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# Characterizing the Antibacterial Properties and Transcriptional Alterations Induced by Lemongrass oil in *Staphylococcus aureus*

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**Essential oils have risen in popularity as "all natural" alternatives used to treat a myriad of conditions. To begin to elucidate the antibacterial properties of essential oils, we tested the effectiveness of lemongrass oil (LGO), tea tree oil (TTO), and willow bark extract (WBE) against *Staphylococcus aureus* growth. To do so, a Methicillin-Resistant strain of *Staphylococcus aureus* (USA300) was exposed to each oil using disk diffusion assays. Of the oils, LGO had the greatest zone of inhibition. The Minimum Inhibitory Concentration (MIC) of both LGO and citral (the primary chemical component of LGO) was determined in macro-broth cultures; exposure to increased concentrations of each resulted in dramatic cell death as determined by cell growth assays. To begin to determine the molecular mechanisms underlying the observed antibacterial effects, we exposed cells to a sub-inhibitory concentration of citral and hybridized the RNA to Affymetrix GeneChips®. Transcripts differentially affected in citral- versus mock-treated cells represent virulence factors, hypothetical proteins, and intergenic regions. Taken together, these results demonstrate that LGO exhibits antibacterial properties against a highly pathogenic bacterial species that is exceedingly resistant to the currently available antibiotics.**

The use of essential oils (EOs) to promote health and wellness is increasing in popularity as consumers seek to utilize more "natural" means to stay healthy and treat disease. Consumers are inhaling these oils through the use of in-home diffusers and applying the oils cutaneously to treat a variety of ailments from anxiety to wounds. Several studies have indicated the effectiveness of essential oils against a wide variety of microbial agents (1,2). More specifically, naturally occurring compounds, including EOs, inhibited the growth of *Mycobacterium avium* subsp. p*aratuberculosis* in macro-broth growth conditions (3). Moreover, practical application of EOs as antibacterials has been suggested for use in food preparation, preservation, and packaging (4), as cleaners for athletic equipment (5), and in wound treatment (6.). The precise mechanisms of the antibacterial activities of EOs are beginning to be elucidated at both the cellular and molecular levels (7,8). Those data indicate that EOs possess both bacterio-static and –cidal activity as well as affect transcription of genes involved in a variety of stress responses and essential cellular functions (7).

*Staphylococcus aureus* is a common cause of hospital- and community-acquired bacterial infections ranging in severity from superficial skin lesions to invasive infections of the heart and bones (9). In fact, the Centers for Disease Control and Prevention estimates that more people die from invasive *S. aureus* infections (mortality rate of 6.3 per 100,000) than HIV/AIDS each year (mortality rate of 2.1 per 100,000) (10). Compounding the seriousness of these infections, *S. aureus* is highly resistant to currently available antibiotics making infections increasingly difficult to treat (10,11). The current study aimed to identify the antibacterial properties of essential oils in a clinically-relevant, methicillin-resistant strain of *S. aureus* (MRSA). To do so, disk diffusion assays were performed with EOs. Lemongrass oil (LGO) demonstrated the most potency against *S. aureus* and was the focus of this study. Bacterial growth characteristics were determined in the presence of LGO and its primary chemical component, citral. In addition, molecular targets of lemongrass oil were identified via Affymetrix GeneChip® analysis. Results demonstrated that LGO may represent a novel therapeutic agent for treatment of MRSA by inhibiting bacterial growth. Moreover, LGO may target the expression of virulence factors and additional proteins at

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the molecular level which may, in turn, explain the mechanism of growth inhibition.

## MATERIALS AND METHODS

**Bacterial strain and growth conditions.** USA300, a community-acquired, methicillin-resistant strain of *Staphylococcus aureus*, has been previously described (36). Bacteria were grown in Tryptic Soy Broth (TSB) or Agar (TSA; Carolina Biological; Burlington, NC) at 37°C unless otherwise noted.

**Disk Diffusion Assays.** Lawns of USA300 were swabbed onto Mueller Hinton (Carolina Biological) agar plates from overnight cultures. Ten µl of serially-diluted Lysol (control), Lemongrass oil, Tea Tree oil, or Willow Bark Extract (all essential oils used were Aura Cacia brand purchased from Whole Foods; Ann Arbor, MI) were pipetted onto 6-millimeter (mm) filter paper disks. Disks were placed on agar and plates were incubated at 37°C for 24 hours. Zones of inhibition were measured in mm. The average and standard deviation of three independent experiments are represented in Table 1.

**Minimum Inhibitory Concentration determination.** Minimum Inhibitory Concentrations (MIC) of lemongrass oil and its primary chemical component citral (Sigma Aldrich; St. Louis, MO) were determined using standard macro-broth dilution assays with modification (37,38). Briefly, the optical density (OD; $A_{600}$) of overnight cultures of USA300 was measured. Cells were diluted to an OD of 0.5 in Mueller Hinton Broth supplemented with 5% Tween-80 (13,34); Sigma Aldrich; St. Louis, MO] as an emulsifier. 5 milliliters (ml) of culture was pipetted into glass test tubes and serially-diluted LGO or citral was added to each. ODs were measured following 24 hours of growth at 37°C with shaking. The MICs were defined as the lowest concentration that inhibited growth as measured by OD. All tests were performed in triplicate.

**Growth- and kill-curve analysis.** Overnight cultures of USA300 were diluted 1:100 into TSB supplemented with 5% Tween-80 and grown to mid-exponential phase (OD=0.3; T0) followed by the addition of LGO or citral at varying concentrations. For growth curves, the OD was measured at T0, T30 min, T1 hr, T2 hr, T3 hr, and T4 hr. For kill curves, colony forming units were enumerated at the same time points following serial dilution in 0.8% NaCl and growth on TSA plates at 37°C for 24 hr. Experiments were repeated in triplicate and data was statistically analyzed via t-test (p=0.05).

**GeneChip® Analysis.** Overnight cultures of USA300 were diluted 1:100 into TSB supplemented with 5% Tween-80 and grown to mid-exponential phase (OD=0.3; T0) followed by the addition of a sub-inhibitory concentration of citral (0.5X MIC) for 30 min. Twenty ml of either mock- or citral-treated cells were then added to an equal volume of ice-cold acetone:ethanol (1:1) solution and stored at -80°C until RNA isolation. RNA was isolated and prepared for GeneChip® analysis as previously described (39). Briefly, aliquots of cells were pelleted by centrifugation at 3000 RPM for 10 min at 4°C. Cell pellets were washed twice in TE buffer and disrupted in a FastPrep120 shaker (MP Biomedical; Santa Ana, CA). Cell debris was collected by centrifugation and supernatants were used for RNA isolation with Qiagen RNeasy® Mini Kits (Valencia, CA) following manufacturer's recommendations. RNA concentrations were measured spectrophotometrically at $OD_{260}$. Ten micrograms (□g) of total RNA was labeled and hybridized to a *S. aureus* GeneChip® following manufacturer's recommendations for antisense prokaryotic arrays (Affymetrix; Santa Clara, CA). RNA was reverse-transcribed and cDNA was purified using Qiagen PCR Clean-Up kits, fragmented by DNase I (Amersham BioSciences; Pittsburgh, PA) and 3' end-labeled with biotin using the Enzo Bioarray Terminal Labeling Kit (Enzo Life Sciences; Farmingdale, NY). A total of 1.5 µg of labeled cDNA was hybridized to an Affymetrix *S. aureus* Genechip®, washed, stained, and scanned as previously described (39). Commercially available GeneChips® were used in this study representing > 3,300 *S. aureus* open reading frames (ORF) and >4,800 intergenic regions from strains N315, Mu50, NCTC 8325, and COL (Affymetrix). GeneChip® signal intensity values for each ORF and intergenic region at each replicate time point (n ≥ 2) were averaged and normalized to controls using GeneSpring software (Agilent Technologies; Redwood City, CA). A comparison of signal intensity values with mock-treated samples allowed identification of transcripts that increased or decreased ≥ 2-fold in citral-treated cells. GeneChips® were processed in duplicate for each sample (mock- and citral-treated) from two independent experiments. Data was statistically analyzed via t-test (p=0.05).

## RESULTS

**Methicillin Resistant *S. aureus* is susceptible to treatment with essential oils.** Disk diffusion assays were performed to determine whether lemongrass oil (LGO), willow bark extract (WBE), and/or tea tree oil (TTO) could inhibit the growth of *S. aureus* strain USA300. Lawns of USA300 were swabbed onto Mueller Hinton Agar plates followed by the addition of varying concentrations of essential oils or Lysol® (control). Zones of inhibition were measured and compared to zones produced by Lysol®, an Environmental Protection Agency-registered disinfectant and known anti-staphylococcal agent (12). Though we cannot rule out the possibility that other commercial brands of essential oils may produce different results, the experiments presented here were conducted with Aura Cacia brand EOs purchased from Whole Foods. As seen in Table 1, results indicated that lemongrass oil produced a zone of inhibition 40% larger than the control at full potency. Lysol produced zones of inhibition at all concentrations tested whereas LGO was only effective at 100, 50, and 25% potency (Table 1). Tea tree oil was moderately effective producing a zone size that was reduced by 18% compared to the control at full potency (Table 1). Willow bark extract was not able to inhibit USA300 at any concentration tested (Table 1). Due to the increased size of the zone of inhibition produced by LGO, all further experiments were conducted using only this oil and the purified chemical component of LGO, citral (13,14).

**Optical density and cell viability are inhibited by lemongrass oil and citral.** To begin to elucidate the mechanism by which LGO inhibits staphylococcal growth, we conducted growth and kill curves with both LGO and citral. The Minimum Inhibitory Concentration (MIC) of both LGO and citral was determined to be 0.04% (v/v; data

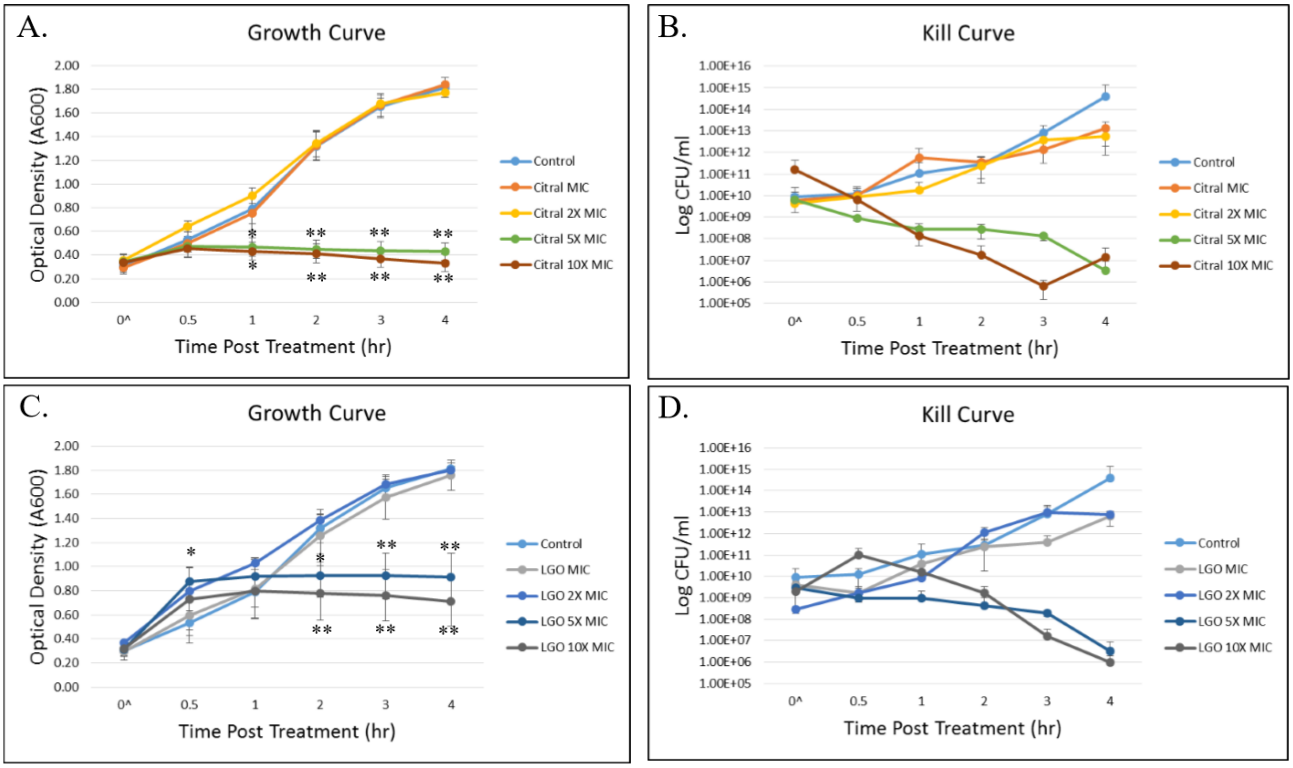


**FIG. 1 Citral and LGO inhibit growth of USA300 cells.** USA300 cells were grown to mid-log phase ($OD_{600}$=0.3) and then were either mock-treated (control), treated with the MIC of Citral (A, B) or LGO (C, D), or increasing concentrations of each. Citral or LGO was added at 0^. Optical density (A, C), and colony forming units (cfu/ml, B, D) were measured at indicated time points (hr). Data represents the average of 3 independent experiments; standard deviation is represented by the error bars at each data point. *p≤0.05; **p≤0.01

not shown) via macro-broth dilution method of MIC determination. As shown in Figure 1, cells were grown to mid-exponential phase ($OD_{600}$=0.3) and were then exposed to increasing concentrations of citral (MIC, 2X, 5X, or 10X MIC). Growth was measured spectrophotometrically and the optical density demonstrated that cell growth was significantly inhibited by citral at 5X and 10X MIC (Fig. 1A). Measurement of colony forming units of cells exposed to increasing concentrations of citral indicated a decrease in viability (Fig. 1B). Similar results were observed for cells exposed to increasing concentrations of LGO for both growth (Fig. 1C) and viability (Fig. 1D). Results were generated from 3 independent experiments. Data was statistically analyzed via a t-test (p=0.05) comparing each data point for cells treated with lemongrass oil or citral to those of the control.

**Citral exposure both up- and down-regulates transcription.** To better understand the underlying molecular mechanisms of LGO and citral's abilities to inhibit staphylococcal growth, Affymetrix GeneChips® were used to determine which transcripts were up- or down-regulated in response to citral treatment. To do so, mid-exponential phase cells were exposed to a sub-inhibitory concentration of citral (0.5X MIC) for 30 min at which point cell growth was arrested followed by total RNA isolation and hybridization to microarrays. GeneChips® were processed in duplicate for each sample and data was statistically analyzed via t-test (p=0.05). As seen in Table 2, nine transcripts were down-regulated (at least 2-fold) when compared to mock-treated cells. Of these, at least 1 transcript is a known virulence factor (*efb*), 3 are hypothetical proteins, and 3 are intergenic regions. As seen in Table 3, eight transcripts were upregulated (at least 2-fold) when compared to mock-treated cells. Of these, 1 transcript is an ABC transporter, 1 a transcriptional regulator (TetR family), 2 hypothetical proteins, and 1 intergenic region. One of the upregulated hypothetical proteins represents the transcript encoding VraX protein. The function of VraX is as yet unknown but its upregulation has been shown to occur in other essential oil-exposed cells (see below).

## DISCUSSION

Previously, we demonstrated the effectiveness of essential oils alone and in combination at inhibiting staphylococcal growth (5). The strain used in that study was an attenuated, laboratory strain of the bacteria. The strain used in the current study is a community-acquired, methicillin-resistant isolate of *S. aureus*. We wanted to determine if the effect we previously measured with the weakened strain could be replicated

**Table 1. Zones of Inhibition measured (mm) in response to Lysol (control) or Essential Oils***

| | 100.00% | 50.00% | 25.00% | 12.50% | 6.25% | 3.13% | 1.56% | 0.78% | 0.39% |
|---|---|---|---|---|---|---|---|---|---|
| **Lysol** | 20.33±1.53 | 21.33±1.15 | 20.33±2.52 | 16.33±0.58 | 14±1 | 12±1 | 10.33±1.53 | 9.33±0.58 | 8.33±0.58 |
| **Lemongrass Oil** | 28.33±1.53 | 16±5.29 | 9.33±2.08 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Tea Tree Oil** | 16.67±3.06 | 17±3.46 | 5.33±4.62 | 8±0 | 8±0 | 8±0 | 8±0 | 8±0 | 5.33±4.62 |
| **Willow Bark Extract** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*Measurements represent the average and standard deviation of three replicates

in a clinically-relevant strain of the same species. Indeed, we measured susceptibility to LGO and TTO in USA300 (Table 1). Due to the large size of the zone of inhibition produced by LGO, we performed all other experiments with this essential oil or its primary chemical component, citral.

To begin to understand the underlying antibacterial mechanisms of LGO, growth and kill curve assays were performed following MIC determination for both LGO and citral. Interestingly, LGO and citral exhibited the same MIC against USA300. This result is similar to what has previously been demonstrated for the inhibitory action of these chemicals against *S. aureus*; MICs for LGO and citral were within .01 µl of each other (13.). LGO or citral was added with increasing concentrations (MIC, 2X, 5X, and 10X MIC) to mid-exponentially growing cells. Results demonstrated that within 1 hour of treatment with citral and 2 hour treatment with LGO cell growth was arrested in cells treated with 5X and 10X MIC (Fig. 1A,C), whereas cell growth mirrored untreated cells for those exposed to MIC or 2X MIC LGO or citral. To understand the mechanism of cell growth interference, kill curve assays were performed to enumerate colony forming units following exposure to the compounds. Arrests in cell growth can be explained by a loss of cell viability as demonstrated by a drop in colony forming units at 1 hour post- citral and 2 hours post-LGO exposure in 5X and 10X MIC treated cells (Fig. 1B, D). Based on these results, it can be concluded that LGO and citral affect *S. aureus* through a bactericidal mechanism.

Lemongrass oil is an essential oil made from *Cymbopogon citratus*, and it is widely used in perfumes and fragranced cleaning products. Studies have shown that this essential oil, which is composed mainly of monoterpene aldehydes, has antibacterial and antifungal properties (15,16). Additional studies have demonstrated the antibacterial action of monoterpenes (17). Due to the hydrophobicity of these compounds, it has been hypothesized that their action involves disruption of the lipids within the plasma membrane. Likewise, LGO and citral are highly hydrophobic hence the necessity of using Tween-80 as an emulsifier in the present study. It is possible that LGO and citral interfere with cell viability by affecting the integrity of the plasma membrane.

Transcriptional profiling identified 9 transcripts that were down-regulated (Table 2) at least 2-fold in citral-treated as compared to mock-treated cells. Conversely, 8 transcripts were up-regulated (Table 3) in response to citral treatment. Though the precise mechanism underlying the bactericidal effect observed here is unknown, previous reports have shown essential oils to inhibit virulence factors thus weakening the disease-causing potential of these organisms (18,22). Indeed, our transcriptional profiling data indicate that the expression of virulence factors, including Efb, AgrC, and a TetR transcriptional regulator, is modulated by exposure to citral (Tables 2 and 3). Of the 8 transcripts that were upregulated, 2 have been previously shown to be upregulated in *S. aureus* cells treated with tea tree oil (7). One of these, a transcript from the short chain dehydrogenase/reductase family (locus SACOL2594) was upregulated 2.4 fold in our cells and 10.7 fold in the TTO-treated cells suggesting that terpene-based essential oils may play a role in regulating enzymatic activity within *S. aureus* cells (19) or the microbe may upregulate this redox enzyme in order to metabolize the essential oil (20). Notably, the gene encoding the VraX protein (locus SACOL0625) increased 2.3-fold in our cells and 39 fold in the TTO cells (7). *vraX* encodes the Vancomycin-Resistance Associated gene and has been implicated in the response to cell wall stress (21) and may be up-regulated by cell wall and plasma membrane targeting compounds. The lipophilic nature of essential oils could explain the increase in expression of both of these transcripts as follows: in response to essential oil exposure, the cells induced a stress response mediated by *vraX* to combat cell membrane damage/stress which, in turn, affected the oxidoreductases which are themselves embedded in the plasma membrane.

Treatment options for infections caused by USA300 are limited as this bacterium is a multi-drug resistant strain of *S. aureus*. However, a previous study investigating the growth characteristics of *Listeria monocytogenes* and *S. aureus* exposed to eugenol and

**Table 2. Fold Decrease in Gene Expression of Citral-Treated Cells Compared to Mock-Treated**

| Fold Decrease in Citral-Treated Cells | Locus | Gene | Description |
|---|---|---|---|
| -2.59 | SAS076 | *efbC* | fibrinogen-binding protein |
| -2.5 | SACOL0768 | | conserved hypothetical protein |
| -2.27 | SACOL0767 | | conserved hypothetical protein |
| -2.26* | SACOL0480 | | conserved hypothetical protein |
| -2.07 | SAR2036 | | chemotaxis-inhibiting protein |
| -2.23* | SA2002 | *map* | map protein, authentic frameshift |
| -2.60* | Intergenic | | intergenic upstream of ORF sa_c6262s5443 |
| -2.97 | Intergenic | | intergenic upstream of ORF sa_c8934s7849 |
| -2.11* | Intergenic | | intergenic upstream of ORF sa_c1011s797 |

p≤0.05

**Table 3. Fold Increase in Gene Expression of Citral-Treated Cells Compared to Mock-Treated**

| Fold Increase in Citral-Treated Cells | Locus | Gene | Gene Description |
|---|---|---|---|
| +2.56 | SACOL2593 | | transcriptional regulator, TetR family |
| +2.06 | SACOL2356 | | Hemin ABC transporter, ATP-binding protein |
| +2.30 | SACOL2591 | | NmrA (trancriptional regulator)/HSCARG (NADPH sensor) family protein |
| +2.37* | SACOL2594 | | short chain dehydrogenase |
| +2.33 | SACOL0625 | *vraX* | conserved hypothetical protein; vancomycin-resistance associated protein |
| +2.19* | SACOL0063 | | conserved hypothetical protein; dihydroneopterin aldolase |
| +2.28* | SA2421 | *agrC* | accessory gene regulator protein C |
| +3.18 | Intergenic | | reverse complement of intergenic downstream of ORF sa_c10252s10668 |

p≤0.05

citral, the main components of clove and citrus essential oils, respectively, showed that resistance to the oils did not develop over time (23). EOs have also been shown to work synergistically with traditional antibiotics by reversing bacterial resistance (24,25,26) through several mechanisms including efflux inhibition (27,28) and quorum sensing interference (29). On the contrary, other studies have demonstrated reduced antibiotic susceptibility of pathogens, including MRSA, that were continuously exposed to a sub-inhibitory concentration of tea tree oil (30,31). This decrease in susceptibility may pose a risk to human health and treatment of infection as bacteria are continually exposed to sub-inhibitory concentrations of EOs in cosmetics, cleaning products, and other commercial products (32).

The data presented here supports previous studies that have demonstrated the effectiveness of lemongrass oil at inhibiting a variety of bacterial species including *S. aureus* (5,13,33-35). The data presented here further those studies to show the bacteriocidal effect high concentrations of LGO and its primary chemical component citral have on *S. aureus.* The data from our transcriptional profiling study may provide some insight into the mechanism by which these compounds interfere with cellular viability. Further studies are needed to understand the precise molecular mechanisms and to determine if lemongrass oil could be translated into a novel therapeutic to be used either alone or in combination with traditional antibiotics.


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