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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.