Major components of orange oil inhibit *Staphylococcus aureus* growth and biofilm formation, and alter its virulence factors

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INTRODUCTION

Bovine mastitis is inflammation of the udder tissue in at least one quarter and is usually caused by a bacterial pathogen (Contreras & Rodríquez, 2011; Wilson et al., 1997). Treatment for bovine mastitis can be expensive depending on the type of infection. On a national level, the USA dairy industry loses $2 billion in costs associated with mastitis each year (National Mastitis Council, 1996). On a farm level, the cost per a cow can vary depending on severity and chronicity of infection. Clinical infections present with symptoms of localized and systemic inflammation, including swelling and redness of one or more udder quarters, depression, anorexia, fever and reduced milk quality and production. Clinical cases of mastitis cost $179 per cow (Bar et al., 2008). However, subclinical cases are more prevalent (Barlow, 2011; Haveri et al., 2005). Subclinical cases may only present with reduced milk quality and production, as well as an elevated somatic cell count (SCC). With a medium SCC, subclinical cases of mastitis cost $108 per cow (Ott, 1999). However, subclinical cases with high SCC can cost dairy producers $295.24 per cow (Ott, 1999). In addition to economic losses, mastitis can have a long-term impact on the health of dairy cattle. The bacterial infections can cause permanent damage to the mammary tissue, which reduces milk production and quality for a lifetime and decreased reproductive efficiency (Harmon, 1994).

The most common causative agent of mastitis is *Staphylococcus aureus* (Contreras & Rodríquez, 2011; Makovec & Ruegg, 2003; Reyher et al., 2012; Wilson et al., 1997). It is an infectious bacterial pathogen, which is usually spread between cows during the milking process. The most common form of treatment is antibiotic therapy with pirlimycin. However, antibiotics often fail to cure *S. aureus*-associated mastitis, particularly in subclinical and chronic cases (Barkema et al., 2006). This, combined with pressure from the FDA as well as consumers to reduce antibiotic usage, makes it urgent to search for alternatives in the treatment of mastitis.

Essential oils have been studied for their antimicrobial properties (Kim et al., 1995; Lis-Balchin & Deans, 1997;
Smith-Palmer et al., 1998; Alzoreky & Nakahara, 2003). One commonly studied essential oil is orange/citrus oil. Orange oil has been demonstrated to inhibit methicillin-resistant S. aureus (MRSA) growth (Muthaiyan et al., 2012). Since orange oil is generally recognized as a safe product, the FDA allows its use in human and animal feed. In addition, it can be cheaply extracted, making it a cost-effective antimicrobial product. However, orange oil contains many compounds not all of which display antimicrobial properties. The major components of terpenes, cold-pressed Valencia orange oil are 20.2 % linalool, 18.0 % decanal, 14.1 % citral, 5.8 % α-terpineol, 5.2 % valencene, 4.1 % dodecanal, 3.9 % citronellal and 0.3 % limonene (Nannapaneni et al., 2009). Both linalool and decanal have demonstrated inhibition of S. aureus growth (Liu et al., 2012). Citral has been studied for its ability to inhibit a wide range of bacterial pathogens, such as S. aureus, as well as pathogenic fungi (Saddiq et al., 2010). As a result, these compounds are of interest as antibiotic alternatives.

In this study, we aimed to investigate the potential role of the most prevalent components of cold-pressed, terpeneless Valencia orange oil, linalool, decanal, citral, and valencene as an antibiotic alternative in the treatment of S. aureus-associated bovine mastitis. Their interaction with S. aureus and bovine mammary epithelial (MAC-T) cells was examined.

METHODS

Bacterial strains and growth conditions. S. aureus strain ATCC 29740, isolated from mastitic milk, was used for this study. S. aureus was grown and maintained on nutrient agar or in nutrient broth (Gibco). Prior to each experiment, S. aureus from the frozen (~80°C) stock was sub-cultured on nutrient agar plates and grown for 18–24 h at 37°C.

Preparation of stock solution orange oil components. Citral, linalool, decanal and valencene (Sigma-Aldrich) were used for this study. A 10% stock solution for these components in 1% Tween 80 in distilled water was used for all experiments.

Determination of minimum inhibitory concentrations. Minimum inhibitory concentration (MIC) was determined using the broth dilution method described by Muthaiyan et al. (2012). Concentrations of citral, linalool, decanal and valencene ranging from 0.01% to 2.5% were tested for antimicrobial activity against S. aureus. These concentrations were added to 10⁵ c.f.u. ml⁻¹ S. aureus in a 24-well plate (Greinerbio-one). Plates were incubated overnight at 37°C, and visually inspected for growth inhibition of S. aureus to determine the MIC.

Growth inhibition assay. These methods were adapted from Yang et al. (2014). S. aureus was diluted to 10⁵ c.f.u. ml⁻¹ in LB or nutrient broth (Difco) after being adjusted to OD₆₀₀ ~ 0.08–0.12, which is equal to 10⁵ c.f.u. ml⁻¹. The LB or nutrient broth was either supplemented with citral (0.02 or 0.04 %) or linalool (0.12 or 0.24 %). A control was used, which contained unsupplemented LB or nutrient broth. Cultures were incubated for 72 h at 37°C in cell culture tubes, and enumeration took place every 24 h. If no bacteria were detectable after 24 h for any concentration, the experiment was repeated except with incubation for 3 h and enumeration occurring every 1 h.

Inhibition of pre-formed biofilms. Methods were adapted from literature previously described by Karolis et al. (2005), O’Toole (2011) and Ma et al. (2012). S. aureus was adjusted to 10⁶ c.f.u. ml⁻¹ by diluting to OD₆₅₀ ~ 0.08–0.12. This was then further diluted to 10⁷ c.f.u. ml⁻¹. Aliquots of 200 µl were added into each well of a 96-well polystyrene flat-bottom microtitre plate (Greinerbio-one), and plates were incubated for 24 h at 37°C. Supernatants of the 24-well plate were then removed and discarded, and the biofilms were then treated with unsupplemented nutrient broth or nutrient broth supplemented with either citral (0.02, 0.04 or 0.08 %) or linalool (0.12, 0.24 or 0.48 %). These plates were sealed with Parafilm (Sigma-Aldrich) to prevent evaporation of citral and linalool and incubated for 24 h at 37°C. Supernatants were shaken out, and the 96-well plates were rinsed by being submerged in water. Plates were rinsed three times, with water being shaken out after each rinse. A 200 µl aliquot of 0.1% crystal violet was added to each biofilm and incubated for 10–15 min at room temperature. The plates were then rinsed again three times, with water being shaken out after each rinse, and allowed to dry overnight. To each well, 200 µl of 30% acetic acid in distilled water was added and the plates were shaken for 1 h, before the acetic acid solution was removed to a new plate and the absorbance of the solution was read at 540 nm using a spectrometer (Thermo Scientific).

Mammalian cell culture and monolayer formation. The MAC-T cell line is an immortalized bovine mammary gland epithelial cell line and was used for all cell culture work. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 100 µg gentamicin ml⁻¹ in 75 cm² cell culture flasks incubated at 37°C overnight in a 5% CO₂ incubator.

Detection of cytotoxicity on MAC-T cells. Methods were adapted from Mubarak et al. (2015) and Youselzadi et al. (2011) and used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for determining cytotoxicity. Aliquots of 200 µl of a 2 × 10⁴ MAC-T cell suspension were added to each well of a 96-well plate and incubated for 24 h at 32°C in a 5% CO₂ incubator. The media were removed and the cells washed three times with DMEM. Cells were then treated with DMEM supplemented with 10% FBS, 100 µg ml⁻¹ gentamicin and either citral (0.02, 0.04 or 0.08 %) or linalool (0.12, 0.24 or 0.48 %). A control of only DMEM supplemented with 10% FBS and 100 µg ml⁻¹ gentamicin was included. The cells were incubated again for 1 h at 32°C in a 5% CO₂ incubator, after which the media were removed and cells washed three times with DMEM. Cells were then incubated with 200 µl DMEM supplemented with 10% FBS and 100 µg ml⁻¹ gentamicin, and 20 µl of 5 mg ml⁻¹ MTT, reconstituted in 1× PBS for 2 h at 37°C in a 5% CO₂ incubator. The media were then removed. The 96-well plate was shaken for 10–15 min. The absorbance was read at 492 nm on a spectrometer with a blank of 100% DMSO.

S. aureus association and invasion assay for MAC-T cells. The methods of Biswas et al. (2000) and Salahsheen et al. (2014a) were adapted for this assay. Aliquots of 500 µl of a suspension of 2 × 10⁴ MAC-T cells ml⁻¹ in DMEM supplemented with 10% FBS were added to a 24-well plate and incubated for 18–24 h at 37°C in 5% CO₂ incubator. The cells were washed three times with DMEM, then 1 ml DMEM supplemented with 10% FBS was added to the cells, and 10 µl of 10⁶ c.f.u. ml⁻¹ S. aureus, for a final concentration of 10⁵ c.f.u. ml⁻¹ S. aureus, was used to infect the cells. The cells were incubated at 37°C in a 5% CO₂ incubator for 1 h. Afterwards, cells were washed three times with DMEM and treated with DMEM supplemented with 10% FBS and either 0.02% citral, 0.04% citral or 0.12% linalool. A control of only DMEM supplemented with 10% FBS was used. The cells were incubated again for 1 h at 37°C in a 5% CO₂ incubator and were then washed three times with DMEM. To enumerate associated bacteria, cells were incubated with 0.1% Triton X-100 (Sigma) for 15 min at 37°C in a 5% CO₂ incubator, and the associated bacteria were plated on LB agar to count colonies. To enumerate invaded bacteria, cells were first treated with DMEM supplemented with 10% FBS and 100 µg ml⁻¹ gentamicin for 1 h at 37°C in a 5% CO₂ incubator. Afterwards,
cells were incubated with 0.1 % Triton X-100) for 15 min at 37°C in a 5 % CO₂ incubator, and the invaded bacteria were plated on LB agar to count colonies.

**Effect on gene expression of S. aureus virulence and biofilm genes.** For expression of S. aureus virulence genes, cells were infected and treated in a similar fashion to the association and invasion assay, but scaled up to 6 ml in 25 cm² flasks. RNA was extracted using methods described in Ahn et al. (2014), and a NanoDrop ND-1000 was used to measure the concentration of the extracted RNA. One microgram of RNA was used to create cDNA using the instructions provided in the Verso cDNA Synthesis kit (Thermo Scientific). All quantitative PCR used the methods described in Ahn et al. (2014) and Salaheen et al. (2014b), except that cDNA was diluted 10-fold. The virulence genes measured were *agrA*, *sodA*, *sirA*, *hla*, *hil*, *spa*, *sae*, *sarA*, *sarB*, *sarS* and *sigB*, and the reference gene used was the 16S rRNA gene. The genes and primers used are listed in Table 1.

To determine expression of S. aureus biofilm genes, S. aureus was formed and treated in the same way as in the inhibition of pre-formed biofilms assay. However, treatments only included an LB broth control, 0.02 % citral, 0.04 % citral and 0.12 % linalool. After 24 h of treatment, RNA extraction, cDNA synthesis and qPCR were carried out using the same methods described for the virulence genes above, except cDNA synthesis used qScript cDNA SuperMix (Quanta Biosciences). The biofilm genes measured were *icaA*, *icaB*, *icaC*, *icaD*, *fnbA*, *fnbB*, *clfA*, *clfB* and *ebpS*, and the 16S rRNA gene was used as a reference. All genes and primers used are listed in Table 1.

**Statistical analysis.** Statistical significance between treatments was determined by two-way ANOVA analysis and Tukey’s HSD, using the general linear model in Statistical Analysis System (SAS). When P<0.05, mean differences were considered statistically significant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Reference</th>
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<tr>
<td><em>agrA</em></td>
<td>CAAGAGAAAACATGTGTTACCATTATAA</td>
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<td><em>sodA</em></td>
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<td>CGCCATTTTCTGATTATGTTAAC</td>
<td>Ster et al. (2005)</td>
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<tr>
<td><em>hil</em></td>
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<td>TTTGCCACCTGTTAGTGAAGG</td>
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<td><em>spa</em></td>
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<td>GCATCACTTTTCTGAGTATGAG</td>
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<td><em>sbi</em></td>
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<tr>
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<td><em>sigB</em></td>
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<td><em>icaC</em></td>
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<td><em>icaD</em></td>
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<tr>
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<td><em>clfA</em></td>
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<td>CCGATGCCTGTCAGGCTGAG</td>
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</tr>
<tr>
<td><em>ebpS</em></td>
<td>GGTTGACGGCTTGTAATGGGTGT</td>
<td>GCGGCTGACCCGACACCT</td>
<td>Atshan et al. (2013)</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>TATGGAAGGAAACACCTGGCAAG</td>
<td>TCATCGTTAAGGCCGTTGACAT</td>
<td>Ster et al. (2005)</td>
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</table>

**RESULTS**

**MICs of citral, linalool, decanal and valencene required to inhibit S. aureus**

The MICs of citral, linalool, decanal and valencene are shown in Table 2. Only citral and linalool were found to have an inhibitory effect on S. aureus. The MICs for citral and linalool were 0.02 % and 0.12 %, respectively. A concentration of 2.5 % was not even sufficient to inhibit growth of S. aureus for either decanal or valencene. Due to higher MIC values and cytotoxic effects, decanal and valencene were excluded from the remainder of the study.

**Time-dependent inhibitory effects of citral and linalool on S. aureus growth**

Growth inhibition of S. aureus by various concentrations of citral and linalool over 72 h is shown in Fig. 1a. Concentrations of 0.04 % citral and 0.24 % linalool inhibited the growth of S. aureus below detectable limits after 24 h of treatment. The minimum concentration, 0.02 % citral, inhibited growth by 3.76 log c.f.u. ml⁻¹, and 0.12 % linalool inhibited growth by 2.67 log c.f.u. ml⁻¹. The difference in inhibitory effect between citral and linalool after 24 h of treatment was not statistically significant. This inhibitory effect was maintained for 72 h for both citral and linalool. However, the inhibitory effect was reduced after 48 h for both citral and linalool.
Table 2. Minimum inhibitory concentrations (MICs) for citral, linalool, decanal and valencene against S. aureus

<table>
<thead>
<tr>
<th>Component</th>
<th>MIC (%)</th>
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<tbody>
<tr>
<td>Citral*</td>
<td>0.02</td>
</tr>
<tr>
<td>Linalool</td>
<td>0.12</td>
</tr>
<tr>
<td>Decanal</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Valencene</td>
<td>&gt;2.5</td>
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</tbody>
</table>

*A 10% stock solution of citral, linalool, decanal and valencene was used, with each component dissolved in 1% Tween 80 in distilled water.

A shorter 3 h growth inhibition assay was used for 0.04% citral and 0.24% linalool, and the results are shown in Fig. 1b. A concentration of 0.04% citral decreased S. aureus concentration below that of the initial inocula after 1 h and eliminated S. aureus below detectable limits by 3 h. A concentration of 0.24% linalool also demonstrated inhibition of S. aureus growth after 1 h. However, 0.24% linalool did not decrease S. aureus concentration below that of the initial inocula until 3 h of treatment.

Fig. 1. (a) A 72 h growth inhibition assay of S. aureus after being treated with only LB broth (control), 0.02% citral, 0.04% citral, 0.12% linalool or 0.24% linalool in LB broth. The assay was performed for 72 h with bacterial enumeration occurring every 24 h. The starting inoculum of S. aureus was 1.9 x 10^6 c.f.u. ml^-1. The data illustrate the mean at each time point, with bars representing the standard deviation. For each time point, statistical significance was determined by Tukey’s HSD test. Statistically significant differences are denoted by different letters.

Fig. 2. A concentration of 0.04% citral decreased S. aureus growth after 1 h. However, 0.24% linalool did not decrease S. aureus concentration below that of the initial inocula after 3 h. A concentration of 0.12% citral, 0.24% linalool and 0.48% linalool in LB broth. The assay was performed for 3 h with bacterial enumeration occurring every 1 h. The starting inoculum of S. aureus was 1.9 x 10^4 c.f.u. ml^-1. The data illustrate the mean at each time point, with bars representing the standard deviation. For each time point, statistical significance was determined by Tukey’s HSD test. Statistically significant differences are denoted by different letters.

Inhibition of pre-formed S. aureus biofilms with citral and linalool

Fig. 2 illustrates the inhibition of S. aureus pre-formed biofilms by both citral and linalool. All concentrations tested, which included the MIC values above, were capable of inhibiting pre-formed biofilms. The most effective concentrations were 0.08% citral, 0.24% linalool and 0.48% linalool, which had A540 values of 0.079, 0.075 and 0.067, respectively. The difference in absorbance between these concentrations was not statistically significant. However, they were significantly lower than the A540 values of the control and 0.02% citral, which were 0.104 and 0.090, respectively.

Cytotoxic effects of minimum concentration of citral and linalool on MAC-T Cells

Cytotoxicity of the minimum concentrations of citral and linalool on MAC-T cells is shown in Fig. 3. The relative cytotoxicity for citral is depicted in Fig. 3(a) and the relative cytotoxicity for linalool is depicted in Fig. 3(b). Both citral and linalool showed cytotoxicity for all concentrations tested. The relative cytotoxicities for 0.02%, 0.04% and 0.08% citral were 29.8%, 47.7% and 59.6%, respectively. The relative cytotoxicities for 0.12%, 0.24% and 0.48% linalool were 31.9%, 72.4% and 74.7%, respectively. Only concentrations with relative cytotoxicities below 50% cytotoxic concentration (CC50), which were 0.02% citral, 0.04% citral, and 0.12% linalool, were used in further experiments.
Role of citral and linalool in adhesion to and invasion into MAC-T cells by S. aureus

The effect of citral and linalool on S. aureus association with MAC-T cells is shown in Fig. 4(a). All concentrations of citral and linalool tested reduced S. aureus adherence to MAC-T cells compared to the control. A concentration of 0.02 % citral had the lowest reduction, at 4.5×10³ c.f.u. ml⁻¹. A concentration of 0.12 % linalool reduced adherent S. aureus by 6.0×10⁴ c.f.u. ml⁻¹, which was not significantly different from 0.02 % citral. A concentration 0.04 % citral produced the highest reduction, at 7.2×10⁴ c.f.u. ml⁻¹, which was not significantly different from 0.12 % linalool.

The ability of citral and linalool to inhibit S. aureus invasion of MAC-T cells is shown in Fig. 4(b). Concentrations of 0.02 % and 0.04 % citral were able to reduce invaded S. aureus by 5.2×10⁵ c.f.u. ml⁻¹ and 9.7×10⁵ c.f.u. ml⁻¹, respectively. There was a statistically significant difference between 0.02 % citral and 0.04 % citral. The lowest reduction in invasion was 1.0×10⁶ c.f.u. ml⁻¹ with 0.12 % linalool; this was not significantly different from 0.04 % citral.

Effect of citral and linalool on expression of S. aureus genes involved in virulence and biofilms

Fig. 5 shows the impact of citral and linalool on S. aureus virulence gene expression. All tested concentrations of citral (0.02 % and 0.04 %) and linalool (0.12 %) decreased the expression of sirA, hla, spa and sarS. Both concentrations of citral also decreased expression of agrA, sodA, hlb and sarR. In addition, a concentration of 0.04 % citral reduced expression of sbi, sarA and sigB. Fig. 6 illustrates the impact of citral and linalool on biofilm gene expression. While citral had no impact on any of the biofilm genes examined, 0.12 % linalool decreased expression of icaA, icaB, fnbB, clfA, clfD and efb by greater than 2-fold. The expression of icaC, icaD and fnbA was not impacted by citral and linalool.

DISCUSSION

The major components of terpeneless, cold-pressed Valencia orange oil – linalool, citral, decanal and valencene – were examined for their inhibitory effects on S. aureus growth, and ability to alter S. aureus–MAC-T cell interactions. Of the four components, only citral and linalool were capable of inhibiting S. aureus growth, having MIC values of 0.02 % and 0.12 %, respectively. Another study has examined the MIC of these components on S. aureus as well, and determined them to be 0.06 % for citral and 0.125 % for linalool (Fisher & Phillips, 2006). These results are similar to our own findings. In addition, both citral and linalool were able to decrease S. aureus growth after exposure for 24 h at their MIC values, and could completely eliminate S. aureus below detectable limits at twice that value. Citral and linalool, in addition to many other terpenes, have been
Components of orange oil inhibit *Staphylococcus aureus*

![Graph 1](attachment:genes_involved_in_biofilmformation.png)

**Fig. 5.** Gene expression of *S. aureus* virulence genes after 1 h of infection of MAC-T cells and 1 h of treatment with either a control of DMEM supplemented with 10% FBS, or with 0.02% citral, 0.04% citral or 0.12% linalool in DMEM supplemented with 10% FBS. Asterisks denote statistically significant differences from baseline.

![Graph 2](attachment:virulence_genes.png)

**Fig. 6.** Gene expression of *S. aureus* biofilm genes after 24 h of growth and 24 h of treatment with either a control of LB broth, or with 0.02% citral, 0.04% citral, or 0.12% linalool in LB broth. Asterisks denote statistically significant differences from baseline.

researched for their antimicrobial properties. Their mode of action is theorized to be due to damage to the bacterial cell membrane, which leads to K⁺ ion leakage and reduced membrane potential (Inoue *et al.*, 2004).

The effects of citral and linalool on pre-formed biofilms were also examined. Linalool appeared to be stronger than citral at inhibiting biofilms. Linalool at the MIC value of 0.12% reduced pre-formed biofilms beyond that of the MIC value of citral (0.02%). It should be noted that all concentrations tested resulted in statistically significant reductions of pre-formed biofilms compared to control. We were able to find no other studies that examined the impact of citral and linalool on pre-formed biofilms. Yet, we can infer some information based on studies that examined the ability of other terpenes to disrupt biofilms. Other terpenes have been found to possess anti-biofilm agents against *S. aureus* biofilms (Kuzma *et al.*, 2007), meaning our results are consistent with past research. As for the mode of action, one study found that terpenes disrupted biofilms by promoting cell separation, caused by terpenes reducing the fatty acid composition of the cell membrane and increasing hydrophobicity (de Carvalho & da Fonseca, 2007). It is possible that citral and linalool act in a similar manner, but further study is required.

Cytotoxicity was tested on MAC-T cells for both citral and linalool. All concentrations tested for both citral and linalool were found to be cytotoxic. There were only three concentrations found to be below CC50, 0.02% citral, 0.04% citral, and 0.12% linalool. As a result, only these concentrations were used for the remainder of the study. In one previous study, citral was determined to have a high cytotoxicity in human skin cells (Hayes & Markovic, 2002). Yet, it should be noted that in vivo toxicity in rat models was found to be 100-fold lower than in vitro results (Hayes & Markovic, 2002). In addition, it was only at concentrations above 1% that citral caused any skin sensitization (Hayes & Markovic, 2002). Therefore, concentrations of 0.02% and 0.04% citral are probably safe for *in vivo* use. Linalool has also been tested for cytotoxicity in a previous study, but again on human skin cells (Prashar *et al.*, 2004). Previous results are consistent with our own; in the former study a large increase in cytotoxicity occurred between 0.125% and 0.25% (Prashar *et al.*, 2004). We found a large increase in relative cytotoxicity between 0.12% linalool (relative cytotoxicity of 31.9%) and 0.24% linalool (relative cytotoxicity of 72.4%). Concentrations of 0.12% linalool and lower should be safe for use, but it is not advised to use higher concentrations. More studies would be needed to determine the safe dosages for use *in vivo*.

The study then determined whether citral and linalool impacted *S. aureus* association with and invasion of MAC-T cells. All of the concentrations below CC50 were capable of decreasing association and invasion. The most effective concentrations were 0.04% citral and 0.12% linalool, with there being no statistically significant difference between the two. While the mechanism is unclear, our results on expression of virulence genes may offer some insight into the effects of citral and linalool. Both citral and linalool downregulated expression of *sirA*, *hla*, *spa* and *sarS*. However, citral alone reduced expression of *agrA*, *sodA*, *hlb* and *sarR*. In addition, only a concentration of 0.04% citral resulted in decreased expression of *sbi*, *sarA* and *sigB*.

AgrA is transcriptional regulator for virulence gene expression and is considered the sole quorum-sensing regulator (Lowy, 1998; Queck *et al.*, 2008). It is possible that the downregulation of *agrA* could contribute to the downregulation of the other genes examined.

The Sar protein family, SarA, SarR and SarS, are regulatory proteins for cell wall and extracellular proteins. Both SarA and SarS work antagonistically while SarR modulates the two. Reduced expression of all three may result in the reduced expression of other proteins. SigB acts as another
regulatory factor that impacts both virulence and stress responses. Downregulation of sigB may serve to increase susceptibility to stressful environmental conditions (Ster et al., 2005).

SirA is involved in iron acquisition for Staphylococcus aureus (Heinrichs et al., 1999). As iron is a critical nutrient for infection and is often sequestered in cells, methods of iron acquisition are critical (Trivier et al., 1996). Due to the down-regulatory effects of citral and linalool on sirA, S. aureus may have problems obtaining iron for infection.

Sbi is an immunoglobulin-binding protein. It plays a role in the evasion of adaptive immunity by binding to IgG as well as the evasion of innate immunity by binding complement (Burman et al., 2008).

SpA, surface protein A, also binds IgG (Burman et al., 2008). While this would not affect the in vitro work done, it may serve to limit infections in vivo. The genes hla and hlb both encode haemolysins (Ster et al., 2005); with lowered expression of these genes, cell damage may be reduced. This downregulation of major virulence genes may serve to inhibit S. aureus pathogenesis and infection.

However, for biofilm genes, only linalool appeared to be able to downregulate expression. A concentration of 0.12% linalool was capable of reducing expression of icaA, icaB, fnbB, clfA, clfB and ebpS. The genes icaA and icaB are part of a larger ica operon, which also includes icaC and icaD. The ica operon (icaABCD) facilitates cell-to-cell adhesion in biofilm formation. This is accomplished by the production of polysaccharide intercellular adhesin, which is translocated to the cell surface and deacetylated for attachment. The role of IcaA in conjunction with IcaD is to produce polysaccharide intercellular adhesin, while the role of IcaB is to deacetylate it to attach to surfaces and other cells. Downregulating icaA and icaB may interfere with cell-to-cell attachment and the growth of biofilms (O’Gara, 2007).

FnB and FnB can also initiate biofilm development (Houston et al., 2011). However, they appear to play larger roles in the persistence of biofilms (Atshan et al., 2013; Houston et al., 2011). They also help facilitate adherence to surfaces, as do products encoded by clfA and clfB (Götz, 2002) and ebpS (Downer et al., 2002). Downregulation of fnbA and fnbB would interfere with another mode of biofilm development as well as initial attachments. Adherence would be further reduced by the downregulation of clfA, clfB and ebpS. Reduction of expression of these genes would interfere with biofilm production and maintenance, which could serve to limit S. aureus colonization and infection.

Citral and linalool should be considered for further research in the treatment of S. aureus-associated mastitis. Any issues with cytotoxicity should not present any problems in vivo according to past research in mouse models. Concentrations with relative cytotoxicity below the CC50 may have greater benefits than risks. Both citral and linalool reduced in vitro growth and pre-formed biofilms. For linalool treatments, this decrease in pre-formed biofilms may be due in part to downregulation of genes that play a role in biofilm formation and development. Both citral and linalool also decreased S. aureus association and invasion, which may be due to their downregulation of virulence genes. Future directions include determining the MIC, MIC90, minimum bacteriostatic concentration (MBC), MBC90 and time kill curves of citral and linalool on other strains of S. aureus found in bovine mastitis. In addition, the impact of multiple treatments, as well as safety and efficacy in vivo, should be examined.

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REFERENCES


