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# Synergistic Antimicrobial Activity by Light or Thermal Treatment and Lauric Arginate: Membrane Damage and Oxidative Stress

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**ABSTRACT** The need for more effective antimicrobials is critical for the food industry to improve food safety and reduce spoilage of minimally processed foods. The present study was initiated to develop an efficient and novel antimicrobial approach which combines physical treatments (UV-A or mild heat) and generally recognized as safe lauroyl arginate ethyl (LAE) to inactivate surrogate strains, including *Escherichia coli* and *Listeria innocua*. Synergistic inactivation of bacteria resulted in an ~6-log reduction of target bacteria, while individual treatments resulted in <1.5-log inactivation under the same set of conditions. In addition, the synergistic mechanism between LAE and UV-A/mild heat was evaluated by supplementing with a variety of antioxidants for suppressing oxidative stress and measurement of cell membrane damage by nucleic acid release. These results demonstrate that the synergistic antimicrobial activity of LAE and mild physical stresses was suppressed by supplementation with antioxidants. The research also compared LAE with another membrane-targeting lipopeptide antimicrobial agent, polymyxin B, to understand the uniqueness of LAE-induced synergy. Briefly, differences in modes of action between LAE and polymyxin B were characterized by comparing the MIC, damage to liposomes, and oxidative stress generation. These differences in the mode of action between LAE and polymyxin B suggested that both compounds target cell membrane but significantly differ in mechanisms, including membrane disruption and oxidative stress generation. Overall, this study illustrates synergistic antimicrobial activity of LAE with light or mild heat and indicates a novel oxidative stress pathway that enhances the activity of LAE beyond membrane damage.

**IMPORTANCE** This study highlights an effective antimicrobial processing approach using a novel combination of lauroyl arginate ethyl (LAE) and two different physical treatments, light (UV-A) and mild heat. Both combinations demonstrated synergistic inactivation against a model Gram-negative bacterium or a Gram-positive bacterium or both by a >5-log reduction. Further mechanistic study revealed that oxidative stress is responsible for synergistic inactivation between LAE and UV-A, while both membrane damage and oxidative stress are responsible for the synergistic combination between LAE and mild heat. The mode of action of LAE was further compared to that of polymyxin B and analyzed using artificial membrane model systems and the addition of antioxidants. The proposed combination of LAE and common physical treatments may improve food preservation, food safety, and current sanitation processes for the food industry and the inactivation of pathogenic strains in biomedical environments.

**KEYWORDS** lauric arginate, mild heat, oxidative stress, polymyxin B, synergism, UV-A

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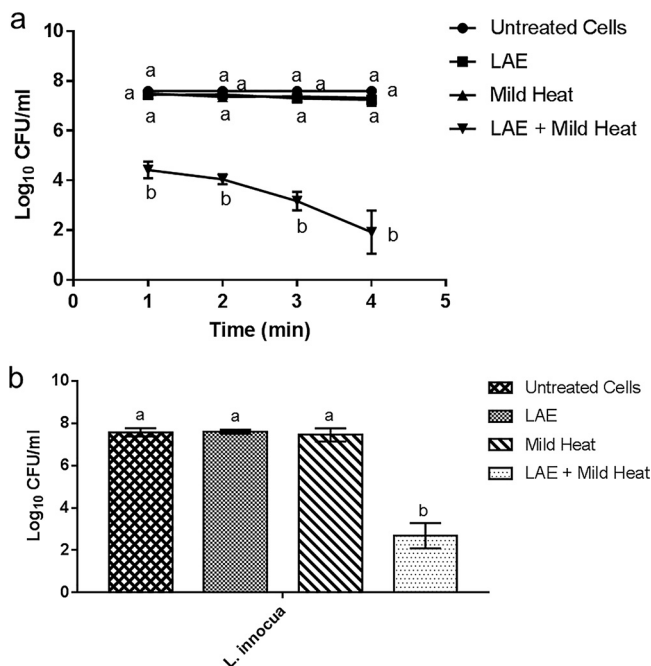
The need for more effective antimicrobials is critical for the food industry to improve food safety and reduce the spoilage of minimally processed foods. In the food sector, microbial spoilage results in wastage of over 30% of fresh produce (1). In addition, according to the Centers for Disease Control and Prevention, every one in six Americans gets sick, including 128,000 hospitalizations and 3,000 deaths each year, as a result of foodborne illness. Foodborne pathogens such as *Escherichia coli* O157 are among the leading causes of foodborne diseases that require hospitalization (2, 3). Furthermore, the emergence of bacterial resistance against sanitizers commonly used in the food industry enhances the risk of lethal infections. For example, *E. coli* O157 and *Listeria monocytogenes* strains have been reported to develop resistance to common sanitizers (4, 5). The prevalence of spoilage/pathogenic foodborne microorganisms and the emergence of sanitizer resistance among many microbes highlight a critical need to develop novel approaches to reduce/eliminate spoilage/pathogenic microorganisms in food systems.

To address the need for more effective antimicrobials, one strategy is to develop a combination of two complementary antimicrobial approaches, such as a combination of mild physical treatment and a bioactive compound. Using these approaches, recent studies have illustrated the potential to synergistically kill a variety of bacteria, including *E. coli* and *Listeria* spp. (6–9). This synergistic antimicrobial treatment approach could be promising for food and medical applications since mild physical treatments such as light, mild heat, and ultrasound are commonly used for both food processing and medical applications, and the use of food-grade compounds can reduce regulatory constraints.

Lauroyl arginate ethyl (LAE) is a generally recognized as safe (GRAS) antimicrobial preservative used in the food industry with a maximum allowable concentration of 200 ppm on a per-gram weight basis (10). LAE is potent against a broad spectrum of foodborne and clinical pathogens, including *L. monocytogenes*, *Staphylococcus aureus*, *E. coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa* (11, 12). Several studies have demonstrated the mechanism of LAE binding and disruption of the bacterial cell membrane. This binding and disruption leads to rapid bacterial inactivation without any cell lysis (13, 14). In addition, LAE has been reported to have low toxicity, since it can be hydrolyzed in the human body into two natural components, lauric acid and L-arginine, and both components can either enter into the urea cycle or serve as a human dietary component (15). Furthermore, as a cationic surfactant, LAE has been reported to improve the efficiency of removing bacteria from leafy greens at 0.1% concentration (16).

Synergistic applications of LAE with carvacrol have been reported to reduce *Salmonella* in ground turkey (17). LAE was also studied in combination with leaf oil, eugenol, and cinnamon oil, which showed synergism against *L. monocytogenes* (14, 18). However, a minimum of 4 h was required to observe the antimicrobial synergy between LAE and essential oils (18). The relatively long treatment time suggests that the combination of LAE and essential oils may not be an ideal processing strategy. In addition, essential oils can impact the flavor of foods (19). Therefore, a more rapid and effective processing technology is evaluated here based on the synergistic combination of LAE with mild physical treatments, including UV-A and mild thermal processing.

The overall motivation in the present study was to discover a novel antimicrobial approach to control spoilage/pathogenic foodborne microorganisms. The novel approach is based on a combination of LAE as a GRAS additive with selected mild physical treatments (i.e., mild heat and light) to achieve the rapid inactivation of *E. coli* O157:H7 and *Listeria innocua*. This study also focuses on understanding the mechanism of synergy between LAE and mild physical treatments and compares the mechanistic differences between LAE and a common clinical antibiotic, polymyxin B. The current research illustrates a potential of applying a food-grade antimicrobial compound with multiple physical treatments and evaluates possible synergistic mechanisms between LAE and these physical processes.



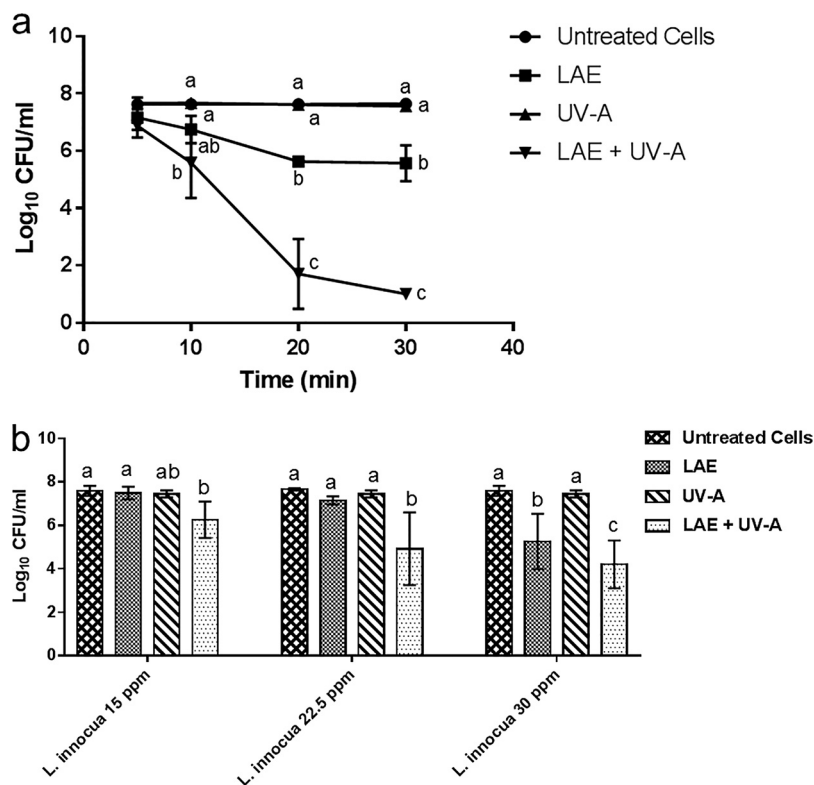
**FIG 1** Synergistic inactivation of *E. coli* O157:H7 and *L. innocua* using a combination of LAE and mild heat. (a) Inactivation of *E. coli* O157:H7 treated with the combination of LAE and mild heat for 1, 2, 3, and 4 min. (b) Inactivation of *L. innocua* treated with the combination of LAE and mild heat for 4 min. The controls included untreated cells and single treatments with LAE or mild heat. Statistical analysis results were compared for each time point/condition. Different letters indicate significant differences among the treatments.

## RESULTS

**Synergistic combination between LAE and light or mild heat with isobolograms.** Isobologram studies were conducted to select synergistic combination between LAE and UV-A light or mild heat using *E. coli* as a model system. The results presented in Fig. S1 in the supplemental material demonstrate that the combination of LAE (15 ppm) and UV-A is synergistic compared to individual treatments. Furthermore, the synergistic combination requires half the concentration of LAE and a 4-fold lower fluence level of UV-A light to achieve the same 6-log reduction of the inoculated *E. coli* using individual treatments. Similarly, the results in Fig. S2 demonstrate that the combination of LAE (15 ppm) and mild heat (55°C) is synergistic compared to individual treatments. Furthermore, the synergistic combination requires a 2.3-fold lower concentration of LAE and >27-fold less thermal energy to achieve the same 5-log reduction of the inoculated *E. coli* using individual treatments. In summary, combinations of LAE and UV-A light/mild heat under the selected conditions demonstrated synergistic inactivation against *E. coli*.

**Synergistic inactivation of *Escherichia coli* and *Listeria innocua* using a combination of mild heat and LAE.** Figure 1a shows the inactivation kinetics of a model Gram-negative bacterium, *E. coli*, upon treatment with mild heat, LAE, and mild heat plus LAE. The results highlight the synergistic inactivation of *E. coli* using a combination treatment of mild heat and LAE in contrast to limited antimicrobial activity of mild heat or LAE treatment alone. The results show that the combined treatment of mild heat and LAE inactivated *E. coli* by 3-log ( $P < 0.05$ ) within 1 min of treatment and an overall 5-log reduction was observed during 4 min of treatment. In contrast, LAE or mild heat alone failed to demonstrate significant reduction of *E. coli* within 4 min of treatment. The bacterial count in the case of synergistic treatment was statistically compared to bacterial counts after individual treatments at each time point.

Synergistic combination of LAE and mild heat was also tested against a model Gram-positive bacterium, *L. innocua*, using the same experimental condition as tested



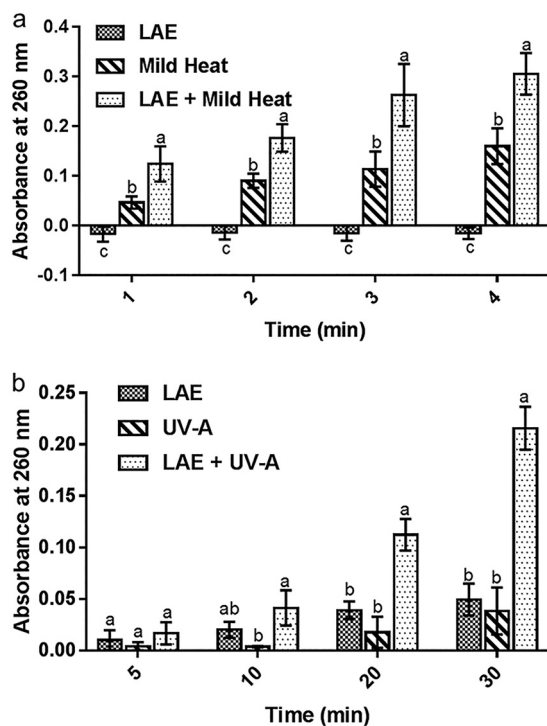
**FIG 2** Synergistic inactivation of *E. coli* O157:H7 and *L. innocua* using a combination of LAE and UV-A. (a) Inactivation of *E. coli* O157:H7 treated with a combination of LAE and UV-A for 5, 10, 20, and 30 min. (b) Inactivation of *L. innocua* treated with a combination of LAE (15, 22.5, or 30 ppm) and UV-A for 30 min. The controls included untreated cells and single treatments with LAE or UV-A. Statistical analysis results were compared for each time point/condition. Different letters indicate significant differences among the treatments.

for *E. coli*. As shown in Fig. 1b, 5-log antimicrobial synergy was achieved when LAE and mild heat were applied simultaneously for 4 min against *L. innocua*, while the mild heat or the LAE treatment alone resulted in insignificant reduction of the initial bacterial load. In summary, the results indicate significant synergistic inactivation of a model Gram-negative and a Gram-positive bacterium using a combination of mild heat and LAE.

**Synergistic inactivation of *E. coli* and *L. innocua* using a combination of UV-A and LAE.** Figure 2a shows the inactivation kinetics of *E. coli* upon treatment with UV-A, LAE, and both UV-A and LAE at room temperature. The results highlight the synergistic inactivation of *E. coli* using a combination treatment of UV-A and LAE in contrast to limited antimicrobial activity of UV-A or LAE treatment alone. UV-A plus LAE reduced the *E. coli* population by 6.5 logs in 30 min. In contrast, UV-A treatment alone did not induce any significant inactivation of the initial inoculum, whereas LAE treatment alone inactivated 2 logs of *E. coli* after 30 min of incubation.

Synergistic combination of UV-A and LAE was also tested against *L. innocua* for 30 min, with LAE at various final concentrations, i.e., 15, 22.5, or 30 ppm. These concentrations were selected based on similar isobologram studies for *E. coli* (data not shown). As shown in Fig. 2b, the antimicrobial synergies were not significant compared to individual concentrations (LAE or UV-A) at any tested concentrations except for the 22.5-ppm LAE concentration. In summary, the results illustrate significant synergistic inactivation of a model Gram-negative bacterium in contrast to reduced synergism in antimicrobial activity observed in the case of a model Gram-positive bacterium using a combination of LAE and UV-A.

**Nucleic acid leakage assay.** Nucleic acid release induced by mild heat and LAE against *E. coli* is shown in Fig. 3a. Bacterial cells treated only with LAE did not cause



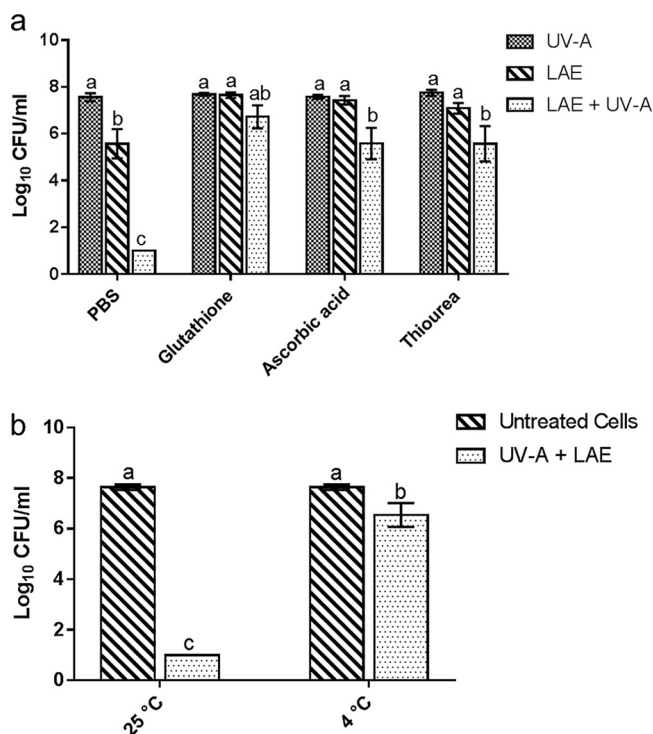
**FIG 3** Nucleic acid release from *E. coli* cells. (a) Nucleic acid leakage measured for individual treatments, i.e., mild heat or LAE, and a combination of mild heat and LAE treatment using absorbance measurement at 260 nm. (b) Nucleic acid leakage measured for individual treatments with UV-A or LAE and for the combination treatment of UV-A plus LAE using an absorbance measurement at 260 nm. Statistical analysis was conducted at each time point.

release of nucleic acids compared to the blank sample ( $P > 0.05$ ). In contrast, cells treated with mild heat alone or in combination with LAE significantly increased nucleic acid release over time (4 min) ( $P < 0.05$ ). The statistical comparison illustrated that the combination of LAE with mild heat significantly ( $P < 0.05$ ) increased the leakage of nucleic acids compared to mild heat treatment alone.

As shown in Fig. 3b, neither UV-A nor LAE significantly increased nucleic acid leakage during the 30-min treatment against *E. coli*. In comparison, UV-A plus LAE significantly ( $P < 0.05$ ) increased nucleic acid release at 20 and 30 min, suggesting increased bacterial cell membrane permeabilization over time. The increased release of nucleic acid absorbance is expected since enhanced inactivation of cells by the combination of LAE and UV-A may result in increased permeability of the bacterial cell membrane (20).

**Role of oxidative stress in synergistic inactivation of LAE and UV-A against *E. coli*.** The results presented in Fig. 4a show that antimicrobial synergy between UV-A and LAE was completely depleted upon treatment with glutathione. Similarly, treatment of cells with other antioxidants, such as ascorbic acid or thiourea, significantly reduced the synergy between UV-A and LAE compared to the controls without antioxidants. These results suggested that oxidative stress is one of the major mechanisms responsible for the observed antimicrobial synergy between UV-A and LAE.

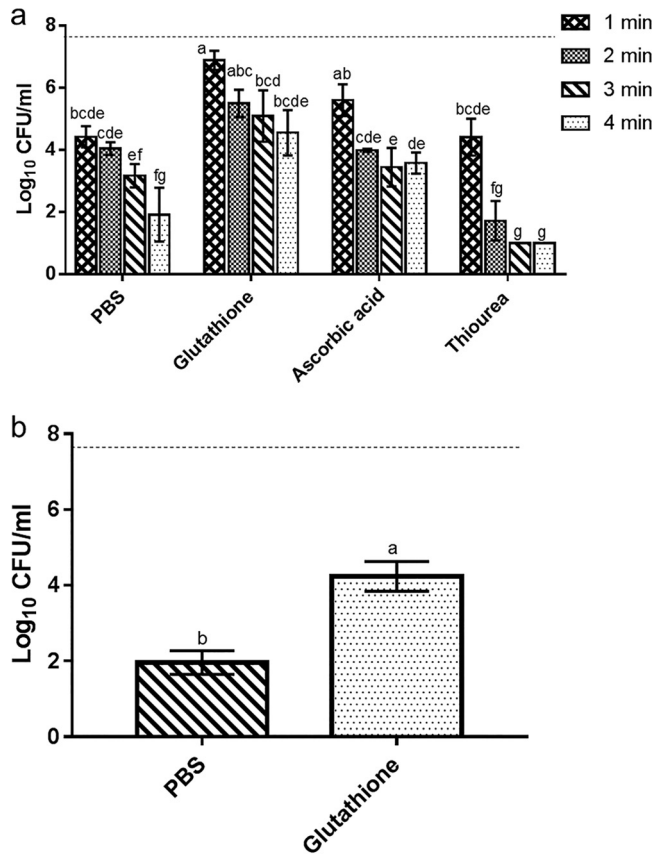
In addition, antimicrobial synergy between UV-A and LAE was associated with bacterial cell metabolism. As shown in Fig. 4b, antimicrobial synergy was reduced when bacterial cells were treated by the combination of LAE and UV-A at 4°C compared to the same treatment at room temperature. This result suggests that the observed antimicrobial synergy was dependent on the active metabolic state of bacterial cells and that the oxidative stress induced by the synergistic combination of LAE and UV-A was potentially dependent on the metabolic activity of cells.



**FIG 4** Influence of antioxidants or temperature on the synergistic antimicrobial activity of the combination of UV-A and LAE. (a) Reduction in the synergistic activity of UV-A and LAE upon the addition of glutathione, ascorbic acid, or thiourea at room temperature. (b) Influence of temperature (without antioxidants) on synergistic antimicrobial activity of UV-A and LAE. Different letters indicate significant differences among the treatments.

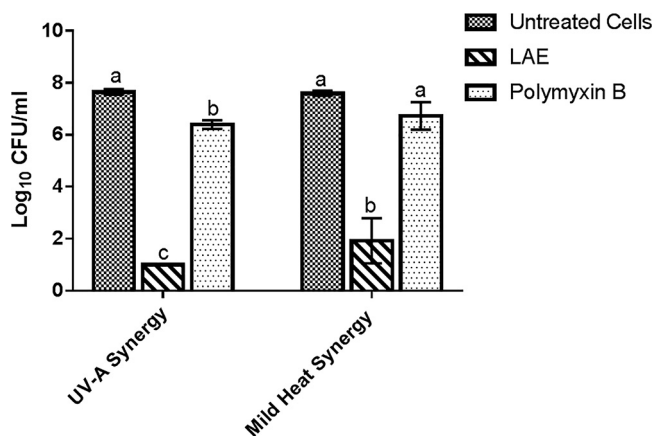
**Investigation of synergistic mechanism between LAE and mild heat against *E. coli*.** To evaluate the role of oxidative stress in the observed synergistic interactions between LAE and mild heat, the combination of antioxidants with LAE and mild heat was tested using an approach similar to that described for Fig. 5. As shown in Fig. 5, the synergistic interaction between mild heat and LAE decreased significantly ( $P < 0.05$ ) in the presence of glutathione compared to the controls without glutathione. The level of bacterial inactivation with glutathione was significantly reduced at 1, 3, and 4 min compared to the controls. Similarly, the level of inactivation of bacteria was also significantly ( $P < 0.05$ ) reduced in the presence of ascorbic acid at 4 min compared to the controls without ascorbic acid. In contrast to the results obtained with ascorbic acid and glutathione, the addition of thiourea enhanced the antimicrobial synergy between LAE and mild heat at 2, 3, and 4 min. In summary, oxidative stress may have a role in the synergistic antimicrobial activity generated by the combination of mild heat and LAE.

**Inactivation of *E. coli* by polymyxin B combined with physical treatments (UV-A and mild heat).** Polymyxin B was selected in this study to verify the observed synergistic combination between amino acid-based antimicrobial compounds and mild physical stress. The rationale for selecting polymyxin B is presented in Materials and Methods. Polymyxin B was combined with either UV-A or mild heat to investigate possible antimicrobial synergies. As shown in Fig. 6, the combination of polymyxin B and UV-A at 30 min induced a 1.2-log reduction in the inoculated *E. coli* population, which is significantly less ( $P < 0.05$ ) than the synergistic inactivation of bacteria achieved using a combination of LAE and UV-A ( $>6.5$ -log reduction) under the same set of experimental conditions. In addition, no antimicrobial synergy between polymyxin B and mild heat was observed at 4 min ( $P > 0.05$ ). This result is in contrast to the 5.5-log inactivation of *E. coli* by synergistic treatment with a combination of LAE and mild heat under the same set of experimental conditions. As indicated by the results shown in



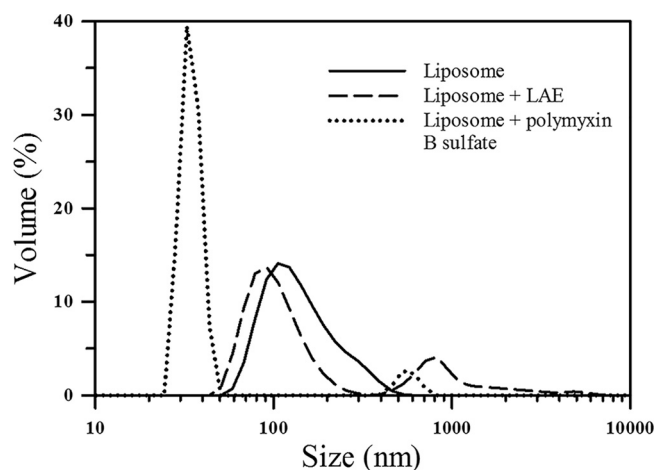
**FIG 5** Reduction in the synergistic activity of mild heat- and LAE-treated *E. coli* for 1 to 4 min (a) or *L. innocua* for 4 min (b) in the presence of the selected antioxidant. The controls (PBS) were treated with mild heat plus LAE without the presence of any antioxidants. Different letters indicate significant differences among the treatments. The dotted line indicates the inoculum size.

Fig. 6, permeabilization of the bacterial cell membrane may not be the only factor responsible for antimicrobial synergy between LAE and multiple physical treatments. These observations led to further evaluation of the unique factors that are required for the synergistic antimicrobial effect observed using a combination of LAE with mild physical treatments (UV-A and mild heat) in *E. coli*. Since polymyxin B alone does not



**FIG 6** Comparison of microbial inactivation of *E. coli* O157:H7 cells using synergistic combinations of LAE with physical treatments and of polymyxin B with physical treatments. The control included untreated cells. Different letters indicate significant differences among the treatments.





**FIG 7** Particle size distribution of model liposomes treated with polymyxin B or LAE; the control included untreated liposome.

induce any significant bactericidal activity, the data obtained with polymyxin B alone are not presented in Fig. 6.

**Comparison between polymyxin B and LAE to understand the uniqueness of LAE-induced synergy.** Since polymyxin B and LAE demonstrated different levels of synergistic interaction with physical treatments (UV-A or mild heat), several experiments were conducted to characterize differences in the antimicrobial mechanisms of LAE and polymyxin B. First, the MICs of these antimicrobial compounds were compared using *E. coli* O157:H7 as a model target strain. The MIC values for LAE and polymyxin B were 15 and 3.75  $\mu\text{g/ml}$ , respectively. Based on the MIC, polymyxin B can be considered a more potent antimicrobial bacteriostatic compound than LAE. In summary, despite stronger bacteriostatic activity, polymyxin B fails to exhibit an increased level of antimicrobial synergy with physical treatments compared to LAE.

Both LAE and polymyxin B were then analyzed for their mode of action on a model cell membrane using a unilamellar liposome. The zeta potential of the liposomes decreased significantly from  $-48\text{ mV}$  to  $-2.01$  and  $-0.93\text{ mV}$  upon incubation with LAE and polymyxin B, respectively. In addition, reduction in interparticle electrostatic repulsions resulted in flocculation of the liposomes, as shown by the particle size distribution curves in Fig. 7 and the value of polydispersity index (PDI) and average peak size (nm) given in Table 1. However, in the presence of LAE, the average volume mean diameter of the unilamellar vesicles did not change significantly ( $P > 0.05$ ) (Fig. 7). On the other hand, interaction of liposomes with polymyxin B created a significant fraction of colloidal particles ( $<35\text{ nm}$ , Fig. 7 and Table 1) smaller than intact liposomes. This evidence suggests that polymyxin B caused lysis of the liposomes based on its surfactant properties and potentially promoted the formation of mixed micelles, whereas LAE did not lyse the liposomes. It is important to note that the concentration of both LAE and polymyxin B is greater than the critical micelle concentrations (21, 22). This suggests that LAE binds and potentially inserts itself in a phospholipid membrane, whereas polymyxin B disrupts the bilayer membrane and promotes the formation of mixed micelles. In addition, the levels of oxidative stress generated by LAE and

**TABLE 1** Size distributions, polydispersity indices, and zeta potentials of model liposomes in the presence or absence of LAE and polymyxin B

Sample (ppm)	Avg size(s) (nm)	PDI <sup>a</sup>	Zeta potential (mV)
Liposome	162.6	0.144	-48.1
Liposome and LAE (15)	Peak 1, 133.4; peak 2, 1,375	0.702	-2.01
Liposome and polymyxin B (15)	Peak 1, 34.9; peak 2, 559.3	0.826	-0.93

<sup>a</sup>PDI, polydispersity index.

polymyxin B in bacterial cells were investigated. The results showed that LAE is more potent in generating oxidative stress, which may be correlated with its rapid bactericidal activity compared to polymyxin B. Detailed results are shown in the supplemental material and Fig. S3.

## DISCUSSION

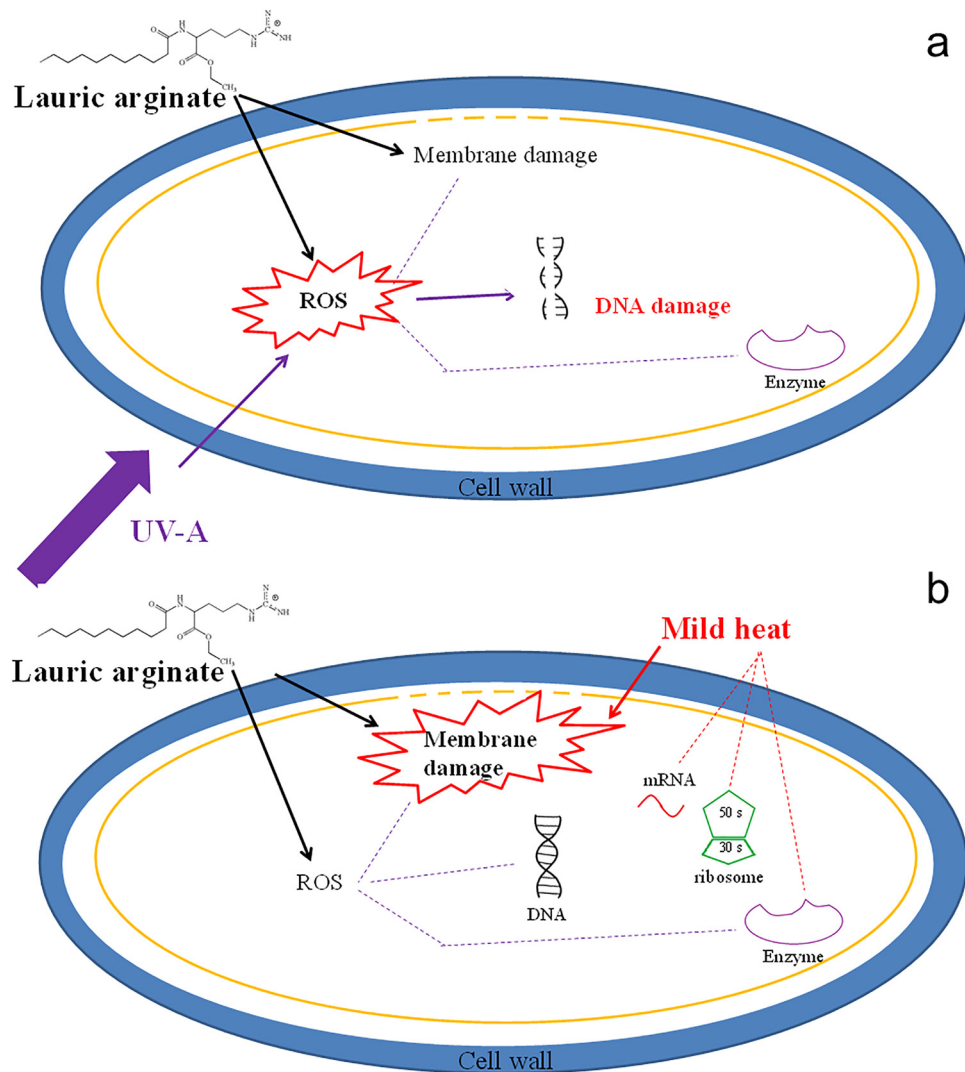
An antimicrobial approach based on a synergistic novel combination of LAE with multiple physical treatments was reported in this study. The combination of LAE with mild heat or UV-A light achieved a 5- to 6-log reduction in an *E. coli* O157:H7 strain in contrast to a 1- to 2-log reduction of bacteria by individual treatments. Combination of LAE with mild heat also demonstrated a 5-log synergistic reduction against *L. innocua*, a Gram-positive bacterium. To the best of our knowledge, the synergistic combination between an amino acid-derived antimicrobial compound and UV-A or mild heat has not been heavily investigated. Results from prior studies have focused only on synergistic antimicrobial activity using a combination of GRAS polyphenolic compounds and light (6, 7, 9, 23, 24). Furthermore, the results also show the significance of these mechanistic differences in the synergistic antimicrobial activity of these compounds with physical treatments (UV-A or mild heat). The results also illustrate the mechanistic differences in the antimicrobial activity of LAE compared to that of polymyxin B.

Both LAE and polymyxin B are well-established membrane-active antimicrobial agents. The results of this study demonstrate significant differences in the fate of membrane structures upon interaction with these agents. For instance, the results illustrate that both antimicrobial compounds electrostatically interacted with model cell membranes which influenced the net charge on liposomes. However, polymyxin B significantly reduced the size of liposomal particles, suggesting lysis of the membrane, whereas LAE had a minimal effect on the size of model liposomes. These results are in agreement with the literature (13, 14, 25). Despite the membrane activity and bacteriostatic properties of polymyxin B, antimicrobial synergy with light or mild heat was not observed with polymyxin B, in contrast to the results observed with LAE. This comparative evaluation suggested that membrane damage by itself is not a sufficient condition for the synergistic interaction of LAE with light or mild heat.

We proposed here that the oxidative stress may be the key difference between LAE and polymyxin B. Oxidative stress generation has been recently reported as one of the potential pathways that may contribute to the antimicrobial activity of diverse antibiotics (26), although some studies have also presented counterevidence to this hypothesis (27, 28). In the present study, bactericidal activity and oxidative stress generation from LAE or polymyxin B were evaluated. As shown in Fig. S3, the stronger bactericidal activity of LAE compared to polymyxin B was correlated with oxidative stress generation, since LAE bactericidal activity was depleted at lower temperatures and in the presence of antioxidants such as glutathione. A similar finding was reported by other researchers (26), where oxidative damage induced by certain antibiotics was among the lethal factors responsible for cell inactivation. In comparison, no oxidative stress was observed for polymyxin B, even during extended incubation.

The potential mechanism of antimicrobial synergy between LAE and physical treatments is illustrated in Fig. 8. Figure 8a shows the potential mechanism for the synergistic antimicrobial activity of LAE and UV-A. UV-A alone has been reported to generate reactive oxygen species (ROS), which can damage bacterial DNA, as well as the cell membrane and intracellular proteins (29). As shown in Fig. 8a, oxidative stress generated by a combination of UV-A and LAE is a leading factor for antimicrobial activity. This enhanced oxidative stress can lead to bacterial DNA damage, as well as oxidation of the cell membrane and proteins. This physical perturbation of the bacterial cell membrane plus enhanced oxidation stress may explain the synergistic interaction of bacteria. Interestingly, the synergistic influence of this treatment was dependent on temperature. This suggested the role of the metabolic activity of cells in increasing the susceptibility of cells to oxidative and membrane damage.

As illustrated in Fig. 8b, the synergistic mechanism between mild heat and LAE can



**FIG 8** Proposed synergistic antimicrobial mechanisms between LAE and mild physical treatments. (a) The synergistic combination of LAE and UV-A may induce enhanced oxidative stress (ROS), in addition to membrane damage, resulting in enhanced inactivation of bacterial cells. (b) The synergistic combination of LAE and mild heat may induce enhanced membrane damage, in addition to oxidative stress generation. Solid lines indicate pathways evaluated in this study, while dotted lines indicate additional pathways that may contribute to the synergistic inactivation of bacteria.

be attributed to oxidative stress generation by LAE, as well as the dual stress of mild heat and LAE, which induces enhanced membrane damage. The enhanced membrane damage was expected since even under mild heat conditions, bacterial membrane permeation increases, which results in the release of intracellular components (30), and the supplementation of LAE may exacerbate the membrane leakage based on enhanced pore formation. As shown in Fig. 5, oxidative stress-generated antimicrobial synergy can be neutralized with antioxidant supplementation, and the residual synergistic antimicrobial activity can be attributed to enhanced membrane damage induced by LAE and mild heat.

The synergistic combination between LAE and physical treatments may provide an effective, rapid, and low-cost antimicrobial processing approach that can be easily transferred to the food industry or medical applications. Examples may include incorporating LAE into packaging films (31, 32) and coupling LAE with UV-A treatment at room temperature to obtain surface sanitation of fresh produce (33). The application of LAE and mild heat could also be used to surface pasteurize cabbage, tomatoes, and cantaloupes (33–35).

In conclusion, antimicrobial synergies against the Gram-negative bacterium *E. coli* O157:H7 and the Gram-positive bacterium *L. innocua* were observed between the GRAS antimicrobial compound LAE and two mild physical treatments. Enhanced oxidative stress generation and exacerbated membrane damage resulted in a synergistic mode of action between LAE and mild physical treatments. Mechanistic differences between LAE and polymyxin B were compared based on the MIC, artificial cell membrane disruption, and oxidation stress generation. The synergistic application of mild physical treatments and LAE can be utilized in agriculture, the food industry, and the medical field as an alternative sanitizing approach that is safer, faster, and more efficient than some of the current processes.

## MATERIALS AND METHODS

**Reagents.** Lauroyl arginate ethyl (LAE; CytoGuard LA20, 20% [vol/vol]) was kindly provided by A&B Ingredients (Fairfield, NJ). Phosphate-buffered saline (PBS) was purchased from Fisher Bioreagent (Pittsburgh, PA). Tryptic soy broth (TSB), tryptic soy agar (TSA), glutathione, ascorbic acid, and L- $\alpha$ -phosphatidylcholine from egg yolk (egg PC) were purchased from Sigma-Aldrich (St. Louis, MO). Polymyxin B sulfate, thiourea, and rifampin were bought from TCI America (Portland, OR). Ultrapure water was filtrated using a Milli-Q filtration system (EMD Millipore, Billerica, MA).

**Microbial culture.** A rifampin-resistant *L. innocua* mutant (ATCC 33090; ATCC, Manassas, VA) and a Shiga toxin knockout rifampin-resistant *E. coli* O157:H7 mutant (ATCC 700728) were kindly provided by Trevor Suslow (University of California—Davis) and Linda Harris (University of California—Davis). Enumeration of both bacteria was performed on TSA supplemented with rifampin at a final concentration of 50  $\mu\text{g/ml}$ .

**Synergistic combination between LAE and light or mild heat based on isobolograms.** Synergistic combination between the selected concentration of LAE and UV-A light was evaluated using isobolograms, as described by Markovsky et al. (36), with some minor modifications. For this analysis, *E. coli* inactivation was measured by varying the concentration of LAE incubated with bacterial cells for a fixed amount of time without UV-A treatment. Similarly, inactivation of bacterial cells was evaluated with the variation in the total fluence of UV-A exposure of cells without the presence of LAE. Each synergistic combination was selected and tested for bactericidal activity, where LAE was fixed at 15  $\mu\text{g/ml}$  and UV-A treatment for 30 min, corresponding to a fluence of 8.64 J/cm<sup>2</sup>. Similarly, the synergistic combination between LAE and mild heat was also evaluated using isobolograms. In this analysis, LAE at different concentrations was selected to inactivate *E. coli* for a fixed amount of time (4 min), whereas for mild heat treatment the temperature was fixed at 55°C, but the incubation time was varied. A synergistic combination was selected and tested for bactericidal activity with an LAE concentration of 15  $\mu\text{g/ml}$  and mild heat treatment for 4 min at 55°C.

**Inactivation of *E. coli* and *L. innocua* by individual treatments (mild heat or LAE) and their combination.** Overnight cultures of *E. coli* or *L. innocua* were grown in TSB for 18 to 22 h at 37 or 30°C, respectively. Both cultures were then centrifuged (1 min, 16,100  $\times g$ ) and washed twice in sterile PBS to a final concentration of  $5.0 \times 10^7$  CFU/ml. For mild heating, the solution was heated to 55°C. At this temperature and after a short incubation time, there is minimal deterioration of food quality. The glass scintillation vials (total volume, 20 ml) were preheated in a 55°C water bath for a minimum of 10 min. The *E. coli* and *L. innocua* cells were then treated with mild heat, LAE, or mild heat plus LAE for 4 min. Final concentrations of LAE were supplemented at 15 ppm, and the survivor populations were enumerated on rifampin-supplemented TSA plates.

**Inactivation of *E. coli* and *L. innocua* by individual treatments (UV-A or LAE) and their combination.** Both cell cultures were prepared, centrifuged, washed, and resuspended in PBS, as described earlier. The *E. coli* or *L. innocua* cells were then treated at room temperature with UV-A, LAE, or UV-A plus LAE. LAE was supplemented at a final concentration of 15 ppm for testing against *E. coli*, whereas three different final concentrations (15, 22.5, and 30 ppm) were evaluated for testing against *L. innocua*. These concentrations were selected based on similar isobologram studies for *E. coli* (data not shown). The survivor population of *E. coli* after each treatment was enumerated on rifampin-supplemented TSA at 5, 10, 20, and 30 min, whereas the survivor population of *L. innocua* was only enumerated after 30 min.

The UV-A treatment was conducted in a UV-A chamber, which consisted of a closed plastic box (Suncast Corporation, Batavia, IL) and four UV-A lamps (320 to 400 nm, 18 W; Actinic BL, Philips, Holland) installed on the underside of the top lid of the chamber (37). Both UV-A-treated and UV-A/LAE-treated samples were set on a plastic rack at the center of the UV-A chamber, with a distance of 0.5 cm to the UV-A lamps. The average light intensity for the combination of four UV-A lamps was  $4.8 \pm 0.1 \text{ mW} \cdot \text{cm}^{-2}$ . Experiments were performed in triplicates.

**Nucleic acid leakage assay.** Since LAE was reported as a membrane-disrupting antimicrobial compound (13, 14), damage to bacterial cell membrane was analyzed in the synergistic combination. A disrupted cell membrane structure will usually lead to the diffusion of small molecules, including potassium ions and nucleic acid, from the cytoplasm to the outer environment (20, 38). *E. coli* was chosen as a model microorganism for the results presented in Fig. 3 through 8. *E. coli* was selected since it had synergistic interactions with both selected physical treatments, in contrast to *Listeria*. Briefly, *E. coli* cells treated under all of the previously described conditions were centrifuged at  $16,162 \times g$  for 5 min and 4°C. The supernatant was taken and measured in a Genesys 10S UV-Vis

spectrophotometer (Thermo Scientific, Waltham, MA) in UV cuvettes (Brand, Wertheim, Germany) at 260 nm (maximum nucleic acid absorption wavelength). Control bacterial cells without any treatment were also spun down, and the supernatant was used as a blank. All DNA leakage assays were repeated at least three times.

**Investigation of the synergistic mechanism between LAE and physical treatments (UV-A and mild heat).** The experiments followed the same scheme described earlier but were supplemented with the antioxidants glutathione, ascorbic acid, and thiourea at final concentrations of 8, 4, and 80 mM, respectively. Antioxidants were applied to evaluate whether oxidative stress generation is one of the mechanisms for the observed antimicrobial synergy.

In order to determine whether the same antimicrobial synergy persisted, UV-A and LAE were combined to treat *E. coli* according to the same experimental setup at a refrigeration temperature (4°C). Survivor population was enumerated after treatment for 30 min.

**Inactivation of *E. coli* by polymyxin B combined with physical treatments (UV-A and mild heat).** Verification of the observed synergistic combination between LAE and mild physical processing was conducted by replacing LAE with polymyxin B. Polymyxin B was selected due to its structural and mechanistic similarity to LAE: (i) both compounds have a long lipid chain at the N-terminal end, (ii) both compounds are peptides or amino acids in nature and carry a positively charged amino acid(s), and (iii) both compounds have been well documented for their ability to disrupt the bacterial cell membrane. For instance, polymyxin B can interact with lipopolysaccharide on the outer membrane of Gram-negative bacteria through replacement of Ca<sup>2+</sup> and Mg<sup>2+</sup> and subsequently disrupt both outer (39) and cytoplasmic (40) membranes, resulting in cell lysis (25). Polymyxin B treatments combined with physical treatments (UV-A and mild heat) were conducted as described earlier against *E. coli*. The survivor population of *E. coli* was enumerated on TSA agar.

**MICs for LAE and polymyxin B.** The MICs for LAE and polymyxin B were tested in a 96-well plate according to Clinical and Laboratory Standards Institute recommendations (41). The final concentration of LAE was tested at 7.5, 15, and 30 µg/ml, while the final concentration of polymyxin B was tested at 3.75, 7.5, and 15 µg/ml. The 96-well plate was then incubated at 37°C for 16 to 20 h and observed for turbidity. The MIC was determined as the lowest concentration of an antimicrobial compound that will not lead to visible growth of a microorganism. The MIC experiments were conducted in triplicates.

**Preparation of model cell membrane.** Unilamellar liposome as a model cell membrane was created using a dehydration and rehydration method (42). In this method, thin film of egg L- $\alpha$ -phosphatidylcholine was obtained by dissolving the lipid in chloroform and evaporating the solvent under a vacuum using a rotary evaporator. Thin film was further kept under a vacuum to remove all traces of solvents. The dried thin film was rehydrated in PBS to achieve a 1-mg/ml total lipid concentration and then vortexed to prepare multilamellar vesicles. The multilamellar vesicles were extruded 15 times (mini-extruder; Avanti Polar Lipids, Inc.) through 400-nm pores, followed by the use of 200-nm polycarbonate track-etched membranes to obtain unilamellar liposomes of ~160 nm (Table 1).

**Size distribution and zeta potential of model cell membrane.** Size distributions (nm) and zeta potentials (mV) of liposome model cell membranes in the presence or absence of both antimicrobials (LAE and polymyxin B) were acquired using a dynamic light scattering system (Malvern Zetasizer Nano, Westborough, MA). Both treated and untreated liposome solutions were diluted 10-fold in PBS and suspended in a polystyrene cuvette with a standard path length of 10 nm for size distribution and in disposable folded capillary zeta cells for zeta potential measurements. Solutions were equilibrated at room temperature for 2 min before data acquisition. Scattered light was detected at 90° relative to the incident laser (633-nm He-Ne laser) light for 12 to 20 runs of 10 to 20 s each, with a medium viscosity of 0.89 cP and a refractive index of 1.33.

**Oxidative stress generation from polymyxin B and LAE against *E. coli*.** Both polymyxin B and LAE were prepared at a final concentration of 15 µg/ml either in PBS or in PBS supplemented with 10 mM glutathione. Supplementation of antioxidants (e.g., glutathione) can quench oxidative stress potentially generated from antimicrobial compounds, preventing bacterial cells from being inactivated (43). The *E. coli* cells were then inoculated to a final concentration of  $5.0 \times 10^7$  CFU/ml. All treatments were incubated at room temperature or 4°C for 30 min or 2 h, followed by enumeration of *E. coli* survivors on rifampin-supplemented TSA plates.

**Statistical analysis.** All experiments were conducted in triplicates. The bacterial population and absorbance at 260 nm were compared statistically, using analysis of variance by SAS 9.4 (SAS Institute, Inc., Cary, NC). Tukey's honest significant difference test was used to evaluate the significance of the difference among means ( $P < 0.05$ ).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01033-19>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

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