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Antimicrobial efficacy of aqueous ozone in combination with short chain fatty acid buffers

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SUMMARY

Background: Mitigating surface contamination by microbes such as *S. aureus*, *Salmonella enterica*, or *Klebsiella pneumoniae*, is an ongoing problem in hospital and food production environments.
Aim: To determine whether addition of buffering solution to source water used for manufacture of aqueous ozone increases ozone efficacy against ozone-resistant bacterial species.
Methods: Antimicrobial effects of aqueous ozone were studied in combination with acetate, propionate, or butyrate short chain fatty acids (SCFA) as well as citrate or oxalate buffer formulations against *Staphylococcus aureus* on glass coupons. Aqueous ozone combined with an acetate buffer was also evaluated against *Salmonella enterica* and *Klebsiella pneumoniae*.
Findings: The acetate, propionate, and butyrate buffered aqueous ozone combinations had a significant 3–4 log reduction of *S. aureus* ($P<0.05$) colony forming unit (CFU), while citrate or oxalate buffered aqueous ozone, although statistically significant versus buffer alone, had less activity. Treatment of *S. aureus*, *S. enterica*, or *K. pneumoniae* with acetate buffered aqueous ozone also resulted in a 4 log or greater reduction in CFUs post-treatment for all three species, versus treatment with water alone.
Conclusions: All buffer systems tested had a significantly greater reduction in CFUs following treatment with the combination of buffer and ozone, compared to treatment with buffer or ozone individually, which has not been previously reported for hard surfaces. These results suggest that SCFA buffered ozone has greater anti-bacterial activity relative to either agent alone, and the activity is independent of the buffering activity. Thus, these formulations have potential to sanitize without residues, using an environmentally conscious formulation.

Introduction

A variety of disinfectant types are currently used in the hospital setting, including high level disinfectants such as peracetic acid (PAA) for surgical instruments, and intermediate

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and low level disinfectants for non-critical instruments such as thermometers and bedside tables [1]. Unfortunately, traditional hospital cleaning and disinfection practices are not always effective, although new "no-touch" automated disinfection technologies such as UV/narrow spectrum light systems and aerosol and vaporized hydrogen peroxide can eliminate human error [2]. A standard disinfectant in common use is sodium hypochlorite or bleach. Sodium hypochlorite is cost effective with broad antimicrobial activity and is likely to continue as the standard hospital antimicrobial solution [3]. However, when sodium hypochlorite interacts with organic nitrogen or other water contaminates, byproducts such as chloroamines, chlorophenols, and trihalomethanes form as a result, causing changes in taste, odor, and the accumulation of disinfectant residue. The food and water treatment industries have a growing concern about these byproducts following disinfection [4,5]. There is interest in ozone as a disinfectant, as it breaks down into oxygen without a residue, and interactions with organic compounds result in non-toxic byproducts [6]. The use of ozone as a sanitizer or disinfectant agent, while leaving fewer and less toxic residues, may also be used as an adjunct to traditional disinfectants while mitigating their side effects. Combining ozone with chlorine disinfection has shown a synergistic effect [7], and mixing the high level disinfectant PAA with aqueous ozone has documented bactericidal activity against *Campylobacter jejuni*, *Salmonella typhimurium*, and *Escherichia coli* while reducing ambient PAA, and health risks to food handlers by 90%, without loss of efficacy [8]. However, the effect of ozone with SCFAs for use as a disinfectant on hard, non-porous surfaces used in the hospital and food industry, has yet to be examined.

Byproducts of disinfection are not the only concern. The rise of drug resistant microbes means that sanitizers and disinfection techniques require an increased focus on the ability to control resistant organisms [9]. Additionally, sources of contamination such as water fixtures should be targets, such that preventative measures for maintaining clean hospital environments should include water fixtures and the water distribution system. Contaminated systems are sources of waterborne organisms such as *Legionella* and other Gram-negative bacteria [10], and aqueous ozone would thus be a solution for sanitation. Aqueous ozone has been shown to reduce microbial contamination of water system tubing by 65% [11], and by several logs in sink and p-traps [12].

Aqueous ozone is capable of reducing contamination from a wide variety of microorganisms, such as those important for dental instruments including *Streptococcus mutans* [13], as well as organisms important to the food industry such as *E. coli*, *Staphylococcus aureus*, *Candida albicans*, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Alcaligenes faecalis*, and *Bacillus atrophaeus* [14—17], including strains that are drug resistant [18]. Further, aqueous ozone is able to bypass some microorganism defensive mechanisms, such as biofilm and spore formation. Aqueous ozone is able to reduce *S. aureus* biofilm CFU to background levels in as little as 30 seconds of exposure. Longer exposure to aqueous ozone, up to 4 minutes, is able to reduce CFU of *Pseudomonas* biofilms. Some strains are more susceptible to ozone than others, and older biofilms are more sensitive than younger biofilms [19]. Ozone is capable of reducing spore populations, such as *Aspergillus nidulans* and *Aspergillus ochraceus* when exposed to different levels of gaseous ozone [20] or *Bacillus subtilis*, as a model for disinfecting river water contaminated with organic material [21]. However, the killing of bacterial spores does not necessarily appear to be due to DNA damage. In experiments using *B. subtilis*, spores were not disabled via DNA damage, as strains of *B. subtilis* with or without DNA protective mutations were inactivated with similar rates by ozone. Instead, the mechanism for ozone inactivation appears to be related to altering spores so that they are unable to germinate. This may have been due to damage to the spore's inner membrane [22].

Ozone's efficacy is not limited to vegetative bacteria and spores. Aqueous ozone can inactivate Norwalk virus, poliovirus, and coliphage MS2, as detected by viral infectivity assays and RT-PCR [23]. Ozone has two-stage viral inactivation kinetics, with 99—99.5% of poliovirus inactivation occurring in the first 8 seconds or less. Ozone concentrations of 1.5 mg/L and higher, increase the rate of inactivation in the second stage. However, concentrations of less than 0.15 mg/L do not have a consistent ability to cause viral inactivation [24]. Ozone can cause viral inactivation by damaging the lipid envelope of herpes simplex virus, vesicular stomatitis Indiana virus, vaccinia virus, adenovirus type 2, and influenza A by lipid peroxidation [25]. Viruses may also be affected by the ability of ozone to cleave the deoxyribose-phosphate backbone of DNA by hydroxyl radicals and direct modification of DNA bases by ozone itself [26,27].

While aqueous ozone can be an effective cleaner, there are several factors that can affect its performance; including pH, temperature, and organic material [28]. pH affects the decomposition of ozone, with a greater rate of degradation occurring in the neutral to alkaline pH range as opposed to an acidic pH, due to the slower rate of hydroxyl radical generation [29]. The stability of ozone is also dependent on temperature with increased stability at lower temperatures [30]. In our examination of the role of pH in the efficacy of aqueous ozone, we studied several SCFA buffering systems that maintain an acidic pH. Acetic acid is known to have antimicrobial properties [31,32], including activity against *Mycobacterium tuberculosis* [33]. Propionic acid, as well as other SCFAs, have shown activity against food-borne organisms such as *Salmonella*, *Listeria*, and *E. coli* [34,35], as well as activity against fungi [36]. Butyric acid has also been shown to have antibacterial activity against *E. coli* O157:H7 when used to treat drinking water [37]. Our comparative studies also included citric acid, a naturally occurring component in citrus fruits that have antimicrobial properties [38,39]. Similarly, ascorbic acid is another component of citrus fruits, and a known anti-oxidant with weak antimicrobial activity [40,41]. 2-(N-morpholino) ethane sulfonic acid (MES), is a synthetic buffer used in protein research [42], and oxalic acid, an acid found in some plants and vegetables has activity against yeast and fungi in combination with other cleaners [43]. Although there are multiple studies examining the efficacy of ozone in conjunction with compounds such as chlorine dioxide, PAA, or hydrogen peroxide, little research has been performed on the efficacy of ozone with SCFAs. The initial hypothesis of our study was that by creating an acidic environment using buffer systems, aqueous ozone would show greater efficacy against organisms that exhibit greater resistance to ozone and other reactive oxygen species, such as *S. aureus*, and aid in the development of an effective, inexpensive, and environmentally friendly surface sanitizing system.

Materials and methods

Culture of test organisms and establishment of treatment coupons

Freeze-dried stocks of *Staphylococcus* (*S. aureus* subspecies *aureus* Rosenbach, strain FDA 209, ATCC 6538), *Salmonella* (*S. enterica* subspecies *enterica* (Kauffman and Edwards) Le Minor and Popoff serovar Montevideo, strain G4639, ATCC BAA-710), and *Klebsiella* (*K. pneumoniae* subspecies *pneumoniae* (Schroeter) Trevisan, strain CIP 104216, NCIB 10341, ATCC 4352) were obtained from ATCC. After rehydration and growth in appropriate culture medium, both plated stocks and stocks frozen in glycerol were created. Test organisms were qualified for absorbance at 600 nm versus CFU by serially diluting inoculum and reading absorbance followed by plating of dilutions onto agar plates. Prior to buffered aqueous ozone testing, growth medium was inoculated and incubated in a shaker incubator at 37° C for 24 hours, then transfer cultured two additional times with a final incubation of 48 hours. Twenty microliters of the resulting inoculum were used to coat glass slides, also known as test squares or coupons, with 10^5 to 10^7 cells, as calculated from the standard curve generated from the absorbance qualification data, and allowed to dry in a 37° C incubator for 40 minutes following a modification of the ASTM method E1153-14.

Creation of buffered aqueous ozone

Briefly, buffered aqueous ozone was generated using the free standing CleanCore CCT 1.0 unit, mounted in a kiosk enclosure, at a concentration of 1.5 ppm (±0.2 ppm) as measured using an AT1 model Q454 dissolved ozone monitor. The water source utilized by the CCT 1.0 was clean, cold, softened Omaha municipal tap water. Buffers made with a combination of an acid (acetic acid, propionic acid, or butyric acid) with its sodium salt, were added to the source water to provide buffering capability and keep the pH slightly acidic at approximately pH 5.5—6. Citrate buffer and oxalate buffer were also created with citric acid and oxalic acid respectively, with their corresponding sodium salts, at approximately pH 5.5. Chemicals were sourced from Fisher Scientific or Sigma. Buffer concentration was 0.05M, although some preliminary testing using acetate buffers also tested a concentration range of 0.01 and 0.1M.

Experimental treatment of test coupons

Buffered aqueous ozone was collected in a biological safety cabinet before being applied to the appropriate coupons contained within 50 ml conical vials using a pipet-aid. As a control, the same buffer without aqueous ozone was used. In the first series of experiments, water was used as an additional control to examine the effect of the buffer separate from the combination treatment. Following a five minute incubation at room temperature, the supernatant in the vial was sampled and placed on test agar plates at a volume of 0.2 ml, as well as an aliquot taken for serial dilution at 1:10, 1:100, and/or 1:1,000 in nutrient broth, which were also placed on test agar plates. All test plates were plated with 0.2 ml spread onto two replicates using a glass spreader and a Bel-Art inoculating turntable. All plates were then incubated at 37°C for 48—54 hours. CFUs for each plate were counted, recorded and averaged for each sample. Test validity required survival of a minimum of 7.5 x 10^6 CFU on the control coupons for *S. aureus* and *K. pneumoniae*, or at minimum sufficient control CFU to show a 99.9% reduction for *S. enterica*. Sample CFUs were analyzed for statistical significance using the Mann Whitney U test, (as not all the data was normally distributed) with significance level α=0.05.

Results

Effect of buffer pH and molarity on reduction of S. aureus by ozone

In order to determine the optimal buffer pH, to be used in combination with ozone, a narrow range of pH values were tested using acetate buffer. As the pH of the acetate buffer increased towards neutral pH (7), the survival of *S. aureus* on untreated control coupons increased until it reached the required number for test validity under the ASTM E1153-14 method (7.5 x 10^5) at pH 6 (Figure 1A).

In addition, three concentrations of acetate buffer, 0.01, 0.05, and 0.1M, were tested with or without 1.5 ppm aqueous ozone (Figure 1B). Aqueous ozone, generated in acetate buffer at 0.05M or 0.1M, showed an overall 4 log or greater reduction of *S. aureus* CFU following treatment. This decrease in the CFU seen in coupons treated with the acetate buffered aqueous ozone was significant when compared to the buffer control coupons (Mann Whitney U test, P=0.00) for both 0.05M and 0.1M acetate buffered 1.5 ppm aqueous ozone. The difference in bacterial survival between coupons treated with 0.05M and 0.1M acetate buffered 1.5 ppm ozone was not significant (P=0.94).

When the molarity of the buffer was reduced to 0.01M, the average reduction was also reduced, from a 4 log to a 2 log reduction. The standard error for this percent reduction was largely due to the variable percent reduction seen in individual experiments (between 51-100% versus buffer alone). This reduction of CFU on coupons treated with 0.01M acetate buffered aqueous ozone versus treatment with buffer alone was significant (P=0.00), but it was also a significantly lower reduction than the reduction seen with 0.05M acetate buffered ozone (P=0.01) and 0.1M acetate buffered ozone (P=0.01).

Reduction of S. aureus by buffered aqueous ozone

Glass coupons coated with an *S. aureus* inoculum were treated with 1.5 ppm aqueous ozone formulated in 0.05M acetate, propionate, butyrate, citrate, or oxalate buffer, using coupons treated with buffer alone as controls (Figure 2). After an exposure time of five minutes at room temperature, there was a statistically significant decrease in *S. aureus* CFU on coupons treated with aqueous ozone made with any of the five buffer systems versus buffer alone, but only the acetate buffered aqueous ozone reached an average of greater than 4 log reduction, which was statistically significant (P=0.00). Coupons treated with 1.5 ppm aqueous ozone formulated in pH 5.5 propionate or butyrate buffer showed an average of greater than 3 log reduction of *S. aureus* in the experimental group versus the control groups treated with buffer alone. This

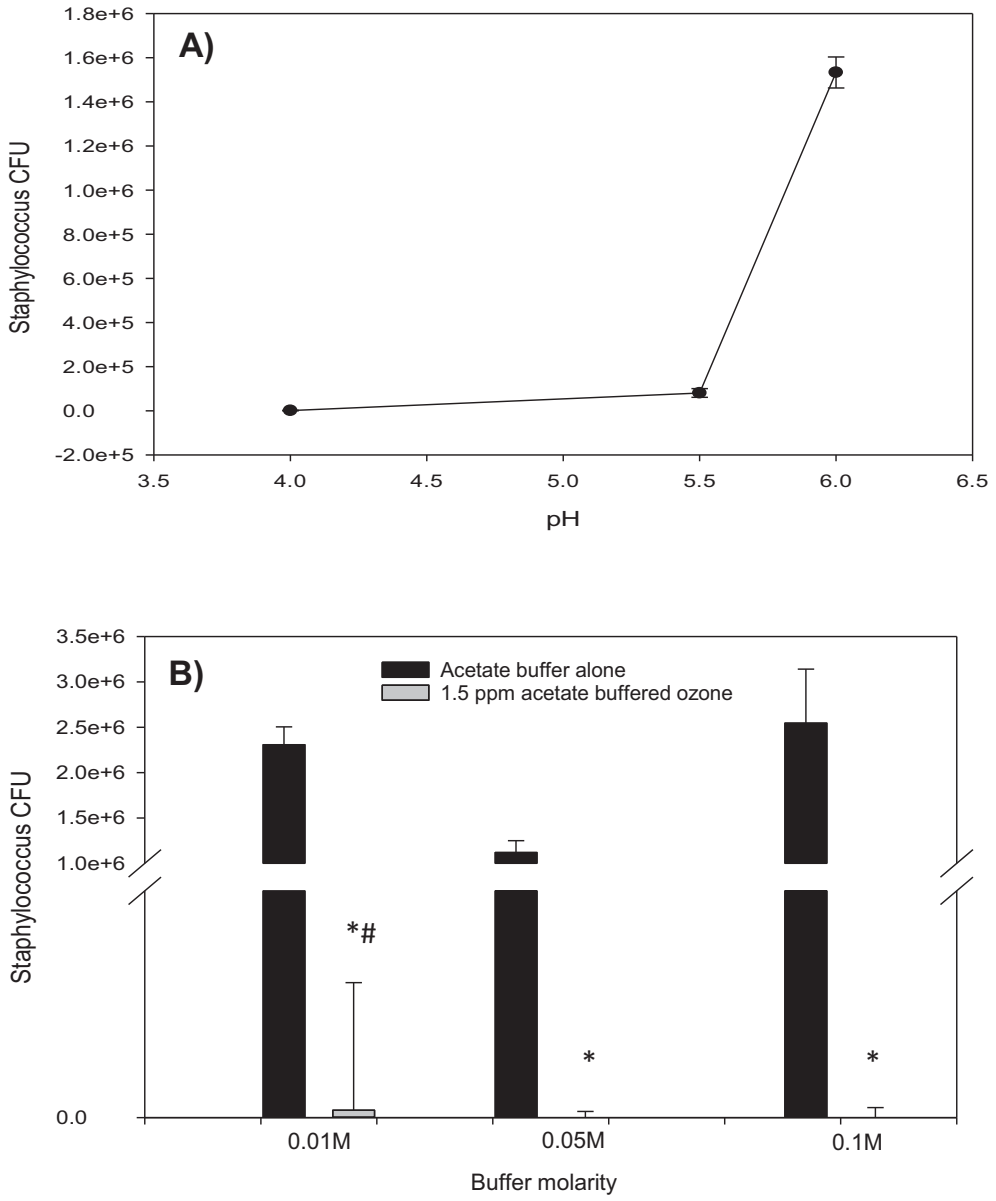

Figure 1. Effect of buffer molarity or pH on *S. aureus* survival following treatment with acetate buffer alone or 1.5 ppm acetate buffered ozone. **A)** Effect of buffer pH on survival of *S. aureus* on treated coupons. Coupons were treated with 0.05M acetate buffer alone, at a pH of 4, 5.5, or 6. Data is taken from a single experiment with three replicates. **B)** Test coupons coated with *S. aureus* were treated with 0.01M, 0.05M, or 0.1M acetate buffer alone or combined with 1.5 ppm aqueous ozone. Data columns are geometric mean CFU counts representative of three independent experiments with 3—6 replicates. *, statistical significant difference in CFU reduction versus corresponding buffer alone control ($P<0.05$, Mann Whitney U test). #, statistical significant difference in CFU reduction versus other tested buffer concentrations.

reduction was statistically significant for both propionate buffered aqueous ozone and butyrate buffered aqueous ozone ($P=0.00$). However, although butyrate buffered aqueous ozone showed a 3 log reduction in *S. aureus* CFU following treatment, the reduction by butyrate buffered ozone was significantly less effective than acetate buffered ozone ($P=0.02$).

In addition to the SCFA buffers, during preliminary studies, ascorbate and MES buffer systems were also tested, but were unable to exhibit measurable ozone concentrations above background during ozone generation by a digital ozone detection sensor (data not shown), and were eliminated from testing. However, other compounds with similar buffering properties, namely citrate and oxalate, were studied in combination with ozone, and found to be unable to achieve the same level of CFU reduction on treated coupons, relative to buffer alone. Citrate buffered aqueous ozone had a 0.3 log reduction and oxalate buffered aqueous ozone had a 0.5 log reduction versus buffer alone, both of which were statistically significant ($P=0.00$). Both citrate and oxalate buffered aqueous ozone were significantly less efficient than acetate, propionate, or butyrate buffered ozone ($P=0.00$) at reducing *S. aureus* CFU. Additionally, oxalate buffer alone was

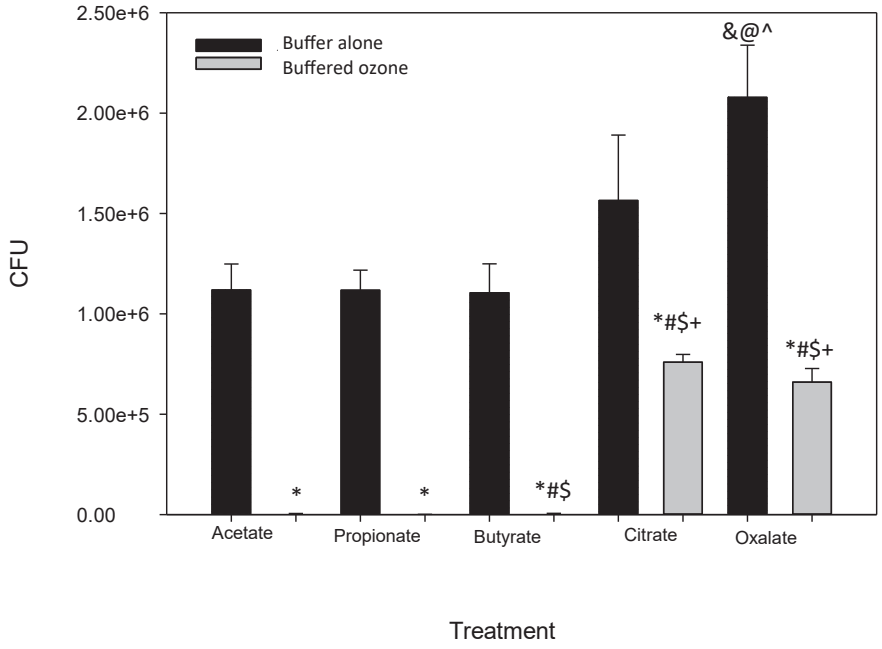

Figure 2. Reduction of *S. aureus* CFU by buffered aqueous ozone. Test coupons were coated with *S. aureus* inoculum, dried, and then treated with 1.5 ppm aqueous ozone in combination with acetate, propionate, butyrate, citrate, or oxalate buffer, or buffer alone. *, significantly different from corresponding buffer control alone. #, significantly different from acetate buffered 1.5 ppm aqueous ozone. $, significantly different from propionate buffered 1.5 ppm aqueous ozone. +, significantly different from butyrate buffered 1.5 ppm aqueous ozone. &, significantly different from acetate buffer alone. @, significantly different from propionate buffer alone. ^, significantly different from butyrate buffer alone.

significantly less efficient than acetate buffer alone (P=0.004), propionate buffer alone (P=0.004), or butyrate buffer alone (P=0.008).

Reduction of Staphylococcus, Salmonella, and Klebsiella by acetate buffered ozone

In experiments with *S. aureus,* treatments using 1.5 ppm aqueous ozone in combination with 0.05M acetate buffer, as compared to a water control, acetate buffered 1.5 ppm aqueous ozone had a 4 log reduction of *S. aureus* CFU post treatment, which was statistically significant (P=0.00). In contrast, a treatment of buffer alone had a less than 1 log reduction (Figure 3). Although this reduction was significant (P=0.00) versus the water control, it was also significantly lower than the acetate buffered 1.5 ppm aqueous ozone combination treatment (P=0.00).

When used to treat test coupons coated with *S. enterica*, 0.05M acetate buffer alone had a 1 log reduction of CFU versus water. However, acetate buffered 1.5 ppm aqueous ozone showed a 6 log reduction in *Salmonella* CFU which were statistically significant (P=0.00). Both the combination treatment and the acetate buffer alone was also significantly effective versus *Klebsiella*. Acetate buffer alone showed a 1 log reduction in *Klebsiella* CFU versus the water control. However, the acetate buffered 1.5 ppm aqueous ozone had a 6 log average reduction in *Klebsiella* CFU following treatment, which is statistically significant (P=0.00). Acetate buffer alone in this experiment was also significantly better in CFU reduction in comparison to water (P=0.00), but the combination of aqueous ozone and acetate buffer was significantly more effective than buffer alone (P=0.00).

Discussion

In these experiments, we utilized glass coupons coated with *S. aureus*, *S. enterica*, or *K. pneumoniae* to examine the antimicrobial efficacy of buffered aqueous ozone on hard surfaces, which has been yet been fully explored in the existing literature. We note that a study testing a solution of ozonated 1% acetic acid (pH 2) resulted in a 4.1 $\log_{10}$ reduction in yeast and mold counts and a 3.2 $\log_{10}$ reduction in aerobic plate counts following treatment of durum wheat [44]. The reduction in the yeast and mold count (0.3 $\log_{10}$) with the ozonated acetic acid was significant relative to 1% acetic acid a difference not observed with the aerobic plate count that resulted in a 0.2 $\log_{10}$ reduction. In our studies we first qualified the effect of buffer pH and molarity on antimicrobial performance. As the pH became more acidic, we saw a reduction in *S. aureus* survival. Weakly acidic solutions can pass through the cell membrane of bacteria and affect the cytoplasm directly [45], and the relationship between pH and *S. aureus* growth has been modeled, with growth at acidic pH being shown to be lower than at a neutral pH [46]. Using the acetic acid buffer system, it appears the optimum acetate buffer pH for *S. aureus* survival on control coupons is pH 5.5 to 6. We used this as the standard pH for the remainder of the experiments and were able to ensure that sufficient organisms survived to maintain test validity following the ASTM E1153-14 test method while controlling for the antimicrobial effect of buffer alone.

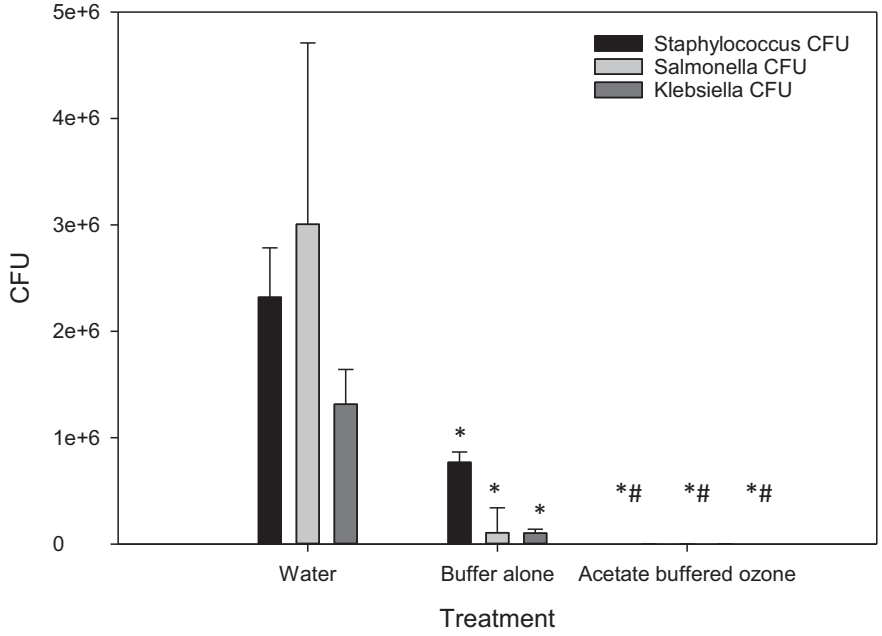

Figure 3. Survival CFU of *S. aureus*, *Salmonella enterica*, or *Klebsiella pneumoniae* following treatment with 0.05M acetate buffer alone or in combination with 1.5 ppm aqueous ozone. Data columns are geometric mean CFU counts representative of three separate experiments with 3—5 replicates each. *, statistically significant difference in CFU versus water control. #, statistically significant difference in CFU versus buffer alone control.

As the concentration of hydroxide ions (and thus the pH) increases, so does the rate of ozone decay [28]. In our testing, using an acidic pH of 5.5—6, we found that this allows the survival of the required number of bacteria on the control coupons when used alone. However, increased killing of *S. aureus* occurred with 1.5 ppm aqueous ozone buffered with 0.05M, pH 5.5—6.0 acetate buffer. This could be due to the increased stability of the ozone under acidic conditions, combined with the ability of some SCFAs to affect the cellular machinery of bacteria [47].

As many of our buffer selections are antimicrobial in their own right, testing of buffer concentration was critical to separate their antimicrobial effects from effects found in the combination treatment, as well as ensure adequate support of ozone stability. The acetate buffer system we tested (and potentially other SCFA-based buffer solutions) with molarity of 0.01—0.1M creates a slightly acidic solution with a pH ~6 that accepts and holds ozone in solution. The buffer alone was able to reduce *S. aureus* CFU, but not to the same extent as the combination treatment at these concentrations. We selected the 0.05M buffer concentration for downstream testing, as this concentration allows for sufficient organism survival on control coupons while showing an additive effect on *S. aureus* CFU when combined with ozone.

Once the parameters for buffer pH and concentration were established, we qualified the effects of a variety of buffer systems in combination with ozone against *S. aureus*. Our data documents that the combination of aqueous ozone in acetate, propionate, and butyrate buffer produces a more effective antimicrobial activity as opposed to combinations with other buffers at the same pH and molarity. While citrate and oxalate buffers can maintain the buffered ozone solution at an acidic pH, similar to the acetate buffer, they do not increase the antimicrobial activity of aqueous ozone to the same level of CFU reduction and are significantly less efficient. This suggests that a mechanism other than pH is responsible for the increased antimicrobial efficacy of the combination of ozone in acetate, propionate, or butyrate buffer against *S. aureus*.

Other buffers that we tested, such as MES and ascorbic acid, are difficult to ozonate. MES buffer is a zwitterionic compound used as a running buffer for gel electrophoresis, and has been shown to interfere with oxidation reactions via interaction with oxidative radicals [48]. Ascorbic acid, or Vitamin C, is a known antioxidant that can attack reactive oxygen species such as hydrogen peroxide [49]. Although these buffers can buffer a solution at pH 5.5 to 6, we were unable to ozonate them. The anti-oxidant properties of these buffering solutions make it likely that these compounds react with the ozone at time of generation or otherwise interfere with ozone generation, leading to the lack of aqueous ozone output that we observed during our testing.

Both the acetate and propionate buffers had a similar reduction of *S. aureus* CFU, which was statistically higher than the reduction by ozone in combination with butyrate buffer. Butyric acid has a one carbon longer chain in its structure in comparison to propionic acid, and two more in comparison to acetic acid. This corresponded to the range of log reduction of *S. aureus* in this study, with butyric having the lowest overall reduction of CFU and acetate having the highest overall reduction of CFU.

In our final studies, we chose a 1.5 ppm aqueous ozone formulated in 0.05M acetate buffer to treat coupons coated with *S. aureus*, *S. enterica*, or *K. pneumoniae*. In our previous unpublished studies, aqueous ozone alone was able to reduce *S. aureus* CFU by 0.4 logs, *Salmonella* by 3 logs, and *Klebsiella* by 2 logs in comparison to a water control. After a five minute

incubation with 1.5 ppm aqueous ozone in combination with 0.05M acetate buffer, we saw these reductions change to a 6 log reduction of *Salmonella* and *Klebsiella* CFU in comparison to treatment with water alone. Additionally, *S. aureus*, which is more resistant to aqueous ozone, had a 4 log CFU reduction which is a ten-fold increase in CFU log reduction versus aqueous ozone alone. Further, this greater reduction is not solely due to the inherent antimicrobial effects of acetic acid, which resulted in a 0.5 log reduction against *S. aureus*. The reduction of *Salmonella* and *Klebsiella* CFU by acetate buffer alone in these experiments was a 1 log reduction, i.e. less than half of the reduction seen by aqueous ozone. Thus, the antimicrobial activity by the combination treatment is greater than the antimicrobial efficiency of aqueous ozone or acetate buffer individually.

In conclusion, in these experiments using 1.5 ppm aqueous ozone with SCFA buffers, including acetic, propionic, and butyric acid, we observed an increased antimicrobial activity against *S. aureus*, *S. enterica*, and *K. pneumoniae*. These results have not been previously reported for this range of species following treatment of hard surfaces. Additionally, although SCFA have been shown to have germicidal activity alone, the exposure times are generally longer (up to 30 minutes) in previous work. Additionally, in our studies there was significantly less antimicrobial activity following treatment with SCFAs alone than with the buffered aqueous ozone. In summary, our data document that the addition of aqueous ozone to SCFA buffers results in a significant increase in antimicrobial activity with a five minute contact time. We conclude that this novel combination provides an approach for the sanitation of food handling and hospital environments using less toxic ingredients compared to traditional sanitizers.

Conflict of interest statement

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