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# Ethyl Acetoacetate Added to the Wash Water of Chicken Reduces Spoilage Bacteria, as well as Externally Added *Salmonella* Spec. and *Campylobacter* Spec

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## Abstract

Ethyl acetoacetate (EAA) is a food anti-microbial with a previously demonstrated efficacy in reducing spoilage bacteria when used as a treatment for ground beef. In this study, we tested the effectiveness at reducing naturally occurring spoilage bacteria and externally added pathogens when used as a processing aid for chicken. Boneless and skinless chicken thighs were first immersed in bacteria of the genus *Salmonella* or *Campylobacter* for 5 min and then washed either in $H_2O$ or 10% EAA in $H_2O$ for 5 min, followed by 30 min of incubation at 4°C. Control meat pieces did not receive the bacterial inoculum, the EAA treatment, or either.

The wash with 10% EAA in $H_2O$ reduced the total number of naturally occurring bacteria by 1.2 log, which is equivalent to 94%. Pseudomonads were reduced by 1.03 log (= 90.7%). Lactobacilli were increased by 10% in response to the EAA treatment. Externally added *S. enterica* subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium (ATCC 19585) was reduced by 0.6 log (=75%). *S. enterica* serovar Typhimurium FSL R6-0020 (TB0041) was reduced by 0.35 log (=48%). *C. jejuni* subsp. *jejuni* (Jones *et al*.,) Veron and Chatelain (ATCC 700819) was reduced by the EAA treatment by 0.45 log (=51%) and *C. coli* (Doyle) Veron Chatelain (ATCC33559) by 0.6 log (=76%). We believe that EAA is as effective or more effective in reducing the number of spoilage and pathogenic bacteria on chicken than currently existing processing aids.

***Keywords:*** *Salmonella Enterica Serovar Typhimurium; Campylobacter Jejuni; Campylobacter Coli; Chicken; Food Anti-Microbial*

## Abbreviations

EAA: Ethyl Acetoacetate; AAA: Acetoacetate; TB: Tryptone Broth; TSB: Tryptic Soy Broth; BHI: Brain Heart Infusion Broth; MRD: Maximum Recovery Diluent; SSA: Salmonella Shigella Agar; MHA: Mueller-Hinton Agar

## Introduction

Ethyl acetoacetate (EAA) is a food anti-microbial that was described as growth and biofilm inhibitor in our own research. EAA is chemically similar to acetoacetate (AAA), the compound we originally identified as reducer of live bacterial counts and biofilm amounts in liquid beef broth [21]. EAA is more cost effective than AAA and the better anti-microbial against *Yersinia enterocolitica*, *Serratia marcescens*, and *Cronobacter sakazakii* [14]. EAA has since been used in one clinical application that was modeled after the antibiotic lock treatment of urinary catheters [29], where it reduced biofilm in silicone tubings [30]. As an example of a food safety application, EAA reduced spoilage bacteria when used as a treatment for ground beef at a concentration of 0.5% [13].

EAA should be safe to use as a food anti-microbial, as it has FDA approval as flavoring agent under 21CFR172.515 and is used under Flavis No. 9.402. To determine whether EAA might change to a toxic compound during the heating process, we investigated the heat sta-

bility of EAA in liquid beef extract. EAA was heated to 165 F (inside of ground beef) and 190 F (outside of ground beef) and analyzed by a combination of gas chromatography and mass spectrometry [12]. Under numerous conditions tested, we never saw a difference in the chromatograms and spectrograms between the untreated and the heat treated samples of EAA. We conclude that EAA is heat stable up to 190 F.

During our efforts to commercialize our pending patent [28], several companies commented that we needed to expand the list of food applications for EAA before they would further investigate our technology. Chicken was recommended for this purpose. The Centers for Disease Control and Prevention (CDC; www.cdc.gov) list two outbreaks of bacterial infectious disease associated with chicken in 2021 so far, one by *Listeria monocytogenes*, and one by *Salmonella enteritidis*. Earlier outbreaks associated with chicken include an outbreak by *Salmonella infantis*, one by *Salmonella* Typhimurium 1, 4,[5],12:i:, and a third by *Salmonella Typhimurium* in 2018. Between 2015 and 2019, Canada reported 18 outbreaks of *Salmonella* that were associated with breaded chicken products and a total of 584 confirmed cases [25]. A recent review in Avian Pathology summarizes the chicken-*Campylobacteri jejuni* interaction [1]. The authors describe chicken as a predominant source of *C. jejuni*, and question the common belief that the bacteria are just commensal bacteria that colonize the chicken intestinal tract. *C. jejuni and Campylobacter coli* are prevalent in chicken all over the world (*e.g.* Malaysia [33], Italy [5], Spain [7], Brazil [6]) and commonly recognized as a primary infectious agent for chicken in addition to *Salmonella* spec.

Interventions to prevent food borne disease outbreaks associated with chicken are diverse. In the past, a combination of lactic acid and sodium benzoate in the wash solution was considered effective at reducing numerous pathogenic bacteria on raw chicken [15]. These have been replaced by chlorine and peroxyacetic acid to be used at the reprocessing and chill stages, as well as irradiation at packaging; however, a recent study describes that none of the single interventions complied with the standards set by the Food Safety and Inspection Service (FSIS; www.fsis.usda.gov) [9]. Likewise, a study on *C. jejuni* investigated the effect of chlorine on the morphology of the bacteria and found that chlorine treated bacteria divided into two sub-populations; one that exhibited changes in cell shape and showed cellular degradation and a second one that remained unchanged and could be resuscitated [26]. Altogether, the list of chemicals that is used to prevent food borne disease outbreaks associated with chicken is long and yet, outbreaks continue to happen. With this study, we introduce EAA as novel intervention to reduce *Salmonella* spec. and *Campylobacter* spec. on chicken. EAA is cost effective and reduces the number of some naturally occurring background bacteria on the chicken, as well as live bacterial counts of several externally added bacterial strains of the genus *Salmonella* and *Campylobacter.* We believe that EAA is a food anti-microbial that deserves further investigation as a processing aid for chicken.

## Materials and Methods

### Bacterial strains

Two different strains of each of *Salmonella* spec. and *Campylobacter* spec. were tested. The two *Salmonella* strains were *S. enterica* serovar Typhimurium FSL R6-0020, also designated TB0041 and *S. enterica* subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium, also designated LT2. The two *Campylobacter* strains were *C. jejuni* subsp. *jejuni* (Jones *et al.*) Veron and Chatelain ATCC 700819, also designated NCTC11168 and *C. coli* (Doyle) Veron Chatelain ATCC33559, also designated CIP 7080. *S. enterica* serovar Typhimurium FSL R6-0020 has been provided by Dr. Teresa Bergholz (Michigan State University, East Lansing MI). The remaining strains were obtained from the American Type Culture Collection (ATCC; www.atcc.org). The basic characteristics of these bacterial strains are summarized in table 1.

| Bacterial strain | Alternative designation | ATCC # | Characteristic | Reference |
|---|---|---|---|---|
| *S. enterica* serovar Typhimurium FSL R6-0020 | TB0041 | Not deposited | Source: bovine feces<br>Genome sequenced: no | www.foodmicrobe-tracker.com [38] |
| *S. enterica* subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium | LT2 | ATCC 19585 | Source: lab modified<br>Genome sequenced: yes | [18; 19; 27] |
| *C. jejuni* subsp. *jejuni* (Jones *et a*l.,) Veron and Chatelain | NCTC 11168 | ATCC 700819 | Source: human feces<br>Genome sequenced: yes | [32] |
| *C. coli* (Doyle) Veron Chatelain | CIP 7080 | ATCC 33559 | Source: pig feces<br>Genome sequenced: yes | [34] |

***Table 1:*** *Bacterial strains used for this study.*

The four bacterial strains were made resistant towards 50 µg/ml of nalidixic acid, using the method that was described previously [36]. Bacteria were stored at -80°C. Prior to each experiment, *Salmonella* spec. was plated onto Luria Bertani broth (LB) agar plates, supplemented with 50 µg/ml nalidixic acid, and incubated at 34°C overnight. *Campylobacter* spec. was plated onto tryptic soy broth (TSB) agar plates, supplemented with 50 µg/ml nalidixic acid and incubated for a week under microaerophilic conditions at 42°C. To prepare the inocula for the chicken experiments, *Salmonella* spec. was grown overnight in liquid brain-heart infusion (BHI), supplemented with 50 µg/ml nalidixic acid at 34°C. The overnight culture was diluted 1:10 into BHI and incubated at 34°C for 2 h. The culture was then adjusted to an $OD_{600}$ of 1.0 with BHI. The actual inoculum was 200 ml of a 1:100 dilution of this culture in PBS and contained between 1.58 x $10^9$ and 6.75 x $10^9$ colony forming units (CFU) of *S. enterica*. *Campylobacter* spec. was grown for three days in liquid BHI under microaerophilic conditions at 42°C. The inoculum for the chicken experiment was 200 ml of this culture, containing between 1.5 x $10^9$ and 5.3 x $10^9$ CFU of *C. jejuni* and between 3 x $10^6$ and 1 x $10^7$ for *C. coli. C*ompositions of LB, TSB, and BHI are included in table 2.

| Name | Abbrev. | Purpose | Composition | Brand |
|---|---|---|---|---|
| Luria Bertani agar | LB | *Salmonella* growth | 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar | Difco BD |
| Tryptic soy broth agar | TSB | *Campylobacter* growth | 17 g/l pancreatic digest casein, 3 g/l papaic digest of soybean, 2.5 g/l dextrose, 5 g/l NaCl, 2.5 g/l $K_2PO_4$, 15 g/l agar | Difco BD |
| Brain heart infusion | BHI | *Campylobacter* growth | 7.7 g/l calf brain infusion solids, 9.8 g/l beef heart infusion solids, 10 g/l protease peptone, 5 g/l NaCl, 2 g/l glucose, 2.5 g/l $Na_2HPO_4$ | Difco BD |
| Maximum recovery diluent | MRD | Diluent | 1 g/l peptone, 8.5 g/l NaCl, pH 7.0 | Becton Dickinson |
| Plate count agar | PCA | Total bacterial counts | 5 g/l tryptone, 2.5 g/l yeast extract, 1 g/l glucose, 15 g/l agar, pH 7.0 | Difco BD |
| Pseudomonas agar | PSA | Detection of pseudomonads | 16 g/l gelatin peptone, 10 g/l casein hydrolysate, 10 g/l $K_2SO_4$, 1.4 g/l $MgCl_2$, 0.5 mg/ml cetrimide, 0.5 mg/ml fucidin, 2.5 mg/ml cephalosporin, 11 g/l agar, pH 7.1 | Oxoid |
| All purpose tween agar | APT | Detection of lactobacilli | 7.5 g/l yeast extract, 12.5 g/l pancreatic digest of casein, 10 g/l dextrose, 5 g/l sodium citrate, 0.001 g/l thiamine HCl, 5 g/l NaCl, 5 g/l $K_2HPO_4$, 0.14 g/l $MnSO_4 \cdot H_2O$, 0.8 g/l $MgSO_4 \cdot 7H_2O$, 0.04 g/l $FeSO_4$, 0.2 g/l polysorbate, 15 g/l agar, pH 6.7 | Difco BD |
| Shigella Salmonella agar | SSA | Detection of *Salmonella* | 5 g/l beef extract, 2.5 g/l pancreatic digest of casein, 2.5 g/l peptic digest of animal tissue, 10 g/l lactose, 8.5 g/l bile salts mixture, 8.5 g/l sodium citrate, 8.5 g/l sodium thiosulphate, 1 g/l ferric citrate, 0.025 g/l neutral red, 15 g/l agar, 0.33 mg/l brilliant green, pH 7.0 | Difco BD |
| Müller Hinton agar[1] | MHA | Detection of *Campylobacter* | 2 g/l beef extract, 17.5 g/l acid hydrolysate of casein, 1.5 g/l starch, 12.5 mg/l sodium pyruvate, 12.5 mg/l ferrous sulfate, 12.5 mg/l sodium metabisulphite, 5,000 IU/l polymyxin B, 10 mg/l rifampicin, 10 mg/l trimethoprim lactate, 10 mg/l amphotericin B, 17 g/l agar, pH 7.3 | DifcoBD/ Oxoid HiMedia Laboratories |

***Table 2:*** *Composition of the bacterial growth media and selective agar plates.*

Note that sodium pyruvate, ferrous sulphate, and sodium metabisulphite were added as *Campylobacter* growth supplement (liquid) SR0232E from Oxoid. Polymyxin, rifampicin, trimethoprim lactate, and amphotericin were added as Campylobacter selective supplement IV, modified (Preston Selective Supplement) from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

## Inoculation and treatment of chicken

For a complete work flow of the experiment and the enumeration of the bacteria, please, see Fig. 1. We purchased boneless chicken thighs from a local grocery store and weighed the pieces, which usually were between 75 and 95 g of weight. Weighed chicken thighs were put into freezer bags and 200 ml of inoculum were added. For the negative control chicken, 200 ml of PBS (*Salmonella*) or BHI (*Campylobacter*) was added. Chicken thighs were incubated in the bacterial suspension for 5 min. Chicken thighs were then drained on sterile mash racks into collection trays for 10 min before being transferred into a new freezer bag, to which 200 ml of either $H_2O$ or 10% EAA in $H_2O$ were added. Chicken thighs were incubated in this solution for 5 min and then drained as before. Chicken thighs were transferred to stomacher bags (VWR, Radnor, PA), weighed, and incubated at 4°C for 30 min. To determine the effect of EAA on the naturally occurring flora on the chicken, the initial experiment was done without adding bacteria at 5%, 7.5%, and 10% of EAA.

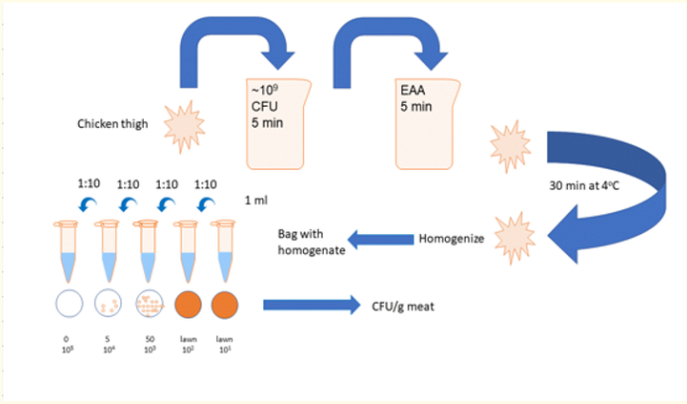


***Figure 1:*** Workflow of the chicken experiment. Individual components of the figure were taken from Motifolio (Motifolio Inc., Ellicott City, MD).

## Enumeration of bacteria

Live bacterial counts on the chicken were determined after adding maximum recovery diluent (MRD) to the chicken pieces in the stomacher bags at an amount of 1 ml per 5 g of chicken. Chicken was homogenized in a Seward Stomacher 400 Circulator (Cole Parmer, Vernon Hills, IL). Bacteria were enumerated by plating serial dilutions onto selective agar plates. To produce two technical replicates for each of the biological replicates, we plated each dilution onto two separate agar plates. Total bacterial counts were determined on PCA plates, incubated for two days at room temperature. Pseudomonads were enumerated on PSA plates, incubated for two days at room temperature. Lactobacilli were determined on APT plates, incubated anaerobically at 30°C for two days. *Salmonella* spec. was enumerated on SSA plates, incubated at 37°C for 2 days. For the enumeration of *Campylobacter* spec., we used MHA plates, incubated under microaerophilic condition at 42°C for two days. Each experiment was performed in four biological replicates. Media compositions for the selective agar plates are included in table 2.

## Data analysis

Each experiment was performed in four biological (different bacterial cultures and chickens) and two technical (homogenates were plated twice) replicates. Using the dilution series, colony counts were converted first to CFU/g of chicken and then to $\log_{10}$ CFU/g of chicken. Next, the average for the two plate replicates was calculated. We determined log reductions by subtracting the $\log_{10}$ CFU/g of chicken data for the EAA treated chicken pieces from those of the $H_2O$ treated chicken pieces. In a final step, average and standard deviation were calculated across the four biological replicates. Log reductions were converted to percent reductions as follows: $100-(1/10^{\text{log reduction}}) \times 100$.

For the analysis of the spoilage bacteria, statistical analysis was performed with a one-way ANOVA for the combination of treatment and concentration. For the analysis of the externally added pathogens, statistical analysis was performed with a paired *t*-test to compare $\log_{10}$ CFU/g of chicken data of EAA treated chicken with those from $H_2O$ treated samples. For both analyses, a *p*-value below 0.05 was considered the threshold for a statistical significance of the difference.

## Results and Discussion

### EAA reduces spoilage bacteria on chicken by approximately 1 log

The determination of the effect of EAA on the live bacterial counts of the naturally occurring microflora on chicken was performed in three separate experiments at different concentrations of EAA (5%, 7.5%, 10%). At each of these concentrations, the $\log_{10}$ CFU/g of chicken were compared between pieces of chicken that were treated with $H_2O$ and those that were obtained from the EAA treatment. Bacteria were enumerated on PCA to determine the total bacterial counts on the chicken, PSA to determine Pseudomonads, and APT for the determination of Lactobacilli. Pseudomonads and Lactobacilli are among the predominant naturally occurring bacteria in chicken and considered spoilage bacteria [20]. The data are summarized in Fig. 2. The results from the statistical analysis are presented in figure 3.

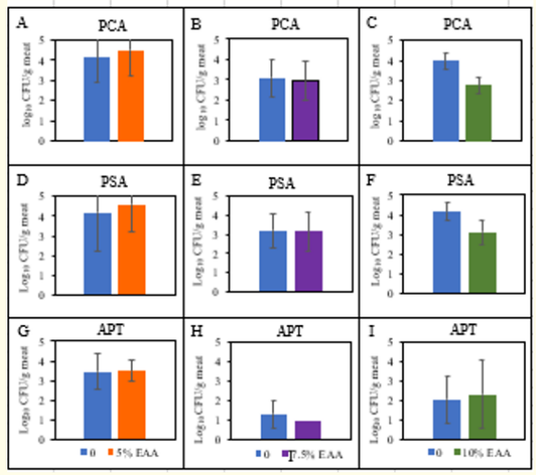


***Figure 2:*** Live bacterial counts of naturally occurring spoilage bacteria after treatment with EAA. Panel A; total bacterial count from the PCA plates, comparing 5% EAA in H2O to H2O (0% EAA). Panel B; PCA count, comparing 7.5% EAA to 0% EAA. Panel C; PCA counts, comparing 10% EAA to 0% EAA. Panels D through F; comparison of 5% EAA, 7.5% EAA, and 10% EAA to the 0% EAA control samples for the Pseudomonad count from the PSA plates. Panels G through I; comparison of 5% EAA, 7.5% EAA, and 10% EAA to the 0% EAA control samples for the Lactobacilli count from the APT plates.

| | | PCA | | | | PSA | | | | APT | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0% | 5% | 7.5% | 10% | 0% | 5% | 7.5% | 10% | 0% | 5% | 7.5% | 10% |
| PCA | 0% | | ***** | | | | | | | | | | |
| | 5% | ***** | | ***** | ***** | | | | | | | | |
| | 7.5% | | ***** | | | | | | | | | | |
| | 10% | | ***** | | | | | | | | | | |
| PSA | 0% | | | | | | | | | | | | |
| | 5% | | | | | | | | ***** | | | | |
| | 7.5% | | | | | | | | | | | | |
| | 10% | | | | | | ***** | | | | | | |
| APT | 0% | | | | | | | | | | | ***** | |
| | 5% | | | | | | | | | | | ***** | |
| | 7.5% | | | | | | | | | ***** | ***** | | |
| | 10% | | | | | | | | | | | | |

***Figure 3:*** Outcome of the one-way ANOVA for the data from the spoilage bacteria. The dark blue fields mark identical plates/concentrations on the x-axis and the y-axis. Light blue fields mark fields that contain data from the same plates (e.g. we don't compare PSA to PCA plates), but at different concentrations. Asterisks signify a statistically significant difference between bacterial counts at the two concentrations on the x-axis and the y-axis.

The total bacterial count did not get reduced until a concentration of 10% of EAA was reached (Figurre 2A-C). At 5% EAA, the total bacterial count was even higher than the one that was obtained from the water treated chicken (Figure 2A, orange bars). At 7.5% EAA, we observed a log reduction of 0.11 (Figure 2B, purple bars). At 10% EAA, the log reduction was 1.2 on the PCA plates (Figure 2C, green bars). The difference between the total bacterial count at 10% (Figure 2C, green bar) and that at 5% (Figure 2A, orange bar) was statistically significant (Figure 3). It is noteworthy that for the PCA counts, the difference between 10% (Figure 2C, green bar) and water (Figure 2A-C, blue bars) is not statistically significant. It is possible that the statistical significance of the difference between the 5% and 10% treatments is due to the slightly increased cell counts at 5% relative to the water treatment. However, the error bars in Figure 2C don't overlap, increasing confidence into the difference between the 10% EAA and the water treatment.

The pattern was very similar for the Pseudomonad counts from the PSA plates. At 5% EAA, the Pseudomonad count was higher for the EAA treatment than for the water treatment (Figure 2D, orange bar). At 7.5% EAA, the count from the PSA plate was very similar to the PSA count from the water treatment (Figure 2E, purple bar). At 10% of EAA, the log reduction was 1.03 (Figure 2F, green bar). The difference between the Pseudomonad count at 10% is different from that at 5% with statistical significance (Figure 3), while the difference between the 10% EAA and the water treatment is not. It is noteworthy that the water treated chicken yielded similar counts from the PCA plates and the PSA plates. This confirms that Pseudomonads are the largest population of bacteria on untreated chicken, which is consistent with current literature [22]. The differences in both PCA and PSA counts from the water treated chicken between the three experiments can be explained by fluctuations in the naturally occurring microflora on the chicken. We have seen this in our ground beef experiments as well [13].

The Lactobacilli counts from the APT plates was different. At 5%, there was no difference in APT counts from the water treatment (Figure 2G, orange bar). At 7.5%, we did achieve a log reduction of 0.37. While the reduction does not seem very high, statistical analysis yielded a statistical significance of this difference (Figure 3). Intriguingly, the APT counts were higher for 10% EAA treatment than for the water treatment. Since Lactobacilli can exhibit an inhibitory effect on pathogens, including *Salmonella* spec and *Campylobacter* spec. [4;23;35], a treatment that reduces the more critical Pseudomonad count, while increasing the Lactobacilli count, might be successful at reducing the number of pathogens on the chicken.

Altogether, EAA reduces total bacterial counts and Pseudomonad counts by a little more than 1 log when used as a treatment in the wash water at a concentration of 10%. This reduction is much lower than what we saw when EAA was used as a treatment for ground beef [13]. However, the chicken experiment was done under different conditions than the beef experiment. For chicken, we added 10% EAA to the wash water, followed by 30 min of incubation. This application is an example of using EAA as a processing aid for chicken and we rely on the anti-microbial to kill the bacteria. For ground beef, EAA was added at a 0.5% concentration to the meat, followed by up to 5 days of incubation. This previous application is an example of using EAA as treatment, where the anti-microbial was used to inhibit bacterial growth in addition to killing them. The short incubation time in the chicken experiment caused a lack of opportunity for bacterial growth, thus the total bacterial counts in the water treated samples were much lower (~4 log) (this study) than in the previous beef experiment (~8 log) [13]. On chicken, 8 $\log_{10}$ CFU/g of *Pseudomonas* spec. is considered spoilage [3], but it can take 7 days of storage for *Pseudomonas* counts to get to 8 log [8].

To evaluate the data from this current study of using EAA as a processing aid for chicken, one has to compare the data from this study to the effect of other anti-microbials that were added to the wash water of chicken for a short period of time (min or s). As one example, using plasma-activated water on chicken breasts for 60 s reduced the live bacterial counts of *Pseudomonas deceptionensis* by approximately 1 log [16], which is similar to our log reduction on the Pseudomonad count. In another example, chlorinated wash water or electrolyzed oxidizing water reduced psychrotrophic bacteria on chicken from 1.54 + 0.56 $\log_{10}$ CFU/g for chicken that had been washed with water to 0.99 + 0.01 $\log_{10}$ CFU/g for chicken that had been washed with either chlorinated or electrolyzed oxidizing water. These bacterial counts were determined immediately after washing [11] and equal a log reduction of 0.55, which is less than the reduction of total bacterial counts by EAA that we observed in this study. We believe EAA is as effective or more effective than current processing aids at reducing total bacterial counts and Pseudomonad counts.

### EAA reduces externally added *S. enterica* on chicken

The effect of 10% EAA added to the wash water of chicken on *S. enterica* was determined with two externally added strains, here designated ATCC 19585 (*S. enterica* subsp. enterica (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium) and TB0041 (*S. enterica* serovar Typhimurium FSL R6-0020) (Figure 4). EAA caused a log reduction of 1.3 for the total bacterial count on the PCA plates for ATCC 19585 with a *p*-value from the paired *t*-test of 0.000318, as well as a log reduction of 0.35 for the total bacterial count for TB0041 with a *p*-value of 0.005574 from the *t*-test. On the SSA plates that selectively allow for *S. enterica* to grow, we obtained log reductions of 0.62 and 0.36, respectively. Both these reductions were statistically significant with *p*-values of 0.000333 and 0.007432, respectively. Converting log reductions to percent reductions, we obtained a 92% reduction for ATCC 19585 on the PCA plates, a 45% reduction for TB0041 on the PCA plates, a 75% reduction for ATCC 19585 on the SSA plates and a 48% reduction for TB0041 on the SSA plates.

In comparison, a solution containing 0.5% lactic acid and 0.05% sodium benzoate reduced *S. enterica* by approximately 1 log after washing for 30 min [15]. While the log reduction is somewhat larger than the one we obtained for EAA, the 30 min washing time is also much longer than what we used for EAA (this study). An intriguing study investigated chlorine resistance in dependence on the exposure time among Salmonella Kentucky that had been isolated from chicken [24]. After 10 min of exposure to 30 ppm of chlorine, the most resistant strain had a log reduction of 0.55, the most susceptible strain of 3.11. The time it took to achieve a log reduction of 1 was 10 min for the most resistant strain and 1.5 min for the least resistant strain. For comparison, the exposure time to treat the chicken with EAA in this study was 5 min.

Combination treatments to reduce *Salmonella* on chicken have been tested as well. A solution of 0.5% SDS in combination with 0.0005% chlorine reduced *Salmonella* on chicken skin between 13% (skin mechanically de-feathered) and 34% (skin hand de-feathered) [39]. In comparison, the reductions caused by the EAA wash in this study yielded percent reductions of 75% and 48% for the two *S. enterica* strains. It seems like the reductions in response to the EAA treatment are similar or better than those for existing treatment options.

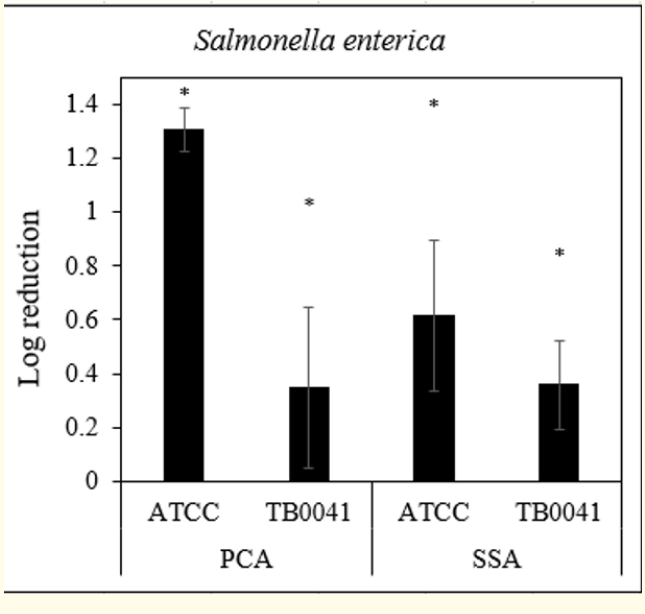


***Figure 4:*** Effect of 10% EAA on externally added S. enterica ATCC 19585 and S. enterica T0041. On the left are the total bacterial counts from the PCA plates, on the right the Salmonella counts from the SSA plates. An asterisk above the bar indicates a statistically significant difference between EAA treated and the H2O treated chicken.

### EAA reduces externally added *Campylobacter* spec. on chicken

The effect of 10% EAA added to the wash water of chicken had an effect on *C. jejuni* subsp. *jejuni* (Jones *et al*.,) Veron and Chatelain (NCTC 11168; ATCC 700819) that was similar to that observed for *S. enterica*. EAA caused a log reduction of 1.09 for the total bacterial counts from the PCA plates with a *p*-value from the paired *t*-test of 0.0033. On the MHA plates that selectively allow for *Campylobacter* spec. to grow, we obtained a log reduction of 0.45 with a *p*-value of 0.016774. These *p*-values indicate a statistically significant difference between the treated and untreated chicken on both, PCA and MHA plates. Converting log reductions to percent reductions, we obtained an 90.5% reduction on the PCA plates and a 51.1% reduction on the MHA plates.

For the *C. coli* (Doyle) Veron Chatelain strain (CIP 7080; ATCC33559), EAA caused a log reduction of 0.45 for the total bacterial counts from the PCA plates with a *p*-value from the paired *t*-test of 0.07296. On the selective MHA agar plates, the log reduction was 0.62 with a *p*-value of 0.079469. Percent reductions were 62.6% on the PCA plates and 76% on MHA plates. Altogether, the effect of EAA on *Campylobacter* spec. appears similar to that on *S. enterica*, though better for *C. jejuni* than for *C. coli.*

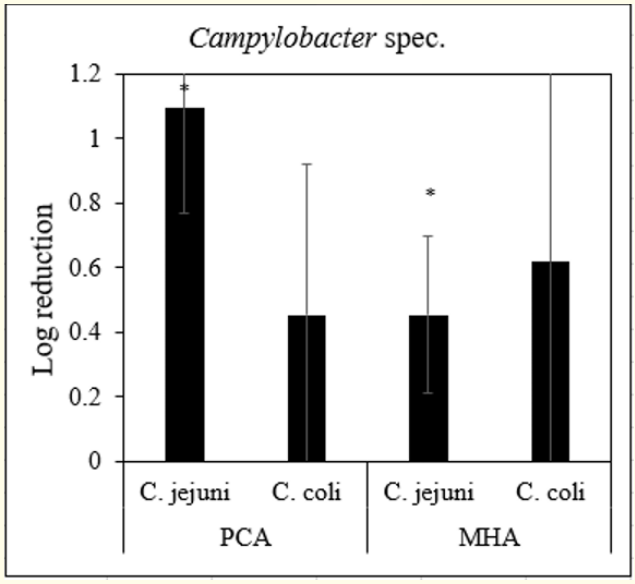


***Figure 5:*** Effect of 10% EAA on externally added C. jejuni and C. coli. On the left are the total bacterial counts from the PCA plates, on the right the Campylobacter counts from the MHA plates. An asterisk above the bar indicates a statistically significant difference between EAA treated and H2O treated chicken.

In comparison, applying up to 500 pm of peroxyacetic acid to chicken wings resulted in a 2 log reduction in *C. coli* at 60 min incubation, but the effect on *S. enterica* was pH dependent with a highest reduction at pH 10 [17]. Additional disadvantages of the peroxyacetic acid treatment, especially when used in combination with chlorine are an off-color and off-odor physical appearance of the chicken [2], increase in resistance towards medically relevant antibiotics which may facilitate adaptation of *Campylobacter* in the food-processing environment [31], and the formation of *Campylobacter* biofilm which could not be completely inactivated by treatment with chlorine or peroxyacetic acid [37].

To overcome resistance, modern treatments with lytic phage have been postulated [10]. To aid with the biofilm problem, EAA might provide a successful alternative. Our initial anti-microbials AAA and PEA were identified during a screen for growth and biofilm inhibition of *E. coli* O157:H7 [21]. EAA was described as an inhibitor of growth and biofilm for *Yersinia enterocolitica*, *Serratia marcescens*, and *Cronobacter sakazakii* [14]. Biofilm of numerous pathogens, including several uropathogenic *E. coli*, *P. aeruginosa*, and *S. aureus* were reduced in silicone tubing after three applications of 100 mg/ml EAA over the course of two weeks [30]. One of the proposed purposes for EAA in our pending patent is the use of EAA as a sanitizer for surfaces to reduce biofilm [28]. We believe that EAA has benefits beyond the mere reduction in live bacterial counts on the chicken.

## Conclusion

This study investigates the effectiveness of EAA as a food anti-microbial in reducing spoilage bacteria and externally added pathogens on boneless chicken thighs. Adding 10% EAA to the wash water, EAA reduced the total number of spoilage bacteria by > 1 log and Pseudomonads by ~1 log. The number of Lactobacilli was increased at 10% EAA, relative to the water wash. The numbers of externally added *Salmonella* were reduced by 75% and 48% for two different *S. enterica* strains. The number of externally added *C. jejuni* were reduced by 51%, those of externally added *C. coli* were reduced by 76%. Comparing these numbers with reductions by existing treatments for chicken, the effectiveness of EAA seems similar or slightly better than other treatments. Among the advantages of EAA as a processing aid for chicken, i) EAA is very cost effective, ii) it has a demonstrated heat stability, which is essential for such foods that need to be cooked (*e.g.* chicken) [12], iii) has been described as a biofilm inhibitor [14;30], and iv) already has FDA approval as a flavoring agent (21CFR172.515). We believe that further investigation of EAA as a processing aid for chicken in a real-life company food processing chain will be a meaningful next step.

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## Conflict of Interest

The authors declare no financial interest.

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**Volume 17 Issue 12 December 2021**