Methods:

Three isolated bacterial strains were obtained from the Deitsch lab including Escherichia coli strain K12 substr. MG1655/ATCC 700926, Enterococcus faecalis OG1RF ATCC 47077, Bacillus subtlis subsp. subtilis str. 168 ATCC 23857 and one isolated strain Deinococcus radiodurans R1. Single colonies were picked from each agar plate using a pipette tip and were placed in 5 mls each of tryptic soy broth for E.coli K12, B. subtlis, and E. faecalis. D.radiodurans was placed in 5 mls of nutrient broth supplemented with 1% glucose. Bacteria were grown overnight for 12 hrs in 15ml falcon tubes inside shaking incubator at 200 RPM and 37°C.

From these fresh grown stock solutions, 100ul of cultured bacteria were obtained and placed in 900ul of appropriate broth (tryptic soy broth or nutrient broth with 1% glucose) in 1.5ml eppendorf tubes. From this 1:10 dilution the bacteria were serially diluted to 1:100, 1:1000, 1:10,000, 1:100,000, 1:1,000,000, and 1:10,000,000. 100ul from each dilution was then plated on corresponding tryptic soy agar plates and nutrient broth with 1% glucose agar plates using Zymo glass shaker beads to randomly distribute colonies across the plate. Plates were then grown at 37°C overnight for 12 hrs.

Colony forming units (c.f.u's) were counted and serial dilutions to be used for the Sanisport experiment were chosen based on choosing plates that had about 300 c.f.u's. In order to obtain statistically significant results most methods suggest choosing dilutions that produce between 30 and 800 c.f.u's so as to be able to distinguish between single colonies.

Using the correct dilution, 1 ml each of a single strain of bacteria was placed in triplicates in a 12 well plate so as to have enough surface area exposed to ozone treatment. 3 samples were used for each time point of 0mins, 30mins, 60mins, and 120mins. Ozone treatment was done on separate days for each strain to reduce chance of cross contamination. The 12 well plate was placed uncovered inside of Sanisport and for the 0min time point, 100ul was taken from the 1 ml sample and placed in a labeled ependorf tube to be plated at the end of the experiment. The remaining 900ul was placed in a cryotube with 70ul of DMSO added and then placed on dry ice before moving into -80°C freezer.

Sanisport doors were closed and the cycle button was pushed. Each 16min cycle consists of a 10min phase where purified air is blown inside the machine from fans and followed by a 6min phase of 20-25ppm ozone treatment according to manufacturer's instructions. Ozone is generated each cleaning cycle inside the machine by reacting oxygen available in the air with ultraviolet light. At the end of the 5^{th} cycle 100ul of sample is taken from the three corresponding 30min wells and saved for plating at the end of the experiment while 900ul is frozen down as described above. This is done for 5 more cycles for the 1hr time point and then 10 more cycles for the 2hr time point. After the 2hr time point the saved 100ul samples are plated on appropriate agar plates and labeled. C.f.u's are then counted the next day after a 12hr incubation at 37°C.

The same methods are followed for the different surfaces tested (syringe, stethoscope, etc.) with minor modifications. 100ul of bacteria sample were added to each surface via pipette in triplicates and then for each time point 0min, 30min, 60min, and 120min the surface was swabbed for 3 mins using isohelix buccal swabs. For dry surfaces swabs were wetted with respective media and then used for

swabbing. Swabs were then placed in 1.5ml eppendorf tubes and suspended in 100ul of corresponding media. After the 2hr time point the each eppendorf tube containing swab and media was vortexed for 10 seconds at max speed to shake bacteria off of swab and into surrounding media at bottom of tube. The tubes were then centrifuged at 6000 rpm for 10 mins at room temperature to collect bacteria at bottom of tube. Swabs were removed using sterile tweezers and media was pipetted up and down 10 times to resuspend bacteria at the bottom of the tube. 100ul of media was then plated onto respective agar plates, labeled, and incubated at 37°C overnight for 12 hours. C.f.u's were counted the following day.

Results:

For the 12 well plate experiments c.f.u's were counted, c.f.u/ml calculated, and the triplicates were averaged with SEM calculated to obtain ozone kill curves. Ozone treatment for 2hrs led to a 98.2% reduction in D.radiodurans, 99.5% reduction in E.coli k12, 90% reduction in B.subtlis, and 99.4% reduction in E.faecalis.

For the syringe experiments ozone treatment for 2 hrs led to a 94.2% reduction in D.radiodurans, 99.6% reduction in E.coli K12, 94.6% reduction in B.subtlis, and 99.2% reduction in E.faecalis.

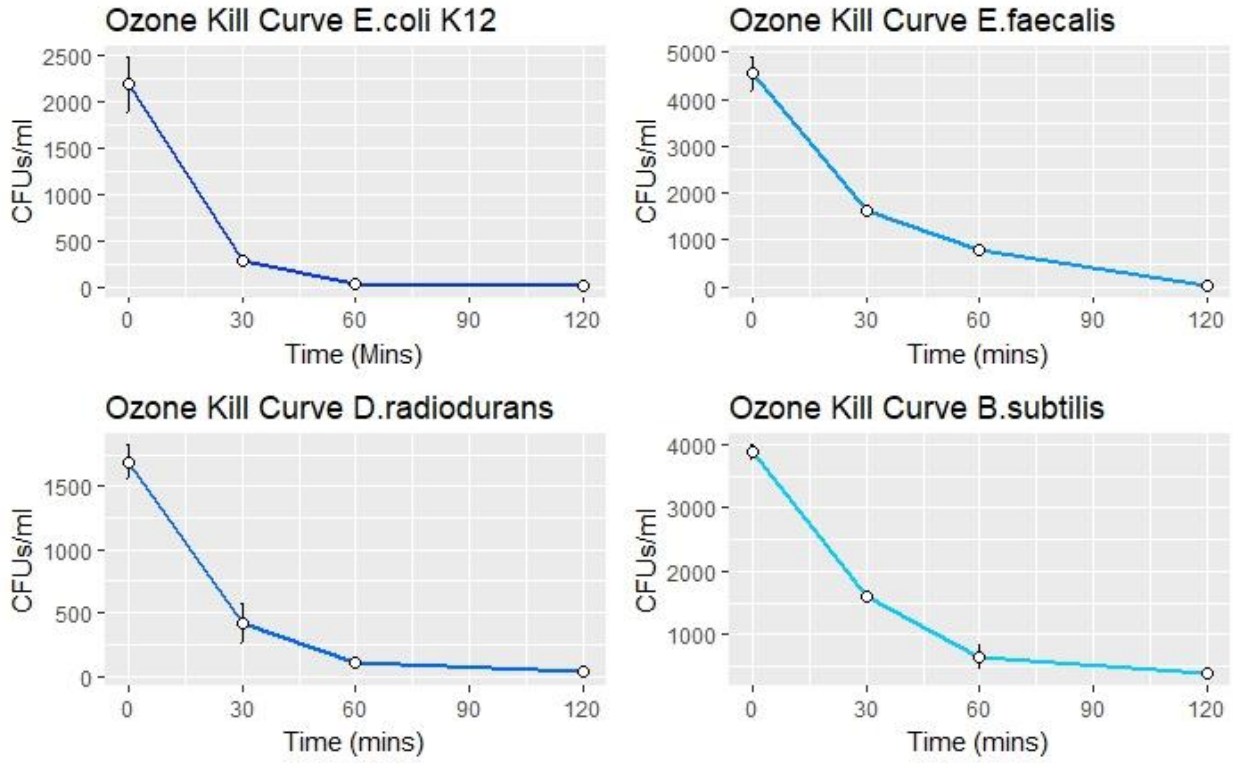


Figure 1. Ozone kill curves for syringe experiment. 1ml of bacteria was pipetted into wells of 12 well plates in triplicates and 100ul was collected at each time point for plating. Colony forming units were

then counted following plating and 12hr incubation. CFUs are converted into CFUs/ml. Error bars represent standard error of the mean.

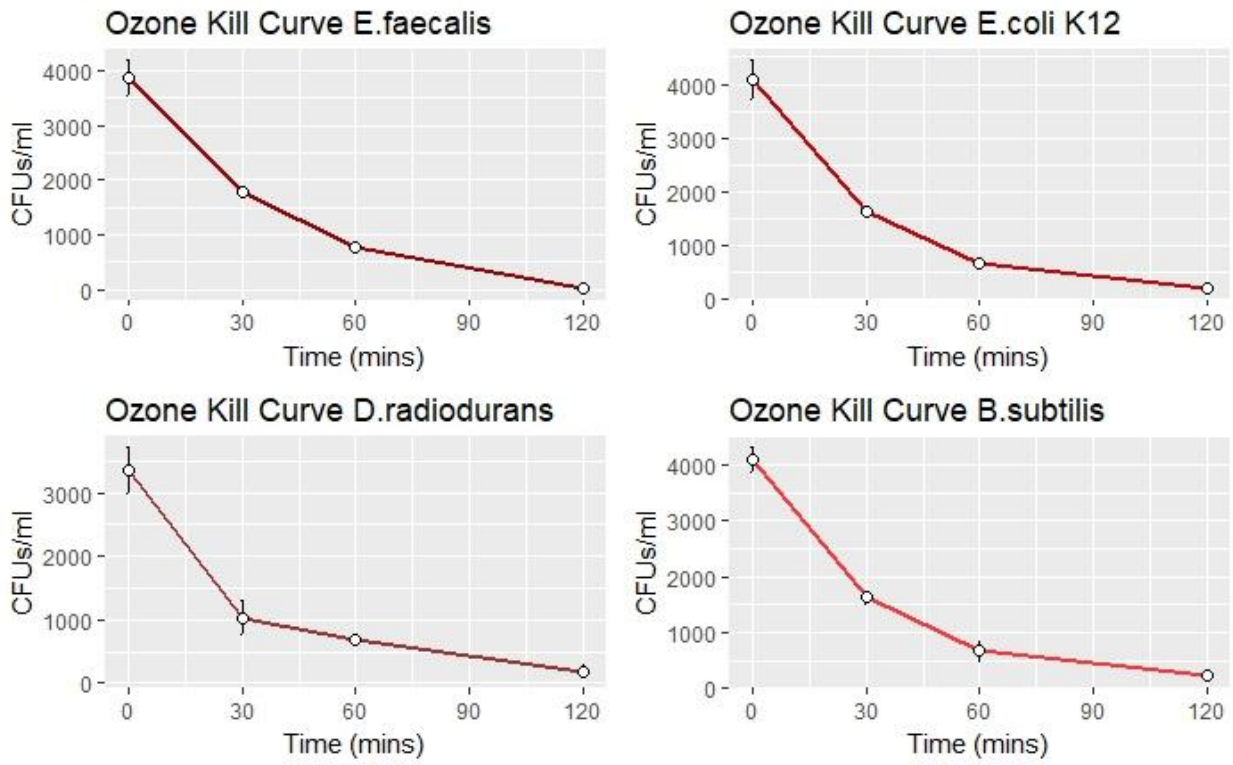
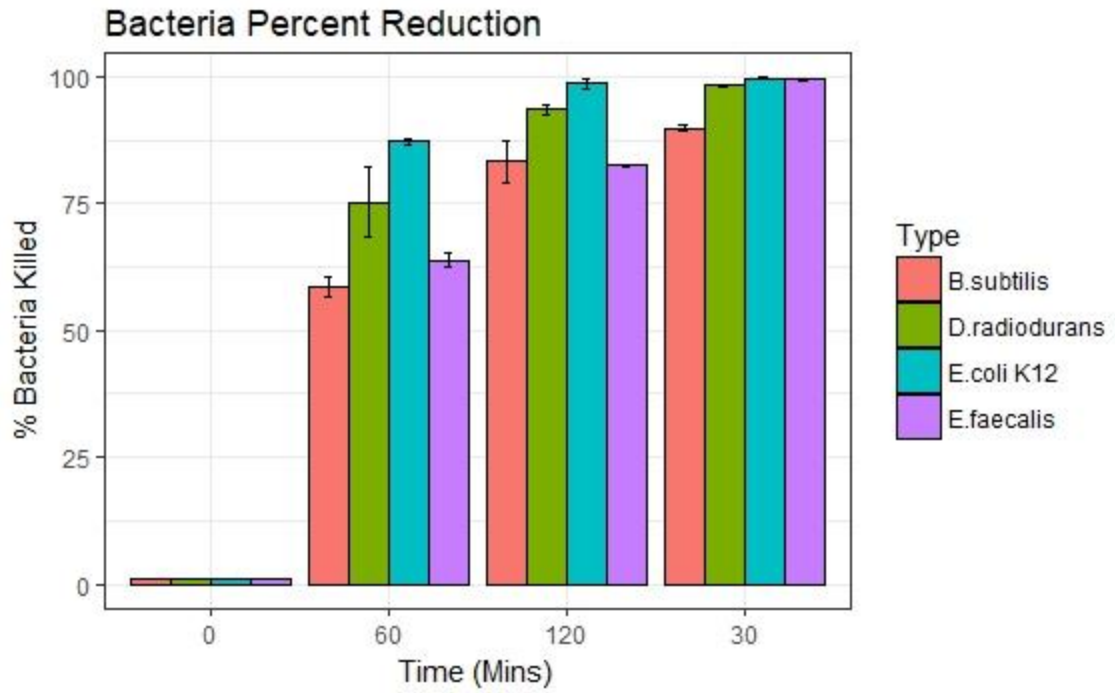


Figure 2. Ozone kill curves for syringe experiment. 100ul of bacteria was pipetted into the inner tubing of 5cc syringes in triplicates and swabbed for 3 mins at each time point. Colony forming units were then counted following plating and 12hr incubation. CFUs are converted into CFUs/ml. Error bars represent standard error of the mean.

a)



b)

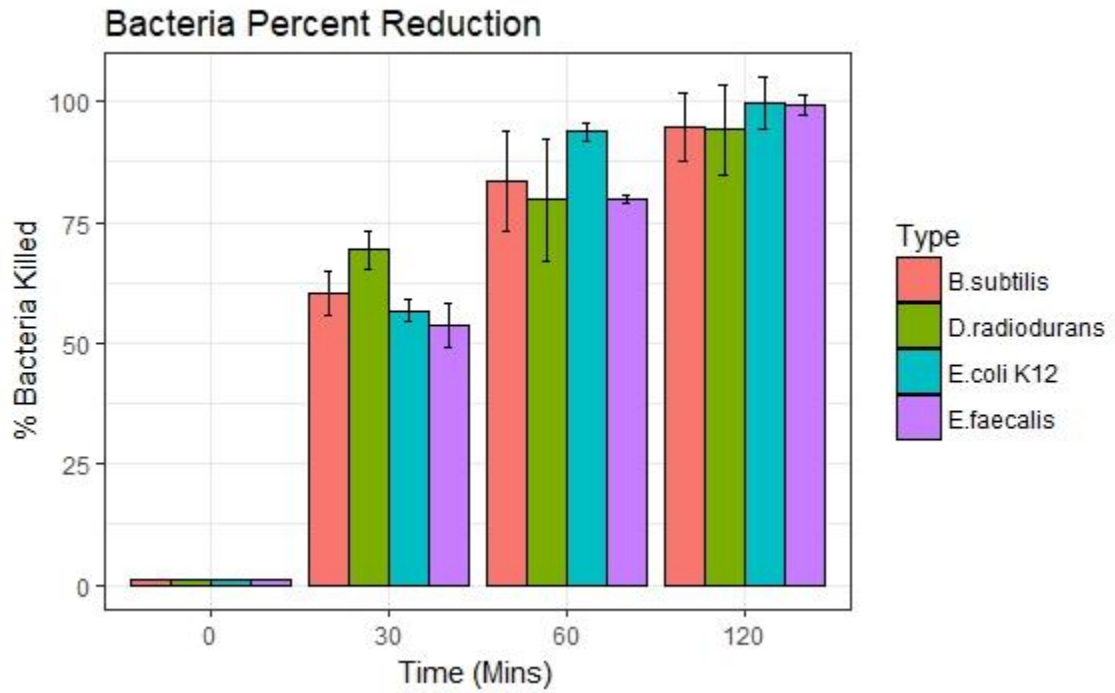


Fig 3. Bacterial percent reductions from ozone treatment, error bars represent standard error of the mean. **A)** Represents the exposure of bacteria in 12 well plates to ozone. The 0 time point represents

the initial stage of the experiment with no ozone exposure. At the 30 minute time point B.subtilis, D.radiodurans, E.Coli K12, and E.faecalis experienced a 58.66%, 75.34%, 87.2%, & 63.86% reduction respectively as compared to the 0 time point. At the 60 minute time point B.subtilis, D.radiodurans, E.Coli K12, and E.faecalis experienced a 83.29%, 93.49%, 98.62%, & 82.48% reduction respectively as compared to the 0 time point. At the 120 minute time point B.subtilis, D.radiodurans, E.Coli K12, and E.faecalis experienced a 89.75%, 98.23%, 99.70%, & 99.41% reduction respectively as compared to the 0 time point. **B)** Represents the exposure of bacteria inside 5cc syringes to ozone. The 0 time point represents the initial stage of the experiment with no ozone exposure. At the 30 minute time point B.subtilis, D.radiodurans, E.Coli K12, and E.faecalis experienced a 60.26%, 69.31%, 56.79%, & 53.79% reduction respectively as compared to the 0 time point. At the 60 minute time point B.subtilis, D.radiodurans, E.Coli K12, and E.faecalis experienced a 83.71%, 79.74%, 93.78%, & 79.82% reduction respectively as compared to the 0 time point. At the 120 minute time point B.subtilis, D.radiodurans, E.Coli K12, and E.faecalis experienced a 94.62%, 94.24%, 99.57%, & 99.22% reduction respectively as compared to the 0 time point.

Table 1.

12 Well Plate Ozone Treatment				
	B.subtilis	D.radiodurans	E.coliK12	E.faecalis
0min	-	-	-	-
30min	58.66% ± 1.92%	75.34% ± 6.97%	87.20% ± .71%	63.86% ± 1.45%
60min	83.29% ± 4.17%	93.49% ± 1.1%	98.62% ± .11%	82.48% ± .21%
120min	89.75% ± .58%	98.23% ± 1.1%	99.70% ± .11%	99.41% ± .29%
5cc Syringe Ozone Treatment				
	B.subtilis	D.radiodurans	E.coliK12	E.faecalis
0min	-	-	-	-
30min	60.26% ± 4.71%	69.31% ± 4.04	56.79% ± 2.32%	53.79% ± 4.40%
60min	83.71% ± 10.33%	79.74% ± 12.52	93.78% ± 1.75%	79.82% ± .81%
120min	94.62% ± 7.06%	94.24% ± 9.29	99.57% ± 5.41%	99.22% ± 2.17%