The Efficacy of Peracetic Acid against *Salmonella* Heidelberg and *Campylobacter jejuni* at Various Solution pH Levels

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Summary

In previous studies performed by this laboratory on the efficacy of PAA against microorganisms at different pH levels, the work was done with planktonic (free floating) organisms. This study evaluates the efficacy of PAA against sessile (attached) bacteria on the surface of poultry at a given PAA concentration and at several increasing pH levels.

Recently, this laboratory also released a new study showing the dramatic reduction of PAA vapor evolution from water at various increasing pH levels, which is a major advantage in improving worker and inspector comfort and safety in industrial environments that utilize PAA as the antimicrobial. This current study confirms the associated efficacy of PAA at increasing pH levels over a fixed 60-minute time frame, which would be an average exposure time for the poultry carcass in most chiller operations.

The efficacy against both Salmonella and Campylobacter at 40 ppm over 60 minutes is equally effective at pH levels of 6, 7.5, 8.5 and 9.5, and temperatures < 2.2° C. Therefore, increasing pH levels will reduce fugitive PAA vapors from the poultry chiller without compromising the intended efficacy of the antimicrobial.

Purpose

In poultry processing, one of the key antimicrobial intervention locations for the reduction of *Salmonella* and *Campylobacter* spp. is in the pre-chiller and main-chiller. The main-chiller is the intervention location where poultry carcasses are submerged for a prolonged period of time (30-120 minutes on average) which makes it an ideal location for treatment with an antimicrobial agent such as peracetic acid (PAA). In recent years, poultry pre-chillers have become increasingly popular. The main purpose of a poultry pre-chiller is to reduce the microbial load on the carcasses heading into the main chiller. Reducing the microbial load on the carcasses in the pre-chillers aids in ensuring a more adequate reduction in *Salmonella* and *Campylobacter spp*. in the main-chiller as well as in further processing.

Adjusting the pH of the PAA solution in the pre-chiller and main-chiller has become common practice. pH adjusting the pre-chiller and main-chiller between 7.6-9.5 potentially decreases PAA vapor evolution, aids in increasing the aesthetics of the poultry, and increases the sensitivity of *Salmonella spp*. to PAA. The pKa (acid dissociation constant) of peracetic acid is 8.2

at 25°C and 8.4 at 0°C. While it is hypothesized that increasing the pH of a poultry chiller will increase the *Salmonella* susceptibility, *Campylobacter jejuni may* be more resistant to pH changes, thus decreasing the end-point efficacy of PAA at elevated pH levels.

The purpose of this study is to determine the effects of PAA against *Salmonella* Heidelberg and *Campylobacter jejuni* at elevated pH levels in a laboratory simulated chiller.

Materials and Methods

A total of five 55-gallon blue HDPE drums were cut in half. The drums were fitted with a circular ½ inch line composed of polyethylene (PE) connected to a rotary screw air compressor. A total of 10 holes were drilled into the PE tubing using a 3/32 drill bit approximately 15 cm apart. The drum would serve as the simulated main-chiller with the tubing providing air agitation. The drums were filled with 85 kg of potable city water and ice achieving a temperature between 0-2.2°C.

A freeze-dried pellet of *Salmonella* Heidelberg (ATCC 8326) was reconstituted in 100 mL of sterile Brain Heart Infusion (BHI) Broth (Criterion, Cat No.: C5140). The culture was incubated in a microaerophilic atmosphere at 35°C for 24 hours. After the 24-hour incubation period, aliquots were taken from the culture and plated on 20 Hardy Diagnostic's Tryptic Sot Agar with 5% Sheep Blood (Blood agar, Cat No.: A10). The cultured plates were incubated in a microaerophilic atmosphere at 35°F for 24 hours. After the incubation period, the bacteria were separated from the agar plates using a sterile L-shaped bacterial spreader and transferred to 10L of sterile Butterfield's Buffer. The solution was mixed to ensure homogeneity. This solution would serve as the *Salmonella* Heidelberg inoculum for the study.

Two *Campylobacter jejuni* (ATCC 33291) freeze-dried pellets were cultured in two vials containing approximately 100 mL of Bolton Broth (Sigma Aldrich, lot number BCBB7257) containing 5% defibrinated sheep blood (Hardy Diagnostics). The cultured broth was incubated under an anaerobic atmosphere at 40° C for 48 hours. The bacteria were separated from the nutrient broth by centrifugation. One mL of the concentrated bacteria mixture was removed from the cultured broth and plated on Campy Cefex Agar (Hardy Diagnostics). This was repeated 20 times to achieve a total of 20 Campylobacter inoculated Campy Cefex Agar plates. The plates were kept under anaerobic atmosphere and incubated for 48 hours at 40°C. After the 48-hour incubation period, the surfaces of the 20 *Campylobacter* inoculated Campy Cefex Agar plates were aseptically scraped using a sterile L-shaped spreader and transferred to 10 L of sterile Butterfield's Buffer. The solution was mixed to ensure homogeneity. This solution would serve as the *Campylobacter jejuni* inoculum for the study.

Sixty freshly slaughtered boiler chickens (post evisceration, pre-processed) were obtained from a local processor located in Modesto, CA. The chicken carcasses were separated into two groups consisting of 30 carcasses per group. Thirty of the chicken carcasses were individually submerged in the *S.* Heidelberg culture for five seconds. The remaining 30 carcasses were individually submerged in the *Campylobacter jejuni* culture for 5 seconds. The inoculated carcasses were allowed to sit undisturbed for 45 minutes to ensure bacterial attachment.

Perasan MP-2C (Lot # 844-092815-1) was analyzed via iodometric titration to yield a PAA concentration of 22.61% and a hydrogen peroxide concentration of 5.81%. Four of the five chillers were dosed with 13.5 mL of Perasan MP-2C to achieve a nominal PAA concentration of 40 ppm. The concentration of PAA in the chillers was analyzed using the Palin Modified DPD Methodology (Enviro Tech US Patent 7,651,860 B2) to verify the concentration was 40 ppm ± 3 ppm. PAA was not added to one of the simulated chillers and the pH was not adjusted. This chiller would serve as the water treated control for the study. The pH of the of the four PAA dosed chillers were adjusted using 18M sodium hydroxide. The four pH levels that were tested were 6, 7.5, 8.5, and 9.5 (± 0.2).

Five each of the *S.* Heidelberg and *C. jejuni* inoculated carcasses were transferred to individual sterile stomacher bags along with 400 mL of sterile Neutralizing Buffered Peptone water (nBPW)[1]. The contents of the bags were agitated vigorously for 60 seconds to ensure adequate removal of surface bound bacteria. Aliquots were taken from each bag and plated on their respective media. The *S.* Heidelberg samples were plated on 3M Enterobacteriaceae Petrifilms™ and the *C. jejuni* samples were plated on Campy Cefex Agar plates. These samples would serve as the untreated control samples.

The remaining 25 *Salmonella* inoculated carcasses were submerged into the five different air agitated chillers (five carcasses per chiller). The temperature of each chiller was maintained in a temperature range of between 0-2.2°C using additional ice. The concentration of PAA was maintained at 40 ± 3 ppm using additional Perasan MP-2C. The pH was also maintained for each of the respective chillers using additional 5M sodium hydroxide. After 60 minutes of treatment, the carcasses were removed and transferred to individual sterile stomacher bags along with 400 mL of sterile nBPW. The bags were agitated vigorously for 60 seconds to ensure adequate removal of surface bound bacteria. Aliquots were taken from each bag and plated on 3M Enterobacteriaceae Petrifilms™.

[1] nBPW formulating procedure: https://askfsis.custhelp.com/app/answers/detail/a_id/2007 Accessed September 21, 2016

The water from each chiller was discarded and the chillers as well as the tubing were pressure washed with a mild detergent then treated with a nominal 1000 ppm PAA solution for 20 minutes to ensure complete eradication of residual *Salmonella*. The chillers were then refilled with water and ice. PAA was added to the fresh chiller solutions and the pH of the four PAA chillers were adjusted using 18M sodium hydroxide.

Next, the remaining 25 *Campylobacter* inoculated carcasses were submerged into the five different air agitated chillers (five carcasses per chiller). The temperature of each chiller was maintained at a temperature range of between 0-2.2°C using additional ice. The concentration of PAA was maintained at 40 ± 3 ppm using additional Perasan MP2C. The pH was also maintained for each of the respective chillers using additional 5M sodium hydroxide. After 60 minutes of treatment, the carcasses were removed and transferred to individual sterile stomacher bags along with 400 mL of sterile nBPW. The bags were agitated vigorously for 60 seconds to ensure adequate removal of surface bound bacteria. Aliquots were taken from each bag and plated on Campy Cefex Agar plates.

Petrifilms were incubated at 35°F under a microaerophilic atmosphere for 24 hours then enumerated. Campy Cefex Agar plates were incubated under anaerobic conditions at 40°C for 48 hours then enumerated.

Results and Discussion

Table 1 shows the average remaining *S.* Heidelberg colonies on the poultry carcasses treated with water and PAA at various pH levels in a stimulated chiller for 60 minutes.

Sample ID	Avg. $\log_{10}$ (CFU/mL)	Avg. $\log_{10}$ Reduction (CFU/mL)	STDev (CFU/mL)	n
Control (untreated)	6.60	NA	0.08	5
Water only	6.35	0.25	0.05	5
40 ppm PAA pH 6	5.07	1.53	0.13	5
40 ppm PAA pH 7.5	5.11	1.49	0.20	5
40 ppm PAA pH 8.5	5.05	1.55	0.19	5
40 ppm PAA pH 9.5	4.93	1.67	0.21	5

The untreated control carcasses had an average $\log_{10}$ *S.* Heidelberg count of 6.60 CFU/mL. The carcasses treated with water only for 60 minutes at 0-2.2°C decreased *S.* Heidelberg the $\log_{10}$ counts by 0.25 CFU/mL. Treatment with a nominal 40 ppm PAA at pH 6, 7.5, 8.5, and 9.5 yielded very similar $\log_{10}$ reductions. While the microbial reductions at the elevated pH levels do not appear drastically different, there's a clear trend that suggests *Salmonella* Heidelberg is more susceptible to PAA at elevated pH levels.

Table 2 shows the average remaining *C. jejuni* colonies on the poultry carcasses treated with water and PAA at various pH levels in a stimulated chiller for 60 minutes.

Sample ID	Avg. log_{10} (CFU/mL)	Avg. log_{10} Reduction (CFU/mL)	STDev (CFU/mL)	n
Control (untreated)	5.99	NA	0.38	5
Water only	5.79	0.20	0.04	5
40 ppm PAA pH 6	4.81	1.18	0.06	5
40 ppm PAA pH 7.5	4.78	1.20	0.09	5
40 ppm PAA pH 8.5	4.82	1.17	0.08	5
40 ppm PAA pH 9.5	4.84	1.15	0.04	5

The untreated control poultry carcasses had an average log_{10} of *C. jejuni* of 5.99 CFU/mL. The carcasses treated with water only for 60 minutes at 1°C decreased the *C. jejuni* log_{10} counts by 0.20 CFU/mL. The carcasses treated with a nominal 40 ppm PAA for 60 minutes showed relatively similar decreases in *C. jejuni* counts.

Given a pKa of PAA is 8.4 at 0°C, the actual PAA concentration changed depending on the pH levels. Commercially available titration kits do not reflect this change because all commercially PAA test kits use an acid reagent thus dissociated PAA (sodium peracetate), reverts back to the undissociated PAA.

Table 3 shows the undissociated (free) PAA concentrations at 0°C at the various pH levels.

pH	Nominal PAA Conc. (ppm)	% Undissociated PAA	Actual PAA Conc. (ppm)
6	40	100%	40.0
7.5	40	90%	36.0
8.5	40	45%	18.0
9.5	40	10%	4.0

Conclusions

It was hypothesized that increasing the pH of a poultry chiller will increase *Salmonella spp*. sensitivity to PAA thus achieving similar reductions at higher pH levels in *Salmonella* counts compared to PAA solutions at pH 6 and 7.5. While the results presented in Table 1 do not show a drastic reduction in *Salmonella* Heidelberg counts at elevated pH levels, there is a clear trend that suggests *S.* Heidelberg is more sensitive to PAA at elevated pH levels in poultry chillers. Considering the data presented in Table 3, and the fact that the pKa of PAA at 0°C is pH 8.4, the undissociated PAA concentration at elevated pH levels (8.5 and 9.5) are significantly

lower than at lower pH levels (6 and 7.5), yet the resulting efficacy is similar at all pH levels tested, indicating that *Salmonella* is indeed more sensitive to eradication by PAA.

While *Salmonella* Heidelberg demonstrated to be more susceptible to PAA at elevated pH levels, *Campylobacter jejuni* reductions remained about the same across the various pH levels. Considering again that the concentration of undissociated PAA is drastically different at pH 8.5 and 9.5 compared to pH 6 and 7.5, the *C. jejuni* efficacy results suggest that increasing the pH of a poultry chiller will not affect the efficacy against *Campylobacter spp*. compared to lower pH levels. In previous studies done by this laboratory, Campylobacter exhibited a MEC (minimum effective concentration) against PAA of approximately 15-20 ppm at common pH ranges.

It should be noted that in actual poultry applications there are a wide variety of variables that can affect microbial efficacy in poultry chillers. If poultry chillers are adjusted to a pH >9, it is recommended to increase PAA concentrations a minimum of 20-30% to account for the decrease in undissociated (free) PAA at these elevated pH levels to ensure microbial efficacy is not compromised.