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Research Study:

**Comparison of methods for quantitating *Salmonella enterica*
Typhimurium and Heidelberg strain attachment to reusable
plastic shipping container coupons and preliminary
assessment of sanitizer efficacy**

BY:

Zhaohao Shi, Christopher A. Baker, Sang In Lee, Si Hong Park, Sun Ae Kim, and
Steven C. Ricke

Center for Food Safety and Department of Food Science, University of
Arkansas, Fayetteville, Arkansas, USA

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Comparison of methods for quantitating *Salmonella enterica* Typhimurium and Heidelberg strain attachment to reusable plastic shipping container coupons and preliminary assessment of sanitizer efficacy

Zhaohao Shi, Christopher A. Baker, Sang In Lee, Si Hong Park, Sun Ae Kim, and Steven C. Ricke

Center for Food Safety and Department of Food Science, University of Arkansas, Fayetteville, Arkansas, USA

ABSTRACT

Salmonella serovars, one of the leading contributors to foodborne illness and are especially problematic for foods that are not cooked before consumption, such as fresh produce. The shipping containers that are used to transport and store fresh produce may play a role in cross contamination and subsequent illnesses. However, methods for quantitatively attached cells are somewhat variable. The overall goal of this study was to compare conventional plating with molecular methods for quantitating attached representative strains for *Salmonella* Typhimurium and Heidelberg on reusable plastic containers (RPC) coupons, respectively. We attached *Salmonella enterica* serovar Typhimurium ATCC 14028 and serovar Heidelberg SL486 (parent and an antibiotic resistant marker strain) to plastic coupons (2.54 cm²) derived from previously used shipping containers by growing for 72 h in tryptic soy broth. The impact of the concentration of sanitizer on log reductions between unsanitized and sanitized coupons was evaluated by exposing attached *S. Typhimurium* cells to 200 ppm and 200,000 ppm sodium hypochlorite (NaClO). Differences in sanitizer effectiveness between serovars were also evaluated with attached *S. Typhimurium* compared to attached *S. Heidelberg* populations after being exposed to 200 ppm peracetic acid (PAA). Treatment with NaClO caused an average of 2.73 ± 0.23 log CFU of *S. Typhimurium* per coupon removed with treatment at 200 ppm while 3.36 ± 0.54 log CFU were removed at 200,000 ppm. Treatment with PAA caused an average of 2.62 ± 0.15 log CFU removed for *S. Typhimurium* and 1.41 ± 0.17 log CFU for *S. Heidelberg* (parent) and 1.61 ± 0.08 log CFU (marker). Lastly, scanning electron microscopy (SEM) was used to visualize cell attachment and coupon surface topography. SEM images showed that remaining attached cell populations were visible even after sanitizer application. Conventional plating and qPCR yielded similar levels of enumerated bacterial populations indicating a high concordance between the two methods. Therefore, qPCR could be used for the rapid quantification of *Salmonella* attached on RPC.

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Introduction

Salmonella is a major cause of foodborne illness in the United States (US), resulting in an estimated 20,000 hospitalizations and 400 deaths per year, the most of any foodborne bacterium.^[1] *Salmonella* is a Gram-negative enteropathogenic bacterium that can cause a range of illnesses from gastroenteritis to potentially life threatening conditions such as bacteremia if an infection becomes invasive in at risk population.^[2] In 2014, various *Salmonella* species caused 10 multistate outbreaks in the US with approximately 1,000 outbreak cases.^[3] One characteristic associated with *Salmonella* is the ability to form biofilms as a protective adaptation against environmental challenges, with this being the preferred state in their natural habitats.^[4,5]

Biofilms are composed of communities of bacterial cells within an extracellular matrix that can adhere to biotic as well as abiotic surfaces.^[6] Aggregation into biofilms allows these communities to tolerate greater stresses and persist in hostile environments, which presents a problem to the food industry as potential reservoirs of contamination.^[7,8] Numerous studies have been conducted on major foodborne bacteria known to form biofilms such as *Salmonella* Typhimurium,^[9–12] *Listeria*

monocytogenes,^[13–15] and *Escherichia coli* O157:H7^[16] to evaluate attachment to abiotic surfaces including stainless steel and plastics, which are often encountered in food processing and transportation systems. It can be assumed that these surfaces could pass pathogenic cells to food products with the sloughing off of cells from biofilm matrices.^[17]

It has been suggested that determining the efficacy of sanitizers to remove attached microorganisms from food storage and transportation equipment will be important to determine the extent to which attached microorganisms can persist in food processing and shipping environments.^[18,19] Recent reports have resulted in a heightened recognition and debate on the risk that may be associated with reusable plastic containers (RPCs) due to cell attachment, biofilm formation, and fresh produce contamination.^[19,20] The RPCs can retain considerable levels of bacteria, for example in survey studies 37.5% and 8.3% of the RPCs from the field contained $> \log 5$ CFU/swab and $> \log 6$ CFU/swab, respectively.^[20] RPCs are most often used in the harvesting, processing, packing and shipping of fresh produce, which may be problematic as any microbial contamination could eventually

be transferred to fresh produce.^[17,21] These RPCs are designed for several cycles of use, are often placed directly on soil, and because of that, there is a potential risk of cross contamination, especially if not thoroughly sanitized.^[22] A previous report based in Italy indicated that their RPCs could hypothetically be reused on average, 200 times over a lifetime of 10 years before being removed from circulation.^[23]

This research is unique in that the RPCs evaluated in this study were previously in the distribution stream. Upon receipt, some of these containers had visually discernible surface wear, which may provide a more realistic model for evaluating the efficacy of sanitizer treatments on containers that have been through several cycles of use, sanitization, and reuse. The challenge was to adopt and develop methods for direct quantitation of laboratory attached *Salmonella* to these complex materials that already contained a background of unidentified bacteria. The primary objective of study was to compare standard plating methodology with qPCR for recovery and quantitation of *Salmonella* that were attached to these surfaces in the laboratory. While this was not an attempt to assess the broader aspects of RPC contamination, general industry sanitizer conditions were simulated as a part of this initial methodology development study. We chose to look at the best characterized representative strains of two serovars (*S. Typhimurium* and *S. Enteritidis*) that have been identified as commonly observed in *Salmonella* outbreaks in produce.^[24] The quantitative methods represented a comparison of independent experimental approaches ranging from standard selective plate enumeration of both serovars, generation of a specific *S. Heidelberg* marker strain that allowed direct recovery, and finally a quantitative PCR assay based on primers specific for *S. Heidelberg*.

Materials and methods

Bacterial growth conditions and marker strain preparation

Isolated colonies of *S. Typhimurium* ATCC 14028, *S. Heidelberg* SL486 (parent strain), or a *S. Heidelberg* nalidixic acid (NA) resistant marker strain derived from SL486 were added to 5 mL of tryptic soy broth (TSB) (Neogen, Lansing, MI, USA) and incubated for 18 h at 37°C, 110 rpm. The marker strain was generated by daily subculture of SL486 into growth media containing increasing amounts of NA over the course of seven days until a final resistance concentration of 20 µg/mL NA was achieved.

Sanitizer preparation

Both sodium hypochlorite (NaClO, Sigma-Aldrich, St. Louis, MO, USA) and peracetic acid (PAA, Sigma-Aldrich) were prepared by diluting stock sanitizer in sterile deionized water to a final concentration of 200 ppm, the maximum residue allowed on food contact surfaces without further removal.^[25] In addition, NaClO was prepared at 200,000 ppm for comparison of effectiveness with 200 ppm.

Coupon preparation and attachment of cells

Coupons of 1 × 1 inch (2.54 × 2.54 cm) size were cut from RPCs provided by a commercial company using a band saw

with uniform coupons without holes selected for use. Attachment of cells on coupons was based on procedures described previously^[19] with some modifications. Initially, coupons were scrubbed in distilled water and soaked in 70% ethanol (5 min exposure with agitation) to remove surface contamination. These were subsequently dried for 2 min and placed in sterile 90 mL specimen cups (Clarity Diagnostics, Boca Raton, FL, USA) with 40 mL TSB and 0.5 mL of overnight culture for an initial inoculum level of approximately 10⁷ colony forming units (CFU). Two coupons were placed in each cup with one designated for sanitization and one remaining unsanitized for comparison. Cups were incubated at 37°C at 110 rpm for 24 h to initiate RPC surface attachment. Following 24 h incubation, the coupons were rinsed thoroughly with deionized water to remove planktonic cells, dried for 2 min, and placed in new sterile specimen cups. Forty mL of new TSB was added to each cup and the coupons were incubated at 37°C at 110 rpm for 72 h to generate the final population level of attached bacterial cells.

Bacterial enumeration

Coupons were rinsed with deionized water, dried for 2 min, and transferred to sterile 50 mL centrifuge tubes (VWR, Radnor, PA, USA). Twenty mL of phosphate buffered saline (PBS, pH 7.4) and 3 g of glass beads (3 mm, EMD Millipore, Billerica, MA, USA) were added to the tubes to facilitate removal of attached bacterial cells. Tubes were vigorously shaken for 1 min to remove attached cells as previously described by Park and Kang.^[12] Rinsates were serially diluted with PBS to produce 10-fold diluted samples and spread-plated on tryptic soy agar plates (Neogen) in duplicate. Nonselective TSA was used to reduce stress caused by selective agents present in other media. Plates were incubated at 37°C for 24 h to determine the CFU per coupon.

Sanitizer treatment

Out of the two coupons from each specimen cup, one was designated to be sanitized and one to remain unsanitized. The unsanitized coupons were removed from their cups and rinsed with 40 mL of distilled water to remove residual TSB and planktonic cells before proceeding to bacterial enumeration. The sanitized coupons were first sprayed five times on each side with 43°C tap water to simulate how the RPC would be sprayed with water in a commercial environment before treatment with sanitizer. Afterwards, they were transferred to cups containing 150 mL of NaClO at 200 ppm or 200,000 ppm, or to cups containing PAA at 200 ppm and were subjected to vigorous agitation for 30 s at room temperature. Coupons were subsequently removed from the sanitizer, dried for 2 min, and enumerated using the previously described procedure in subsection "bacterial enumeration". Antibacterial treatments were performed on five biological replicates for NaClO and on five replicates for PAA.

DNA extraction and *Salmonella* confirmation by conventional PCR

Conventional PCR was performed to confirm *Salmonella* presence in the rinsate. Fifteen mL of PBS containing detached cells

was centrifuged for 10 min at 11,000 rpm and 14 mL of the supernatant removed. The remaining 1 mL was centrifuged with 950 μ L of supernatant removed to obtain a concentrated DNA sample. Samples were subsequently boiled and placed in ice to extract DNA from the cells. The PCR reaction volume consisted 1 μ L of sample DNA, 500 nM of each primer (F: TTT GGC GGC GCA GGC GAT TC; R: GCC TCC GCC TCA TCA ATC CG),^[26] which amplifies the 423 bp fragment within the genomic DNA of *Salmonella*, 10 μ L of 2X premix ExTaq (Takara, Mountain View, CA), and 7 μ L of distilled water. The PCR steps included initial denaturation of 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s followed by a 5 min elongation step at 72°C. The amplified product was electrophoretically separated on 1% agarose gel in 1X TAE buffer.

DNA extraction and enumeration of *S. Heidelberg* in rinsate by quantitative PCR

Quantitative PCR with a Mastercycler[®] ep realplex (Eppendorf, Hauppauge, NY, USA) was used to quantify detached cells and confirm plate enumerations of the *S. Heidelberg* samples. Extraction of DNA from the PBS rinsate containing detached cells was performed with a Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Each duplicate PCR aliquot consisted of 5 μ L of DNA, 500 nM of each primer (F: TGT TTG GAG CAT CAT CAG AA; R: GCT CAA CAT AAG GGA AGC AA),^[27] 10 μ L of SYBR[®] Premix Ex Taq[™] II (Takara, Shiga, Japan), and nuclease free water to bring to a final reaction volume of 20 μ L. Aliquots were subsequently denatured at 95°C for 2 min and followed by 40 cycles of 95°C for 15 s, annealing at 60°C for 15 s, and extension at 68°C. Melting curve analysis consisted of an increasing temperature of 0.5°C per min for 20 min from 60 to 95°C.

Scanning electron microscopy (SEM) imaging

The evaluation of coupons via scanning electron microscopy (SEM) was performed based on Clayborn et al.^[19] Briefly, coupons were attached to an aluminum specimen mount with a double-coated carbon conductive tab (Ted Pella Inc., Redding, CA, USA) and viewed with a Philips SL 30 ESEM (FEI Company, Hillsboro, OR, USA) in a low vacuum mode.

Statistical analysis

Plate counts were performed in duplicate, and the average and standard error log CFU per coupon were determined by averaging all biological replicates subjected to the same experimental conditions. A Student *t*-test ($P \leq 0.05$) was performed to compare differences with JMP[®] Genomics 7.0 (SAS Institute Inc., Cary, NC, USA).

Results

Effect of concentration on NaClO sanitizer efficacy on *S. Typhimurium*

Salmonella Typhimurium ATCC 14028 attached cells were exposed to 200 and 200,000 ppm NaClO treatments and the difference in lethality between concentrations in decreasing cell numbers was compared (Fig. 1). The *S. Typhimurium* exposed

to 30 s of 200 ppm NaClO sanitizer exhibited an average baseline cell population of 7.32 ± 0.14 log CFU recovered per coupon from unsanitized coupons and an average cell population of 4.59 ± 0.14 log CFU recovered from sanitized coupons for a log reduction of 2.73 ± 0.23 due to sanitizer activity. Application of 200,000 ppm sanitizer resulted in baseline cell populations of 6.92 ± 0.31 log CFU and sanitized treatment counts of 3.57 ± 0.31 log CFU for a reduction of 3.36 ± 0.54 log CFU per coupon due to sanitizer activity. The log CFU reduction values were significantly different between the two sanitizer concentrations ($P < 0.05$).

Response of serovar representative strains to PAA sanitizer efficacy

Salmonella Typhimurium ATCC 14028 and *S. Heidelberg* SL486 (parent and derivative marker strain) attached cells were exposed to 200 ppm PAA treatments and the efficacy of sanitizer was compared between the two serovars (Fig. 2). *S. Typhimurium* attached cells treated with 200 ppm PAA sanitizer for 30 s exhibited baseline plate populations of 7.56 ± 0.10 log CFU per coupon and sanitized coupon cell populations of 4.93 ± 0.13 log CFU for a log reduction of 2.62 ± 0.15 . The parent strain of *S. Heidelberg* attached cells exhibited unsanitized treatment population levels of 7.19 ± 0.27 log CFU per coupon and sanitized treatment population levels of 5.78 ± 0.13 log CFU for a log reduction of 1.41 ± 0.17 . The marker strain yielded an unsanitized treatment population level of 7.52 ± 0.12 log CFU per coupon, a sanitized treatment population level of 5.91 ± 0.14 log CFU per coupon, and a log reduction of 1.61 ± 0.08 . There was no significant difference in the reduction rate between parent and the marker strain ($P > 0.05$).

Salmonella confirmation by PCR

As a non-selective media was used, conventional PCR was used to confirm the presence of *Salmonella* on sanitized coupons as well as unsanitized coupons. Samples (PBS rinsates from bead exposed coupons) were confirmed to consist of *Salmonella*, regardless of sanitization treatment. Each aliquot successfully amplified a 423 bp region of the targeted gene from *Salmonella*.

Salmonella Heidelberg enumeration by quantitative PCR

The log CFU per coupon for samples before and after treatment with PAA (200 ppm) for 30 s was also evaluated using qPCR to confirm the cell number on each coupon. The efficiency and correlation coefficient (R^2) obtained from the standard curves were 96% and 0.998, respectively. Based on the qPCR analysis, the average log population of the parent strain was 6.12 ± 0.26 log CFU/coupon (unsanitized coupon) and 5.52 ± 0.22 log CFU/coupon (sanitized coupon) for PAA treatment (200 ppm for 30 s). In the case of the marker strain, before and after log populations with PAA treatment were 7.07 ± 0.21 and 6.26 ± 0.17 log CFU/coupon, respectively (Table 1).

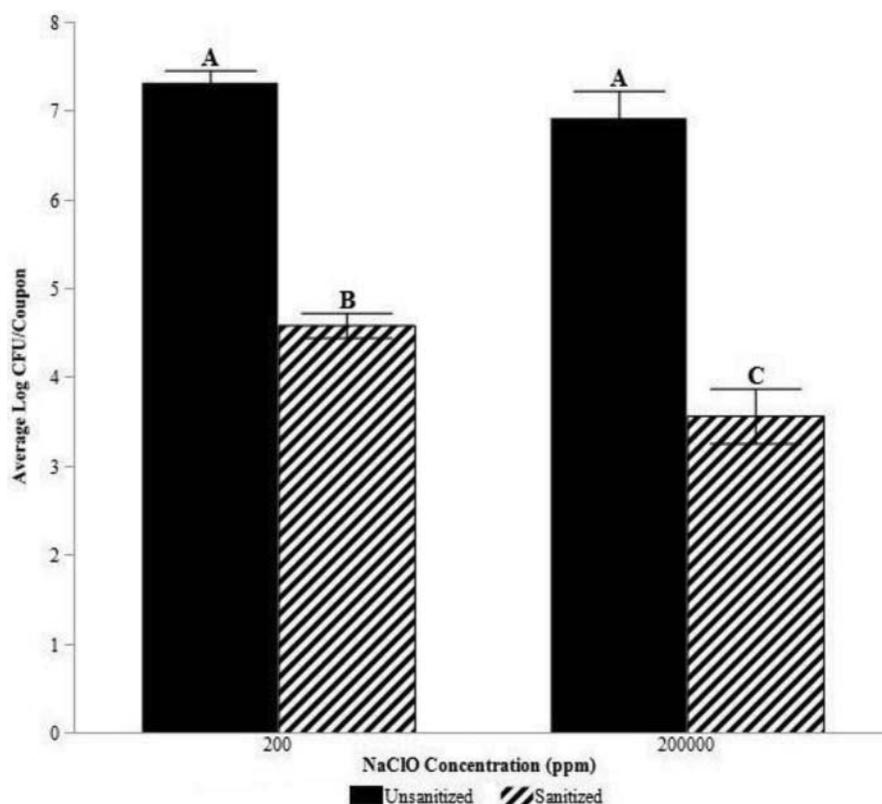


Figure 1. Average log CFU *Salmonella* Typhimurium ATCC 14028 cell populations recovered from unsanitized and sanitized coupons treated with 200 ppm and 200,000 ppm sodium hypochlorite (NaClO) ($n = 5$). Different letters above each bar indicate statistically significant differences between values ($P < 0.05$).

Scanning electron microscopy (SEM) images

Scanning electron microscopy images were generated from selected coupons throughout the experimental process. An inoculated *S. Typhimurium* coupon was examined by SEM to evaluate the topography of the coupons (Fig. 3A). Cells were shown to be attached following 72 h of growth (Fig. 3B) with the attached cell matrix revealed upon additional magnification (Fig. 3C). Some residues appeared to remain following sanitization with 200 ppm PAA (Fig. 3D).

Discussion

Both NaClO and PAA are representative sanitizers commonly used to disinfect equipment/utensil including plastic containers and other food contact surfaces in the food industry.^[27–30] Previous studies have investigated the efficacy of these sanitizers for removal of attached cells from a variety of possible food contact surfaces from stainless steel to concrete to the produce itself.^[18,31–33] These studies are difficult to compare due to the different materials tested as well as the contrasting experimental approaches that have been used among laboratories.^[18] However, based on the current study, usage of these sanitizers with protocols derived from industry standards^[34] appears to be insufficient for removal of all laboratory attached *Salmonella* cells from the RPCs (Figs. 1 and 2). Even when NaClO was applied at a level a thousand times greater (200,000 ppm) than the recommended concentration (200 ppm), the

reduction in *S. Typhimurium* population between treated and untreated samples was only increased by 0.63 log CFU. The ability of the attached *Salmonella* cells to persist even when exposed to such a high concentration highlights the necessity of maintaining clean containers and preventing attachment from occurring.

Some potential differences in sanitizer effectiveness between the strain of *S. Typhimurium* and the strain of *S. Heidelberg* were also observed. *S. Typhimurium* attached cells exhibited a tenfold greater reduction due to sanitizer than the *S. Heidelberg* cells (Fig. 2). This result suggests that there may be differences in sanitizer effectiveness against *S. Typhimurium* and *S. Heidelberg*. However, more strains of each serovar would need to be tested to confirm this as being a serovar difference. Different serovars of *Salmonella* are known to associate with different poultry products^[35] as well as exhibit differences in attachment to produce.^[31,36] This indicates that there may be differences between serovars due to adapting to different environments as González-Gil et al.^[37] found with different serovars showing differences in virulence gene responses while under acid stress. It is possible that this specific strain was particularly resistant to the activity of PAA which highlights the issue of different bacterial contaminants on RPCs possibly requiring different treatments. Thus, introduction of effective interventions or multiple hurdles in the sanitization stages with the use of several sanitizers with different modes of action may be necessary to eliminate attached cells from plastic shipping containers. These methods along with different bacterial strains and serovars combined as a mixture or cocktail of serovars would

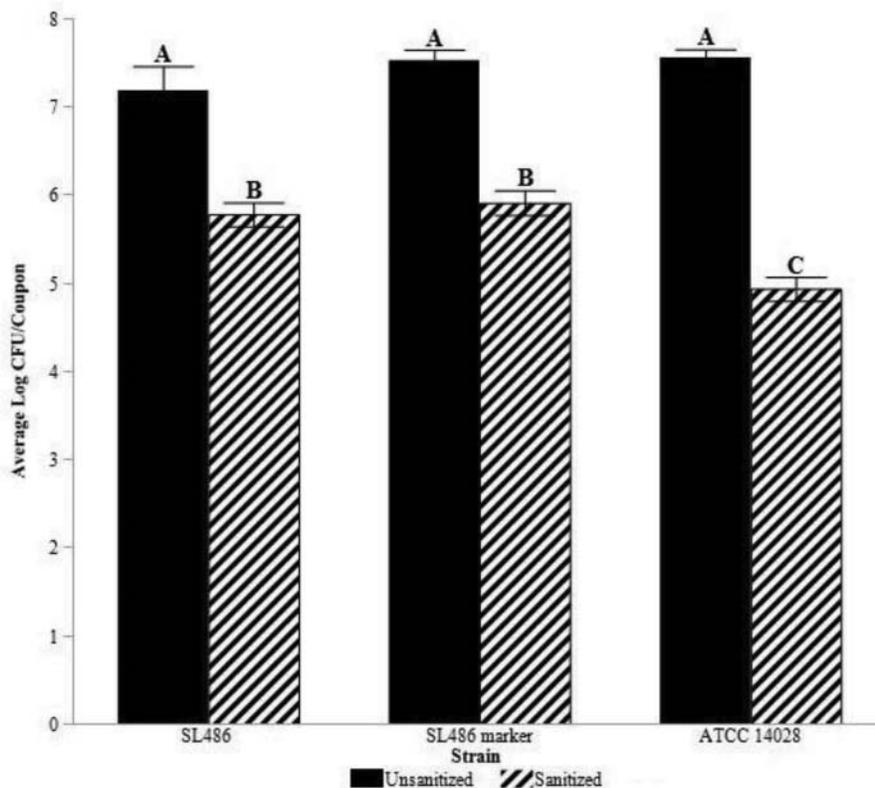


Figure 2. Average log CFU *Salmonella* Heidelberg SL486 (parent and marker) and *Salmonella* Typhimurium ATCC 14028 cell populations recovered from unsanitized and sanitized coupons treated with 200 ppm peracetic acid (PAA) ($n = 5$). Different letters above each bar indicate statistically significant differences between values ($P < 0.05$).

probably need to be employed for routine testing to ensure maximum efficacy of a corresponding sanitizer against a range of possible *Salmonella* responses.

In general, recovered populations were not significantly different between the two enumeration methods (conventional plating method and qPCR) indicating a high concordance between the two methods. Only one case (parent strain for unsanitized coupon) resulted in a minimal difference ($P = 0.02$). Some variations of means were observed; however, most of the data exhibited similar bacterial cell populations. Some variance of means was to be expected because the two methods are based on different targets, namely recovery of viable cells versus DNA.

The SEM images demonstrated that residues and bacterial cells remained after sanitizer treatment and mechanical agitation (Fig. 3). The SEM images revealed the extent to which these plastic materials are a potential reservoir for microbial contamination. Coupons appeared to be rough and worn after many cycles of use

thus providing a potentially better environment for *Salmonella* to attach. The coupons evaluated in this study consisted of a diverse topography with considerable variation in the surface characteristics. Any microbial populations that became embedded into these cracks could potentially escape the action of sanitizers and provide a reservoir, which after repeated contact with food products, may lead to consumer illness and a lower overall product yield. This is consistent with the previous surveys of RPC used in the field, where RPCs contained $> \log 5$ CFU/swab (9 out of 24 or 37.5% of the RPCs) and $> \log 6$ CFU/swab (2 out of 24 or 8.3%).^[20] These results suggest that RPC surfaces could play an important role in cross-contamination of bacteria to the corresponding food products transported in RPCs. RPCs which escape full cleaning where dirt and organic matter remain may protect any organisms which are attached. Nyeleti et al.^[38] observed that *Salmonella enterica* appeared to survive better against ultraviolet radiation treatment on stainless steel surfaces after coating with bovine serum albumin. In the current study, treatment with 200 ppm NaClO and PAA caused an average of 2.73 and 2.62 log CFU of *S. Typhimurium*, respectively. For *S. Heidelberg*, only a 1.41 and 1.61 log reduction was obtained with 200 ppm PAA. If an RPC contains more than 3 log CFU/coupon of the respective pathogenic bacteria, they may not be entirely eliminated with current industry methods for sanitizing containers. Indeed, the attached *Salmonella* cells were recovered with standard plating methods and confirmed visually upon SEM examination.

In summary, it appears that standard food contact surface sanitizers may be insufficient for eradicating microorganisms from certain food equipment surfaces,^[18] and that these

Table 1. Quantitative PCR and plate log population comparisons of *S. Heidelberg* parent and marker strains on reusable plastic container (RPC) coupons.

| Strain | Unsanitized coupon | | Sanitized coupon* | |
|---------------|--------------------------|--------------------------|-------------------|-------------|
| | Plating | qPCR | Plating | qPCR |
| Parent strain | 7.19 ± 0.27 ^a | 6.12 ± 0.26 ^b | 5.78 ± 0.13 | 5.52 ± 0.22 |
| Marker strain | 7.52 ± 0.12 | 7.07 ± 0.21 | 5.91 ± 0.14 | 6.26 ± 0.17 |

*Treated with 200 ppm of peracetic acid (PAA) for 30 s.

Results are expressed as the mean ± standard error ($n = 5$).

^{a-b}Different letters indicates significant difference in recovered population between two enumeration methods within each strain ($P < 0.05$).

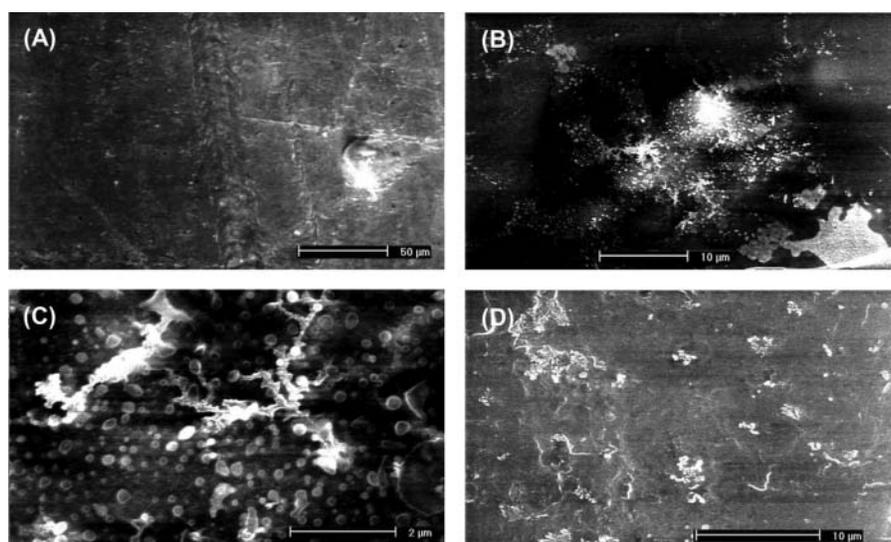


Figure 3. Scanning electron microscopy images of (A) an uninoculated reusable plastic containers (RPC) coupon, (B) attached cell prior to sanitization, (C) attached cell at a higher magnification, and (D) a RPC coupon post sanitization with 200 ppm peracetic acid (PAA) and attached cell removal.

surfaces should be evaluated to develop proper risk assessments and subsequently reduce foodborne illness. Due to the nature of fresh produce, contamination may enter a supply system and increase the likelihood of cross contamination while in storage and during transportation if microorganisms are allowed to attach to containers.^[21,39-41] The attachment of bacterial cells on food contact surfaces such as RPCs is especially concerning from a food safety aspect for food items such as fresh produce as these foods do not undergo a heat treatment step during preparation that occurs in other food products such as meat.^[42] However, predictive modeling and risk assessment may be difficult for these shipping containers due to the variability of the RPC surfaces among a set of containers which may affect attachment and cleaning efficacy. Future studies should compare the level of bacterial cell attachment and sanitizer efficacy against completely new RPCs versus RPCs after different cycles of reuse. Future research must also focus on a variety of conditions that mimic fluctuating environmental conditions such as those brought about by temperature or humidity due to seasonal changes.^[42] The environments in which the containers are exposed should also be assessed to determine factors (high-risk areas, environmental contamination, among others) that may impact container handling equipment as well as contamination occurring during transportation and microbial interactions since many factors can contribute to cell attachment.^[11,18] Additionally, research should be performed to examine various aspects of transfer rates from attached cells on shipping containers to fresh produce. Finally, field studies to determine the prevalence of foodborne pathogens before and after sanitation are needed to assess the frequency and potential risk.

In conclusion, we demonstrated that qPCR could offer a reasonable estimate to quantify *Salmonella* populations attached on RPC when compared with standard plating methodology. The distinct attribute of qPCR is that it represents a much more rapid quantification method (requiring time for whole process: < 4 h) since no further confirmation step is needed. This may be important for the produce and food industry to routinely screen the contamination levels of bacteria to assure

limited exposure to cross contamination of their products during manufacturing, transportation and distribution. Thus, it appears that molecular quantification can be utilized for the rapid quantification of *Salmonella* and other foodborne pathogens attached on RPC and may also be helpful for maintaining hygienic quality of RPC as well as food safety of products which are in contact with RPCs.

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Author contributions

Zhaohao Shi, Christopher A. Baker, and Sang In Lee performed experiments, drafted the manuscript, collected test data, and analyzed the data. Sun Ae Kim revised the manuscript. Zhaohao Shi, Si Hong Park, and Steven C. Ricke designed the study and revised the manuscript.

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