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Biofilm formation by *Salmonella* Enteritidis in a simulated liquid egg processing environment and its sensitivity to chlorine and hot water treatment

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ABSTRACT

This study investigated the biofilm formation by three *S.* Enteritidis strains in a simulated liquid egg processing environment using 0.1% peptone water (PW) (control), 10% whole eggs (WE), 10% egg yolks (EY) and 10% egg whites (EW) as growth media, and evaluated the effectiveness of chlorine (200 ppm, 5 min) and hot water (71 °C, 30 s) treatments against *S.* Enteritidis biofilms. The results showed that *S.* Enteritidis formed significantly ($P < 0.05$) denser biofilms in PW and EW compared to those in WE and EY. However, biofilms formed in PW were less resistant to chlorine treatment than those formed in WE, EY, and EW, with average log reductions of 6.34, 2.28, 0.67 and 0.95 CFU/cm^2, respectively. Microscopic observation showed that biofilm morphology was greatly affected by the growth medium, and the egg matrices might act as protective barriers, contributing to the greater chlorine resistance. All biofilms were very sensitive to hot water treatment, which reduced the cell populations by 4.30–7.08 log CFU/cm^2. This study could advance our understanding towards the biofilm forming abilities of *S.* Enteritidis in liquid egg processing environments and the effectiveness of sanitation methods against *S.* Enteritidis biofilms, which may aid in the development of better sanitation strategies.

1. Introduction

Eggs are extensively consumed in the world due to their exceptional nutritious values and relatively cheap prices (Muñoz, Dominguez-Gasca, Jimenez-Lopez, & Rodriguez-Navarro, 2015; Surai & Sparks, 2001). However, consumption of raw or undercooked eggs may cause illnesses if eggs are contaminated with *Salmonella*. An outbreak caused by *S.* Enteritidis in Singapore that sickened 216 people was linked to the consumption of cream cakes, which were made of unpasteurized egg whites (Solhan et al., 2011). Besides, a multistate outbreak occurred in the United States, which resulted in 1939 reported illnesses, was associated with shell eggs that were contaminated with *S.* Enteritidis (CDC, 2010).

To ensure egg safety, pasteurization is often used to reduce the level of *S.* Enteritidis in liquid eggs. However, *Salmonella* cells were detected even in the pasteurized liquid egg samples (Hara-Kudo & Takatori, 2009; Kim et al., 2015), which was possibly due to insufficient thermal processes or recontamination after pasteurization. The presence of *Salmonella* in the shell egg processing environment, such as floor drains, breaker egg diverter, breaker egg belt surface, and wash tanks, has been well documented (Musgrove & Berrang, 2008; Musgrove, Ingram, Hinton, & Liljebjelke, 2010). The persistence might be attributed to the ability of *Salmonella* to form biofilms on these surfaces (Wang, Ding, Wang, Xu, & Zhou, 2013), which create potential sources of recontamination of pasteurized liquid eggs.

Biofilms are aggregates of surface-attached microbial cells enclosed within an extracellular polymeric substance (EPS) matrix (Donlan, 2002). The capabilities of *Salmonella* spp. to form biofilms on contact surfaces have been mostly investigated in laboratory media, and only a few studies used food matrices (chicken, beef, turkey, or lettuce broth) as growth media (Kim & Wei, 2007; Wang

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et al., 2013). To the best of our knowledge, no study has been carried out to investigate the biofilm formation by *S.* Enteritidis using eggs as the media. During liquid egg production, eggs can be processed either as a whole or as separated egg yolks and whites (Prochaska, Carey, & Shafer, 1996). A study that investigates the biofilm formation by *S.* Enteritidis using whole eggs, egg yolks, and egg whites as the nutrient sources would better reflect the actual situation in liquid egg processing environments.

To reduce or eliminate biofilm cells on food contact surfaces, cleaning and sanitizing are the most conventional approaches (Simões, Simões, & Vieira, 2010). The United States Department of Agriculture requires that equipment that contacts with liquid eggs should be cleaned to remove any egg residues, and if hypochlorites are used for the surface sanitation, the concentration of free chlorine should be within the range of 100–200 ppm (FSIS, 2011). Apart from chemical sanitizers, hot water has also been approved by the United States Food and Drug Administration (FDA) for surface decontamination (FDA, 2009). However, so far, little is known about the effectiveness of chlorine and hot water against *Salmonella* biofilms in liquid egg processing environments. Therefore, the objective of this study was to evaluate the biofilm forming abilities of three *S.* Enteritidis strains in a simulated egg processing environment using 0.1% peptone water (control) (PW), whole eggs (WE), egg whites (EW) and egg yolks (EY) as growth media, and to determine the resistance of biofilms against chlorine and hot water treatment.

2. Material and methods

2.1. Bacterial strains and culture conditions

Salmonella Enteritidis ATCC13076 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and *S.* Enteritidis 124 (phage type 8, Maryland Department of Health and Mental Hygiene, MD, USA) and *S.* Enteritidis 125 (phage type 13A, U.S. Department of Agriculture, Washington DC, USA) were obtained from Dr. Kunho Seo of Konkuk University in Republic of Korea. The three *S.* Enteritidis strains expressed two different morphotypes on Congo red agar plates. *S.* Enteritidis ATCC13076 exhibited brown, dry and rough (bdar) morphotype, while the other two strains displayed red, dry and rough (rdar) morphotype (Yang et al., 2016).

Each *S.* Enteritidis strain was cultivated twice in tryptic soy broth (TSB) (Oxoid, Hampshire, UK) at 37 °C for 18 h. Subsequently, each cell suspension was centrifuged at 3500×*g*, 4 °C for 10 min, washed twice with 0.1% (w/v) peptone water (PW) (Oxoid) and resuspended in 0.1% PW to obtain a working culture with a concentration of approximately 10^9 CFU/mL.

2.2. Media preparation

The four organic substrates used in this study were 0.1% PW, 10% (v/v) WE, 10% (v/v) EY, and 10% (v/v) EW. Chicken eggs (Seng Choon Farm Pte Ltd, Singapore) purchased from a local supermarket were stored at 4 °C until use. The surfaces of eggs were sanitized by soaking in 70% (v/v) ethanol for 1 min and air dried in a biosafety cabinet. Eggs were manually broken and blended with a sterilized hand blender (Braun, Kronberg, Germany). For egg yolks and egg whites, they were directly withdrawn by inserting the pipette tips into cracked eggs. The egg media were prepared by adding 5 mL of blended whole eggs, egg yolks or egg whites into 45 mL of 0.1% peptone water, and the mixtures were homogenized by stirring.

2.3. Biofilm formation

Stainless steel (grade 304) coupons (2.5 cm × 1 cm × 0.2 cm) were used as the tested surfaces. Prior to use, coupons were washed and autoclaved as previously described (Yang, Kumar, Zheng, & Yuk, 2015). The working culture was inoculated into each medium to achieve an initial cell count of 10^7 CFU/mL. Five milliliters of the inoculated media were separately transferred into 15 mL of sterile centrifuge tubes (Greiner Bio-one, PA, USA). Each centrifuge tube contained a sterile stainless steel coupon, which was completely submerged in the media. Centrifuge tubes were then incubated at 25 °C under static condition for 2, 4 and 7 days.

2.4. Chlorine treatment

Chlorine solution was freshly prepared by diluting sodium hypochlorite solution (Bleach, Hygold Chemical Supplies, Singapore) with potassium phosphate buffer solution (0.05 mol/L, pH 6.8) to achieve a final concentration of 200 ppm. The concentration of free chlorine in the chlorine solution was determined using MQuant™ chlorine test strips (Merck, Darmstadt, Germany) and an RQflex® 10 reflectometer (Merck) according to the manufacturer's instructions. To determine the sensitivity of biofilm cells towards chlorine, coupons were aseptically removed from the tube with sterile forceps and rinsed twice with phosphate buffer saline (PBS) (Vivantis Inc., CA, USA) to remove loosely attached cells and egg residues. Subsequently, each coupon was transferred to a sterile plastic tube containing 5 mL of 200 ppm chlorine. After 5 min of treatment, the coupon was immediately placed in a centrifuge tube containing 5 mL of D/E neutralizing broth (Acumedia, lansing, MI, USA).

2.5. Hot water treatment

To determine the sensitivity of biofilm cells towards hot water, plastic tubes containing 5 mL of sterilized deionized (DI) water was heated in a circulating water bath (PolyScience, IL, USA). The temperature of the DI water was monitored by Fluke 54 II thermocouple (Fluke corporation, WA, USA). When the temperature was stable at 71.1 ± 0.5 °C, coupons after PBS rinses were submerged into the DI water for 30 s. Subsequently, each coupon was transferred to a centrifuge tube containing 5 mL of TSB that was chilled in an ice bath.

2.6. Enumeration of planktonic and attached cells

To enumerate planktonic cells, cell suspension from each tube was diluted with 0.1% peptone water and spread plated onto tryptic soy agar (TSA) (Oxoid) plates with appropriate dilutions. All plates were then incubated at 37 °C for 24 h, followed by colony counting.

To enumerate attached cells without treatment, coupons after PBS rinses were transferred into centrifuge tubes containing 5 mL of 0.1% peptone water. The tubes were then subjected to sonication (57H, Ney Dental International, CT, USA) at 48 kHz for 3 min, followed by vigorous vortex for 30 s to remove and disaggregate biofilm cells from the coupons. To enumerate attached cells after chlorine or hot water treatments, coupons in centrifuge tubes with 5 mL of D/E neutralizing broth or TSB were directly subjected to sonication and vortex. Cell suspensions in the centrifuge tubes were diluted, spread plated, and the number of cells was enumerated after incubation as described above. Pour plating method was also used if the number of attached cell after treatment was expected to be low.

2.7. Characterization of biofilms with a fluorescence microscope

The morphology of biofilms was investigated by staining the coupons with the LIVE/DEAD® *Bac*Light Bacterial Viability Kit (Molecular Probes Eugene, OR, USA). The staining solution in the LIVE/DEAD® *Bac*Light Bacterial Viability Kit was freshly prepared by adding 3 μL of SYTO®9 dye and 3 μL of propidium iodide (PI) to 1 mL of sterilized DI water. Each biofilm sample was rinsed twice with DI water and stained with 200 μL of the staining solution for 30 min in dark. After staining, samples were gently washed and air-dried. The biofilms were observed using an Olympus BX51 fluorescence microscope (Olympus corporation, Tokyo, Japan) with appropriate filter cubes for SYTO®9 dye (WB, DM500, BP450-480, BA515) and PI (WG, DM570, BP510-550, BA590). All images were taken by an attached Olympus DP71 camera (Olympus corporation).

2.8. Statistical analysis

Mean values were calculated from three independent experiments with duplicate samples (n = 6). Significant differences were evaluated by independent samples *t*-test or one-way analysis of variance (ANOVA) using SPSS software (Statistical Package for the Social Sciences, version 18.0, IBM, NY, USA). The difference was considered as statistically significant if $P < 0.05$.

3. Results

3.1. Planktonic cell counts

The number of planktonic cells in each medium at day 2, 4 and 7 is shown in Fig. 1. Regardless of incubation period and bacterial strain, the number of planktonic *S.* Enteritidis cells grown in PW (6.52–7.96 log CFU/mL) was significantly ($P < 0.05$) lower compared to those grown in egg media (8.26–8.75 log CFU/mL), while no significant ($P \geq 0.05$) difference in the number of planktonic cells was observed among different egg media. As the incubation time increased, the number of planktonic cells was relatively constant in egg media, but it decreased by 0.37–0.91 log CFU/mL in PW for all tested strains. Besides, bacterial strains did not significantly affect the number of planktonic cells in egg media. However, in PW, *S.* Enteritidis ATCC 13076 reached significantly higher cell population than *S.* Enteritidis 124 and 125 throughout the period examined. The average cell counts, which were the mean values of the number of planktonic cells at day 2, 4 and 7, were 7.78, 6.80, and 6.96 log CFU/mL for these three strains, respectively.

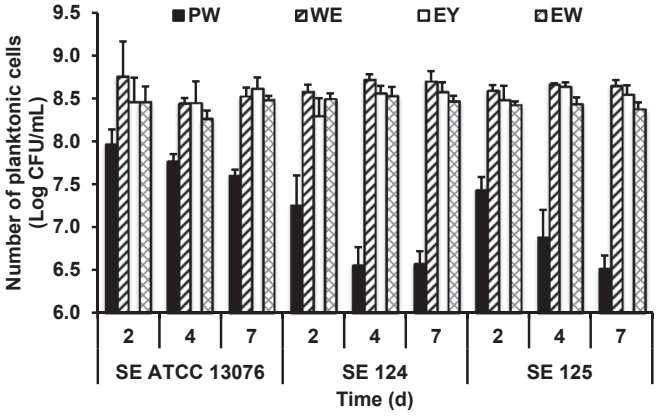

Fig. 1. The numbers of planktonic *S.* Enteritidis (SE) cells in 0.1% peptone water (PW), whole liquid egg (WE), egg yolk (EY) and egg white (EW) at 25 °C for 2, 4, and 7 days.

3.2. Attached cell counts

The number of attached cells to stainless steel coupons at day 2, 4 and 7 is shown in Fig. 2. All tested *S.* Enteritidis strains attached and formed biofilms onto the coupons submerged in PW, WE, EY, and EW at 25 °C, with cell numbers of 4.49–7.08 log CFU/cm^2. In addition, there was no significant ($P \geq 0.05$) strain variation in biofilm density, except that denser biofilms (0.58–0.88 log higher) were formed by *S.* Enteritidis 124 in PW at day 4 and 7 than those formed by the other two strains. Within the same strain, biofilms formed in PW and EW were significantly ($P < 0.05$) denser than those formed in WE and EY. For example, the biofilm densities of *S.* Enteritidis ATCC 13076 at day 2 were 5.78, 4.68, 4.81, and 6.27 log CFU/cm^2 in PW, WE, EY, and EW, respectively. Moreover, no significant difference in biofilm densities was found between WE and EY, or between PW and EW, with the only exception of *S.* Enteritidis 124, which formed denser biofilms in PW (6.82 and 7.08 log CFU/cm^2) than those in EW (6.10 and 6.06 log CFU/cm^2) at day 4 and 7. Regardless of bacterial strain and growth medium, the number of attached cells was not influenced by the incubation time.

3.3. Biofilm resistance to chlorine treatment

As incubation time did not alter *S.* Enteritidis biofilm formation, biofilms formed for 2 and 7 days, which represented the early and late stage of biofilm formation in this study, were used to determine biofilm resistance to chlorine treatment. The log reductions of biofilms formed under different conditions to chlorine treatment at 200 ppm, pH 6.8 for 5 min are shown in Fig. 3. Biofilm cells formed in PW were completely inactivated by chlorine treatment, with the highest reductions of 5.78–7.08 log CFU/cm^2, while those formed in egg media were more resistant, with reductions of only 0.07–4.68 log CFU/cm^2. Among the three egg media, cell populations of biofilms formed in WE were generally reduced to a greater extent by chlorine than those formed in EY and EW, with average reductions of 2.28, 0.67, and 0.95 log CFU/cm^2, respectively. Although the log reductions of biofilm cells formed in EY were either similar or lower than those formed in EW, the surviving populations of biofilms formed in EW (4.68–5.48 log CFU/cm^2) were generally significantly higher than those formed in EY (3.23–5.08 log CFU/cm^2) due to their higher biofilm densities. Therefore, biofilms formed in EW exhibited greater chlorine resistance than those formed in EY under most tested conditions. The effect of bacterial strains or incubation time on biofilm resistance

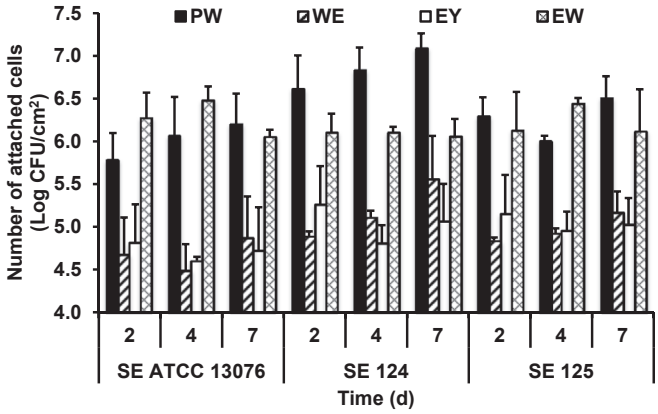

Fig. 2. The numbers of attached *S.* Enteritidis (SE) cells to stainless steel coupons submerged in 0.1% peptone water (PW), whole liquid egg (WE), egg yolk (EY) and egg white (EW) at 25 °C for 2, 4, and 7 days.

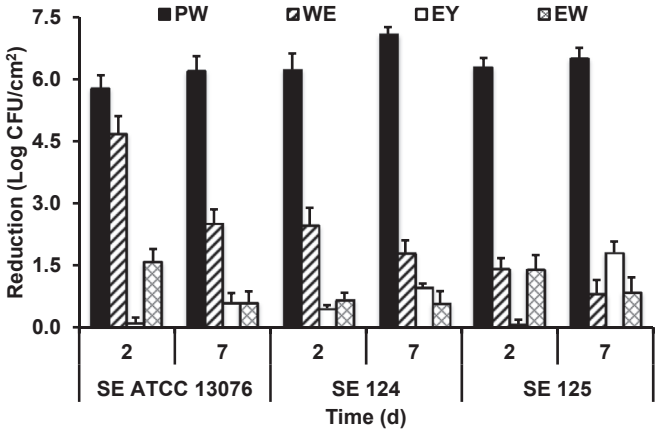

Fig. 3. Log reductions of *S.* Enteritidis biofilms formed in 0.1% peptone water (PW), whole liquid egg (WE), egg yolk (EY) and egg white (EW) at 25 °C for 2 and 7 days to chlorine treatment at 200 ppm, pH 6.8 for 5 min.

was also analyzed, and the results showed that biofilm resistance to chlorine treatment was not dependent on these two factors.

3.4. Biofilm resistance to hot water treatment

The log reductions of biofilms formed under different conditions to hot water treatment at 71.1 °C for 30 s are shown in Fig. 4. Hot water treatment was effective in inactivating biofilm cells under all tested conditions, reducing the populations of biofilm cells by 4.3–7.08 log CFU/cm^2. Out of 24 tested conditions, biofilm cells were completely eliminated under 12 conditions, while the surviving populations of biofilm cells under the rest of conditions were 0.06–1.43 log CFU/cm^2 (data not shown). In general, higher log reductions were observed in biofilms formed in PW and EW. However, the results might be due to the higher cell populations of biofilms formed in these two media rather than their greater heat sensitivity. Thus, no comparison of the heat resistance of biofilms formed in different media was made. In addition, bacterial strains and incubation time did not significantly ($P \geq 0.05$) affect biofilm resistance to heat treatment, except that the biofilm formed by *S.* Enteritidis 125 in EW for 7 days (4.68 log reduction) was more heat tolerant compared to that formed for 2 days (5.91 log reduction), or those formed by *S.* Enteritidis ATCC 13076 and 124 (5.89 and 5.84 log reduction) at day 7.

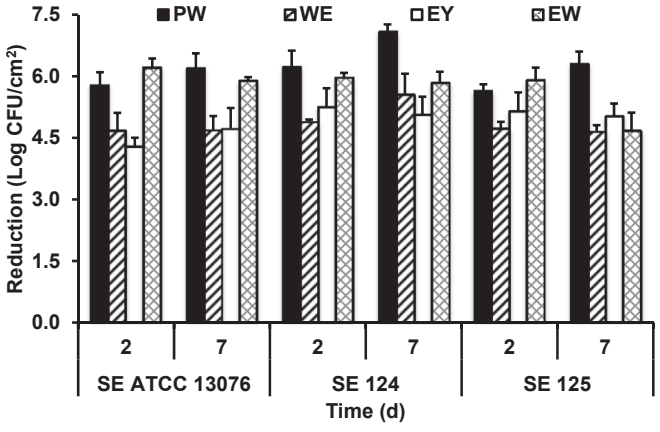

Fig. 4. Log reductions of *S.* Enteritidis biofilms formed in 0.1% peptone water (PW), whole liquid egg (WE), egg yolk (EY) and egg white (EW) at 25 °C for 2 and 7 days to hot water treatment at 71.1 °C for 30 s.

3.5. Biofilm morphology

Biofilms formed by *S.* Enteritidis 124 in different media for 7 days were observed with the aid of SYTO®9/PI staining under a fluorescence microscope (Fig. 5). Although all coupons were washed to remove any visible residues, coupons in PW were found to be free of organic matters (Fig. 5A), while those in egg media were randomly covered with egg residues that were stained either yellow or green as indicated by arrows (Fig. 5B–D). Interestingly, residues of WE and EY were either dot-like or irregular shaped with a collection of small dots, while those of EW were cloud shaped. The adsorption of different organic materials onto contact surfaces greatly affected biofilm morphology as biofilm cells in PW, WE and EY formed clusters but those in EW were individually scattered within EW tissues (Fig. 5E–H). Compared to PW, biofilm cells in WE and EY were less seamlessly attached to each other and formed smaller clusters, which were present either above or under the egg residues.

4. Discussion

This study was conducted to evaluate the biofilm-forming abilities of *S.* Enteritidis strains in a simulated liquid egg processing environment, and to ascertain whether chlorine and hot water treatments are effective in the subsequent inactivation of *S.* Enteritidis biofilm cells. Three *S.* Enteritidis strains with different morphotypes (bdar and rdar) were used because biofilm resistance to sanitizer has been reported to be dependent on the morphotypes of *S.* Enteritidis strains (Yang et al., 2016). In addition, strains were directly used rather than adapted to antibiotics because the result of the preliminary experiment showed that eggs were almost free from background microbiota contamination (less than 2 CFU/mL of whole egg samples). Moreover, egg media were diluted because our previous study found that low nutrient condition (20 times diluted TSB) promoted *Salmonella* biofilm formation as well as enhanced biofilm resistance to sanitizing agents (Yang et al., 2016).

In this study, planktonic cell counts in each tested medium were investigated since bacterial planktonic growth was believed to be a contributing factor of biofilm formation (Wen, Yates, Ahn, & Burne, 2010). As expected, the present study demonstrated that PW did not support the growth of *S.* Enteritidis well, and this was possibly due to that PW had limited amount of nutrient. However, similar level of population densities was observed in WE, EY, and EW, which was contradictory to the fact that egg white was a poor medium for bacterial growth due to its nutrient deficiencies, high viscosity, antimicrobial molecules (ovotransferrin, cystatin, and ovalbumin related protein X), and alkaline pH (Baron et al., 2016). One possible explanation for this phenomenon was that EW in this study was diluted, which might result in lower viscosity and lower concentration of antimicrobial molecules. Besides, alkaline pH was not a challenge to *S.* Enteritidis as the initial pH of 10% egg white was 8–9 (data not shown), which was within the growth range (pH 5.3–9.0) of *S.* Enteritidis (Yang et al., 2014).

Interestingly, *S.* Enteritidis formed denser biofilms in PW and EW compared to those in WE and EY, although it grew poorly in PW and reached similar planktonic cell density in EW, WE and EY. Therefore, no correlation between planktonic growth and biofilm formation was observed in this study. The higher number of biofilm cells found in PW compared to those in WE and EY contradicts with the results obtained by Dourou et al. (2011) who reported that biofilm formation by *E. coli* O157:H7 was stimulated when the surface was conditioned with food matrices. However, a study conducted by Wang et al. (2013) showed that biofilm density of

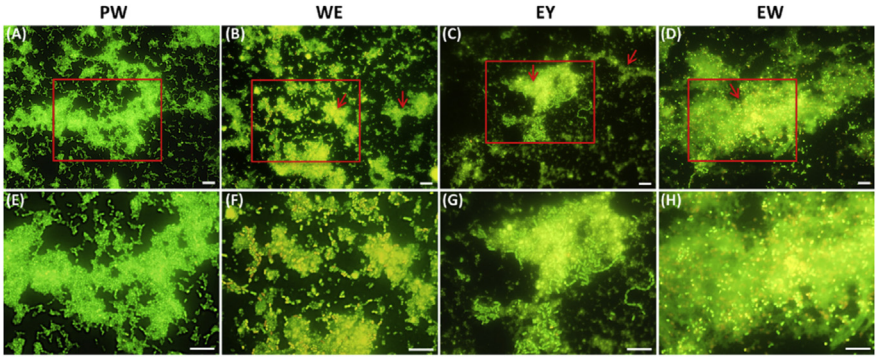

Fig. 5. SYTO®9 and propidium iodide (PI) staining of biofilms formed by *S.* Enteritidis 124 in 0.1% peptone water (PW) (A and E), whole liquid egg (WE) (B and F), egg yolk (EY) (C and G), and egg white (EW) (D and H) for 7 days. (A–D) representative images of biofilms observed under 100× objective lens, and arrows indicate egg residues. (E–H) enlarged images of the highlighted areas in pictures A–D. Scale bars represent 10 μm.

Salmonella spp. was much lower if the surface was immersed in meat broth compared to that in a laboratory medium. On the other hand, no significant difference in biofilm formation between PW and EW was observed in the present study. Therefore, the influence of food matrices on biofilm formation might be a complex issue, relying on both bacterial strains and the type of organic materials.

The greater biofilm densities in PW and EW are possibly due to their lower nutrient contents as compared to those of WE and EY, which have been reported as a stimulating factor for *Salmonella* biofilm growth (Stepanović, Ćirković, Ranin, & Švabić-Vlahović, 2004; Yang et al., 2016). The enhanced attachment under low nutrient conditions might be explained by the fact that available nutrients in a low nutrient medium are concentrated at the substratum surface instead of the bulk fluid and consequently the bacteria will migrate towards the surface and attach to it (Brown, Ellwood, & Hunter, 1977). In addition, biofilm formation is influenced by the type of organic material adsorbed onto the surface because it could modify the properties of contact surface (Whitehead & Verran, 2015). The conditioning film resulted from the residues of EW exhibited a three dimensional structure (Fig. 5), which might increase the total area of the contact surface for *S.* Enteritidis attachment.

To evaluate the efficiency of chlorine against *S.* Enteritidis biofilms, biofilms formed under different conditions were treated with 200 ppm of chlorine for 5 min. The results showed that the chlorine treatment was very effective in the complete elimination of biofilm cells formed in PW, but it could only partially reduce the populations of biofilms formed in egg media. The lower efficiency of chlorine against biofilms formed in egg media might not be due to the organic matters present in egg media that can react with chlorine solution since the free chlorine concentration in the solution remained unchanged after 5 min of treatment. However, the presence of organic materials in egg media might contribute to the difference in chlorine efficiency because they affected *S.* Enteritidis biofilm morphology (Fig. 5).

The egg residues that covered the biofilm cells might protect the cells from chlorine treatment by acting as physical barriers (Rutala et al., 2008). The phenomena that almost all biofilm cells in EW resided within the cloud-shaped biofilm structure, but some of the biofilm cells in WE and EY were exposed to their surrounding environments (Fig. 5) might explain why biofilm cells formed in EW were more resistant to chlorine than those in WE and EY. Besides, the three-dimensional structure of biofilms formed in EW might further contribute to their greater chlorine resistance because the structure might hinder the penetration of chlorine into the biofilm, and thus cells deep within the biofilms might be protected from being attacked by chlorine (Mah & O'Toole, 2001).

The present study also investigated whether the morphotype of *S.* Enteritidis would affect its biofilm formation and resistance to sanitizers. The results showed that there was no significant difference in biofilm densities among different strains, although *S.* Enteritidis ATCC10376 produced less cellulose compared to the other two strains. The result is consistent with our previous finding (Yang et al., 2016) using laboratory media as nutrient source. Therefore, the capability of *S.* Enteritidis strains to produce cellulose may not affect their capability to form biofilms. Furthermore, no strain variation was observed in biofilm resistance to chlorine in this study, but our previous results (Yang et al., 2016) demonstrated that *S.* Enteritidis strains with rdar morphotype (*S.* Enteritidis 124 and 125) were more resistant to chlorine treatment compared to *S.* Enteritidis ATCC 13076 with bdar morphotype. The different results were probably because that different media were used for biofilm formation. As cellulose production was highly dependent on the growth medium (Chawla, Bajaj, Survase, & Singhal, 2009), the media tested in this study might not induce cellulose production of *S.* Enteritidis biofilms due to their high content of protein but low content of carbohydrate (Li-Chan & Kim, 2008).

Hot water treatment was performed at 71 °C for 30 s, which is the basic requirement for the sanitization of equipment and utensils as recommended by FDA (FDA, 2009). The results of the present study demonstrated that hot water was more effective than chlorine in eliminating *S.* Enteritidis biofilms. The greater bactericidal effect of heat might be attributed to its greater ability to penetrate barriers, such as biofilms and food matrices, compared to that of chlorine (Rutala et al., 2008). However, under certain conditions, a few biofilm cells still survived the hot water treatment. Thus, higher temperature or prolonged treatment time should be applied to achieve the complete killing effect. Besides, it should be noted that organic materials that are not removed during the cleaning process might be cooked and firmly attached to the surface during hot water treatment (Chmielewski & Frank, 2003), resulting in a greater difficulty in the removal of food debris later. Therefore, hot water sanitation is preferably used on open surfaces where scrubbing and brushing can be performed beforehand.

5. Conclusion

This is the first study that investigates the effect of different egg media on *S.* Enteritidis biofilm formation and comparatively evaluates the effectiveness of chlorine and hot water treatments against *S.* Enteritidis biofilms in liquid egg processing environments. The results of the present study showed that biofilm formation by *S.* Enteritidis was significantly greater in EW compared to those in WE and EY. The greater biofilm density was possibly due to its low

nutrient content as well as that the organic matters of EW modified the properties of the contact surface. In addition, chlorine treatment was insufficient to inactivate *S.* Enteritidis biofilms formed in egg media even if the maximum allowed concentration was applied. Biofilms formed in EW was more tolerant to chlorine than those in WE and EY. On the other hand, hot water treatment was very effective to eliminate *S.* Enteritidis biofilm cells. However, higher temperature or longer treatment time is suggested to ensure adequate surface sanitation. Therefore, the present study demonstrated the potential value of using hot water for the inactivation of *S.* Enteritidis biofilms in liquid egg processing environments.

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