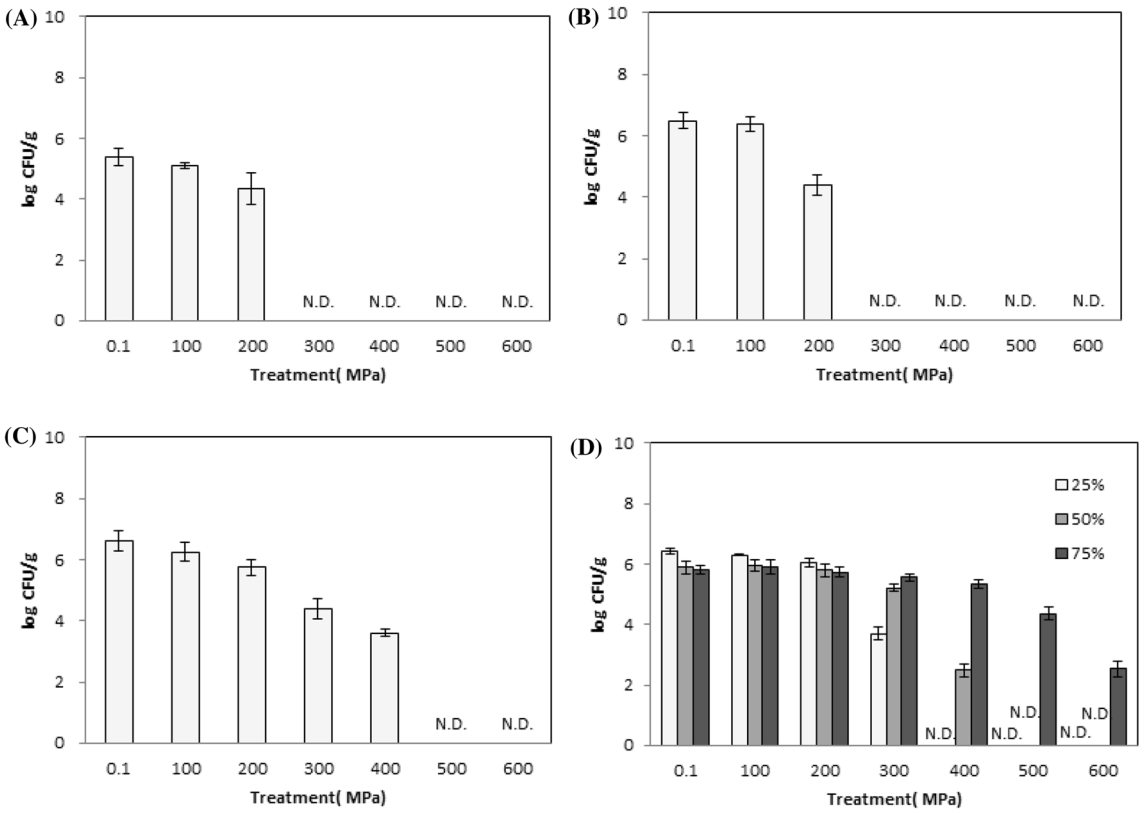

Fig. 2 Changes of the *Staphylococcus aureus* colony counts after hydrostatic pressure (HP) treatment in each ingredient for salted-fermented-seasoned short-neck clam, *Jogaejeot-muchim*. (**A**) Crushed garlic, (**B**) crushed ginger, (**C**) red pepper, and (**D**) starch syrup (25, 50 and 75 % (w/w)). *N.D.* 'not detected'

Table 1 Parameters in each process step of *Jogaejeot-muchim*

Process steps	Parameters		
	pH	Salt con. (%)	Aw
Raw short-neck clam	6.2 ± 0.1[a]	0.9 ± 0.1	0.97 ± 0.01
Salted-fermented short-neck clam	6.1 ± 0.1	8.5 ± 0.2	0.92 ± 0.01
Salted-fermented-seasoned short-neck clam	6.0 ± 0.1	7.0 ± 0.2	0.90 ± 0.01

[a] Values are the mean ± SD

salt or sucrose (composed of glucose and fructose) concentrations affected the HP-resistance of bacteria. At the same a_w value of salt and sucrose, the HP-resistance effect of bacteria in sucrose was higher than that in salt. The salt concentration of the SFS short-neck clam (7 %) was slightly lower than that of the SF short-neck clam (8.5 %) (Table 1). However, the a_w of the SFS short-neck clam was lower than that of the SF short-neck clam, due to the inclusion of the starch syrup (composed of glucose and maltose) in the SFS short-neck clam. The lower a_w in the SFS short-neck clam than in the raw and SF short-neck clam might affect the resistance to HP in the inactivation of *S. aureus*.

HP treatment should be effective in a packaged final product to eliminate the risk of a cross-contamination. Higher intensity HP treatment was effective to inactivate bacteria. In general, previous studies indicated that 600 MPa is needed to sufficiently reduce the levels of pathogens and to prevent their outgrowth during subsequent refrigerated storage (Aymerich et al. 2005; Marcos et al. 2008). However, for efficient processing in industry, it is necessary to reduce HP intensity. Therefore, since HP treatment should be complemented with the use of additional hurdles to reduce HP intensity and to maintain microbial safety of the final product, HP treatment with antimicrobial additions was considered.

Table 2 Antimicrobial activity (diameter of inhibition zone) of thiamine dilaurylsulfate depending on the pH and salt concentrations of agar plates against *Staphylococcus aureus* (Units: mm)

Con. (μg/mL)[1]	pH and salt			
	pH 5		pH 6	
	4 %	7 %	4 %	7 %
100	22.1[2]ab[3]	23.2a	21.6b	22.6ab
10	17.4b	20.8a	16.1c	18.2ab
1	11.1b	13.6a	8.7c	9.7bc
0	8a	8a	8a	8a

Final cell concentration for microorganism was approximately 6 log CFU/plate

[1] Ten ul of thiamine dilaurylsulfate in 30 % ethanol solution was applied to paper disk (8 mm, diameter)

[2] The diameter (mm) of the clear zone including the paper disk diameter. The value, 8 mm, means no activity against *S. aureus*

[3] Different letters in each row are significantly different ($P < 0.05$)

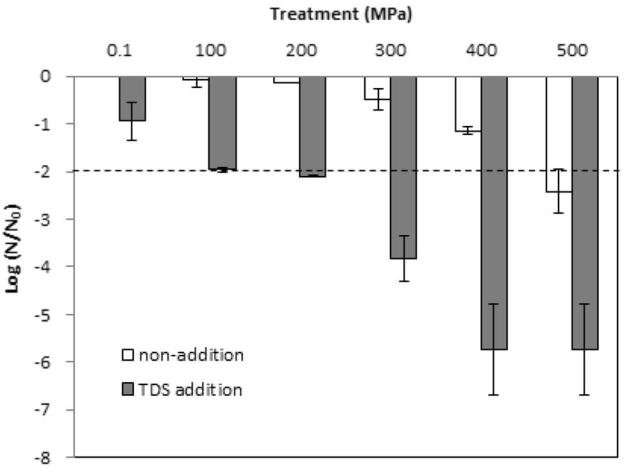

Fig. 3 Effects of the hydrostatic pressure treatment with/without thiamine dilaurylsulfate addition (1 g/kg) on the inactivation of *S. aureus* in salted-fermented-seasoned short-neck clam, *Jogaejeot-muchim*

Effect of HP treatment with TDS for the inactivation of *S. aureus* in SFS short-neck clam step

To evaluate the inhibition effect of *S. aureus* by antimicrobials and TDS, the disk diffusion method was performed on an agar plate with adjusted pH and salt concentrations (Table 2). The physical parameters of the SFS short-neck clam were pH 6 and 7 % salt concentration (Table 1). Therefore, the conditions of the agar plate were adjusted to be similar to the SFS short-neck clam (a pH of 5 or 6 and a salt concentration of 4 or 7 %). The inhibition effect of TDS against *S. aureus* was observed as a clear zone on the agar plate. A clear zone by 30 % ethanol (0 μg/mL) was not observed. The largest clear zone in each concentration (1, 10, and 100 μg/mL) of TDS was observed on the agar plate adjusted pH 5 and 7 % salt concentration. On the agar plate adjusted pH 6 and 4 % salt concentration, the smallest clear zone in each concentration of TDS was observed. A clear zone on the agar plate was observed at the minimum concentration of TDS used in this study (1 μg/mL).

TDS is composed of sodium lauryl sulfate and a thiazole ring, which affect the antimicrobial effect. Sodium lauryl sulfate damages biological membranes and inhibits the proliferation of bacteria resulting by disrupting the conformation of protein molecules (Rykke et al. 1990). The quaternary amine group in the thiazole ring can perturb cytoplasm membranes (Thorsteinsson et al. 2003). Therefore, TDS has been used as an antimicrobial in various studies to inhibit pathogens and mold in food (Wei et al. 2014; Choi et al. 2015).

TDS (1 g/kg) was added to the SFS short-neck clam, and the reduction effect of *S. aureus* by HP was measured (Fig. 3). The *S. aureus* counts induced by the addition of TDS compared with non-addition showed 1.0 log CFU/g reductions without HP treatment. In TDS added samples, 2.0 log CFU/g reductions were achieved by 100 MPa treatment. The reduction effect of 200 MPa treatment with/without TDS was not significantly different from 100 MPa with/without TDS. Although the 100 and 200 MPa treatments were weak to inactivate *S. aureus* in the SFS short-neck clam step, the reduction by 100 and 200 MPa with TDS was approximately 2.0 log CFU/g (Fig. 3). The reduction effect of the 300 MPa treatment was less than 1 log CFU/g in the SFS short-neck clam step. However, 300 MPa treatment with TDS resulted a dramatic reduction of approximately 4 log CFU/g. In the 400 and 500 MPa treatment with TDS, the *S. aureus* counts were below the detection limit (2 log CFU/g) in the SFS short-neck clam step. The intensity of HP was reduced to 100 MPa with TDS addition for a 2 log reduction of *S. aureus* in the SFS short-neck clam step. However, 300 MPa treatment with TDS (4 log reduction of *S. aureus*) is recommended for microbial safety assurance for SFS short-neck clam.

HP and TDS played a role in a hurdle to inactivate *S. aureus* in SFS short-neck clam. Leistner (1978) introduced multi-target preservation and synergistic effect by hurdles. Synergistic reduction effects were estimated by the procedure described by Koivunen and Heinonen-Tanski (2005) (Table 3). In the result of the synergy values for HP treatment with TDS, positive values (0.9–3.1 log CFU/g) were observed (Table 3). A positive value results when the inhibitory action of the hurdle combination is higher than the addition of each one applied separately. The synergy values for 100 and 200 MPa treatments with TDS addition were approximately 1 log CFU/g. In the 300–500 MPa treatments with TDS addition, the synergy value was 2.4–3.1 log CFU/g. The synergy value for over 300 MPa with TDS addition

Table 3 Synergy values of the hydrostatic pressure treatment and thiamine dilaurylsulfate addition on the reductions of *S. aureus* depending on pressure in salted-fermented-seasoned short-neck clam, *Jogaejeot-muchim* (Units: log CFU/g)

Pressure (MPa)	Antimicrobial (MPa) Thiamine dilaurylsulfate
100	0.9 ± 0.3[1]b[2]
200	1.1 ± 0.4b
300	2.4 ± 0.6ab
400	3.1 ± 1.2a
500	2.6 ± 1.0a

[1] Values are the mean ± SD of the synergistic value for the reduction of *S. aureus* (log CFU/g)

[2] Different letters are significantly different ($P < 005$)

was higher than that for 100–200 MPa with TDS. The synergistic effect of inactivation induced by HP treatment with antimicrobial is due to the combined factor destabilization of the membrane structure or function, although the specific modes of action are different (Ross et al. 2003). HP caused membrane injuries, making the pathogen more sensitive to antimicrobials due to the increase of the cell penetrability (Valero and Salmeron 2003).

HP treatment with TDS achieved the desired effect for microbial quality of SFS short-neck clam. For consumer acceptability, color is probably the important quality factor. Hunter's color value (*L*, a*, b**) of SFS short-neck clam by HP treatment with TDS was not changed (data not shown). In general, TDS addition was not affected physicochemical properties such as color and texture (Oh et al. 2014; Wei et al. 2014). In the SFS short-neck clam, HP treatment was not affected color parameters.

To improve the microbial safety of SFS short-neck clam, the final product of the Korean *Jogaejeot-muchim* manufacturing process, a reduction of the effects of *S. aureus* by HP treatment with TDS, was investigated. For the inactivation of pathogens in the SFS short-neck clam step, higher intensity of HP treatment is more effective in microbial reduction. However, reduction of the HP intensity is required for industrial application. The addition of TDS as an antimicrobial was used to reduce the intensity of HP treatment for reduction of *S. aureus* on the SFS short-neck clam step. In the SFS short-neck clam step, HP treatment with TDS addition showed a synergistic effect, and the HP intensity for a 2 log reduction was reduced from 500 to 100 MPa treatment with TDS addition, and a 4 log reduction was achieved by the 300 MPa treatment with TDS addition. This study concluded that the TDS addition contributed to reducing the intensity of HP treatment required for 99 % inactivation of *S. aureus* and that combined treatment (300 MPa treatment with TDS) can be used to provide microbial safety assurance in Korean *Jogaejeot-muchim*.

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