ATTACHMENT OF *SALMONELLA* AND OTHER FOODBORNE PATHOGENS TO REUSABLE PLASTIC CONTAINERS

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Abstract

Reusable Plastic Containers (RPC) were used for a study to determine the ability of bacteria to adhere and form biofilms on the RPCs being used in commercial settings. The three biofilm groups of interest were *Salmonella* spp*., Listeria monocytogenes*, and *E. coli* O157:H7. The RPC coupons served as the platform for generation of bacteria biofilms of these bacteria. After biofilm formation on RPC coupons by the respective bacteria the coupons were examined using Scanning Electron Microscopy (SEM) for presence of bacteria. In a second study the RPC coupons were subjected to a bacteria biofilm growth process then sanitized using methods and sanitizing agents typically found in commercial and industrial settings. In a third study the RPC coupons were exposed to a bacteria biofilm growth process then swabbed using methods that closely mimic scrubbing actions performed during sanitation processes typically used in commercial and industrial settings. In all cases bacteria not only attached to the RPC but could not be dislodged by either sanitizers or physical scrubbing.

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 et al., 2014). Numerous food sources of *Salmonella* exist with produce and most meat proteins being identified as major contributors (Hanning et al., 2009; Finstad et al., 2012; Howard et al., 2012; Foley et al., 2008, 2011, 2013). Poultry broiler meat and eggs have always been considered primary sources and continue to be fairly prominent (Finstad et al., 2012; Howard et al., 2012; Galiş et al., 2013; Painter et al, 2013; Ricke et al., 2013a,b; Pires et al., 2014;). In particular table shell eggs and layer farms have been associated with *Salmonella* outbreaks (Ricke, 2003, Dunkley et al., 2009; Howard et al., 2012; Martelli and Davies, 2012; Galiş et al., 2013; Ricke et al. 2013a,b). The number of eggs processed and shipped for retail, involves equipment capable of washing, candling, sizing, and packaging over 180,000 eggs per hour (Musgrove, 2011). Eggs produced at the farm can enter the egg processing system either in an "in-line" production system where eggs are directly moved via conveyor belts from the layer farm where they are produced directly to an egg processing facility or as an "off-line" production system where eggs are collected at the farm and subsequently transported to another site for processing (Musgrove, 2011).

However, potential contamination issues remain with certain segments of the egg retail market. Historically in the U.S., used cases, fillers and flats were considered available for reuse (Eggleton and Carpenter, 1961). However, Board et al. (1963) surveyed new, used, and dirty egg flats and observed that they could become heavily contaminated especially if they had egg albumen or yolk material remaining on them. Banwart, (1964) demonstrated that *Salmonella* and other egg contaminant bacteria could attach to these egg flats and that only autoclaving the flats

completely eliminated them. This issue has re-emerged in the U.S. for certain local markets where retail egg containers can be reused and there is the potential for contamination to occur over time if these are not properly sanitized. There is evidence for this potential risk from studies conducted on retail egg markets in other countries. Based on the recovered levels of *Salmonella* from egg shells, egg contents and egg trays in South India, Suresh et al. (2006) concluded that reused egg trays were a potential risk for exposure to *Salmonella*. After examining eggs transported from farms to wholesale and retail markets located in North India Singh et al., (2010) found *S.* Typhimurium to be the predominant serovar with a higher incidence from eggs collected in the retail markets leading them to suggest that surface contamination must have occurred during handling, storage, and transportation of the eggs from the farms to the market. 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.

From what is known the question arises as to whether *Salmonella* and other foodborne pathogens that might come in contact with surfaces such as RPC materials can attach to these surfaces and once attached, can these organisms be dislodged from such surfaces. Certainly, foodborne pathogens such as *Salmonella* and *Listeria monocytogenes* are known to attach to a variety of surfaces and furthermore can become part of communities encased in polymeric substances forming difficult to remove biofilms (Kalmokoff et al. 2001; de Oliveira et al., 2010; Steenanckers et al., 2012). The objectives in the current study were to initially determine and confirm the ability of *Salmonella spp*., *Listeria monocytogenes*, and *E. coli* O157:H7 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.

Materials and Methods

Bacterial strains used in these studies

Five *Salmonella* strains, namely, *Salmonella* Kentucky*, S. Newport, S.* Enteritidis*, S.* Heidelberg and *S.* Typhimurium were obtained from the WBA culture collection. Five strains of *Listeria monocytogenes* were obtained either from the American Type Culture Collection (ATCC) or the Tyson laboratory, namely, ATCC #19111 (SPR-CULRF-504), ATCC #19115 (SPR-CULRF-500), ATCC #43257 (SPR-CULRF-502), ATCC #49594 (SPR-CULRF-501), and Tyson #2926 (human isolate from lunchmeat) (SPR-CULRF-503). The *E. coli* O157:H7 strain used in this study was a non-toxin forming isolate, ATCC# 19206 (SPR-CULQC-552).

Bacterial inocula preparation

All five strains of *Salmonella spp.* were streaked onto TSA plates for isolation followed by incubation at 35 ± 1°C for 18 hours. Likewise all five strains of *L. monocyogenes* were streaked onto TSA plates for isolation and incubated at 35 ± 1°C for 18 hours. The *E. coli* O157:H7 was streaked onto a TSA plate for isolation and incubated at 35 ± 1°C for 18 hours. After incubation, an isolated colony was picked from each TSA to 10 ml of BHI broth and incubated at 35 ± 1°C for 18 hours. After incubation, 0.5 ml from each 10 ml BHI was transferred to a 40 ml BHI broth and incubated at 35 ± 1°C for 18 hours. After the final incubation all five *Salmonella* serovar inocula were combined and mixed in a sterile jar. This was also done for the five *L. monocytogenes* inocula but was not required for *E. coli* O157:H7 since only strain was used.

RPC sample preparation and biofilm formation (Study I)

Six RPC coupons were prepared by sanitizing each coupon and allowing each coupon to dry. Two coupons for each bacteria was prepared for testing. Of the two coupons, one coupon was used for testing and one coupon was retained for backup purposes if needed. Each coupon was triple rinsed thoroughly with sterile DI water to ensure no sanitizer residue was lingering. Three 90 ml sterile specimen cups with the respective bacterial isolate name was labelled as follows: *Salmonella spp*. – RPC, *L. monocytogenes* – RPC**,** *E. coli* O157:H7 – RPC. Each coupon was inserted into its respective cup along with a sterile magnetic stir bar. The stir bar was used to create extra motion within the cup during incubation. A 40 ml aliquot of appropriate growth medium was aseptically dispensed into each cup. For this study TSB was used for the *Salmonella* samples and BHI was used for both the *L. monocytogenes* and *E. coli* O157:H7 samples. A 0.5 ml aliquot of each inoculum was dispensed into appropriate cup containing coupons. The three cups were placed onto a platform shaker (set at a rotation of 110 rpm) that had been positioned in a 35±1°C incubator and incubated for 18 to 24 hr. After incubation, all cups were removed and the coupons and stir bars were individually and aseptically removed from the respective cups. The cups and inoculated growth media were discarded.

Using a sterile 25 ml pipette, the coupons were rinsed with sterile DI water to remove any loose planktonic cells. Even though they are the same organism planktonic cells were considered physiologically distinct from the cells growing in a biofilm because rather than attaching they either float or swim in the liquid growth medium. The rinsed coupons and stir bars were placed into labeled sterile 90 ml specimen cups and the above rinsing steps were repeated for each coupon individually to avoid cross contamination. Once all coupons were rinsed and placed into their respective specimen cups, 40 ml of the appropriate growth media was aseptically dispensed into each cup and coupons were confirmed as being submerged. All

three cups were incubated on the platform shaker (set at a rotation of 110 rpm) at 35±1°C for 72 hours. After the final incubation, each coupon was aseptically removed, rinsed with sterile DI water. and placed in individual sterile cups. Each coupon was examined using scanning electron microscopy (SEM) for visual confirmation of attachment and biofilm development.

Sanitizer application-*Salmonella* (Study II)

All five *Salmonella* serovars were prepared as a cocktail as described previously in the biofilm study I. In this study six, 90 ml sterile specimen cups per treatment group were used, namely 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 been covered with foil and sanitized with isopropyl alcohol. The corner of each coupon was grasped with sanitized forceps and sterile DI water dispensed over the coupon to remove loose 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. The hot water used in each treatment group measured 123.5°F. Water pressure used for the spray was not measured; however the water flow was set to "full force". Treatment 1 (Hot Water + Alkaline Detergent) was conducted as follows: The corner of the coupon was grasped and each side of the coupon was sprayed for 5 seconds with hot water using a spray nozzle attached to the sink faucet. After the hot water spray, the coupon was dipped in the alkaline detergent mixture and aggressively moved back and forth for 5 seconds, then placed on a wire rack and allowed to dry for two minutes. The coupon was subsequently placed in a sterile stomacher bag.

Treatment 2 (Hot Water + Alkaline Detergent + 200 ppm to 400 ppm)was conducted as follows: The corner of the coupon was grasped and each side of the coupon sprayed for 5 seconds with hot water using a spray nozzle attached to the sink faucet. After the hot water spray, the coupon was dipped in the alkaline detergent mixture and aggressively moved back and forth for 5 seconds. After removal, the coupon was quickly shaken to remove excess detergent mixture. Next, the coupon was dipped in the quaternary ammonium mixture and aggressively moved back and forth for 5 seconds. For this treatment the concentration of the quaternary ammonium was set at 250 ppm. Again, after removal, the coupon was shaken to remove excess sanitizer followed by placement on a wire rack, allowed to dry for two minutes and placed in a sterile stomacher bag.

Treatment 3 (200 ppm to 400 ppm quaternary ammonium) was conducted as follows: The corner of the coupon was grasped, dipped in the quaternary ammonium mixture, aggressively moved back and forth for 5 seconds, and shaken to remove excess. For this treatment, the concentration of the quaternary ammonium was 250 ppm. Once again the coupon was placed on a wire rack, allow to dry for two minutes and then placed in a sterile stomacher bag. Treatment 4 (Hot Water + Alkaline Detergent + approximately 200ppm Chlorine Solution) was conducted as follows: The corner of the coupon was grasped and each side of the coupon sprayed for 5 seconds with hot water using a spray nozzles attached to the sink faucet. After the hot water spray, the coupon was dipped in the alkaline detergent mixture and aggressively moved back and forth for 5 seconds then shaken to remove excess detergent mixture. Next, the coupon was dipped in a chlorine and water mixture, aggressively moved back and forth for 5 seconds, then shaken to remove the excess. For this study the concentration of the chlorine solution was 205

ppm. The coupon was placed on a wire rack, allowed to dry for two minutes followed by placement in a sterile stomacher bag.

Treatment 5 (approximately 200 ppm chlorine solution) was conducted as follows: The corner of the coupon was grasped, dipped in the chlorine solution, aggressively moved back and forth for 5 seconds, and finally shaken to remove excess. For this treatment the concentration of the chlorine solution was 200 ppm. The coupon was placed on a wire rack, allowed to dry for two minutes 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 instead transferred directly to a sterile stomacher bag. The extra coupons needed for SEM imaging were removed from the treatment groups and held at a refrigerated temperature.

A PC1 Master Test Kit (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 mLs of sterile buffered peptone water was added and they were shaken vigorously for 30 seconds. All samples were incubated at 35±1°C for 18 to 24 hours. After incubation, the coupon samples were tested for the presence of *Salmonella spp.* using the BAX® PCR system. Each coupon was examined using SEM for visual confirmation of attachment and potential biofilm formation.

***Salmonella spp.* Biofilm Formation Process and Impact of Swabbing (Study III)**

All five *Salmonella* serovars were prepared as a cocktail as described previously in the biofilm study I. The RPC coupons were prepared by sanitizing each coupon with 70% isopropyl alcohol and allowed to dry. Each coupon was aseptically and thoroughly rinsed with sterile DI water to remove any sanitizer residue. Five, 90 ml sterile specimen cups were labelled and RPC

coupons inserted into each cup. Aliquots (40 ml) of Tryptic Soy Broth (TSB) were aseptically dispended into each cup followed by adding a 0.5 ml inoculum into each cup containing the coupon and TSB. The inoculated cups were placed onto a platform shaker that had been positioned in a 35 ±1°C incubator, started (set at 110 rpm) and incubated for 18 hours. After the 18 hours incubation, all coupons were removed individually and aseptically from the respective cups. Cups and inoculated growth medium were discarded. Using a sterile 25 ml pipette, the coupon were rinsed with sterile DI water to remove any loose planktonic cells placed into a labeled sterile 90 ml specimen cup and the above rinsing steps were repeated for each cup individually to prevent any type of cross contamination during the biofilm formation process. Once all coupons were rinsed and placed into specie cups, 40 ml of the TSB was aseptically dispensed into the cup and ensured that the coupon was submerged in broth. All cups were incubated on the platform shaker at 35±1°C for 72 hours.

After the final incubation each coupon was aseptically removed and transferred to a tray that has been covered with foil and sanitized with isopropyl alcohol. Using sanitized forceps, the corner of the coupon was grasped and sterile DI water dispensed over the coupon to remove loose cells. Each coupon was placed into individual sterile cups and allow coupons to dry. Coupons were picked up with sterile gloves and the entire coupon surface was swabbed using a PUR-Blue™ DUO™ swab that was moistened with buffered peptone water. Swabbing was done aggressively and with pressure with the intent of removing as much *Salmonella* biofilm as possible. The swab was returned to its corresponding tube filled with 9 ml of buffered peptone water. The swabbing was repeated two more times (for a total of three swabs per coupon) changing swabs for each repetition and was repeated for each of the 5 coupons. Once all swabs were performed, the RPC coupons were placed into a sterile stomacher bag and 20 ml of sterile

buffered peptone water was added. A negative control was prepared by pouring 20ml of the buffered peptone water into a sterile stomacher bag. A positive control was prepared by pouring 20 ml of the buffered peptone Water into a sterile stomacher bag. One Salmonella Bioball® was added to the buffered peptone water. All samples (swabs and coupons) were incubated at 35±1°C for 18 to 24 hours. After incubation, test samples and controls were tested for the presence of *Salmonella* using the BAX®system.

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 the project, 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 MSDS and actions taken was noted in the Research Project Design Form.

RESULTS

Biofilm formation for Multiple Foodborne Pathogens (Study I)

Reusable Plastic Containers were used for a study to determine the ability of different foodborne pathogenic bacteria to adhere and form biofilms on the RPCs being used in commercial settings. The three biofilm groups of interest were *Salmonella* spp*., Listeria monocytogenes*, and *E. coli* O157:H7. The *Salmonella spp*. biofilm was comprised of serovars *S.* Newport, *S.* Kentucky, *S.* Heidelberg, *S.* Enteritidis, and *S.* Typhimurium. The *L. monocytogenes* biofilm consisted of one poultry isolate and four human isolates while the *E. coli* O157:H7 was a non-toxin forming strain. The RPC's were disassembled and cut into 1 in^2 pieces (referred to as coupons). Preliminary work using Scanning Electron Microscopy (SEM)

provided visual confirmation of *S.* Enteritidis adhering to the RPC and stainless steel coupons. An SEM examination of *Listeria monocytogenes* and *E. coli* O157:H7 inoculated RPC coupons indicated that they were able to attach to RPC as well (see figures with corresponding SEM pictures attached for study I).

Sanitizer application-*Salmonella* (Study II)

A study was performed to evaluate the ability of five treatment methods typically used in commercial/industrial settings for sanitation to disrupt and remove *Salmonella spp*. biofilms on the RPC. The *Salmonella spp*. 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 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.

After the incubation, all coupons were analyzed using BAX® PCR for the detection of *Salmonella spp*. In the case of this study, all RPC coupons from all treatment groups tested positive for the presence of *Salmonella* serovars (see Table 1). Simultaneously, the extra coupons from each treatment group were examined using SEM to confirm the presence of *Salmonella spp*. biofilm on coupons from each group. Based on SEM and PCR analyses, *Salmonella* cells were still attached even after administration of the respective sanitizers. All SEM images confirmed that a *Salmonella* biofilm-like structure remained intact after administration of the various sanitizers (see figures with corresponding SEM pictures attached for study II).

***Salmonella spp.* Biofilm Formation Process and Impact of Swabbing (Study III)**

Reusable Plastic Containers were used for a study to determine the ability of repeated swabbing to disrupt and remove *Salmonella* biofilms that are formed on the RPCs. The

Salmonella spp. biofilm were comprised of *S.* Newport, *S.* Kentucky, *S.* Heidelberg, *S.* Enteritidis, and *S.* Typhimurium. The RPC's were disassembled and cut into 1 in² coupons. Preliminary work using scanning electron microscopy (SEM) provided visual confirmation of *Salmonella* serovars adhered to the RPC coupons (data not shown). After each coupon was subjected to a biofilm formation process, the coupons were swabbed three consecutive times, using a different swab each time, to determine if the repeated swabbing action could remove the *Salmonella* biofilm from the RPC coupons.

After the incubation, all coupons and swabs were analyzed using BAX® - based PCR analyses for the detection of *Salmonella spp*. In the case of this study, all RPC coupons and swabs tested positive for the presence of *Salmonella* serovars (data not shown). A positive control and a negative control were run along with the coupon and swab samples to eliminate the suspension of false positives that could occur due to contaminated media. Also, internal positive controls were contained in the BAX® system to assure PCR success. Based on SEM and PCR analyses the *Salmonella* serovars remained attached after repeated swabbing.

DISCUSSION and CONCLUSIONS

The SEM images gave evidence that each bacteria (*Salmonella*, *Listeria* and *E. coli*) were capable of attaching to the RPC and forming biofilms. Likewise the PCR detection analyses confirmed that at least in the case of the *Salmonella* attachment studies that the bacteria showing up on the SEM were indeed *Salmonella*. While certain *Salmonella* serovars such as *S.* Enteritidis are well known for being present in egg production and processing (Howard et al., 2012) there is precedent for foodborne pathogens such as *Listeria* to also occur in these environments. *Listeria* spp. can be found in many food processing plant environments (Milillo et al. 2012a) but have isolated and characterized in poultry, eggs, egg wash water and egg processing equipment (Laird

et al, 1991; Farber et al., 1992; Jones et al, 2006; Jones and Musgrove, 2008a,b; Milillo et al. 2012b). Likewise, Jones and Musgrove (2008a,b) also observed the presence of *Escherichia* isolates from these characterized microbial populations in the egg processing samples but these isolates were not further identified to determine if they were foodborne pathogen species *Escherichia*. Given the high frequency of *Listeria* species (particularly *L. innocua*) after characterizing the microbial populations in rinsates from egg vacuum loaders in mixed and off-site egg production plants, Jones and Musgrove (2008a,b) speculated that egg vacuum loaders were a potential contamination site for *Listeria*. From these studies it is not clear whether they originally were attached to incoming egg flat surfaces or were part of the plant facility environment microflora and this remains to be determined. However, the current SEM studies do suggest that *L. monocytogenes* certainly can attach and form biofilms on RPC materials that would compose egg flats. This would certainly be consistent with *Listeria*'s ability to form biofilms on other surfaces (Kalmokoff et al. 2001; de Oliveira et al., 2010).

In study II, the SEM images and BAX® results gave evidence that the sanitizing methods and agents used in this study were not effective in disrupting and eliminating *Salmonella spp*. biofilms from RPC surfaces. In this study all coupons were cut from flat, smooth areas of the RPC 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 high concern due to the ability of biofilms to form in these areas and 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 representing materials all commonly encountered in food processing environments, but

bacteria in general that exist as a biofilm community are well protected against environmental stresses such as disinfectants.

The SEM images provide evidence that the selected sanitizer treatments administered in this study (chlorine and quaternary ammonium) did not effectively remove the developed *Salmonella spp*. biofilms on the RPC. When sanitizers are employed in an egg processor 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 (Berardinelli et al., 2011; Howard et al., 2012; Galiş et al. 2013). Not only sanitizers based on botanical compounds, enzyme catalyzed bactericidal reactions, or electrolyzed water (Kuo et al., 1997b; McKee et al., 1998; Knape et al., 1999, 2001; Russell, 2003; Bialka et al., 2004; Park et al., 2005; Cao et al., 2009; Upadhyaya et al., 2013) have been examined but exposing shell eggs or egg processing equipment to ultraviolet light, non-thermal atmospheric gas plasma, ozone, or ionizing radiation has also been also assesse for their relative effectiveness (Gao et al., 1997; Kuo et al., 1997a,c; Chavez et al., 2002; Rodriguez-Romo & Yousef, 2005; Keklik et al.,2010; Ragni et al., 2010). 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 pathogens after they have formed biofilm communities on the surfaces of egg processing equipment and egg handling materials. The relative effectiveness of the respective sanitizer 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 spp.* biofilms from the surfaces of RPC's. The swabbing methods used were 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 were ineffective in *Salmonella* removal 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* in these biofilms would not only be capable of attaching and remaining on surfaces but would they also shed cells onto anything that may come in contact with the biofilm (such as hands during transport or objects transported or stored in the RPC). In addition, little is known about the interaction between the type of packaging and the cross contamination that may occur between it and the table shell egg. At least in the processing plant there is some indication that cross contamination does occur between contaminated equipment and the eggs during transient processing (Davies and 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.

Finally, microbial contamination on surfaces such as RPC materials will most likely consist of more than one bacterial species and will probably be a fairly complex microbial consortia. How this microbial composition influences the before and after biofilm formation by organisms such as Salmonella may impact not only the extent of biofilm formation but the ability to not only 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* spp. Microbiome

sequencing offers opportunities to much more thoroughly characterize these microbial population 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 and which non-*Salmonella* microbial species are most likely to favor *Salmonella* establishment in the biofilm matrix.

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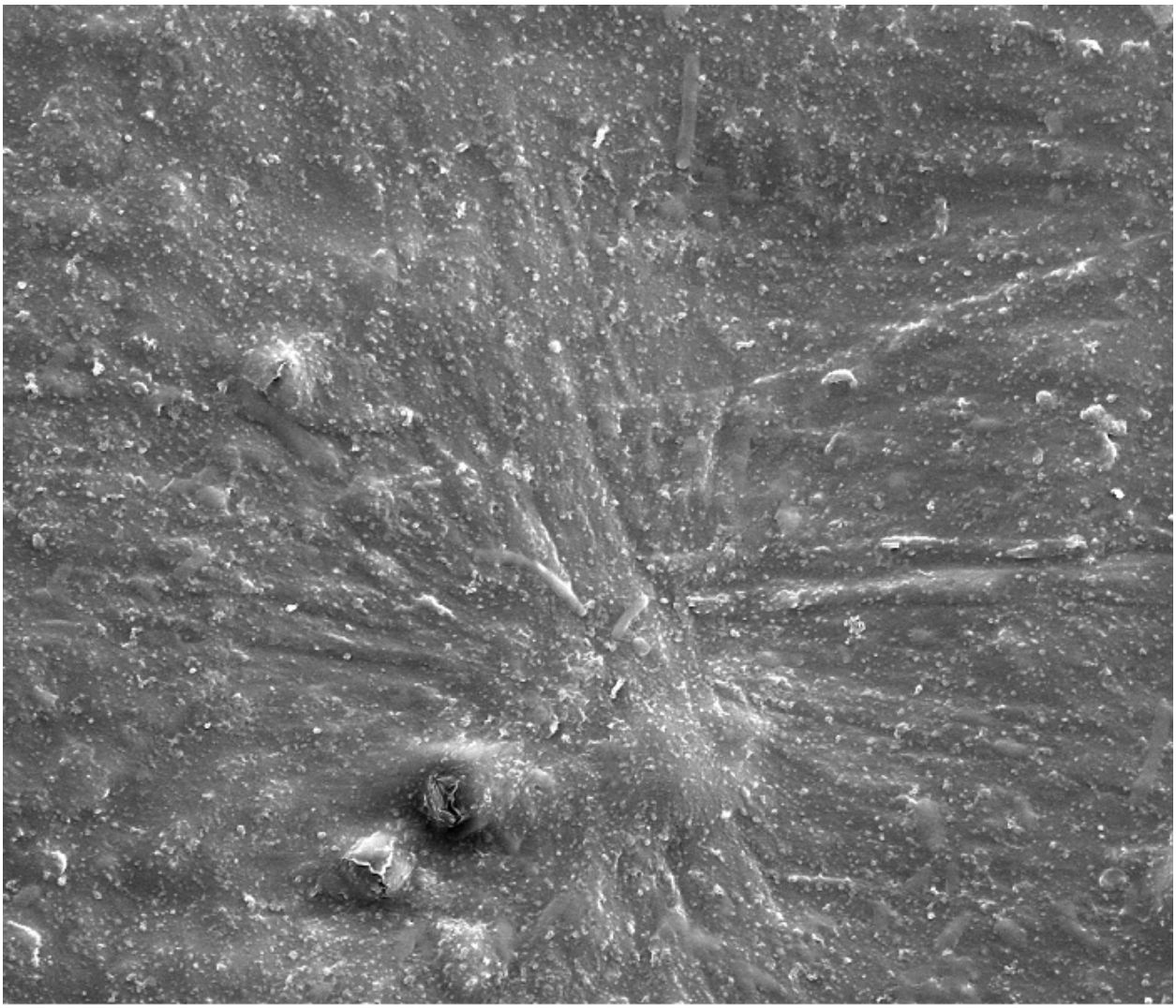
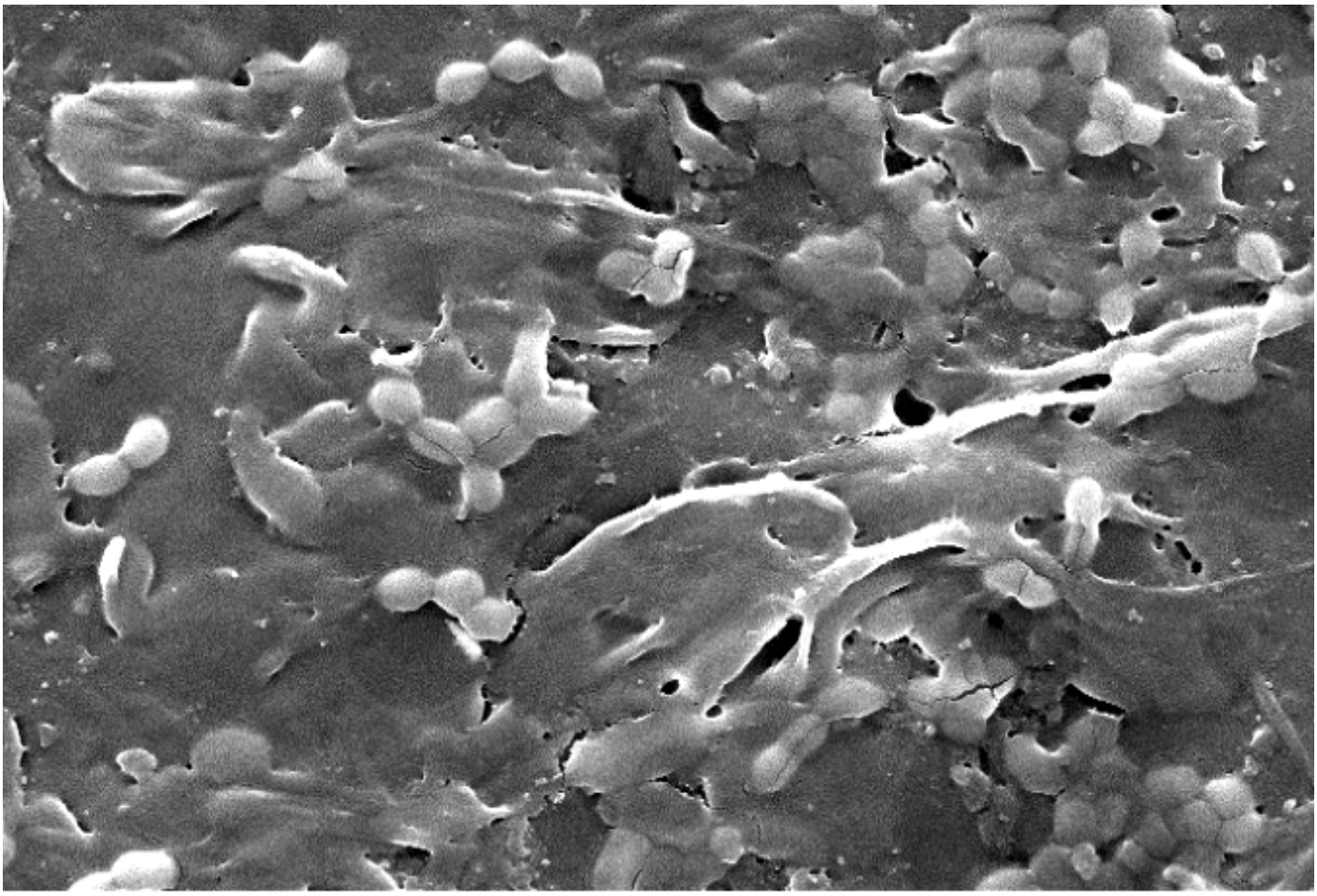
Table 1- BAX PCR Results – Study II

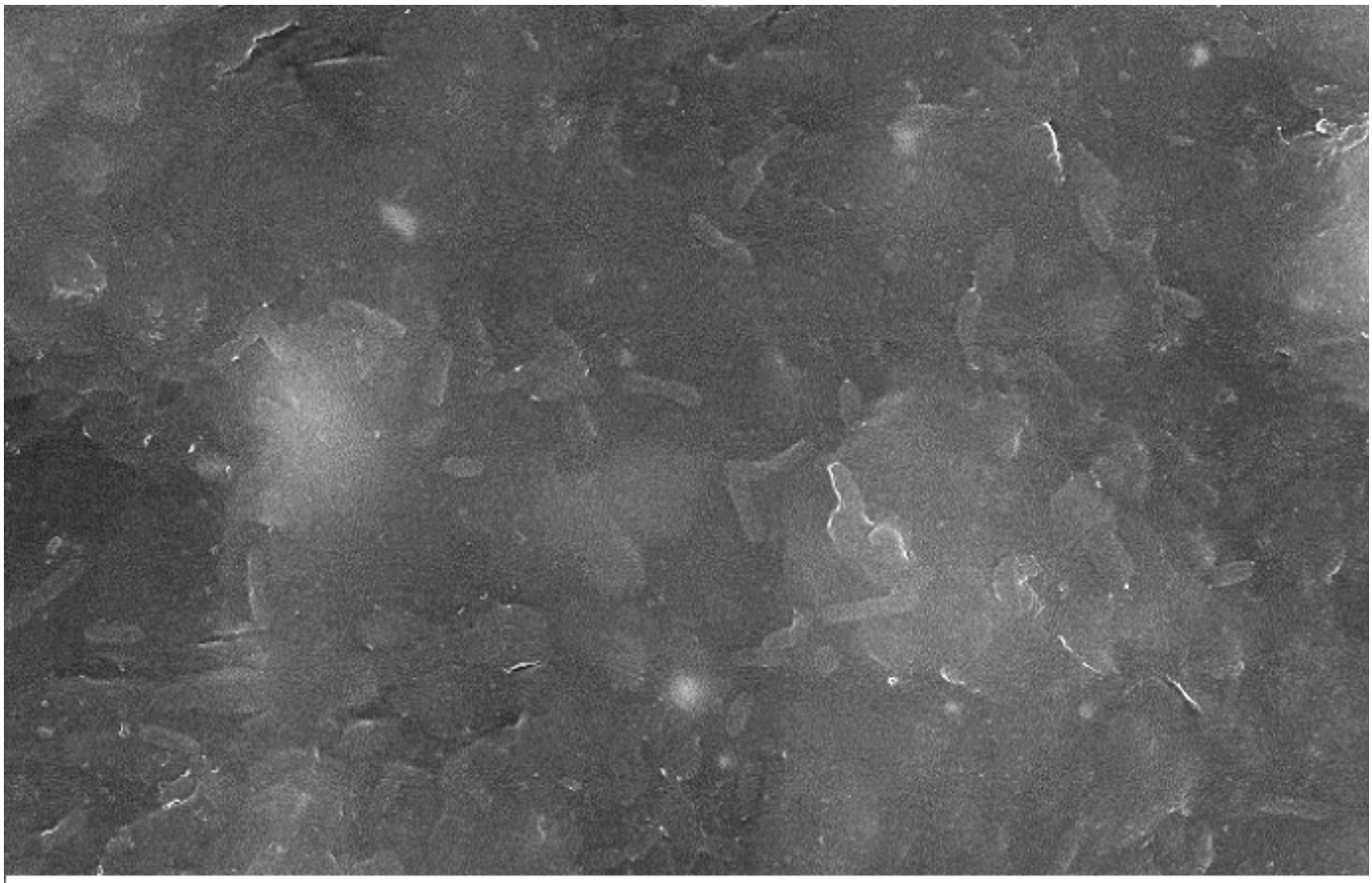
Treatment: **n=5**	Salmonella Result
Treatment 1: RPC 1 to RPC 5	POSITIVE
Treatment 2: RPC 1 to RPC 5	POSITIVE
Treatment 3: RPC 1 to RPC 5	POSITIVE
Treatment 4: RPC 1 to RPC 5	POSITIVE
Treatment 5: RPC 1 to RPC 5	POSITIVE
Treatment 6: RPC 1 to RPC 5	POSITIVE



Salmonella Biofilm on RPC
Study 1



E. coli O157:H7 Biofilm on RPC
Study 1



Listeria monocytogenes Biofilm
Study 1

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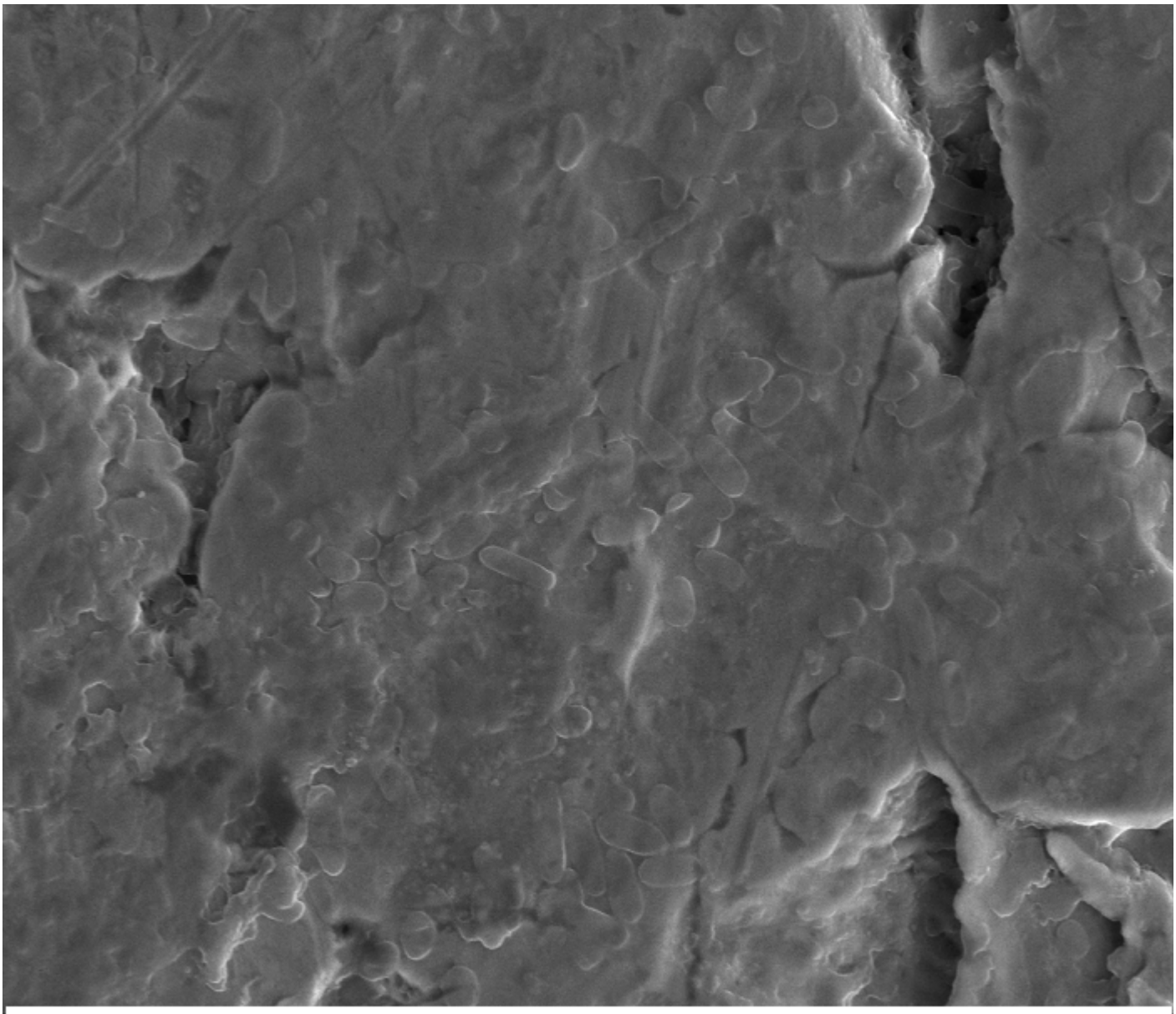
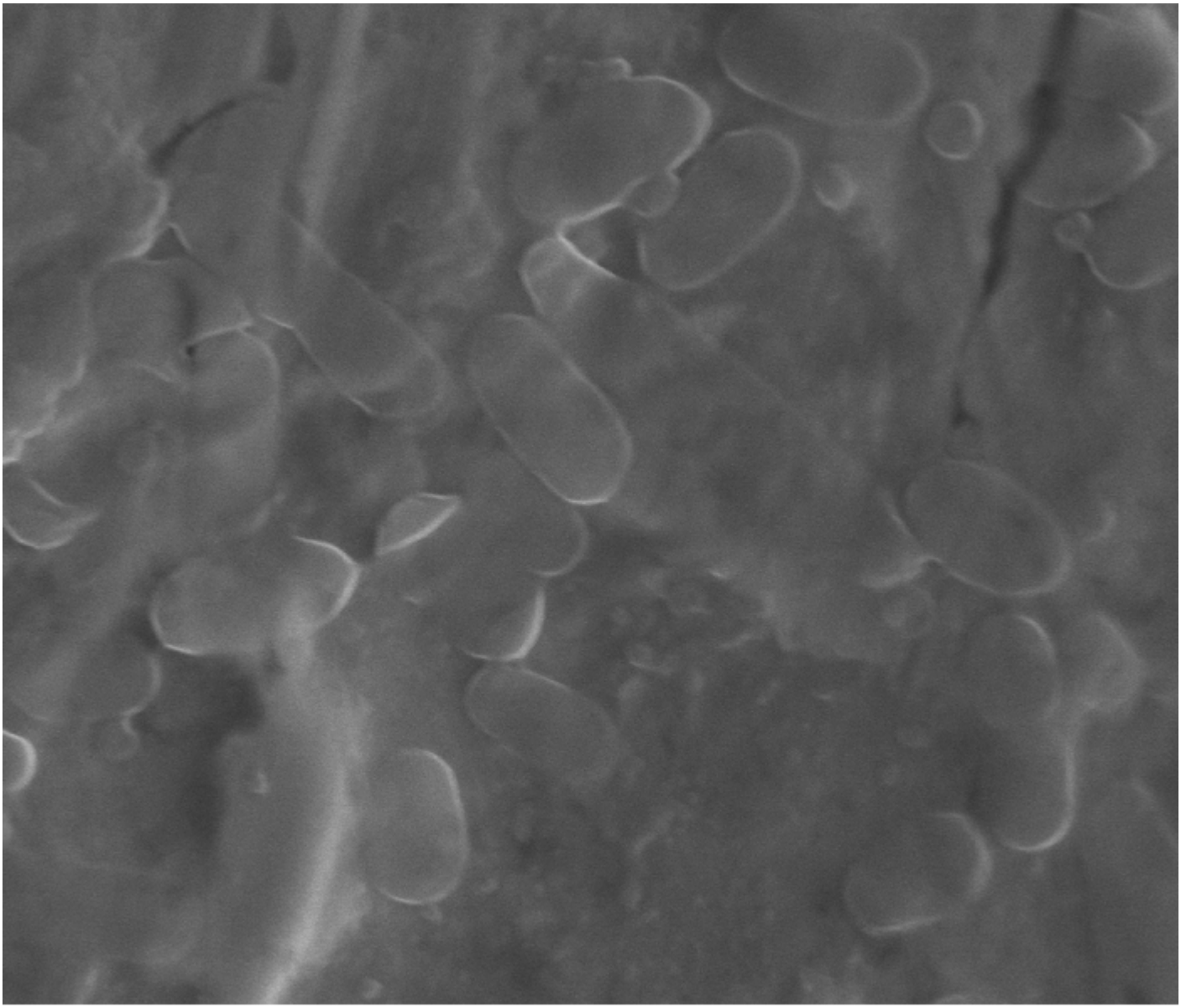
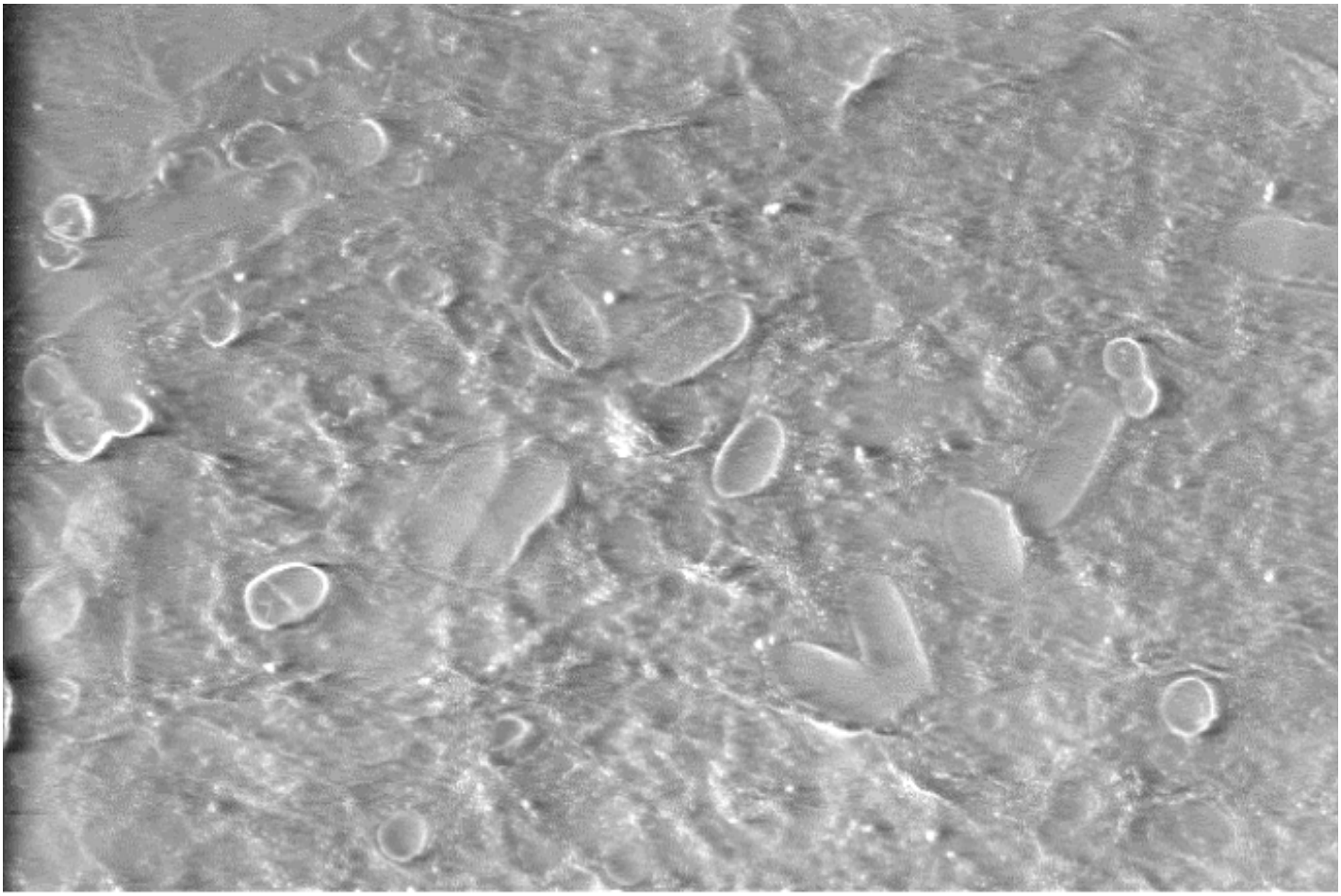


Salmonella Biofilm on RPC - Study 2
Treatment 1: Hot Water + Alkaline Detergent

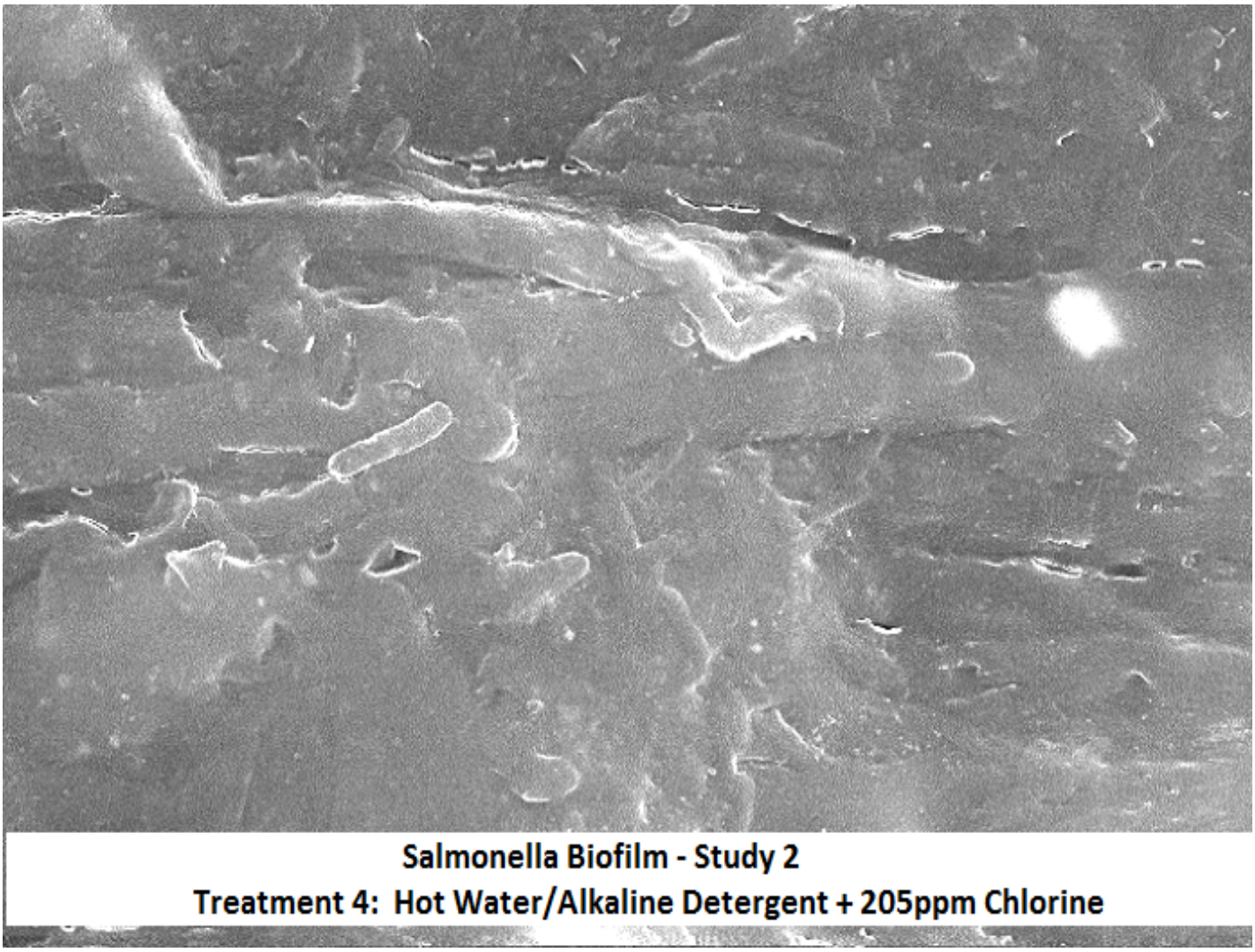


Salmonella Biofilm - Study 2

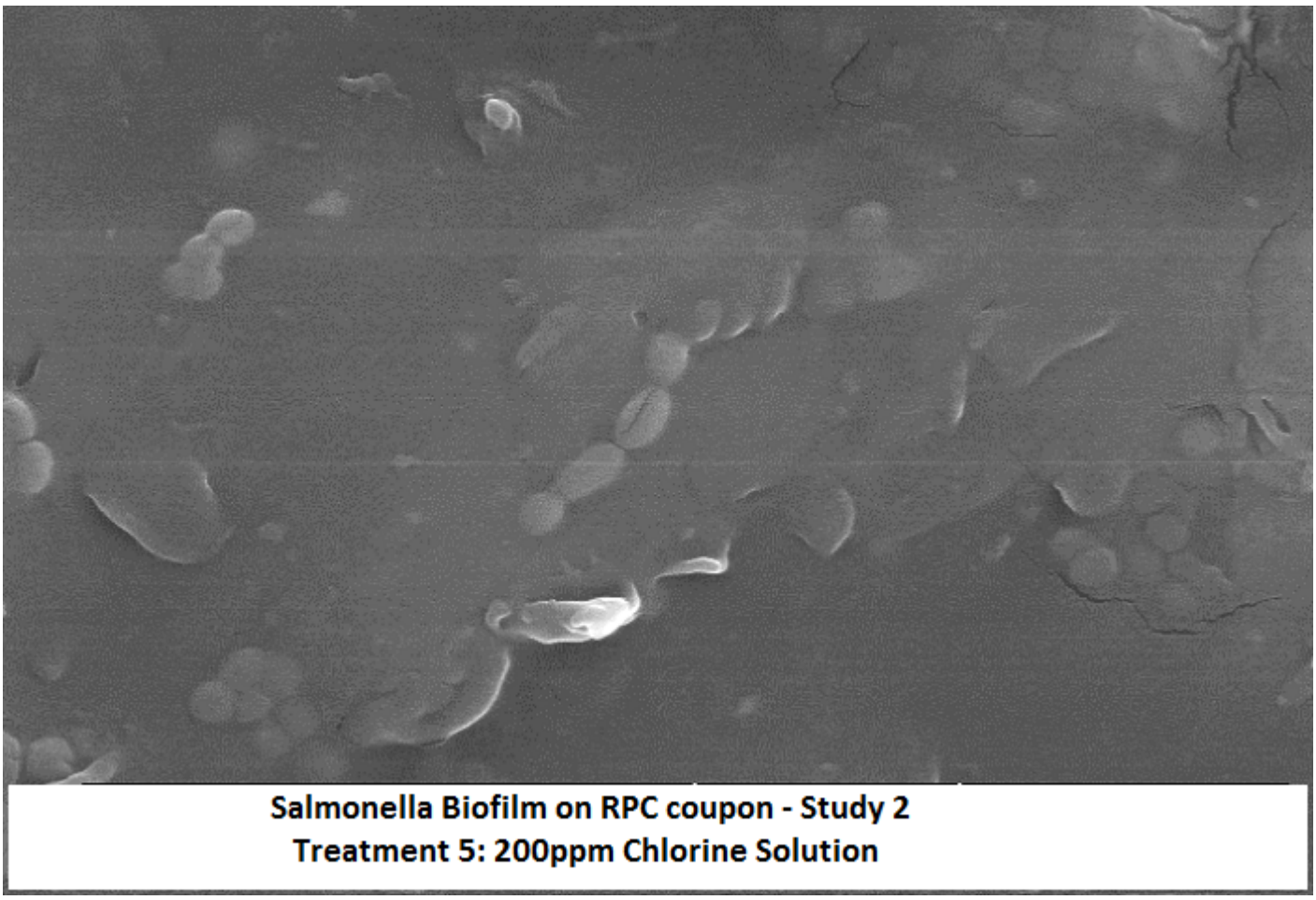
Treatment 2: Hot Water/Alkaline Detergent + Quaternary Ammonium



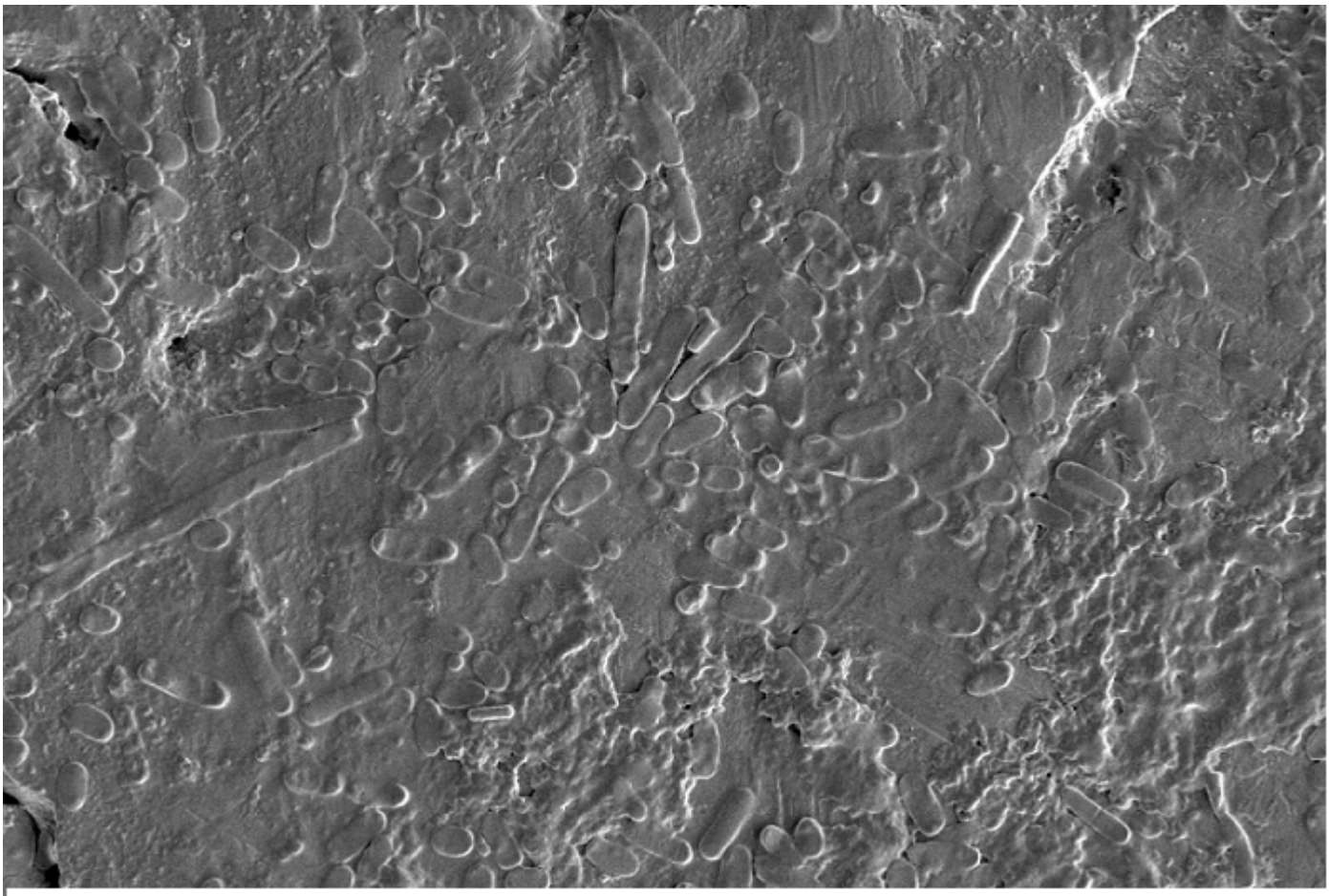
Salmonella Biofilm - Study 2
Treatment 3: Quaternary Ammonium



Salmonella Biofilm - Study 2
Treatment 4: Hot Water/Alkaline Detergent + 205ppm Chlorine



Salmonella Biofilm on RPC coupon - Study 2
Treatment 5: 200ppm Chlorine Solution



Salmonella Biofilm - Study 2
Treatment 6: Untreated Control

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