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Full-Length Research Paper

Understanding the Antimicrobial Effects of Lauric Arginate, Ethylenediaminetetraacetic Acid (EDTA), and a Vinegar Based Ingredient against *Salmonella* Spp. using Transmission Electron Microscopy and Flow Cytometry

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ABSTRACT: Transmission electron microscopy inspection of lauric arginate, a vinegar-based ingredient, and lauric arginate in combination with EDTA did not show any disruption of the cytoplasmic membrane. Instead, all of the micrographs showed an intact cell with no leakage compared to the negative control. This study indicated that more work needs to be conducted and an alternative method needs to be evaluated in order to evaluate the cell morphology. When staining cells with PI and SYTO9, the membrane was affected. Results showed that there was a population of dead cells distinguished from the live cells. Although you can distinguish the populations, the density of the area represents only a certain quadrant of the total volume of the cells.

Keywords: Antimicrobial mechanism, *salmonella*, cells

INTRODUCTION

Mechanisms of antimicrobials have been studied by many methods including measuring the change in cell homeostasis using fluorescent probes, relative change of membrane potential and ATP synthesis, and oxygen consumption (Raafat et al., 2008; Nowotarska et al., 2017). Changes in cell morphology after antimicrobial treatment can be observed using transmission electron microscopy (Raafat et al., 2008; Nowotarska et al., 2017). In order to develop new antimicrobial processes, the mechanism is very important to understand. Flow cytometry measures the changes in the physiological cell properties. The dyes that are used aid in the identification of the cell viability. The cells can be enumerated and distinguished using different fluorescent dyes. These dyes allow scientists to differentiate cell structure function (Léonard et al., 2016). In order to determine the efficacy

of an antimicrobial solution, there needs to be a concise method to demonstrate the interruption of the cellular membrane that coincides with the known mechanisms of the membrane. The aim of this study were 1) to determine the effectiveness of Lauric Arginate, EDTA, and a Vinegar Based Ingredient, against S. Typhimurium *in vitro* and a meat matrix, and 2) to determine the mechanism of action of these solutions using transmission electron microscopy and flow cytometry.

MATERIALS AND METHODS

Transmission electron microscopy (TEM)

Inoculum preparation

Salmonella Typhimurium strain ATCC 13311, kept frozen at -80ºC, was thawed at room temperature under running water. Tryptic soy broth (BD Bacto Tryptic Soy Broth 211825, Sparks, MD, USA) cultures were prepared by transferring 100µL into 9mL of the TSB. The culture was incubated at 35°C for 18h. The inoculum was washed with 9mL of sterile 0.1% peptone water (Thermo Scientific Oxoid CM0009 Lenexa, Kansas, USA) three times using centrifugation at 5,000g for 10 min at 4°C. The suspension was placed onto XLT-4 agar. The initial concentration of the culture was 10^7 cfu/ml. A colony was selected from the agar plate and regrown in the TSB. The inoculum was used for the treated ground chicken breast inoculation. The chicken breasts were coarse- ground through a 5- mm plate and separated into five 25-g batches for each treatment. All breast meat batches (25 g per batch) were inoculated with 0.25 mL of *Salmonella* Typhimurium and mixed manually. The inoculated meat was stored at 4°C for 10 min to allow for bacterial attachment. The treatments included; the control without meat, 2% VBI, 0.25% EDTA, 1% LAE and 0.25% EDTA, and 0.25% EDTA and 2% LAE. The cells were regrown and washed using the same procedure as earlier and used for the Transmission Electron Microscopy procedure. The treated cells were re-centrifuged at 12000g for 10 min and fixed in 4% paraformaldehyde and 2% glutaraldehyde mixed with 0.1% sodium cacodylate. These cells were microwaved (Pelco Microwave), washed in 0.1% sodium cacodylate buffer and phosphate buffered saline, and encapsulated in melted 3% low- temperature gelling agarose Type IV (A3643 Sigma- Aldrich, Saint-Louis, MO) fixed with 1% osmium tetroxide, washed with water, then dehydrated in an ethanol series followed by 100% acetone. The samples were cured at 60°C in epoxy resin. The resin blocks were trimmed and collected on mesh grids stained with 2% uranyl acetate. The sections were examined with the Hitachi H-7000 TEM. These samples were analyzed at the Interdisciplinary Center for Biotechnology Research, Gainesville, FL.

Flow cytometry

Inoculum preparation

Salmonella Typhimurium strain ATCC 13311, kept frozen at -80ºC, was thawed at room temperature under running water. Tryptic soy broth (BD Bacto Tryptic Soy Broth 211825, Sparks, MD, USA) cultures were prepared by transferring 100 µL of the bacteria into 9mL of TSB and incubated for 18h at 35°C. The inoculum was washed with 9mL of sterile 0.1% peptone water (Thermo Scientific Oxoid CM0009 Lenexa, Kansas, USA) three times using centrifugation at 5,000g for 10 min at 4° C. The initial concentration of the culture was 10^7 cfu/ml. The bacterial suspension was used as the inoculum for the study. Propidium Iodine (PI) and SYTO9 were used to differentiate between live and dead bacterial cells. This dye is normally excluded from viable cells. The cells were treated with solutions of 2% Vinegar Based Ingredient, 0.5% LAE, 1% LAE, and 2% LAE, a combination of 1% LAE and 0.25% EDTA, and a combination of 2% LAE and EDTA. After the cells were treated, each sample was diluted to 10^6 in Phosphate Buffered Saline (PBS). One mL was stained with 2.5 µL of PI and SYTO9 in combination and incubated at room temperature for 30 min in the dark. Samples were run in the Accuri C6 (New Jersey) Flow Cytometer with the Forward Scatter area that measures the size of the cell. The flow cytometry was analyzed using duplicate samples with the FlowJo Software.

RESULTS AND DISCUSSION

Transmission electron microscopy

The effect of LAE on *Salmonella* Typhimurium has been documented by electron microscopy for over ten years. It is known that the use of Lauric Arginate and Acetic Acid have shown efficacy against gram negative organisms (Bonnaud et al., 2010; Terjung et al., 2014; Husna et al., 2015). When LAE is incorporated into to food products, lower efficacy has been documented because of the addition of complex food matrices such as polysaccharides, fat, and protein (Loeffler et al., 2014; Ma et al., 2020). This was validated by Sharma et.al when the researchers determined that LAE had no effect at 200 ppm on *Salmonella* Typhimurium in ground chicken breast (Sharma et al., 2013). In order to enhance the effect of LAE, scientists have incorporated chelating agents to aid in altercations of membrane permeability of

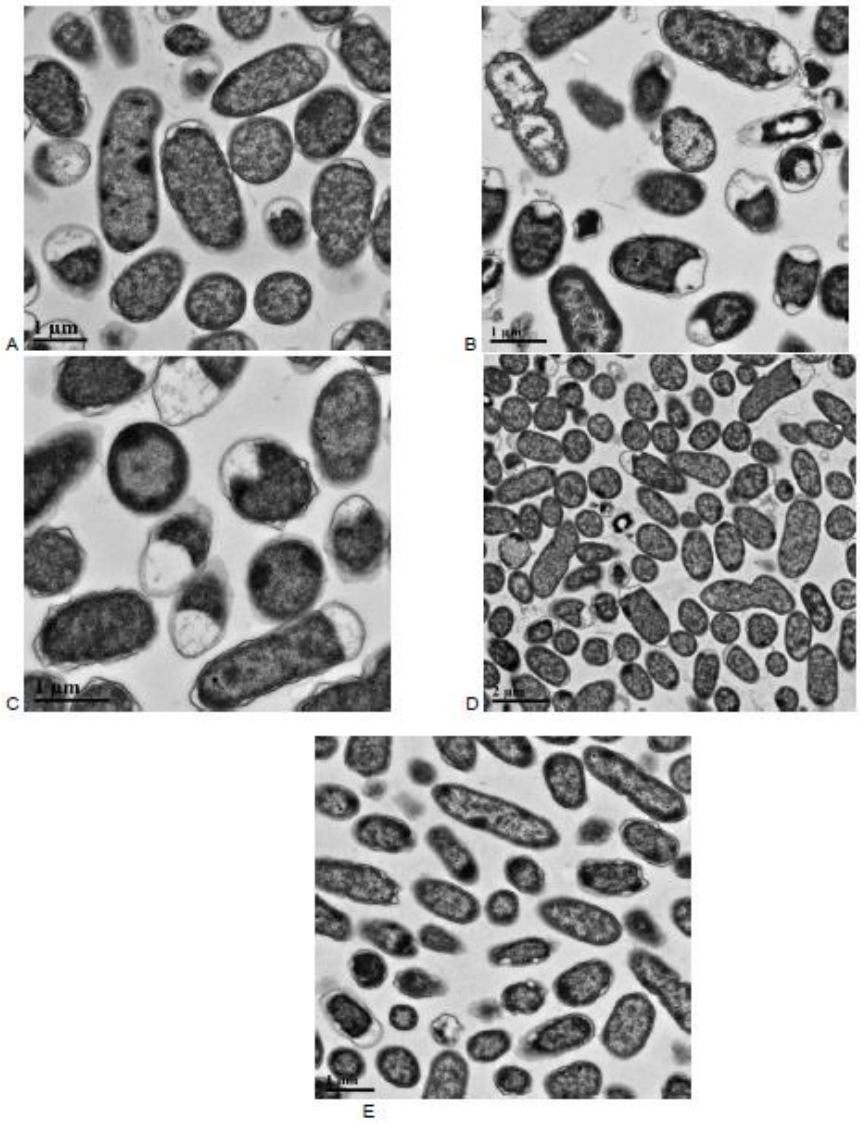

Figure 1: Transmission electron micrographs of S. Typhimurium a) control with no meat b) 2% VBI in ground chicken, c) 0.25% EDTA in ground chicken, d) 1% LAE and 0.25% EDTA, e) 2% LAE and 0.25% EDTA.

gram negative organisms (Economou et al., 2009). EDTA can also limit cation availability that is responsible for destabilization of the membrane. Ethylenediaminetetraacetic acid (EDTA) enhanced the antimicrobial activity of LAE and essential oils combination against gram negative bacteria (Ma et al., 2020). Organic acids' antimicrobial activity is proposed to be due to the ability of undissociated acids to cross the membrane and release protons inside the cell (Bae and Lee, 2015). When this process occurs, it causes cytoplasmic leakage. At this point, the cell has no membrane functionality. The proposed mechanisms have also been documented throughout the years and have extensively been shown to be two mechanisms-cytoplasmic acidifications, and disassociation accumulation of acid anions (Mani-Lopez et al., 2012). This study showed that there was not an immediate inactivation of *Salmonella* Typhimurium with any of the treated ground chicken cells (B-E) when compared to the positive control, *Salmonella* Typhimurium only (Figure 1). The cells appear to be intact, with minimum deformities within the cross-section. Cells that were treated with a Vinegar based ingredients showed that *Salmonella* Typhimurium could survive refrigerated temperatures.

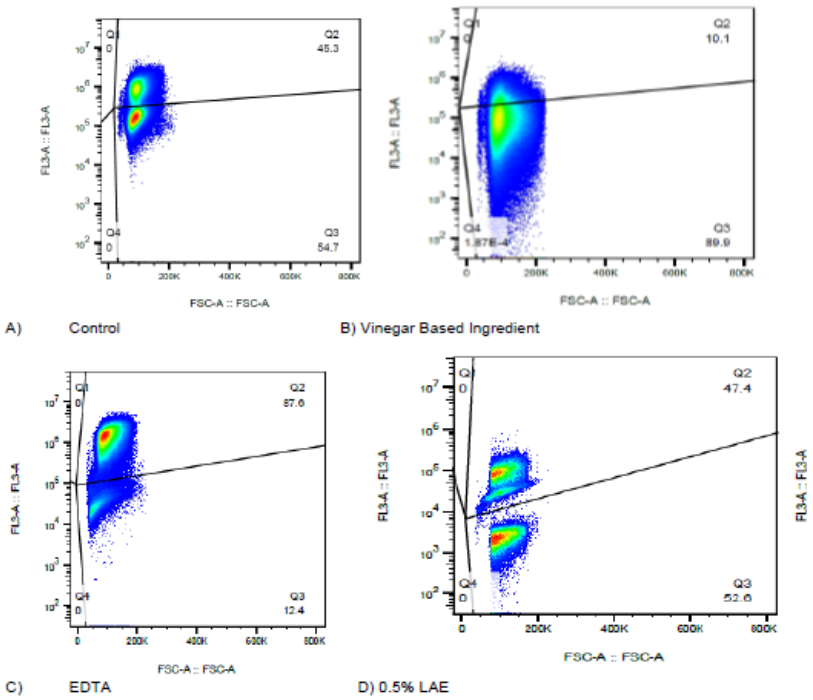

Figure 2: Flow cytometry analysis of *Salmonella* Typhimurium treated with the labeled solutions and stained with PI and SYTO9.The grid regions represent PI positive cells (dead cells) and the lower region represents PI negative Cells (Live cells).

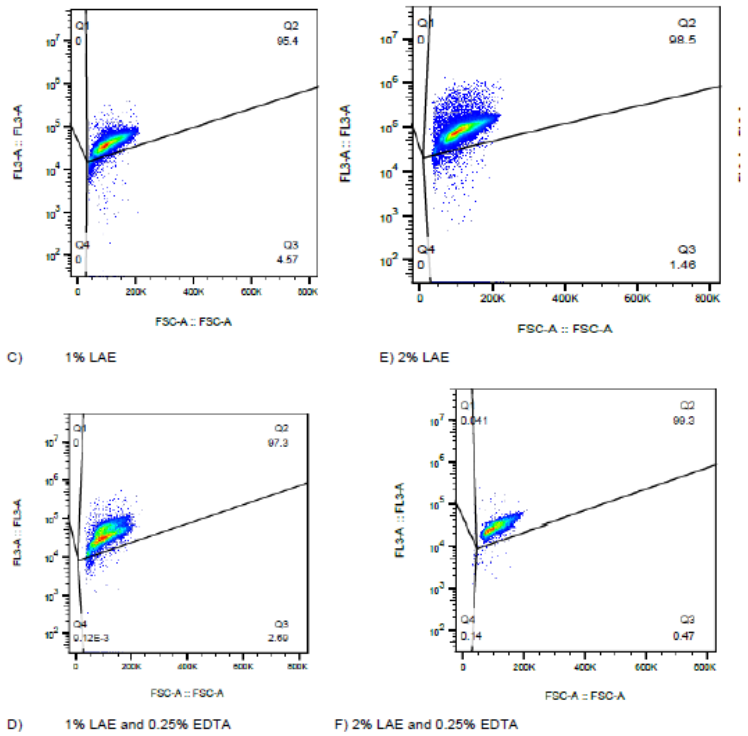

Figure 2. Contd.

Salmonella can grow in a wide range of pH conditions varying from 3.8-9.5 (Food Standards Australia, 2013). There were no structural differences between the LAE treatments alone (1% and 2%) and the 1% and 2% LAE and 0.25% EDTA combinations. The EDTA results are similar to what was found in a previous study with the application of LAE and eugenol with EDTA and there was no effect (Manrique et.al., 2017).

Flow cytometry

Flow cytometry measures the changes in the physiological cell properties. The dyes that are used aid in the identification of the cell formality. The cells can be enumerated and distinguished using different fluorescent dyes. These dyes allow scientists to differentiate cell structure function (Léonard et al., 2016). The micrographs in this study distinguish two dense populations of cells. Cells can be identified as live or dead by the location on the quadrant shown in (Figure 2). In the upper quadrant, cells were positive for PI which means that they are dead. The lower quadrant displays the living cells. The color areas of the graphs represent the density of the selected cell population. Identification may be accurate, but some of the compromised bacterial cells could be considered dead using flow cytometry (Berney et al., 2007). Overall, the results from the flow cytometry can only be compared to the 1% and 2% LAE treatments as well as these treatments in combination with 0.25% EDTA.

Conclusion and applications

Future work is needed in order to understand the best mechanism applications and the dosage rates, levels, and compounds of the antimicrobials need to be evaluated. Cell morphology is essential in determining the most effective solution for the organism. Other antimicrobial mechanism methods need to be evaluated in order to determine a standard for every sample that is tested.

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