Sanitizer Application for Biofilm Removal

Objectives:

- Determine cocktail/culture concentration
- Biofilm formation on stainless steel coupons
- Sanitizer application and evaluation
- CryoSEM confirmation for presence of biofilms and impact on biofilm removal following treatment

Culture concentration:

The strains of bacteria used in this study were *Salmonella enterica* s.v Typhimurium, *Listeria monocytogenes* and *E. coli* O157:H7. *E coli* O157:H7 and *Salmonella* Typhimurium were grown in LB broth and *Listeria monocytogenes* was grown in BHI broth to a final concentration of 10^8 to 10^9 CFU/ml after 24 hours of incubation with rotation at 37°C. Liquid cultures were washed three times with 0.1 M phosphate buffer, pH 7.0 (PB) to remove the growing medium by centrifuging the samples (6,000 rpm, 5 min). The bacteria were resuspended in PB and mixed to form a cocktail of similar concentration of each bacterium for biofilm formation.

Biofilm formation and detachment:

For biofilm formation on stainless steel coupons, multi-well plates were filled with 1 mL of inoculum mix culture and the stainless-steel coupons were placed into each well (Figure 1). Plates were incubated with shaking at 50 rpm at 32°C for 5 days, adding 0.5 mL of LB broth every 24 hours. The coupons were removed from the multi-well plate and washed with 1 mL of distilled water to remove any unattached cells. The coupons were then dried at room temperature for 5-10 minutes. To remove the biofilm from the stainless-steel coupons, each coupon was immersed into sterile tubes containing 1 mL PB, sonicated for 5 minutes, and then vortexed for 20 seconds. The samples were serially diluted using PB and spread plated on selective media for *E. coli* (MacConkey Agar), *Salmonella* (XLT4 Agar) and *Listeria* (Modified Oxford Medium). The bacteria were enumerated following incubation at 37°C for 48 hours.

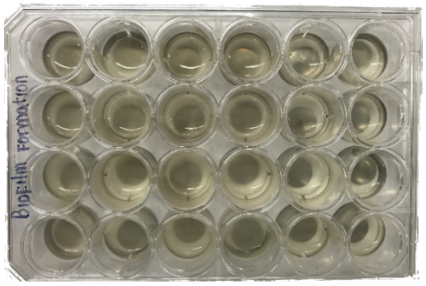

Figure 1. Multi-well plate used for biofilm formation of *E. coli, Listeria and Salmonella* on stainless steel coupons.

Table 2. Enumeration of bacteria (CFU/mL; SD = Standard Deviation) from stainless steel coupons in phosphate buffer.

BIOFILM FORMATION ON STAINLESS STEEL COUPONS			
Replicate	***E. coli***	***Salmonella***	***Listeria***
1	4.00E+07	1.00E+07	5.00E+06
2	8.00E+07	7.00E+07	2.00E+06
3	6.00E+07	5.00E+07	7.00E+06
4	3.00E+07	1.00E+07	5.00E+06
5	1.10E+08	2.00E+07	1.00E+07
AVERAGE	***6.40E+07***	***3.20E+07***	***5.80E+06***
SD	***2.87E+07***	***2.40E+07***	***2.64E+06***

Table 3. Enumeration of the bacteria in biofilms (CFU/mL; SD = Standard Deviation) from stainless steel coupons after water application for 10 minutes.

WATER TREATMENT CONTROL (PHOSPHATE)			
Replicate	***E. coli***	***Salmonella***	***Listeria***
1	5.00E+05	3.00E+04	3.00E+04
2	2.00E+05	2.00E+05	1.00E+05
3	3.00E+05	3.00E+05	1.00E+05
4	1.00E+05	1.00E+05	2.00E+04
5	3.00E+04	2.00E+05	1.00E+05
AVERAGE	***2.26E+05***	***1.66E+05***	***7.00E+04***
SD	***1.65E+05***	***9.29E+04***	***3.69E+04***
LOG reduction	***2.45***	***2.29***	***1.92***

Table 3 represents the concentration of bacteria after a 10-minute water application to remove biofilm. The water treatment achieved an ~2 log reduction in the concentration of viable *Salmonella, E. coli* and *Listeria* cells that were present on the stainless steel coupon.

Sanitizers application

After biofilm formation, the stainless-steel coupons were immersed into each sanitizer. Enviro Tech Perasan A and Perafoam was applied at room temperature for 5 minutes at three different concentrations. The first treatment had a final concentration of 500 ppm PAA (2,321 ppm hydrogen peroxide, and 16,200 ppm Perafoam); the second treatment was 2,000ppm PAA (9,109ppm hydrogen peroxide and 8,200ppm Perafoam); and the third treatment was 3,000ppm PAA solution, (13,663ppm hydrogen peroxide and 8,200ppm Perafoam). A final set of coupons containing bacterial biofilms were exposed to the Sterilex sanitizer (equal volumes of Solution 1 and Solution 2 (12.8 ounces) in 1 gallon of water) for 10 minutes at room temperature. To remove the biofilm from the stainless-steel coupons following treatment, each coupon was immersed into sterile tubes containing 1mL PB and sonicated for 5 minutes and vortexed for 20 seconds. The samples were serially diluted using PB and spread plated on selective media for *E. coli* (MacConkey Agar), *Salmonella* (XLT4 Agar) and *Listeria* (Modified Oxford Medium). The bacteria were enumerated following incubation at 37°C for 48 hours.

Table 4. Bacterial concentration after 5 minutes of Perasan and Perafoam application (500ppm PAA).

ENVIRO TECH	***PERASAN+PERAFOAM***		
Replicate	***E. coli***	***Salmonella***	***Listeria***
1	0.00E+00	0.00E+00	0.00E+00
2	0.00E+00	0.00E+00	0.00E+00
3	0.00E+00	0.00E+00	0.00E+00
4	0.00E+00	0.00E+00	0.00E+00
5	0.00E+00	0.00E+00	0.00E+00
AVERAGE	***0.00E+00***	***0.00E+00***	***0.00E+00***
SD	***0.00E+00***	***0.00E+00***	***0.00E+00***
LOG red NTC	***7.81***	***7.51***	***6.76***
LOG red WTC	***5.35***	***5.22***	***4.85***

No viable bacteria were detected following treatment with 500 ppm PAA (2,321 ppm hydrogen peroxide, and 16,200 ppm Perafoam; Table 4). Therefore, application of treatment 2 and 3 (2000 and 3000 ppm of PPA, respectively) were focused on only the cryo-SEM imaging for examination of the extent of biofilm removal (SEM images below).

Table 5. Bacterial concentration after Ultra Disinfectant (phosphate buffer) application.

STERILEX	***ULTRA DISINFECTANT***		
Replicate	***E. coli***	***Salmonella***	***Listeria***
1	0.00E+00	0.00E+00	0.00E+00
2	0.00E+00	0.00E+00	0.00E+00
3	0.00E+00	0.00E+00	0.00E+00
4	0.00E+00	0.00E+00	0.00E+00
5	0.00E+00	0.00E+00	0.00E+00
AVERAGE	***0.00E+00***	***0.00E+00***	***0.00E+00***
SD	***0.00E+00***	***0.00E+00***	***0.00E+00***
LOG red NTC	***7.81***	***7.51***	***6.76***
LOG red WTC	***5.35***	***5.22***	***4.85***

No viable bacteria (*Salmonella* Typhimurium*, E. coli* O157:H7, or *Listeria monocytogens*) were recovered following treatment with Ultra Disinfectant for 10 minutes at room temperature.

CryoSEM Images:

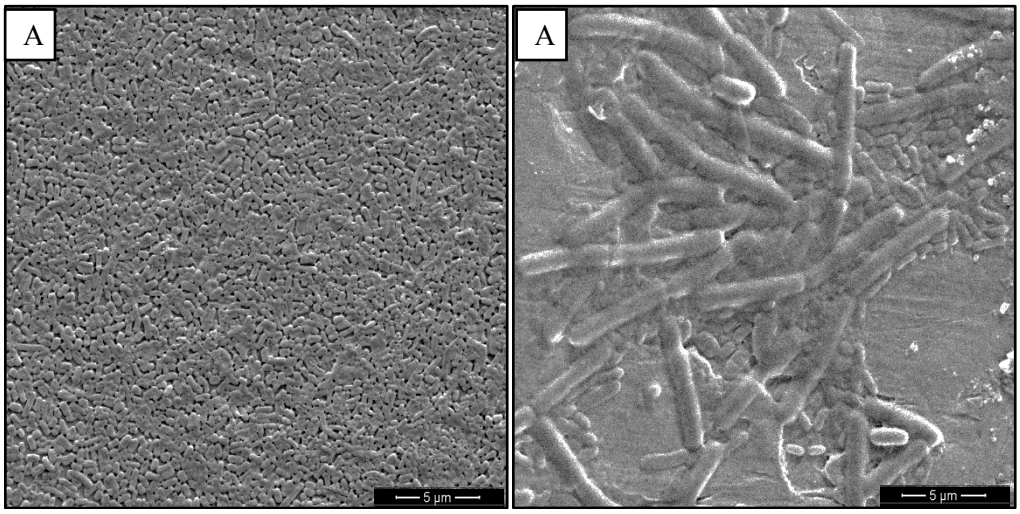

Fig 2. Representative cryo-SEM Images of biofilm formation on stainless steel panels (NTC).

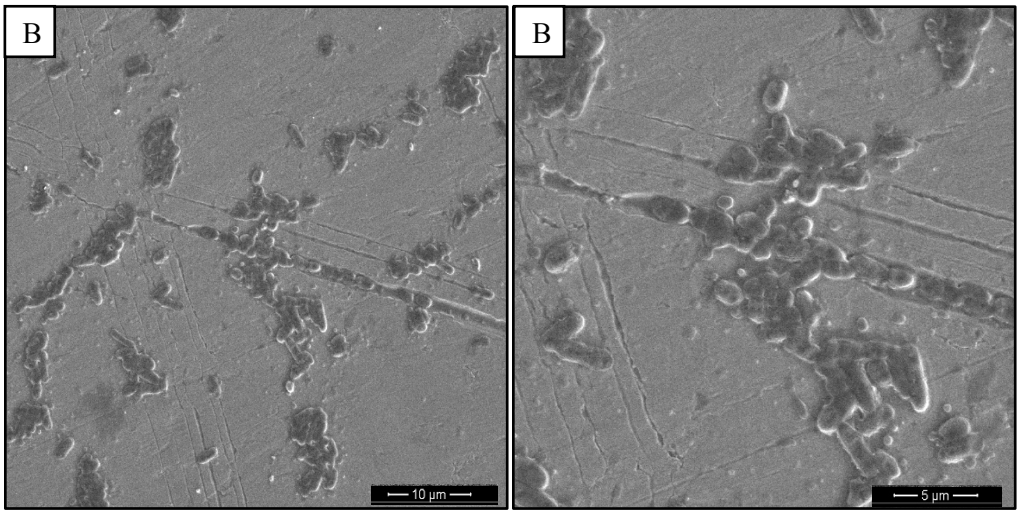

Fig 3. Representative cryo-SEM Images of biofilm on stainless steel panels after water application (WTC).

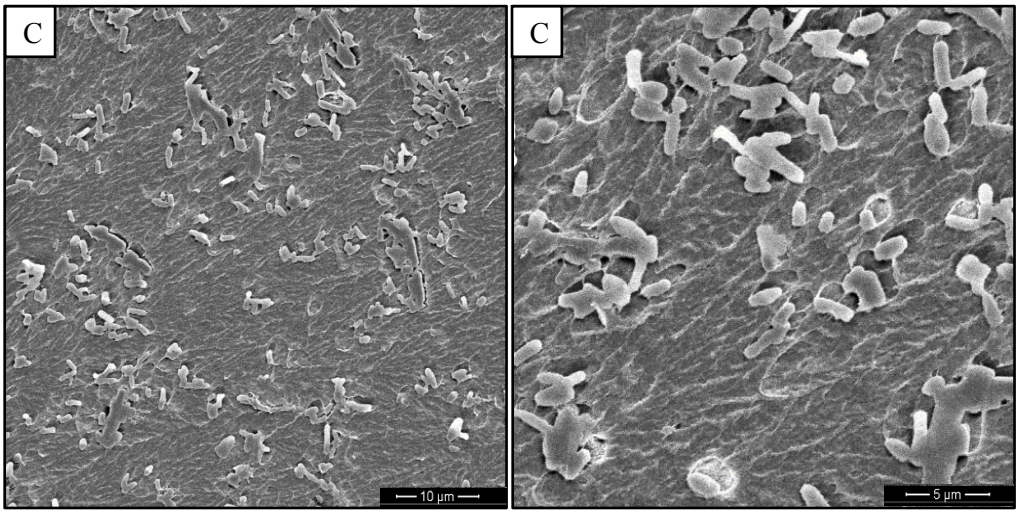

Fig 4. Representative cryo-SEM Images of biofilm on stainless steel panels after application of 500 ppm Perasan and Perafoam.

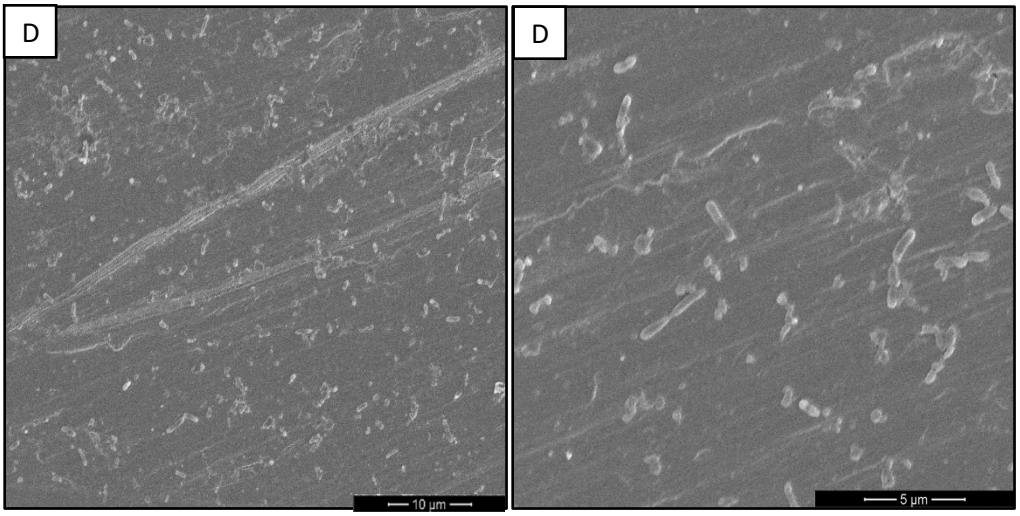

Fig 5. Representative cryo-SEM Images of biofilm on stainless steel panels after application of 2,000 ppm Perasan and Perafoam.

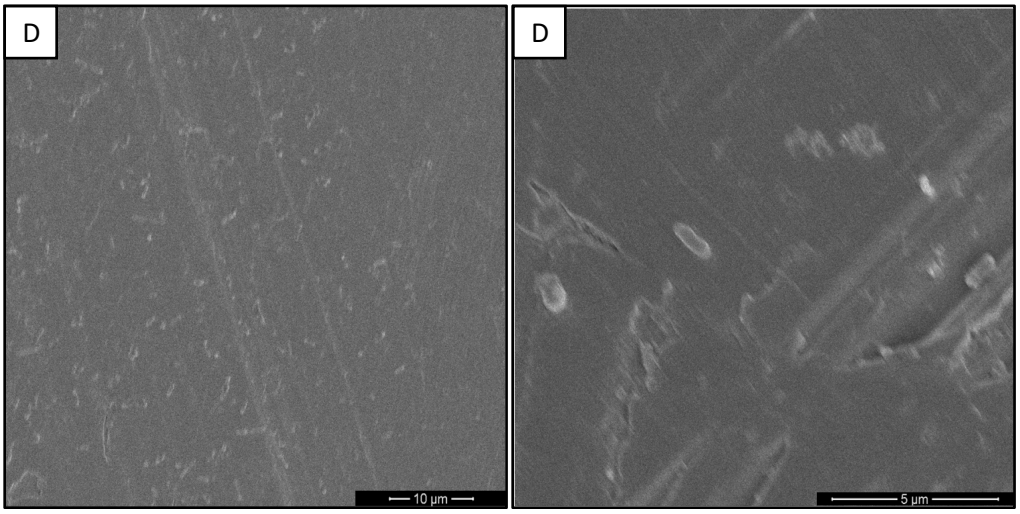

Fig 6. Representative cryo-SEM Images of biofilm on stainless steel panels after application of 3,000 ppm Perasan and Perafoam.

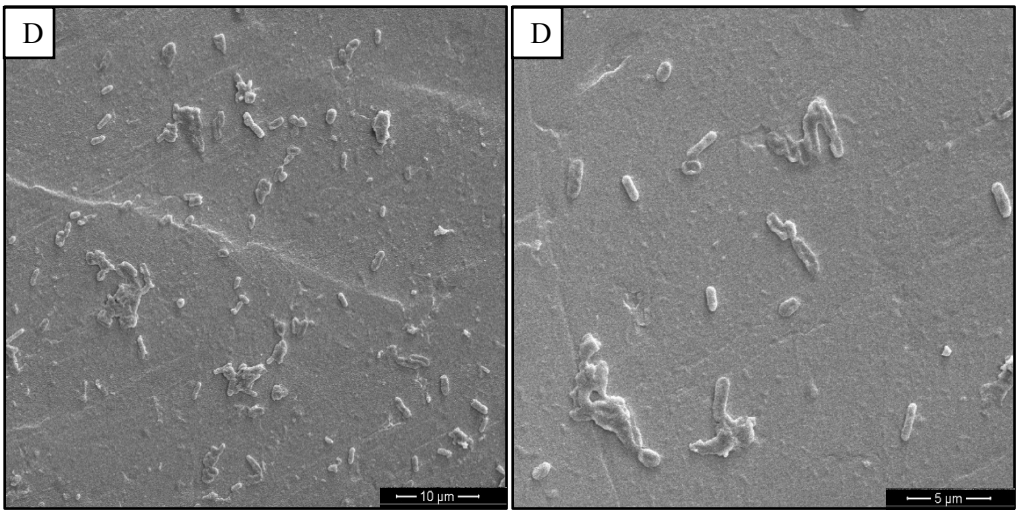

Fig 7. Representative cryo-SEM Images of biofilm on stainless steel panels after Ultra Disinfectant application.

Conclusion

The application of Perasan A and Perafoam (Enviro Tech), and Ultra Disinfectant (Sterilex) achieved a total reduction of viable *E. coli* O157:H7*, Salmonella* Typhimurium and *Listeria monocytogenes* on stainless steel coupons. Therefore, application of Perasan A and Perafoam has the same effect in bacteria inactivation compared with the application of Ultra Disinfectant for the same exposure time. Residual bacteria and/or biofilm were still present following the application of all sanitizers tested, however, the extent of the presence of residual bacteria/biofilm decreased when higher concentrations of Perasan A and Perafoam were used. The residual bacteria/biofilm that were present may be because no physical action (such as scrubbing) was applied during the sanitizer treatment that would have aided in biofilm disruption. The higher concentrations of PAA that were used likely resulted in more disruption and removal of the biofilms that were present (Figures 4-6). Combining physical/mechanical forces with chemical sanitizers will likely assist in the disruption and removal of biofilms on stainless steel surfaces.