Antimicrobial Efficacy of Pelargonic Acid Micelles against *Salmonella* varies by Surfactant, Serotype and Stress Response

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The antimicrobial properties of Pelargonic acid (PA), a component of tomatoes, makes it an attractive candidate as a food additive and sanitizer. The antimicrobial efficacy of PA emulsions generated using surfactants: Tween 80, Triton X100, Sodium Dodecyl Sulfate (SDS) and Quillaja Saponin was evaluated against *Salmonella* serotypes Newport, Oranienburg and Typhimurium. Micelle/droplet size, and minimal inhibitory concentrations (MIC) were determined. Surfactant type and concentration significantly influenced the antimicrobial efficacy of PA \((p < 0.05)\). Overall, *Salmonella* Newport was the most \((p < 0.05)\) susceptible serotype to PA emulsions. PA emulsions generated with 1.00% SDS had the highest \((p < 0.05)\) antimicrobial activity, with MIC of 7.82 mM against *S*. Newport and 15.62 mM against *S*. Oranienburg/ *S*. Typhimurium, respectively. Addition of PA to Trypticase Soy Broth resulted in a decreased growth rate and an increased lag phase duration. Cells exposed to PA formed elongated filaments \((>5 \mu m)\). Additionally, *Salmonella* serotypes Typhimurium and Newport also formed floccular biofilms. PA emulsions at a concentration of 31.25 mM generated using 1% SDS and 1% Quillaja saponin resulted in >6 log CFU/ml reduction in *Salmonella* population. Although all PA emulsions evaluated inhibited *Salmonella*, morphological changes to this antimicrobial varied substantially among the *Salmonella* serotypes tested.

Non-typhoidal *Salmonella* are one of the most commonly implicated bacterial agents of foodborne infection in the United States. A wide variety of *Salmonella* serotypes have been responsible for outbreaks associated with poultry, produce, beef, low moisture food and pet food. The heterogeneity among food matrices indicates that different levels of stress tolerances could occur among *Salmonella* serotypes. Further, the rise of Anti-Microbial Resistance (AMR) in *Salmonella* is threat of immediate concern with dire ramifications that requires the exploration of antimicrobial agents and a better understanding of antimicrobial resistance strategies in *Salmonella*.

Antibiotic resistant bacteria result in 700,000 deaths every year globally and have been on the rise. Sanitizers, such as quarternary ammonium compounds (QACs), when used at sub-lethal concentrations, can contribute to the development of antibiotic resistance in bacterial pathogens. There is a strong evidence that antibiotic resistance and biocide resistance in bacteria are linked; the phenomenon is referred to as biocide-antibiotic cross-resistance or cross-tolerance. Thus, the possibility that human enteric pathogens could develop resistance against conventional biocides along with multidrug-resistance, calls for the development of new sanitizers and antimicrobial compounds.

Plants have been used for millennia and remain a promising source of antimicrobial compounds to substitute for currently used sanitizers and antibiotics. Plant derived fatty acids such as lauric acid, oleic acid, palmitoleic acid, caproic, caprylic, and capric acid have demonstrated antimicrobial activity against both Gram-positive and Gram-negative foodborne bacterial pathogens. The antimicrobial activities of certain fatty acids result from their ability to disrupt bacterial membrane lipids, alter membrane fluidity or form hydroperoxides leading to oxidative damage. Pelargonic acid (PA) is a commonly used antifungal agent with a Generally Recognized as
Safe (GRAS) status and is a component of the tomato exometabolome. Recently the antimicrobial activity of PA against *Salmonella* was demonstrated\(^1\) indicating that the fatty acid had better antimicrobial activity when dispersed as a micelle using Quillaja saponin than its unmodified form, hence highlighting its potential as a GRAS antimicrobial food additive or as a sanitizer.

Through a variety of fatty acids demonstrate antimicrobial activity, their efficacy is often diminished by their poor solubility in water and phase separation in an aqueous medium\(^2\). Emulsification of fatty acids can increase their miscibility and dispersion in water,\(^2\) resulting in improved antimicrobial activity\(^1,11,12\). Surfactants such as Tween 80, SDS and Quillaja saponin, a triterpene glycoside from the bark of the *Quillaja saponaria* Molina can be used to produce emulsions of fatty acids and essential oils that have antimicrobial properties. Surfactants can influence the antimicrobial efficacy of an emulsion due to their charges and their placement on the colloidal particle, thereby influencing the particle size and the amount of antimicrobial compound that is in contact with the target bacterial cell\(^14-17\).

The surfactant choice and concentration can either, synergistically strengthen or impede the antimicrobial activity of an emulsion\(^16,18\). Previous research indicated that exposure to sublethal concentration (20 mM) of PA induced a transitional morphological change (filament formation) in *Salmonella*\(^11,19,20\). Formation of diverse phenotypes by bacteria in challenging environments is a strategy to overcome antimicrobial hurdles\(^21-23\). Determining morphological changes in *Salmonella* during the exposure to PA emulsions formed using different surfactants could provide a better understanding of the antimicrobial’s mechanistic action of and means of adaptation by the pathogen.

The minimal inhibitory concentrations (MIC) of aqueous emulsions of PA generated using Tween 80, Triton X100, SDS and Quillaja saponin were determined against *Salmonella* serotypes Newport isolated from cattle, *Oranienburg* isolated from nut and a reference Typhimurium strain for antimicrobial testing. Bactericidal efficacy of the most potent emulsions was evaluated against a cocktail of the *Salmonella* serotypes after treatment durations of 30 s and 5 min. Growth kinetics and cell physiology of the *Salmonella* serotypes after treatment durations of 30 s and 5 min. Growth kinetics and cell physiology of the *Salmonella* serotypes were tested in presence of inhibitory PA emulsions to study adaptive mechanisms to the antimicrobial. The results of this study are intended to bridge the current knowledge gap on the role of surfactant type in antimicrobial efficacy of PA to aid in the development of fatty acid-based washes and rinses for the food industry.

**Materials and Methods**

**Micelle and droplet size measurements.** Droplet size distribution and polydispersity (intensity-based size distribution) measurements of emulsions were conducted at 20 °C by Dynamic Light Scattering that examines the fluctuations in light scattering due to brownian motion of the emulsion droplets, using a Zetasizer Nano ZS device (Malvern, Southbough, MA). Briefly, 5 ml of emulsion samples were diluted in 50 ml of deionized water. Then, 10 ml of the diluted samples were filtered through a 0.40 μm nylon filter (Syringe-filters, Cole Parmer, UK) and vortexed. Mean hydrodynamic diameter of emulsion droplets was calculated via cumulative analysis (z-average) and the size distributions were defined by the polydispersity index (PdI) and the size distribution graph.

**Bacterial Culture.** *Salmonella enterica* subsp. *enterica* serotype Newport (11590 K, beef isolate, GFP labelled), S. Oranienburg (1839, pecan isolate) and S. Typhimurium (CDC 6516-60/ATCC 14028) were obtained from the Center for Food Safety, University of Georgia, Culture Collection. Cultures were revived by two successive 24 h transfers in 10 ml Tryptic Soy Broth (TSB, Acumedia Neogen Corporation, Lansing, MI) with antibiotics (100 μg/ml Ampicillin for *S. Newport* and Typhimurium and 50 μg/ml Nalidixic acid for *S. Oranienburg*), at 35 °C. Then, the cultures were streaked on Tryptic Soy Agar (TSA, Acumedia, Neogen Corporation. Lansing, MI) with antibiotics and incubated at 35 °C for 24 h. Individual colonies were then confirmed serologically using latex agglutination tests in the cultures were streaked on Tryptic Soy Agar (TSA, Acumedia, Neogen Corporation. Lansing, MI) with antibiotics (Oxoid, Ogdensberg, NY). Isolated colonies were then streaked on XLT4 (Xylose Lysine Deoxycholate agar with 0.5% Xylose) at 35 °C for 24 h and observed for a change in color from blue to pink indicating bacterial growth. The lowest dilution at which no bacterial growth was determined (blue well) was considered the minimum inhibitory concentration. The MICs of the individual surfactants at concentrations of 1%, 0.1% and 0.01% (w/v) and PA without surfactant that was suspended in water by vortexing for 1 min were determined as comparative controls using the modified 96 well plate Resazurin assay. Each test was performed three times and an average of the three MIC values were used.
**Bactericidal activity.** Concentration of PA emulsions that were inhibitory were evaluated for their bactericidal activity against *Salmonella* serotypes. After determining the MIC as described in the previous section, wells containing the lowest inhibitory concentration of the pelargonic acid emulsion were neutralized using Dey-Engley neutralizing broth (Neogen Corp., Lansing, MI) and enumerated on TSA by spread plating with appropriate dilutions. Emulsions that resulted in a reduction of bacterial populations ≥ 3 log CFU/ml were considered bactericidal. Each experiment was performed three times and an average of the three MIC values were used.

**Antimicrobial efficacy against Salmonella cocktail.** A PBS suspension consisting of a cocktail of *Salmonella* serotypes Newport, Typhimurium and Oranienburg at a concentration of 9 Log CFU/ml was exposed to 31.25 mM and 15.62 mM of PA-Quillaja Saponin (1%) and PA – SDS (1%). The concentrations of emulsions selected were inhibitory to all three serotypes of *Salmonella*. Exposure durations tested were for 30 s and 5 min. Following exposure, the fatty acid was neutralized using Dey-Engley neutralizing broth and enumerated by spread plating after appropriate dilutions. Each experiment was performed three times.

**Growth Kinetics of Salmonella in presence of PA emulsion.** *Salmonella* serotypes Newport, Typhimurium and Oranienburg were grown in presence of TSB at 37°C with 15.6 mM of PA emulsion generated using 1% Quillaja saponin and TSB with 7.80 mM of PA emulsion generated using 1% SDS. The concentration of the emulsion used was inhibitory to S. Newport (p < 0.05) and was sub-inhibitory to S. Typhimurium and S. Oranienburg. Initial population of 5 log CFU/ml of *Salmonella* serotypes were grown in 50 ml centrifuge tubes (VWR International, Radnor, PA) of TSB at 37°C. The cells were neutralized using Dey-Engley neutralizing broth and enumerated at 0, 2, 4, 8, 12, 16, 20 and 24 h by droplet plate method on TSA with appropriate serial dilutions. Controls for the experiment were grown without the presence of PA emulsions with identical culture conditions. Growth curves were plotted and analyzed using MATLAB (The Math Works, Natick, MA, Version 2017). Baranyi model was used as the primary model to predict the bacterial population as a function of time.

**Assessment of morphology.** Cell morphology of *Salmonella* serotypes grown in TSB amended with 15.62 mM of PA emulsion generated using 1% Quillaja saponin and TSB with 7.80 mM of PA emulsion generated using 1% SDS at, 37°C after 24 h of growth were evaluated through phase contrast microscopy (Nikon Eclipse Ci-L; Nikon Corp., Japan). Wet mount preparations of 20 µl and studied using a Nikon 100X Plan Apo objective (Nikon Eclipse 50i; Nikon Corp., Japan) with the Nikon Elements software through with a scale of 5 µm to differentiate and enumerate cells that were larger than 5 µm (filaments, F) and cells that were smaller than 5 µm (regular, R). and aggregates of cells (>5 cells) were considered as floccules. Direct counts of filamentous cells and regular-sized cells were determined from 3 experimental replicates (3 fields per slide) for each treatment and time point. The average counts of filamentous cells (F) and regular cells (R) were used to determine the F:R ratio. Each field had between 50 to 200 cells. The structure of the floccular biofilms was studied using Confocal Laser Scanning Microscopy (CLSM). Floccular biofilm was further confirmed by staining with FilmTracer calcein red-orange (Molecular Probes, Eugene, OR) biofilm stain. Speciminet staining with FilmTracer calcein red-orange was conducted according to manufacturer’s instructions. After an hour of incubation at room temperature in the dark, the sample was observed using a Zeiss 880 laser scanning confocal microscope with a 63 ×/1.4 NA oil immersion Plan Apochromatic objective at 576/590 nm.

**Statistical analysis.** The experiments for this study were designed using a randomized blocked design factorial treatment arrangement, repeated measures with sampling, blocked on replication (Table 1). The statistical analysis of the data was performed with analysis of variance (ANOVA) using JMP Pro 14 (SAS Institute, Cary, NC). Tukey-Kramer honestly significant difference test was used to determine differences between mean values of the MIC and droplet size of different tests at a 95% confidence level. To evaluate the correlation between antimicrobial efficacy of PA and average micellar/droplet diameter of PA emulsions, the ordinary least squares regression analysis was applied to the MIC values and the corresponding average micellar/droplet diameter.

**Results**

**Micelle characteristics.** The micelle size of PA emulsions varied depending on the type and concentration of surfactant. In PA emulsions generated using SDS, micelle diameter was positively associated with surfactant concentration, specifically 1.00% SDS emulsion had the largest (3220.50 ± 133.50 nm) micelles, significantly (p < 0.05) smaller micelles (1499.67 ± 112.90 nm) in emulsions produced with 0.10% SDS, and those generated with 0.01% SDS had the smallest micelles diameters (289.70 ± 27.44 nm) (Table 1). In contrast, in all other surfactants tested, no positive association between micelle diameter and surfactant concentration was observed. Nevertheless, the diameter of micelles varied significantly (p < 0.05) with surfactant concentration, except for Quillaja saponin and TritonX 100 at 1.00 and 0.10% (Table 1). The antimicrobial activity of PA emulsions generated using Quillaja saponin against all three *Salmonella* strains indicated that a positive correlation existed between higher micellar size and inhibitory activity. This correlation was highest for S. Newport as compared to other two serotypes. For Tween treatment, there was very weak correlation for S. Newport and S. Oranienburg, whereas the ‘r’ value was 0.38 for S. Typhimurium. For Triton, the correlation coefficient ranged between −0.25 and 0.10, whereas for SDS, the correlation coefficient was strongly negative for S. Newport (−0.86) (Fig. 2). The correlation coefficient could not be calculated for the other two strains as the MIC values were constant for all the experimental micellar sizes.

The average pH of 1 M stock solutions of the pelargonic acid emulsions generated using the various surfactants was 3.55 ± 0.18. Polydispersity index varied with both, the type and concentration of the surfactant. The positive
The inhibitory activity of PA emulsions against Salmonella was determined. The MIC of PA against all three Salmonella serotypes was 125 ± 0.00 mM. Quillaja Saponin did not inhibit Salmonella growth at 1%, 0.1% and 0.01%. When emulsions of PA were formed using Quillaja saponin, the antimicrobial activity, as denoted by a decrease in MIC, increased significantly (p < 0.05). Of the three serotypes of Salmonella tested, S. Newport was most susceptible to PA (p < 0.05). The lowest concentration of PA emulsions that was inhibitory against S. Newport was 7.82 ± 0.00 mM and was produced using 1% SDS (p < 0.05). The MIC of PA emulsion produced using 1% SDS for S. Oranienburg and S. Typhimurium, was 15.62 ± 0.00 mM (Fig. 1). No significant difference in the MIC of PA emulsions produced using 1%, 0.1% and 0.01% SDS was observed against S. Oranienburg and S. Typhimurium (Fig. 1).

Similar to SDS-based emulsions, PA emulsions generated using 1% and 0.1% Quillaja saponin were significantly more inhibitory to S. Newport with an MIC of 15.62 ± 0.00 mM and 13.02 ± 4.51 mM respectively (p < 0.05) (Fig. 1). Against serotypes Oranienburg and Typhimurium, PA emulsions generated with 1% and 0.1% Quillaja saponin had an average MIC of 29.42 ± 6.98 mM.

No significant differences in susceptibility was observed among the Salmonella serotypes when treated with 0.01% Quillaja saponin generated PA emulsions (MIC of 27.77 ± 6.88 mM) (Fig. 1).

Non-ionic surfactants, Tween 80 or Triton X 100 were not as effective as SDS and Quillaja saponin in improving the antimicrobial activity of PA. Emulsions of PA generated using 1.00% Tween 80 or 1.00% Triton X 100 were inhibitory to the Salmonella serotypes tested at 31.25 ± 9.02 mM (Fig. 1). PA emulsions generated using 0.1% Tween 80 had an average MIC of 48.61 ± 16.47 mM against the Salmonella serotypes tested. MIC of PA emulsions

Table 1. Characteristics of PA emulsions generated using 0.01, 0.1 and 1% surfactants. Z-average diameter values that are preceded by a different letter are significantly (p < 0.05) different from each other for the same surfactant.
generated using 0.1% SDS and Quillaja saponin were significantly \( p < 0.05 \) more efficacious against \textit{Salmonella} than those of Tween 80 and Triton X100 (39.62 ± 15.37 mM) (Fig. 1).

\[ S. \text{ Newport} \] was significantly more susceptible to PA emulsions generated using 0.01% Tween 80 (39.79 ± 15.51 mM) and TritonX 100 (39.62 ± 15.37 mM) than other serotypes \( p < 0.05 \). The average MIC of PA emulsions generated using 0.01% Tween 80 and Triton X100 against \textit{S. Oranienburg} and Typhimurium was 57.29 ± 12.16 mM.

**Bactericidal activity of emulsions.** Suspensions generated using Triton X100 and Tween 80 were not bactericidal at their MIC. Bactericidal activity was observed for suspensions of PA using 0.1% (10.93 ± 0.00 mM) and 1% SDS (7.82 ± 0.00 mM) respectively, against \textit{S. Newport} (Fig. 1). Quillaja saponin (0.01%) generated PA suspensions were bactericidal to \textit{S. Typhimurium} and \textit{S. Oranienburg} at a concentration of 31.25 ± 0.00 mM (Fig. 1). Quillaja saponin 1%, generated PA suspensions were bactericidal to \textit{S. Newport} and \textit{S. Oranienburg} at 15.62 ± 0.00 mM and 31.25 ± 00 mM respectively. Emulsion generated using 0.1% Quillaja saponin was bactericidal at a concentration of 31.25 ± 00 mM against \textit{S. Typhimurium}, indicating that serotype and surfactant-based variation significantly affected bactericidal activity of the emulsions \( p < 0.0001 \).

**Growth kinetics of \textit{Salmonella.}** \textit{Salmonella} serotypes Newport, Oranienburg and Typhimurium were grown in TSB containing PA emulsions generated using Quillaja saponin and SDS. The emulsions were used at a concentration that was inhibitory to \textit{S. Newport}, 5.62 mM, PA with 1% Quillaja saponin and 7.82 mM PA with 1% SDS. Growth rates and physiological adaptations to PA were compared among the three serotypes of \textit{Salmonella} to understand the effects of PA on bacterial growth and the physiological adaptations undergone by the organisms to overcome PA associated challenge.

Figure 3 shows the fitted Baranyi models (Baranyi and Roberts, 1994) for \textit{Salmonella} serotypes Newport, Oranienburg, and Typhimurium, grown in TSB (control) and TSB amended with 15.62 mM PA with 1% Quillaja saponin and 7.82 mM PA with 1% SDS. While the control samples demonstrated a full growth curve with the maximum population density of 10.57 log CFU/ml, none of the PA treated samples could reach the maximum population density after the initial growth. As such, a full Baranyi model was fitted to the control sample growth data, whereas a reduced Baranyi model was fitted to the growth data showing a growth pattern in both the treated
samples. As it is evident from the growth curves, the lag phase duration was the minimum for control samples for all three strains (2.90, 2.72, and 2.54 h for \textit{S}. Newport, \textit{S}. Typhimurium, and \textit{S}. Oranienburg, respectively). For the same strains and for samples treated with SDS and pelargonic acid, the lag phase durations were calculated as 3.36, 2.53, and 5.84 h, respectively. The maximum growth rates (log CFU/h) for these treatments were estimated as 0.99, 0.97, and 0.58, as compared with the control sample growth rates of 1.26, 1.62, and 0.87 log CFU/h for \textit{S}. Newport, \textit{S}. Typhimurium, and \textit{S}. Oranienburg, respectively. These values suggest that although lag phase duration was not affected much by SDS generated PA emulsion, the maximum growth rate was reduced considerably. The samples treated with Quillaja Saponin generated PA emulsions were further impacted, as reflected by the lag phase durations of 4.84, 1.09, and 5.84 h, and maximum growth rates of 0.42, 0.30, and 0.43 log CFU/h for \textit{S}. Newport, \textit{S}. Typhimurium, and \textit{S}. Oranienburg, respectively. All three strains had a considerable drop of population within 12-hours of treatment with either PA emulsion.

**Figure 3.** Fitted Baranyi models for the growth data of three \textit{Salmonella} strains at 37°C. Solid lines and triangular markers (Controls: no antimicrobial; 100µl 7-log inoculum + 9.9 ml TSB); Dotted lines and circular markers (100µl 7-log inoculum + 9.9 ml TSB + 156.25µl 1 M PA with 1% saponin); Dashed lines and square markers (100µl 7-log inoculum + 9.9 ml TSB + 78.25µl SDS-PA).
Morphological diversity. Filament formation was significantly influenced by the type of Salmonella serotype (p < 0.05) exposed to PA emulsions with S. Oranienburg forming the significantly higher number of filaments (p < 0.05) (Table 2). Exposure of S. Oranienburg cells to 7.80 mM of PA emulsion generated using 1% SDS for 24 h resulted in the highest number of filament formation with an F:R of 2.18 ± 1.53 (p < 0.05).

S. Typhimurium formed the lowest number of filaments after exposure to the PA emulsions generated using both surfactants (p < 0.05) with F:R of 0.24 ± 0.18 and 0.07 ± 0.03 for Quillaja saponin and SDS generated PA emulsions respectively (Table 2).

Floccule formation was significantly influenced by both the serotype (p < 0.0001) of Salmonella and the PA emulsions (p < 0.05) the cells were exposed to. Exposure of S. Typhimurium to 7.80 mM of PA emulsion generated using 1% SDS resulted in significantly higher floccules than all other treatments (p < 0.05) that resulted in floccule formation. S. Oranienburg did not form floccules upon exposure to both SDS and Quillaja saponin generated PA emulsions (Table 2, Fig. 7).

Microscopy analysis of PA-challenged S. Typhimurium cells after 24 h growth in TSB indicated that the cells formed aggregates and were encased in a film of exopolymeric substance (Fig. 4). Observation using confocal laser scanning microscopy using optical Z sectioning demonstrated that cellular aggregates (herein referred as floccules) had a thickness of 10–20 µm and consisted of Salmonella cells and EPS (Figs. 4-panel E and F, 5, 6).

Table 2. Differences in the ratio of regular cells:filaments and floccule formation by S. Oranienburg, S. Newport and S. Typhimurium in response to PA (7.80 mM) + 1% SDS emulsions and PA (15.62 mM) + 1% Quillaja saponin. Treatments resulting significantly different ratios of filaments to regular cells (F:R) and floccule formation (p < 0.05) have been indicated by different alphabet.

| Salmonella Serotype | PA emulsion | F:R       | Floccules       |
|---------------------|-------------|-----------|-----------------|
| Oranienburg         | 7.80 mM PA emulsion + 1% SDS | 0.53 ± 0.23ab | 0.53 ± 0.23ab  |
| Oranienburg         | 15.62 mM PA + 1% Quillaja saponin | 2.18 ± 1.53 | 0.53 ± 0.23ab  |
| Typhimurium         | 7.80 mM PA emulsion + 1% SDS | 0.07 ± 0.03 | 18.3 ± 1.52ab  |
| Typhimurium         | 15.62 mM PA + 1% Quillaja saponin | 0.24 ± 0.18 | 8.33 ± 6.65c   |
| Newport             | 7.80 mM PA emulsion + 1% SDS | 0.63 ± 0.16a | 2.33 ± 0.57ac  |
| Newport             | 15.62 mM PA + 1% Quillaja saponin | 0.32 ± 0.08b | 2.66 ± 0.57bc  |

Figure 4. Phase contrast micrographs of (A) Salmonella Newport cells grown for 24 h in TSB, (B) TSB with 7.81 mM PA emulsions generated using 1% SDS, (C) TSB with 15.62 mM PA emulsions generated using 1% Quillaja Saponin. (D) Salmonella Typhimurium cells grown for 24 h in TSB, (E) TSB with 7.81 mM PA emulsions generated using 1% SDS, (F) TSB with 15.62 mM PA emulsions generated using 1% Quillaja Saponin. (G) Salmonella Oranienburg cells grown for 24 h in TSB, (H) TSB with 7.81 mM PA emulsions generated using 1% SDS, (I) TSB with 15.62 mM PA emulsions generated using 1% Quillaja saponin. Bars at the bottom right corners represents 10 µm in length.
CLSM micrographs of the stained floccules further confirmed that both filaments and regular size cells were constituents of these floccular biofilms (Figs. 5, 7).

*Salmonella* Newport was the most susceptible serotype to the concentration of PA used in TSB. In response to both Quillaja saponin- and SDS-generated emulsions, *S*. Newport formed filaments (> 5 µm) and regular sized cells (Figs. 4, 7). The filaments formed by *S*. Newport were thicker than filaments formed by other serotypes with certain filaments displaying cellular dysplasia indicating structural damage to certain filaments (Fig. 4, panel b, c).

**Antimicrobial efficacy of PA emulsions against a *Salmonella* cocktail.** Emulsions containing 31.25 mM PA generated using 1% SDS was most effective against the *Salmonella* cocktail after an exposure duration of 30 s, resulting in a 4.7 log CFU/ml reduction (p < 0.05). Quillaja saponin (1%) based PA emulsions (31.2 mM) had significantly lower antimicrobial efficacy and reduced *Salmonella* by 2.15 log CFU/ml (p < 0.05).
No significant differences were observed in antimicrobial activity between PA emulsions generated using 1% SDS (1.62 log CFU/ml) or 1% Quillaja saponin (0.77 log CFU/ml) when used at a concentration of 15.62 mM (Fig. 8).

After 5 min of exposure no significant differences were observed in antimicrobial activity between PA emulsions generated using 1% SDS (6.85 log CFU/ml) or 1% Quillaja saponin (6.21 log CFU/ml) when used at a concentration of 31.25 mM (Fig. 9). The antimicrobial efficacy of PA emulsions tested at 31.25 mM was significantly higher than PA emulsions tested at 15.26 mM (p < 0.05) (Fig. 9). PA (15.26 mM) emulsions generated using 1% SDS or Quillaja saponin resulted in 4.61 and 3.87 log CFU/ml reduction of Salmonella population in the cocktail.

Figure 7. Confocal micrograph demonstrating differences in (A) S. Newport (B) S. Typhimurium and (C) S. Oranienburg response to PA emulsions. Bars at the bottom right corners represents 10 μm in length.
PA concentration and duration of exposure significantly influenced antimicrobial activity of the emulsions ($p < 0.05$) (Fig. 9).

**Discussion**

PA is a fatty acid commonly found in plants such as tomatoes and is an FDA approved food additive. PA was found to have inhibitory activity against *Salmonella* at a concentration of 125 mM when dispersed in water by physical agitation. When emulsified using Quillaja saponin, the inhibitory activity of PA significantly increased. Hence in the current study, emulsions of PA were formed using a variety of surfactants at different concentrations and evaluated for their inhibitory activity against *Salmonella* Serotypes Newport, Typhimurium and Oranienburg. Of the surfactants tested in the study Quillaja saponin, SDS and Tween 80 are approved by the FDA as GRAS and TritonX 100 is approved for the dispersal of pesticides and applications associated with food packaging.

All the surfactants used in the current study improved the inhibitory activity of PA against *Salmonella* in comparison to the control (125 mM) (Fig. 1). The antimicrobial properties of a micelle depends on its availability in the aqueous phase. Emulsions of essential oils generated using surfactants such as Tween 80 or Triton X100, which are non-ionic in nature, had lower antimicrobial efficacy against *Salmonella Enteritidis, Escherichia coli O157:H7*, and *Listeria monocytogenes* than the unmodified essential oils. The current study determined that PA emulsions formed using Tween 80 and Triton X100 had lower ($p < 0.05$) inhibitory activity against all three *Salmonella* serotypes, in comparison to Quillaja saponin and SDS (Fig. 1). It was observed that surfactant type and concentration significantly affected both, inhibitory and bactericidal activities of PA ($p < 0.05$). The concentration of surfactant at which its molecules aggregate to form micelles is known as the critical micelle point (CMC). The CMC of SDS is 2.1 g/l, Tween 80 is 0.013 g/l, Triton X100 is 0.15 g/l and Quillaja saponin is 0.8 g/l. Surfactants used at 1% were all above the CMC and could have contributed to the antimicrobial activity of micelles. Tween 80, Triton X100 and Quillaja saponin exceeded the CMC at 0.1%.

Among the surfactants tested, SDS based emulsions had the largest PA micelles, as well as a high level of variation in the size of the colloidal particles generated (as denoted by the polydispersity index) compared to emulsions generated using other surfactants.

*S. Newport* was generally more sensitive to PA emulsions than serotypes Typhimurium and Oranienburg ($p < 0.05$). Differences in the sensitivity of *Salmonella* serotypes to antimicrobial compounds can occur and can result in higher incidences of specific serotypes in certain food commodities. For instance, *S. Oranienburg* has been isolated from dry spices and has a higher tolerance to natural antimicrobial compounds in spices.
Pelargonic acid exposure to bacterial cells at lethal concentrations results in cell membrane damage and lysis\textsuperscript{11}, similar to bacterial cells exposed to carvacrol, the active component of Oregano oil\textsuperscript{11,13}. Analysis of growth curves of the *Salmonella* serotypes exposed to MIC of PA emulsions generated using 1% SDS and Quillaja saponin respectively, resulted in a reduction in growth rate and an increase in lag phase duration (Fig. 3). The formation of bacterial filaments, which are elongated structures containing many cells that remain attached due to a block in the divisome, could contribute to the reduction in growth rate as filaments are detected as a single colony during plating on bacteriological media\textsuperscript{19,38}. Microscopy indicated that while cells of all three serotypes formed filaments when exposed to PA emulsions, the ratios of filaments to regular cells were different (Table 2). Further, S. Typhimurium and S. Newport cells formed free-floating aggregates of floccular biofilms, whereas S. Oranienburg did not, indicating that stress response to PA varied among *Salmonella* serotypes. Changes in bacterial morphology among *Salmonella* serotypes occurred both during the use of Quillaja saponin and SDS as surfactants for the formation of PA emulsions. S. Oranienburg cells formed filaments that were longer than 5 µm and several filaments exceeded 100 µm in length (Fig. 4). Filaments are transient phenotypes that contain chains of cells connected one to another due to incomplete cell division\textsuperscript{39}. Filaments disintegrate when cell division is completed and result in spontaneous release of many individual cells\textsuperscript{40,41}. S. Typhimurium and S. Newport also formed filaments, but they occurred less frequently and were usually shorter (30 µm) than S. Oranienburg filaments (100 µm) (Table 2, Figs. 4, 7). Apart from filaments, S. Typhimurium and less frequently S. Newport formed aggregates that were encased in EPS (Figs. 5, 6). Autoaggregation of cells is an important precursor to biofilm formation\textsuperscript{42,43}. The free-floating aggregates of S. Typhimurium and S. Newport were encased in a dense polymeric matrix that was observed through fluorescent staining and the use of LSCM (Figs. 5, 6). The formation of floccular biofilms as well as filamentation suggest another, a less explored bacterial strategy to mitigate antimicrobial exposure (Fig. 6)\textsuperscript{40}. Filaments formed by S. Newport, the serotype most susceptible to PA, often appeared to be disfigured indicating possible structural damage to the cells (Fig. 3). All three serotypes displayed phenotypic diversity by forming filaments (Figs. 4, 5, 7), floccular biofilms and regular size cells when exposed to PA indicating that the formation of diverse phenotypes might be a strategy employed by *Salmonella* to overcome stressors such as sanitizer exposure\textsuperscript{38}.

Emulsions have many applications in the food industry and can be used in antimicrobial formulations and coatings\textsuperscript{41–44}. Antimicrobial emulsions can improve the safety of foods, feeds and food contact surfaces. PA emulsions (31.2 mM) generated using 1% SDS resulted in a 4.7 log CFU/ml reduction of the *Salmonella* cocktail containing all three serotypes in 30 s and 6.8 log CFU/ml in 5 min (Figs. 8, 9). A similar reduction of *Salmonella* serotypes was observed using a PA emulsion generated using Quillaja saponin after 5 min but not at 30 s, indicating that the type of surfactant selected might also effect the time taken for the interaction between the fatty acid and the target pathogen.

Conclusions

The current study demonstrates the antimicrobial activity of PA against *Salmonella* and for the first time reveals that surfactant type plays an important role in influencing the antimicrobial efficacy of PA emulsions. Further, the observation that *Salmonella* employs multiple stress responses among a clonal population of cells when challenged with an antimicrobial, indicates that phenotypic diversity may play an important role in antimicrobial resistance. This new information will help industry better evaluate their choices of antimicrobials, as inhibitory concentrations of certain sanitizers could vary greatly from bactericidal concentrations, depending on the type of surfactant and surfactant concentration chosen. The finding that the use of surfactants can reduce the amount of PA required for inhibitory activity against *Salmonella* by 50% or greater, could help in reducing the environmental footprint, the cost associated with sanitizer production and the development of alternatives to biocides currently used by the food industry.

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Competing interests

The authors declare no competing interests.

Additional information

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