MICROBIOLOGY AND FOOD SAFETY

Application of calcium oxide (CaO, heated scallop-shell powder) for the reduction of *Listeria monocytogenes* biofilms on eggshell surfaces

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ABSTRACT This study investigated bactericidal activity of 0.05 to 0.50% calcium oxide (CaO) against planktonic cells in tryptic soy broth (TSB) and biofilms of *Listeria monocytogenes* on eggshell surfaces. The bactericidal activity of CaO against planktonic cells and biofilms of *L. monocytogens* significantly ($P < 0.05$) increased log reductions with increasing concentrations of CaO. Exposure to 0.05 to 0.50% CaO for one min reduced planktonic cells in TSB cell suspensions by 0.47 to 3.86 $\log_{10}$CFU/mL and biofilm cells on the shell surfaces by 0.14 to 2.32 $\log_{10}$CFU/cm^2. The Hunter colors of eggshells ("L" for lightness, "a" for redness, and "b" for yellowness), shell thickness (puncture force), and sensory quality (egg taste and yolk color) were not changed by 0.05 to 0.50% CaO treatment. The nonlinear Weibull model was used to calculate $C_R = 3$ values as the CaO concentration of 3 log (99.9%) reduction for planktonic cells ($R^2 = 0.96$, RMSE = 0.26) and biofilms ($R^2 = 0.95$, RMSE = 0.18) of *L. monocytogens*. The $C_R = 3$ value, 0.31% CaO for planktonic cells, was significantly ($P < 0.05$) lower than 0.57% CaO for biofilms. CaO could be an alternative disinfectant to reduce planktonic cells and biofilms *L. monocytogenes* on eggshell surface in egg processing plants.

Key words: *Listeria monocytogenes*, biofilms, calcium oxide, eggshell surface, Weibull model

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INTRODUCTION

Fresh eggs are an inexpensive daily source of proteins, fats, and micronutrients that are consumed throughout the world (Miranda et al., 2015). Fresh raw egg is also commonly used as a major or minor ingredient in the preparation of diverse processed food products, such as breads, snacks, noodles, and sauces. Furthermore, in recent decades, there has been increasing interest in developing functional egg-derived foods to obtain profitability for egg producers and the food industries and to improve the overall public health condition for consumers (Miranda et al., 2015). Fresh eggs can also act as a vehicle for numerous pathogenic microorganisms such as *Salmonella*, *Escherichia coli*, *Campylobacter* spp., and *Listeria* spp. (Adesiyun et al., 2005). Contamination of the eggshell can occur at processing facilities through contact with transfer belts and packaging materials. Microorganisms that adhere to the eggshell surface can also penetrate into the interior of the egg through microscopic pores on the eggshell surface, resulting in the contamination of egg contents, including the albumen and yolk.

For decades, *Listeria monocytogenes* has been regarded as a global human foodborne pathogen. *L. monocytogenes* has been isolated from a wide range of raw and ready-to-eat meats, poultry, egg and egg products, seafood, and vegetables and from various food-processing environments. However, *L. monocytogenes* infections have never been reported to be associated with eggs, despite the fact it has been isolated from the laying environment and eggshells (Chemaly et al., 2008). Sayed et al. (2009) reported that up to 7% of eggshells were contaminated by *Listeria* spp., and that eggshells were contaminated with *L. monocytogenes* more frequently than egg contents. A study in France demonstrated that the rates of *L. monocytogenes* contamination of raw and pasteurized egg products were 17 and 2%, respectively (Rivoal et al., 2010). Thus, egg products are susceptible to contamination by *L. monocytogenes* if not prepared and handled properly (Hwang and Marmer, 2007).

The persistence of *L. monocytogenes* biofilms on food surfaces and surfaces that contact food is the main attribute facilitating its environmental spread and subsequent contamination of ready-to-eat meat products (Keskinen et al., 2008). Kim et al. (2016) recently noted

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that in the egg processing environment, *L. monocytognes* can form the biofilms more easily because of a large amount of water. They also noted that peeled, boiled egg products are mostly in contact with water, and peeled eggshells also can enter into a processing container without difficulty. Thus eggshell remained in the processing container as the major surface can form biofilm. Biofilms are regarded as architecturally three-dimensional structures of aggregated bacteria. Biofilm formation comprises bacteria that attach irreversibly to the surface and produce exopolymeric substances. It is well documented that biofilms have been playing an important role as a barrier for microorganisms against unfavorable environmental conditions, such as UV rays, drying, disinfectants, and antibiotics (Milanov et al., 2009). Thus, biofilm formation on inert surfaces is known to protect *L. monocytogenes* from various chemical and physical stresses.

In the United States and Korea, eggshells can be washed to remove pathogenic bacteria to avoid food poisoning related to egg processing plants. The prevalence of egg washing for egg and egg-related products was 50% in the United States and 9% in Korea in 2002 (Lee and Hong, 2005). However, this washing step to eliminate microorganisms on eggs can damage the cuticle layers of the shell. Thus, it is important that egg product processors are aware of conditions that enhance or limit the effectiveness of sanitization methods while retaining the quality of the eggshell.

Scallop shells are waste products of scallop harvesting in districts of Korea and Japan. When scallop shells are heated to $> 700^{\circ}C$, calcium carbonate ($CaCO_3$), the main component of scallop shells, is converted to calcium oxide (CaO), which has potent bactericidal activity (Sawai et al., 2001, 2003). The antibacterial activity of scallop shell powder heated to 1,000°C is comparable to that of pure CaO (Sawai et al., 2001). Heated scallop shell powder effectively reduces the indigenous aerobic bacterial population in shredded cabbage (Sawai et al., 2001) and kills *Bacillus subtilis* spores (Sawai et al., 2003). Moreover, previous studies have reported that biofilms of *Staphylococcus aureus* (Sawai et al., 2013) and *Escherichia coli* O157: H7 on glass plates (Kubo et al., 2013) can be eliminated by heated scallop shell powder. Bodur and Cagri-Mehmetoglu (2012) also reported that scallop shell power slurries could significantly reduce the numbers of *L. monocytogenes*, *S. aureus*, and *E. coli* O157: H7 on stainless steel surfaces.

There is a need to further examine the bactericidal activity of CaO (heated scallop shell powder) against planktonic cells and biofilms of *L. monocytogenes* grown on inert surfaces, especially during washing. Therefore, in the present study, we investigated the bactericidal activity of CaO (0.05 to 0.50%) against *L. monocytogenes* biofilms on the eggshell surface, which represents the major surface in egg processing plants.

MATERIALS AND METHODS

Bacterial Strain

L. monocytogenes ATCC 19,113 (serotype 3b), isolated from a human patient in Denmark, was obtained from the American Type Culture Collection (Manassas, VA). This strain, as opposed to strains isolated directly from eggs or its environment, was used because of its ability to readily form biofilms (results not shown). Bacteria were recovered from a −80°C frozen stock, and 100 μL of the stock was inoculated into 10 mL of tryptic soy broth (**TSB**: Difco Laboratories, Detroit, MI, USA). The solution was incubated for 24 h at 30°C. After vortexing, 100 μL of the incubated culture was pipetted into 10 mL of fresh TSB and incubated for another 24 hours.

Preparation of Inoculum

After subculturing the strain, the suspension was centrifuged at 10,000 × *g* for 15 min at 4°C, and the pellet was resuspended and washed twice with Dulbecco's phosphate-buffered saline (**DPBS**; Sigma Aldrich, St. Louis, MO, USA). The strain was suspended in DPBS to a final cell density of $OD_{600} = 1.5$. OD_{600} is the optical density of a sample measured at a wavelength of 600 nm. The cell density was determined by serial dilution and plating onto PALCAM Agar (Oxoid, Basingstoke, England). Inocula (approximately 5 to 6 log CFU/mL) were used to form biofilms on eggshell steel surfaces.

Preparation of Eggshell and Removal of Background Microfloras

The preparation of samples was performed following procedures detailed in Srey et al. (2014), with some modifications. Fresh brown eggs were purchased from a local grocery store in Anseong-si, Korea. Shell fragments were obtained by breaking the whole egg and cutting crude fragments into 2 × 2 cm^2 pieces using a knife. Prepared eggshell pieces were soaked in distilled H_2O (dH_2O) for one h to remove residues, such as dust, and then rinsed with dH_2O. After rinsing, the prepared shell pieces were soaked in 100% ethanol at room temperature for one d to remove background microfloras before inoculating with *L. monocytogenes*.

Inoculation for Planktonic Cells and Biofilm Formation

Bacteria were grown, and the inoculation of sample surfaces was performed as previously described (Patel and Sharma, 2010), with some modifications. A 40-μL bacterial suspension and an eggshell coupon were added to a sterile 50-mL Falcon tube, which contained l0 mL

of TSB, to make a final inoculum of approximately 6 to 7 log CFU. Planktonic cells of *L. monocytogenes* were grown in broth at 30°C for 24 h under static conditions in a closed container. Biofilms of *L. monocytogenes* were formed on sample surfaces at 30°C for 24 h under static conditions in a closed container. After forming biofilms, each coupon was washed twice by dipping in 50 mL of sterile dH_2O to remove unattached or loosely attached cells.

CaO Treatment

For CaO treatments, 10 mL of 9 different CaO concentrations (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, and 0.50% [w/v]), were prepared by diluting 96% CaO (Samchun Pure Chemical Co., Ltd, Pyeongtak, Korea) with sterile dH_2O and stirred using a magnetic stirrer. Each of the coupons was dipped in CaO solutions for one minute. We used samples treated with sterile dH_2O as a negative control.

Quantification of Surviving Planktonic Cells from Cell Suspensions and Biofilms From Samples

Planktonic cells in the TSB cell suspension (500 μL) were transferred to 4.5 mL of each concentration of CaO solution. After each CaO treatment, *L. monocytogenes* planktonic cells were quantified by spread plating 10-fold serial dilutions of cells onto PALCAM Agar plates and incubating at 30°C for 48 hours.

After each CaO treatment, the sample coupon was washed once by dipping in 50 mL of sterile dH_2O in order to remove residual CaO on the sample. The CaO-treated eggshell piece was then placed in 10 mL of 0.1% peptone water (**PW**; Oxoid, UK) in a sterile 50-mL Falcon tube and vortexed with glass beads for 2 minutes. *L. monocytogenes* biofilms were quantified by spread plating 10-fold serial dilutions of cells onto PALCAM Agar plates and incubating at 30°C for 48 hours.

Determination of C_R = 3 Values (99.9% Reductions) Using the Weibull Model

The Weibull model, a 2-parameter nonlinear model, can be expressed as

$$\mathrm{Log}\left(\frac{Nt}{N0}\right) = -bt^n \qquad (1)$$

Where: Nt is the population of planktonic cells or biofilm after being exposed to a concentration (%) of CaO (t), No is the initial population of planktonic cells (CFU/mL) or biofilm (CFU/cm^2), b is the scale (a characteristic concentration), and n is the shape parameter as a behavioral index, respectively (van Boekel, 2002). The b value represents the concentration needed to reduce the population by one log unit, and the n parameter indicates the shape of the survival curve. An n value of 1 corresponds to a linear survival curve, whereas n values > 1 or < 1 indicate downward or upward concavity, respectively. For the calculation of C_R (analogous to the traditional D-value) from the Weibull parameters, equation (2) was used, as expressed by Buzrul and Alpas (2007)

$$C_R = \left(\frac{a}{b}\right)^{1/n} \qquad (2)$$

Where: C_R = CaO concentration (%) required to reduce planktonic cells or biofilms by 99.9% (3 log). To determine the concentration dependence of inhibition, the modified Weibull model was fit by nonlinear regression analysis using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA).

Field Emission Scanning Electron Microscopy

The shells treated with 3 different CaO concentrations (0.10, 0.30, and 0.50% [w/v]) were chosen for evaluation using field emission scanning electron microscopy (**FESEM**) (Sigma, Carl Zeiss, Jena, Germany) by comparing with the control (treated with sterile dH_2O). *L. monocytogenes* biofilm on the shells was observed using FESEM after each treatment. FESEM shell processing was conducted according to the procedures described by Srey et al. (2014). The eggshells were fixed in 2% glutaraldehyde (Sigma Aldrich, St. Louis, MO) in phosphate-buffered saline (**PBS**, pH 7.2) for 4 h at 4°C, and then washed 3 times with PBS (15 min each). The fixed biofilms were serially treated with 50, 60, 70, 80, and 90% (v/v) ethanol, and twice with 100% ethanol (15 min each) then dehydrated in 33, 50, 66, and 100% (v/v) hexamethyldisilazane (Sigma Aldrich) in 100% ethanol for 15 min each. The same location on each shell was aseptically cut, and the dehydrated samples were sputter-coated with platinum and visualized using FESEM. FESEM was operated at an accelerated voltage of 5 kV and 5 mm working distance.

Color Evaluation

To assess the quality of eggshells, the color of treated samples was evaluated before and immediately after CaO treatment (0.10, 0.20, 0.30, 0.40, and 0.50%). All samples were not inoculated for color evaluations. The treated sample (2 × 2 cm^2) was placed in a petri dish (20 × 12 mm), and the color of the treated sample was measured using a color-difference meter (UltraScan

PRP, Hunterlab Co., Reston, VA, USA). Color was measured at 5 locations on each sample and was expressed by the Hunter color parameters "L" (lightness), "a" (redness +, greenness −), and "b" (yellowness +, blueness −) (Hunter and Harold, 1987). The standard plate was 97.47 "L", −0.22 "a," and 0.01 "b."

Puncture Force Evaluation

The thickness of the eggshells was determined by texture analysis (TAHDi/500, TAHD) with an adaptor No. 5 (2 mm diameter). Each egg sample after CaO treatment (0.10, 0.20, 0.30, 0.40, and 0.50%) was mounted on a platform and the eggshells were punctured at the top (small end) and bottom (large end). The force required to compress an eggshell by 3% of its diameter was recorded at a strain rate of 60 mm/min. Samples were measured right after combined treatments, and the evaluations for the puncture tests was recorded as the newton (N) required to puncture the whole egg. The mean of 5 measurements was recorded for each sample. Texture data were analyzed via SAS (Version 9.2, SAS Institute Inc., Cary, NC).

Sensory Evaluation

Twenty untrained panelists evaluated the sensory quality of the treated samples. The samples were evaluated for egg taste and yolk color by using the hedonic scale—a 7-point scale graded with "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" like; "6" for much like; and "7" for extreme like, essentially free from any defect, original quality preserved. A rating of >4 indicates greater consumer acceptability of the food product. Prior to sample evaluation, the panelists participated in orientation sessions to familiarize themselves with the attributes of the hedonic scale and the related quality assessment. Each panelist was furnished with a set of 6 samples (0, 0.1, 0.2, 0.3, 0.4, and 0.5% CaO treatment) coded with 3-digit random numbers, and then independently evaluated the samples in identical environments.

Statistical Analysis

All experiments were performed in triplicate and repeated 2 times. The average reduction in microbes was converted to log values. One-way ANOVA was used to determine whether any differences in the reduction of planktonic cells and biofilms, color ("L," "a," and "b"), shell thickness (puncture force), or sensory (egg taste and yolk color) changes due to CaO treatments were significant. Differences among CaO treatments were compared using Duncan's new multiple range test. In addition, the reduction of cell numbers between 2 types of cells at the same concentration of CaO and $C_R = 3$ values between 2 cell types were analyzed with t tests using the SAS statistical software program. All statistical analysis was done using the SAS program version 6.11 (SAS Institute Inc.), and statistical significance was determined as $P < 0.05$.

Table 1. Effects of CaO treatment on the reduction of *L. monocytogenes* planktonic cells in TSB and biofilms on eggshell surface.

CaO (%)	Log_{10} reduction of planktonic cells in TSB	Log_{10} reduction of biofilms on eggshell surface
0.05	$0.47 \pm 0.06^{a,H}$	$0.14 \pm 0.06^{a,E}$
0.10	$0.98 \pm 0.04^{a,G}$	$0.23 \pm 0.02^{b,E}$
0.15	$1.45 \pm 0.02^{a,F}$	$0.28 \pm 0.17^{b,E}$
0.20	$1.63 \pm 0.05^{a,F}$	$0.57 \pm 0.02^{b,D}$
0.25	$1.86 \pm 0.12^{a,E}$	$0.62 \pm 0.06^{b,D}$
0.30	$3.02 \pm 0.12^{a,D}$	$1.21 \pm 0.15^{b,C}$
0.35	$3.30 \pm 0.13^{a,C}$	$1.55 \pm 0.03^{b,B}$
0.40	$3.61 \pm 0.10^{a,B}$	$1.68 \pm 0.03^{b,B}$
0.50	$3.86 \pm 0.03^{a,A}$	$2.32 \pm 0.01^{b,A}$

Egg shell coupon was dipped in CaO solutions for 1 min.

Within the same row, log reduction of *L. monocytogenes* means with different letters (^{a} or ^{b} for each CaO concentration) differ significantly ($P < 0.05$) by t-test.

Within the same column, log reduction of *L. monocytogenes* means with different letters ($^{A\sim H}$ for planktonic cells or $^{A\sim E}$ for biofilms) differ significantly ($P < 0.05$) by Duncan's multiple range test.

RESULTS

Bactericidal Activity of CaO Against *L. Monocytogenes* Planktonic Cells in TSB Cell Suspension and Biofilms on Eggshell Surfaces

As shown in Table 1, the bactericidal activity of different concentrations of CaO against *L. monocytogenes* planktonic cells in TSB cell suspension and biofilms present on eggshell surfaces was analyzed. The bactericidal activity of CaO against planktonic cells increased ($P < 0.05$) with increasing concentrations of CaO (0.05 to 0.50%) (Table 1). No significant difference ($P > 0.05$) in planktonic cell numbers was observed between 0.15 and 0.20% CaO. Each increase in CaO concentration, except for at 0.05% CaO, reduced planktonic cell numbers by approximately 1 log or >1 log (90%). Reductions in planktonic cell numbers by more than 3 log (>99.9%) were observed after treating with ≥0.30% CaO.

The bactericidal activity of CaO against biofilms also increased ($P < 0.05$) with increasing concentrations of CaO (0.05 to 0.50%) (Table 1). No significant difference ($P > 0.05$) in bacterial cell numbers in biofilms on eggshell surfaces was observed between 0.05 and 0.15%, 0.20 and 0.25%, or 0.35 and 0.40% CaO. In contrast to the potent bactericidal activity exhibited against planktonic cells, a reduction of bacterial cell numbers in biofilms by 1 log and >1 log was observed only after 0.25% CaO treatment and ≥0.30% CaO treatment, respectively.

Table 2. $C_R = 3$ values (CaO %) of *L. monocytogenes* planktonic cells in TSB and biofilms on eggshell surface exposed to CaO treatment.

Parameter of Weibull model	Planktonic cells	Biofilms
b[1] ± S.D.	7.77 ± 0.55	4.206 ± 0.49
n[2] ± S.D.	0.90 ± 0.06	0.55 ± 0.04
Correlation coefficient (R^2)	0.96	0.95
Root mean square error (RMSE)	0.26	0.18
$C_R = 3$ values of CaO concentration (%)	0.31 ± 0.02[B]	0.57 ± 0.08[A]

[1]b = scale parameter, CaO concentration (%).
[2]n = shape parameter, concave upward (tailing) survival curve if n < 1, concave downward (shoulder) if n > 1, and linear if n = 1.
Within the same row, $C_R = 3$ value means with different letters ([A] or [B] for cell type) differ significantly ($P < 0.05$) by *t*-test.

Data for the survival of *L. monocytogenes* planktonic cells in the cell suspensions and biofilms on eggshell surfaces treated with different concentrations of CaO were fit using the Weibull model, which is commonly used for nonlinear microbial survival. The concentration of CaO required to reduce cell numbers by 3 log ($C_R = 3$) was predicted based on fits using the Weibull model (Table 2). The Weibull model fit the experimental data well at all tested CaO concentrations (0.05 to 0.50%), as reflected by R^2 values of 0.96 and 0.95 and root mean square errors (**RMSE**) of 0.26 and 0.18 for planktonic cells and biofilms, respectively (Table 2). The concentrations of CaO required to reduce cell numbers by 3 log were 0.31 and 0.57% for planktonic cells and biofilms, respectively. The $C_R = 3$ value (0.31% CaO) predicted for planktonic cells, based on fits using the Weibull model, nearly matched the $C_R = 3$ value observed empirically; 0.30% CaO treatment reduced planktonic cell numbers by 3.02 log. These data demonstrate that the bactericidal activity of CaO against *L. monocytogenes* in planktonic cells in cell suspensions and biofilms on eggshell surfaces was successfully modeled using the Weibull model.

The bactericidal activity of different concentrations of CaO against *L. monocytogenes* planktonic cells in TSB cell suspensions and biofilms on eggshell surfaces were also compared (Table 1). CaO bactericidal activity was significantly greater against planktonic cells than biofilms ($P < 0.05$) at nearly all CaO concentrations tested, except at the lowest concentration tested (0.05%) (Table 1). Specifically, CaO bactericidal activity was 4.26-, 5.18-, 2.86-, 3.00-, 2.50-, 2.13-, 2.15-, and 1.66-fold greater against planktonic cells than biofilms treated with 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, and 0.50% CaO, respectively. Moreover, the concentration of CaO required to reduce bacterial cell numbers by 3 log was 1.84-fold lower for planktonic cells than biofilms ($P < 0.05$) (Table 2). These data suggest that biofilms are generally more tolerant to CaO bactericidal activity than planktonic cells.

Effect of CaO on Surface Color and Thickness of Eggshell, and Sensory Quality of Egg Taste and Yolk Color

To determine the effect of CaO treatment on the overall shell quality, Hunter color values ("L" for lightness, "a" for redness/greenness, and "b" for yellowness/blueness) and puncture force (shell thickness) were measured on the egg shell surface (Table 3). Results of sensory analysis for egg taste and yolk color after CaO treatment (0.05 to 0.50%) are also shown in Table 2. No significant changes ($P > 0.05$) in mechanical Hunter colors, shell thikness, or sensory analysis were observed among all the samples treated with CaO. Therefore, CaO treatment (0.05 to 0.50%) can be used to control the growth of *L. monocytogenes* biofilms on eggshells without affecting their visual or sensory quality.

FESEM of L. Monocytogenes Biofilm Formation on CaO-treated Eggshell Surfaces

L. monocytogenes biofilms on CaO-treated eggshell are illustrated in Fig. 1. The FESEM image of control shows that the presence of aggregated clusters of 3-dimensional compact biofilms or mushroom-like structure embedded in large amounts of mesh-like extracellular polymeric substances (**EPS**) was clearly observed on the surface (Fig. 1A). The presence of aggregation of bacterial cells was observed in 0.1%

Table 3. Comparison of Hunter color ("L", "a," and "b"), shell thickness, and sensory quality of egg taste and yolk color after CaO treatment.

	Hunter color on the eggshell			Shell thickness	Sensory quality of egg	
CaO (%)	"L"	"a"	"b"	Puncture force (N)	Egg taste	Yolk color
0.00	81.10 ± 0.03	4.76 ± 0.03	9.91 ± 0.05	9.66 ± 1.67	5.62 ± 0.87	5.62 ± 0.82
0.10	79.88 ± 0.05	4.89 ± 0.06	9.99 ± 0.06	9.77 ± 2.08	5.46 ± 0.88	5.62 ± 1.04
0.20	80.11 ± 0.04	5.02 ± 0.05	9.78 ± 0.07	9.42 ± 1.61	5.50 ± 0.90	5.54 ± 0.97
0.30	80.28 ± 0.02	4.97 ± 0.04	10.01 ± 0.08	9.67 ± 2.11	5.67 ± 0.78	5.46 ± 0.65
0.40	79.71 ± 0.06	5.13 ± 0.08	10.10 ± 0.09	9.30 ± 1.69	5.62 ± 0.71	5.54 ± 0.97
0.50	81.03 ± 0.07	4.85 ± 0.06	9.89 ± 0.05	9.60 ± 1.63	5.60 ± 0.97	5.60 ± 0.82

"L" values = lightness (0 = dark, 100 = bright).
"a" values = redness/greenness (+ = red,— = green).
"b" values = yellowness/blueness (+ = yellow,— = blue).
There was no significant difference in all treatments on Hunter color on eggshell surface, shell thickness, or sensory quality of eggs ($P > 0.05$).

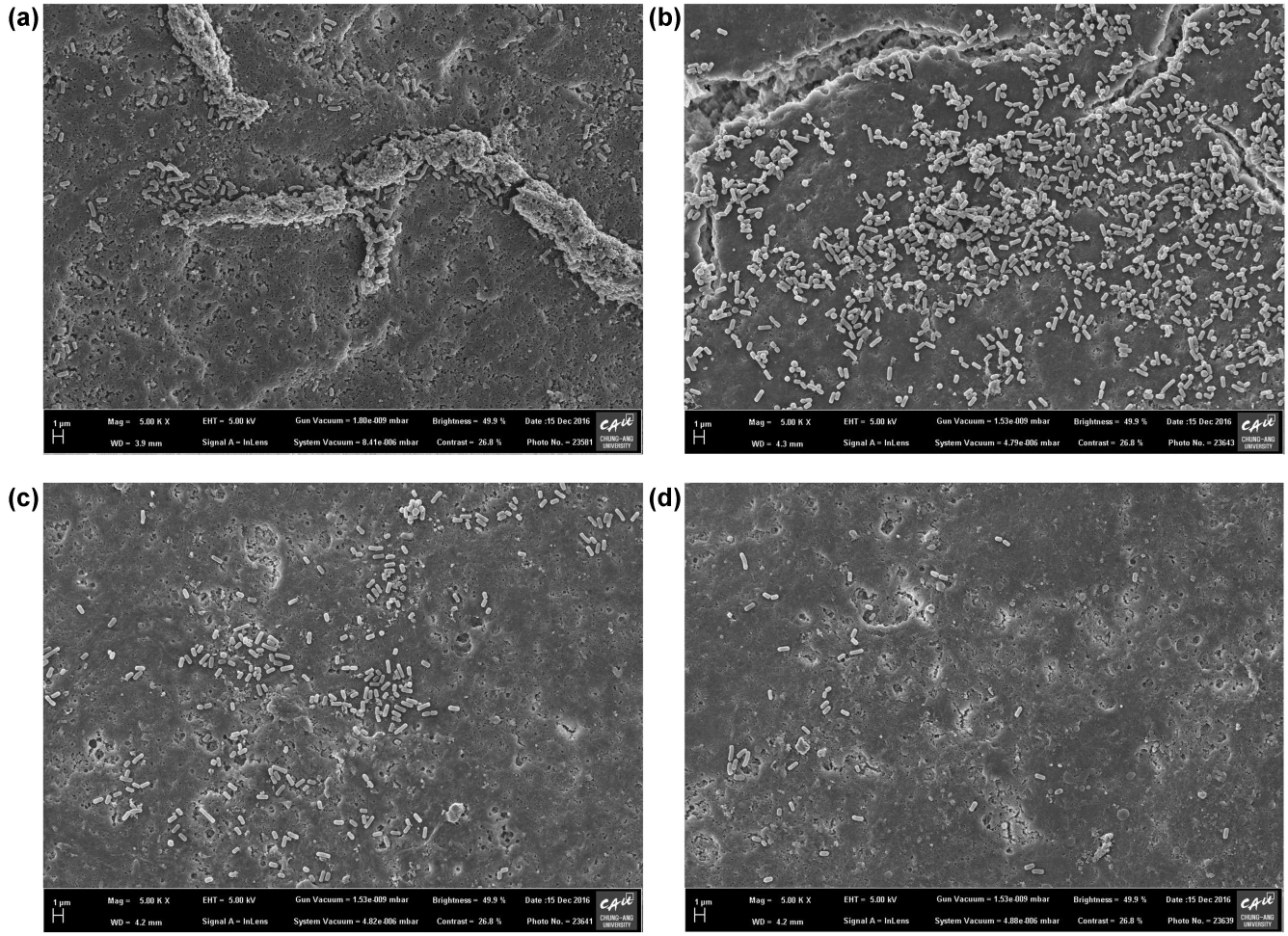

Figure 1. Field emission scanning electron microscopy images of *L. monocytogenes* biofilms on eggshell surfaces: (A) Control (5,000 x magnification). (B) After treatment with 0.1% CaO (5,000 x magnification). (C) After treatment with 0.3% CaO 5,000 x magnification). (D) After treatment with 0.5% CaO (5,000 x magnification).

CaO-treated shell surfaces (Fig. 1B). Destruction of the biofilm community and comparatively fewer bacterial cells were observed in 0.3% CaO-treated shell surfaces (Fig. 1C). However, the compact or weak biofilms were not observed in 0.5% CaO-treated shell surfaces (Fig. 1D). The single bacterial attached cells and complete removal of biofilm with very few cells remaining were clearly observed in 0.5% CaO-treated shell surfaces (Fig. 1D).

DISCUSSION

Biofilms that can form on diverse surfaces of food processing equipment can cross-contaminate fresh food surfaces, such as of vegetables and meat. The ability of *L. monocytogenes* to form biofilms allows their persistence at contamination sites in food processing environments. Nilsson et al. (2011) reported that mature biofilms of *L. monocytogenes* can form within 12 to 24 hours. Therefore, the use of disinfectants is key to interrupting the transmission of *L. monocytogenes* biofilms from equipment surfaces to eggshell surfaces. In our preliminary study, the ability of *L. monocytogenes* strains to form a biofilm was determined using the microtiter plate biofilm assay based on the procedure of Naves et al. (2008) who used the biofilm index (**BFI**) to express biofilm formation of bacteria under different environmental conditions. According to Naves et al. (2008) classification, the BFI readings are strong biofilm (≥1.10), moderate biofilm (0.70 to 1.09), weak biofilm (0.35 to 0.69), and no biofilm (<0.35). BFI of *L. monocytogenes* ATCC 19,113, 19,115, 19,116, and 19,118 were 0.72, 0.15, 0.24, and 0.59, respectively (data not shown). Therefore, *L. monocytogenes* ATCC 19,113 was used as a representative strain of biofilm formers in the current study.

Sodium hypochlorite (NaOCl) is easy to handle, cheap, and effective in reducing microorganisms (Peng et al., 2002). Chlorine concentrations of 50 to 200 mg/L are recommended for eggshell washing in commercial egg processing (USDA, 2001); however, Bialka et al. (2004) noted that high levels of chlorine can be detrimental to the quality of eggs. In particular, 50 mg/L of chlorine in the washing solution reacted with specific amino acids present in the cuticle layer of the shell surface and caused a change to the color of the

white eggshell (Park et al., 2003). Other studies have noted that NaOCl can cause deterioration of its observable quality, unpleasant odors, deposition of residual chlorine, and production of byproducts, such as trihalomethanes (THM), which are carcinogenic at high concentrations (Kim et al., 2008).

Therefore, there is a need to develop an alternative disinfectant to reduce or eliminate planktonic cells and biofilms of pathogenic bacteria, including *L. monocytogenes,* on eggshell surfaces in egg processing facilities. An alternative disinfectant is crucial to food safety and human health. Calcium oxide (CaO), commonly known as lime, quick lime, or burnt lime, is a widely used chemical compound. It is produced from calcium carbonate ($CaCO_3$) found in scallop shells by calcination at 700 to 1000°C ($CaCO_3 \rightarrow CaO + CO_2$). It is relatively inexpensive and exhibits antibacterial (Sawai et al., 2001) and anti-biofilm activity (Bodur and Cagri-Mehmetoglu, 2012; Kubo et al., 2013; Sawai et al., 2013). The use of this chemical in food processing is therefore expected to not only prolong the shelf life of food products but also act as a source of minerals. CaO is "Generally Recognized As Safe" (**GRAS**) for human consumption by the FDA (21CFR184.1210).

Based on the above observations, we evaluated CaO as an alternative disinfectant against *L. monocytogenes* planktonic cells and biofilms on eggshell. A direct comparison of the bactericidal activity of CaO against planktonic cells and biofilms has not been previously reported. In the present study, the bactericidal activity of CaO was 2.97-fold greater against planktonic cells than biofilms on eggshell surfaces (data not shown). This result is consistent with previous findings that biofilms, compared to planktonic cells, are more resistant to diverse kinds of environmental stresses, including starvation (Spector and Kenyon, 2012), nutritional and oxidative stresses (Hingston et al., 2013), dry conditions (Hingston et al., 2013), cold oxygen plasma (Jahid et al., 2015), ultraviolet light radiation (Jahid et al., 2014), and chemical sanitizing agents (Møretrø et al., 2012). Bae et al. (2006) previously reported that the bactericidal activity of CaO solutions increased with increasing CaO concentrations and exposure times, and that *L. monocytogenes* planktonic cells were more resistant to CaO than *E. coli* and *S. typhimurium*. They also reported that *L. monocytogenes* planktonic cell numbers were reduced by 1.44 log (CFU/mL) when treated for 10 min with 0.05% CaO. The bactericidal activity of 10-minute CaO treatments was greater than that of the present study. *L. monocytogenes* planktonic cells were reduced by 0.47 log when eggshells were treated with 0.05% CaO for one minute. The difference in bactericidal activity can be explained by the difference in the time of exposure to CaO. Kim et al. (2016) recently reported that *L. monocytogenes* biofilms on eggshell surface with 50, 100, 150, and 100 ppm NaOCl for one min were reduced by 0.24, 1.07, 1.14, and 1.26 log CFU/cm^2, respectively. The reductions (~1.14 to 1.26 log) of *L. monocytogens* biofilms treated with NaOCl at ~150 to 200 ppm can correspond to our result, which was 1.21 log reduction by CaO at 0.3% (=3000 ppm).

Because our empirical data could be better fit using a nonlinear model, we used the Weibull model to assess concentration-dependent bactericidal activity. Based on fits using the Weibull model, the concentration of CaO required to reduce bacterial cell numbers by 3 log (C_R = 3; 99.9% reduction) was determined. Although the bacteria are usually present in foods in relatively low numbers (<100 g), we chose to analyze C_R = 3 instead of D = 1 (1 log reduction) because the probability of exposure to a higher dose (>1000 CFU) is large enough to account for the observed rate of listeriosis (Hitchins, 1996). The concentration of CaO required to reduce *L. monocytogenes* cell numbers in biofilms by 3 log was higher on eggshell surfaces (0.57% CaO) than on stainless steel surfaces (0.46% CaO) (unpublished data), indicating that the sensitivity of biofilms to CaO on stainless steel surfaces was greater than on eggshell surfaces. This difference might be explained by the surface properties of the 2 substrates; eggshell surfaces are porous, rough, uneven, and nutritious, whereas stainless steel surfaces are nonporous, smooth, even, and non-nutritious. As a result, biofilms are harder to remove from eggshell surfaces than stainless steel surfaces. Moreover, biofilms can penetrate inside the eggshell, contaminating egg contents, such as the yolk and albumen, through microscopic pores.

Controlling the outbreak of pathogens on foods is a major research challenge. The use of any method to ensure the safety of food, including eggs, should also preserve its quality. When we examined the visual quality of CaO-treated eggs, we did not observe any visual changes in the color or appearance of the eggshell. In addition to these qualitative observations, we objectively instrumnet-measured the Hunter color of treated eggshells to determine whether CaO treatment changed the lightness, green/redness, or yellow/blueness of the eggshell. Consistent with our qualitative observations, no changes in the Hunter color of eggshells was apparent.

The mechanism(s) underlying the bactericidal activity of CaO against planktonic cells and biofilms has not been elucidated. The pH of 0.05, 0.10, 0.20, 0.30, 0.40, and 0.50% CaO solutions was 11.91, 12.14, 12.29, 12.35, 12.36, and 12.37, respectively (data not shown). The high alkalinity of CaO solutions is likely responsible for the observed bactericidal activity. This mechanism is consistent with previous results by Bodur and Cagri-Mehmetoglu (2012) who reported that the CaO solution they used was pH 12.5. Another possible mechanism for bactericidal activity of CaO is associated with the generation of superoxides (O_2^-) (Sawai et al., 2001).

CONCLUSION

The results of the present study provide additional support of the bactericidal activity of CaO against *L. monocytogenes* planktonic cells in TSB cell suspensions and biofilms on eggshell surfaces. CaO treatment at

0.05 to 0.50% for one min reduced planktonic cell numbers in the cell suspensions by 0.47 to 3.86 log CFU/mL and biofilm cell numbers on the surfaces by 0.14 to 2.32 log CFU/cm^2. No change of shell color or shell thickness, or sensory quality (egg taste and yolk color) was observed by treating with 0.05 to 0.50% CaO. The concentration of CaO required to reduce cell numbers by 3 log ($C_R = 3$) was estimated based on fits using the Weibull model and were significantly different ($P < 0.05$) for planktonic cells in cell suspension (0.31% CaO) and biofilms on eggshell surfaces (0.57% CaO). CaO, prepared from natural materials, could be a potential substitute for chemical disinfectants that are currently used on eggs in egg processing plants.

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