Viability of *Salmonella* Typhimurium biofilms on major food-contact surfaces and eggshell treated during 35 days with and without water storage at room temperature

Ki-Hoon Lee,*1 Ji-Young Lee,* Pantu Kumar Roy,* Md. Furkanur Rahaman Mizan,*1 Md. Iqbal Hossain,* Si Hong Park,* and Sang-Do Ha*,2

*Department of Food Science and Technology, Advanced Food Safety Research Group, Chung-Ang University, Anseong, Gyeonggi-do 456-756, South Korea; and 1Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331, USA

**ABSTRACT** *Salmonella* is one of the main foodborne pathogens that affect humans and farm animals. The *Salmonella* genus comprises a group of food-transmitted pathogens that cause highly prevalent foodborne diseases throughout the world. The aim of this study was to appraise the viability of *Salmonella* Typhimurium biofilm under water treatment at room temperature on different surfaces, specifically stainless steel (SS), plastic (PLA), rubber (RB), and eggshell (ES). After 35 D, the reduction of biofilm on SS, PLA, RB, and ES was 3.35, 3.57, 3.22, and 2.55 log CFU/coupon without water treatment and 4.31, 4.49, 3.50, and 1.49 log CFU/coupon with water treatment, respectively. The dR value (time required to reduce bacterial biofilm by 99% via Weibull modeling) of *S*. Typhimurium without and with water treatment was the lowest on PLA (176.86 and 112.17 h, respectively) and the highest on ES (485.37 and 2,436.52 h, respectively). The viability of the *S*. Typhimurium on ES and the 3 food-contact surfaces was monitored for 5 wk (35 D). The results of this study provide valuable information for the control of *S*. Typhimurium on different surfaces in the food industry, which could reduce the risk to consumers.

**Key words:** biofilm, eggshell, *S*. Typhimurium, survival ability, Weibull model

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1These authors contributed equally to this work.
2Corresponding author: sangdoha@cau.ac.kr

**INTRODUCTION**

The bacteria on surfaces in food-processing environments are a potential source of cross-contamination and can lead to food spoilage or transmission of disease, through scratched or unclean food-contact surfaces in processing lines (Nidaullah et al., 2017; Coradini et al., 2019). Owing to condensation, these surfaces are favorable sites for bacteria to grow in static biofilms (Petridis et al., 2019). Biofilm do not possess a uniform structure, and bacterial species as well as several extrinsic factors (temperature, flow conditions, pH, presence of salts, nutrients, and so on) play a major role in influencing biofilm formation and the degree of attachment (Jain and Chen, 2007; Hannig et al., 2018). Biofilm formation is strongly influenced by the food-contact surfaces (Jeon et al., 2018; Mizan et al., 2018).

In a biofilm, cells can initiate attachment on food products and food-contact surfaces (Jeon et al., 2018; Mizan et al., 2018), which provide a potential transmission route for foodborne pathogens (Hald et al., 2016). In the food industry, water cleaning is vital to control and remove biofilms (Liu et al., 2016; Esbelin et al., 2018). Nevertheless, poor or ineffective cleaning processes can increase the risk of foodborne outbreaks leading to public health concerns. In addition, the food industry may incur economic losses due to product recalls as well as legal and customer claims (Davey et al., 2013). Biofilms are involved in over 80% of all microbial diseases according to the US National Health Institute and the Centers for Disease Control and Prevention (Khatoon et al., 2018). It has been inferred that biofilms formed on surfaces at various food-processing locations are a crucial source of *Salmonella* contamination of food (Khieu et al., 2013; Lamas et al., 2018). One of the most extensively used materials in machinery and food-contact surfaces in the food industry is stainless steel (SS). Schlisselberg
and Yaron (2013) described how various treatments of SS could affect the biofilms formation of *S. Typhimurium*. The crucial problem of biofilms on food industry surfaces is their transfer to food and resulting contamination of foodstuffs. In this point of view, Wang et al. (2015) evaluated the transfer of *Salmonella* biofilms formed on the food-contact surface of SS to foodstuff (meat). *Salmonella* spp. can contaminate fresh produce during any stage, from farm to table, through cross-contamination by washing with water, handling by workers, and contact with food surfaces (Kroupitski et al., 2009). *Salmonella* biofilm on eggshell (ES) can cause cross-contamination of *Salmonella* (Carrasco et al., 2012; Pande et al., 2016) onto other food-contact surfaces on egg-processing lines and can finally lead to food contamination (Carrasco et al., 2012).

More than 70% of human salmonellosis cases in the United States have been attributed to the consumption of contaminated chicken, turkey, and eggs with 175, 133, and 45 illness outbreaks involving 1,003, 358, and 11 people, respectively (CDC, 2019). Egg products associated with *S. Typhimurium* outbreaks have been frequently reported in Australia (Group, 2012; Kirk et al., 2014). Numerous egg-related human *S. Typhimurium* outbreaks have garnered significant interest from the general public, public health authorities, and egg industry (Chousalkar et al., 2017).

The major materials used for food-contact surfaces are known to be Teflon and nitrile butyl rubber, SS, glass, rubber (RB), and polyurethane (Chia et al., 2009; Fink et al., 2017). In the present study, the viability of *S. Typhimurium* biofilm was evaluated when stored under room temperature for a long period (35 D) on ES and 3 different surfaces (SS, plastic [PLA], and RB) treated with and without water. Indeed, the ability of pathogenic bacteria, including *S. Typhimurium*, to form biofilms on various food-contact surfaces and under different conditions for different periods has been investigated in several research studies (Lamas et al., 2016). In a review, Mizan et al. (2015) stated that *Salmonella* spp. biofilms self-gather and form flat or mushroom-shaped 3D structures on SS. Other studies have examined the viability of *Salmonella* spp. on polypropylene surfaces (Iibuchi et al., 2010), *Salmonella* spp. on ES surfaces at different temperatures (Park et al., 2015b), and specifically, *S. Typhimurium* on ES (McAuley et al., 2015).

In spite of the boundless appliance for forecasting mathematical models of survival and growth and the significant amount of scientific literature on the modeling of biofilm survival ability (Giertsen et al., 2011; Dimakopoulou-Papazoglou et al., 2016), no attempt has been made to study the effects of environmental factors during biofilm formation to establish and apply mathematical models. Therefore, the aim of this study was to establish and appraise predictive mathematical models for the effect of water treatment on the viability of *S. Typhimurium* biofilm on SS, PLA, RB, and ES.

The fitting and performance of linear and modified Weibull models were assessed to estimate the survival behavior of *Salmonella* spp. on SS, PLA, RB, and ES. Survival modeling of *S. Typhimurium* on table egg during storage at different temperatures (Pasquale et al., 2016) as well as the modeling of biofilm formation by *Salmonella enterica* ser. Newport as a function of pH and water activity (Dimakopoulou-Papazoglou et al., 2016) have been reported.

Many investigations have been carried out on food-contact surfaces concerning viability and biofilm attachment ability. However, there is still a need to explore the survival time and mathematical modeling of *S. Typhimurium* biofilm (the major cause of human bacterial gastroenteritis) on the 3 types of food-contact surface (SS, PLA, and RB), especially focusing on egg processing line materials and cookware utensils. Thus, the purpose of this study was to appraise the viability of *S. Typhimurium* on SS, PLA, RB, and ES surfaces.

### MATERIALS AND METHODS

#### Bacterial Strains and Growth Conditions

*S. Typhimurium* ATCC14028 was used in this study to evaluate the viability of *S. Typhimurium* on food-contact surfaces (SS, PLA, RB) and ES with and without water treatment. Bacterial stock culture was maintained at −70°C in Tryptic Soy Broth (TSB; Difco Laboratories Detroit, MI) supplemented with 15% (vol/vol) glycerol (Fisher Scientific, Itasca, IL). The strain was consecutively subcultured twice aerobically at 37°C for 24 h in TSB. Cultured cells were centrifuged at 11,000 × g at 4°C for 10 min and washed twice with sterile phosphate buffered saline (PBS; pH 7.2). The pellets were resuspended in peptone water (PW; Oxoid, Basingstoke, Hampshire, England). The bacterial cell suspension was diluted in 0.1% PW to yield the final cell concentration (10^5–10^6 CFU/mL) for inoculation to make biofilm on coupons. By plating on xylose lysine deoxycholate agar (Difco Laboratories) plates and incubating at 37°C for 24 h, microbial numbers were determined.

#### Preparation of SS/PLA/RB/ES Coupons, Inoculation, and Biofilm Formation

SS (SUS 304 2B; Posco Co., Ltd., Pohang, Korea), PLA (egg packaging; Join Co., Ltd., Eumseong, Korea), and RB (Komax Industrial Co., Ltd., Goyang-ro, Korea) were selected as delegate surfaces used in the food industry. In this study, we used SS, PLA, and RB coupons (2 cm × 2 cm × 0.1 cm) that were processed as earlier addressed (Sadekuzzaman et al. 2018; Hossain et al., 2020). ES coupons were processed as previously described by Park et al. (2018). Briefly, eggs were collected from a local grocery store in Anseong-Si, South Korea (Eggs with a remaining shelf life of at least 40 D were selected and stored at 4°C until use.). Each egg was gently broken, and the ES was cut into 2 cm × 2 cm × 0.1 cm coupons using a sterilized knife. Immediately after, the ES was soaked into 70% alcohol for 10 min, washed 3 times with sterilized deionized water, and then treated with...
UV in a laminar flow biosafety hood for 15 min on each side to remove background flora before inoculation. The bacterial cell suspension was diluted at 1:50 into 50-mL Falcon tubes with each coupon completely submerged in 10-mL TSB. The 50-mL Falcon tubes were incubated without shaking to form biofilms on the coupons at 37°C for 24 h.

**With or Without Water Treatment, Storage and Biofilm Detachment**

After incubation of biofilm formation, half of the *S. Typhimurium* biofilms on SS, PLA, RB, and ES coupons were washed under running sterile water with swirling of 10 s (3 times) and stored at room temperature in a humidity chamber (relative humidity 50%; V8111H-150; Vision Scientific Co., Ltd., Gyeonggi-do, Korea). The remaining half of each sample was stored at room temperature in a humidity chamber (relative humidity 50%) without water treatment except for an initial wash process for removing planktonic cells. The viability of the *S. Typhimurium* on ES and the 3 food-contact surfaces was monitored for 5 wk (35 D). Biofilm cells detachment was done according to the study by Jahid et al. (2014) with minor modifications. After incubation, each coupon expect ES was shifted in a small petri dish (55 mm × 12 mm) containing 2 mL of 0.1% PW and agitated by holding the SS, PLA, and RB coupons on the petri dish using sterile tweezers at the same time to rotate clockwise and anticlockwise. Agitation was always performed by the same person to ensure the same amount of pressure was applied to all the coupons. The suspension was then transferred to a test tube and ultrasonicated for 2 min in a sonicator (380 W, 37 kHz, Elmasonic P; Elma Schmidbauer, GmbH, Singen, Germany) to disperse the biofilm population. Each sample of the ES coupon was vortexed with 10 glass beads and 10-mL PW in a 50-mL Falcon tube for 1 min. In PW, the dispersed biofilm cells were serially diluted for cells counting by plating on xylose lysine deoxycholate agar and incubated for 24 h at 37°C.

**The Modified Weibull Model**

The modified Weibull model (a two-parameter nonlinear model) can be expressed as

\[ \log \left( \frac{N_t}{N_0} \right) = N_0 - 1 / 2.303 \times (t/b)^n \]  

where \( N_t \) is the concentration of biofilm (CFU/coupon) after exposure time \( t \), \( N_0 \) is the initial concentration of biofilm (CFU/coupon), \( t \) is the exposure time, and \( b \) and \( n \) are the scale (characteristic time) and shape parameters as a behavior index, respectively (van Boekel, 2002). To reduce the first log cycle, the value of \( b \) represents the required time to reduce the bacterial biofilm population, while that of \( n \) indicates the shape of the survival curve (\( n = 1 \) corresponds to a linear survival curve, while \( n > 1 \) and \( n < 1 \) correspond to downward and upward concavity, respectively). For the calculation of \( d_{R} \) (analogous to the traditional \( D \) value) from the Weibull parameters, the following equation is used (Buzruñ and Alpas, 2007):

\[ d_{R}(t) = b \times 4.606^{1/n} \]  

where \( d_{R} \) is the time required to reduce the bacterial biofilm by 99%. GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA) was used to appraise the inactivation kinetics for nonlinear regression which was fitted by the modified Weibull method.

**Field Emission Scanning Electron Microscopy**

Field emission scanning electron microscopy images of the *S. Typhimurium* biofilms on SS, PLA, RB, and ES coupons were obtained according to previously reported procedures (Mizan et al., 2018; Hossain et al., 2020) with some modifications. The adhered cells on coupons were fixed for 24 h with 2% glutaraldehyde (Sigma Aldrich, St. Louis, MO) in PBS and then washed 3 times with PBS. The fixed cells were serially treated with ethanol (50, 60, 70, 80, and 90% for 15 min, respectively, and 100% 2 times for 15 min each) and with 33, 50, 66, and 100% hexamethyldisilazane (Sigma Aldrich) in ethanol and successively dehydrated for 15 min each to observe the dehydrated samples which were coated with platinum and visualized on field emission scanning electron microscopy (FE-SEM; Carl Zeiss, Oberkochen, Germany) with an accelerating voltage of 5 kV and 5 nm working distance.

**Statistical Analysis**

Data were analyzed via one-way analysis of variance (Duncan’s test) using SAS version 9.2 (SAS Institute Inc., Cary, NC). Three independent trials were used in all experiments repeated 3 times. Data were considered to be statistically significant at \( P < 0.05 \).

**RESULTS AND DISCUSSION**

**Viability of *S. Typhimurium* biofilm on Food-Contact Surfaces and ES With and Without Water Treatment Stored Under Room Temperature**

The viability of *S. Typhimurium* biofilm was measured at predetermined times (0, 1, 2, 3, 5, 7, 14, 21, 28, and 35 D), after inoculation on SS, PLA, RB, and ES surfaces. The amounts of *S. Typhimurium* biofilm detected from the surfaces with and without water treatment significantly decreased over time (\( P < 0.05 \); Figure 1).

The viability of *S. Typhimurium* biofilm on food-contact surfaces without and with water treatment at room temperature is shown in Figures 1A and 1B, respectively. The reduction of *S. Typhimurium* biofilm after 35 D on food-contact surfaces without water treatment (Figure 1A) was the highest on PLA (3.57
log_{10} CFU/coupon) and the lowest on ES (2.55 log_{10} CFU/coupon). For SS and RB, the corresponding values were 3.35 and 3.22 log_{10} CFU/coupon, respectively. The amounts of S. Typhimurium biofilm detected on the surfaces of ES and the 3 food-contact surfaces significantly decreased over time ($P < 0.05$). From these data, S. Typhimurium biofilm survived for the shortest period on PLA among the 3 food contact surfaces, and the viability was maintained for the longest period on ES among all the surfaces. The characteristics of the food-contact surfaces could be important for bacteria attachment and viability (Stepanović et al., 2004). The bacterial biofilm viability on different food-contact surfaces depends on various factors, including temperature, humidity, and nutrient availability for bacteria (Lamas et al., 2018). According to Brankatschk et al. (2014), Salmonella uses its genetic marker to effectively attach to surfaces and finally colonize.

The viability of the S. Typhimurium biofilms is rated from the highest to the lowest as ES $>$ RB $>$ SS $>$ PLA (Figure 1A), due to the properties of the surfaces. According to our FE-SEM study, the surface roughness is rated from highest to lowest as RB $>$ ES $>$ SS $>$ PLA. FE-SEM images of the S. Typhimurium biofilm formation on the study surfaces are depicted in Figure 2. FE-SEM images reveal that S. Typhimurium colonized and consequently formed compact or dense biofilms on the surfaces. The adhesion forces for Streptococcus spp. on composite resins with different surface roughness values were evaluated by Mei et al. (2011), who confirmed that they increased with increasing surface roughness. It was also reported that the surface roughness of a polyester urethane conveyor belt had a significant influence on the biofilm-forming ability of Listeria monocytogenes (Stepanović et al., 2004). For this reason, S. Typhimurium could make a stronger biofilm on RB than other surfaces (Ronner and Wong, 1993). However, the viability was higher on ES than on RB (Figures 1A, 1B). ES has a surface roughness and porosity similar to those of wood, which allow the gas and water exchange necessary for the developing chick embryo but also microbial ingress and contamination of the egg contents. Thus, S. Typhimurium (1 μm) can enter through an ES pore (15–65 μm) and make a strong biofilm (Ghaneian et al., 2011; Abramian and El-Rassy, 2012). According to our FE-SEM study, we observed pore on ES (Figures 2E, 2F).

After 35 D, the reduction of the S. Typhimurium biofilms on food-contact surfaces with water treatment was the highest on PLA (4.49 log_{10} CFU/coupon) and the lowest on ES (1.49 log_{10} CFU/coupon) (Figure 1B). The reduction values for SS and RB were 4.31 and 3.50 log_{10} CFU/coupon, respectively. The amounts of S. Typhimurium biofilm detected on the 3 surfaces (SS, PLA, and RB) decreased significantly over time ($P < 0.05$), but they decreased significantly less ($P < 0.05$) on the surface of ES than on the other surfaces. The order of viability of the biofilms on the washed surfaces was the same as that on an unwashed surface, with the S. Typhimurium biofilm on ES being the highest (Kim et al., 2016; Jung et al., 2018). This difference could have been due to ES absorbing water through its pore during the 3 times washing step. Although the ES was dried and stored for 24 h in a humidity chamber before washing, its interior region could have remained wet and thus absorbed most of the biofilm. This may explain the high observed viability of the S. Typhimurium biofilm on the ES surface (Kim et al., 2016). Similarly, it has been suggested that wood encourages biofilm formation because of its porosity and absorbency, which can entrap organic material and bacteria (Adetunji and Isola, 2011; Al-kafaween et al., 2019). Moreover, moisture retention on the ES could also account for the greater viability of S. Typhimurium at low temperatures because the eggs were stored while still wet (Rizk et al., 1966).

Overall, it was found that the viability of S. Typhimurium biofilm (rated from highest to lowest: ES $>$ RB $>$ SS $>$ PLA) showed several sharp decreases after 1, 2, 3, 5, and 7 D, but after 7 D, the decrease in the rate of viability was slight (Figure 1). It was also determined that the biofilms on the 3 food-contact surfaces (except for ES) were more viable when not washed with water, although the difference in the amount of S. Typhimurium biofilm was not significant ($P > 0.05$). The viability of S. Typhimurium biofilm on washed ES

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**Figure 1.** The viability of *Salmonella* Typhimurium biofilm formation on different food-contact surfaces on different time intervals without (A) and with (B) water treatment at room temperature.
was significantly higher in value than that on unwashed ES (Wolf-Hall and Nganje, 2017), and the amount of S. Typhimurium biofilm was significantly different ($P < 0.05$). This could have been due to ES absorbing water via its pores during the washing step (Pande et al., 2016).

After 35 D with and without water treatment on all food-contact surfaces, the results of the present study show that the viability of S. Typhimurium biofilms was maintained. McAuley et al. (2015) found that S. Typhimurium biofilm was attached early to the ES surface after exposure for only 20 min but was not maintained after 2 wk. It was also recently reported that the viability of S. enterica on ES surfaces was maintained after 3 wk (Park et al., 2015a). The results of these 2 studies are contradictory because S. Typhimurium cells attached to an ES surface had weaker adhesive power than S. enterica biofilm (exposure for 24 h). The results of the study by Park et al. (2015a) showed that the validity curve for S. enterica on ES was similar to that for S. Typhimurium biofilm in the present study. Consequently, the viability of S. Typhimurium on food-contact surfaces is variable according to the biofilm state and biofilm formation ability.

**Weibull Modeling to Obtain Survival $d_R$ Value Against S. Typhimurium Biofilms on the Food-Contact Surfaces**

In food-contact surfaces, the viability data of S. Typhimurium biofilm were fitted with the Weibull model for nonlinear microbial survival. The values of the Weibull model parameters ($b$, $n$, $d_R$, and $R^2$) are reported in Table 1. The survival curves were a good fit for this model by using $R^2$ to estimate the goodness of fit of the model, whereas the values were over 0.96. Microbial inactivation has commonly been modeled using a first-order kinetics process (Whiting et al., 1996; Pankaj et al., 2013) fitted for comparison with the Weibull model. Moreover,
many researchers have revealed that the Weibull model might have a better fit than first-order models for kinetic viability of bacteria and viruses, such as mixed-culture biofilms of S. Typhimurium (Jahid et al., 2015), reduction of Cladosporium cladosporioides and Penicillium citrinum (Park and Ha, 2015), and survival of norovirus (Kim et al., 2014) and the hepatitis A virus (Bae et al., 2007; Mucha and Cole, 2000; Mattick et al., 2001). The Weibull model arrived at the same result as the experiments. In summary, the viability of S. Typhimurium biofilm on unwashed surfaces (RB, SS, and PLA) except for ES were significantly higher than those on washed surfaces ($P < 0.05$). However, the $d_R$ values of S. Typhimurium biofilm on washed ES were higher than those on unwashed ES. The amounts of S. Typhimurium biofilm on the unwashed and washed surfaces of ES were significantly different ($P < 0.05$). From this result, it can be determined that S. Typhimurium biofilm inactivation on the surface of PLA requires less time than on the other surfaces. The S. Typhimurium biofilm inactivation on the surface of ES was estimated to take a longer time than on the other surfaces. Consequently, the results indicate that the Weibull modeling arrived at the same result as the experiments.

In summary, the viability of S. Typhimurium biofilm on food-contact surfaces without and with water treatment was investigated. The reduction of S. Typhimurium biofilm without and with water treatment was highest on PLA (3.57 and 4.49 log$_{10}$ CFU/coupon, respectively) and lowest on ES (2.55 and 1.49 log$_{10}$ CFU/coupon, respectively). The order of reduction value (from highest to lowest) was PLA > SS > RB > ES. After 35 D treated with and without water, the viability of S. Typhimurium biofilm was maintained on all food-contact surfaces.

Weibull modeling was conducted to obtain survival $d_R$ values for S. Typhimurium biofilms on the food-contact surfaces without and with water treatment. The $d_R$ values of S. Typhimurium biofilm without and with water treatment were lowest on PLA (176.86 and 112.17 h, respectively) and highest on ES (485.37 and 2,436.52 h, respectively). Overall, it was found that the $d_R$ values for S. Typhimurium biofilm from highest to lowest were in the order of ES > RB > SS > PLA (Hingston et al., 2013). It was also found that the $d_R$ values of S. Typhimurium biofilm on the unwashed RB, SS, and PLA (not ES) were less than those on the washed surfaces. The result of this study suggests that washing ES every day does not reduce S. Typhimurium biofilm viability significantly.

**CONCLUSION**

The viability of S. Typhimurium on food-contact surfaces varies according to the biofilm state and formation ability. The viability of S. Typhimurium biofilm over
time depends on the surface roughness and porosity. The results of this study show that *S. Typhimurium* biofilm formed during food formulation and processing can survive persistently in factories and cookware; thus, ensuring a safe environment is very crucial during these procedures. Reducing *S. Typhimurium* biofilm contamination during food processing will reduce the occurrence of associated diseases. The findings in this study provide valuable information for the control of *S. Typhimurium* on different food-contact surfaces in the food industry to prevent foodborne disease. However, the experimental scope of the study is limited to only one strain of *Salmonella*. Further studies are urged to extend the application of this study in food quality and safety regulations.

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