Note

Effects of ε-polylysine and Milk Serum Protein on the Attachment and Decontamination of Salmonella Enteritidis on Lettuce and Radish Sprouts

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Received March 10, 2016; Accepted May 19, 2016

To understand the effects of pretreatment of lettuce during cultivation with 0.001% ε-polylysine (PL) in combination with 0.25% milk serum protein (MSP) on the attachment of Salmonella Enteritidis, lettuce leaves were contaminated with S. Enteritidis after a 1-day treatment with food additives, and harvested after cultivation for 1 day. Viable S. Enteritidis counts on lettuce leaves pretreated with the PL and MSP mixture were significantly reduced from 5.7 log CFU/g to 1 log CFU/g after decontamination by washing with water and a subsequent treatment with NaClO. The viable S. Enteritidis counts on radish sprouts grown for 7 d in the presence of 0.01% PL from seeds inoculated with S. Enteritidis were reduced to 3.1 log CFU/50 sprouts after NaClO decontamination. These counts were significantly lower (< 0.05) than those of plants without additive(s). The treatment with additive(s) did not affect the ascorbic acid and chlorophyll contents of both plants.

Keywords: Salmonella Enteritidis, ε-polylysine, milk serum protein, lettuce, radish sprouts, decontamination

Introduction

Foodborne illness outbreaks linked to the consumption of fresh produce have rapidly increased in recent years (Warriner et al., 2009). Among the pathogenic bacteria, Salmonella and Escherichia coli O157:H7 are the major pathogens causing the foodborne illness outbreaks associated with fresh produce (Buck et al., 2003). Vegetables and fresh-cut produce are typically consumed raw without heat treatment or sanitation processes. Therefore, any safe intervention method to ensure the safety of produce is essential (Schuenzel and Harrison, 2002). Secondary microbial contamination can occur at various stages during cultivation, harvesting, processing, and transportation, and this contamination can arise from environmental, animal, or human sources (WHO/FAO, 2008). Moreover, the application of processing technologies such as cutting, slicing, or skinning can disrupt the natural barriers of intact plants, releasing nutrients, and as a result, accelerating the growth of inhabitant and secondary contaminant bacteria (Harris et al., 2003).

Salmonella contamination may occur in fresh produce at any point, from farm to fork, through incidental contact with the organism, and the sources may include soil, organic manure, irrigation or wash water, handling by workers, and contact surfaces (Beuchat and Ryu, 1997). However, the persistence and survival of Salmonella on fresh produce is dependent on its ability to adapt to new ecological environments (Beuchat, 2002). Soil contamination can lead to the contamination of several crops, including lettuce, parsley, carrot, and radish, up to six months after the contamination event, which shows the highly persistent nature of Salmonella in plant environments (Islam et al., 2004a, 2004b).

Washing and disinfection have been considered the only...
operations actively aimed at reducing microbial populations on fresh-cut vegetables (Pirovani et al., 2004). However, post-harvest washing of fresh produce with tap water cannot be trusted for the complete removal of pathogenic and naturally occurring bacteria (Nguyen-the and Carlin, 1994). A variety of disinfectants, such as chlorine, hydrogen peroxide, organic acids, and ozone have been used to reduce bacterial populations on fruits and vegetables (European Commission, 2002). However, besides their potential toxicity, they have proven incapable of completely removing or inactivating microorganisms on fresh produce (Koseki et al., 2001). In the fresh-cut produce industry, chlorinated water (mainly sodium hypochlorite, NaClO) is the most frequently used disinfectant for the washing of fresh produce (Warriner et al., 2009). The Food and Drug Administration (FDA) allows the use of sodium hypochlorite at concentrations ranging from 50 to 200 mg/L as a disinfection agent for surfaces that come into contact with food (Beuchat, 1998). However, a number of factors may limit its effectiveness, such as the internalization of pathogens within the plant tissue, the hydrophobicity of plant surfaces, and the biofilm-forming ability of some bacteria (Burnett and Beuchat, 2000; Whipps et al., 2008).

Salmonella enterica has been shown to form biofilms on several surfaces, including food contact surfaces, food, and plants (Giaouris and Nesse, 2015). Salmonella enterica is able to colonize various parts of the plant, including the seeds, sprouts, leaves, roots, and fruits, which make the plants important vectors for Salmonella transmission (Steenackers et al., 2012). The attachment strength of Salmonella was shown to be higher in lettuce than in cabbage (Patel and Sharma, 2010). Therefore, it is essential to understand the initial stages of Salmonella attachment to various plant tissues so that effective intervention strategies can be utilized to decrease the contamination.

On the other hand, seeds are considered the most significant primary source of pathogens on sprouted seeds for foodborne illnesses associated with sprout consumption (NACMCF, 1999). It is assumed that Salmonella enterica is spread from the contaminated seeds to the entire batch of sprouts through irrigation water (Barak, 2002). Several decontamination methods including chemical disinfectants (hydrogen peroxide, hypochlorite, chlorine dioxide, calcium hydrogen peroxide, ethanol), physical treatment (heat, irradiation), and their combination have been used to reduce or eliminate pathogenic bacteria from sprout seeds (Kochuranchitt et al., 2009). Seed decontamination using calcium hypochlorite (Ca(OCl)₂) at a concentration of 20,000 mg/L for more than 15 min has been recommended to reduce the risk of bacterial foodborne illness in sprouts production (NACMCF, 1999). However, such high levels of chlorine can be detrimental to the seed quality (Kim et al., 2003) and are not completely acceptable due to the chemical residues and their adverse environmental impact. Therefore, it is crucial to develop new decontamination methods to effectively reduce the pathogens on seeds and sprouts.

In a previous study, the adhesion inhibitory effects of several GRAS (generally recognized as safe) substances, including ε-polylysine (PL), sucrose fatty acid ester (SE) with a fatty acid of C8 to C18, and milk serum protein (MSP), on the microtiter plate attachment of several pathogenic bacteria have been shown (Miyamoto et al., 2011). Moreover, the combination of PL and MSP effectively inhibited the attachment of both Gram-positive and Gram-negative bacteria onto microtiter plates, and this combination was also effective against Salmonella Enteritidis on cabbage leaves (Islam et al., 2014). PL, a polymer of a positively charged lysine produced by Actinobacteria, is a naturally occurring substance that was used in our experiment. It is reported that PL inhibited biofilm formation by S. aureus, even at low concentrations such as 0.001% (Furukawa et al., 2010). PL seems to bind strongly to the negatively charged cell surface of both Gram-positive and Gram-negative bacteria using electrostatic interaction and shows antibacterial activity (Hansen and Gill, 2000). The decrease in the negative charges on the surface of bacterial cells caused by the binding of PL seems to reduce the attachment force of bacterial cells onto the surface of the plants. On the other hand, MSP is a mixture of milk proteins found in cheese whey (Evans et al., 2010). It contains whey proteins such as β-lactoglobulin and α-lactoalbumin. These proteins also seem to be involved in the inhibition of bacterial attachment. It seems that the different mechanisms for the interaction between bacterial cells and basic proteins or MSP can be attributed to their combined effects on the inhibition of attachment of both Gram-positive and Gram-negative bacteria to microtiter plates (Islam et al., 2014).

Therefore, the practicability of treatments with these GRAS substances on the decontamination of foodborne pathogens in vegetables was investigated. The effects of the pretreatment of lettuce leaves with a combination of PL and MSP during cultivation on the attachment and decontamination of Salmonella Enteritidis, inoculated onto the surface of lettuce after the pretreatment, were investigated. Radish sprouts were also cultivated in the presence of PL from seeds inoculated with S. Enteritidis to evaluate the effects of pretreatment.

Materials and Methods

Bacterial culture and media preparation S. Enteritidis NBRC3313 purchased from Biological Resource Center, NITE (NBRC), Chiba, Japan was transformed with a pEGFP vector (BD Biosciences, San Jose, CA, USA), which encodes for ampicillin resistance and carries the enhanced green fluorescent protein (EGFP) gene (Duffy et al., 2005). The transformed strain was designated as S. Enteritidis-EGFP.

A loopful of S. Enteritidis-EGFP from the stock plate culture was inoculated into 5 mL of Luria-Bertani broth (LB; Becton, Dickinson and Company, MD, USA) supplemented with 100 µg/mL ampicillin and incubated overnight at 30°C with shaking at 130 rpm. Then, 10 µL of 1000-fold diluted culture broth was...
transferred into 5 mL of LB supplemented with 100 μg/mL ampicillin. The bacterial cells were cultured at 30°C for 21–24 h with shaking at 130 rpm to obtain cells in the stationary phase of growth. The bacterial cells were harvested by centrifugation at 13,400 × g for 5 min at 4°C. The cell pellet was suspended in sterile water. Optical density at 660 nm (OD_{660}) of the cell suspension was adjusted to 0.12, to prepare a cell suspension at 10^7 CFU/mL, with sterile water. The suspension was used for the inoculation of lettuce leaves and radish seeds.

**Treatment of lettuce leaves and radish seeds** The PL was obtained from Chisso Corporation, Tokyo, Japan, and the MSP was obtained from Asama Chemical Co., Ltd., Tokyo, Japan. PL and MSP were dissolved in water and filter-sterilized with EB-DISK 25 (pore size 0.2 μm, Kanto Chemical Co., Ltd., Tokyo, Japan) and the pH of the PL solution was adjusted to 7 with 0.1 N HCl. The mixture of 0.001% PL and 0.25% MSP was used for the pretreatment of lettuce leaves prior to inoculation with S. Enteritidis-EGFP. For the treatment of radish seeds inoculated with S. Enteritidis-EGFP and growing sprouts, 0.01% PL was used. The concentrations used in this study were based on the effective concentrations to reduce biofilm formation of several pathogenic bacteria onto microtiter plates and cabbage leaves (Islam et al., 2014; Miyamoto et al., 2011).

**Cultivation of lettuce** The seeds of red leaf lettuce (Lactuca sativa var. crispa, variety name: Banchu Red Fire lettuce) were obtained from TAKII & Co., Ltd., Kyoto, Japan. The seeds were surface disinfected with 70% ethanol for 10–15 s, and dipped in a 0.2% sodium hypochlorite solution for 10 min. After disinfection, the seeds were rinsed three times with sterile water and planted in a container, 7.5 cm in diameter, containing a commercially available soil mixture (peat-moss/perlite/vermiculite; pH 5.5–6.5, water content 10%) (Corporation Oishi Bussan, Fukuoka, Japan) that was autoclaved at 121°C for 21 min. Plants were grown in a Biophotochamber (LX-3000, Taitec, Tokyo, Japan) at 20°C with a 16 h photo-period and an 8 h dark period for 2 months. Irrigation was carried out daily using 20 to 30 mL of sterilized tap water to maintain water content. The bacterial cells were harvested by centrifugation at 13,400 × g for 5 min at 4°C. The cell pellet was suspended in 100 mM phosphate buffer (pH 7.4), and 0.1 mL of the diluted solution was spread on a tryptic soy agar (TSA; Becton, Dickinson and Company) plate and incubated at 37°C for 48 h.

Viable bacterial counts were measured immediately after inoculation, after incubation for 24 h, after washing with water, and after NaClO treatment to show the effect of pretreatment on the attachment and decontamination of S. Enteritidis-EGFP on lettuce leaves. In the case of sprouts, viable bacterial counts were measured after the overnight drying of inoculated seeds, after PL treatment of seeds, after cultivation for 7 d, after washing with water, and after NaClO treatment to show the effects of PL on the attachment and decontamination of S. Enteritidis-EGFP on seeds and sprouts. At each sampling, duplicate bags from each treatment were analyzed for viable S. Enteritidis-EGFP and viable resident bacterial counts. The lettuce leaves and sprouts were transferred to stomacher bags with a filter containing a 9-fold amount of buffered peptone water (BPW) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and were then homogenized for 30 s with the masticator paddle blender (IUL S. A., Barcelona, Spain). The homogenate was 10-fold serially diluted with phosphate-buffered saline (PBS, 137 mM NaCl, 8.10 mM NaHPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4), and 0.1 mL of the diluted solution was spread on a tryptic soy agar (TSA; Becton, Dickinson and Company) plate and incubated at 37°C for 48 h.
After incubation, the number of green colonies was measured as viable <i>S. Enteritidis-EGFP</i> and other colonies as viable resident bacteria.

Measurement of ascorbic acid content Ascorbic acid content was analyzed according to the 2,4-dinitrophenylhydrazine method described by Rahman et al. (2007) with some modifications. Lettuce leaves pretreated with the combination of 0.001% PL and 0.25% MSP (and surface dried overnight at room temperature) and sprouts grown for 7 d from seeds treated with 0.01% PL were used for measurement of ascorbic acid contents. About 5 g of lettuce leaves and sprouts were cut separately and ground with a simple mortar and pestle in 50 mL of 5% (w/v) metaphosphoric acid (Nacalai Tesque, Inc., Kyoto, Japan). The homogenate was centrifuged at 800 × g for 10 min at room temperature and the resulting supernatant was filtered with filter paper (No.1, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Filtrates were used for the determination of total ascorbic acid (ascorbic acid and dehydroascorbic acid). A few drops of hydrated 2,6-dichlorophenolindophenol sodium salt (Nacalai Tesque, Inc.) solution was added to 2 mL of the test solution and then 2 mL of 5% metaphosphoric acid solution containing 2% (w/v) thiourea (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added. Furthermore, standard solutions (1, 5, 10, 25, and 50 mg/L) of L-ascorbic acid (Kishida Chemical Co., Ltd., Osaka, Japan) were prepared with 5% metaphosphoric acid solution. Then, 1 mL of 2% 2,4-dinitrophenylhydrazine solution was added to all standard and sample tubes. For the completion of the reaction, all of the tubes for standards, samples, and blank were kept at 50°C for 1 h in a water bath. After incubation, they were cooled on ice and mixed with 5 mL of 85% H₂SO₄ with constant manual shaking. At this stage, the blank solutions were treated with 1 mL of 2,4-dinitrophenylhydrazine. After incubation at room temperature for 30 min, the absorbance at 540 nm was measured using a spectrophotometer (UV-1800; Shimadzu Corp., Kyoto, Japan) to determine the difference between the samples and the blank. The total ascorbic acid concentration was determined using a calibration curve with L-ascorbic acid as a standard.

Measurement of chlorophyll content The chlorophyll content was measured according to the method described by Porra et al. (1989) with some modifications. The lettuce and sprouts used for chlorophyll measurement were the same as those used for the measurement of ascorbic acid. About 5 g of lettuce leaves were cut and ground in 5 mL of pure water with a simple mortar and pestle, and then the homogenates were mixed with 20 mL of 80% acetone (Nacalai Tesque, Inc.) with stirring. In the case of sprouts, leaves from about 40 radish sprouts (about 0.2 g) were cut and ground in 0.4 mL of pure water with a simple mortar and pestle, and then the homogenates were mixed with 1.6 mL of 80% acetone with stirring. The mixtures were then centrifuged at 800 × g for 5 min at room temperature, and the resulting supernatants were diluted 10-fold with 80% acetone to determine the chlorophyll content. The 80% aqueous acetone was used as the blank. The absorbance of the supernatants was measured at 663.6 nm and 646.6 nm using a spectrophotometer (UV-1800; Shimadzu Corp.). The total chlorophyll content was obtained by the sum of the chlorophyll <i>a</i> and chlorophyll <i>b</i> contents. The chlorophyll content (μg/mL) was determined using the following equations (Eq. 1-3).

\[
\text{Chl} \ a \ (\mu g/mL) = 12.25 \ A_{663.6} - 2.55 \ A_{646.6} \quad \cdots \text{Eq. 1}
\]

\[
\text{Chl} \ b \ (\mu g/mL) = 20.31 \ A_{646.6} - 4.91 \ A_{663.6} \quad \cdots \text{Eq. 2}
\]

\[
\text{Ch}a + b \ (\mu g/mL) = 17.76 \ A_{663.6} + 7.34 \ A_{646.6} \quad \cdots \text{Eq. 3}
\]

Statistical analysis All experiments were conducted in triplicate, and a minimum of two samples were analyzed at each sampling time. The student’s <i>t</i>-test (one-tailed distribution, two sample assuming equal variance) was performed to determine significant differences (<i>P</i> < 0.05) between the means. All statistical analyses were performed using Microsoft Excel for Mac 2011 (V 14.3.1, Microsoft Corporation, Redmond, WA, USA).

Results Effects of pretreatment with the 0.001% PL and 0.25% MSP mixture on attachment and decontamination of Salmonella Enteritidis-EGFP on lettuce leaves Based on our previous study using microtiter plates and cabbage leaves (Islam et al., 2014), the effects of pretreatment with one of the effective combinations of PL and MSP were investigated in lettuce leaves. Figure 1 shows the effects of pretreatment of lettuce leaves with the combination of 0.001% PL and 0.25% MSP during cultivation on the attachment and decontamination of <i>S. Enteritidis-EGFP</i> inoculated onto the surface of lettuce after the pretreatment. After 24-h incubation, the viable <i>S. Enteritidis-EGFP</i> counts were decreased by 0.2 log on lettuce leaves pretreated with the 0.001% PL and 0.25% MSP mixture compared to those of lettuce without pretreatment, indicating the low antibacterial activity of this combination of GRAS substances (Fig. 1a). Viable counts of <i>S. Enteritidis-EGFP</i> on lettuce leaves pretreated with the 0.001% PL and 0.25% MSP mixture were reduced from 5.7 log CFU/g to 2.8 log CFU/g after washing with water. The counts were significantly lower by 1.4 log (<i>P</i> < 0.05) than those of lettuce without the pretreatment. Furthermore, viable <i>S. Enteritidis-EGFP</i> counts were decreased to 1 log CFU/g by combined washing with water and treatment with 200 mg/L of NaClO for 5 min in lettuce pretreated with PL and MSP. The counts were significantly lower by 1.2 log (<i>P</i> < 0.05) than those of lettuce without the pretreatment. These results clearly showed that the pretreatment of lettuce leaves with PL and MSP facilitated the decontamination of <i>S. Enteritidis-EGFP</i> inoculated after pretreatment. On the other hand, no significant changes in viable resident bacterial counts were observed on lettuce leaves pretreated with PL and MSP after washing with water and a
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**Fig. 1.** Effects of pretreatment with a mixture of 0.001% PL and 0.25% MSP on the attachment and decontamination of Salmonella Enteritidis-EGFP inoculated on lettuce leaves. Lettuce plants were pretreated with a mixture of 0.001% PL and 0.25% MSP before the inoculation with S. Enteritidis-EGFP. Salmonella (a) and resident bacterial (b) counts were determined immediately after inoculation, after incubation for 24 h, after washing with water, and after decontamination with NaClO. Values represent the mean ± SD of three independent experiments. (*, *P* < 0.05).

combination of washing and NaClO treatment compared to those of the samples without pretreatment (Fig. 1b). However, there was a clear tendency for decreases in both the viable S. Enteritidis-EGFP counts and the resident bacterial counts by washing and subsequent combination of washing and NaClO treatment on lettuce leaves with and without pretreatment. Therefore, pretreatment of the surface of vegetables with 0.001% PL and 0.25% MSP during cultivation significantly promoted the decontamination of foodborne pathogens inoculated after pretreatment by the combination of washing with water and NaClO treatment.

**Effects of 0.01% PL treatment in seeds contaminated with Salmonella Enteritidis-EGFP on attachment and decontamination of the cultured sprouts** In a preliminary experiment, the combined use of 0.001% PL and 0.25% MSP showed no effects on the decontamination of S. Enteritidis-EGFP on radish sprouts during cultivation. MSP seemed to promote the growth of S. Enteritidis-EGFP during cultivation for 7 d. Based on the results of the experiment using microtiter plates (Miyamoto et al., 2011), the effects of one of the most effective substances, PL, was investigated alone at 0.01% for the decontamination of S. Enteritidis-EGFP in radish sprouts. Figure 2 shows the effects of 0.01% PL treatment during cultivation on the attachment and decontamination of S. Enteritidis-EGFP on radish sprouts raised from S. Enteritidis-EGFP-inoculated seeds. The viable counts of S. Enteritidis-EGFP on radish sprouts grown in the presence of 0.01% PL, from seeds inoculated with S. Enteritidis-EGFP at 3.7 log CFU/50 seeds, increased to 6.4 log CFU/50 sprouts after cultivation.
(Fig. 2a). The S. Enteritidis-EGFP viable counts were reduced from 6.4 to 5.5 log CFU/50 sprouts after washing with water, which was significantly lower ($P < 0.05$) than those of sprouts cultivated in the absence of PL by 1.1 log. Furthermore, viable S. Enteritidis-EGFP counts were decreased to 3.1 log CFU/50 sprouts by the subsequent treatment with 200 mg/L NaClO for 5 min, which was significantly lower ($P < 0.05$) than those of sprouts without treatment by 1.6 log. On the other hand, no significant changes in viable resident bacterial counts were observed on sprouts grown in the presence of PL after washing with water and the combination of washing and NaClO treatment compared to those of the sprouts without treatment (Fig. 2b). Therefore, the 0.01% PL treatment during cultivation significantly promoted the decontamination of S. Enteritidis-EGFP by the combination of water washing and subsequent NaClO treatment in sprouts raised from inoculated seeds.

**Effects of PL and MSP on ascorbic acid and chlorophyll contents** In this study, the ascorbic acid contents were determined to be 13.0 and 18.3 mg/100 g on lettuce without pretreatment and that pretreated with 0.001% PL and 0.25% MSP, respectively. On the other hand, the ascorbic acid contents were 50.8 and 41.3 mg/100 g in sprouts cultivated in the absence and in the presence of 0.01% PL, respectively. The chlorophyll contents of lettuce leaves were determined to be 23.7 and 20.6 mg/100 g in lettuce without pretreatment and that pretreated with PL and MSP, respectively. The chlorophyll contents in the radish sprouts were 23.9 and 27.1 mg/100 g in sprouts cultivated in the absence and in the presence of 0.01% PL, respectively. There were no significant differences ($P < 0.05$) in the chlorophyll and ascorbic acid contents in treated samples compared to untreated samples for both the seeds.

Fig. 2. Effects of treatment with 0.01% PL during cultivation on the attachment and decontamination of sprouts raised from *Salmonella* Enteritidis-EGFP inoculated seeds. The seeds of radishes were inoculated with *S. Enteritidis*-EGFP, and the contaminated seeds were germinated in the presence of 0.01% PL and cultivated for 7 d. *Salmonella* (a) and resident bacterial (b) counts were determined after inoculation, after treatment with PL for 4 h, after cultivation for 7 d, after washing with water, and after decontamination with NaClO. Values represent the mean ± SD of three independent experiments. (*, $P < 0.05$).
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serovars and is used (20,000 mg/L, to disinfect the surface of leaves is significantly lower in the PL solution and cultivation in the presence of PL leads to a reduction in both the multiplication and viable counts of *S. Enteritidis*-EGFP after decontamination in radish sprouts.

The decontamination process should not affect the quality of the final product. The ascorbic acid and chlorophyll contents are major quality attributes of green vegetables and should be taken into consideration. The ascorbic acid and chlorophyll contents are linked to the light intensity during the cultivation of vegetables. The ascorbic acid contents were reported to be in the range of 3 to 41 mg/100 g (average 17 mg/100 g) and of 21 to 80 mg/100 g (mostly from 27 to 51 mg/100 g) in lettuce and radish sprouts, respectively, depending on the part of the plant and the level of maturity (Izaki et al., 1984; Obi, 1990; i). Similarly, the chlorophyll contents were reported to be 15.1 mg/100 g and in the range of 0 to 111 mg/100 g in red leaf lettuce and radish sprouts, respectively, depending on the light intensity during cultivation (Izaki et al., 1986; Izumi et al., 1984). The wide range in the initial ascorbic acid content in lettuce leaves can be attributed to various factors, such as the lettuce variety, climate conditions, cultivation practices, maturity at harvest, harvesting method and post-harvest handling conditions (Lee and Kader, 2000). The ascorbic acid and chlorophyll contents of the red leaf lettuce and radish sprouts were in the range of those reported previously. Therefore, the presence of PL alone and in combination with MSP at the concentration used in this study did not affect the contents of ascorbic acid and chlorophyll in sprouts and lettuce, respectively.

The results obtained in this study showed the applicability of produce pretreatment with the GRAS compounds PL and MSP during cultivation for the effective decontamination of *Salmonella* on the surfaces of fresh produce by washing with water and subsequent NaClO treatment, although the study of additional applications to various types of produce and food-borne pathogens is required.

**Acknowledgements** This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (research project for ensuring food safety from farm to table, DI-7201). The authors are grateful to Asama Chemical Co., Ltd. and Chisso Corporation for providing the milk serum protein and e-polysyline, respectively.

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