Meat and Muscle Biology™

Utilizing a Combined Approach to Assess the Antimicrobial Efficacy of Peracetic Acid on Chicken Thighs and Beef Trim

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Abstract: Antimicrobial interventions are widely utilized in the meat and poultry industry as a way to reduce foodborne pathogens; however, little is known about their overall impact on the microbiota. The objective of this study was to evaluate the impact of peracetic acid (PAA), when used as a short-duration antimicrobial spray, on the pathogen load and microbiota of inoculated chicken thighs and beef trim. Thighs were inoculated with a cocktail of *Salmonella* spp. and *Campylobacter jejuni* and trim with a cocktail of *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (*E. coli*). Inoculated thighs and trim were either not treated (NT) or independently sprayed in a modified spray cabinet with one of the following treatments: 0, 200, 400, and 800 ppm PAA. Samples were rinsed and subsequently used for pathogen detection and microbiota analyses. Pathogens were enumerated by spread plating on selective media, and genomic DNA was extracted for 16S rRNA gene sequencing. Pathogen data were analyzed using ANOVA and linear regression, with means separated by Tukey's Protected honestly significant differences (HSD; $P \leq 0.05$). Microbiota data were analyzed using the QIIME2 pipeline, with data considered significant at $P \leq 0.05$ for main effects and $Q \leq 0.05$ for pairwise differences. Results from this study demonstrate that a spray, with and without PAA, effectively lowered the level of pathogens compared to NT ($P < 0.05$). Increasing PAA concentrations resulted in lower levels of *Salmonella* and *Campylobacter* on thighs ($P < 0.05$, $R^2 = 0.44$ and 0.55) and *Salmonella* and *E. coli* on trim ($P < 0.05$, $R^2 = 0.18$ and 0.17). The microbiota remained mostly unchanged, with pairwise differences being observed between 0 and 400 ppm and 0 and 800 ppm ($P < 0.05$,$Q < 0.05$) on the β-diversity metric Bray Curtis. The application of PAA as a short-duration antimicrobial spray is an effective intervention strategy to reduce pathogen load; however, efficacy may vary between meat product and target pathogen.

Key words: poultry, beef trim, peracetic acid, microbiota, foodborne pathogens
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Introduction

Despite improvements, foodborne illness continues to pose a major threat to global public health (Gizaw, 2019). In the United States, it is estimated that pathogenic bacteria, such as nontyphoidal *Salmonella* spp. and *Campylobacter*, cause 3.6 million cases of foodborne illness each year (Scallan et al., 2011b). Animal products remain one of the top commodity groups associated with bacterial foodborne illness (64%), followed by plant products (32.1%) and seafood (3.9%) (Painter et al., 2013). National prevalence data from the 2021 calendar year estimated that *Salmonella* and *Campylobacter* prevalence on chicken parts was 6.69% and 16.20%, respectively, and *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC) prevalence in beef trim was 0.75% and 1.64%, respectively (USDA FSIS, 2022a). In the same year, there were a total of 47 recalls, 6 of which were

due to *Salmonella* and STEC contamination in beef, pork, and chicken products (USDA FSIS, 2022b). With the estimated cost of a recall being $10 million, not including the lasting damage done to the brand and the impact on future sales, there is a clear incentive for food manufacturers to utilize antimicrobials or other pathogen inactivation strategies to mitigate these risks.

Antimicrobial interventions can be applied at various points during harvest, fabrication, and processing, most commonly in the form of sprays, dips, or immersion chilling systems (Winkler and Harris, 2009; Zhang et al., 2019). Peracetic acid (PAA) first gained popularity in the poultry industry as a replacement for chlorine. Historically chlorine was considered the industry standard for preventing cross-contamination; however, the efficacy of chlorine is heavily impacted by organic load and increases in pH (Byrd and McKee, 2005). Peracetic acid has become a more widely accepted antimicrobial with a broad range of approved usages (USDA FSIS, 2023). Most commercial PAA contains 3 chemicals, PAA, acetic acid, and hydrogen peroxide at varying concentrations, held in an aqueous solution with the addition of a stabilizer (Organic Materials Review Institute, 2000). The primary properties responsible for its antimicrobial efficacy are its acidic nature and ability to oxidize. However, studies have shown that the efficacy can vary between providers and formulation (Micciche et al., 2019; Cano et al., 2021).

Validation studies are considered the standard for evaluating the effect of specific antimicrobials on a microbial population (Shintani, 2015). However, while the primary goal of an antimicrobial is to reduce pathogenic bacteria, it is also important to consider the unintended effects it may have on the microbial community as a whole (Yang et al., 2021). In recent years, 16S rRNA gene sequencing has become a valuable tool to assess the changes in microbiota and the presence of potential spoilage organisms (Lee et al., 2017; Cauchie et al., 2020; Wythe et al., 2022). Therefore, the objective of this study was to determine the effect of PAA, at various concentrations, on the pathogen load and the microbiota of inoculated meat products when applied as a short-duration antimicrobial spray. The effect on pathogen load was assessed by plating for enumeration on selective media, and the microbiota was characterized using 16S rRNA gene sequencing. By evaluating the effects of PAA at various concentrations, this study aims to determine the optimal usage rates and characterize the microbial community after treatment.

Materials and Methods

Preparation of inoculum

Two cocktail cultures were prepared for the inoculation of chicken thighs and beef trim. The inoculum for chicken thighs consisted of 5 strains of *Salmonella enterica* (Typhimurium S9, Typhimurium M-09-0001A-1, Heidelberg S13, Enteritidis E40, and Enteritidis 6424) and one strain of *Campylobacter jejuni* (NCTC 11168). The inoculum for beef trim consisted of the same 5 strains of *Salmonella enterica* and 7 strains of *Escherichia coli* (*E. coli*; O26:H11, O111:H8, O103:H2, O45:H2, O145:NM, O121:H19, and O157:H7). All *Salmonella* and *E. coli* strains were from the University of Wisconsin-Madison Food Research Institute stock culture collection. Isolates were maintained in individual CRYOBANK™ vials (COPAN Diagnostics Inc., Murrieta, CA, USA) and stored at −80°C. Separate frozen cultures of *Salmonella*, *E. coli*, and *Campylobacter* were individually streaked for isolation on xylose lysine deoxycholate (XLD) agar (Difco, BD Biosciences, Sparks Glencoe, MD, USA), MacConkey agar (BD Biosciences), and modified Charcoal-Cefoperazone-Deoxycholate agar (mCCDA; HiMedia, Mumbai, India), respectively. The XLD and MacConkey plates were incubated aerobically at 37°C for 24 h, and mCCDA plates were incubated under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C for 48 h using the Anoxomat III system (Advanced Instruments, Norword, MA, USA). A single colony from each XLD and MacConkey plate was transferred to 40 mL of TSB (BD Biosciences) and incubated aerobically for 12 h at 37°C. A single colony from the mCCDA plate was transferred to 40 mL of Bolton Broth (Oxoid Ltd, Altrincham, Cheshire, England) and incubated microaerophilically for 24 h at 42°C using the Anoxomat III system. The cultures were centrifuged at 13,500 × g for 2 min, decanted, and subsequently washed twice with 1× Phosphate Buffered Saline (PBS; VWR Life Science, Radnor, PA, USA). After the final wash, cocktails were prepared by resuspending individual pellets in 1× PBS, combining the resuspended pellets, and bringing the final volume to 40 mL. The final inoculation concentration of *Salmonella* and *E. coli* were 10^8 colony forming units (CFU)/g, whereas the final inoculation concentration of *Campylobacter jejuni* was 10^6 CFU/g.

Inoculation of meat products

A total of 26 skin-on, bone-in chicken thighs ($n = 5$, k = 5) and 26 pieces of beef trim ($n = 5$, k = 5)

were used to conduct this experiment, including one uninoculated untreated sample ($n = 1$) for each product type to be used for microbiota analysis. Chicken thighs, weighing on average 186.9 ± 5.9 g, were inoculated with a cocktail of *Salmonella* and *Campylobacter*. Beef trim, with an average weight of 103.3 ± 0.7 g, was inoculated with a cocktail of *Salmonella* and *E. coli*. Both meat products were spot inoculated on the upward facing surface at a rate of 1 mL per 25 g, and the inoculum was then spread using the side of a sterile pipette tip and set aside to rest for 60 to 90 min at 4°C. Attachment levels of 10^7 CFU/mL of *Salmonella* and *E. coli*, and 10^5 CFU/mL of *Campylobacter*, were obtained.

Peracetic acid treatment

A (5 gal or 18.9 L) stock solution of 800 ppm was prepared using tap water and PAA (Hydrite Chemical Co., Brookfield, WI, USA). The stock solution was subsequently diluted down to generate a series of working solutions with a PAA concentration of 400 and 200 ppm. Concentrations were verified using a Hydrishield PA22HP titration kit (Hydrite Chemical Co., Brookfield, WI, USA). A modified spray cabinet (Figure 1) was utilized for the application of each spray treatment. The spray cabinet contained a single hook, in which the meat product was hung on, surrounded by 12 nozzles set at 30 psi. Inoculated chicken thighs and beef trim were either not treated with anything (NT) or subjected to one of the following levels of PAA: 0 (tap water), 200, 400, or 800 ppm. Chicken thighs were sprayed for 15 s, and beef trim was sprayed for 10 s to mimic industry standard practices and remain in accordance with FSIS Directive 7120.1 (USDA FSIS, 2023). After treatment, samples remained on the hook for 2 min to drip before being transferred to sterile rinse bags.

Microbial analyses

Chicken thighs were rinsed in 150 mL of neutralized buffered peptone water (nBPW; Hardy Diagnostics, Santa Maria, CA, USA) for 1 min before being aliquoted for microbiota analysis (40 mL) and serially diluted (1:10) in 1× PBS for the enumeration of *Salmonella* and *Campylobacter* (USDA FSIS, 2021). Beef trim was homogenized in 100 mL of DE Neutralizing broth (Remel, Lenexa, KS, USA) for 1 min at 200 rpm (Stomacher 80, Seward, West Sussex, UK) before being aliquoted for microbiota analysis and serially diluted (1:10) in 1× PBS for the enumeration of *Salmonella* and *E. coli*. Enumeration was performed by spread plating 100 µL of diluted homogenate onto XLD, MacConkey, and mCCDA for *Salmonella*, *E. coli*, and *Campylobacter*, respectively. XLD and MacConkey plates were incubated aerobically for 24 h at 37°C, and mCCDA plates were incubated microaerophilically for 48 h at 42°C before being manually counted within the range of 30 to 300 CFU (Breed and Dotterrer, 1916).

DNA extraction

Rinsate homogenates that were collected on the day of sampling were stored at −80°C until DNA

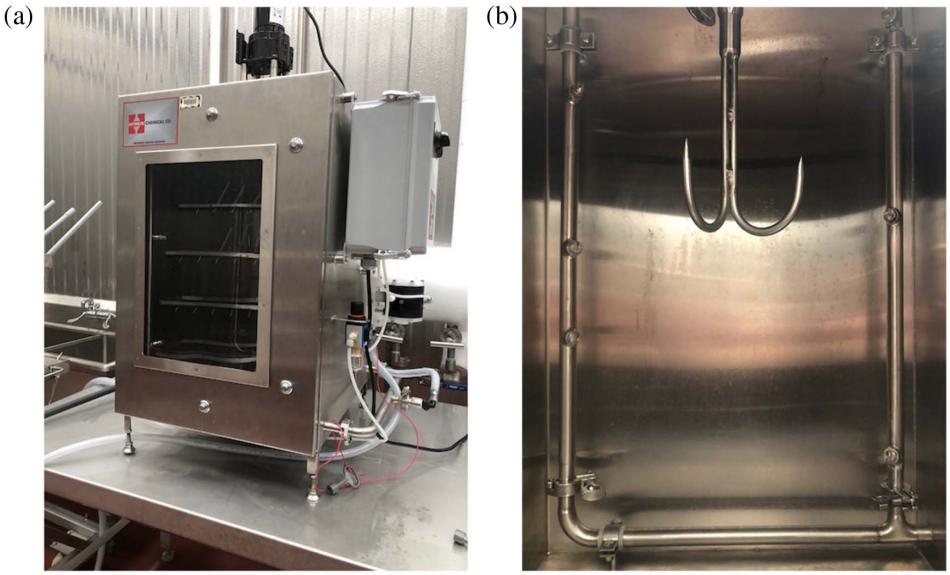

Figure 1. Modified spray cabinet. View of the (a) outside and (b) inside of the modified spray cabinet used to apply the tap water and peracetic acid treatments.

extraction could occur. Rinsates were thawed at room temperature, pelleted for 10 min at 5000 × g, and total genomic DNA was extracted from the resulting pellet using the QIAGEN DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the standard spin-column protocol with the recommended pretreatment for gram-negative bacteria. Extracted samples were eluted by hydrating the spin column with 50 µL of Buffer AE, incubating at ambient temperature for 10 min, and centrifuging for 1 min and 30 s at 8,000 rpm. All eluted samples were analyzed using a NanoQuant plate on an Infinite 200 PRO plate reader (Tecan Trading AG, Männedorf, Switzerland) to measure DNA concentration and 260/280. The eluted samples with a DNA concentration above 15 ng/µL were then diluted to 10 ng/µL in Buffer AE and stored at −20°C until the library could be prepared.

Library preparation

Using the custom primers designed by Kozich et al. (2013), a sequencing library was prepared based on the V4 regions of the 16S rRNA gene. DNA samples (2 µL) were amplified with dual-indexed primers (1 µL of 10 mM forward, 1 µL of 10 mM reverse), including 8 unique nucleotide barcode sequences, using a high-fidelity Accuprime Pfx DNA polymerase (19 µL; Invitrogen, Waltham, MA, USA) and dimethyl sulfoxide (2 µL; DMSO; Fisher Scientific, Waltham, MA, USA). Amplification was verified using gel electrophoresis (1.5%). PCR products were normalized in equimolar concentrations (20 µL) using a SequalPrep™ Normalization kit (Invitrogen, Waltham, MA, USA), and pooled libraries were created containing 5 µL of each normalized sample. Pooled library concentration was determined using a KAPA library quantification kit for Illumina platforms (Kapa Biosystems, Woburn, MA, USA) and a Qubit 1× dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA). The pooled library and PhiX Control v3 (Illumina, Carlsbad, CA, USA) were denatured with freshly made 0.2 N NaOH, incubated for 5 min, and subsequently diluted to 20 pM in Hyb buffer (Illumina, Carlsbad, CA, USA). The denatured 20 pM library and PhiX control were subsequently diluted in Hyb buffer to a final concentration of 6 pM. The resulting 6 pM library was combined with the 6 pM PhiX control (30%, v/v) and loaded into a 2 × 250 cycle v2 MiSeq cartridge (Illumina, Carlsbad, CA, USA). The resulting sequences were uploaded into BaseSpace (Illumina, Carlsbad, CA, USA) and demultiplexed. Demultiplexed reads were uploaded into NCBI (PRJNA906192) and github (https://github.com/RickeLab-UW/Microbiota-of-Meat-Treated-with-PAA).

Statistical and bioinformatic analyses

At the onset of the study, inoculated chicken thighs and beef trim were randomly assigned to a treatment group. The CFU of *Salmonella*, *Campylobacter*, and *E. coli* were Log_{10} transformed and reported on a CFU/g of meat basis (Dittoe et al., 2019). The logarithmic data were analyzed in RStudio (Version 4.2.1) as a linear model using a one-way ANOVA with PAA treatment as a categorical main effect (R Core Team, 2022). Pairwise differences were determined using Tukey's Protected honestly significant differences (HSD) with effects considered significant at $P \leq 0.05$. Further analysis was performed by filtering out the NT control observations and analyzing the remainder of the data in a linear regression model using PAA treatment as a continuous fixed effect. The main effects were considered significant at $P \leq 0.05$.

Sequencing data were analyzed using the QIIME2 pipeline (version 2022.8). Demultiplexed paired-end reads were downloaded from Illumina BaseSpace (Illumina, Carlsbad, CA, USA), formatted, and imported into the QIIME2 pipeline using Casava 1.8 (Bolyen et al., 2019). Imported sequences were filtered and denoised for quality with DADA2 via the q2-dada2 plugin (Callahan et al., 2016). Alignment of the Amplicon Sequence Variant (ASV) was performed using MAFFT, a multiple alignment program (version 7), and rooted and unrooted phylogenetic trees were generated with fasttree2 using the q2-phylogeny plugin (Price et al., 2010; Katoh and Standley, 2013). The ASV were then classified using a SILVA classifier (Silva 138 99% OTU full-length sequences; MD5: b8609f23e9b17bd4a1321a8971303310) with a specified confidence level of 95% via q2-feature-classifier plugin (Quast et al., 2013; Yilmaz et al., 2014; Bokulich et al., 2018). Taxonomic based filtering was performed on the feature tables to exclude chloroplasts and mitochondria from downstream analysis. The α- and β-diversity core metrics were generated through the q2-diversity plugin (McKinney, 2010). Main effect and pairwise comparisons of α-diversity metrics, Shannon's diversity index, and Pielou's Evenness were determined using Kruskal-Wallis (Shannon, 1948; Kruskal and Wallis, 1952; Pielou, 1966). Additionally, main effect and pairwise comparison of β-diversity metrics, Bray-Curtis, and Weighted UniFrac were determined using ANOSIM (Sørensen, 1948; Lozupone and Knight, 2005; Lozupone et al., 2007;

Hamady et al., 2010; Lozupone et al., 2011; McDonald et al., 2018). Subsequently, an analysis of the composition of microbiomes (ANCOM) was performed to distinguish between differentially abundant taxa using the q2-composition plugin (Mandal et al., 2015). Taxonomic means and α-diversity tables were visualized in Microsoft Excel (Version 16.66.1) (Microsoft Corporation, 2018). Main effects were considered significant at $P \leq 0.05$ and pairwise differences at $Q \leq 0.05$.

Results

Chicken

Treatment had a significant effect on the survival of *Salmonella* and *Campylobacter* on skin-on bone-in chicken thighs ($P < 0.01$; Figure 2). The application of 200, 400, and 800 ppm PAA (6.38, 6.37, and 6.03 Log CFU/g) resulted in lower levels of *Salmonella* compared to the NT control (7.14 Log CFU/g; $P < 0.05$). Thighs treated with 200 and 400 ppm PAA (6.38 and 6.37 Log CFU/g) did not result in differing *Salmonella* loads compared to those treated with 0 ppm PAA (tap water; 6.79 Log CFU/g; $P > 0.05$). Application of 800 ppm PAA (6.03 Log CFU/g) was the only treatment that resulted in a lower level of *Salmonella* compared to the thighs treated with 0 ppm PAA (6.79 Log CFU/g; $P < 0.05$). Compared to the NT control (5.33 Log CFU/g), treatment with 0 (tap water), 200, 400, and 800 ppm PAA (4.71, 4.31, 4.17, and 3.79 Log CFU/g) resulted in lower levels of *Campylobacter* ($P < 0.05$). The application of 200 ppm PAA (4.31 Log CFU/g) did not result in differing levels of *Campylobacter* compared to thighs treated with 0 ppm PAA (4.71 Log CFU/g; $P > 0.05$). However, unlike *Salmonella*, the application of both 400 and 800 ppm PAA (4.17 and 3.79 Log CFU/g) resulted in lower levels of *Campylobacter* compared to those treated with 0 ppm PAA (4.71 Log CFU/g; $P < 0.05$). To determine the linear relationship between pathogen load and PAA concentration, the data were analyzed without the NT control. Significant negative relationships ($P < 0.01$) were detected between PAA concentration and the load of both *Salmonella* ($R^2 = 0.44$) and *Campylobacter* ($R^2 = 0.55$; Supplemental Figure 1). With every 100 ppm increase in PAA concentrations, up to 800 ppm, a reduction of 0.09 and 0.11 Log CFU/g of *Salmonella* and *Campylobacter*, respectively, would be expected.

Treatment did not have a significant impact on the microbial diversity of the inoculated chicken thighs. There was no main effect of treatment on either the α-diversity, Shannon's diversity index ($P = 0.72$), and Pielou's Evenness ($P = 0.88$) or on the β-diversity, Bray-Curtis ($P = 0.71$), and Weighted Unifrac ($P = 0.72$; Figure 3; Supplemental Tables 1 and 2). The ANCOM analysis determined that there were no significantly different abundant taxa at any taxonomic level. At the phyla level, the main taxonomic group in the inoculated chicken thighs was Proteobacteria (Figure 4). In contrast, Firmicutes and Proteobacteria were the main taxa present in the uninoculated chicken thighs (Supplemental Figure 3).

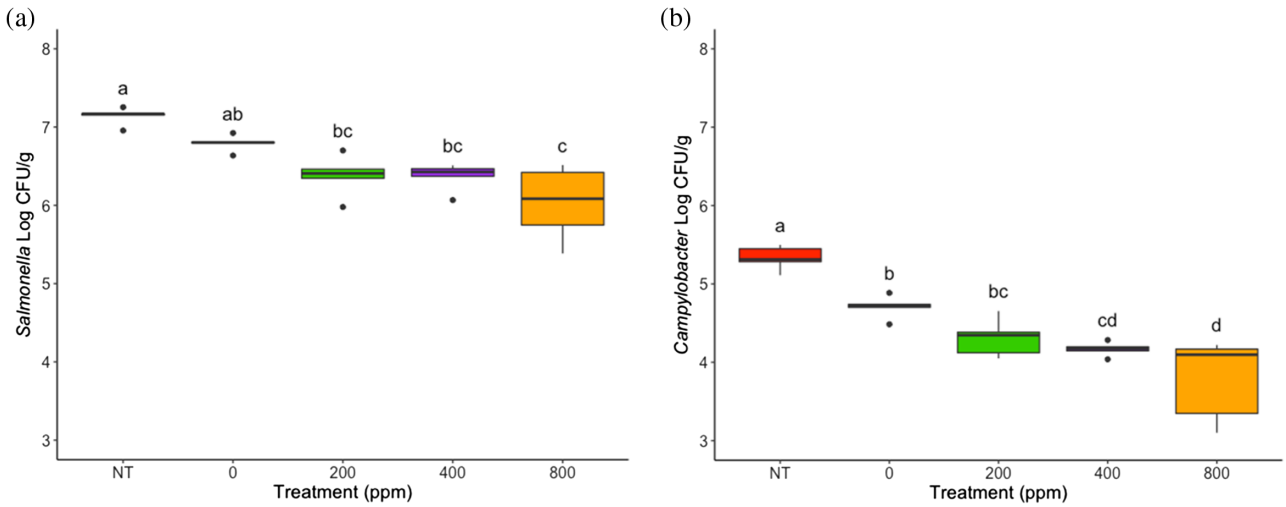

Figure 2. Effect of peracetic acid (PAA) application on the survival of *Salmonella* and *Campylobacter* on chicken thighs. Chicken thighs inoculated with (a) *Salmonella* and (b) *Campylobacter* were placed in a modified spray cabinet where they were either not treated (NT) or sprayed with 0 ppm (tap water), 200 ppm, 400 ppm, or 800 ppm of PAA for 15 s. Products were homogenized in 150 mL of neutralizing buffered peptone water (nBPW) and subsequently plated on selective media. Each letter denotes a significant difference between treatment ($P < 0.0001$, $N = 25$, $n = 5$, $k = 5$).

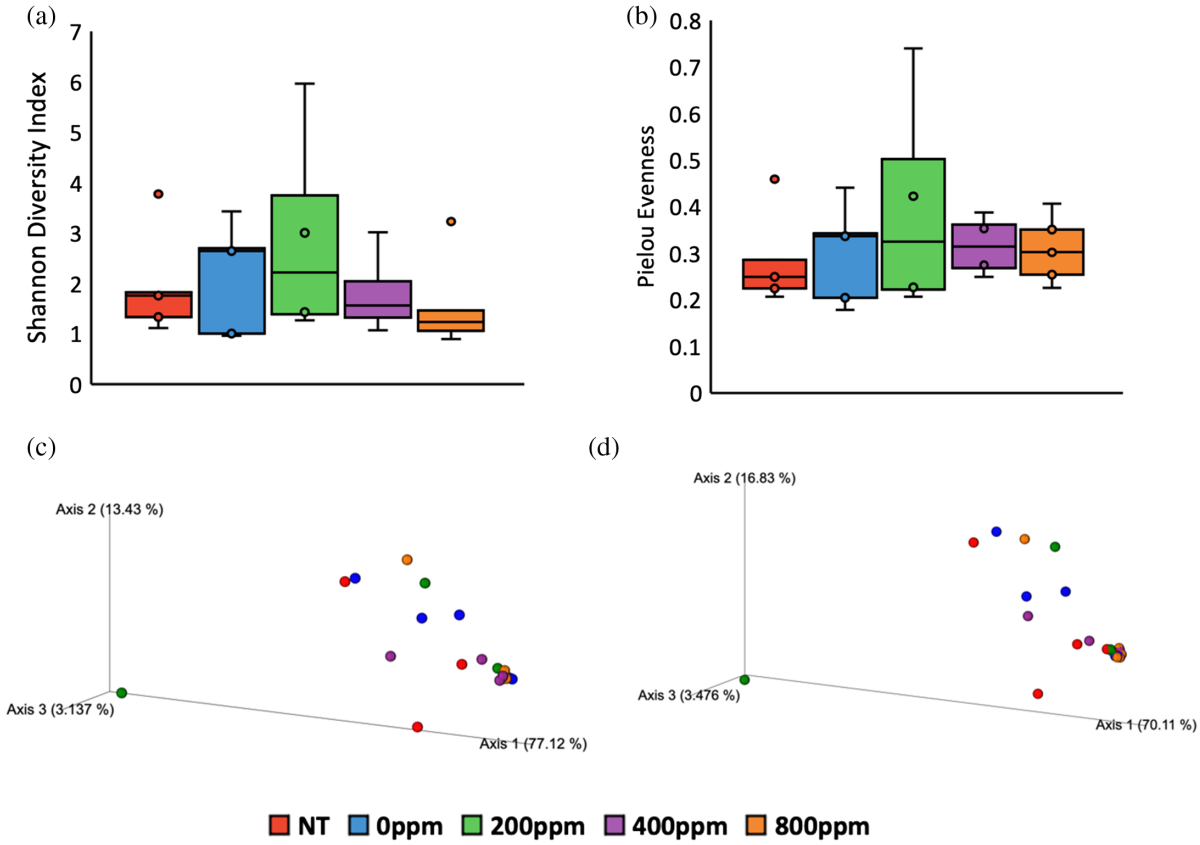

Figure 3. Alpha and beta diversity metrics of inoculated chicken thighs. Total genomic DNA was extracted from inoculated chicken thighs either not treated (NT) or sprayed with 0 ppm (tap water), 200 ppm, 400 ppm, or 800 ppm of peracetic acid (PAA) for 15 s. Main effect of treatment on α-diversity metrics, (a) Shannon's Diversity Index and (b) Pielou's Evenness, were determined using Kruskal-Wallis. β-diversity metrics, (c) Bray-Curtis and (d) Weighted Unifrac, were determined using ANOSIM. There was no treatment effect ($P > 0.05$, $Q > 0.05$).

Beef

There was a significant treatment effect on the survivability of *Salmonella* and *E. coli* on beef trim ($P < 0.01$; Figure 5). The application of 0, 200, 400, and 800 ppm PAA (6.69, 6.72, 6.74, and 6.47 Log CFU/g) all resulted in lower levels of *Salmonella* compared to the NT control (7.25 Log CFU/g; $P < 0.05$). However, the load of *Salmonella* on beef trim treated with 0 ppm PAA (6.69 Log CFU/g) did not differ from those treated with 200, 400, or 800 ppm PAA (6.72, 6.74, and 6.47 Log CFU/g; $P > 0.05$). The *E. coli* results followed a similar trend in which the application of 0, 200, 400, and 800 ppm PAA (7.06, 7.04, 6.95, and 6.83 Log CFU/g) resulted in lower levels of *E. coli* compared to the NT beef trim (7.55 Log CFU/g; $P < 0.05$), while no differences were observed between the trim treated with 0, 200, 400, and 800 ppm PAA ($P > 0.05$). However, significant linear relationships ($P < 0.05$) were observed between PAA concentration and pathogen load, *Salmonella* ($R^2 = 0.18$) and *E. coli* ($R^2 = 0.17$), when data were analyzed without the inclusion of the NT control (Supplemental Figure 2). A reduction of 0.03 Log CFU/g is expected for both *Salmonella* and *E. coli* with every 100 ppm increase in PAA concentration.

There was no main effect of PAA treatment on the α-diversity, Shannon's diversity index ($P = 0.32$), or Pielou's Evenness ($P = 0.28$; Figure 6; Supplemental Table 3). Treatment was not found to have a significant effect on the β-diversity metric Weighted Unifrac ($P = 0.24$); however, a significant effect of treatment was observed on the β-diversity metric Bray-Curtis ($P = 0.01$; Figure 6; Supplemental Table 4). In the Bray-Curtis distance metric, significant pairwise differences were observed between 0 and 400 ppm PAA and the 0 and 800 ppm PAA treatments ($P < 0.01$, $Q < 0.05$). Using ANCOM, it was determined that there were no significantly different abundant taxa at any taxonomic level. Proteobacteria was more than 80% of the microbial community present on the inoculated beef trim regardless of treatment (Figure 7), while the microbial community of the uninoculated beef trim primarily consisted of Firmicutes and Actinobacteriota (Supplemental Figure 3).

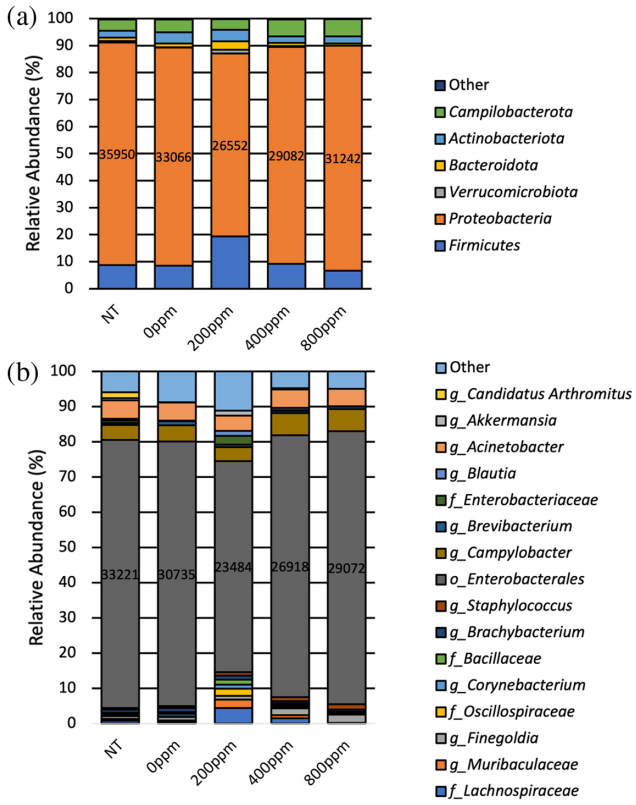

Figure 4. Taxonomic bar plots of the microbial community on inoculated chicken thighs at the phylum and genus level. Mean relative abundance for (a) phyla and (b) genus by treatment. Data labels indicate the mean number of observed features.

Discussion

Numerous studies have demonstrated the efficacy of PAA to reduce the microbial load on meat and poultry products; however, the reported results have varied greatly based on the target organism and product type (Ellebracht et al., 2005; Kim et al., 2017; Ramirez-Hernandez et al., 2018; Zhang et al., 2019; Britton et al., 2020; Kataria et al., 2020; Kumar et al., 2020a). The majority of the research thus far has focused on the application of PAA during poultry processing, evaluating various delivery systems, concentrations, and exposure times (Cano et al., 2021). Immersion interventions (dip/chill tank) have been shown to be the most effective application of PAA, as they generally allow for the longest exposure time and most complete coverage (Cano et al., 2021; Gonzalez et al., 2021). Despite the increased lethality associated with immersion interventions, there are situations in which it is more cost-effective and/or operationally feasible to implement a PAA spray rather than a dip. The current study aimed to evaluate the antimicrobial effects of a short-duration PAA spray on *Salmonella*, *Campylobacter*, *E. coli*, and the overall microbiota of inoculated chicken thighs and beef trim. In general, a short-duration spray effectively lowered the level of inoculated pathogens compared to the NT control, and as the concentration of PAA increased, the level of pathogens was also reduced.

While the application of a spray, at all tested levels (0, 200, 400, and 800 ppm), successfully reduced the load of *Campylobacter* on chicken and *Salmonella*

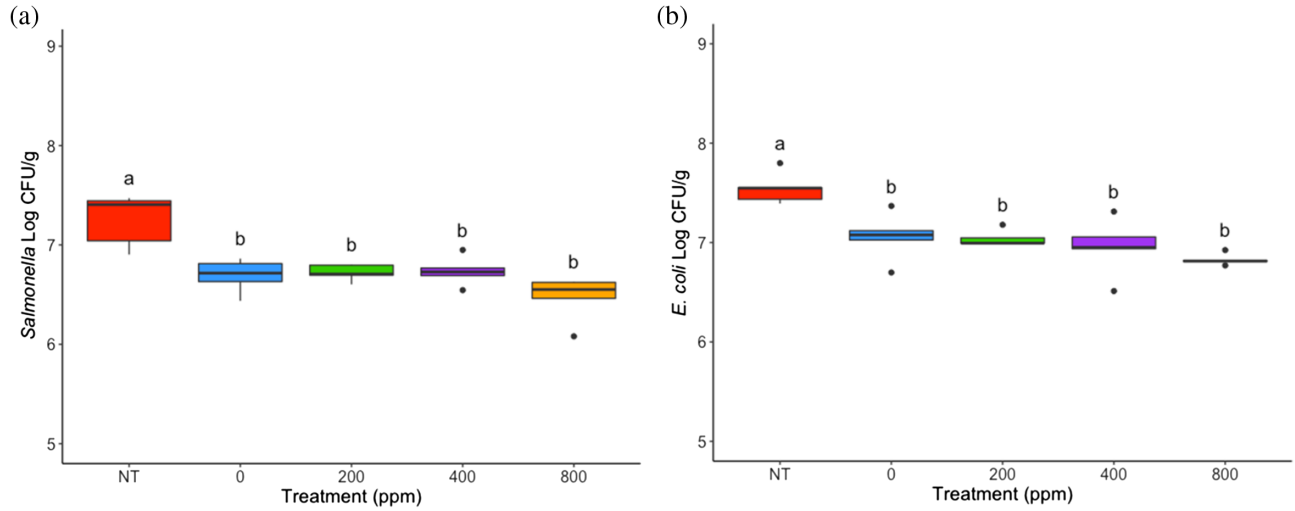

Figure 5. Effect of peracetic acid (PAA) application on the survival of *Salmonella* and *E. coli* on beef trim. Beef trim inoculated with (a) *Salmonella* and (b) *E. coli* was placed in a modified spray cabinet where they were either not treated (NT) or sprayed with 0 ppm (tap water), 200 ppm, 400 ppm, or 800 ppm of PAA for 10 s. Samples were homogenized in 100 mL of DE Neutralizing broth and subsequently plated on selective media. Each letter denotes a significant difference between treatment ($P < 0.0001$, $N = 25$, $n = 5$, k = 5).

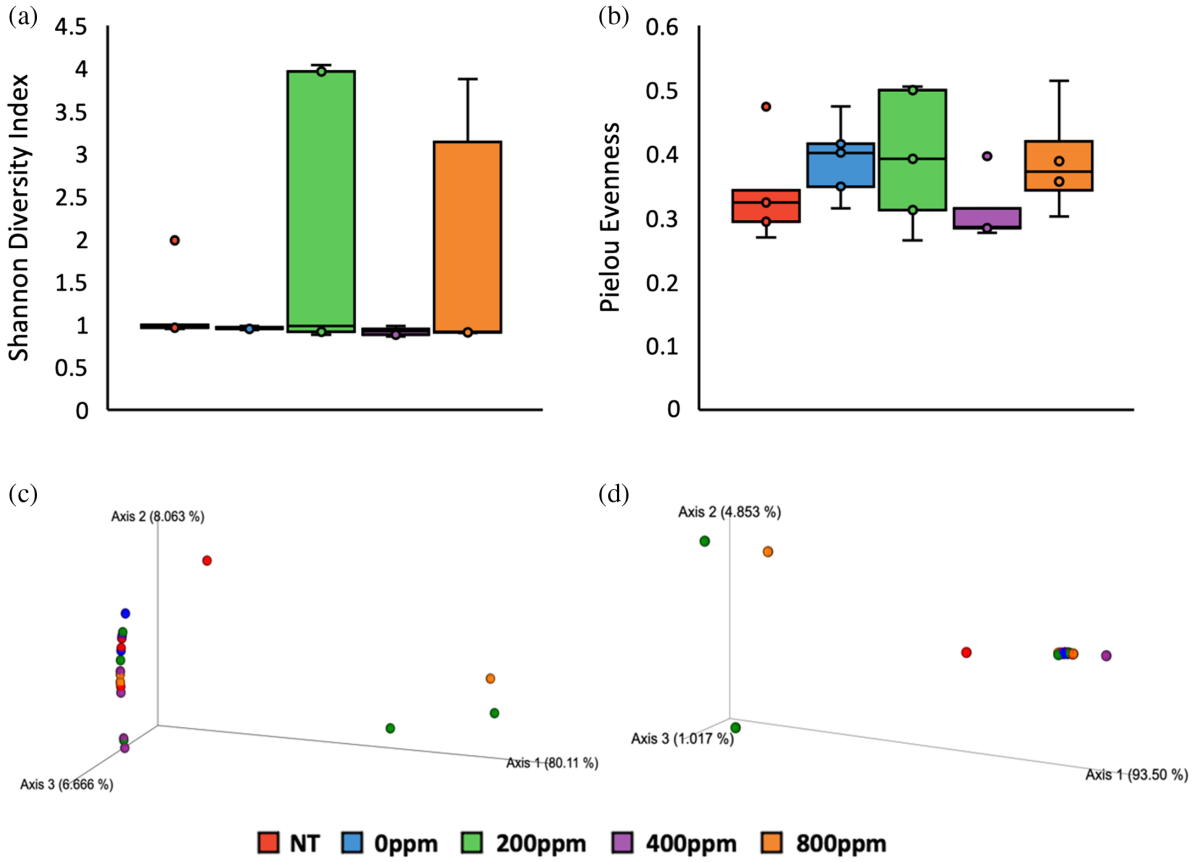

Figure 6. Alpha and beta diversity metrics of inoculated beef trim. Total genomic DNA was extracted from inoculated beef trim either not treated (NT) or sprayed with 0 (tap water), 200, 400, or 800 ppm of peracetic acid (PAA) for 10 s. Main effect of treatment on α-diversity metrics, (a) Shannon's Diversity Index and (b) Pielou's Evenness, were determined using Kruskal-Wallis. Main effect and pairwise comparisons of β-diversity metrics, (c) Bray-Curtis and (d) Weighted Unifrac, were determined using ANOSIM. There was no effect of treatment on α-diversity metrics or the β-diversity metric, Weighted Unifrac ($P > 0.05$, $Q > 0.05$). Pairwise differences were observed in Bray-Curtis β-diversity and are outlined in Supplemental Table 4 ($P < 0.05$, $Q < 0.05$).

and *E. coli* on beef compared to the untreated control group, a rinsing effect of approximately 0.5 Log CFU/g was observed when the inoculated meat products were treated with 0 ppm PAA (tap water). Similar effects were reported by Ramirez-Hernandez et al. (2018) where a 0.3 Log CFU/mL reduction in *Salmonella* was observed after skin-on chicken thighs were sprayed with tap water for 15 s. In another study, beef trim was sprayed for 10 s with tap water, and a 0.4 Log CFU/cm^2 was reported (Britton et al., 2020). For a PAA treatment to be considered effective, the level of recoverable pathogens after treatment must be significantly lower than that of those treated with tap water. Using this definition, only the 800 ppm PAA treatment effectively reduced *Salmonella* on chicken thighs and the 400 and 800 ppm PAA treatment effectively reduced *Campylobacter*. The inability of a short-duration PAA spray to effectively reduce *Salmonella*, at concentrations at or below 400 ppm, has been previously documented. Ramirez-Hernandez et al. (2018) found no significant differences in the amount of recoverable *Salmonella* on chicken thighs treated with either 200 or 400 ppm PAA compared to those treated with tap water. However, *Campylobacter* has been shown to be less tolerant of PAA exposure. Joo et al. (2020) reported that PAA could effectively reduce *Campylobacter* at concentrations as low as 50 ppm when chicken skins were fully submerged for 5 min. Extended exposure time and delivery method (submersion) likely contributed to the superior antimicrobial efficacy demonstrated in this study. However, the current study and others have shown that applying PAA as a short-duration antimicrobial spray can also be an effective delivery system to reduce *Campylobacter* populations on chicken products (Gonzalez et al., 2021).

The application of PAA as a short-duration antimicrobial spray has been shown to be viable intervention strategy for the poultry industry; however, the results from this study suggests that it may have limited benefits when applied to the surface of beef trim (Ramirez-Hernandez et al., 2018; Kumar et al., 2020b; Gonzalez

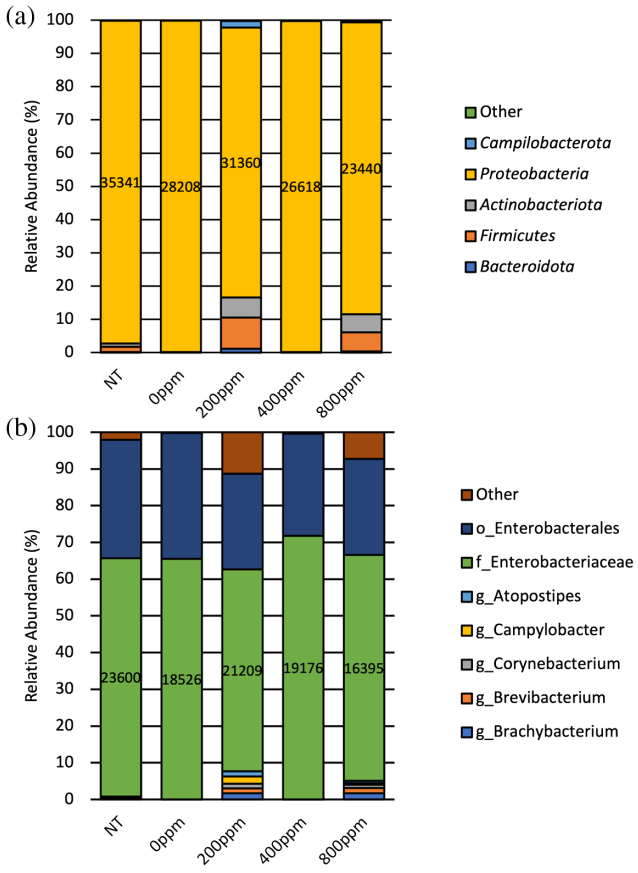

Figure 7. Taxonomic bar plots of the microbial community on inoculated beef trim at the phylum and genus level. Mean relative abundance for (a) phyla and (b) genus by treatment. Data labels indicate the mean number of observed features.

et al., 2021). While most of the validation work has been focused on poultry, several studies have evaluated the efficacy of PAA on both the surface of beef carcasses and on beef trim (Gill and Badoni, 2004; Ellebracht et al., 2005; King et al., 2005; Britton et al., 2020). Unlike poultry, these studies offer somewhat contradictory results. Some studies report that on beef trim the application of a 350 and 400 ppm PAA spray (10 s) can reduce Enterobacteriaceae populations by 1.7 and 1.9 Log CFU/cm^2, respectively (Britton et al., 2020), while others report reductions of 1 Log CFU/cm^2 or less of *E. coli* O157:H7 and *S.* Typhimurium after submerging fresh beef trim in various concentrations of PAA for 15 s. Another study evaluated the effects of a 15 s PAA spray applied to the surface of beef carcasses at two temperatures (45°C and 55°C) and found that even at the highest concentration (1000 ppm), they were only able to reduce *E. coli* O157:H7 and *S.* Typhimurium by 0.9 and 0.6 Log CFU/cm^2 at 45°C and 1.7 and 1.3 Log CFU/cm^2 at 55°C, respectively (King et al., 2005). For comparison, the same study evaluated the efficacy of a 4% lactic acid spray at 55°C and achieved reductions of 2.7 and 3.4 Log CFU/cm^2 of *E. coli* O157:H7 and *S.* Typhimurium, respectively (King et al., 2005). Hot water wash, acidified sodium chlorite, and organic acids (lactic, acetic, citric, etc.) are all common antimicrobial interventions applied either independently or in combination with other antimicrobial treatments to reduce the pathogen load on meat products (Mani-López et al., 2012; Cano et al., 2021). While each of these interventions may have value when used independently, great success has been observed using a combined approach (Leistner and Gorris, 1995; Dittoe et al., 2019; Joo et al., 2020; Olson et al., 2020). When choosing an antimicrobial intervention strategy, it is important to maintain the balance between product safety and product quality. While shelf life and quality were not directly evaluated in this study, PAA acid has been shown to both positively and negatively impact both attributes. Yang et al. (2021) suggest that by reducing the initial microbial load and favoring the growth of lactic acid–producing bacteria, PAA can extend the shelf life. However, other studies have observed that applying PAA at high concentrations has the potential to negatively impact quality attributes, such as color and pH, on both beef and poultry products (Ellebracht et al., 2005; Bauermeister et al., 2008; Chen et al., 2014).

Traditionally, targeted pathogen inactivation studies have been considered the golden standard to validate the efficacy of an antimicrobial intervention. However, in recent years the increased accessibility to next-generation sequencing (NGS) technologies has enabled researchers to look beyond the traditional targeted approach and examine the impact of antimicrobials on the microbial ecosystem as a whole (Park et al., 2023). Microbiota analysis has proved to be a valuable tool for process biomapping, in-line intervention validation, and evaluating the effect of storage conditions on shelf life (Handley et al., 2018; Wages et al., 2019; Weinroth et al., 2019). Yang et al. (2021) demonstrated the utility of this approach from a shelf-life perspective by identifying unique genera associated with PAA treatment, in addition to characterizing the changes in the microbial community of vacuum-packed beef subprimal cuts after treatment and storage. The inclusion of microbiota analysis into traditional validation studies has not come without challenges. Validation studies often require products or surfaces to be inoculated with a target organism, at an artificially high level (~10^8 CFU/mL), to demonstrate a measurable reduction (National Advisory Committee on

Microbiological Criteria for Foods, 2010). Unsurprisingly, studies have shown that this type of inoculation can have significant impacts on the microbial composition, making it difficult to determine the true treatment effect on the native microbial population (Liu et al., 2018; Wythe et al., 2022). Firmicutes, Proteobacteria, and Actinobacteriota make up a large majority of the genera found on raw poultry and beef product (Weinroth et al., 2019; Park et al., 2023). After inoculation, the proportionality of the microbial community shifts and becomes predominantly composed of Proteobacteria (Wythe et al., 2022). Our results showed no effect of treatment on α-diversity (evenness or richness) among inoculated samples. Similar results were observed by Wythe et al. (2022), in which treatment did not impact the richness or evenness within the inoculated treatment groups; however, the microbial population of the non-inoculated not treated group was found to be richer and more even. The same study reported minimal variation in β-diversity among inoculated treatments, further supporting our findings. This lack of treatment effect on the microbial composition may be partially explained by the inability of 16S rRNA gene sequencing to distinguish between viable and non-viable DNA molecules. This inability to differentiate viability status may result in the overestimation of a microbial population (Rudi et al., 2005). As the public health concern surrounding food safety continues to rise, the demand for rapid, sensitive, and accurate pathogen detection methods is higher than ever (Scallan et al., 2011a).

Conclusions

The application of a short-duration antimicrobial spray resulted in a reduction of target pathogens on chicken and beef. However, variations in efficacy were observed between target pathogens and meat matrixes. *Campylobacter* exhibited the least resistance to PAA treatment, resulting in the greatest reductions of the artificially inoculated population. The efficacy of PAA toward *Salmonella* appeared to be more strongly correlated to the meat matrices it was applied to. Peracetic acid treatments were not effective at reducing the population of *Salmonella* and *E. coli* on beef trim; however, they were somewhat effective at reducing the level of *Salmonella* and *Campylobacter* on chicken thighs. These findings suggest that certain properties of beef trim may be providing a protective advantage to foodborne pathogens, enabling them to persist on the product after antimicrobial intervention. In the future, NGS technologies will become a valuable tool in assessing the true impact of antimicrobial interventions on both target organisms and the microbial community as a whole; however, further refinement associated viability and quantitation will be necessary to unlock its full potential. Using this information in combination with traditional validation studies will enable processors to better optimize chemical usage and improve their process control.

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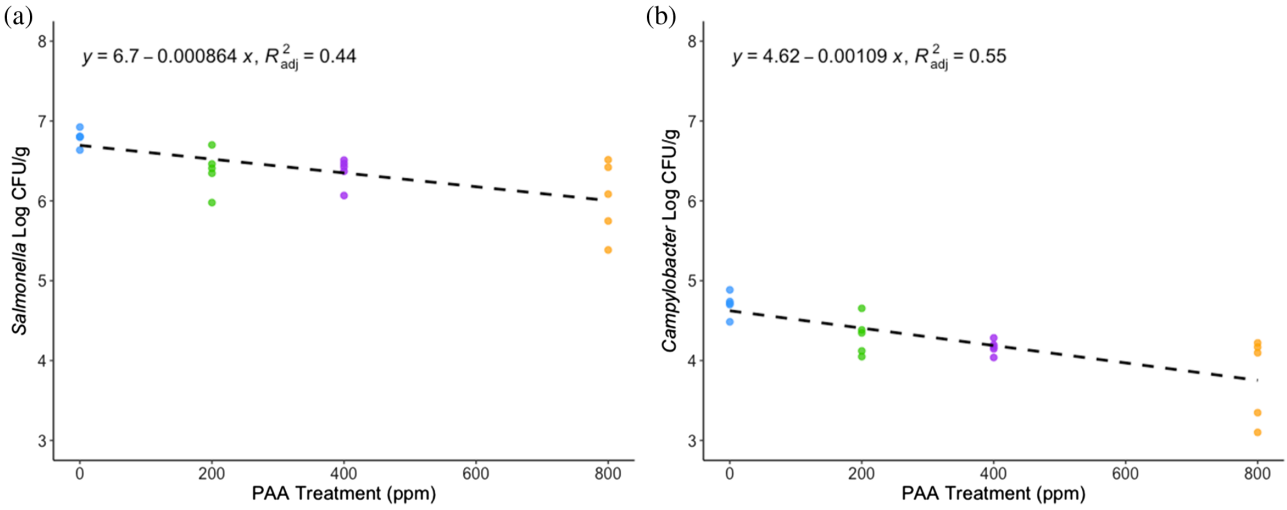
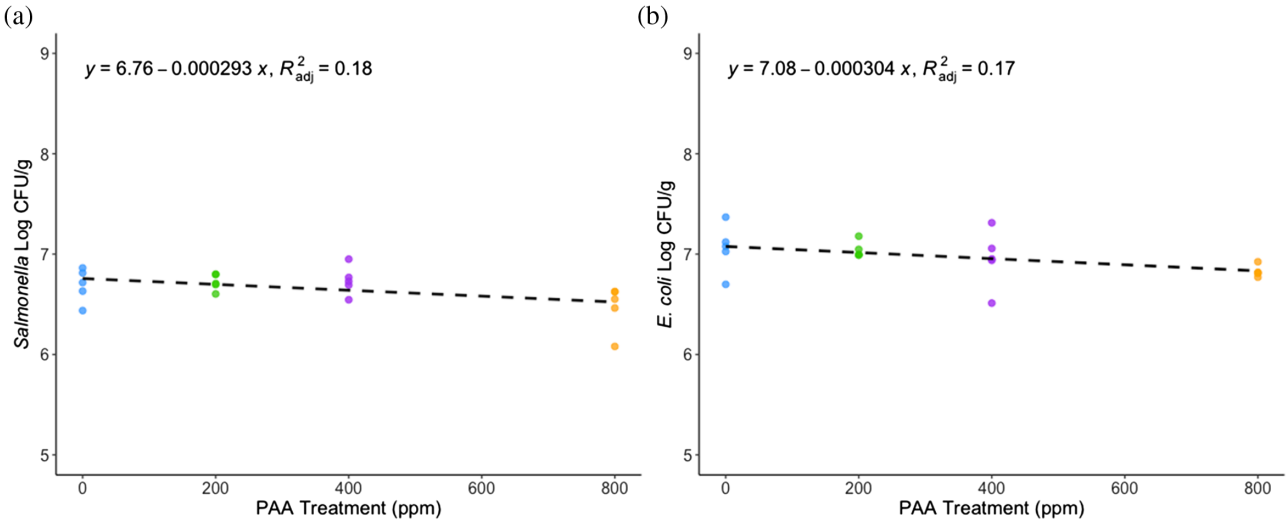
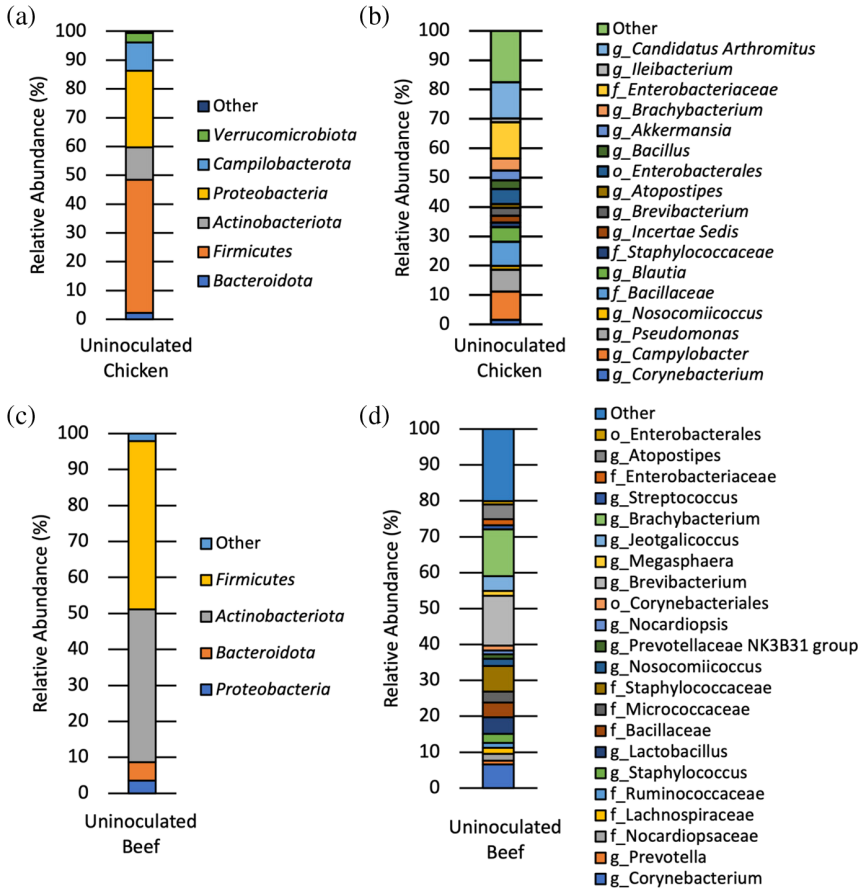
Supplemental Material



Supplemental Figure 1: Linear Effect of PAA concentration on the survival of *Salmonella* and *Campylobacter* on chicken thighs. Chicken thighs inoculated with (a) *Salmonella* and (b) *Campylobacter* were placed in a modified spray cabinet and sprayed with 0 ppm (tap water), 200 ppm, 400 ppm, or 800 ppm of PAA for 15. Thighs were homogenized in 150 mL of NBPW and subsequently plated on selective media. Each point represents one replicate (N = 20, n = 5, k = 4), dashed lines represent the line of best fit, and the regression equation and adjusted R^2 values are provided in the top left corner of each plot ($P < 0.001$).



Supplemental Figure 2: Linear Effect of PAA concentration on the survival of *Salmonella* and *E. coli* on beef trim. Beef trim inoculated with (a) *Salmonella* and (b) *E. coli* were placed in a modified spray cabinet and sprayed with 0 ppm (tap water), 200 ppm, 400 ppm, or 800 ppm of PAA for 10 seconds. Samples were homogenized in 100 mL of DE Neutralizing broth and subsequently plated on selective media. Each point represents one replicate (N = 20, n = 5, k = 4), dashed lines represent the line of best fit, and the regression equation and adjusted R^2 values are provided in the top left corner of each plot ($P < 0.05$).



Supplemental Figure 3: Taxonomic bar plots of the mean relative abundance at the (a & c) phylum and (b & d) genus level for uninoculated (a-b) chicken thigh (n = 1) and (c-d) beef trim (n = 1).

Supplemental Table 1: Inoculated chicken thigh α-diversity metrics, Shannon Entropy ($P = 0.72$) and Pielou's Evenness ($P = 0.88$). Main effects were determined by Kruskal-Wallis[1] with differences being significant at $P \leq 0.05$ and pairwise differences being significant at $Q \leq 0.05$.

		Shannon Entropy[2]			Pielou's Evenness[3]		
Group 1	Group 2	H[4]	p-value	q-value	H[4]	p-value	q-value
0 ppm (n = 5)	200 ppm (n = 4)	0.54	0.46	0.78	0.54	0.46	1.00
	400 ppm (n = 4)	0.00	1.00	1.00	0.24	0.62	1.00
	800 ppm (n = 5)	0.27	0.60	0.78	0.10	0.75	1.00
	NT (n = 5)	0.01	0.92	1.00	0.01	0.92	1.00
200 ppm (n = 4)	400 ppm (n = 4)	0.33	0.56	0.78	0.00	1.00	1.00
	800 ppm (n = 5)	2.16	0.14	0.78	0.06	0.81	1.00
	NT (n = 5)	0.24	0.62	0.78	0.24	0.62	1.00
400 ppm (n = 4)	800 ppm (n = 5)	0.54	0.46	0.78	0.06	0.81	1.00
	NT (n = 5)	0.24	0.62	0.78	0.96	0.33	1.00
800 ppm (n = 5)	NT (n = 5)	1.32	0.25	0.78	0.88	0.35	1.00

[1]No significance effect of treatment ($P > 0.05$, $Q > 0.05$).

[2]Shannon Entropy - α-diversity metric that quantitatively measures community richness.

[3]Pielou's Evenness - α-diversity metric that measures community evenness.

[4]H value: test statistic for Kruskal-Wallis.

Supplemental Table 2: Inoculated chicken thigh β-diversity metrics, Bray-Curtis ($P = 0.71$) and Weighted Unifrac ($P = 0.72$). Main effects and pairwise[1] differences were determined using ANOSIM[2] with significance at $P \leq 0.05$ and $Q \leq 0.05$.

		Bray-Curtis[3]			Weighted Unifrac[4]		
Group 1	Group 2	R[5]	p-value	q-value	R[5]	p-value	q-value
0 ppm (n = 5)	200 ppm (n = 4)	−0.03	0.53	0.80	−0.06	0.65	0.84
	400 ppm (n = 4)	−0.09	0.69	0.80	−0.06	0.52	0.84
	800 ppm (n = 5)	0.00	0.38	0.80	−0.02	0.47	0.84
	NT (n = 5)	−0.08	0.72	0.80	−0.05	0.58	0.84
200 ppm (n = 4)	400 ppm (n = 4)	−0.05	0.64	0.80	−0.05	0.56	0.84
	800 ppm (n = 5)	0.05	0.31	0.80	0.05	0.29	0.84
	NT (n = 5)	−0.03	0.60	0.80	−0.06	0.68	0.84
400 ppm (n = 4)	800 ppm (n = 5)	−0.11	0.82	0.82	−0.12	0.84	0.84
	NT (n = 5)	−0.07	0.69	0.80	−0.09	0.81	0.84
800 ppm (n = 5)	NT (n = 5)	0.04	0.32	0.80	0.02	0.38	0.84

[1]No significance effect of treatment ($P > 0.05$, $Q > 0.05$).

[2]ANOSIM – analysis of similarities is a non-parametric statistical test used to determine whether two or more groups are significantly different.

[3]Bray-Curtis - β-diversity metric that quantitatively measures community dissimilarities without the incorporation of phylogenetic relationships.

[4]Weighted Unifrac - β-diversity metric that quantitatively measures community dissimilarities by incorporating phylogenetic relationships.

[5]R value: Test statistic for ANOSIM.

Supplemental Table 3: Inoculated chicken thigh α-diversity metrics, Shannon Entropy ($P = 0.32$) and Pielou's Evenness ($P = 0.28$). Main effects were determined by Kruskal-Wallis[1] with differences being significant at $P \leq 0.05$ and pairwise differences being significant at $Q \leq 0.05$.

		Shannon Entropy[2]			Pielou's Evenness[3]		
Group 1	Group 2	H[4]	p-value	q-value	H[4]	p-value	q-value
0 ppm (n = 5)	200 ppm (n = 5)	0.10	0.75	0.84	0.01	0.92	1.00
	400 ppm (n = 5)	1.84	0.17	0.44	4.81	**0.03**	0.28
	800 ppm (n = 4)	1.50	0.22	0.44	0.06	0.81	1.00
	NT (n = 5)	1.84	0.17	0.44	1.84	0.17	0.55
200 ppm (n = 5)	400 ppm (n = 5)	0.88	0.35	0.58	0.88	0.35	0.66
	800 ppm (n = 4)	0.54	0.46	0.66	0.00	1.00	1.00
	NT (n = 5)	0.01	0.92	0.92	0.53	0.46	0.66
400 ppm (n = 5)	800 ppm (n = 4)	0.24	0.62	0.78	2.16	0.14	0.55
	NT (n = 5)	3.94	**0.05**	0.44	0.53	0.46	0.66
800 ppm (n = 4)	NT (n = 5)	1.50	0.22	0.44	1.50	0.22	0.55

[1]Bolded values denote significance ($P \leq 0.05$, $Q \leq 0.05$).

[2]Shannon Entropy - α-diversity metric that quantitatively measures community richness.

[3]Pielou's Evenness - α-diversity metric that measures community evenness.

[4]H value: test statistic for Kruskal-Wallis.

Supplemental Table 4: Inoculated beef trim β-diversity metrics, Bray-Curtis ($P = 0.01$) and Weighted Unifrac ($P = 0.24$). Main effects and pairwise[1] differences were determined using ANOSIM[2] with significance at $P \leq 0.05$ and $Q \leq 0.05$.

		Bray-Curtis[3]			Weighted Unifrac[4]		
Group 1	Group 2	R[5]	p-value	q-value	R[5]	p-value	q-value
0 ppm (n = 5)	200 ppm (n = 5)	0.17	0.05	0.12	0.11	0.15	0.51
	400 ppm (n = 5)	0.60	**0.01**	**0.04**	0.07	0.23	0.51
	800 ppm (n = 4)	0.55	**0.01**	**0.04**	0.34	0.03	0.31
	NT (n = 5)	−0.05	0.77	0.77	−0.03	0.59	0.65
200 ppm (n = 5)	400 ppm (n = 5)	0.07	0.26	0.40	0.02	0.38	0.51
	800 ppm (n = 4)	−0.04	0.43	0.47	−0.13	0.82	0.82
	NT (n = 5)	0.02	0.28	0.40	0.02	0.30	0.51
400 ppm (n = 5)	800 ppm (n = 4)	0.00	0.41	0.47	0.06	0.25	0.51
	NT (n = 5)	0.22	0.05	0.12	0.01	0.41	0.51
800 ppm (n = 4)	NT (n = 5)	0.11	0.21	0.40	0.02	0.35	0.51

[1]Bolded values denote significance ($P \leq 0.05$, $Q \leq 0.05$).

[2]ANOSIM – analysis of similarities is a non-parametric statistical test used to determine whether two or more groups are significantly different.

[3]Bray-Curtis - β-diversity metric that quantitatively measures community dissimilarities without the incorporation of phylogenetic relationships.

[4]Weighted Unifrac - β-diversity metric that quantitatively measures community dissimilarities by incorporating phylogenetic relationships.

[5]R value: Test statistic for ANOSIM.