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Assessment of *Salmonella* spp. Attachment to Reusable Plastic Containers Based on Scanning Electron Microscopy and BAX® PCR

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Abstract

Reusable Plastic Containers (RPC) coupons were used to determine the ability of *Salmonella* spp. to adhere and form potential biofilms on commercial RPCs. Attachment of *Salmonella* serovars Kentucky, Newport, Enteriditis, Heidelberg, and Typhimurium was evaluated. The RPC coupons served as a platform for generating biofilms of these microorganisms. Following biofilm formation on the RPC coupons, scanning electron microscopy (SEM) was performed to examine the coupons for bacterial presence. Additionally, the RPC coupons were subjected to a bacterial biofilm growth process and were subsequently sanitized using methods and sanitizing agents that are commonplace in commercial and industrial settings. Lastly, the RPC coupons were exposed to a bacterial biofilm growth process and swabbed using methods that closely mimic scrubbing actions performed during sanitation processes typically used in commercial and industrial settings. In all cases based on SEM assessment, bacteria not only attached to the RPC, but also could not be dislodged by the sanitizers or physical scrubbing that was applied.

Keywords: *Salmonella*, reusable plastic containers, attachment, scanning electron microscopy

1. Introduction

Foodborne *Salmonella* continue to be a public health problem that results in illness and represents a tremendous economic cost on an annual basis (Scallan et al., 2011; McLinden, Sargeant, Thomas, Papadopoulos, & Fazil, 2014). Numerous food sources have been associated with *Salmonella* while produce and most meat proteins have been identified as major contributors (Foley, Lynne, & Nayak, 2008; Hanning, Nutt & Ricke, 2009; Foley et al., 2011; Finstad, O'Bryan, Marcy, Crandall, & Ricke, 2012; Foley, Johnson, Ricke, Nayak, & Danzeisen, 2013). Poultry broiler meat and eggs have long been considered primary sources of *Salmonella*, and continue to be a prominent reservoir (Finstad et al., 2012; Howard et al., 2012; Galiş et al., 2013; Painter et al, 2013; Ricke et al., 2013a,b). In particular, table shell eggs and layer farms have been associated with *Salmonella* outbreaks (Howard et al., 2012; Martelli & Davies, 2012; Galiş et al., 2013; Ricke, Dunkley, & Durant, 2013a; Ricke, Jones, & Gast, 2013b). A large number of eggs are simultaneously processed and shipped for retail, which involves equipment capable of washing, candling, sizing, and packaging over 180,000 eggs per hour (Musgrove, 2011).

Surface contamination of equipment and the potential risks associated with egg processing in general has been investigated (Suresh, Hatha, Sreenivasan, Sangeetha, & Lashmanaperumalsamy, 2006; Singh, Yadav, Singh, & Bharti, 2010; Utrarachkij et al., 2012). For example, in a study on Thailand egg farms and markets, Utrarachkij et al., (2012) concluded that reusable egg trays used for these eggs could serve as a potential source of horizontal *Salmonella* transmission.

Based on past research, the question arises as to whether *Salmonella* and other foodborne pathogens can attach to surfaces such as RPC materials that they might come in contact with during transportation of eggs, and once attached, if these microorganisms can be dislodged from such surfaces. Certainly, foodborne pathogens such as *Salmonella* are known to attach to a variety of surfaces, and can become part of communities encased in polymeric substances resulting in difficult to remove biofilms (Kalmokoff et al. 2001; de Oliveira, Brugnera, Alves, & Piccoli, 2010; Steenanckers, Hermans, Vanderleyden, & De Keersmaecker, 2012). The objectives in the

current study were to determine and confirm the ability of *Salmonella* to adhere and produce bacterial biofilms on RPC. A second objective was to determine the ability of sanitizing procedures to disrupt and eliminate *Salmonella* biofilms on RPC. A final objective was to determine the ability of repeated swabbing to disrupt and eliminate *Salmonella* spp. biofilms on RPC.

2. Materials and Methods

2.1 Bacterial Strain and Inoculum Preparation

Five *Salmonella*, each of which represents a distinct serovar (i.e. Kentucky, Newport, Enteritidis, Heidelberg and Typhimurium), were obtained from the WBA Analytical Laboratories (WBA) (3609 Johnson Road, Springdale, AR 72762) frozen culture collection. All five isolates of *Salmonella* were streaked onto tryptic soy agar (TSA) (Thermo Fischer Scientific, M.A.) plates for isolation and incubated at 35 ± 1 °C for 18 h, and an isolated colony of each serovar was inoculated into a respective 10 mL of Tryptic Soy broth (TSB) (Thermo Fischer Scientific, M.A.) and incubated at 35 ± 1 °C for 18 h. Following incubation, 0.5 mL from each culture was transferred to a 40 mL TSB broth and incubated at 35 ± 1 °C for 18 h. After the final incubation all five *Salmonella* serovar cultures were combined and mixed in a sterile jar.

2.2 RPC Sample Preparation and Biofilm Formation (Study I)

Six RPC coupons were prepared by sanitizing each coupon and each coupon was allowed to dry in preparation for biofilm construction (Figure 1).

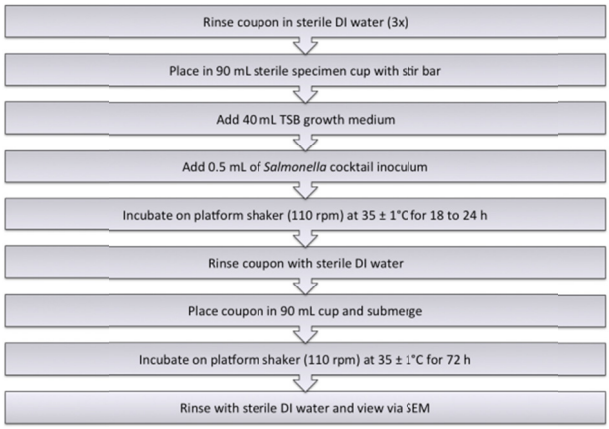

Figure 1. Steps taken to form a biofilm to a RPC coupon

Two coupons 1 in^2 (25.4 mm^2) for each *Salmonella* serovar were prepared for testing – one coupon was used for testing and one coupon was retained for backup purposes if needed. Each coupon was triple rinsed thoroughly with sterile deionized (DI) water to ensure the removal of any sanitizer residue. Each coupon was inserted into a 90 mL sterile specimen cup along with a sterile magnetic stir bar similar to previous research (de Oliveira et al., 2010). The stir bar was used to create additional shaking within the cup during incubation. A 40 mL aliquot of TSB growth medium was aseptically dispensed into each cup, and a 0.5 mL aliquot of the inoculum prepared as previously mentioned was added into each cup containing its respective coupon. The cup was placed onto a platform shaker (110 rpm) and was incubated at 35 ± 1 °C for 18 to 24 h. Following incubation, the coupon and stir bar were individually and aseptically removed from the cup.

Using a sterile 25 mL pipette, the coupons were rinsed with sterile DI water to remove any planktonic cells. Although they are the same organism, planktonic cells were considered physiologically distinct from the cells in the biofilm since they are suspended in the liquid growth medium. The rinsed coupon and stir bar were placed into a sterile 90 mL specimen cup and the previously mentioned rinsing steps were repeated. Once rinsed, the coupon was placed in its cup, and 40 mL of TSB was aseptically dispensed into the cup and the coupon was confirmed as submerged. The cup was placed on the platform shaker (110 rpm) and was incubated at 35 ± 1 °C for 72 h similar to previous research (Kalmokoff et al., 2001). After the final incubation, the coupon was aseptically removed, rinsed with sterile DI water, and placed in a sterile cup. The coupon was examined using scanning electron microscopy (SEM) for visual confirmation of attachment and biofilm development. The coupon was mounted on a aluminum specimen mount using double-coated carbon conductive tabs (Ted Pella,

Inc. Redding, CA), sputter-coated in an EMTECH SC7620 Sputter Coater (Emtech Electronics, Orem, UT), and viewed with a Philips SL 30 ESEM (Philips Electronics, N.V.) in a low vacuum mode.

2.3 Sanitizer Application-Salmonella (Study II) (Figure 2)

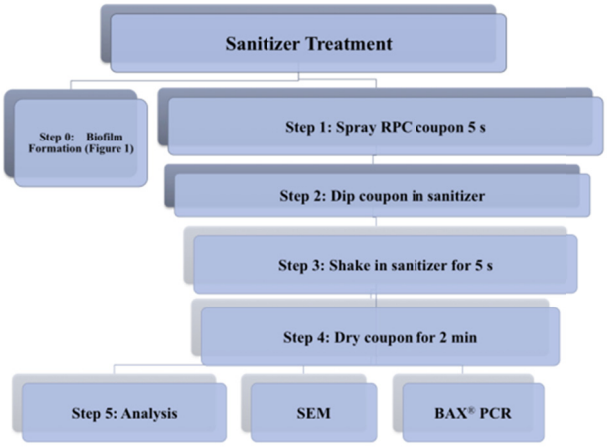

Figure 2. Steps taken for treatment with sanitizers

All five *Salmonella* serovars were prepared as a cocktail as previously described in the biofilm study. Six 90 mL sterile specimen cups per treatment group were used with 5 coupons (sanitized by isopropyl prior to the study) per treatment group and an extra coupon per group that was used for SEM imaging. After the final incubation, each coupon was aseptically removed and transferred to a tray that had previously been covered with foil and sanitized with isopropyl alcohol. The corner of each coupon was grasped with sanitized forceps and sterile DI water was dispensed over the coupon to remove planktonic cells. Each coupon was placed into individual sterile cups with assurance that the respective coupons remained in its assigned group.

For the sanitizer treatments, the respective concentration and water temperature was based on typical commercial and/or industrial standard limits for sanitization processes (USDA, 2006; GPO, 2011). The hot water used in each treatment group measured as 123.5°F (50.83 °C). Water pressure used for the spray was not measured, but the water flow was set to "full force". Treatment 1 (hot water + alkaline detergent (Contrad 70, Decon Labs, Inc. King of Prussia, PA) was conducted as follows: the corner of the coupon was grasped and each side of the coupon was sprayed for 5 s with hot water. After the hot water spray, the coupon was dipped in the alkaline detergent mixture, aggressively shaken for 5 s, placed on a wire rack, and dried for two minutes before being placed in a sterile stomacher bag.

Treatment 2 (hot water + alkaline detergent + quaternary ammonium) was conducted as follows: the corner of the coupon was grasped and each side of the coupon sprayed for 5 s with hot water, dipped in the alkaline detergent mixture, and aggressively shaken for 5 s. After removal, the coupon was quickly shaken to remove excess detergent mixture, dipped in the quaternary ammonium (Decon Quat, EcoLab, St. Paul, MN) mixture (250 ppm), and aggressively shaken for 5 s. After removal, the coupon was shaken to remove excess sanitizer, placed on a wire rack, dried for two min, and placed in a sterile stomacher bag.

Treatment 3 (quaternary ammonium) was conducted as follows: the corner of the coupon was grasped, dipped in the quaternary ammonium mixture (250 ppm), aggressively shaken for 5 s, and shaken to remove excess sanitizer. The coupon was placed on a wire rack, dried for two min, and placed in a sterile stomacher bag. Treatment 4 (hot water + alkaline detergent + 200 ppm chlorine solution (Chlorox® Bleach + water)) was conducted as follows: the corner of the coupon was grasped and each side of the coupon sprayed for 5 s with hot water. After the hot water spray, the coupon was dipped in the alkaline detergent mixture and aggressively shaken for 5 s, and shaken to remove excess detergent mixture. Next, the coupon was dipped in a chlorine and water mixture (205 ppm), aggressively shaken for 5 s, and shaken to remove the excess sanitizer. The coupon was placed on a wire rack, allowed to dry for two min, and was placed in a sterile stomacher bag.

Treatment 5 (200 ppm chlorine solution) was conducted as follows: the corner of the coupon was grasped, dipped in the chlorine solution, aggressively shaken for 5 seconds, and shaken to remove excess. The coupon was placed on a wire rack, dried for two min, and placed in a stomacher bag. Treatment 6 (untreated control) was conducted as follows: the corner of the coupon grasped but not exposed to treatment, and transferred directly to a sterile stomacher bag. The extra coupons needed for SEM imaging were removed from the treatment groups and held at 4 °C.

A PC1 Master Test Kit (Packers Chemical, Inc., Cuba City, WI) (titration kit to test concentration of quaternary ammonium and chlorine) was used to determine the actual level of quaternary ammonium and chlorine for the respective treatment. Once all treatments were performed and all coupons were in their corresponding stomacher bags, 20 mL of buffered peptone water (BPW) (Becton Dickinson and Company) was added, and they were shaken vigorously for 30 s. All samples were incubated at 35 ± 1 °C for 18 to 24 h. After incubation, the coupon samples were tested for the presence of *Salmonella* using the BAX® PCR system according to the manufacturer's instruction (Dupont, 2014). Each coupon was examined using SEM for visual confirmation of attachment and potential biofilm formation.

2.4 Salmonella Biofilm Formation Process and Impact of Swabbing (Study III)

All five *Salmonella* serovars were prepared as a cocktail as described previously in biofilm study I. The RPC coupons were prepared by sanitizing each coupon with 70% isopropyl alcohol and were subsequently dried. Each coupon was aseptically and thoroughly rinsed with sterile DI water to remove any sanitizer residue. Five 90 mL sterile specimen cups were labeled and RPC coupons were inserted into each cup. Aliquots (40 mL) of TSB were aseptically dispensed into each cup and 0.5 mL of inoculum was added into each cup containing the coupon and TSB. The cups were placed onto a platform shaker (110 rpm) and were incubated at 35 ± 1 °C for 18 h. The coupons were removed individually and aseptically from the respective cups, and the coupons were rinsed with sterile DI water to remove planktonic cells. The coupons were placed into a labeled sterile 90 mL specimen cup and rinsed as previously described. Once all coupons were rinsed and placed into the respective cups, 40 mL of the TSB was aseptically dispensed into the cup, the coupon was ensured to be submerged, and the cups were incubated on the platform shaker (110 rpm) at 35 ± 1 °C for 72 h.

After the final incubation step, each coupon was aseptically removed and transferred to a tray that had previously been covered with foil and sanitized with isopropyl alcohol. Using sanitized forceps, the corner of the coupon was grasped and sterile DI water was dispensed over the coupon to remove planktonic cells, and each coupon was placed into an individual sterile cup.

The entire surface of each coupon was swabbed using a PUR-Blue™ DUO™ swab (World Bioproducts) that was moistened with BPW. Swabbing was done aggressively and with pressure to remove as much of the *Salmonella* biofilm as possible. The swab was returned to its corresponding tube containing 9 mL of BPW. For each of the five coupons, the swabbing was repeated two more times (three swabs per coupon) changing swabs for each swab. Once all swabs were performed, the RPC coupons were placed into a sterile stomacher bag and 20 mL of sterile BPW was added. A negative control was prepared by pouring 20 mL of the BPW into a sterile stomacher bag. A positive control was prepared by pouring 20 mL of the BPW into a sterile stomacher bag, and one *Salmonella* Bioball® was added to the BPW. All samples (swabs and coupons) were incubated at 35 ± 1 °C for 18 to 24 h. After incubation, treatment samples and controls were tested for the presence of *Salmonella* using the BAX® PCR system.

2.5 Disposal Protocols for Samples and Chemicals

Samples and testing materials were disposed of at completion of analysis with the approval of the WBA project's team leader and reference to WI-A-011 (Laboratory Waste and Disposal) for disposal procedures. When chemicals were used in this study, they were held on site for future use, returned to the customer, or discarded. Handling, storage, and/or disposal of all chemicals were performed appropriately according to the material safety data sheets (MSDS) and the actions taken were noted in the Research Project Design Form.

3. Results

3.1 Biofilm Formation for Multiple Salmonella Serovars (Study I)

Reusable Plastic Containers were used in this study to determine the ability of *Salmonella spp.* to adhere to and form biofilms on the RPCs being used in commercial settings. The *Salmonella* biofilm was comprised of serovars Kentucky, Newport, Heidelberg, Enteritidis, and Typhimurium. The RPC's were disassembled and cut into 1 in² pieces (referred to as coupons). Preliminary studies using SEM provided visual confirmation that *S.* Enteritidis adhered to the RPC (Figure 3a), and an uninoculated RPC coupon was viewed using SEM to confirm

bacterial absence (Figure 3b).

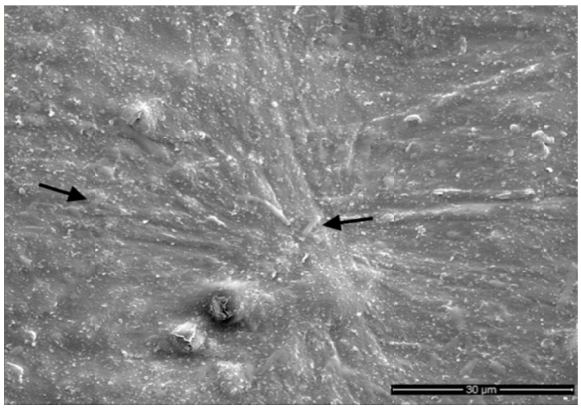

Figure 3a. Study I – SEM image confirming *Salmonella* adhering to a RPC coupon. Magnification 3500×. Arrows indicate attached *Salmonella* cells

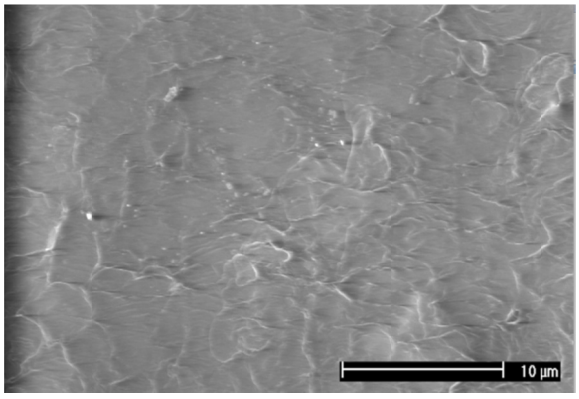

Figure 3b. SEM image of uninoculated RPC coupon. Magnification 3500×

3.2 Sanitizer Application-Salmonella (Study II)

This study was performed to evaluate the ability of five sanitation methods typically used in commercial/industrial settings to disrupt and remove *Salmonella* biofilms on RPCs. The *Salmonella* biofilm was comprised of *S.* Newport, *S.* Kentucky, *S.* Heidelberg, *S.* Enteritidis, and *S.* Typhimurium. The RPC's were disassembled and cut into 1 in^2 (25.4 mm^2) coupons. After each coupon was subjected to a biofilm formation process, the coupons were cleaned/sanitized using products (quaternary ammonium and chlorine) and methods typically used in commercial/industrial settings to sanitize equipment and supplies.

Following incubation, all coupons were analyzed using BAX® PCR for the detection of *Salmonella.* All RPC coupons from all treatment groups tested positive for the presence of *Salmonella* serovars in this study. An enrichment step prior to BAX® PCR ensured that if a positive test resulted then at least one viable cell was present on the coupon. The extra coupons from each treatment group were simultaneously examined by SEM to confirm the presence of *Salmonella* biofilms on the coupons from each group. Based on SEM and PCR analyses, *Salmonella* cells were still present and apparently attached after administration of the sanitizers. All SEM images confirmed that a *Salmonella* biofilm-like structure remained intact after administration of the sanitizers (Figure 2).

3.3 Salmonella Biofilm Formation Process and Impact of Swabbing (Study III)

Reusable Plastic Containers were used in this study to determine if repeated swabbing disrupts and removes *Salmonella* biofilms that are formed on RPCs. The biofilms were comprised of *Salmonella* serovars Kentucky, Newport, Heidelberg, Enteritidis, and Typhimurium. The RPCs were disassembled and cut into 1 in^2 (25.4 mm^2) coupons. Preliminary work using SEM provided visual confirmation that the *Salmonella* serovars adhered to the RPC coupons (data not shown). After each coupon was subjected to the biofilm formation process, the coupons were swabbed three consecutive times using separate swabs each time to determine if the repeated swabbing action could remove the *Salmonella* biofilm from the RPC coupons.

Following incubation, all coupons and swabs were analyzed for the presence of *Salmonella* using BAX® PCR. All RPC coupons and swabs tested positive for the presence of *Salmonella* (data not shown). Positive and negative controls were performed to account for false positives that could occur due to contaminated media. Also, internal positive controls were performed in the BAX® PCR assay to assure successful PCR amplification. Based on SEM and PCR analyses, the *Salmonella* serovars remained attached after repeated swabbing.

4. Discussion

The SEM images provided evidence that *Salmonella* was capable of attaching to the RPCs to form biofilms. The BAX® PCR system is commonly used for *Salmonella* detection in commercial settings (Dupont, 2014), and has previously been evaluated for detection in a variety of food matrices and environments (Bailey, 1998; Tice et al., 2009; Frausto, Alves, & Oliveira, 2013). The PCR results confirmed that at least in the case of the *Salmonella*

attachment studies that the bacteria observed by SEM were indeed *Salmonella*. The low vacuum mode that was used for SEM is advantageous due to the fact that the attached bacterial cells being viewed are less likely to be disrupted due to the vacuum pressure that is associated with typical SEM protocols. Additionally, the biofilm surface does not contact the biofilm in this mode, which negates the need for fixation procedures that may alter the biofilm structure.

In study II, the SEM images and BAX® PCR results suggest that the sanitizing methods and agents used in this study were not effective in disrupting and eliminating *Salmonella* biofilms from RPCs (Figures 2-6). In this study all coupons were cut from flat, smooth areas of the RPCs, which represent areas that should be easily cleaned during sanitation. Areas of the RPC that have raised edges, textured surfaces, and hard to access recessed areas would be of considerable concern due to the ability of biofilms to form in these areas as well as the inability of typical sanitizing methods to reach these areas. In summarizing what is known about *Salmonella* and biofilm formation, Steenackers et al. (2012) noted that *Salmonella* are not only capable of forming biofilms on a wide range of abiotic surfaces including plastic, rubber, cement, glass, and stainless steel which all represent materials commonly encountered in food processing environments, but bacteria that exist as part of a biofilm are well protected against environmental stresses such as disinfectants.

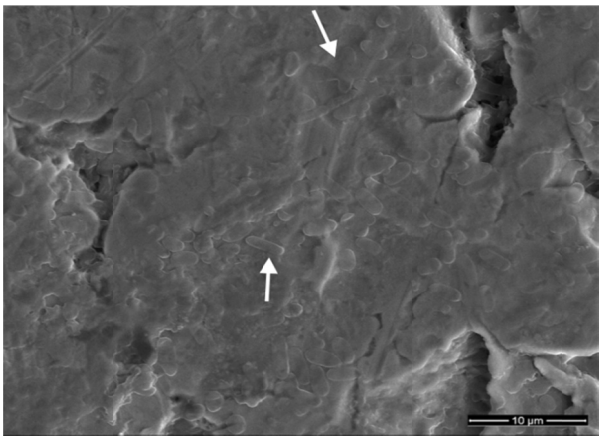

Figure 4. Study II – SEM image of *Salmonella* biofilm following treatment 1 – hot water + alkaline detergent. Magnification 6500×. Arrows indicate attached *Salmonella* cells

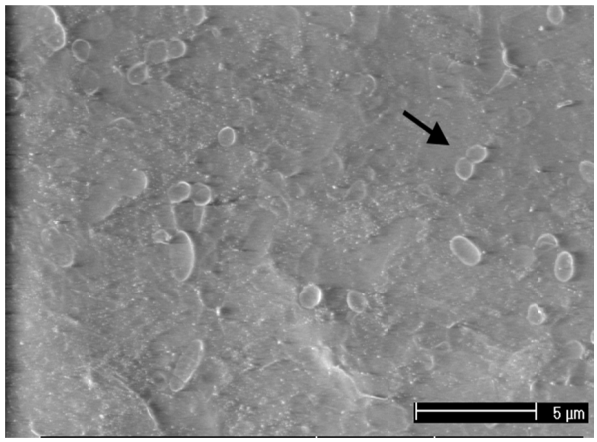

Figure 5. Study II – SEM image of *Salmonella* biofilm following treatment 2 – hot water/alkaline detergent + quaternary ammonium. Magnification 5000×. Arrow indicates attached *Salmonella* cells

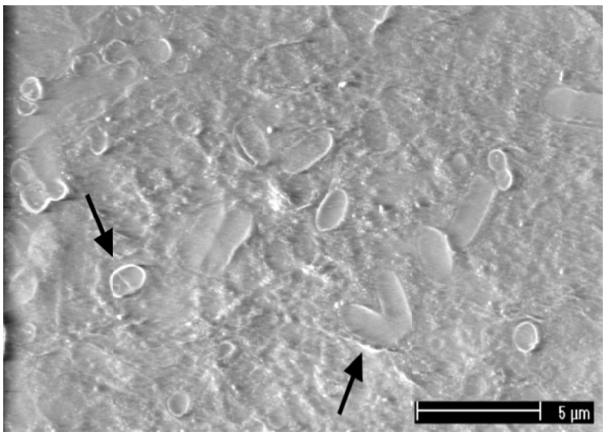

Figure 6. Study II – SEM image of *Salmonella* biofilm following treatment 3 – quaternary ammonium. Magnification 5000×. Arrows indicate attached *Salmonella* cells

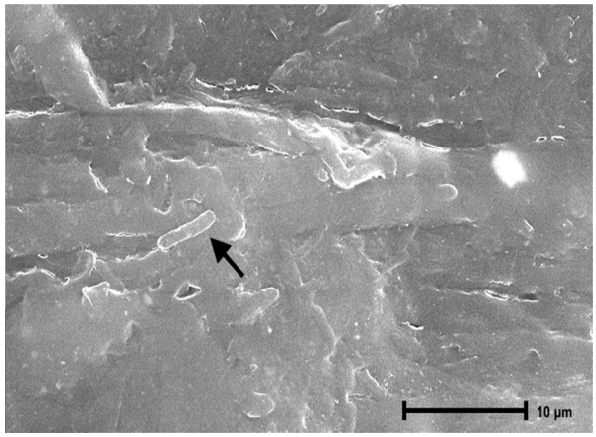

Figure 7. Study II – SEM image of *Salmonella* biofilm following treatment 4 – hot water/alkaline detergent + 205 ppm chlorine. Magnification 2500×. Arrow indicates attached *Salmonella* cell

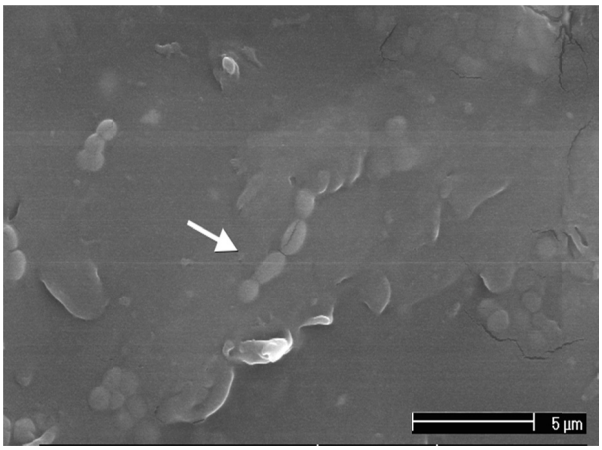

Figure 8. Study II – SEM image of *Salmonella* biofilm following treatment 5 –200 ppm chlorine. Magnification 5000×. Arrow indicates attached *Salmonella* cells

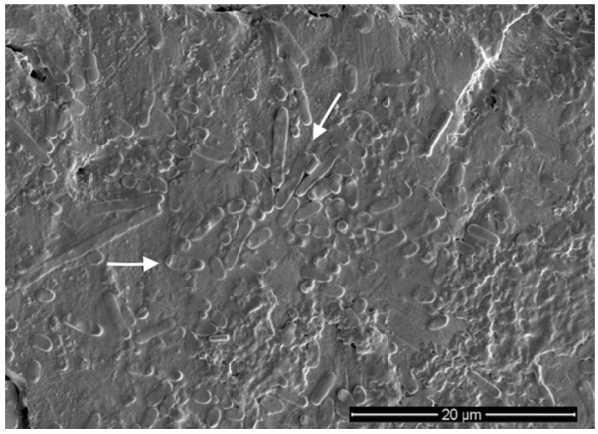

Figure 9. Study II – SEM image of *Salmonella* biofilm following no treatment. Magnification 5000×. Arrows indicate attached *Salmonella* cells

The SEM images provide evidence that the selected sanitizer treatments administered in these study (chlorine and quaternary ammonium) did not effectively remove all of the attached *Salmonella* cells on the RPCs. When sanitizers are employed in an egg processing facility this generally occurs as a rinse solution containing a chlorine concentration of 100 to 200 ppm, or a quaternary ammonium-based compound that is administered immediately after the alkaline egg wash cleaning step as a rinse solution (Hutchinson et al., 2003; Howard et al., 2012). However, given the constant search for improved efficacy coupled with reduced costs, a wide range of sanitizers have been examined for potential use in egg processing (Gao, Stewart, Joseph, & Carr, 1997; Kuo, Cary, & Ricke, 1997a; Kuo et al., 1997b; Kuo, Ricke, & Cary, 1997c; McKee, Kwon, Carey, Sams, & Ricke, 1998; Knape et al., 1999, 2001; Russell, 2003; Bialka, Demirci, Knabel, Patterson, & Puri, 2004; Rodriguez-Romo & Yousef, 2005; Ragni et al., 2010; Berardinelli et al., 2011; Howard et al., 2012).

Steam pasteurization has been evaluated for the efficacy of biofilm reduction of *S.* Typhimurium, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 on stainless steel and polyvinyl chloride (PVC) coupons (Park & Kang, 2014). These authors observed a greater log CFU/coupon reduction of biofilms formed on stainless steel coupons in comparison to PVC coupons (Park & Kang, 2014), which suggests that microorganisms may be more likely to attach to certain food processing surfaces. Additionally, steam pasteurization may be one of the most effective sanitizing techniques, and these results suggest that cell attachment and biofilm formation may play a crucial role in food product contamination.

In future studies, it will be critical to examine whether any of these alternative sanitizing or disinfectant approaches have the potential efficacy against *Salmonella* and other foodborne pathogen biofilms on the surfaces of egg processing equipment and egg handling materials. The relative effectiveness of the sanitizer(s) in question may be the best predictor for potential success against biofilms in these types of environments.

In study III, the BAX® PCR results provided evidence that the repeated swabbing methods used in this study were not effective in eliminating *Salmonella* biofilms from RPC surfaces. The swabbing methods were used to mimic a typical scrubbing action that may be used during sanitation in a commercial and/or industrial setting. This is consistent with the results of study II, indicating that typical sanitizers are ineffective in removal of *Salmonella* and would suggest that general efforts to clean and disinfectant these types of surfaces may not be sufficient. However, several issues remain to be resolved. For example, the question remains as to whether *Salmonella* biofilms would provide a source of contamination by shedding onto anything that it may contact such as human hands during transport or food transported or stored in the RPC. In addition, little is known about the interaction between packaging materials and table shell eggs, which could result in contamination. At least in the processing plant there is some indication that cross contamination does occur between contaminated equipment and eggs during transient processing (Davies & Breslin, 2003). Certainly, it is conceivable that potential microbial cross contamination could occur depending on the type of packaging material, particularly if it is reused and not properly cleaned.

5. Conclusion

Microbial contamination on surfaces such as RPC materials will most likely consist of more than one bacterial species and will probably be fairly complex. How this microbial composition influences the before and after biofilm formation by organisms such as *Salmonella* may impact the extent of biofilm formation as well as the ability to clean and sanitize surfaces containing these biofilms. More comprehensive microbial studies need to be conducted to better identify the dynamics of microbial diversity and their potential interactions with foodborne pathogens such as *Salmonella*. Microbiome sequencing offers opportunities to more thoroughly characterize these microbial populations and detect patterns that may contribute to the more persistent contamination problems. Elucidating these microbial populations may allow for an assessment of the sequence of events that initiates biofilm formation as well as which non-*Salmonella* microorganisms are most likely to favor the establishment of *Salmonella* biofilms.

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