Reduction in Microbial Survival on Food Contact Surfaces by a Spray Coated Polymerized Quaternary Ammonium Compound

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To be sent to Journal of Food Safety for publication

Abstract

Using polymerization and immobilization techniques, the loss of antimicrobial efficacy of a quaternary ammonium compound (QAC) can be minimized by decreasing its solubility and crosslinking it to metal substrates. The survivability of *Listeria innocua* and *Escherichia coli* K12 inoculated to silane QAC coated metal surfaces were compared with uncoated metal surfaces at different treatment conditions for up to 6 months storage. The resilience of the coating material to repeated cleaning procedures, up to 20 washing and rinsing cycles, was also investigated. No evidence of bacteria viability (>5 log reduction of colony forming unit) was observed for *L. innocua* when they were inoculated onto coated surfaces stored for 3 months, whereas *E. coli* was reduced by 3 to 4-logs. For the viable *L. innocua* cells on the coated surfaces, >5 log reductions were achieved even after the coated metal surfaces were cleaned by 20 washing and rinsing cycles prior to the cells inoculation. For the *E. coli* cells, they were not affected much (<1 log reduction) by the coated surfaces after 10 or more cleaning procedures were applied. Overall, the results showed that the coating had potential antimicrobial activity against Gram positive bacteria but it showed moderate activity to Gram negative bacteria.

Introduction

Cross-contamination with undesirable microorganisms during food preparation was identified in many reports as a major factor associated with foodborne illnesses and spoilage. In food processing facilities, reduced survivability of these organisms on food contact surfaces would help to mitigate the risk of subsequent microbial transfer (Kusumaningrum et al. 2003; Podolak et al. 2010). Therefore, various chemical sanitizing agents have been used to prevent the spread of microbial contaminants on various food contact surfaces. Among those sanitizing agents, oxidizing chemicals such as hypochlorites, chlorine dioxide, peracetic acid, hydrogen peroxide, ozone, and aldehydes are widely used on food preparation surfaces since they strongly inactivate microorganisms by inducing chemical oxidation of the cellular components in the organisms (Cortezzo et al. 2004). Used at the correct concentration/time/temperature combination on food contact surfaces with moderate organic matter and bacterial loads, these oxidizing agents have shown the ability to reduce bacterial numbers to levels acceptable to the food processing industry. However, some of these agents are corrosive to equipment at the concentrations required for microbial inactivation and they produce harmful and/or unknown byproducts when they cause the oxidation of organic matter. Furthermore, studies have showed that these chemicals have the potential to produce carcinogenic free radicals (Langlais et al. 1991; Bull, & Cotruvo. 2011). On the other hands, sanitizing agents containing quaternary ammonium compounds (QAC) are relatively safe within prescribed limits and cause no problems to humans. Unlike oxidizing chemicals, QAC are solid compounds that can be easily dissolved and they do not readily evaporate. Therefore, QAC are considered to be an excellent candidate for the polymerization of antibiotics which have prolong activity. Additionally, since the cellular membranes of most bacteria are

64 negatively charged, they are very sensitive to cationic sanitizers such as QAC (Chen & Cooper
65 2002).

66 On food contact surfaces, the efficacy of an antimicrobial agent incorporated into a
67 biopolymer is mainly related to the good film forming property of the polymer and its ability to
68 sequester and subsequently release the antimicrobial agent. Many techniques can be used to attach
69 antimicrobial polymers to food contact surfaces using electrostatic or exclusion steric repulsion
70 mechanism, and thus prevent microbial cells from attaching to these surfaces (Tiller 2010).
71 However, since major microbial contamination in food processing facilities occurs from food
72 sources, organic matter such as food particles have helped to shield contaminating bacteria from
73 the direct repelling activity of antimicrobial biopolymers. Therefore, using innovative
74 polymerization and immobilization techniques, our study was conducted to preserve the
75 antimicrobial efficacy of a sanitizing agent by decreasing its solubility and crosslinking it to food
76 contact surfaces. The active ingredient was 3-trihydroxysily-propyldimethly-octadecly ammonium
77 chloride and the active antimicrobial agent was a silane quaternary ammonium (silane QAC) salt.
78 Therefore, the actual structure was comprised of silane, nitrogen and carbon atoms. These
79 ingredients were certified by the EPA (2016) and allowed to be used on food contact surfaces. The
80 surface coating was developed and applied as a silane QAC salt in a spray format. It was an
81 electrostatic spray application which atomized and applied a positive charge to the droplets as they
82 come out of the nozzle of the sprayer. The droplets come out at 30-60 microns which was roughly
83 900 times smaller than conventional sprayers. This positive charge allowed the solution to create
84 a cohesive 360 degree coverage on the targeted substrates, which upon drying, created a long-term
85 resilient coating on the surface. This unique bonding characteristic allowed the silane QAC salt to
86 remain on the surface even under harsh conditions.

87 The main object of this study was to investigate the growth and/or survival of foodborne
88 microorganisms transferred to coated when compared with uncoated metal surfaces at different
89 environmental conditions. The antimicrobial resilience of the coating material to repeated cleaning
90 procedures, up to 20 washing and rinsing cycles, was also studied. *Listeria innocua* (Gram-positive
91 bacteria) and *Escherichia coli* K12 (Gram-negative bacteria) were tested as surrogates for *Listeria*
92 *monocytogenes* and *Escherichia coli* O157:H7, respectively. The use of surrogates has been
93 proven to be a practical alternative for foodborne pathogen testing (Moce-Livina et al. 2003).

94

95 **Materials and Methods**

96 **Metal surface fabrication**

97 The test metal surfaces were 5 cm x 2.5 cm pieces of stainless-steel sheets for testing and
98 storage at room temperature (23^{o}C). One set of the metal sheets received one coat of the silane
99 QAC and the other set was uncoated. For the coated samples, once the metal surfaces were
100 sterilized, the QAC solution was applied to them and allowed to dry for thirty minutes prior to
101 storage. After drying, the average thickness of the coated layer on each metal sheet was <5 nm.
102 The samples were then stored at 23^{o}C for up to six months.

103 **Bacteria Preparation**

104 *Listeria innocua* Seeliger (ATCC 33090) and *Escherichia coli* K12 (ATCC 29181) cells
105 were separately grown on Tryptic soy agar (TSA) slants and then transferred to Tryptic soy broth
106 (TSB), followed by incubation at 37^{o}C for 21 h. The final cell concentrations in the broth were 9-
107 10 log colony forming units (CFU)/ml. The cells were then harvested by centrifugation (Sovall® RC5C Plus, Newtown, CT) at 10,000 g for 10 min at 4^{o}C. The supernatant was decanted, and the

pelleted cells were re-suspended in 20 ml of sterile phosphate buffer solution (PBS, pH 7.2) to obtain viable cell populations of approximately 9 log CFU/ml. The cell suspensions were serially inoculated into sterile PBS and mixed to give the desired initial numbers.

Antimicrobial activity testing

An industrial antimicrobial method (ISO 22196) was used, with modifications, to evaluate the efficacy of the test metal sheets. At each testing period (after 0, 3, and 6 month storage), each metal sheet was placed in separate sterile Petri dishes with the test surface uppermost. A 0.1 ml aliquot of bacterial cell suspension (~10^6 CFU/ml) in a 1/500 dilution of nutrient broth was inoculated onto both metal sheet surfaces (coated and non-coated). Each sample was covered with a piece of cleaned film measuring 20 mm × 20 mm and gently press down so that the bacterial inoculum could spread to the edges of the sample. All samples were stored in sealed jars at 23 ± 1^{o}C (RT) and cold temperature (10 ± 1^{o}C, CT) at ~95 ± 2% relative humidity (RH) for 24h. The survivors were collected using the hygiene swab method and transferred to test-tubes containing diluent water and neutralizing chemicals. For the plate counting method, tryptic soy agar was used as the growth medium for enumeration of the bacteria survivors.

The resilience of the coating to repeated cleaning

After two months of storage, one set of coated metal sheets was cleaned by washing (horizontally for three times + vertically for three times by scrubbing with a heavy duty sponge) in a washing sink containing soft tap water (43^{o}C, pH ~7.9) and 100 ppm Monsoon detergent (Ecolab Inc., St. Paul, MN), rinsed for 10 s by dipping into fresh soft tap water, then air dried for 1 h. The process was repeated up to 5, 10, 15, and 20 times prior to the bacterial inoculation. Following the bacterial enumeration procedures described in the antimicrobial activity testing

section, the efficacy of the coated sheets was compared with the result obtained for the unwashed (0 cycle) coated sheet.

Microbiological and statistical analysis

The detection limit for the test organisms were 2 CFU/surface for the hygiene swab method. No less than three replicates were tested in each experiment. Variances of the microbial viability were analyzed by equal-variance testing at a significance level of $P<0.05$.

Results and Discussion

Survival of the microorganisms on the coated metal surfaces

The effectiveness of the sprayed coating material for the reduction of viable bacterial cells attached to the metal sheets is shown in Fig 1. for *L. innocua* and in Fig. 2 for *E. coli*. After less than a week (0 month) of storage at room temperature (23^{o}C) for the sprayed metal sheets, almost no evidence of the bacterial viability (>5 log reduction of CFU) was observed for *L. innocua* when the cell suspension was inoculated onto the coated surface for 24 h. For *E. coli*, it was reduced by 3 to 4-logs. It is generally believed that impermeability of the outer membrane in the cell wall of Gram-negative bacteria such as *E. coli*, does not allow many types of chemical agents to reach the inner cytoplasmic membrane (Sundheim, Langsrud, Heir, & Holck, 1998). Previous studies also observed that Gram negative bacteria were less sensitive than Gram positive bacteria when QAC based sanitizers were applied to inactivate microbial contaminants on the surfaces of various table-ware items (Lee et al. 2007; Handojo et al. 2009). The viable cell numbers of the inoculum on the uncoated (control) surfaces remained the same after each test trial for both organisms. Also, there

was no significant difference ($P > 0.05$) in the viability of the microbial numbers found on the coated surfaces after been treated for 24 h at cold (10^{o}C, CT) and room temperature (23^{o}C, RT) conditions for each bacterial strain. This indicated that the antimicrobial compounds in the coating were not significantly weakened at the cold testing temperature. Previous food preservation studies have also shown that the antimicrobial activities of coating materials based on organic chemical compounds, were not affected by relatively low ambient temperatures (<10^{o}C) that are maintained at some food processing and preservation facilities (Campaniello et al, 2008; Sogvar et al., 2016).

When the coated metal sheets were tested after three months storage, their efficacies for cell reductions of both bacterial stains remained the same as the results obtained from the metal sheets at 0 month storage (Figs1a,b; Figs 2a,b). Although the mean reductions of the *E. coli* cell viability were slightly greater than the reductions obtained at 0 month storage, the disparities were not significant ($P< 0.05$). The results suggested that although the thickness of the coated layer was thinner than that of conventionally sprayed coatings, the active compounds remained on the surface without loss of their antimicrobial properties during the three-month storage period.

Six months of storage for the coated samples significantly lowered the antimicrobial properties of the compounds against both bacterial strains. It was shown that after treatment for 24h, less than a two log reduction of the *L. innocua* viability was achieved for the samples tested at CT condition, while ~4 log reductions were observed at RT condition (Fig. 1c). The results implied that temperature conditions at testing can be a significant factor when longer term storage of coated samples are to be used for psychrotropic or cold tolerant microorganisms like *L. innocua*. For the *E. coli*, <1 log reduction (<90% reduction) was observed on the six-month old coated surfaces at both testing temperatures.

Determination of the resilience of the coating material to repeated cleaning procedures

The typical manual-cleaning procedure (washing, rinsing) was used to clean the coated metal sheets at each cleaning cycle. This procedure has been generally applied in food preparation facilities to remove undesirable organic matters on food contact surfaces prior to sanitation (Lee et al., 2007; Handojo et al., 2009). The bacterial survivabilities after repeated cleaning procedures on the coated metal surfaces (without additional spray) are shown in Figures 3 (for *L. innocua*) and 4 (for *E. coli*), respectively. For the viable *L. innocua* cells on the coated surfaces, very small numbers were detected even after the coated metal surfaces were cleaned by 20 washing and rinsing cycles. On the other hand, the *E. coli* cells were not affected much (<1 log reduction) by the coated surfaces after 10 or more cleaning procedures were applied.

As can be seen in the first part of this study, *E. coli* showed much stronger resistance to the coating compounds during each cleaning cycle when compared with *L. innocua*. The results are not surprising since Gram negative bacteria (such as *E. coli* strains) are protected by an additional cell membrane called "The outer membrane (OM)" which acts as an intrinsic barrier to hydrophobic antimicrobial agents such as QAC (Helander et al., 1999, Bore et al., 2007). Since many spoilage and potential pathogens in food processing facilities belong to the Gram negative bacteria group, many studies have been conducted on how to best destroy or disintegrate their OM structure. In the OM structures, lipopolysaccharide (LPS) molecules in the outer leaflet of the membrane are considered to create resistance to hydrophobic antimicrobial agents (Nikaido, 2003). The LPS is composed of three parts; lipid A, O-antigenic polysaccharides and a core oligosaccharide. Previously, studies were conducted to weaken the OM using permeabilizers,

196 which induced damage to the LPS layer. This then allowed an increase in the permeability of the
197 OM to hydrophobic agents. In the use of mechanical methods, Lee and Kaletunc (2009) used high
198 hydrostatic pressure for mechanical disintegration of the OM of Gram negative *Salmonella*
199 *enteritidis,* then observed increasing sensitivity to nisin. They also examined the disintegration of
200 the LPS component of the bacteria using differential scanning calorimetric (DSC) analysis. In the
201 case of chemical permeabilizers, compounds such as EDTA have shown promising results. Also,
202 undissociated forms of weak acids such as benzoic, citric, lactic, sorbic and acetic acids have been
203 considered as good permeabilizers against Gram negative bacteria since the undissociated forms
204 of the acids can pass through both outer and inner cell membranes and consequently lead to cell
205 death (Hirshfield et al., 2003).

206 The next phase of our study will be to reformulate the coating material to incorporate either
207 chelating agents such as EDTA or weak acids, so that it could be more effective in inhibiting the
208 growth of Gram negative bacteria such as *E. coli*.

209

210 **Conclusion**

211 Overall, the results of our study showed that the silane QAC coating technology has potential
212 antimicrobial activity against Gram positive bacteria even in the presence of repeating washing
213 but it showed moderate activity to Gram negative bacteria. Since gram negative bacteria are
214 protected by an additional cell membrane (called the outer membrane) against chemical
215 antimicrobial agents, it will be necessary to modify the technology by using an alternative chemical
216 approach. This approach is currently being developed.

217

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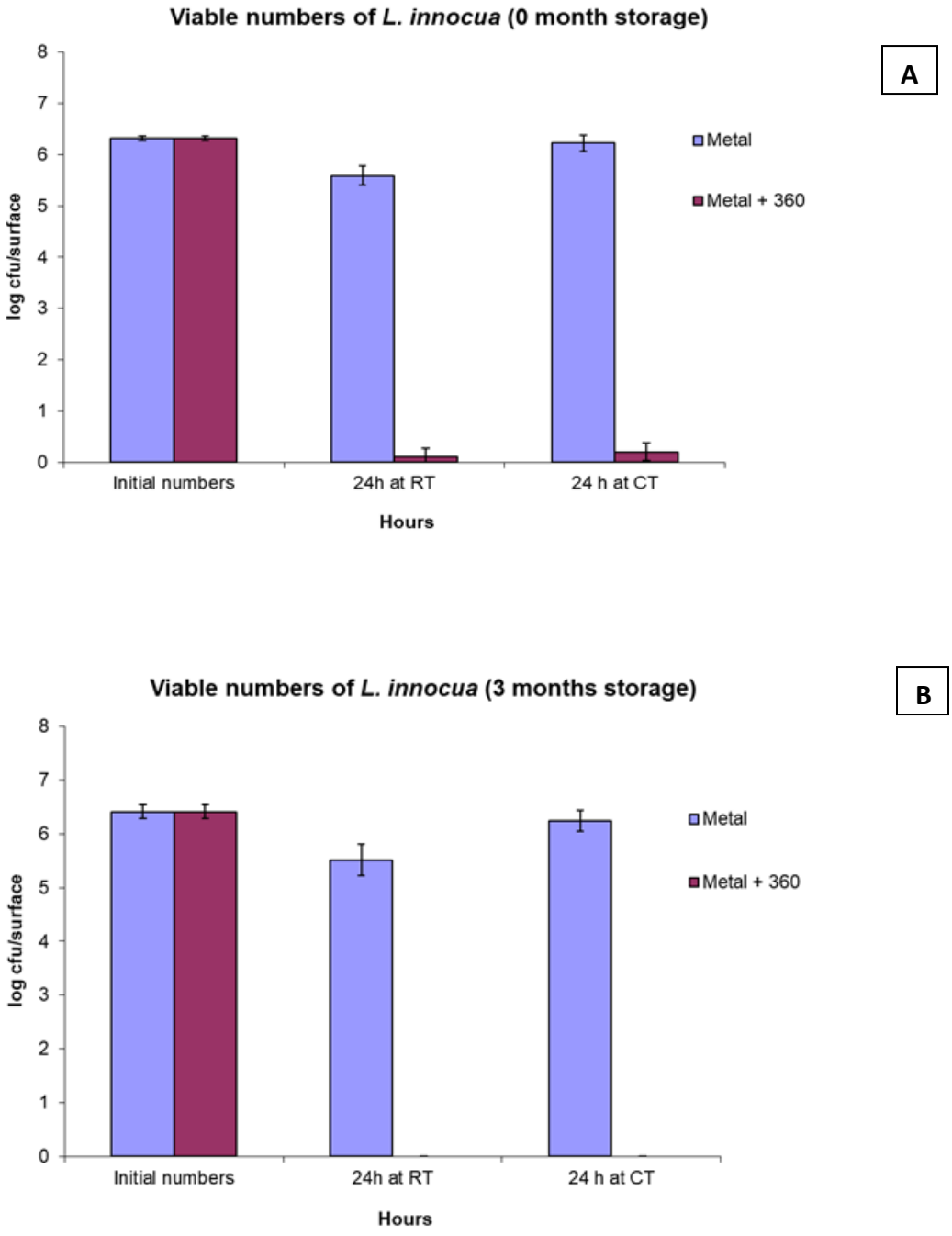
Figure 1.

c)


Viable numbers of L. innocua (0 month storage)
A
log cfu/surface
8
7
6
5
4
3
2
1
0
Metal
Metal + 360
Initial numbers
24h at RT
24 h at CT
Hours
Viable numbers of L. innocua (3 months storage)
B
log cfu/surface
Metal
Metal + 360
Initial numbers
24h at RT
24 h at CT
Hours

c

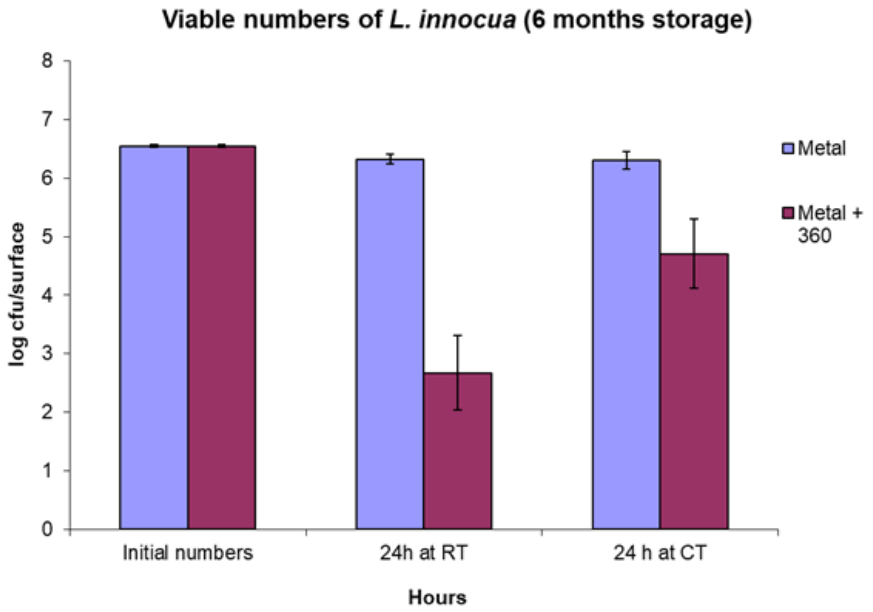
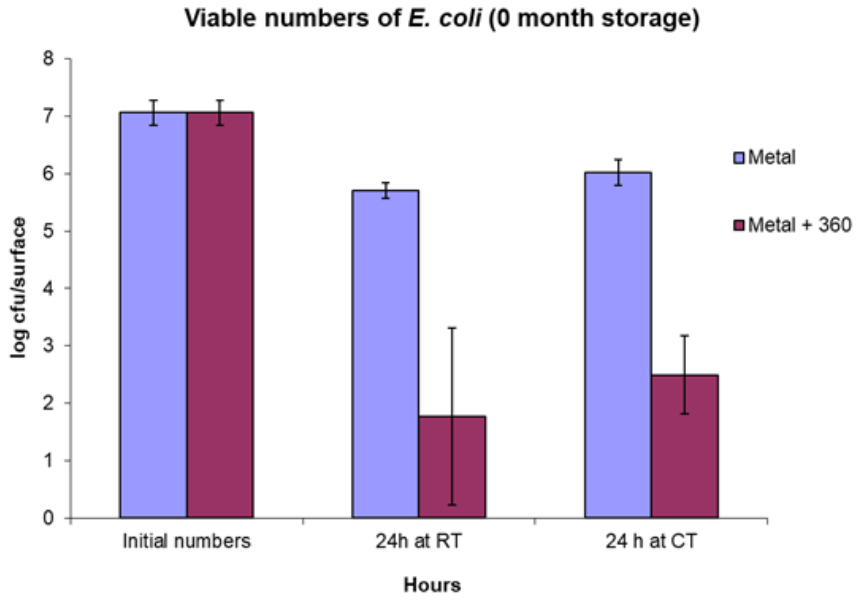
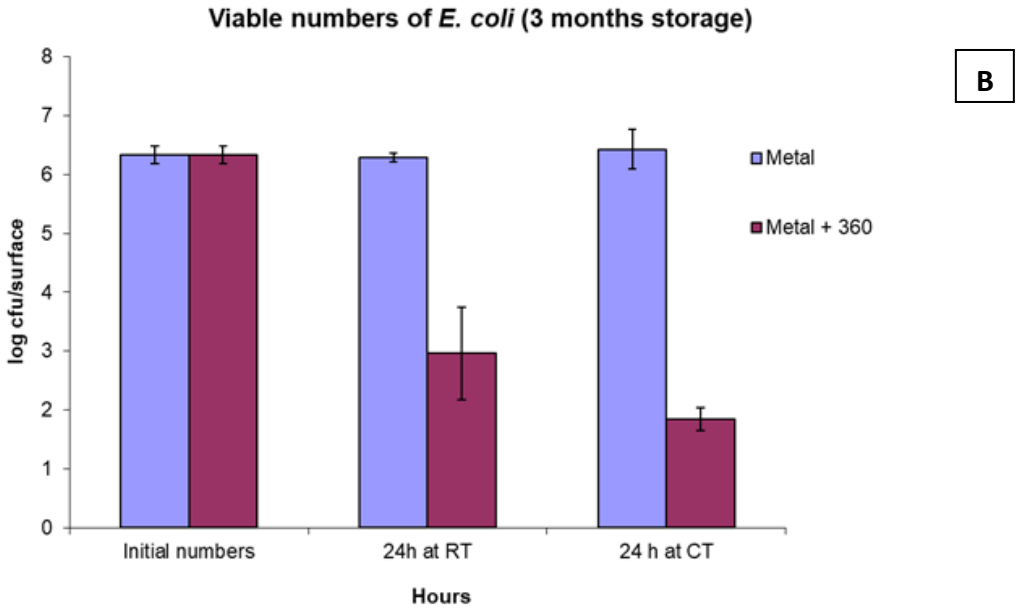
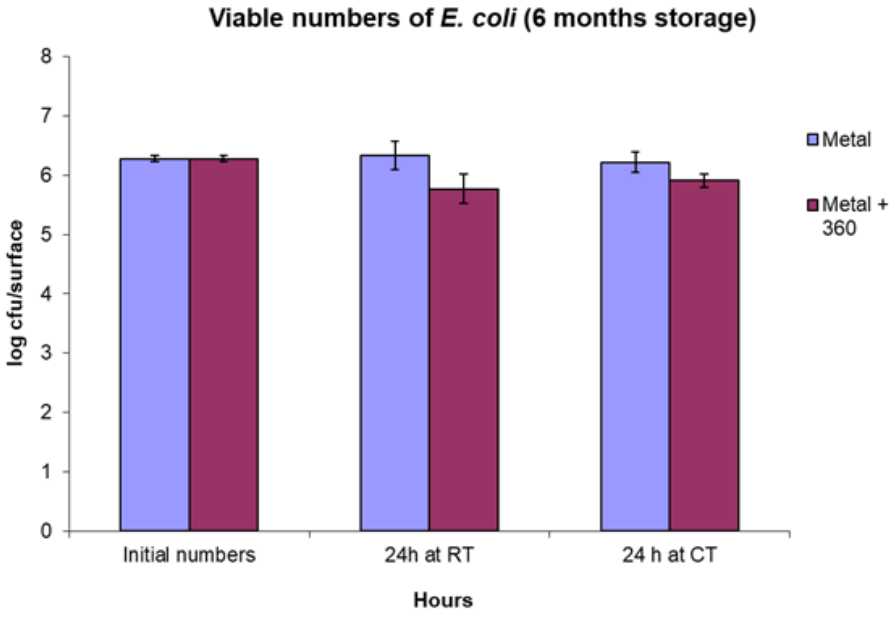

Viable numbers of L. innocua (6 months storage)
log cfu/surface
0
1
2
3
4
5
6
7
8
Initial numbers
24h at RT
24 h at CT
Hours
Metal
Metal + 360

Figure 2.

c)


Viable numbers of E. coli (0 month storage)
A
log cfu/surface
0
1
2
3
4
5
6
7
8
Initial numbers
24h at RT
24 h at CT
Hours
Metal
Metal + 360


Viable numbers of E. coli (3 months storage)
B
log cfu/surface
0
1
2
3
4
5
6
7
8
Initial numbers
24h at RT
24 h at CT
Hours
Metal
Metal + 360


Viable numbers of E. coli (6 months storage)
log cfu/surface
8
7
6
5
4
3
2
1
0
Initial numbers
24h at RT
24 h at CT
Hours
Metal
Metal + 360

C

Figure 3.

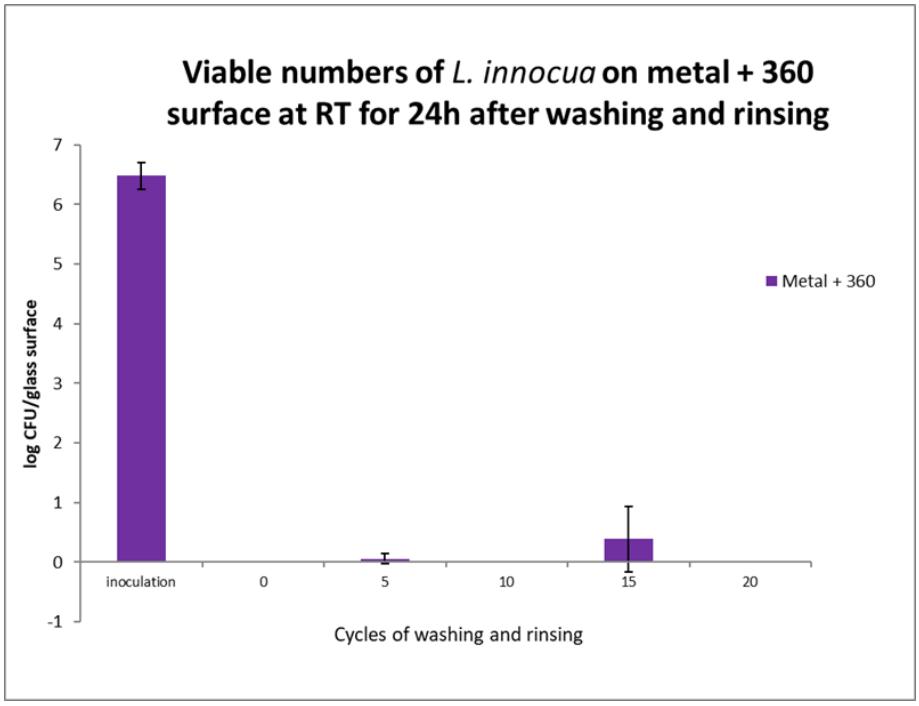
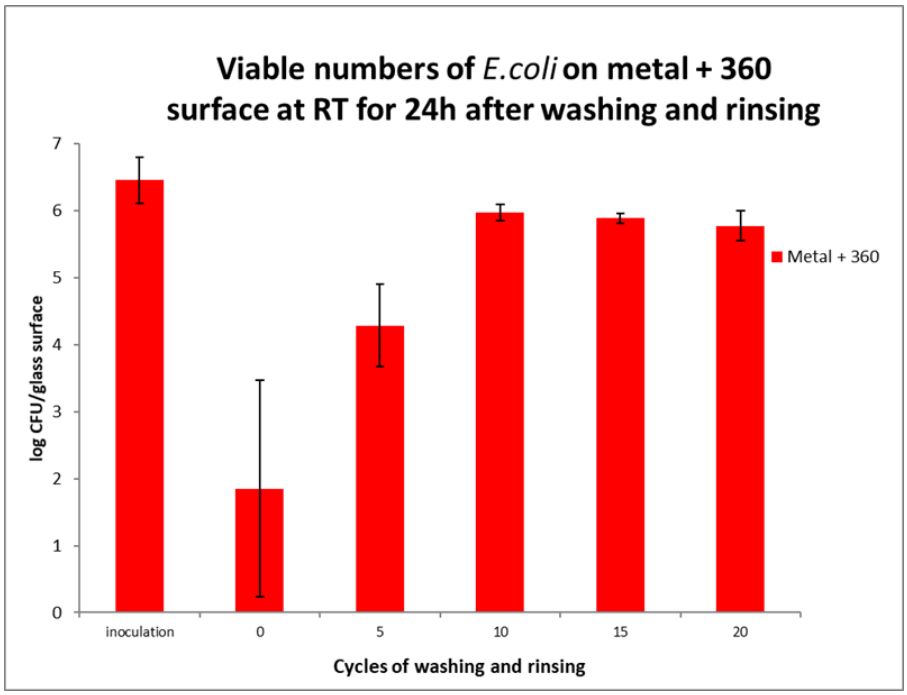

Viable numbers of L. innocua on metal + 360 surface at RT for 24h after washing and rinsing
log CFU/glass surface
7
6
5
4
3
2
1
0
-1
inoculation
0
5
10
15
20
Cycles of washing and rinsing
Metal + 360

Figure 4.


Viable numbers of E.coli on metal + 360 surface at RT for 24h after washing and rinsing
log CFU/glass surface
0
1
2
3
4
5
6
7
inoculation
0
5
10
15
20
Cycles of washing and rinsing
Metal + 360