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Influence of Ethylenediaminetetraacetic Acid (EDTA) on the Ability of Fatty Acids to Inhibit the Growth of Bacteria Associated with Poultry Processing

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Abstract: The effect of Ethylenediaminetetraacetic Acid (EDTA) on the bactericidal activity of Fatty Acids (FA) was examined. A 0.5 M concentration of caproic, caprylic, capric, or lauric acid in 1.0 M Potassium Hydroxide (KOH) was supplemented with 0, 5, or 10 mM of EDTA and adjusted to pH 11.0 with citric acid. FA-KOH-EDTA was added to wells in agar seeded with 10^6 cfu/ml of *Acinetobacter calcoaceticus*, *Aeromonas hydrophila*, *Campylobacter jejuni*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, or *Staphylococcus simulans*. Agar plates were incubated aerobically at 35°C for 24 h, except for *C. jejuni* plates which were incubated microaerophilically at 42°C for 48 h and zones of inhibition around the wells were measured. Results indicated that although caproic acid-KOH inhibited growth of *C. jejuni* only, caproic acid-KOH supplemented with EDTA produced significantly ($p < 0.05$) larger zones of *C. jejuni* as well as zones of inhibition of *A. calcoaceticus*, *E. faecalis* and *P. fluorescens*. Caprylic acid-KOH produced zones of inhibition of all isolates except *A. calcoaceticus*, *L. monocytogenes* and *S. simulans*, but supplementation with EDTA produced zones of inhibition of the 3 isolates in addition to increases in the size of zones of inhibition of *E. faecalis* and *Salmonella Typhimurium*. Capric acid-KOH and lauric acid-KOH inhibited all isolates and supplementing both mixtures with EDTA generally produced larger zones of inhibition. Findings indicate that the addition of EDTA to formulations of FA-KOH may increase the ability of these sanitizers to reduce contamination of poultry processing operations.

Key words: EDTA, fatty acids, bactericidal, agar diffusion assay, poultry processing

INTRODUCTION

Some fatty acids found in animal and vegetable oils possess antimicrobial activity which can be used to inhibit the growth of microorganisms associated with food products (Kabara *et al.*, 1977). The agar diffusion assay has previously been used to demonstrate the antibacterial activity of alkaline salts of caproic, caprylic, capric, lauric and myristic acids towards several bacteria isolated from processed poultry (Hinton and Ingram, 2011). Because of their soap-like qualities, alkaline salts of fatty acids also act as surfactants that may be used to wash away microorganisms from contaminated surfaces (Hinton *et al.*, 2009). Effective formulations of bactericidal surfactants may serve as a basis for the development of alternatives to chemical sanitizers (Oyarzabal, 2005) currently used in poultry processing. Metal chelators are often added to formulations of soaps and detergents to increase the ability of these cleansers to remove dirt and debris from surfaces (Nagarajan and Paine, 1984). When added to surfactant formulations, metal chelators act as detergent builders that sequester or precipitate minerals, such as calcium and magnesium, which can be dissolved in water. High concentrations of these minerals can increase water

hardness thereby decreasing cleansing activity of water (Kovach, 2007). Ethylenediaminetetraacetic Acid (EDTA) is a chelating agent often used as a detergent builder in surfactant formulations. EDTA also possesses bactericidal activity (Chew *et al.*, 1985), which can also inhibit the growth of several bacteria found in poultry processing operations (Hinton and Ingram, 2010). Furthermore, EDTA can increase the sensitivity of some bacteria to other bactericides (Phillips and Duggan, 2001; Russell and Furr, 1977) by increasing the permeability of bacterial cellular membranes (Gray and Wilkinson, 1965; Lieve, 1968; Wilkinson, 1967). The purpose of the current study was to examine the ability of EDTA to increase the antibacterial activity of alkaline salts of fatty acids towards bacteria associated with poultry processing.

MATERIALS AND METHODS

Growth of bacterial cultures and inoculation of agar media: Procedures used for the growth and preparation of suspensions of *Acinetobacter calcoaceticus*, *Aeromonas hydrophila*, *Campylobacter jejuni* 48100, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Salmonella*

typhimurium and *Staphylococcus simulans* cultures were previously described (Hinton and Ingram, 2010). Fresh cultures of the bacterial isolates were used to prepare inoculated agar media by adding suspensions of each culture, except for *C. jejuni*, to separate aliquots of sterile Difco Tryptic Soy Agar (Becton Dickinson and Co., Sparks, MD) which had been tempered to 50°C. Suspensions of *C. jejuni* cultures were added to tempered Blood Agar (Becton Dickinson and Co.) supplemented with 7% lysed horse blood (Lampire Biological Laboratories, Inc., Pipersville, PA). Each inoculated agar media contained 10⁶/colony-forming-units (cfu) of one of the isolates/ml.

Preparation of fatty acid-EDTA mixtures: Alkaline salts of caproic (hexanoic), caprylic (octanoic), capric (decanoic) and lauric (dodecanoic) acids (all obtained from Sigma Chemical Co; St. Louis, MO) were prepared by dissolving 0.5 M of each fatty acid (FA) in separate volumes of 1.0 M potassium hydroxide (KOH). The FA-KOH mixtures were then supplemented with 0, 5, or 10 mM of ethylenediaminetetraacetic acid, dipotassium salt dihydrate (EDTA) (Sigma Chemical Co.) and adjusted to pH 11.0 with citric acid (Sigma Chemical Co.). Prepared solutions were filter sterilized using 0.2 µm filters (Nalge Nunc International, Rochester, NY) and 0.1 ml of the solutions were added to 8 mm wells that had been punched in the agar. All plates were incubated aerobically at 35°C for 18-24 h, except for *C. jejuni* plates which were incubated microaerophilically at 42°C for 48 h. Zones of inhibition around the wells were then measured along a single plane with Traceable[®] Carbon Fiber Digital Calipers (Fisher Scientific, Inc., Pittsburg, PA, USA) (Hinton and Ingram, 2011). Experiment was replicated 12 times.

Statistics: Statistical analyses of differences in the size of zones of inhibition of each bacterial isolate produced by FA-KOH-EDTA were performed using the GraphPad InStat[®] version 3.05 for Windows 95 (GraphPad Software, San Diego, CA). One-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparison tests was performed to determine significant differences in group means. The P value for all ANOVA tests was < 0.05.

RESULTS AND DISCUSSION

Previous studies have demonstrated that solutions of EDTA (Hinton and Ingram, 2011) and solutions of various FA-KOH (Hinton and Ingram, 2011) can inhibit bacteria growth; however, results of the present study indicate that FA-KOH-EDTA solutions may possess greater antibacterial than FA-KOH mixtures. Caproic acid-KOH exhibited bactericidal activity towards *C. jejuni* only; however, caproic acid-KOH mixtures supplemented with 5 mM EDTA also produced zones of inhibition of *P. fluorescens* in addition to significantly larger zones of

inhibition of *C. jejuni* than caproic acid-KOH (Table 1). Furthermore, caproic acid-KOH supplemented with 10 mM EDTA produced significantly larger zones of inhibition of *C. jejuni* and *P. fluorescens* than mixtures supplemented with only 5 mM of EDTA, as well as significant zones of inhibition of *A. calcoaceticus* and *E. faecalis*. The extensively studied sensitivity of some *Pseudomonas* species to the antibacterial activity of EDTA (Wilkinson, 1967) has been linked to the high concentration of phosphorous (Gray and Wilkinson, 1965) and multivalent cations (i. e. magnesium, calcium and zinc) (Asbell and Eagon, 1966) found in the cellular membrane of pseudomonads. All isolates inhibited by caproic acid-KOH-EDTA were Gram negative rods, except for the Gram positive coccus, *E. faecalis*. EDTA has been shown to be an effective disinfectant that prevents infections by *E. faecalis* when used as a dental irrigant during root canal surgery (Bulacio *et al.*, 2006). Previous research has illustrated that *C. jejuni* is the only isolate used in this study that is susceptible to the bactericidal activity of caproic acid (Hinton and Ingram, 2011) while another study indicated that exposure to 10 mM EDTA produced significant zones of inhibition of most of the bacteria used in the present study (Hinton and Ingram, 2010). No zones of inhibition of *E. coli*, *L. monocytogenes*, *Salmonella typhimurium* and *S. simulans* were produced by mixtures of caproic acid-KOH supplemented with 10 mM EDTA, however. Caprylic acid-KOH alone inhibited growth of *A. hydrophila*, *C. jejuni*, *E. faecalis*, *E. coli*, *P. fluorescens* and *Salmonella typhimurium* growth, while caprylic acid-KOH supplemented with 5 mM EDTA produced zones of inhibition of *A. calcoaceticus* and *E. faecalis* that were significantly larger than zones produced by caprylic acid-KOH (Table 2). Furthermore, the size of the zones of inhibition of *L. monocytogenes* formed by caprylic acid-KOH with 10 mM EDTA were significantly larger than zones of inhibition produced by caprylic acid-KOH. Additionally, the zones of inhibition of *A. calcoaceticus*, *E. faecalis*, *Salmonella typhimurium* and *S. simulans* formed by caprylic acid-KOH with 10 mM EDTA were significantly larger than zones of inhibition produced by caprylic acid-KOH with 5 mM EDTA. Although caprylic-KOH without EDTA inhibited the growth of *A. hydrophila*, *C. jejuni*, *E. coli* and *P. fluorescens*, supplementing this mixture with EDTA did not result in larger zones of inhibition of these isolates. A previous study indicated that the Gram negative isolates, *C. jejuni*, *E. coli* and *P. fluorescens* exhibited a relatively high degree of sensitivity towards the antibacterial activity of caprylic-KOH (Hinton and Ingram, 2011); therefore, it is possible that since caprylic-KOH and EDTA cause lysis of bacterial cellular membranes antibacterial synergisms between these two agents might not be evident in highly susceptible isolates.

Capric-KOH (Table 3) and lauric-KOH (Table 4) produced zones of inhibition of all bacterial isolates.

Table 1: Size (mm) of zones of inhibition of bacterial isolates by caproic acid-KOH with various concentrations of ethylenediaminetetraacetic acid (EDTA)^{1,2}

Bacterial isolate									
EDTA									
Conc. (mM)	<i>Acinetobacter calcoaceticus</i>	<i>Aeromonas hydrophila</i>	<i>Campylobacter jejuni</i>	<i>Enterococcus faecalis</i>	<i>Escherichia coli</i>	<i>Listeria monocytogenes</i>	<i>Pseudomonas fluorescens</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus simulans</i>
0	0.00 ^a ±0.00	0.00 ^a ±0.00	12.52 ^a ±1.04	0.00 ^a ±0.00	0.00 ^a ±0.00	0.00 ^a ±0.00	0.00 ^a ±0.00	0.00 ^a ±0.00	0.00 ^a ±0.00
5	0.00 ^a ±0.00	0.00 ^a ±0.00	13.26 ^b ±0.84	0.00 ^a ±0.00	0.00 ^a ±0.00	0.00 ^a ±0.00	0.95 ^b ±0.88	0.00 ^a ±0.00	0.00 ^a ±0.00
10	3.18 ^b ±0.73	0.00 ^a ±0.00	14.63 ^c ±0.34	1.73 ^b ±0.59	0.00 ^a ±0.00	0.00 ^a ±0.00	2.31 ^c ±0.58	0.00 ^a ±0.00	0.00 ^a ±0.00

¹Solution pH adjusted to 11.0 with citric acid.

²Values are averages ± standard deviation. n = 15.

^{a-e}Within columns, different letters indicate significant (p<0.05) differences. Conc. = Concentration

Table 2: Size (mm) of zones of inhibition of bacterial isolates by caprylic acid-KOH with various concentrations of ethylenediaminetetraacetic acid (EDTA)^{1,2}

Bacterial isolate									
EDTA									
Conc. (mM)	<i>Acinetobacter calcoaceticus</i>	<i>Aeromonas hydrophila</i>	<i>Campylobacter jejuni</i>	<i>Enterococcus faecalis</i>	<i>Escherichia coli</i>	<i>Listeria monocytogenes</i>	<i>Pseudomonas fluorescens</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus simulans</i>
0	0.00 ^a ±0.00	1.41 ^a ±0.68	16.59 ^a ±0.78	3.35 ^a ±0.37	1.36 ^a ±0.49	0.00 ^a ±0.00	4.19 ^a ±0.73	0.99 ^a ±0.42	0.00 ^a ±0.00
5	1.67 ^b ±0.99	1.24 ^a ±0.57	16.56 ^a ±0.58	4.03 ^b ±0.36	1.51 ^a ±0.41	0.34 ^{ab} ±0.52	5.19 ^a ±1.00	1.33 ^a ±0.38	0.00 ^a ±0.00
10	3.19 ^b ±0.98	1.59 ^a ±0.83	16.78 ^a ±0.95	4.61 ^c ±0.50	1.55 ^a ±0.38	0.74 ^b ±0.75	4.77 ^a ±1.42	2.14 ^b ±0.42	0.14 ^b ±0.25

¹Solution pH adjusted to 11.0 with citric acid.

²Values are averages ± standard deviation. n = 15.

^{a-e}Within columns, different letters indicate significant (p<0.05) differences. Conc. = Concentration

Table 3: Size (mm) of zones of inhibition of bacterial isolates produced by capric acid-KOH with various concentrations of ethylenediaminetetraacetic acid (EDTA)^{1,2}

Bacterial isolate									
EDTA									
Conc. (mM)	<i>Acinetobacter calcoaceticus</i>	<i>Aeromonas hydrophila</i>	<i>Campylobacter jejuni</i>	<i>Enterococcus faecalis</i>	<i>Escherichia coli</i>	<i>Listeria monocytogenes</i>	<i>Pseudomonas fluorescens</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus simulans</i>
0	11.61 ^a ±0.54	9.23 ^a ±0.70	14.03 ^a ±0.57	14.11 ^b ±0.78	2.59 ^a ±0.24	7.57 ^a ±0.59	12.37 ^a ±0.37	5.77 ^a ±0.53	7.77 ^a ±0.45
5	12.03 ^a ±0.35	9.04 ^a ±0.45	14.22 ^a ±0.51	12.73 ^b ±0.47	2.61 ^a ±0.46	8.17 ^b ±0.51	12.75 ^a ±0.37	5.86 ^a ±0.61	8.24 ^b ±0.33
10	13.26 ^b ±0.61	9.00 ^a ±0.39	15.03 ^b ±0.70	13.80 ^b ±0.63	2.82 ^a ±0.23	8.45 ^b ±0.42	12.44 ^a ±0.63	6.42 ^b ±0.25	8.49 ^b ±0.55

¹Solution pH adjusted to 11.0 with citric acid.

²Values are averages ± standard deviation. n = 15.

^{a-e}Within columns, different letters indicate significant (p<0.05) differences. Conc. = Concentration

Table 4: Size (mm) of zones of inhibition of bacterial isolates produced by lauric acid-KOH with various concentrations of ethylenediaminetetraacetic acid (EDTA)^{1,2}

Bacterial isolate									
EDTA									
Conc. (mM)	<i>Acinetobacter calcoaceticus</i>	<i>Aeromonas hydrophila</i>	<i>Campylobacter jejuni</i>	<i>Enterococcus faecalis</i>	<i>Escherichia coli</i>	<i>Listeria monocytogenes</i>	<i>Pseudomonas fluorescens</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus simulans</i>
0	8.82 ^a ±0.40	5.81 ^a ±0.41	6.93 ^a ±0.78	09.97 ^a ±0.33	1.65 ^a ±0.66	6.42 ^a ±0.35	8.70 ^a ±0.44	3.49 ^a ±0.42	7.48 ^a ±0.44
5	9.91 ^b ±0.81	5.97 ^a ±0.68	8.03 ^b ±0.52	10.41 ^b ±0.32	1.51 ^a ±0.67	6.62 ^{ab} ±0.21	9.29 ^b ±0.49	4.01 ^b ±0.84	7.64 ^a ±0.91
10	9.99 ^b ±0.80	6.81 ^b ±0.55	8.85 ^c ±0.55	10.98 ^c ±0.37	2.06 ^b ±0.56	6.83 ^b ±0.45	9.66 ^b ±0.61	4.34 ^b ±0.39	8.45 ^b ±0.41

¹Solution pH adjusted to 11.0 with citric acid.

²Values are averages ± standard deviation. n = 15.

^{a-e}Within columns, different letters indicate significant (p<0.05) differences. Conc. = Concentration

Zones of inhibition of *L. monocytogenes* and *S. simulans* formed by capric-KOH with 5 mM EDTA were significantly larger than zones produced by the capric-KOH mixtures, while increasing the EDTA concentration of the capric-KOH to 10 mM produced significant increases in the size of the zones of inhibition of *A. calcoaceticus*, *C. jejuni* and *Salmonella typhimurium*, as well as further increases in the size of the zone of inhibition of *E. faecalis*. Also, mixtures of lauric-KOH supplemented with 5 mM EDTA produced significantly larger zones of inhibition of *A. calcoaceticus*, *C. jejuni*, *E. faecalis*, *P. fluorescens* and *Salmonella typhimurium* than lauric-KOH not supplemented with the chelator. Mixtures of lauric-KOH supplemented with 10 mM of EDTA formed inhibition zones of *A. hydrophila*, *L. monocytogenes* and *S. simulans* that were significantly larger than zones produced by lauric-KOH in addition to further increases in the size of zones of inhibition of *C. jejuni* and *E. faecalis* produced by lauric-KOH with 5 mM of EDTA.

EDTA possesses antibacterial activity against Gram negative and Gram positive bacteria (Chew *et al.*, 1985) and the chelator has been shown to increase the bactericidal activity of antimicrobials (Vaara, 1992) because of the ability of the chelator to increase the permeability of cellular membranes (Lieve, 1968; Leive, 1965; Russell and Furr, 1977). Furthermore, strong chelators may inhibit microbial metabolism by binding trace minerals required for cellular reproduction, growth and survival (Bozariis and Adams, 1999). Chelators are routinely added to surfactant formulations to serve as detergent builders (Kovach, 2007). These detergent builders decrease water hardness by binding minerals dissolved in water, thereby increasing the ability of the surfactants to remove dirt and debris during washing operations. Formulations of alkaline salts of fatty acids are currently being examined as microbicidal surfactants that can potentially be used as sanitizers in poultry processing operations (Hinton *et al.*, 2009). Alkaline salts of caproic, caprylic, capric, lauric and myristic acids have exhibited antibacterial activity towards several bacteria associated with poultry processing (Hinton and Ingram, 2011). The addition of chelators to these formulations may increase the ability of these sanitizers to reduce contamination of broiler carcasses by pathogenic, spoilage and indicator microorganisms. Formulations of these antimicrobial surfactants may serve as the basis for the development of more effective poultry processing sanitizers.

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