Assessing the Potential of Reusable Plastic Containers (RPC) to Harbor and Transfer Microbial Loads

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ASSESSING THE POTENTIAL OF RPCs TO HARBOUR AND TRANSFER MICROBIAL LOADS

ARCADIS U.S., Inc. (ARCADIS) performed a third-party review of a program established to assess the potential of reusable plastic containers (RPCs) to harbor significant microbial loads upon reuse.

1. Background

RPCs can be used to store, ship and display food products (primarily produce and poultry). After initial use, RPCs require processes (washing, sanitization, drying, and storage) and chemicals (detergents and sanitizing agents) to maintain the cleanliness of the containers for the shipping and storage of product. Normal use conditions may lead to microbial contamination of, and biofilm establishment on the RPC (Rappusas and Rolle, 2009; Fibre Box Association, 2013). The contamination may result from exposure to environmental conditions or contaminated product. Standard cleaning and sanitization procedures that are not well controlled or not sufficiently rigorous to address the presence of biofilms could result in RPCs with a significant microbiological carryover upon reuse.

Publically available literature and an available field study indicate that bacteria are not only capable of attaching and remaining on surfaces as a biofilm, but that these biofilms may transfer organisms to and from things that they come in contact with, including product. The document, Management of reusable plastic crates in fresh produce supply chains: A technical guide (Rappusas and Rolle, 2009), states that, “Plastic crates must, be appropriately managed and maintained in order to avert any risks associated with their use. Once infected, disease can spread to healthy produce as well as to the contact surfaces of plastic crates.” It further states that, “Proper physical and hygienic management of plastic crates is equally important in order to safeguard against chemical, physical and microbiological risks.”

A recently performed RPC field sanitation study (Warriner, 2013), indicated that, “…it was evident that the sanitary status of the containers was dependent on the batch tested.” The study further concluded that since, “…there was minimal contact of the containers with the workers it can be concluded that the RPC were insufficiently cleaned prior to delivery to the Growers.” Researchers, including Dr. Michelle Danyluk of the University of Florida have investigated the potential transfer of the organisms incorporated within biofilms present on food contact surfaces. Dr. Danyluk investigated the bio-transfer potential of organisms to transfer from tomatoes to shipping cartons and from shipping cartons to produce. Her research showed that under no conditions was the transfer of Salmonella spp. to and from product greater than when used, soiled cartons, were evaluated. She states, “…reused tomato cartons may be a source of contamination for subsequent tomatoes packed in these cartons.” (Danyluk, 2012)
2. Testing Program Review

A testing program was established to test the following hypotheses:

1. Biofilms can form on RPCs used in the food packaging industry;

2. Commercial (unknown) methods for the cleaning and sanitation of RPCs are insufficient to provide clean RPCs to the grower/shipper; and

3. Common (known) methods used for the cleaning and sanitation of RPCs are insufficient to remove bacterial biofilms from RPCs.

The program incorporated both field and bench scale studies, with all testing performed by independent investigators. The field sampling and testing protocols were developed by Dr. Trever Suslow of the University of California – Davis and performed by Primus Labs in Santa Maria, California. The bench scale testing, three unique studies, were established by Dr. Steven Ricke of the University of Arkansas and performed by WBA Analytical Laboratories in Springbrook, Arkansas.

The field study provides a data-based informed view of the cleanliness of RPCs used for the packing and transport of fresh produce by evaluating the presence of Coliforms and Enterobacteriaceae on RPCs used in the United States and cleaned via commercial methods. These bacteria are often evaluated to indicate the evidence of poor hygiene or inadequate processing (especially heat-treatment), process failure and post-process contamination of foods or food contact materials (ILSI Europe, 2011). These indicator organisms may signal an increased likelihood of a pathogen originating from the same source as the indicator organism being present; thus they serve a predictive function (Kornacki et al., 2013). It is further noted that based on historical testing of food and food service environmental samples, the absence of these indicator organisms does not denote the absence of enteric pathogens such as E. coli or Salmonella spp. (Kornacki et al., 2013).

The bench scale studies were performed to provide information and laboratory data to show that bacterial contamination can occur on the RPCs and to evaluate the effectiveness of common (known) sanitization methods used for the cleaning of RPCs.

\[1\] E.coli falls within the Coliform group and Salmonella spp. reside within the family of Enterobacteriaceae.
The relationship between these organism types as well as potential pathogenic organisms that are known members of these groups are noted below.

**Enterobacteriaceae**
- includes *Salmonella* spp.

**Coliforms**
- includes *E.coli*

Figure 1: Test organisms and their relationship with known human health pathogens.
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3. Field Study Review

A field study to provide a more definitive understanding of the contamination of RPCs in the United States was designed by Dr. Trevor Suslow at the University of California – Davis (in conjunction with Primus Labs) and carried out in June, 2014. The sampling and testing protocols for the field study dated May, 2014 (Appendices A and B, respectively) were reviewed. The stated objective of the field study, per the draft protocol, Assessment of General RPC Cleanliness As Delivered for Use in Packing and Distributing Fresh Produce, dated 25 April, 2014 (Appendix C) was to assess the typical microbiological cleanliness and quantitative bacterial profile of a pool of RPCs at the time of delivery to a distribution facility or directly to a harvest location or packing operation. The protocols specify the collection of both at random and for cause RPC samples for evaluation; the samples were evaluated for the presence of the indicator organisms Coliforms and Enterobacteriaceae.

The presence of these indicator organisms are often used to signal the increased likelihood of a pathogen originating from the same source as the indicator organism being present, thus they serve a predictive function. Further, based on historical testing of food and food service environmental samples, the absence of these indicator organisms does not denote the absence of enteric pathogens such as E. coli or Salmonella spp. (Komacki, 2013).

The field study was performed to test the following hypothesis:

- Commercial (unknown) methods for the cleaning and sanitation of RPCs are insufficient to provide clean RPCs to the grower/shipper resulting in containers of varying sanitary status being used for the storage and shipment of food.

3.1 Field Study Sampling and Testing Protocol Review

The field study protocols take into account sample type/size (24 – at random and 10 - for cause RPC samples) per sampling occurrence. The protocol specified the following: sample custody (use of Good Laboratory Practices (GLP)), sample preparation (aseptic technique), sampling method (premoistened

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2 One sample set was also collected on May 30, 2014; however, due to shortcomings at the bacterial testing stage, these data were not included in ARCADIS’ evaluation of the dataset.

3 For cause samples were identified during de-palletizing based on observed adhering soil, plant material, visible dried residues, or other elements (including old labeling) that differentiate these units from random samples chosen at the sampling point.

4 Due to the potential implications of the results, the field study did not include testing for specific pathogens; however it is noted that E.coli O-157 falls within the Coliform group and Salmonella spp. reside within the family of Enterobacteriaceae.
swabs), sample processing, testing and enumeration (standard methods), and documentation (digital imaging and double blind sample coding). Triplicate microbial analysis of each swab sample collected was performed.

The data derived from the field testing provides the necessary data to evaluate the hypothesis presented; the study sampling and testing protocols and the final reports were critically reviewed to assess the rigor in which the field study was carried out.

1. Sampling site/location: Sampling occurred at a singular packing facility over the course of two weeks. The test report specified the sampling date and specific pallet identification for each pallet where samples were taken. Each pallet identification included a prefix of IFCO followed by pallet-specific numeric digits.

2. Sample type/size: Twenty–four (24) at random and ten (10) for cause samples were taken per sampling date. The at random samples were collected from four different pallets on each sampling date, with samples being collected from the top, middle and bottom of the pallets. This sample size was assessed by the primary researcher as sufficient to provide statistically significant results. For cause samples were those that exhibited visible soil.

3. Sample custody: Samples were handled according to standard GLP chain of custody technique. The incorporation of this process ensures sample integrity and identity.

4. Sample documentation: Each RPC chosen for cause sampling was recorded using a specific sample code to identify sampling date and pallet as well as by digital imaging of the visibly soiled area of the RPC. At random RPCs were chosen without consideration given to the presence or absence of visible soil. Samples were taken from the top, middle and bottom of 4 pallets. At random RPCs were coded using similar sample coding as for cause RPCs, but no digital imaging of these samples were performed.
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Examples of a digital image from a for cause sample is presented in Figure 2.

Figure 2: Digital Images of a for cause sample

5. Sampling method: The study protocol indicates that the sampling of the RPCs was performed using aseptic technique including sampling area preparation and techniques to minimize contamination between samples. Three swab samples were collected from each for cause RPC, one of the visibly soiled area, one of the bottom of the RPC and one of the sides and hinges of the RPC. Two swab samples were collected from each RPC chosen at random; one of the bottom of the RPC and one of the sides and hinges of the RPC.

Note: The actual surface area sampled was not provided. As such, results were evaluated on a per sample or per RPC basis, as appropriate.

6. Testing Protocol: All swab samples were processed by the laboratory using standard quantitative microbiological methods for the indicator organisms Enterobacteriaceae and Coliforms.

Testing was performed on the Butterfield’s phosphate buffered saline or Letheen broth extracts of the swab samples. The use of standard methods allowed for the identification of viable vs. non-viable organisms.

3.2 Field Study Test Result Review

Data gathered from the field testing were provided as raw tabular data and digital images from Primus Laboratories. The average data from each swab sample were evaluated to identify trends associated with the presence of the organisms evaluated. Pass/fail levels were established at 1000 (3 log) bacterial colony forming units (CFU)/ per sample or RPC. This level is not only consistent with common food safety standards, but is the same as that used in the Warriner study reference titled, Microbiological Standards for Reusable Plastic Containers within Produce Grower Facilities for testing performed using similar standard
methods. In the study, Dr. Warriner also noted that a 20% failure rate is generally deemed acceptable in the food service industry (Warriner, 2013).

The study results are detailed:

- On a per swab sample basis, considering that multiple swabs were taken from each RPC (Table 1); and
- On a per RPC basis to assess organism loads taking into account results from all swabs from a singular RPC (Table 2).

Additional details regarding levels of each of the indicator organisms (Coliforms and/or Enterobacteriaceae) detected are also presented as part of the overall evaluation.

Table 1: Field Study Results per swab sample

<table>
<thead>
<tr>
<th>Type of Analysis</th>
<th>Number of swab samples analyzed</th>
<th>Highest CFU / swab sample*</th>
<th>Number of RPCs with one or more swab samples ≥ pass/fail criteria</th>
<th>Percentage of RPC with one or more swab samples ≥ pass/fail criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>at random</td>
<td>240</td>
<td>500,000</td>
<td>39/120</td>
<td>33%</td>
</tr>
<tr>
<td>at random**</td>
<td>240</td>
<td>170,000</td>
<td>37/120</td>
<td>31%</td>
</tr>
<tr>
<td>for cause***</td>
<td>150</td>
<td>4,000,000</td>
<td>25/50</td>
<td>50%</td>
</tr>
</tbody>
</table>

* Numbers of both Coliforms and Enterobacteriaceae were considered in the assignment of the highest CFU/swab sample.

** Values normalized to 12 x 12 in area (930 cm²)

*** “for cause” values were not normalized as the soiled areas were not reported

Table 2: Field Study results per RPC

<table>
<thead>
<tr>
<th>Type of Analysis</th>
<th>Number of RPCs analyzed</th>
<th>Highest CFU/RPC</th>
<th>Number of RPCs with a CFU/RPC ≥ pass/fail criteria</th>
<th>Percentage of RPC with one or more samples ≥ pass/fail criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>at random</td>
<td>120</td>
<td>290,000</td>
<td>36/120</td>
<td>30%</td>
</tr>
<tr>
<td>for cause</td>
<td>50</td>
<td>1,400,000</td>
<td>21/50</td>
<td>42%</td>
</tr>
</tbody>
</table>

* Numbers of both Coliforms and Enterobacteriaceae were considered in the assignment of the highest CFU/sample.

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5 Results represent the bacterial levels present on individual swab samples of the RPCs. Two samples swabs were collected per each at random RPC, while three samples swabs were collected per each for cause RPC.

6 Results represent the average bacterial levels from the total number of swab samples taken per RPC.
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1. *for cause* RPC data

In the evaluation of the *for cause* samples, RPC sampling included the sampling of the visibly soiled area, the sides (with hinges) and bottom of the RPC (3 unique samples per RPC).

- As specified in Table 1, twenty-five of the fifty RPCs (50%) swab samples from *for cause* RPCs collected over the five testing dates contained levels of bacteria greater than 1000 CFU/sample. The level of bacteria ranged from <1 CFU/swab sample to as high as 4,000,000 Coliforms CFU/swab sample and as high as 1,500,000 *Enterobacteriaceae* CFU/swab sample.

- As specified in Table 2, twenty-one of the fifty RPC (42%) of the *for cause* RPCs sampled over the five testing dates contained average levels of bacteria (based on two samples per RPC) greater than 1000 CFU/RPC. The average level of bacteria per RPC ranged from <1 CFU/swab sample to as high as 1,355,556 Coliforms CFU/RPC and as high as 500,007 *Enterobacteriaceae* CFU/RPC.

- Further analysis of the *for cause* data indicate that:
  - The visibly soiled area of the RPC did not contain the largest number of bacteria on three of the five sampling dates.
  - The largest number of Coliforms identified on a single RPC swab sample was from an area not visibly soiled.
  - The largest number of *Enterobacteriaceae* identified in a single RPC swab sample was from an area not visibly soiled.

2. *at random* RPC data

In the evaluation of the *at random* samples, RPC sampling included the sampling the sides (with hinges) and bottom of the RPC (2 unique samples per RPC).

- As specified in Table 1, thirty-nine of the 120 RPCs (33%) evaluated *at random* over the five sampling dates had one or more samples that exceeded the 1000 CFU/sample fail rate. The level of bacteria detected from the samples from RPCs selected *at random* ranged from <1 CFU/swab sample to as high as 500,000 CFU/swab sample and as high as 24,667 *Enterobacteriaceae* CFU/swab sample.

- As specified in Table 2, thirty-six of the 120 RPCs (30%) selected *at random* over the five testing dates contained average levels of bacteria (based on two samples per RPC) greater than 1000 CFU/RPC. The average level of bacteria per RPC ranged from <1 CFU/swab sample to as high as 288,334 Coliforms CFU/RPC and as high as 12,339 *Enterobacteriaceae* CFU/RPC.

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7 The fail rate represents the levels of bacteria identified in the evaluation of either Coliforms or *Enterobacteriaceae*. When levels of both organism types exceeded the pass/fail criteria, these failures were only counted a singular time.
4. Bench Scale Studies Review

In preparation for the planned field study, bench scale screening studies were performed. The bench scale study reports were reviewed independently to confirm that they were carried out in accordance with current scientific principles and that the data supported the conclusions developed. The reports reviewed are included as Appendices D, E, and F to this document.

Subsequent to assessing the individual studies and the thoroughness of the reports, the weight of evidence of the bench scale dataset from all three reports was evaluated to ascertain if it would adequately support the hypotheses presented and allow for the program to move to the field testing stage.

4.1 Study #1: WBA Analytical Laboratories, RESEARCH PLANNING (Study I). Study Completion Date: April 28, 2014

The objective of the study was to assess the ability of *Salmonella* spp. (mix of five strains), *Listeria monocytogenes* (mix of 5 strains), or *E. coli* O157:H7 (single non-toxin producing strain) to adhere to and produce bacterial biofilms on RPC coupons. In this study, 1 in\(^2\) sections cut from commercially available RPCs were exposed to three different bacterial cocktails in logarithmic growth phase for 18-24 hours at 35°C.\(^8\)

Based on a scanning electron microscopy (SEM) analysis of the post-exposure RPC coupons, it was determined that biofilms were present. Moreover, the micrographs were consistent with the organisms present in the bacterial cocktail to which the RPC coupons were exposed.

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\(^8\) The 1 in\(^2\) sections of RPC used for testing were identified in the study reports as *coupons*. 

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Figure 3: SEM photomicrographs of RPC post bacterial exposure
4.2 Study #2: WBA Analytical Laboratories, RESEARCH PLANNING (Study II). Study Completion Date: May 8, 2014

The objective of the study was to determine if standard RPC cleaning / sanitizing procedures would disrupt and eliminate *Salmonella spp.* biofilms on RPCs. In this study, 36 coupons were exposed to a cocktail of *Salmonella spp.* in logarithmic growth phase for 18-24 hours at 35°C to establish a biofilm on the coupons. The coupons were subsequently treated with a variety of chemical treatments that could potentially be used for the sanitization of RPCs. Six coupons were left untreated as a control. The test groups included:

1. Hot Water (123.5°F (51°C)) + Alkaline Detergent (6 coupons)
2. Hot Water (123.5°F(51°C)) + Alkaline Detergent + 250 ppm Quaternary Ammonium (6 coupons)
3. 250 ppm Quaternary Ammonium (6 coupons)
4. Hot Water (123.5°F(51°C)) + Alkaline Detergent + 200 ppm Chlorine Solution (6 coupons)
5. 200 ppm Chlorine Solution (6 coupons)
6. Untreated control (6 coupons)

Note: In the test groups above, in which hot water was used, water pressure used was not directly measured; however, water flow was set at full force.

Five coupons from each test group were evaluated after cleaning for the presence of *Salmonella spp.* using the Du Pont BAX® System while one coupon was assessed after cleaning using SEM for the presence of a biofilm.

The SEM analysis showed that none of the cleaning / sanitation systems used were able to completely remove the *Salmonella spp.* biofilm from the RPCs.
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Figure 4: SEM photomicrographs of RPC coupons post cleaning/sanitization
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The Du Pont BAX® System analysis of the peptone broth used to rinse the RPCs indicated the presence of *Salmonella* spp. in all samples. Based on these results, it was concluded that none of the cleaning / sanitation methods evaluated were able to completely remove the bacterial biofilm from the RPCs.

4.3 Study #3 – WBA Analytical Laboratories, RESEARCH PLANNING (Study III). Study Completion Date: May 8, 2014

In the third study, the effects of scrubbing action (physical removal effects) on *Salmonella* spp. biofilms on RPC coupons were evaluated. In this study, five RPC coupons were exposed to a *Salmonella* spp. cocktail in a logarithmic growth phase for 18-24 hours at 35°C to establish a biofilm. These five RPCs were then swabbed three times to mimic a scrubbing action. After the swabbing, samples of the cleaned RPCs, and swabs used for the cleaning were evaluated for the presence of *Salmonella* spp. by the Du Pont BAX® System. Each sample tested positive indicating that although the swabs were successful in removing some *Salmonella* spp. from the RPC coupons, organisms remained on the RPC coupons even after repeated scrubbing.

Taken as a whole, the three bench scale studies confirm that:

- Biofilms of various bacterial species can and do form on RPCs used for food storage and transport; and
- Common (known) methods used for the cleaning and sanitizing (both chemical and physical) of RPCs are insufficient to remove biofilms in a laboratory setting.

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9 Although the DuPont BAX® system identifies DNA from both viable and non-viable organisms, based on the unlikelihood that intact DNA from non-viable organisms would be present post cleaning/sanitation, the positive result was determined to be indicative of the presence of viable organisms. This was based on laboratory experience and information provided by the system developer.
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5. Pathogen Transfer Risk

Recent bench and field testing showed that reusable plastic containers (RPCs) are often colonized by high levels of various microbial populations and that pathogenic organisms such as *Listeria monocytogenes*, *Salmonella spp.* and *E.coli* can and do form biofilms on RPCs. As a natural outcropping of this work, the ability of the organisms present within biofilms to transfer to other surfaces they contact was further evaluated. The presence of biofilms was determined to be a relevant risk factor due to the potential contamination of food products by pathogenic and/or spoilage organisms (Winkelströter, 2013). Further, biofilms have been identified as playing an important role in the dissemination of microorganisms in food processing, packing and shipping environments (Srey, 2013; Winkelströter, 2013). Based on previous studies of biofilms on food contact surfaces, organisms contained within a biofilm matrix have the capacity to transfer from produce via contact to packaging surfaces, and those organisms may subsequently transfer from the packaging surface upon reuse to uncontaminated fresh food product.

Publically available peer-reviewed work detailing the variables associated with biofilm formation was identified; it provides information which must be considered in biofilm formation including the organism and strain (and if it is present as a pure culture, which is unlikely in the natural environment), environmental conditions (i.e., moisture, pH, and temperature), substrate (i.e., stainless steel, polyethylene, etc.) and time. Together these factors determine the likelihood of biofilm formation as well as the potential for organisms to detach from the developed biofilm and contaminate other surfaces. This ability to transfer microorganisms through contact is termed the bio-transfer potential. This potential transfer rate can be quantified by the calculation of the transfer coefficient (Danyluk, 2012). The transfer coefficient is calculated using the following equation:

\[
TC = \frac{P_G}{P_c}
\]

TC = Transfer Coefficient  
\(P_G\) = the organism population on the previously uncontaminated surface.  
\(P_c\) = the organism population on the initial surface source of the pathogen.

The TC quantifies the ability of known biofilm to detach and contaminate a new surface. The higher the transfer coefficient, the more readily organisms can be removed to effect a previously uncontaminated surface. Although many papers were reviewed, for this summary, two papers that focus on organisms and surfaces of interest to the food industry have been summarized; it is felt that these two papers not only provide insights into biofilm formation, but also the bio-transfer potential of common pathogens as they relate to common surfaces in the food industry including single use, reused and soiled packaging.\(^\text{10}\)

\(^{10}\) Dirt consisted of 250 grams of standard tomato debris from the locular cavity, and 50 g of sandy soil. This mixture was placed in a food processor for 1 minute. A standard quantity of the debris was painted onto used carton and allowed to dry overnight.
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Based on the high use level of stainless steel in the food industry and the potential for Listeria contamination, much work has been done with this organism and this surface. The biofilm transfer potential of L. monocytogenes and stainless steel (SS) was evaluated in a study by Mattos de Oliveira, et al. (2010). In this study, organism adherence on food grade SS began to occur as rapidly as 3 hours after exposure. Microbial adhesion occurs generally in two stages: reversible and irreversible adhesion. Mattos de Oliveira et al. (2010) found that the initial adhesion capacity of L. monocytogenes onto SS was 58.75% (+/-0.90%) after 3 hours, with mature biofilms present after 240 hours. The time required for attachment and maturation of a biofilm are highly dependent on the specific serovar of the organism(s) involved, the contact surface and the environmental conditions present. Other studies have shown that L. monocytogenes will adhere to SS in as quickly as 20 minutes (or as long as four hours) at a broad range of temperatures (Hood and Zottola, 1995; Smoot and Pierson, 1998).

Mature biofilms consist of microcolonies within a matrix of extracellular polymeric substances; this matrix is responsible for the structure, cohesion and functional integrity of the biofilm. The heterogeneous composition of the polymeric substances that make up the matrix confer a level of resistance to common sanitization procedures, making the biofilm difficult to remove and the organisms difficult to eliminate. Mattos de Oliviera et al. (2010) note that high levels of L. monocytogenes (>9 Log CFU/mL) were readily removed from SS at a consistent level after 96 hours of exposure, indicating that the biofilm may be sloughing off resulting in a potential for more widespread microbial transfer. It was further stated that biofilm development and transfer could be attributed to adequate conditions for growth (nutrients and environmental conditions). This bio-transfer represents the concern associated with the presence of biofilms on food contact surfaces.

In a Center for Produce Safety study, the risk factors associated with Salmonella spp. and tomato packaging operations were assessed (Danyluk, 2012). One aspect of this study is of particular interest to the evaluation of single use vs. multi-use containers. Dr. Danyluk evaluated new, used, and soiled cartons to assess the bio-transfer potential of these different substrates (clean cartons vs used, soiled cartons). In the study, under no conditions, was the transfer of Salmonella spp. greater than those of used dirty cartons. To quantify the bio-transfer potential of Salmonella spp. to and from single use, and used, dirty cartons, the transfer coefficients as determined by Dr. Danyluk’s research are presented in Tables 3 and 4 (Danyluk, 2014).
### Table 3: Bio-transfer potential between Cartons and Red tomatoes (n=10)

<table>
<thead>
<tr>
<th>Transfer Coefficient</th>
<th>Inoculated Carton to Tomato</th>
<th>Inoculated Tomato to Carton</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12°C 25°C 12°C 25°C</td>
<td>12°C 25°C 12°C 25°C</td>
</tr>
<tr>
<td>Organism Inoculum</td>
<td>0 d 1 d 7 d 0 d 1 d 7 d</td>
<td>0 d 1 d 7 d 0 d 1 d 7 d</td>
</tr>
<tr>
<td>Drying</td>
<td>Single use Cartons</td>
<td>Used Soiled Cartons</td>
</tr>
<tr>
<td>Wet (immediate)</td>
<td>0.2 0.1 0.3 0.2 0.2 0.1</td>
<td>0.5 0.1 0 0.5 0.2 0 0.7 1.0 2.0 0.7 0.6 1.0</td>
</tr>
<tr>
<td>1 hour</td>
<td>0.2 0.1 0.1 0.2 0.1 1.0</td>
<td>0 0 0 0 0 0.6 0.4 1.1 0.6 0.5 0.4</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.1 0.0 0.2 0.1 0.1 1.0</td>
<td>0 0 0 0 0 2.2 0 0 0.2 0 0</td>
</tr>
</tbody>
</table>

### Table 4: Bio-transfer potential between inoculated cartons and mature green tomatoes at different inoculum concentrations, temperatures and drying times (n=6)

<table>
<thead>
<tr>
<th>Transfer Coefficient</th>
<th>12°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 d 1 d 7 d</td>
<td>0 d 1 d 7 d</td>
</tr>
<tr>
<td>Organism Inoculum</td>
<td>8 log</td>
<td>5 log</td>
</tr>
<tr>
<td>Drying</td>
<td>Single Use Carton</td>
<td>Used Soiled Carton</td>
</tr>
<tr>
<td>8 log</td>
<td>0.3±0.1 0.0±0.0 4.5±8.8</td>
<td>0.0±0.0 0.0±0.0</td>
</tr>
<tr>
<td>5 log</td>
<td>0.5±0.2 25.1±43 0.3±0.1 0.2±0.1 0.6±0.5</td>
<td>0.8±0.7</td>
</tr>
<tr>
<td>8 log</td>
<td>0.3±0.1 1.0±0.5 4.8±4.6 3.3±0.7 1.0±0.5</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>5 log</td>
<td>0.3±0.0 175±112 0.3±0.6 2.2±0.2 3.3±3.0</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>
In her work, Danyluk also evaluated the effects of moisture on the bio-transfer rate. In her study, the bio-transfer rate was increased in the presence of moisture at initial inoculum, with the highest variability occurring after 1 hour of contact time. She postulated that this was likely due to variable moisture loads that may be representative of typical packing operations. Further, Danyluk also noted that the presence of debris on used cartons (especially if stored at cooler temperatures) actually increases the survival of *Salmonella spp.*, and that the organism can survive on cartons for as long as seven days even in the absence of debris. She notes, “...*reused tomato cartons may be a source of contamination for subsequent tomatoes packed in these cartons*” (Danyluk, 2012). When coupled with the presence of biofilms, typical sources of moisture found in the produce industry such as wet packing containers (Suslow, 2014), high humidity, flash cooling and misting at the retail level commonly would support the bio-transfer of organisms from established biofilms.

The bio-transfer rate is dependent on multi-variate inputs including the organism(s) and serovars present in the biofilm, contact surface, and environmental conditions (i.e., temperature, pH, moisture). These factors taken together affect the ability of biofilms to form on the surface and to detach and contaminate products and other surfaces with which they come into contact.
6. Summary

Public literature tells us that fresh produce can acquire microorganisms through cross-contamination of the contact surfaces of RPCs during storage and transport (Rappusas and Rolle, 2009). The document, Management of reusable plastic crates in fresh produce supply chains: A technical guide (Rappusas and Rolle, 2009), states that, “Plastic crates must be appropriately managed and maintained in order to avert any risks associated with their use. Once infected, disease can spread to healthy produce as well as to the contact surfaces of plastic crates.” It further states that, “Proper physical and hygienic management of plastic crates is equally important in order to safeguard against chemical, physical and microbiological risks.”

Subsequently, a recently performed RPC field sanitation study (Warriner, 2013), indicated that, “…it was evident that the sanitary status of the containers was dependent on the batch tested.” The study further concluded that since “…there was minimal contact of the containers with the workers it can be concluded that the RPC were insufficiently cleaned prior to delivery to the Growers.”

Researchers, including Dr. Michelle Danyluk of the University of Florida have investigated the potential transfer of the organisms incorporated within biofilms present on food contact surfaces. Dr. Danyluk investigated the bio-transfer potential of organism within a biofilm matrix to transfer from tomatoes to shipping cartons and from shipping cartons to produce. Her research showed that under no conditions was the transfer of Salmonella spp. to and from product greater than when used dirty cartons were evaluated. She states, “…reused tomato cartons may be a source of contamination for subsequent tomatoes packed in these cartons.” (Danyluk, 2012)

A testing program that included both bench and field testing was established to further expand the dataset and confirm the following hypotheses:

1. Biofilms can form on RPCs used in the food packaging industry;
2. Common (known) methods used for the cleaning and sanitation of RPCs are insufficient to completely remove bacterial biofilms from RPCs; and
3. Commercial (unknown) methods for the cleaning and sanitation of RPCs in the field are insufficient to provide clean RPCs to the grower/shipper, resulting in containers of varying sanitary status being used for the storage and shipment of food.

Field testing on RPCs currently in use for the storage and transportation of produce indicates that the RPCs have significant microbial loads after cleaning based on the presence of the indicator organisms evaluated (Coliforms and Enterobacteriaceae). Thirty-three percent (33%) of RPCs selected at random had one or more samples that exceeded the generally accepted pass-fail level of 1000 CFU/RPC, while 50% of RPCs selected for cause had samples where the pass/fail level was exceeded. This pass/fail level of 1000 (3 log) bacterial colony forming units (CFU)/ per sample or RPC is consistent with common food safety standards, and the same used by Dr. Keith Warriner in a similar study conducted in Canada using comparable methodology. When evaluated on an average number of CFU/RPC, thirty percent (30%) of RPCs sampled...
Assessing the Potential of RPCs to Harbor and Transfer Microbial Loads

at random had an average CFU/RPC that exceeded the pass-fail level of 1000 CFU/RPC, while 42% of RPCs selected for cause had average levels of organisms above the pass/fail level. Presence of the indicator organisms evaluated (Coliforms and Enterobacteriaceae) are often used to provide evidence of poor hygiene, inadequate processing or post-process contamination.

The data also show that the visibly soiled areas of for cause RPCs often did not contain the largest number of bacteria (Coliforms or Enterobacteriaceae) identified from the 3 samples collected form the RPC. This indicates that the presence of visible soil on an RPC may be indicative an overall contamination of the RPC and that simple removal of the soiled area may be insufficient to remove the microbial contamination.

The results of the field testing provide data showing the inability of current washing procedures to sufficiently remove the microbial load from RPCs and that residual microbial contamination on RPCs assessed post-cleaning (both for cause and at random) are above acceptable levels (>1000 CFU/RPC) for more than 20% of the RPCs evaluated.

It is noted that the actual microbial load present on the RPCs may have been underestimated in the field study as other organisms not residing within the Coliform or Enterobacteriaceae groupings, such as food spoilage organisms (e.g. yeast and molds) or the pathogen Listeria monocytogenes would not have been accounted for. Thus, the actual microbial load may be even higher.

Further, while the indicator organisms included in the field investigation (Coliforms and Enterobacteriaceae) are often used to signal the increased likelihood of a pathogen, the absence of these indicator organisms does not denote the absence of enteric pathogens such as E. coli or Salmonella spp. (Kornacki et al., 2013).

The review of the bench testing confirmed that RPCs are susceptible to the establishment of biofilms composed of common food-born bacterial pathogens. Further, these biofilms are not readily removed using common cleaning and sanitation practices.

Identified publically available research shows that biofilms can and do form on food and food contact surfaces and that the biofilm matrices often transfer bacteria to other substrates they come into contact with. Further, when used, soiled packaging materials come into contact with produce, they are more susceptible to biofilm formation and subsequent bio-transfer of the organisms present within those biofilms, than when new, clean containers are used.
7. References


RPC Study Sampling Protocol, May 8, 2014

RPC Testing Protocol, May 8, 2014


WBA Analytical Laboratories, RESEARCH PLANNING (Study I), April 28, 2014

WBA Analytical Laboratories, RESEARCH PLANNING (Study II), May 8, 2014

WBA Analytical Laboratories, RESEARCH PLANNING (Study III), May 8, 2014

Appendix A

RPC Study Sampling Protocol
Sampling Protocol for Trevor Suslow’s RPC Cleanliness Survey, May 2014

Sampling will be started in the month of May, 2014. Two types of samples will be taken from the 16 x 25 RPC size:

1. Random Sample - 6 units from 4 pallets.
   - Two samples from the upper region of each pallet
   - Two samples from the middle region of each pallet
   - Two samples from the lower region of each pallet
   - Samples will be selected at random. Do not select only visibly soiled or only visibly clean pallets. Visibly soiled pallets can be selected for sampling as a For Cause sample. Please see instructions below for instructions and classification.

2. For Cause Samples - 10 units per sampling session
   - Samples select For Cause will have visible soiling on the surface of the RPC.
   - Visible soiling is classified as any variety of organic matter (soil, dried and adhered plant matter, old labels, or other residues) present on the RPC
   - Digital documentation of “For Cause” soiling must be taken prior to sampling. The images must be labeled sequentially by date for the duration of the project.

Samples will be taken from the pallet area to a designated sampling area by an individual wearing disposable sterile gloves, using caution to only handle the RPC by the edges to reduce transfer of organisms. Gloves must be changed between samples and between removing exterior wrapping and corner braces of the pallet. This area will ideally be indoors, but if no such facilities exist, may occur in the back of a van or SUV with a plastic barrier to prevent dust interference. A small folding table or other platform capable of disinfection must be provided as sampling area.

Swab and Sponge Sampling Method:
- Prior to, and between each sample, the sampling platform described above must be sprayed with 20% bleach followed by 70% alcohol.
- A new sheet of butcher paper will then be laid out to cover the sampling platform. Paper must be changed between each sample.

For Random Samples:
1. Change gloves. Take one full area swab of the bottom (inside) of the random sample RPC using a hydrosponge. Use both side of the sponge and move in multiple directions to ensure proper sampling. Place sponge into a pre-labeled sample bag and immediately place in an insulated cooler maintained at approximately 10C or lower.
2. Change gloves. Take one full area swab of the side panels and hinges of the RPC using a hydrosponge. Use both side of the sponge and move in multiple directions to ensure proper sampling. Place sponge into a pre-
labeled sample bag and immediately place in an insulated cooler maintained at approximately 10°C or lower. Separate coolers are required for each pallet tested.

- For Cause Samples:
  1. Locate visibly soiled portion on RPC. Create ID for this RPC using sampling guide (suggestion: take picture of sample ID on paper prior to actual picture of soiled RPC). We must be able to determine which picture goes with each sample.
  2. Change gloves. Using a letheen swab, swab the entire surface of the visibly soiled area. Use all sides of the swab and move in multiple directions to ensure proper sampling. Place swab back into pre-labeled tube and immediately place swab in an insulated cooler maintained at approximately 10°C or lower.
  3. Change gloves. Take one full area swab of the bottom (inside) of the random sample RPC using a hydrosponge. Use both side of the sponge and move in multiple directions to ensure proper sampling. Place sponge into a pre-labeled sample bag and immediately place in an insulated cooler maintained at approximately 10°C or lower.
  4. Change gloves. Take one full area swab of the side panels and hinges of the RPC using a hydrosponge. Use both side of the sponge and move in multiple directions to ensure proper sampling. Place sponge into a pre-labeled sample bag and immediately place in an insulated cooler maintained at approximately 10°C or lower. A separate cooler to store the For cause samples is required.

- Fill out the custom chain of custody forms for each sample between sampling. Sample information must include the date and time, sampler initials, and identification code labels on the outside of the sample bags. For Cause samples should have the number of digital images takes for specified sample.

- If the samples are transferred in custody (to the lab, courier, or any other personnel,) the chain of custody must reflect the date and times of transfer as well as the temperature of the cooler. Couriers or lab personnel must check the temperature and add additional icepacks if deemed necessary to keep cooler under 10°C.

- Samples must be brought to the lab immediately after sampling completion for processing. Keep laboratory staff informed of delays or changes in sampling timing.

- Upon receipt, the laboratory staff must take the temperature of the cooler as well as 3 individual samples upon receipt. This information is to be recorded on the chain of custody along with sample IDs of the 3 samples which had temperatures recorded.
Appendix B

RPC Study Testing Protocol
Testing Protocol Based on Trevor Suslow's RPC Cleanliness Survey Study

Testing will be completed in the months of May-June 2014. Samples arriving at the laboratory must have Chain-of Custody (COC) filled out prior to processing. Analyst, media lot numbers, petrifilm lot numbers, incubators, scales and stomachers must be noted on the COC. The temperature of the cooler and 3 sample bags must be recorded upon receipt.

For hydrosponge samples:
- Add 25 mL of BFB (Butterfield’s Phosphate Buffer) directly to the hydrosponge.
- Place sample into the stomacher reflected on the COC. Stomach for 90 seconds.
- Plate sample in triplicate, 3 Enterobacteriaceae petrifilm and 3 TC petrifilm (for Fecal Coliform Analysis).
  1. If necessary, repeat for requested dilutions. Note: Dilutions may be determined necessary based on the preliminary set of results.
     Correspondence with the microbiologists and the team at IP is necessary to determine this information.
- Incubate Enterobacteriaceae petrifilm in accordance with the manufacturer's instructions. Incubate the TC petrifilm at 44.5C for 24 hours.
- After plating, sample is to be held at 2-8 degrees C to maintain integrity in the event of a dilution or concentration step is required.

For letheen swabs:
- Add 5 mLs of BFB and vortex thoroughly prior to plating.
- Vortex for approximately 20 seconds.
- Plate sample in triplicate, 3 Enterobacteriaceae petrifilm and 3 TC petrifilm.
  1. If necessary, repeat for requested dilutions. Note: Dilutions may be determined necessary based on the preliminary set of results.
     Correspondence with the microbiologists and the team at IP is necessary to determine this information.
- Incubate Enterobacteriaceae petrifilm in accordance with the manufacturer's instructions. Incubate the TC petrifilm at 44.5C for 24 hours.
- After plating, sample is to be held at 2-8 degrees to maintain integrity in the event of a dilution or concentration step is required.

Reading Results:
- Report the time of reading on the COC
- Using the manufacturer's instructions and visual guidelines, count colonies typical for Enterobacteriaceae and Fecal Coliforms on their respective petrifilm.
- Record all petrifilm results on the COC (CFU’s only)
- Calculate and record the average for the 3 petrifilm plates according to standard methods for enumeration of colonies.
Appendix C

Assessment of General RPC
Cleanliness As Delivered for Use in
Packing and Distributing Fresh
Produce draft protocol, 25 April, 2014
Assessment of General RPC Cleanliness As Delivered for Use in Packing and Distributing Fresh Produce

Purpose: To assess the typical microbiological cleanliness and quantitative bacterial profile of pool RPC's at the time of delivery to a distribution facility or directly to a harvest location or packing operation.

Background: Growers, handlers, and shippers have expressed concern regarding the microbiological cleanliness of RPC's, as received from the RPC-supplier depot, which they use for ready-to-wash fresh produce. Although there is no direct evidence, at this time, there is a concern for the transfer of microbiological hazards from RPC surfaces, or frequently observed entrapped residues, to their product. A recent survey report from the University of Guelph, on the cleanliness of RPC's in the Canadian pool system delivered for produce packing, presented some outcomes that have stimulated a heightened interest to assess regional situations.

Aim: To determine whether the University of Guelph outcomes represent an outlier situation or are representative of the general sufficiency of washing and sanitizing that occurs at RPC depots. The aim is for produce suppliers to have a data-based and informed view of their packing supplies. In addition, the anticipated outcomes will create an opportunity, if warranted, to engage RPC suppliers in a dialogue for improved sanitization of RPCs between users in the closed pool loop.

Scope: A limited duration baseline survey program will be conducted in CA and FLA on RPCs taken directly following discharge from a delivery vehicle. The microbiological survey will test only for indicator bacteria and will not include the assessment of any pathogen or pathogen virulence-marker testing. If warranted, based on initial test outcomes, retained samples may be evaluated for non-cultural evidence of bacterial pathogens at least 6 months after any individual shipment using the ROC lot sampled would have passed. If this test is agreed to, all analyzed samples will be double-blind coded to prevent association with any specific product or operation.

Precautions: With an unknown microbiological heterogeneity among a population of pool-system RPC's and high potential for an unbalanced quantitative and qualitative distribution of bacteria a specific spatial points among individual RPC units, it is impossible to develop a statistically derived sampling plan. In advance of having a baseline of data against which to derive the predicted standard deviations it becomes necessary to assign practical assumptions that define the survey approach. A decision can be made at this point as to whether the survey will be allowed to evolve or simple stick with a sampling regime as a Phase 1 “scoping” exercise.

Methods:

- **RPC Sample Size** – 34 units per site/visit; Six visits per region
  - Random Sample – 6 units from 4 palletized deliveries selected at random from top, middle, and lower positions (2 per position)
  - If less than 4 pallets are planned for delivery on the coordinated sampling date then the number of units per pallet will be distributed across the load accordingly
For Cause Sample – 10 units per load selected by observation to be visibly ‘soiled’

- Swab Sample Number – This survey is designed to maximize the detection of viable microbial populations on interior surfaces of delivered RPC units.

- Random Sample Units – 2 large area swabs per unit
  - Full area swab of the bottom of the RPC
  - Full area swab of the side-panels and hinge areas of the RPC

- For Cause – 2 large area swabs per unit and 2 cotton-tipped swabs at visible ‘soiled’ target(s)
  - Swab first at For Cause target with small area cotton-tipped swabs
  - Full area swab of the bottom of the RPC
  - Full area swab of the side-panels and hinge areas of the RPC

- Sampling Site Preparation – An on-site area to conduct the swabbing of interior surfaces of each unfolded RPC will be established to minimize interference from on-going local operations, facilitate proper aseptic swabbing and aseptic swab sample handling. Ideally this is in an enclosed room, such as employee break room, or similar protected site. However, reasonable measures to separate swabbing activities from RPC de-palletizing and selection activities are sufficient. If RPC sampling must be at harvest locations, a swabbing station within the rear interior of a van, or equivalent, is acceptable. We have done this multiple times during field and commercial packing operation surveys and lay new plastic sheeting at the rear doors with the vehicle parked in a location and direction to effectively exclude significant dust intrusion.

  - A small folding table should be available to serve as the platform for laying out each RPC for swabbing.
  - Prior to start-up, the table surface should be sprayed with OxiVir or a comparable surface sanitizing antimicrobial and, ideally, a new sheet of butcher paper placed over the surface between each pallet-group being tested.
  - An on-site work-bench or table treated in the same manner is acceptable

- Sample Method – At this time our preference is for the use of sterile pre-wetted cheese cloth swatches. The reason for this is based on our experience that the rougher surface texture, as compared to commercial kit sponge swabs, is better at removing adherent bacteria from surfaces and is comparable in conforming to tight spaces, with pressure, such as joints and flaps. However; we are very cognizant of the need to use standardized materials and are open to a selection of any preferred and familiar sponge (large surface format) swab kit by those responsible for conducting the sampling.

  - Palletized RPC’s in a newly delivered lot will be placed in a protected area, as practical, to prevent dust, debris, or other likely sources of external microbial introduction from affecting results during unstacking and sampling.
  - RPC’s will be removed from the pallet by individuals wearing new sterile disposable gloves; gloves are exchanged between removing exterior stabilizing wrap and corner braces and between de-palletizing to access sampling locations for each pallet.
  - Reasonable care will be taken to ensure handling of the RPCs from edges to minimize possible transfer between unstacking and selection and during observations to separate For Cause units from others
Ideally, RPC’s selected at random will be carried to the swabbing station from each pallet tier position location.

During de-palletizing, For Cause RPC’s will be selected based on observed adhering soil, plant material, visible dried residues, or other elements (including old labeling) that differentiate these units from randomly selected units. The operator will make a decision as to whether glove exchange is prudent after handling these units.

Digital image documentation will be conducted for each For Cause RPC target area and labeled sequentially for each date until the conclusion of the project.

Swabbing Method – Standard swabbing procedures will be utilized. A visual SOP guide will be made available if requested but for this DRAFT we assume that all contracted parties are familiar and proficient with these standard methods.

Rather than a typical 12"x12" area for large format swabbing, we are recommending a full surface swab as described above.

For Cause target area swabbing will be at the point of visible soil/product residue rather than a typical 4"x 4" area.

Swabs will be placed in the pre-labeled sample bag or Whirl-Pak and immediately transferred to an insulated cooler with sufficient frozen gel-ice packs to maintain at least 10°C, or lower, during transport to the analytical lab.

The total time from sample collection to sample processing must be less than 24h.

Sample Custody – Standard GLP practices will apply. All individual sample bags containing swabs must be uniquely labeled with indelible ink or permanent bar-code label and placed in a master container for each RPC pallet location and for each pallet. For Cause swabs should be similarly labeled but placed in a separate master container.

If individuals conducting the sampling are different than the individuals conducting sample processing there should be a signed Sample Log Sheet for each date reflecting the transit time and receiving time.

The temperature of each cooler and three sample bags must be recorded upon receiving.

If samples are held O.N. before processing they must be placed in a secure area within a walk-in cooler or refrigerator at 2.5 to 4.0°C.

Sample Processing – Standard microbiological methods will be applied for quantitative recovery.

25 ml of Butterfield’s Phosphate Buffered Saline, or an equivalent sterile buffered solution, are added directly to the large area swabs ensuring that the entire cheese cloth swatch or sponge is fully covered.

At this time we do not believe it is necessary to add DE Neutralizing Broth to the sample bag at the time of sampling.

5 ml of Butterfield’s Phosphate Buffered Saline, or an equivalent sterile buffered solution, are added directly to the cotton-tipped swab tube if not already included in the swab kit format.

Release of bacteria from the swab will be performed in a Stomacher or Pulsifier, or equivalent apparatus, at a Medium setting for 45 sec.
Sample Enumeration - Standard microbiological methods will be applied for quantitative recovery

For each sample, 200µl of the sample buffer will be plated onto three plates of each media listed below. The media named is simply a suggestion and the contract lab is welcomed to identify a preferred equivalent alternative.

- Violet Red Bile Glucose (VRBG) Agar – Total Enterobacteriaceae
  - Incubate at 35°C for 48h

- CHROM ECC – Coliform/E. coli
  - Incubate at RT (22°C) for 4h and move to 44°C for 36h

All media should be poured at least 3 days in advance and have a well-dried surface to accommodate and absorb the 200 µl

All residual sample buffer solutions should be held at 2.5 to 4.0°C in case a concentration or dilution step is warranted

- TBD – retained sample buffer may be stored in 22% glycerol and held at -80°C for 16s rDNA analysis or ribotyping at a future date

Standard methods for enumeration of colonies will be applied

Notes will be taken of

Documentation - Standard microbiological methods will be applied for quantitative data reporting

Analysis – Raw data and data summaries should be transferred to M. Danyluk and T. Suslow for transformation, statistical analysis, and trending

A real time calculation of standard deviation and power calculation (M. Danyluk) will be conducted during the first three sampling dates to determine whether a significant change in sample number is necessary

Reporting and Control of Data Dissemination - TBD
Appendix D

WBA Analytical Laboratories,
RESEARCH PLANNING (Study I),
April 28. 2014
RESEARCH PLANNING (Study I):

An egg producer supplied Reusable Plastic Containers (RPC) 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 are a *Salmonella* spp., *Listeria monocytogenes*, and *E. coli O157:H7*. The *Salmonella* spp. biofilm will be comprised of *S. newport*, *S. kentucky*, *S. heidelberg*, *S. enteritidis*, and *S. typhimurium*. The *L. monocytogenes* biofilm will be comprised of one poultry isolate and four human isolates. The *E. coli O157:H7* will be a non-toxin forming strain. The RPC’s will be disassembled and cut into 1 in² pieces (referred to as coupons). Preliminary work using Scanning Electron Microscopy (SEM) has provided visual confirmation of *S. enteritidis* adhering to the RPC and stainless steel coupons.

Test Method/Application:
The RPC coupons will be subjected to a bacteria biofilm growth process then examined using Scanning Electron Microscopy (SEM).

Reason for Initiation of Method Validation:
Request by WBA customer.

Study conducted by:
Jacquelyn Adams and John Clayborn

OBJECTIVE:
To determine and confirm the ability of *Salmonella* spp., *Listeria monocytogenes*, and *E. coli O157:H7* to adhere and produce bacterial biofilms on Reusable Plastic Containers provided by an egg producer.

MATERIALS:

Test Product:
Reusable Plastic Containers (RPC) coupons

Supplies Needed:
Sterile specimen cups
Micropipettor
Pipette Aid
Inoculating Loops
TSA agar plates
2ml Pipets
Tryptic Soy Broth
BHI Broth
Sterile stir bars
Autoclave Sterilization Packages
*E. coli O157:H7*, Non-Toxin Forming
ATCC# 19206 (SPR-CULQC-552)
5 strain Salmonella spp. cocktail: (isolates obtained by laboratory)
- Salmonella kentucky
- Salmonella newport
- Salmonella enteritidis
- Salmonella heidelberg
- Salmonella typhimurium

5 strain Listeria monocytogenes cocktail:
- ATCC #19111 (SPR-CULRF-504)
- ATCC #19115 (SPR-CULRF-500)
- ATCC #43257 (SPR-CULRF-502)
- ATCC #49594 (SPR-CULRF-501)
- Tyson #2926 (human isolate from lunchmeat) (SPR-CULRF-503)

Equipment:
- 35°C Incubator
- Platform Shaker
- Electron Scanning Microscope

Sample Group(s) and # per group(s):
- Group A: Salmonella spp. - RPC coupon
- Group B: Listeria monocytogenes – RPC coupon
- Group C: E. coli O157:H7 – RPC coupon

DISPOSAL PLANS (sample disposal, chemical waste streams):
Samples and testing materials will be disposed of at completion of analysis with the approval of project’s team leader. Refer to WI-A-011 (Laboratory Waste and Disposal) for disposal procedures.

Note: If chemicals are used in the project, they will be held on site for future use, returned to the customer, or discarded. Handling, storage, and/or disposal of all chemicals will be performed appropriately according to the MSDS and actions taken will be noted in the Research Project Design Form.

METHODS (Detailed steps to conduct study):

Inoculum preparation (Salmonella spp. cocktail, L. monocytogenes cocktail and E. coli O157:H7):
- Streak all five strains of Salmonella spp. onto TSA plates for isolation and incubate at 35 ± 1°C for 18 hours.
- Streak all five strains of L. monocytogenes onto TSA plates for isolation and incubate at 35 ± 1°C for 18 hours.
- Streak the E. coli O157:H7 onto a TSA plate for isolation and incubate at 35 ± 1°C for 18 hours.
- After incubation, pick an isolated colony from each TSA to 10ml of BHI broth and incubate at 35 ± 1°C for 18 hours.
- After incubation, transfer 0.5ml from each 10ml BHI to a 40ml BHI broth and incubate at 35 ± 1°C for 18 hours.
- After the final incubation combine and mix all five Salmonella inoculums into a sterile jar. Repeat for the five L. monocytogenes inoculums. Since only one E. coli O157:H7 strain was used, no combining is necessary.

Sample preparation and treatment:
- Prepare six RPC coupons by sanitizing each coupon and allowing to dry. Two coupons for each bacteria will be prepared for testing. Of the two coupons, one coupon will be used for testing and one coupon will be for backup purposes if necessary.
• Triple rinse each coupon thoroughly with sterile DI water to ensure no sanitizer residue is lingering.
• Label three 90ml sterile specimen cups with bacteria name. For this study cups were labeled as follows:
  o *Salmonella* spp. – RPC
  o *L. monocytogenes* – RPC
  o *E. coli O157:H7* – RPC
• Insert coupon into each cup along with a sterile magnetic stir bar. The stir bar is used to create extra motion within the cup during incubation.
• Aseptically dispense 40ml of appropriate growth medium 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.
• Aseptically dispense 0.5ml of each inoculum into appropriate cup containing coupons.
• Place the three cups onto a platform shaker (set at a rotation of 110 rpm) that has been positioned in a 35±1°C incubator.
• Start the platform shaker and incubate for 18-24 hours.
• After incubation, remove all cups.
• Individually and aseptically remove the coupon and stir bar from the cup. Discard the cup and inoculated growth medium.
• Using a sterile 25ml pipette, rinse the coupon with sterile DI water to remove any loose planktonic cells. Planktonic cells are phyciologically distinct from the cells growing in a biofilm. They are of the same organism but instead of attaching to a surface they may float or swim in the liquid growth medium.
• Place the rinsed coupon and stir bar into a labeled sterile 90ml specimen cup.
• Repeat the above rinsing steps for each coupon individually to avoid cross contamination.
• Once all coupons have been rinsed and placed into specimen cups, aseptically dispense 40ml of the appropriate growth medium into the cup and ensure coupon is submerged.
• Incubate all three cups on the platform shaker (set at a rotation of 110 rpm) at 35±1°C for 72 hours.
• After the final incubation, aseptically remove each coupon, rinse with sterile DI water and place in individual sterile cups.
• Examine each coupon using scanning electron microscopy (SEM) for visual confirmation of attachment and biofilm development.

REFERENCE MATERIAL (ie. Previous studies, literature review, AOAC, WI#):

BIOFILM FORMATION BY *Listeria monocytogenes* ON STAINLESS STEEL SURFACE AND BIOTRANSFER POTENTIAL
Maira Maciel Mattos de Oliveira1; Danilo Florisvaldo Brugnera1; Eduardo Alves2; Roberta Hilsdorf Piccoli1*

Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions
M.L. Kalmokoff, J.W. Austin, X.-D. Wan, G. Sanders, S. Banerjee, J.M. Farber

Elimination of *Salmonellae* from Inoculated Filler Flats
G. J. BANWART, Purdue University, Lafayette, Indiana

Possible horizontal transmission of Salmonella via reusable egg trays in Thailand
Fuangfa Utrarachkij, Srirat Pornraung Wong, Kanokrat Siripanichgon, Chie Nakajima, Yasuhiko Suzuki, Orasa Suthienkul

WI-A-011 Laboratory Waste and Disposal

SAFETY REQUIREMENTS (ie. MSDS, PPE):
Observe Laboratory Safety Requirements associated with handling *Salmonella* spp., *L. monocytogenes*, *E. coli O157:H7*. 
RESULTS/SUMMARY of DATA:

A study was performed to evaluate the ability of *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157:H7 to attach and form biofilms on the plastic material from disassembled Reusable Plastic Containers provided by an egg producer.

One RPC coupon per bacteria was subjected to a biofilm formation process. After the process, each sample was examined using scanning electron microscopy (SEM) to visually confirm attachment of bacteria as well as biofilm formation. SEM examination was conducted by a third party laboratory.

CONCLUSION:

*Study completion date: April 28, 2014*

The SEM images give evidence that each bacteria was capable of attaching to the RPC and forming a biofilm. See attached images below.
Listeria monocytogenes Biofilm
Study 1
Appendix E

WBA Analytical Laboratories,
RESEARCH PLANNING (Study II),
May 8, 2014
RESEARCH PLANNING (Study II):

An egg producer supplied Reusable Plastic Containers (RPC) for a study to determine the ability of sanitizing agents, typically used in commercial and industrial settings, to disrupt and remove Salmonella spp. biofilms that have formed (in a laboratory setting) on the RPC. The Salmonella spp. biofilm will be comprised of S. newport, S. kentucky, S. heidelberg, S. enteritidis, and S. typhimurium. The RPC’s will be disassembled and cut into 1 in² pieces (referred to as coupons). Preliminary work using scanning electron microscopy (SEM) has provided visual confirmation of Salmonella spp. adhering to the RPC coupons. After each coupon has been subjected to a biofilm formation process, the coupons will be cleaned/sanitized using products and methods typically used in commercial/industrial settings to sanitize equipment and supplies.

Test Method/Application:

The RPC coupons will be subjected to a bacteria biofilm growth process then sanitized using methods and sanitizing agents typically found in commercial and industrial settings.

Reason for Initiation of Method Validation:

Request by a WBA customer.

Study conducted by:

Jacquelyn Adams and John Clayborn

OBJECTIVE:

To determine the ability of sanitizing procedures to disrupt and eliminate Salmonella biofilms on Reusable Plastic Containers provided by an egg producer.

MATERIALS:

Test Product:

Reusable Plastic Containers (RPC) coupons

Supplies Needed:

Sterile specimen cups
Micropipettor
Pipette Aid
Inoculating Loops
TSA agar plates
2ml Pipets
Tryptic Soy Broth
Sterile Water
Buffered Peptone Water
Sterile Stomacher Bags
Sterile Graduated Cylinder
Sterile stir bars
Forceps
Sterile tongue depressors
Metal Bowls
Autoclave Sterilization Packages
Alkaline Detergent
Quaternary Ammonium
Chlorine
5 strain *Salmonella* spp. cocktail: (Isolates obtained by laboratory)
  - *Salmonella kentucky*
  - *Salmonella newport*
  - *Salmonella enteritidis*
  - *Salmonella heidelberg*
  - *Salmonella typhimurium*

**Equipment:**
- 35°C Incubator
- Platform Shaker
- BAX® Machine
- PC1 Master Test Kit (titration kit to test concentration of Quaternary Ammonium and Chlorine)
- Electron Scanning Microscope

**Sample Group(s) and # per group(s):**
- Group A: Treatment 1 – Hot Water + Alkaline Detergent
- Group B: Treatment 2 – Hot Water + Alkaline Detergent + 200ppm – 400ppm Quaternary Ammonium
- Group C: Treatment 3 – 200ppm – 400ppm Quaternary Ammonium
- Group D: Treatment 4 – Hot Water + Alkaline Detergent + ~200ppm Chlorine Solution
- Group E: Treatment 5 – ~200ppm Chlorine Solution
- Group F: Treatment 6 – Untreated Control

**DISPOSAL PLANS (sample disposal, chemical waste streams):**
Samples and testing materials will be disposed of at completion of analysis with the approval of project’s team leader. Refer to WI-A-011 (Laboratory Waste and Disposal) for disposal procedures.

Note: If chemicals are used in the project, they will be held on site for future use, returned to the customer, or discarded. Handling, storage, and/or disposal of all chemicals will be performed appropriately according to the MSDS and actions taken will be noted in the Research Project Design Form.

**METHODS (Detailed steps to conduct study):**

*Inoculum preparation (Salmonella spp. cocktail):*
  - Streak all five strains of *Salmonella* spp. onto TSA plates for isolation and incubate at 35 ± 1°C for 18 hours.
  - After incubation, pick an isolated colony from each TSA to 10mls of BHI broth and incubate at 35 ± 1°C for 18 hours.
  - After incubation, transfer 0.5ml from each 10ml BHI to a 40ml BHI broth and incubate at 35 ± 1°C for 18 hours.
  - After the final incubation, combine and mix all five Salmonella inoculums into a sterile jar.
**Salmonella spp. Biofilm Formation Process and sample preparation:**

- Prepare RPC coupons by sanitizing each coupon with isopropyl alcohol.
- Aseptically and thoroughly rinse each coupon with sterile DI water to remove any sanitizer residue.
- Label six, 90ml sterile specimen cups per treatment group and label with group number.
- Insert RPC coupons into each cup. This study includes 5 coupons per treatment group and an extra coupon per group, that will be used for SEM imaging.
- Aseptically dispense 40ml of Tryptic Soy Broth (TSB) into each cup.
- Aseptically dispense 0.5ml inoculum into into each cup containing the coupon and TSB.
- Place the inoculated cups onto a platform shaker that has been set at 110rpm and has been positioned in a 35 ±1°C incubator.
- Incubate cups for 18-24 hours.
- After incubation, remove all cups.
- Individually and aseptically remove the coupon from the cup. Discard cup and inoculated growth medium.
- Using a sterile 25ml pipette, rinse the coupon with sterile DI water to remove any loose planktonic cells. Planktonic cells are physiologically distinct from the cells growing in a biofilm. They are the same bacteria type but instead of attaching to the surface of the RPC they were floating in the TSB.
- Place the rinsed coupons into a labeled sterile 90ml specimen cup.
- Repeat the above rinsing steps for each cup individually to prevent any type of cross contamination during the biofilm formation process.
- Once all coupons have been rinsed and placed into specimen cups, aseptically dispense 40ml of the TSB into the cup and ensure coupon is submerged in broth.
- Incubate all cups on the platform shaker (set at a rotation of 110rpm) at 35±1°C for 72 hours.
- After the final incubation, aseptically remove each coupon and transfer to a tray that has been covered with foil and sanitized with isopropyl alcohol. Using sanitized forceps, grasp the corner of the coupon and dispense sterile DI water over the coupon to remove loose cells.
- Place each coupon into individual sterile cups while ensuring the coupons remain in its assigned group.

**Sample Treatment:** Treatment concentration and water temperature is based on typical commercial and/or industrial standard limits for sanitization processes.

**Note:** 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 will be conducted as follows:
  - Grasp the corner of the coupon and spray each side of the coupon for 5 seconds with hot water using a spray nozzle attached to the sink faucet.
  - After the hot water spray, dip the coupon in the alkaline detergent mixture and aggressively move the coupon back and forth for 5 seconds.
  - Place the coupon on a wire rack and allow to dry for two minutes.
  - Place each coupon in a sterile stomacher bag.
- Treatment 2 will be conducted as follows:
  - Grasp the corner of the coupon and spray each side of the coupon for 5 seconds with hot water using a spray nozzle attached to the sink faucet.
  - After the hot water spray, dip the coupon in the alkaline detergent mixture and aggressively move the coupon back and forth for 5 seconds. After removal, quickly shake the coupon to remove excess detergent mixture.
  - Next, dip the coupon in the Quaternary Ammonium mixture and aggressively move the coupon back and forth for 5 seconds. For this study the concentration of the quaternary ammonium was 250ppm. Again, after removal, shake to remove excess sanitizer.
  - Place the coupon on a wire rack and allow to dry for two minutes.
  - Place each coupon in a sterile stomacher bag
Treatment 3 will be conducted as follows:
- Grasp the corner of the coupon. Dip the coupon in the Quaternary Ammonium mixture and aggressively move the coupon back and forth for 5 seconds, shake to remove excess. For this study the concentration of the quaternary ammonium was 250ppm.
- Place the coupon on a wire rack and allow to dry for two minutes.
- Place each coupon in a sterile stomacher bag

Treatment 4 will be conducted as follows:
- Grasp the corner of the coupon and spray each side of the coupon for 5 seconds with hot water using a spray nozzles attached to the sink faucet.
- After the hot water spray, dip the coupon in the alkaline detergent mixture and aggressively move the coupon back and forth for 5 seconds. Shake to remove excess detergent mixture.
- Next, dip the coupon in a Chlorine and water mixture. Aggressively move the coupon back and forth for 5 seconds, shake to remove excess. For this study the concentration of the Chlorine solution was 205ppm.
- Place the coupon on a wire rack and allow to dry for two minutes.
- Place each coupon in a sterile stomacher bag.

Treatment 5 will be conducted as follows:
- Grasp the corner of the coupon. Dip the coupon in the Chlorine solution and aggressively move the coupon back and forth for 5 seconds, shake to remove excess. For this study the concentration of the chlorine solution was 200ppm.
- Place the coupon on a wire rack and allow to dry for two minutes.
- Place each coupon in a stomacher bag.

Treatment 6 will be conducted as follows:
- Grasp the corner of the coupon and do not treat, instead transfer directly to a sterile stomacher bag.

The extra coupons needed for SEM imaging were removed from the treatment groups and held refrigerated.

Once all treatments have been performed and all coupons are in corresponding stomacher bags, add 20mLs of sterile Buffered Peptone Water and shake vigorously for 30 seconds.

Incubate all samples at 35±1°C for 18-24 hours.

After incubation, test coupon samples for the detection of Salmonella spp. using the BAX® system. (Appendix A)

REFERENCE MATERIAL (ie. Previous studies, literature review, AOAC, WI#):

BIOFILM FORMATION BY Listeria monocytogenes ON STAINLESS STEEL SURFACE AND BIOTRANSFER POTENTIAL
Maíra Maciel Mattos de Oliveira1; Danilo Florisvaldo Brugnera1; Eduardo Alves2; Roberta Hilsdorf Piccoli1*

Adsorption, attachment and biofilm formation among isolates of Listeria monocytogenes using model conditions
M.L. Kalmokoff, J.W. Austin, X.-D. Wan, G. Sanders, S. Banerjee, J.M. Farber

Elimination of Salmonellae from Inoculated Filler Flats
G. J. BANWART, Purdue University, Lafayette, Indiana

Possible horizontal transmission of Salmonella via reusable egg trays in Thailand
Fuangfa Utrarachkij, Srirat Pornraungwong, Kanokrat Siripanichgon, Chie Nakajima, Yasuhiko Suzuki , Orasa Suthienkul

WI-A-011 Laboratory Waste and Disposal
WI-M-018-01 Salmonella Analysis

SAFETY REQUIREMENTS (ie. MSDS, PPE):
Observe Laboratory Safety Requirements associated with handling Salmonella spp.
RESULTS/SUMMARY of DATA:

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 Reusable Plastic Containers. The RPC’s were cut into 1in² pieces (referred to as coupons) from the flat level surfaces of the RPC’s and divided into groups based on the treatment methods. All coupons were subjected to a biofilm formation process. After the biofilm formation process was complete, the coupons from each group were aseptically rinsed then allowed to dry before being sanitized following the treatment methods used in this study. Once all treatments had been administered, one coupon from each group was examined using SEM the remaining coupons were submerged in Buffered Peptone Water and allowed to incubate. After the incubation, all coupons were analyzed using BAX® 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 spp.*

<table>
<thead>
<tr>
<th>Treatment: n=5</th>
<th>Salmonella Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment 1: RPC 1 to RPC 5</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>Treatment 2: RPC 1 to RPC 5</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>Treatment 3: RPC 1 to RPC 5</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>Treatment 4: RPC 1 to RPC 5</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>Treatment 5: RPC 1 to RPC 5</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>Treatment 6: RPC 1 to RPC 5</td>
<td>POSITIVE</td>
</tr>
</tbody>
</table>

Simultaneously, the extra coupons from each treatment group were examined using scanning electron microscopy (SEM) to confirm the presence of *Salmonella spp.* biofilm on coupons from each group. The SEM examination was conducted by a third party laboratory. All images confirm that a *Salmonella spp.* biofilm was present.

CONCLUSION:

*Study completion date: May 8, 2014*

The SEM images and BAX® results give evidence that the sanitizing methods and agents used in this study were not effective in disrupting and eliminating *Salmonella spp.* biofilms from RPC surfaces. As mentioned above, all coupons were cut from flat, smooth areas of the RPC which represent areas that would be easily cleaned during sanitation. Areas of the RPC that have raised edges, textured surfaces and hard to access recessed areas are 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.

Research has shown that bacteria biofilms are not only capable of attaching and remaining on surfaces but 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).

The SEM images provide evidence that the treatments administered in this study did not effectively remove the developed *Salmonella spp.* biofilms. See attached images below.
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
June 5, 2014

PCR and Dead Cell Detection

One criticism of PCR is that it amplifies DNA from both live and dead cells. This could be problematic if a PCR method reports positive results based on harmless dead cells, and food is then unnecessarily destroyed.

This potential problem is generally not an issue when using the BAX® system because food or environmental samples are enriched prior to testing. Enrichment provides the nutrients for live bacterial growth; dead cells are not affected. Therefore, dead cells would need to be present at a sufficient level prior to enrichment to yield the concentration required for BAX® system detection (approximately 10,000 cells/mL).

Further, the BAX® system protocol calls for 1:10 dilution of original sample into the enrichment broth. Thus, if 100 ml of enrichment broth were used, the true minimum concentration requirement for detection by the BAX® system would be 100,000 dead cells per gram of food or 1,000,000 dead cells per sponge.

Additionally, cells must provide good quality DNA for detection. It is highly unlikely that the DNA of dead cells will remain intact through the industrial processes that lead up to enrichment. Thus, the actual level of dead cells required to yield detectable DNA would need to be even higher.

This calculation applies only to meat or environmental samples tested for Salmonella or E. coli O157:H7. Other food types that are tested for Salmonella require an additional regrowth step and foods tested for Listeria require a secondary enrichment. These samples would need 50-times and 100-times the concentration of dead cells calculated above due to the additional enrichment steps.

Sincerely,
Troy E. Ayers, M.S.
DuPont Nutrition & Health
Molecular Diagnostics
Appendix F

WBA Analytical Laboratories,
RESEARCH PLANNING (Study III),
May 8, 2014
An egg producer supplied Reusable Plastic Containers (RPC) 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 will be comprised of *S. newport*, *S. kentucky*, *S. heidelberg*, *S. enteritidis*, and *S. typhimurium*. The RPC’s will be disassembled and cut into 1 in² pieces (referred to as coupons). Preliminary work using scanning electron microscopy (SEM) has provided visual confirmation of *Salmonella spp.* adhering to the RPC coupons. After each coupon has been subjected to a biofilm formation process, the coupons will be swabbed three consecutive times, using a different swab each time, to determine if the repeated swabbing action can remove the *Salmonella* biofilm from the RPC coupons.

**Test Method/Application:**
RPC coupons will be subjected 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.

**Reason for Initiation of Method Validation:**
Request by a WBA customer.

**Study conducted by:**
Jacquelyn Adams and John Clayborn

**OBJECTIVE:**
To determine the ability of repeated swabbing to disrupt and eliminate *Salmonella spp.* biofilms on Reusable Plastic Containers provided by an egg producer.

**MATERIALS:**

**Test Product:**
Reusable Plastic Containers (RPC) coupons

**Supplies Needed:**
Sterile specimen cups
Micropipettor
Pipette Aid
Inoculating Loops
TSA agar plates
2ml Pipets
Tryptic Soy Broth
Sterile Water
Buffered Peptone Water
Sterile Stomacher Bags
Sterile Graduated Cylinder
Sterile stir bars
Forceps
Sterile tongue depressors
Metal Bowls
Autoclave Sterilization Packages
PUR-Blue™ DUO™ Swabs

5 strain *Salmonella* spp. cocktail: (isolates obtained by laboratory)
- *Salmonella kentucky*
- *Salmonella newport*
- *Salmonella enteritidis*
- *Salmonella heidelberg*
- *Salmonella typhimurium*

**Equipment:**
- 35°C Incubator
- Platform Shaker
- BAX® System

**DISPOSAL PLANS (sample disposal, chemical waste streams):**

Samples and testing materials will be disposed of at completion of analysis with the approval of project’s team leader. Refer to WI-A-011 (Laboratory Waste and Disposal) for disposal procedures.

Note: If chemicals are used in the project, they will be held on site for future use, returned to the customer, or discarded. Handling, storage, and/or disposal of all chemicals will be performed appropriately according to the MSDS and actions taken will be noted in the Research Project Design Form.

**METHODS (Detailed steps to conduct study):**

**Inoculum preparation (*Salmonella* spp. cocktail):**
- Streak all five strains of *Salmonella* spp. onto TSA plates for isolation and incubate at 35 ± 1°C for 18 hours.
- After incubation, pick an isolated colony from each TSA to 10ml of BHI broth and incubate at 35 ± 1°C for 18 hours.
- After incubation, transfer 0.5ml from each 10ml BHI to a 40ml BHI broth and incubate at 35 ± 1°C for 18 hours.
- After the final incubation, combine and mix all five *Salmonella* spp. inoculums into a sterile jar.

**Salmonella spp. Biofilm Formation Process and sample preparation:**
- Prepare RPC coupons by sanitizing each coupon with 70% isopropyl alcohol and allowing to dry.
- Aseptically and thoroughly rinse each coupon with sterile DI water to remove any sanitizer residue.
- Label five, 90ml sterile specimen cups.
- Insert RPC coupons into each cup.
- Aseptically dispense 40ml of Tryptic Soy Broth (TSB) into each cup.
- Aseptically dispense 0.5ml inoculum into each cup containing the coupon and TSB.
- Place the inoculated cups onto a platform shaker that has been positioned in a 35 ±1°C incubator.
- Start the platform shaker (set at 110 rpm) and incubate for 18 hours.
- After the 18 hours incubation, remove all cups.
- Individually and aseptically remove the coupon from the cup. Discard cup and inoculated growth medium.
- Using a sterile 25ml pipette, rinse the coupon with sterile DI water to remove any loose planktonic cells. Planktonic cells are physiologically distinct from the cells growing in a biofilm. They are the same bacteria type but instead of attaching to the surface of the RPC they were floating in the TSB.
Place the rinse coupons into a labeled sterile 90ml specimen cup.

Repeat the above rinsing steps for each cup individually to prevent any type of cross contamination during the biofilm formation process.

Once all coupons have been rinsed and placed into specie cups, aseptically dispense 40ml of the TSB into the cup and ensure coupon is submerged in broth.

Incubate all cups on the platform shaker at 35±1°C for 72 hours.

After the final incubation, aseptically remove each coupon and transfer to a tray that has been covered with foil and sanitized with isopropyl alcohol. Using sanitized forceps, grasp the corner of the coupon and dispense sterile DI water over the coupon to remove loose cells.

Place each coupon into individual sterile cups and allow coupons to dry.

Using sterile gloves pick up the coupon and swab the entire coupon surface using a PUR-Blue™ DUO™ swab that is moistened with buffered peptone water. Swab aggressively and with pressure with the intent of removing as much Salmonella biofilm as possible.

Return the swab to its corresponding tube filled with 9ml of Buffered Peptone Water.

Repeat the swabbing two more times (for a total of three swabs per coupon) changing swabs for each repetition.

Repeat the above swabbing for each of the 5 coupons.

Once all swabs have been performed, place the RPC coupon into a sterile stomacher bag and add 20ml of sterile Buffered Peptone Water.

Prepare a negative control by pouring 20ml of the Buffered Peptone Water into a sterile stomacher bag.

Prepare a positive control by pouring 20ml of the Buffered Peptone Water into a sterile stomacher bag. Add one Salmonella Bioball® to the Buffered Peptone Water.

Incubate all samples (swabs and coupons) at 35±1°C for 18-24 hours.

After incubation, test samples and controls for the detection of Salmonella using the BAX® system (Appendix A).

REFERENCE MATERIAL (ie. Previous studies, literature review, AOAC, WI#):

BIOFILM FORMATION BY Listeria monocytogenes ON STAINLESS STEEL SURFACE AND BIOTRANSFER POTENTIAL
Maíra Maciel Mattos de Oliveira1; Danilo Florisvaldo Brugnera1; Eduardo Alves2; Roberta Hilisdorf Piccoli1*

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WI-A-011 Laboratory Waste and Disposal
WI-M-018-01 Salmonella Analysis

SAFETY REQUIREMENTS (ie. MSDS, PPE):
Observe Laboratory Safety Requirements associated with handling Salmonella spp.
RESULTS/SUMMARY of DATA:

A study was performed to evaluate the ability of repeated swabbing methods to disrupt and remove *Salmonella spp.* biofilms on the Reusable Plastic Containers. The RPC’s were cut into 1in² pieces (referred to as coupons) from the flat level surfaces of the RPC’s. All coupons were subjected to a biofilm formation process. After the biofilm formation process was complete, five coupons were aseptically rinsed then allowed to dry before being swabbed following the methods used in this study. Once all swabs had been administered, the coupons and swabs were submerged in Buffered Peptone Water and allowed to incubate. After the incubation, all coupons and swabs were analyzed using BAX® for the detection of *Salmonella spp.* In the case of this study, all RPC coupons and swabs tested positive for the presence of *Salmonella spp.* A positive control and a negative control were ran 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 are contained in the BAX® system to assure PCR success.

CONCLUSION:

*Study completion date: May 8, 2014*

The BAX® results provide 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.
June 5, 2014

PCR and Dead Cell Detection

One criticism of PCR is that it amplifies DNA from both live and dead cells. This could be problematic if a PCR method reports positive results based on harmless dead cells, and food is then unnecessarily destroyed.

This potential problem is generally not an issue when using the BAX® system because food or environmental samples are enriched prior to testing. Enrichment provides the nutrients for live bacterial growth; dead cells are not affected. Therefore, dead cells would need to be present at a sufficient level prior to enrichment to yield the concentration required for BAX® system detection (approximately 10,000 cells/mL).

Further, the BAX® system protocol calls for a 1:10 dilution of original sample into the enrichment broth. Thus, if 100 ml of enrichment broth were used, the true minimum concentration requirement for detection by the BAX® system would be 100,000 dead cells per gram of food or 1,000,000 dead cells per sponge.

Additionally, cells must provide good quality DNA for detection. It is highly unlikely that the DNA of dead cells will remain intact through the industrial processes that lead up to enrichment. Thus, the actual level of dead cells required to yield detectable DNA would need to be even higher.

This calculation applies only to meat or environmental samples tested for Salmonella or E. coli O157:H7. Other food types that are tested for Salmonella require an additional regrowth step and foods tested for Listeria require a secondary enrichment. These samples would need 50-times and 100-times the concentration of dead cells calculated above due to the additional enrichment steps.

Sincerely,
Troy E. Ayers, M.S.
DuPont Nutrition & Health
Molecular Diagnostics