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Effects of Tea Tree Oil on *Staphylococcus aureus* Virulence Factors

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by KA Hammer, CF Carson & TV Riley

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Researcher Contact Details
Professor T. V. Riley
Microbiology (M502),
The University of Western Australia,
35 Stirling Hwy, Crawley, Western Australia, 6009

Phone: (08) 9346 3690
Fax: (08) 9346 2912
Email: triley@cyllene.uwa.edu.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details
Rural Industries Research and Development Corporation
Level 1, AMA House
42 Macquarie Street
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4819
Fax: 02 6272 5877
Email: rirdc@rirdc.gov.au.
Website: http://www.rirdc.gov.au

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Foreword

S. aureus is an important pathogen of humans, causing diseases ranging from superficial skin and wound infections to severe illnesses such as septicaemia, endocarditis and toxic shock syndrome. *S. aureus* is particularly a problem in hospitals because it spreads easily in these environments and causes potentially fatal infections in immunocompromised hospital patients.

Both laboratory and clinical data indicate that tea tree oil may be a useful agent in the treatment of *S. aureus* infections. The aims of this research are therefore to investigate and characterise the effects of tea tree oil and tea tree oil components on the production of virulence factors by *S. aureus*. Virulence factors are the mechanisms and strategies used by bacteria to initiate and establish infections. If tea tree oil reduces or stops the production of virulence factors this may be another mechanism by which tea tree oil works to prevent or clear *S. aureus* infections. Another aim of this research is to continue and expand on the body of work describing the in vitro and in vivo activity of tea tree oil against *S. aureus*, including methicillin-resistant isolates.

This report describes the results of experiments determining the production of extracellular proteases, coagulase and toxins, and the formation of biofilm in the presence of tea tree oil.

This project was funded from industry revenue from Novasel Australia Pty Ltd which is matched by funds provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1200 research publications, forms part of our Tea Tree Oil R&D program, which aims to support the continued development of an environmentally sustainable and profitable Australian tea tree oil industry that has established international leadership in marketing, in value-adding, and in product reliability and production.

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Peter O'Brien
Managing Director
Rural Industries Research and Development Corporation

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The generosity of Australian Plantations Pty. Ltd., Wyrallah, NSW for providing the batch of tea tree oil that was used throughout this study is acknowledged.

The assistance of the Division of Microbiology and Infectious Diseases of the Western Australian Centre for Pathology and Medical Research for providing recent clinical isolates is acknowledged, as is Mr Geoff Coombs from the Department of Microbiology and Infectious Diseases at Royal Perth Hospital, Perth, for providing strains of MRSA.

We are grateful for the technical, financial and institutional support of the Microbiology Discipline, School of Biomedical and Chemical Sciences, The University of Western Australia, and the Division of Microbiology and Infectious Diseases at The Western Australian Centre for Pathology and Medical Research.

Abbreviations

agr	Accessory gene regulator
AgSD	Silver sulphadiazine
AD	Atopic dermatitis
BHIB	Brain heart infusion broth
cfu	Colony forming units
g	Force of gravity
h	Hour
ISO	International standards organisation
MBC	Minimum bactericidal concentration
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
NB	Nutrient broth
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
OD	Optical density
PIA	Polysaccharide intercellular adhesin
PBS	Phosphate buffered saline
R	Resistant
RPH	Royal Perth Hospital
rpm	Revolutions per minute
S	Susceptible
SASP	*S. aureus* serine protease
SD	Standard deviation
SDW	Sterile distilled water
SSS	Scalded skin syndrome
TSB	Tryptone soya broth
TSBG	Tryptone soya broth with glucose
TSS	Toxic shock syndrome
TSST	Toxic shock syndrome toxin
TTO	Tea tree oil
v/v	Volume for volume
VF	Virulence factor
w/v	Weight for volume

Contents

Executive Summary

A collection of 44 reference, clinical and methicillin-resistant *S. aureus* isolates was obtained. The collection was tested for susceptibility to tea tree oil, and some preliminary tests were conducted to investigate the production of virulence factors.

Tea tree oil susceptibility data showed that minimum inhibitory concentrations (MICs) ranged from 0.12 – 0.5% (v/v), with 90% of isolates inhibited by 0.5%. Minimum bactericidal concentrations (MBCs) ranged from 0.5 - 2.0% with 90% of isolates killed at or below 2.0%.

In addition to susceptibility testing, some simple agar plate assays were used to characterise (or biotype) the isolates by screening for the production of several enzymes or other characteristics. Of the 44 *S. aureus* isolates, 34% showed haemolysis on blood agar, 73% showed strong lipase reactions, 16% were positive for lecithinase, and assays investigating pigment production demonstrated that 57% showed yellow pigment, 34% showed cream pigment and 9% showed white.

Before complex assays investigating the production of virulence factors were conducted, experiments to determine how low, or sub-inhibitory concentrations of tea tree oil affected the growth of *S. aureus* in vitro were performed. These assays showed that growth in the presence of 0.016 and 0.031% tea tree oil was not markedly different from growth in the absence of tea tree oil, whereas the presence of 0.062% tea tree oil inhibited bacterial growth more substantially. From these experiments it was concluded that concentrations of 0.031% or below would be used for assays investigating the effects of tea tree oil on the production of virulence factors. Additional screening assays were conducted to determine the most appropriate isolates for thorough investigation in each of the virulence factor assays. Those isolates producing the relevant virulence factor at high levels in vitro were selected for further investigation.

The first virulence factor to be investigated in depth was the production of the extracellular enzyme protease. Six isolates were investigated by culturing each organism for 24 h with tea tree oil and removing samples at 4, 8 and 24 h to measure levels of protease in the cell-free supernatant. Although most isolates showed lower levels of protease when cultured with tea tree oil, reductions were only statistically significant for three of the six isolates, and were not significant at all time points. The presence of tea tree oil did not significantly affect the numbers of viable organisms present at any time point.

The second virulence factor to be investigated was the production of coagulase, another extracellular protein. Coagulase levels for a total of 12 isolates were measured after 16 h incubation in the presence of 0.031% tea tree oil. For four isolates, significantly higher levels of coagulase were produced in the presence of tea tree oil when compared to control cells. For the remaining eight isolates, coagulase levels were either increased (n = 4), decreased (n = 1) or equivalent (n = 3), but these differences were not significant.

The third virulence factor assay investigated the production of extracellular toxins by *S. aureus*. A total of eight isolates was tested for the production of enterotoxins A, B, C or D and another two isolates were tested for the production of toxic shock syndrome toxin. In general, these experiments did not demonstrate any significant reduction in levels of toxin after growth for 24 h in the presence of 0.016 or 0.031% tea tree oil. As a result, toxin levels after growth in the presence of 0.062% tea tree oil were also investigated. For nine of the 10 isolates toxin levels were reduced when cells were grown with 0.062% tea tree oil, but analysis of viable count data demonstrated a parallel decrease in the numbers of cells present. These decreases in toxin levels may therefore have been a function of reduced cell numbers.

The final characteristic of *S. aureus* to be investigated was the formation of biofilm on polystyrene surfaces. Six isolates were selected for this assay on the basis of preliminary investigations. The

effects of tea tree oil, at concentrations ranging in doubling dilutions from 2 – 0.002% was investigated. Results showed that for most isolates, the presence of 2, 1 and 0.5% tea tree oil completely prevented growth, and very few viable organisms were present at these concentrations. For two isolates, the formation of biofilm was significantly reduced in the presence of 0.016 – 0.25% tea tree oil. For a third isolate, biofilm formation was significantly inhibited by 0.016, 0.031 and 0.062% tea tree oil, but not by 0.12 and 0.25%. For the fourth isolate, biofilm formation was significantly increased in the presence of 0.062, 0.12 and 0.25% tea tree oil, and for the remaining two isolates no significant changes were seen.

To summarise, these assays produced some interesting and disparate findings. The investigation of extracellular proteases and toxins demonstrated that the production of these particular proteins does not appear to be specifically inhibited by tea tree oil. Instead, reductions were for the most part a function of a decrease in cell density. In contrast, investigation of the protein coagulase indicated that levels were increased in the presence of tea tree oil, although the mechanisms by which this occurred remain unknown. Lastly, for some isolates, the formation of biofilm was significantly reduced in the presence of sub-inhibitory tea tree oil, but for other isolates no such effect was seen. This study has demonstrated that the presence of tea tree oil does affect the production of virulence factors by *S. aureus*, but does not necessarily cause a reduction. This work was heavily weighted towards investigating the production of virulence factors that are extracellular proteins. Further studies investigating characteristics that are either distantly or unrelated to the production of extracellular proteins, such as the susceptibility of cells to phagocytosis or changes in adherence characteristics, may reveal more promising results.

Chapter 1: Introduction

1.1 General characteristics of *Staphylococcus aureus*

Staphylococcus aureus is a catalase positive, facultatively anaerobic, Gram-positive coccus (Wilkinson, 1997). Within the genus *Staphylococcus*, *S. aureus* is the most significant of the pathogenic species and is easily distinguished from the other species because it is coagulase positive; the remainder are coagulase negative. The normal microbial habitat of *S. aureus* is warm-blooded mammals, with approximately 20% of humans permanently colonised with this organism, and as much as 50% of the population transiently colonised (Tenover and Gaynes, 2000). The areas of the body most commonly colonised in healthy individuals include the nares, perineum, vagina and axillae (Noble, 1997).

1.2 Infections caused by *S. aureus*

In addition to its role as a commensal organism, *S. aureus* can cause many different infections, ranging from those that are relatively mild and superficial to those that are life threatening or fatal. The particular strain of *S. aureus* causing the infection may be derived from a patient's own flora, or may be community or hospital acquired. In fact, *S. aureus* is one of the most common causes of both community and hospital-acquired infections (Boyce, 1997; Projan and Novick, 1997). Infections caused by antibiotic resistant *S. aureus* strains, such as methicillin resistant *S. aureus* (MRSA) are particularly problematic given that the resistance of the organism to conventional antibiotics leaves few treatment options.

1.2.1 Skin and superficial infections

S. aureus is very quick to opportunistically infect broken or compromised skin and as such causes a wide range of superficial skin infections. *S. aureus* can cause folliculitis, cellulitis, mastitis, furuncles (boils), carbuncles and pyoderma (Noble, 1997). The organism also causes staphylococcal scalded skin syndrome and may cause impetigo or 'school sores'. In several of these infections, research has shown that the bacterial strain causing the infection is indistinguishable from that colonising the nares (Noble, 1997), suggesting that a patient's own flora often serves as the reservoir for infection. In addition to the conditions named above, *S. aureus* is also frequently found as a colonising or infecting organism in the skin lesions associated with ecxema or atopic dermatitis. Skin ulcers in elderly and/or diabetic patients may also be colonised and/or infected with *S. aureus*, as may surgical wounds. The treatment of *S. aureus* skin infections is very much dependant on the type of infection and can vary from surgical drainage only to topical antimicrobial agents to oral antibiotics (Noble, 1997).

1.2.2 Other infections

S. aureus is a very versatile pathogen and is able to infect virtually every human organ system and body site (Archer, 1998). It has caused severe life-threatening infections of the central nervous system, joints and bone, circulatory system, respiratory tract, urinary tract, and gastrointestinal tract. In addition, *S. aureus* can cause endocarditis, meningitis, brain abscesses and infections of the eyes (Ing *et al.*, 1997; Archer, 1998). These severe infections are usually treated with intravenous antibiotics to encourage a rapid and successful treatment outcome. defence

1.3 *S. aureus* virulence factors

One of the key factors enabling *S. aureus* to survive, colonise, proliferate and cause human infections is the expression of virulence factors (VFs). Furthermore, *S. aureus* produces an array of VFs, which can be broadly grouped into those involved in 1) bacterial attachment, 2) evasion of host defenses

and 3) tissue invasion (Projan and Novick, 1997). Work to date suggests that each single VF alone is not sufficient to cause infection and that it is much more likely that several VFs work together in concert to cause infection and disease (Projan and Novick, 1997). It has also been shown that not every *S. aureus* strain produces every VF, or does not produce each to the same degree (Karlsson and Arvidson, 2002), and it is tempting to then assume that those strains expressing more or higher levels of VFs are the more pathogenic. Those VFs analysed in the present study are discussed in depth below.

1.3.1 Proteases

S. aureus produces several extracellular proteases, including metallo-, serine and cysteine proteases (Dubin, 2002). These proteases are thought to be involved in evading host defenses and invading tissue (Archer, 1998). The general function of all proteases is to cleave proteins and in doing so, this may inactivate key proteins and antimicrobial peptides involved in host defenses. An example of this is the serine protease of *S. aureus* that has been shown to cleave IgG antibodies (Projan and Novick, 1997). Finally, the proteases may destroy host tissue proteins, leading to generalised tissue destruction (Projan and Novick, 1997) and at the same time the creation of valuable nutrients for microbial growth. In addition to the functions described above, the expression of proteases may contribute to the severity of superficial *S. aureus* infections, given that *S. aureus* strains isolated from patients with atopic dermatitis have been shown to produce much higher levels of proteases than strains from healthy volunteers (Miedzobrodzki *et al.*, 2002).

1.3.2 Coagulase

Coagulase is produced by *S. aureus* as an extracellular protein and is thought to have a role in cellular attachment. This is important given that attachment is one of the first steps in the pathogenesis of infection (Archer, 1998). However, the role of coagulase as a virulence factor is still a matter of some debate (Projan and Novick, 1997). This is based on studies with animal models showing that coagulase-deficient mutants do not show reduced virulence compared to coagulase-competent strains. Nonetheless, given that most *S. aureus* strains express coagulase, and that this protein binds to and activates prothrombin and causes the coagulation of serum (Projan and Novick, 1997; Carter *et al.*, 2003), it would seem likely that coagulase plays an important role in disease.

1.3.3 Toxins

S. aureus produces a range of extracellular toxins, including toxic shock syndrome toxin-1, enterotoxins A, B, C 1-3, D, E, G, H and I, exfoliative toxins A and B, α-toxin and leukocidin (Dinges *et al.*, 2000). All of these, with the exception of α-toxin, are thought to play a role in evading host defenses (Projan and Novick, 1997). Most of these toxins also have superantigen activity, meaning that they stimulate non-specific proliferation of the T lymphocytes of the immune system (Balaban and Rasooly, 2000).

Several *S. aureus* toxins cause specific illnesses or syndromes, such as food poisoning caused by enterotoxins, toxic shock syndrome (TSS) caused by toxin shock syndrome toxin, and scalded skin syndrome caused by the exfoliative toxins, to name a few. Staphylococcal food poisoning occurs when food containing pre-formed toxin is ingested and the illness is characterised by nausea, vomiting, abdominal pain and diarrhoea, with onset occurring 2-6 hours after ingestion of the contaminated food (Balaban and Rasooly, 2000). The potentially fatal TSS is characterised by a diffuse rash, desquamation, hypotension, high fever and the involvement of three or more organ systems (Dinges *et al.*, 2000). Most cases of TSS are wound or menstruation-associated, the latter linked to the use of tampons in women (Dinges *et al.*, 2000).

Although food poisoning and TSS are important illnesses resulting from exposure to two kinds of *S. aureus* toxins, of greater relevance to this project is the role of *S. aureus* toxins in superficial infections. Two toxins that are known to be expressed by bacteria infecting the skin are exfoliative toxins A and B. These toxins cause scalded skin syndrome (SSS), which is characterised by separation and loss of the epidermal skin layers (Bohach *et al.*, 1997). Toxin-producing *S. aureus* strains have also been shown to have a clear role in the disease process of atopic dermatitis (AD). Patients with AD are often colonised with enterotoxin producing strains and the expression of these toxins is thought to provoke the immune responses that are seen in these patients (Bunikowski *et al.*, 2000; Laouini *et al.*, 2003). Research describing the production and/or effects of toxins in superficial infections has largely focused on strains from AD patients, but it would seem likely that enterotoxins or other toxins are also expressed in non-AD *S. aureus* infections such as boils and wound infections.

1.3.4 Biofilm production

Biofilm has been defined as an accumulated microbial community enmeshed in self-produced extracellular material (Yarwood *et al.*, 2004). Although the phenomenon of biofilm formation has been most widely studied in the coagulase-negative staphylococci, particularly *S. epidermidis*, *S. aureus* is also known to form biofilm in vitro and in vivo. Biofilms are medically important for several reasons. Firstly, biofilm may form on indwelling medical devices such as catheters and this may lead to severe blood-stream infections. Secondly, biofilm-associated bacteria are often less susceptible to antimicrobial agents and host defenses and, as such, infections involving biofilm may be harder to treat and clear (Yarwood *et al.*, 2004). The formation of biofilm has been described as occurring in two major stages. The first involves the attachment of cells to a surface. This is mediated partly by cell wall-associated molecules such as adhesins. The second stage involves microbial multiplication and the formation of an extracellular matrix, composed largely of polysaccharides (Yarwood *et al.*, 2004). Cell-to-cell signalling, also known as quorum sensing, is thought to play a role in the formation of biofilm and it is possible that antimicrobial agents that prevent the formation of biofilm may do so by interfering with this cell-cell signalling.

1.4 Objectives of this study

The in vitro activity of tea tree oil against *S. aureus* has been well established in the scientific literature using standard broth and agar dilution assays (Carson *et al.*, 1995a; Carson *et al.*, 1995b; Raman *et al.*, 1995; Nelson, 1997). Furthermore, a few studies have investigated the mechanism of action of tea tree oil using techniques such as time kill curves and electron microscopy, and by investigating the leakage of intracellular materials (Cox *et al.*, 2000; Carson *et al.*, 2002). Previous studies have demonstrated that conventional antibiotics such as linezolid and tetracycline reduce the production of virulence factors by particular bacteria (Doss *et al*., 1993; Gemmell & Ford, 2002). However, the effects of tea tree oil on the production of VFs by *S. aureus* have not previously been investigated. Therefore, the aim of this research is to investigate and characterise the effects of tea tree oil on the production of VFs by *S. aureus*. As described above, VFs are the mechanisms and strategies used by bacteria to initiate and establish infections. If tea tree oil reduces or stops the production of VFs this may be another mechanism by which tea tree oil works to prevent or clear *S. aureus* infections. Another aim of this research is to expand on the body of work describing the in vitro and in vivo activity of tea tree oil against *S. aureus*, including methicillin-resistant isolates.

Chapter 2: Materials and methods

2.1 Tea tree oil

Melaleuca alternifolia (tea tree) oil was kindly donated by Australian Plantations Pty Ltd., Wyrallah, NSW. Batch 97/1 was used for all studies and the composition, as determined by gas-chromatography mass spectrometry performed by the Wollongbar Agricultural Institute, Wollongbar, NSW, is shown in Table 2.1.

Table 2.1 Composition of *M. alternifolia* oil batch 97/1

Component	Percentage	Component	Percentage
1. terpinen-4-ol	41.5	9. aromadendrene	1.0
2. γ-terpinene	21.2	10. δ-cadinene	1.0
3. α-terpinene	10.2	11. limonene	0.9
4. terpinolene	3.5	12. ledene	0.9
5. α-terpineol	2.9	13. globulol	0.6
6. α-pinene	2.5	14. sabinene	0.4
7. 1,8-cineole	2.1	15. viridiflorol	0.3
8. ρ-cymene	1.5		

2.2 *S. aureus* isolates

2.2.1 Source

A collection of 44 *S. aureus* isolates was obtained. This included five reference isolates *S. aureus* NCTC 10442, NCTC 10443, NCTC 10657, NCTC 7121 and NCTC 6571. The remaining 39 isolates were from clinical sources. Twenty-nine were recent clinical isolates from The Western Australian Centre for Pathology and Medical Research (PathCentre) and the remaining 10 were methicillin-resistant *S. aureus* (MRSA) epidemic strains kindly provided by Mr Geoffrey Coombs from Royal Perth Hospital, Western Australia. Antibiotic susceptibility testing had already been performed on many of these isolates at each respective institute and these details were recorded at the time. Clinical sources and antibiotic susceptibility profiles are shown in Table 2.2.

Table 2.2. Clinical source and antibiotic susceptibilities of *S. aureus* isolates

Isolate number	Clinical source	Antibiotics								
		FL	E	P	V	MU	C	G	FU	ME
NCTC 10442	Reference isolate									**R**
NCTC 10443	Reference isolate			**R**						
NCTC 10657	Reference isolate									
NCTC 7121	Reference isolate									
NCTC 6571	Reference isolate									
6	wound swab	S	S	**R**						
7	skin swab	S	S	**R**	S	S				
8	right eye swab	**R**	**R**	**R**	S	S	**R**	S	S	
9	wound swab	**R**	S	**R**	S	S				
10	wound fluid	S	S	S	S	S				
11	blood culture	S	S	**R**	S					
12	blister fluid swab	S	S	**R**	S	S				
13	urine	S	S	**R**	S					
14	blood culture		S	**R**	S	S				
15	heel swab	S	S	**R**	S	S				
16	swab	S	S	**R**	S					
17	wound swab	S	S	**R**	S	S				
18	wound swab	S	S	**R**	S	S				
19	right eye swab	S	S	**R**	S	S				
20	wound swab	S	**R**	**R**	S					
21	wound swab	S	S	**R**	S	S				
22	knee swab	S	S	**R**	S	S				
23	elbow swab	**R**	S	**R**	S	S	S	S	S	
24	toe swab	**R**	**R**	**R**	S	S	**R**			
25	nasal swab	**R**	**R**	**R**	S			**R**		
26	wound swab	**R**	**R**	**R**						
27	wound swab	**R**	**R**	**R**					**R**	
28	sacral wound swab	S	S	**R**						
29	eye swab	**R**	S	**R**	S	S	S	S		
30	lip lesion swab	S	S	S	S	S				
31	breast swab	S	S	**R**	S	S				
32	foot swab	S	**R**	**R**	S	S				
33	wound swab	**R**	**R**	**R**						
34	leg wound swab	**R**	S	**R**	S	S	S	S	**R**	
35	RPH MRSA									**R**
36	RPH MRSA UK 16									**R**
37	RPH MRSA									**R**
38	RPH MRSA									**R**
39	RPH MRSA Irish 2									**R**
40	RPH MRSA									**R**
41	RPH MRSA UK 15									**R**
42	RPH MRSA WSSP									**R**
43	RPH MRSA AUS-2									**R**
44	RPH MRSA AUS-3									**R**

R: resistant; S: susceptible; blank cells indicate that no data were available.

Key to antibiotics: FL flucloxacillin; E erythromicin; P penicillin; V vancomycin; MU mupirocin; C ciprofloxicin; G gentamicin; FU fucidin; ME methicillin

2.2.2 General culture techniques

All *S. aureus* isolates were cultured routinely on blood agar incubated at 35°C. Long term storage of isolates was at –80°C in brain heart infusion broth (BHIB) containing 20% glycerol as a cryoprotectant.

2.2.3 Preliminary characterisation/biotyping of isolates

All *S. aureus* isolates were characterised and screened for the production of several virulence factors following the methods of Udo & Jacob (2000), as described briefly below. Inocula were prepared by subculturing isolates onto blood agar and incubating overnight at 35°C. Growth from plates was then suspended in 0.85% saline and adjusted to a concentration of approximately 10^8 cfu/ml. A multipoint inoculator was then used to deliver drops of approximately 1-3 µl onto the surface of each pre-dried plate.

Haemolysins
Haemolysins were detected by spot-inoculating isolates onto agar containing 5% horse blood (blood agar) and incubating plates for 24 h at 35°C. After incubation, β-haemolytic isolates showed a zone of clearing in the blood agar adjacent to the bacterial growth.

Lecithinase
Egg yolk agar plates were prepared by adding egg yolk emulsion at a final concentration of 10% (v/v) to nutrient agar supplemented with 1% glucose. Egg yolk emulsion was prepared by adding 1.4 ml sterile distilled water for every 1.1 g of dehydrated egg yolk. Inoculated plates were incubated for 72 h at 35°C and isolates showing clearing adjacent to growth were considered lecithinase positive.

Lipase
Nutrient agar plates containing 1% Tween 80 (v/v) were prepared. Inoculated plates were incubated for 48 h at 35°C and colonies showing an opalescent zone adjacent to growth were recorded as lipase positive.

Pigment
Ultra high temperature milk was added to nutrient agar at a final concentration of 20% (v/v). Inoculated plates were incubated for 24 h at 35°C then for a further 24 h at room temperature. Colonies were recorded as having yellow, cream or white pigmentation.

2.3 In vitro susceptibility testing

2.3.1 Inoculum preparation

Isolates were grown for 24 h on blood agar at 35°C. Colonies were then suspended in sterile distilled water (SDW) and the suspension was adjusted to 0.5 McFarland, corresponding to approximately 10^8 cfu/ml. This was serially diluted in SDW as necessary to correspond to a final inocula concentration of approximately 5×10^5 cfu/ml. Final inocula concentrations were confirmed by Miles-Misra viable counts, by spot inoculating 10 µl volumes in duplicate onto blood agar.

2.3.2 Microdilution testing

Serial two-fold dilutions of tea tree oil ranging from 4 – 0.016% (v/v) were prepared in 100 µl volumes in a 96-well microtitre tray, in Mueller Hinton II broth. A control well containing no tea tree oil was included for each organism. A final concentration of 0.001% Tween 80 (v/v) was included in all dilutions to enhance oil solubility. An equal volume of inocula was added to each well and the 96-

well tray was then incubated at 35°C for 24 h. After incubation, MICs were determined visually as the lowest concentration of oil resulting in no visible growth. MBCs were determined by subculturing 10 μl volumes from each well and spot inoculating onto blood agar. Subcultures were incubated at 35°C and the resulting colonies counted. MBCs were determined as the lowest concentration of oil resulting in the death of 99.9% of the inoculum. Assays were repeated at least twice and modal values were chosen.

2.4 Growth curves in the presence of tea tree oil

Preliminary assays to determine which concentrations of tea tree oil did not significantly inhibit the growth of *S. aureus* were conducted. Overnight cultures were prepared by inoculating 1-2 colonies of *S. aureus* NCTC 10443 and NCTC 6571 into approximately 10 ml of BHIB and incubating at 35°C with shaking for 24 h. This overnight culture was diluted 1 in 50 into fresh BHIB containing 0.016%, 0.031% and 0.062% tea tree oil. A control flask containing no tea tree oil was included. All flasks contained a final concentration of 0.001% Tween 80 to enhance oil solubility. Since the MICs of tea tree oil for *S. aureus* NCTC 10443 and NCTC 6571 were 0.25 and 0.5%, respectively, the concentration of 0.062% tea tree oil corresponds to 1/4 and 1/8th of the MIC for each isolate. After flasks were inoculated, samples were taken at 0, 3, 4, 6, 8 and 24 h. The optical density of each sample was determined at 600 nm, as an estimation of growth. The $\log_{10}$ value of each optical density value was then plotted against time.

2.5 Virulence factor assays

2.5.1 Viable counts

Unless stated otherwise, viable counts were performed by serially diluting samples 10-fold in 0.85% (w/v) saline. Volumes of 100 μl were then spread in duplicate onto nutrient agar. Plates were incubated at 37°C for approximately 24 h. Colonies from plates with between 30 and 300 colonies were counted and colony counts from duplicate plates were averaged. The numbers of viable organisms in each original sample was then calculated.

2.5.2 Measurement of extracellular proteases

The assay for measuring extracellular protease was based on the method described by Miedzobrodzki *et al*. (2002). Using azocasein as a substrate, this assay measures total proteolytic activity, including serine protease and metalloprotease activity. Isolates of *S. aureus* were cultured overnight by inoculating 1-2 colonies into approximately 10 ml of BHIB and incubating for 24 h at 35°C with shaking. These cultures were then diluted 1/20 into culture flasks containing fresh BHIB supplemented with 4% (w/v) soluble casein. Each flask also contained 0.001% (v/v) Tween 80, and tea tree oil at a final concentration of 0.016% or 0.031% (v/v). The control flask contained no tea tree oil. Flasks were incubated at 35°C with shaking at 150 rpm. Samples were taken at 4, 8 and 24 h for determination of extracellular protease levels and viable counts. Viable counts were performed directly from each sample. Supernatants were then obtained by centrifuging samples for 5 min at 13000 U/min. Supernatants were then removed and stored at –20°C until being processed to determine protease levels.

For this, samples were brought to room temperature and 35 μl of supernatant was added to a mixture of 315 μl of TRIS buffer and 210 μl of 1.5% (v/w) azocasein. A blank was prepared with 35 μl of uninoculated culture media instead of supernatant. Mixtures were incubated at 37°C for 3 h and then 600 μl of 6% (w/v) trichloroacetic acid was added to stop the reaction. Mixtures were left at room temperature for 15 min to allow the precipitation of unreacted protein. They were then centrifuged and the absorbance of the supernatants was read at 365 nm. One unit of protease was defined as an increase in absorbance of 0.001 after 3 h incubation at 37°C. Assays were repeated at least three

times for each isolate. Isolates NCTC 6571 and NCTC 7121 were tested as described above whereas additional isolates were tested against 0.031% tea tree oil only, sampled at 8 and 24 h only.

2.5.3 Measurement of coagulase levels

The assay to determine levels of coagulase was based of that described by Gemmell & Ford (2002). Isolates of *S. aureus* were cultured overnight by inoculating 1-2 colonies into approximately 10 ml of nutrient broth (NB) and incubating for 24 h at 35°C with shaking. These cultures were then diluted 1/20 into culture flasks containing fresh NB. Each flask also contained 0.001% (v/v) Tween 80, with either no tea tree oil (control flask) or a final concentration of 0.031% tea tree oil (v/v). Flasks were incubated for 16 h at 35°C with shaking at 150 rpm. After 16 h, samples were taken for determination of coagulase levels and viable counts. Viable counts were performed directly from each sample. Coagulase levels were determined from culture supernatants, which were obtained by centrifuging samples for 5 min at 6000 *g*. Culture supernatants were serially diluted 2-fold in PBS with 10% v/v NB in a 96-well microtitre tray. An equal volume of citrated rabbit plasma that had been diluted 1/10 (v/v) in PBS with 10% NB was then added to each well. The tray was incubated for 6 – 8 h at 37°C. After 6 h each well was checked with the aid of a reading mirror for coagulation or clotting. The titre was determined as the highest dilution of supernatant producing a measurable clot. Assays were repeated at least three times for each isolate and modal titres were selected.

2.5.4 Toxin assays

Overnight cultures were obtained by inoculating 1-2 colonies of each isolate into approximately 10 ml of growth medium and incubating for 18 - 24h at 37°C with shaking. BHIB was used for assays detecting toxic shock syndrome toxin (TSST) and tryptone soya broth (TSB) was used for assays to detect enterotoxins. After incubation, cells were collected and washed twice in 0.85% saline to remove pre-formed toxins. The cell pellet was then resuspended to the original volume in the relevant growth medium.

Washed, resuspended cells were then diluted 1/20 into culture flasks containing fresh growth medium. Each flask also contained 0.001% (v/v) Tween 80, and tea tree oil at a final concentration of 0.016, 0.031 or 0.062% (v/v). The control flask contained no tea tree oil. Flasks were incubated at 37°C with shaking. After 24 h incubation, samples were taken for determining toxin levels and viable counts. Viable counts were performed directly from each sample. Samples were then centrifuged at 6000 *g* for 5 min. Supernatants were removed and stored at –20°C until toxin assays were conducted.

Supernatants were tested for the presence of toxins by using reversed passive latex agglutination (RPLA) toxin detection kits (Oxoid, Basingstoke, UK). The SET-RPLA kit was used to detect staphylococcal enterotoxins A, B, C and D and the TST-RPLA kit was used to detect toxic shock toxin. Assays were performed and results were recorded according to the manufacturer's instructions. The titre endpoint was determined as the lowest dilution of supernatant showing agglutination, not including any '±' results. Experiments were repeated at least twice and modal titre results were selected.

2.5.5 Biofilm assays

Overnight cultures were prepared by inoculating 1-2 colonies into approximately 10 ml of Trypticase soya broth with 0.25% glucose (TSBG) (Heilmann *et al.*, 1996). Cultures were incubated with shaking at 37°C for 18 – 24 h. Cultures were then diluted in TSBG to the opacity of a 0.5 McFarland turbidity standard, corresponding to ~ 10^8 cfu/ml.

Serial dilutions of tea tree oil were prepared in a 96-well polystyrene microtitre tray (Nunc, Roskilde, Denmark) in TSBG. Final concentrations of tea tree oil ranged from 2 – 0.002% (v/v) and each well also contained a final concentration of 0.001% Tween 80 to enhance oil solubility. To six tray rows

were added 100 µl of inocula for each organism. The last two rows served as tea tree oil controls and TSBG was added to these rows instead on inocula. The last column of the tray served as a positive growth control, containing growth medium and Tween 80, but no tea tree oil. The tray was covered and incubated aerobically at 37°C for 24 h (Heilmann *et al.*, 1996).

After incubation, the presence of growth in each well was determined visually with the aid of a reading mirror and 10 µl volumes were subcultured from non-turbid wells onto nutrient agar. Trays were then washed and stained according to previously described methods (Stepanovic *et al.*, 2000). Briefly, the contents of each well were removed and each well wash washed three times with 0.85% saline. Trays were shaken vigorously to remove non-adherent bacteria. Adherent bacteria were fixed by adding 99% methanol to each well and leaving for 15 min at room temperature. Wells were then emptied and left to dry. Biofilm was stained by adding 200 µl of Hucker's 2% crystal violet stain for 5 min. Trays were then rinsed thoroughly under tap water and air dried. After drying, stain was resolubilised by adding 160 µl of 33% glacial acetic acid to each well and agitating gently. The absorbance of each well was then read at 570 nm in triplicate. An average was obtained for the two blank rows in each tray and these values were subtracted from each corresponding absorbance reading. Mean values for the triplicate readings were then obtained.

Twenty isolates were initially screened for biofilm formation. Isolates were ranked according to the degree of biofilm formation in wells containing no tea tree oil and the six isolates producing the highest absorbance readings were selected for further studies.

2.6 Statistical analyses

The student's 2-tailed t-test, assuming unequal variance was used to analyse viable count data, protease levels and absorbance readings from the biofilm assays. Protease levels were also analysed using Dunnett's multiple comparison test (GraphPad Prism). Titres for the coagulase and toxin assays were converted to $\log_2$ values and were then analysed using the Student's 2-tailed t-test. Where each replicate of an experiment gave identical titres no analyses were possible given that there was no variation in the values.

Chapter 3: Results

3.1 Preliminary characterisation (biotyping) of isolates

Results of the screening of isolates for haemolysis, lipase, lecithinase and pigment are shown in Table 3.1. Of the 44 isolates, 15 (34.1%) showed haemolysis, 32 (72.7%) showed strong lipase reactions (with another two isolates showing slight lipase activity), 7 (15.9%) showed lecithinase, and 25 (56.8%) showed yellow, 15 (34.1%) cream and 4 (9.1%) white pigment.

3.2 In vitro susceptibility to tea tree oil

A total of 30 *S. aureus* isolates was tested for their susceptibility to tea tree oil by the broth microdilution method (Table 3.1). MICs ranged from 0.12 – 0.5%, and the MIC_{50} and MIC_{90} were both 0.5%. MBCs ranged from 0.5 – 2.0%, the MBC_{50} was 1.0% and the MBC_{90} was 2.0%.

3.2.1 Growth curves in the presence of tea tree oil

Preliminary growth curves for *S. aureus* isolates NCTC 10443 and NCTC 6571 are shown in Figure 3.1. For both isolates, growth in the presence of 0.016% and 0.031% tea tree oil was not markedly different from controls, whereas growth in the presence of 0.062% tea tree oil was more substantially affected. On the basis of these results, most assays investigating virulence factors were conducted using concentrations of 0.031% or below.

3.3 Production of virulence factors

3.3.1 Extracellular protease

From the 23 isolates screened for protease production, the six isolates producing the highest levels were selected for further comprehensive testing. Results showed that although some reductions in protease levels were seen in the presence of tea tree oil for most of the isolates, whether these reductions were significant or not depended on which statistical test was used for analysis. Significant reductions in protease production were seen for isolates NCTC 6571, NCTC 7121 and 44, but not for isolates 20, 34 and 41 (Figs. 3.2 and 3.3).

Table 3.1. Biotyping and tea tree oil susceptibility of *S. aureus* isolates

Isolate number	Biotyping				Tea tree oil*	
	β-Haemolysis	Lipase	Lecithinase	Pigment	MIC	MBC
10442[a]	-	-	-	Y	0.5	1
10443[a]	-	-	-	C	0.25	1
10657[a]	++	+	+	W	0.25	0.5
7121[a]	++	+	-	W	0.25	0.5
6571[a]	-	-	-	W	0.5	1
6	-	+	-	Y		
7	-	+	+	C		
8	+	+	+	C	0.5	1
9	-	+	-	Y	0.5	2
10	+	+	+	Y	0.5	1
11	-	+	-	C	0.5	1
12	+	-	-	Y	0.5	1
13	-	+	+	C	0.5	1
14	-	+	-	C	0.5	1
15	-	-	-	Y	0.25	1
16	-	+	-	Y		
17	-	+	-	Y		
18	+	+	-	Y		
19	-	slight	+	C		
20	+	+	-	Y	0.5	2
21	-	-	-	C		
22	+	+	-	Y		
23	-	+	-	Y	0.5	1
24	+	+	+	C	0.5	0.5
25	-	+	-	Y	0.5	1
26	+	+	-	Y	0.5	1
27	-	+	-	Y	0.25	1
28	+	-	-	Y		
29	-	+	-	Y	0.5	1
30	-	+	-	Y		
31	-	+	-	Y	0.5	1
32	+	+	-	Y	0.5	2
33	+	slight	-	Y	0.5	2
34	-	+	-	Y	0.5	1
35	-	+	-	C		
36	-	-	-	W	0.12	1
37	-	+	-	Y		
38	-	+	-	Y	0.25	1
39	+	+	-	C	0.25	1
40	-	+	-	C		
41	-	+	-	C	0.12	1
42	-	-	-	Y		
43	+	+	-	C	0.25	1
44	-	-	-	C	0.25	1

[a] Reference strains (NCTC)

* MIC testing was not performed on all isolates. Blank cells indicate that no data were available

Key: + positive reaction; - negative reaction; Y yellow; C cream; W white.

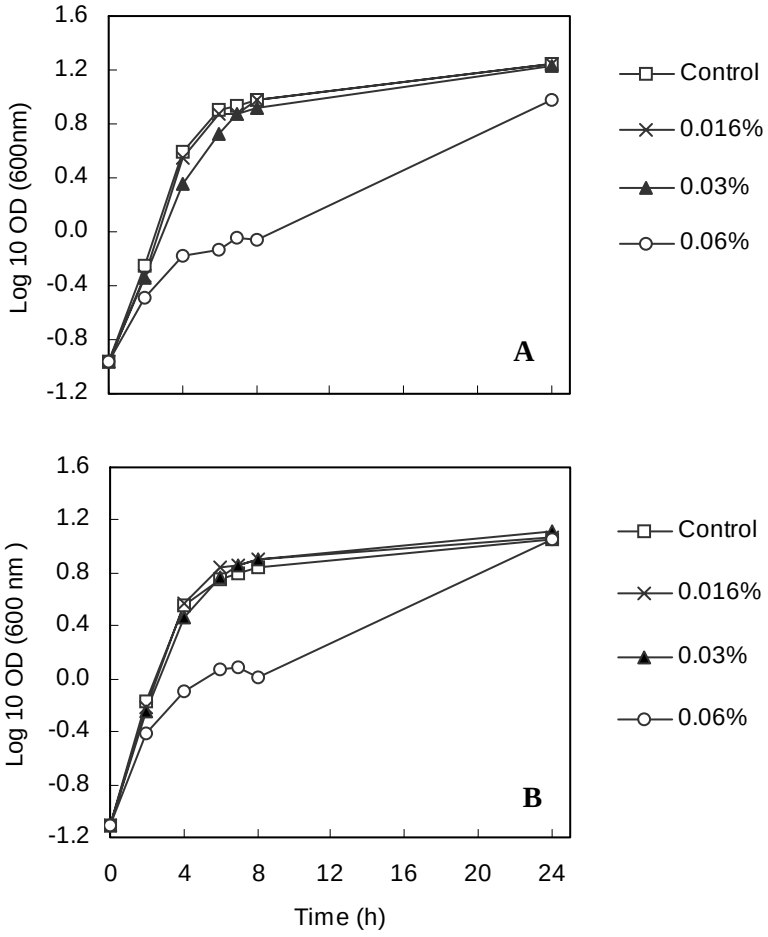

FIG 3.1. Representative growth curves of *S. aureus* NCTC 10443 (A) and NCTC 6571 (B) grown in BHIB in the presence of 0.016, 0.031 and 0.062% tea tree oil. Controls contained no tea tree oil.

For *S. aureus* NCTC 6571, protease levels were significantly reduced in the presence of 0.031 and 0.016% tea tree oil after 24 h, and in the presence of 0.031% tea tree oil after 8 h (2-tailed t-test, Excel). However, using Dunnett's multiple comparison test (GraphPad), the only reduction that differed significantly was that seen after 8 h treatment with 0.031% tea tree oil.

For isolate 44, protease levels in the presence of 0.031% tea tree oil were significantly lower than controls after 8 h only (2-tailed t-test; Dunnett's multiple comparison test).

Finally, using Dunnett's multiple comparison test, protease levels produced by *S. aureus* NCTC 7121 differed significantly from controls at all three time points, after treatment with

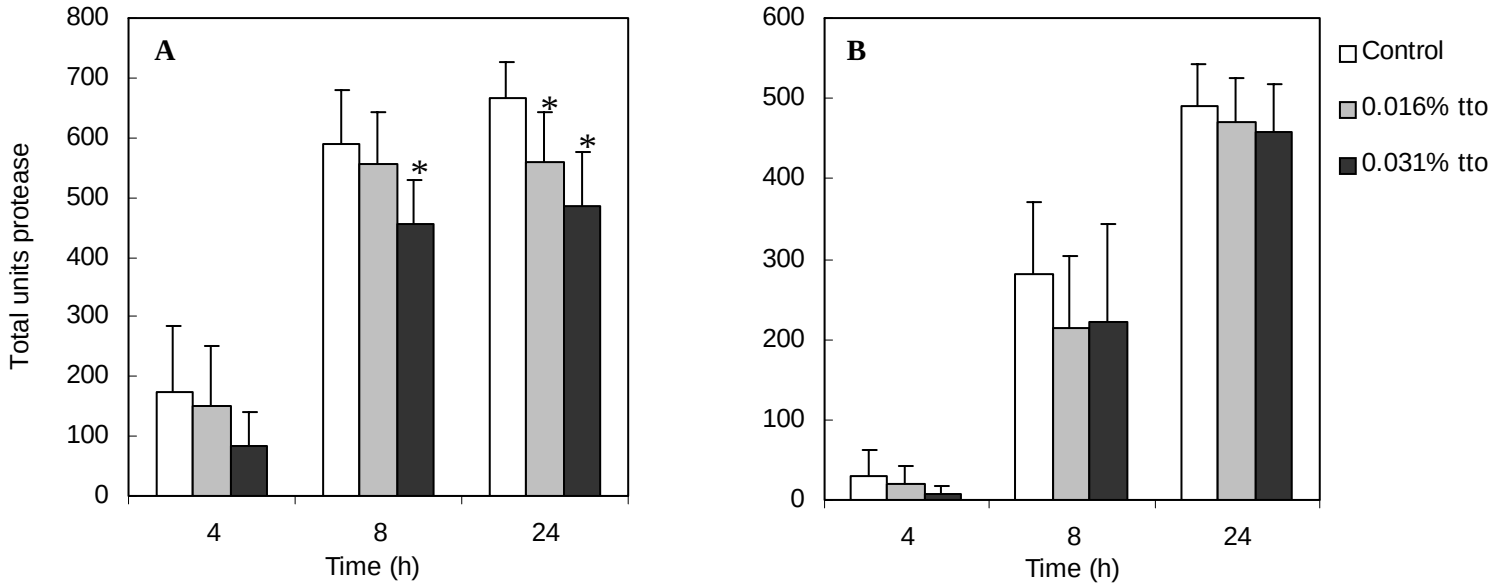

Fig 3.2. Mean (+ SD) protease production by *S. aureus* NCTC 6571 (A) and NCTC 7121 (B). Note that scales differ on each figure. *Values differ significantly from controls (2-tailed t-test).

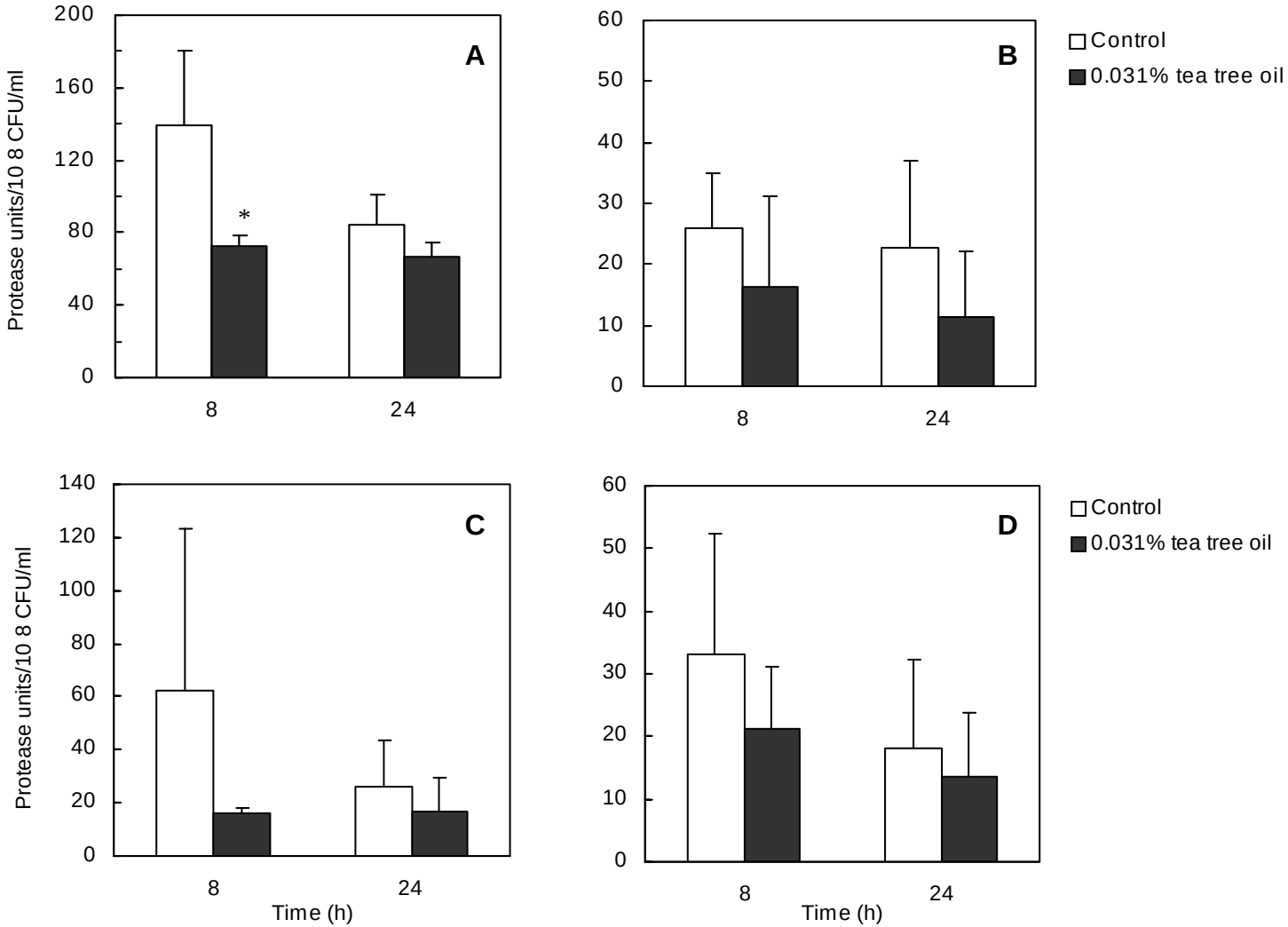

Fig 3.3. Mean (+ SD) protease production by *S. aureus* clinical isolates 44 (A), 34 (B), 20 (C) and 41 (D). Note that scales differ on each figure. No values differed significantly from controls (2-tailed t-test).

both 0.016 and 0.03% tea tree oil. However, these differences were not significant when analysed using t-tests.

The growth of all *S. aureus* isolates in the presence of 0.016 or 0.031% tea tree oil was not adversely affected, as determined by comparing the viable count data from each treatment at each time point to the viable count data for control cells (2-tailed t-test, Excel) (Table 3.2).

Results for total protease production indicated that most isolates produced relatively low levels of protease. Based on these initial results, additional testing to determine levels of *S. aureus* serine protease (SASP), which is a component of the total extracellular protease, was not pursued.

3.3.2 Coagulase assays

A total of 12 isolates was tested for the production of coagulase after incubation in the presence and absence of 0.031% tea tree oil (Table 3.3). Those isolates producing the highest levels of coagulase were isolates 11, 12 and 34. Of all isolates, one produced less coagulase in the presence of tea tree oil, three produced equivalent amounts and the remaining eight produced more coagulase in the presence of tea tree oil, when compared to controls.

Statistical analysis of coagulase titres showed that only four isolates (NCTC 10442, 14, 20 and 33) showed significant differences, and all showed significantly increased coagulase titres in the presence of tea tree oil. Differences in coagulase production were not significant for the remaining isolates. Of the four isolates with significantly increased coagulase, only one also showed a significant difference in viable count. For this organism the count for cells grown with tea tree oil was significantly lower than for control cells, but the coagulase titre was significantly higher in spite of this.

Mean viable counts in the presence of 0.031% tea tree oil were lower than controls for nine isolates. However, this difference was significant for isolates NCTC 7121, 11, 33 and 44 only. Mean viable counts in the presence of 0.031% tea tree oil were higher than controls for the remaining three, although this difference was not significant.

Table 3.2. Mean (SD) viable counts (cfu/ml) for isolates grown in the presence of tea tree oil for protease assays.

Isolate	Treatment	Time (h)		
		4	8	24
NCTC 6571	Control	2.67×10^9 (1.78×10^9)	5.87×10^9 (1.69×10^9)	1.03×10^9 (2.41×10^9)
	0.016%	2.59×10^9 (1.40×10^9)	5.83×10^9 (1.64×10^9)	1.12×10^9 (3.34×10^9)
	0.031%	2.63×10^9 (1.50×10^9)	6.09×10^9 (1.98×10^9)	1.12×10^{10} (3.92×10^9)
NCTC 7121	Control	1.83×10^9 (7.49×10^8)	3.64×10^9 (7.94×10^8)	3.34×10^9 (2.00×10^9)
	0.016%	1.59×10^9 (6.58×10^8)	3.70×10^9 (1.15×10^9)	2.75×10^9 (1.32×10^9)
	0.031%	1.44×10^9 (8.92×10^8)	4.26×10^9 (1.58×10^9)	3.98×10^9 (9.31×10^8)
20	Control	-	7.17×10^9 (2.67×10^9)	1.06×10^{10} (4.22×10^9)
	0.031%	-	6.51×10^9 (2.04×10^9)	8.54×10^9 (3.45×10^9)
34	Control	-	5.54×10^9 (1.81×10^9)	1.31×10^{10} (6.99×10^9)
	0.031%	-	6.94×10^9 (4.86×10^9)	1.30×10^{10} (3.17×10^9)
41	Control	-	4.79×10^9 (9.91×10^8)	5.10×10^9 (1.67×10^9)
	0.031%	-	5.95×10^9 (5.47×10^8)	5.26×10^9 (1.62×10^9)
44	Control	-	5.66×10^9 (1.70×10^9)	8.50×10^9 (2.32×10^9)
	0.031%	-	6.18×10^9 (1.50×10^9)	8.18×10^9 (2.15×10^9)

Table 3.3 Coagulase production and viable counts after incubation for 16 h with 0.031% tea tree oil

Isolate	Coagulase titre*			Viable counts (mean ± SD)		
	Control	0.03% TTO	*P* value[a]	Control	0.031% TTO	*P* value
NCTC 7121	256	128	0.164	2.59×10^9 (7.6×10^8)	1.76×10^9 (5.5×10^8)	**0.032**
NCTC 10442	128	512	**0.037**	1.79×10^9 (2.8×10^8)	1.14×10^9 (4.2×10^7)	0.054
NCTC 6571	64	512	0.608	2.62×10^9 (1.3×10^9)	1.74×10^9 (2.9×10^8)	0.439
9	256	> 2048	0.151	3.45×10^9 (1.0×10^9)	3.60×10^9 (3.9×10^9)	0.943
11	> 2048	> 2048	0.396	3.14×10^9 (9.2×10^8)	1.39×10^9 (1.0×10^9)	**0.045**
12	1024	1024	0.321	2.56×10^9 (4.3×10^8)	1.76×10^9 (2.1×10^8)	0.065
14	32	1024	**0.011**	2.92×10^9 (1.1×10^8)	2.87×10^9 (8.0×10^8)	0.950
20	64	> 2048	**0.003**	1.09×10^9 (1.2×10^9)	1.37×10^9 (2.9×10^8)	0.719
33	32	2048	**0.024**	2.76×10^9 (8.4×10^8)	1.02×10^9 (5.6×10^8)	**0.006**
34	> 2048	> 2048	0.884	3.30×10^9 (9.1×10^8)	2.15×10^9 (3.2×10^8)	0.150
41	128	> 2048	0.246	1.13×10^9 (2.9×10^8)	1.34×10^9 (3.0×10^8)	0.399
44	16	256	ND	3.05×10^9 (7.1×10^7)	1.06×10^9 (2.0×10^8)	**0.026**

* Note that a higher titre indicates more coagulase activity. Statistically significant values are in bold type.

[a] Derived by determining the Log_2 values for each titre and comparing values using the student's 2-tailed t-test. Off-scale values were converted to the next highest value (eg > 2048 was converted to 4096). ND, not determined.

3.3.3 Toxin assays

Results of the screening of isolates for toxin production are shown in Table 3.4. Of the 26 isolates screened, 20 (77%) were positive for one or more toxin (excluding ± reactions). Those isolates negative for the production of any toxin were NCTC 6571, NCTC 7121, 10, 14, 19 and 37. Of toxin positive isolates, 11 (55%) produced only one toxin, five (25%) produced only two toxins and four (20%) produced more than two.

Table 3.4 Screening of *S. aureus* isolates for the production of toxins

Toxin	Isolate number	Number (%) of strains positive
Enterotoxin A	10657, 9, 13, 15, 24, 27, 29, 31, 34, 36	10 (38.5%)
Enterotoxin B	10442, 10443, 10657, 13, 24, 26, 27, 44	8 (30.8%)
Enterotoxin C	13, 24, 25, 26, 27, 32, 33, 41	8 (30.8%)
Enterotoxin D	11, 13, 24, 26, 27	5 (19.2%)
Toxic shock syndrome toxin	12, 13, 15, 26, 32, 36, 41	7 (26.9%)

Each isolate produced toxin at varying levels. Therefore only those producing relatively high levels were selected for further investigation. A total of eight isolates was investigated for the production of enterotoxins (Table 3.5) and two isolates were investigated for the production of TSST (Table 3.6) in the presence of tea tree oil.

Analysis of viable count data showed that for all isolates except isolate 11, counts of cells grown with 0.062% tea tree oil were significantly lower than control cells after 24 h. For isolates 33 and 34 counts were also significantly lower when cells were grown with 0.031% tea tree oil. Lastly, for isolate NCTC 10442, the numbers of cells grown with 0.016% tea tree oil differed significantly from controls ($P = 0.049$), with counts being significantly higher in the presence of tea tree oil than in the absence.

Table 3.5 Production of enterotoxins and viable counts of isolates after 24 h incubation with tea tree oil.

		Toxin production		Viable count (mean (SD))	
Toxin	Treatment	Titre	*P* value[a]	CFU/ml	*P* value[b]
Enterotoxin A					
29	Control	2048		1.80×10^{10} (1.37×10^{9})	-
	0.016%	2048	0.423	2.08×10^{10} (1.92×10^{9})	0.123
	0.031%	1024	0.101	1.56×10^{10} (3.03×10^{9})	0.301
	0.062%	16	**0.001**	2.83×10^{9} (2.29×10^{9})	**0.002**
34	Control	2048		1.97×10^{10} (1.48×10^{9})	-
	0.016%	2048	0.230	2.17×10^{10} (3.09×10^{9})	0.396
	0.031%	1024	0.101	1.59×10^{10} (6.03×10^{8})	**0.034**
	0.062%	128	**0.008**	4.86×10^{9} (4.38×10^{9})	**0.001**
44	Control	256		9.07×10^{9} (2.93×10^{9})	
	0.016%	256	0.423	9.47×10^{9} (1.46×10^{9})	0.846
	0.031%	128	0.184	2.38×10^{9} (3.75×10^{8})	0.056
	0.062%	< 4	**0.002**	7.67×10^{8} (1.08×10^{9})	**0.026**
Enterotoxin B					
10443	Control	131072		1.10×10^{10} (1.70×10^{9})	-
	0.016%	65536	0.057	1.29×10^{10} (1.30×10^{9})	0.107
	0.031%	65536	0.057	8.54×10^{9} (2.14×10^{9})	0.109
	0.062%	128	**0.000**	1.92×10^{8} (9.83×10^{7})	**0.013**
10442	Control	524288		1.13×10^{10} (4.21×10^{9})	
	0.016%	131072	**0.013**	1.39×10^{10} (4.40×10^{9})	**0.049**
	0.031%	131072	**0.003**	8.51×10^{9} (5.51×10^{9})	0.905
	0.062%	32	**0.000**	1.46×10^{9} (1.86×10^{9})	**0.010**

Table 3.5 cont. Production of enterotoxins and viable counts of isolates after 24 h incubation with tea tree oil.

		Toxin production		Viable count (mean (SD))	
Toxin	Treatment	Titre	*P* value[a]	CFU/ml	*P* value[b]
Enterotoxin C					
33	Control	524288		1.39×10^{10} (3.70×10^{8})	
	0.016%	131072	0.193	1.41×10^{10} (2.60×10^{9})	0.868
	0.031%	32768	**0.037**	9.83×10^{9} (4.93×10^{8})	**0.001**
	0.062%	256	**0.002**	2.58×10^{9} (4.26×10^{9})	**0.044**
41	Control	131072		8.82×10^{9} (1.88×10^{9})	
	0.016%	65536	0.101	1.01×10^{10} (1.49×10^{9})	0.404
	0.031%	65536	0.057	9.85×10^{9} (2.86×10^{9})	0.633
	0.062%	4096	**0.000**	3.75×10^{9} (5.93×10^{8})	**0.035**
Enterotoxin D					
11	Control	2048		1.61×10^{10} (9.07×10^{8})	
	0.016%	2048	0.423	1.57×10^{10} (1.87×10^{9})	0.618
	0.031%	2048	-	1.31×10^{10} (2.61×10^{9})	0.492
	0.062%	1024	0.103	6.75×10^{8} (6.86×10^{8})	**0.021**

[a] *P* value obtained by calculating the Log2 value of each titre and analysing data using Student's 2-tailed t-test (treatment versus control)
[b] *P* value obtained by comparing the viable count from each tea tree oil treatment to the control using Student's 2-tailed t-test

Table 3.6 Production of toxic shock syndrome toxin and viable counts of isolates after 24 h incubation with tea tree oil.

			Toxin production		Viable count (mean (SD))	
Toxin		Treatment	Titre	*P* value[a]	CFU/ml	*P* value[b]
TSST						
	15	Control	8192		9.80×10^9 (3.02×10^9)	
		0.016%	2048	**0.025**	1.37×10^{10} (1.99×10^9)	0.139
		0.031%	1024	**0.003**	1.14×10^{10} (1.05×10^9)	0.461
		0.062%	< 4	**0.000**	8.57×10^8 (1.43×10^9)	**0.021**
	36	Control	512		9.35×10^9 (7.05×10^8)	
		0.016%	256	0.057	1.01×10^{10} (1.54×10^9)	0.502
		0.031%	64	**0.010**	4.87×10^9 (2.37×10^9)	0.071
		0.062%	< 4	**0.008**	8.31×10^8 (6.83×10^8)	**0.000**

[a] *P* value obtained by calculating the Log2 value of each titre and analysing data using Student's 2-tailed t-test (treatment versus control)
[b] *P* value obtained by comparing the viable count from each tea tree oil treatment to the control using Student's 2-tailed t-test

Statistical analysis of toxin titres showed that toxin production was significantly reduced in the presence of 0.062% tea tree oil for all isolates except isolate 11. Toxin titres were also significantly lower after growth in the presence of 0.031% tea tree oil for isolates NCTC 10442, 33, 15 and 36. Significant decreases in toxin titre were also seen after growth in 0.016% for isolates NCTC 10442 and 15. In general, toxin titres were significantly reduced in parallel with a significant reduction in viable count. Instances where toxin titre was significantly decreased in the absence of a significant decrease in viable count were NCTC 10442 (ETB) with 0.031% tea tree oil, 15 (TSST) with 0.016 and 0.031% tea tree oil, and 36 (TSST) with 0.031% tea tree oil.

Interestingly, all isolates except isolate 11 showed higher mean viable counts with 0.016% tea tree oil compared to control cells, although this increase was significant for only one isolate. Despite the slightly higher numbers of viable cells, five of the eight isolates tested (15, 36, NCTC 10443, NCTC 10442 and 33) showed decreased toxin titres in the presence of 0.016% tea tree oil compared to controls. This difference in titre was significant for isolates 15 (TSST) and NCTC 10442 (ETB) only. For the remaining three isolates toxin titres for cells grown with 0.016% tea tree oil were the same as those for control cells. No isolate showed increased toxin production in the presence of tea tree oil.

3.3.4 Biofilm assays

Isolates NCTC 6571, 44, 39, 36, 33 and 26 were assessed for biofilm formation in the presence of several concentrations of tea tree oil. The growth of each isolate in the presence of tea tree oil was assessed both visually and by subculture prior to biofilm staining (Table 3.7). For most isolates, growth was not visible at concentrations of 0.5% and above, although subcultures revealed that low numbers of organisms remained viable at concentrations of 0.5 – 2.0% tea tree oil.

A typical representation of a stained biofilm tray is shown in Fig 3.4, and biofilm formation by each isolate (as measured by crystal violet staining) is shown graphically in Fig 3.5. For all isolates, biofilm formation was significantly reduced in the presence of 0.5, 1.0 and 2.0% tea tree oil, which for the most part corresponds to the concentrations of tea tree oil that also prevented visible growth. In addition, several trends were seen with regard to the effects of tea tree oil on biofilm formation. For isolates 36 and 44, biofilm formation was significantly

Table 3.7 Growth of *S. aureus* in the biofilm assay as determined visually and by subculture

Isolate	Highest tea tree oil % with turbid growth (determined visually)		Highest tea tree oil % with growth upon subculture*	
	Range	Mode	Range	Mode
NCTC 6571	0.12 - 0.25	0.25	0.25 – 1	0.25
26	0.12 - 0.25	0.25	0.25 – 2	0.5
33	0.12 - 0.25	0.25	0.25 – 0.5	0.5
36	0.12 - 0.25	0.25	0.25 - 2	2
39	0.25 – 0.5	0.25	0.25 - 1	0.5
44	0.25 - 0.5	0.5	0.5 - 2	2

* More than one colony present

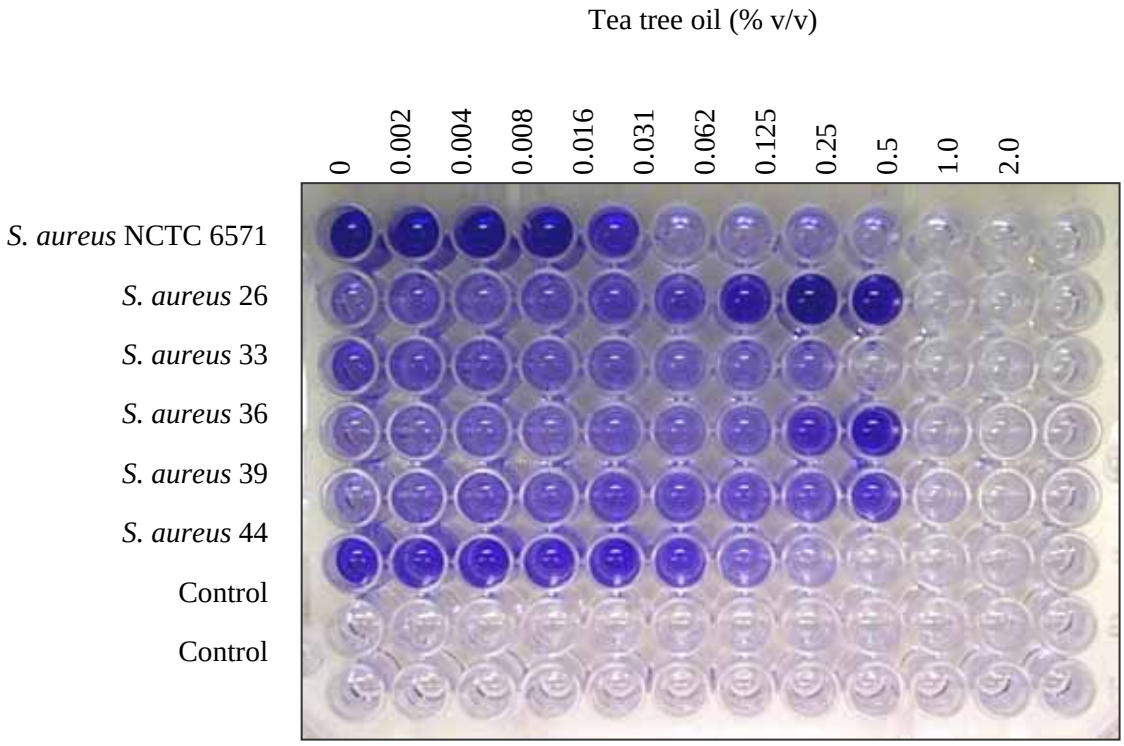

Fig 3.4 Crystal violet staining of biofilm formation in the presence of tea tree oil in a 96-well microtitre tray.

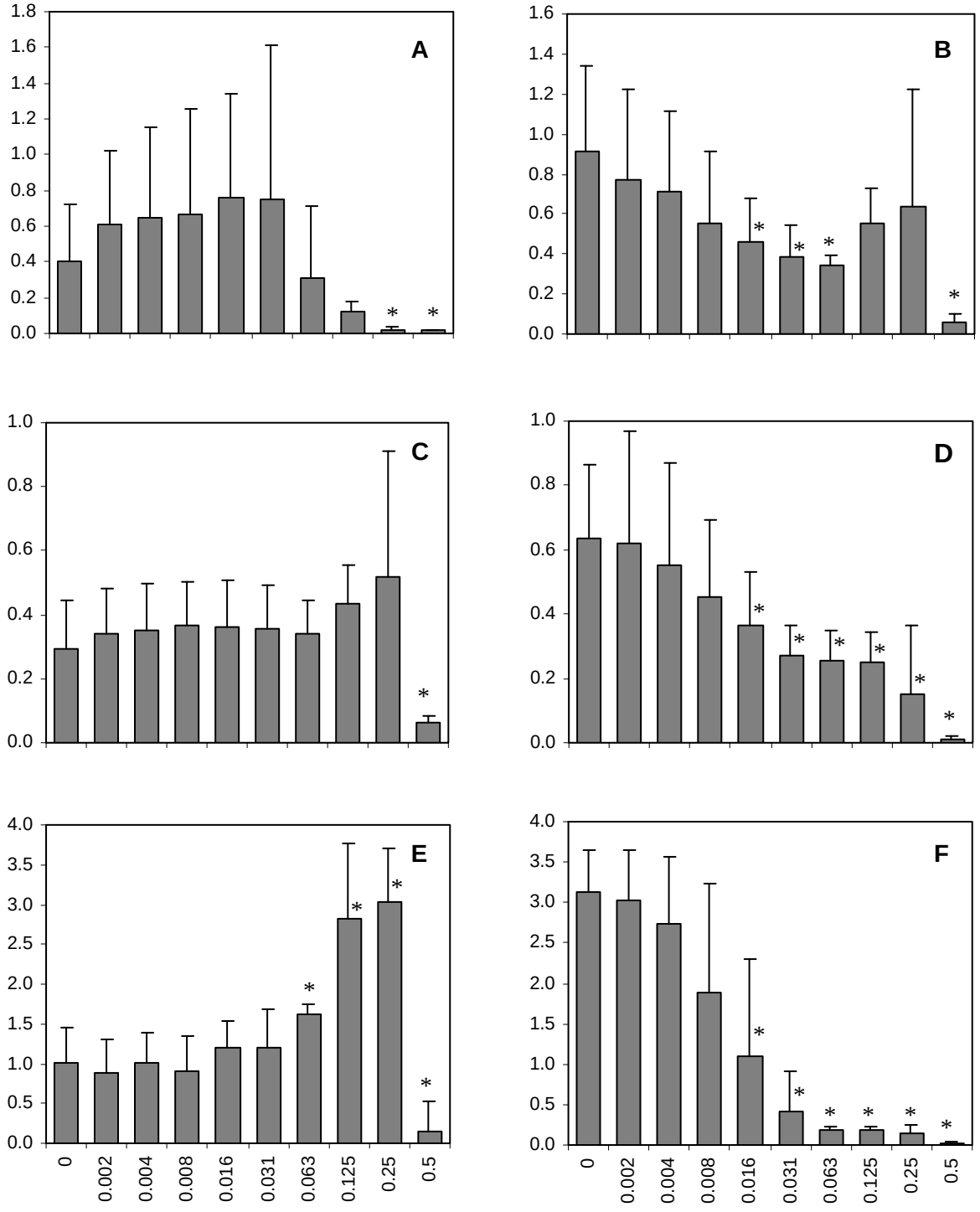

Fig 3.5. Biofilm formation by *S. aureus* isolates NCTC 6571 (A), 33 (B), 26 (C), 36 (D), 39 (E) and 44 (F) in the presence of tea tree oil. Mean + standard deviation. Bars marked with an asterisk differ significantly from controls ($P < 0.05$).

reduced compared to controls at concentrations of 0.016% and above, although turbid growth was apparent at all concentrations up to and including 0.25% for isolate 36 and 0.5% for isolate 44. For isolate 33, biofilm formation was significantly reduced at 0.016, 0.031 and 0.062% tea tree oil, but not at concentrations of 0.125 and 0.25%. For isolate 39, biofilm formation was significantly increased in the presence of 0.062, 0.125 and 0.25% tea tree oil, and was significantly decreased at concentrations above this. Lastly, no dramatic changes in biofilm formation were evident for isolates NCTC 6571 and 26, with significant reductions occurring at concentrations of ≥ 0.25% and ≥ 0.5% for each isolate, respectively.

Chapter 4: Discussion

4.1 Effects of tea tree oil on the production of virulence factors

The VFs produced by *S. aureus* enable this pathogen to cause a wide range of infections and diseases. It was therefore of great interest to establish whether the presence of tea tree oil affected the production of these VFs. Results obtained in the present study showed that the production of protease and toxins by *S. aureus* was reduced in the presence of tea tree oil, although these decreases were generally accompanied by corresponding decreases in cell densities. In contrast, levels of coagulase were increased in the presence of tea tree oil. Analysis of biofilm formation in the presence of tea tree oil showed mixed results, with instances of both increased and decreased biofilm formation. These findings are discussed in depth below.

4.1.1 Protease

The investigation of the production of extracellular protease showed that for some isolates levels in the presence of tea tree oil were significantly lower than control levels, in the absence of any significant decrease in cell numbers. However, this effect varied according to which isolate was tested, with the most reproducible results obtained for those isolates producing protease at the highest levels.

Very few previous studies have been conducted examining the effects of subinhibitory antimicrobial agents on the production of protease. Protease production by the Gram negative bacterium *Shigella* spp. was reduced in the presence of 0.75 and 1.0 μg/ml *Ocimum gratissimum* essential oil (Iwalokun *et al.*, 2003). However, cell density was not measured so no meaningful conclusions can be drawn about whether these results were merely a reflection of a reduction in cell density. Another study, using two *S. aureus* strains, found that the presence of silver sulphadiazine (AgSD) did not significantly alter protease production after 24 h, compared to the controls (Edwards-Jones and Foster, 2002). However, the production of protease appeared to be delayed in the presence of AgSD. Their study also showed that for one of the isolates the proportions of proteases varied greatly, with control cells producing largely metalloprotease whereas cells grown with AgSD produced only a very small amount of metalloprotease. It may therefore be of interest to investigate if any such effects also occur during treatment with tea tree oil.

Investigation of protease production in the present study showed that the presence of tea tree oil did not cause a specific, complete halt to protease production by *S. aureus*. Rather, there was an incremental, dose-dependent decrease in protease production in the presence of increasing tea tree oil. It would therefore seem logical that even less protease would be produced in the presence of an even higher tea tree oil concentration such as 0.062%. However, this concentration is also high enough to become growth inhibiting, and therefore any decreases in protease levels would in all likelihood simply be a reflection of an overall decrease in cell density.

It has been observed previously that the suppression of exoprotein synthesis occurs only with antibiotics that inhibit protein synthesis and that antibiotics with other modes of action may in fact have a stimulatory effect (Herbert *et al.*, 2001). Although tea tree oil is not an antibiotic as such, an extrapolation of the above generalisation is that tea tree oil does not act by inhibiting the synthesis of the extracellular enzyme protease.

4.1.2 Coagulase

In contrast to the results obtained for the production of protease, coagulase levels were increased in the presence of 0.031% tea tree oil for most *S. aureus* isolates, when compared to controls. Previous studies with sub-inhibitory concentrations of other antimicrobial agents have shown either a reduction, or no effect on coagulase levels after incubation with sub-inhibitory levels of each

compound. Those shown to reduce coagulase include the protein synthesis inhibitors chloramphenicol, tetracycline, gentamicin (Doss *et al.*, 1993) and linezolid (Gemmell and Ford, 2002), whilst those showing little or no effect include ciprofloxacin, enoxacin, methicillin (Doss *et al.*, 1993) and silver sulphadiazine (Edwards-Jones and Foster, 2002). In contrast, whilst clindamycin was shown to stimulate the transcription of the gene encoding coagulase (Herbert *et al.*, 2001), no corresponding increase in measurable coagulase levels was seen. Since coagulase is a secreted protein, and several of the antibiotics mentioned above are inhibitors of protein synthesis, it would seem logical that these agents reduce coagulase levels by directly inhibiting protein synthesis. With regard to non-antibiotics, two studies assessing the effects of *Nepeta cataria* (catnip) and *Helichrysum italicum* extracts on coagulase production both found that treatment with these extracts had little effect on coagulase levels (Nostro *et al.*, 2001a; Nostro *et al.*, 2001b). Although these two extracts are not closely related to tea tree oil in terms of their chemical composition, the results are not incompatible with those from the present study, with all results indicating that plant antimicrobial compounds do not appear to reduce the production of coagulase by *S. aureus*.

There are several possible explanations for the apparent increases in coagulase levels after growth in the presence of sub-inhibitory tea tree oil. Exposure to tea tree oil may alter cell permeability leading to the leakage of coagulase. Alternatively, the tea tree oil may stimulate the production and/or cellular export of coagulase, maybe as a protective or defensive measure for the organism. It is also hypothetically possible that the presence of tea tree oil affects the outermost part of the cell wall in such as way as to cause the coagulase to become less highly associated with the cell wall and therefore more coagulase is found free in the supernatant. Increased expression of VFs after treatment with sub-inhibitory levels of antimicrobial agents has been reported previously, although infrequently. *S. aureus* isolates resistant to fluoroquinolones have increased expression of fibronectin-binding proteins after treatment with sub-inhibitory ciprofloxacin (Bisognano *et al.*, 1997). Another example is increased α-toxin production by *S. aureus* after treatment with sub-inhibitory levels of the β-lactam antibiotics nafcillin (Kernodle *et al.*, 1995) and methicillin (Doss *et al.*, 1993; Ohlsen *et al.*, 1998). The mechanisms behind the apparent increase in coagulase seen in the present study may be elucidated by assessing whether or not the gene encoding coagulase is expressed or transcribed more in the presence of tea tree oil. These results would help differentiate between increased export, decreased association with the cell wall, or increased coagulase production due to gene transcription. The investigation of coagulase levels at additional time points may also expand our understanding of this phenomenon, especially since the production of many virulence factors is known to be highly dependent on bacterial growth phase and cell density (Wright and Holland, 2003).

4.1.3 Toxins

Evaluation of toxin production by *S. aureus* showed that levels were generally decreased in the presence of tea tree oil in a dose-dependent manner, similar to that seen for the extracellular proteases. However, decreases in toxin levels were often accompanied by decreases in cell numbers, implying that reductions in toxin levels were most likely to be a reflection of reduced cell growth. Previous studies have shown that sub-inhibitory levels of clindamycin, flucloxacillin and gentamicin reduced levels of TSST (van Langevelde *et al.*, 1997) and linezolid reduced levels of SEA and SEB (Bernardo *et al.*, 2004). Again, these antibiotics act by preventing protein synthesis so a reduction in toxin production would seem logical. However, another study assessing 55 *S. aureus* strains for the production of TSST after treatment with sub-inhibitory levels of several topical antimicrobial agents found considerable strain-dependent variation (Edwards-Jones and Foster, 1994). In response to mupirocin, hydrogen peroxide and povidone iodine, most strains showed either no change or a decrease in production. In response to chlorhexidine, most strains showed no change although some were increased, and strains showed unchanged or increased TSST levels in response to AgSD (Edwards-Jones and Foster, 1994). These results highlight the complexity of the microbial response to subinhibitory levels of antimicrobial compounds.

In addition to studies with antibiotics, changes in toxin production in response to plant oils and extracts have also been investigated. Nostro *et al.* (2002) found that levels of enterotoxins A-D were

all reduced in the presence of *H. italicum* extract, but these reductions were accompanied by decreases in numbers of viable cells. Similarly, the production of enterotoxin by *Bacillus cereus* was decreased in the presence of 0.02, 0.04 and 0.06 mg/ml carvacrol, but these carvacrol concentrations also reduced viable counts and growth rates (Ultee and Smid, 2001). Lastly, the production of the toxin listeriolysin O by the Gram positive organism *Listeria monocytogenes* was reduced in the presence of sub-inhibitory concentrations of bay (*Pimenta racemosa*), cinnamon leaf (*Cinnamomum verum*), clove stem (*Syzigium aromaticum*), thyme (*Thymus vulgaris*) and nutmeg (*Myristica fragrans*) oils (Smith-Palmer *et al.*, 2002). These reductions in toxin levels occurred in the absence of any reduction in viable count.

Previous studies have indicated a range of responses of *S. aureus* to sub-inhibitory antimicrobial agents. Similar to the effects of tea tree oil on protease production, tea tree oil did not appear to cause any specific reduction in toxin production. This again suggests that tea tree oil does not cause a reduction in the expression of the toxin genes, although experiments measuring methods gene expression are required to support or refute this theory.

4.1.4 Biofilm formation

Few studies have assessed the effects of antimicrobial agents on the de novo formation of biofilm by *S. aureus*. Studies have more commonly assessed the effects of antimicrobial agents of pre-formed biofilm (Pitts *et al.*, 2003) or have investigated biofilm formation by *Staphylococcus epidermidis*, since this organism is well known for its slime or biofilm-producing capacity (Rupp and Hamer, 1998; Rachid *et al.*, 2000; Götz, 2002)

The *S. aureus* isolates investigated in this study showed responses ranging from no apparent effect to increased or decreased production, but for three of the six isolates biofilm was significantly decreased at very low, sub-inhibitory concentrations. These decreases may be due to tea tree oil inhibiting one or more of the factors that contribute to the formation of biofilm. These include the initial attraction and adhesion step, cell proliferation, cell-cell signalling and the production of extracellular polysaccharides to create the cellular matrix (Vuong *et al.*, 2000). Furthermore, there are many factors within each of these steps that contribute to the overall effectiveness of each stage. Additional studies to determine which of these stages or factors is most affected by the presence of tea tree oil are necessary before further generalisations about how the presence of tea tree oil reduces the formation of biofilm can be made.

In contrast to decreases in biofilm formation, several of the isolates appeared to have enhanced biofilm formation in the presence of tea tree oil, although increases were only significant for one of the isolates. These increases occurred at concentrations just below those preventing visible growth. The phenomenon of increased biofilm in the presence of particular agents or conditions has been observed previously. Biofilm formation in *Staphylococcus* spp. has been induced by unfavourable environmental conditions or agents such as detergents, urea, ethanol, and oxidative or osmotic stress (Rachid *et al.*, 2000; Götz, 2002). In addition, sub-inhibitory levels of the protein-synthesis inhibitors tetracycline and quinupristin-dalfopristin strongly induced the formation of biofilm by *S. epidermidis* (Rachid *et al.*, 2000). The increased production of biofilm under adverse conditions is postulated to be a survival strategy or a strategy to overcome unfavourable external conditions. By forming biofilm, the organism protects itself against external harm and this correlates with the observation that microbial biofilms are generally less susceptible to antimicrobial agents than planktonic or free bacteria are. In the study above examining the effects of tetracycline and quinupristin-dalfopristin on biofilm formation, they also observed that the *ica* operon of *S. epidermidis*, which is required for the synthesis of polysaccharide intercellular adhesin (PIA), was strongly induced in parallel with biofilm formation (Rachid *et al.*, 2000). Recent evidence has shown that the *ica* operon is also present in *S. aureus* (Vuong *et al.*, 2000) and it would be interesting to investigate whether those strains showing enhanced biofilm formation in the present study also show increased transcription of the *ica* operon.

Another genetic element thought to have a role in *S. aureus* biofilm formation is the

accessory gene regulator (*agr*) (Vuong *et al.*, 2000). This system regulates the synthesis of many virulence factors, and is responsible for down-regulating the production of cell wall-associated proteins and up-regulating secreted proteins in the late exponential growth phase (Mullarky *et al.*, 2001). The *agr* locus is also known as a quorum-sensing system, enabling cell-cell signalling, which is thought to have a critical role in the formation of biofilm. However, the role of *agr* in biofilm formation by *S. aureus* is not clear, with many *agr*-negative isolates producing the highest quantities of biofilm (Vuong *et al.*, 2000). In addition, the role of *agr* in biofilm formation appears to depend on the conditions under which the biofilm is grown (Yarwood and Schlievert, 2003). Although it is intriguing to speculate that tea tree oil may be affecting the formation of biofilm by interfering with cell-cell signalling, more information about whether the isolates used in this study contain the *agr* system, and how it is expressed, is required before any such conclusions can be drawn.

4.1.5 General discussion

Comparison of results from this study with previously published literature is not straightforward since many previous studies have investigated antimicrobial agents with a single, well-characterised mechanism of action, such as the inhibition of protein synthesis. It would perhaps be of greater relevance to compare results from the present study with results for compounds with antimicrobial mechanisms similar to those of tea tree oil, such as compounds known to interfere with cell membranes. Unfortunately only limited studies have been performed with such agents.

The presence of sub-inhibitory tea tree oil did not appear to cause any decreases in protein synthesis independent of decreases in cell density. These results suggest that tea tree oil does not act by inhibiting protein synthesis. However, the results from these assays cannot be extended to the production of all *S. aureus* VFs, and the responses of other VFs to sub-inhibitory tea tree oil remain to be determined. In particular, it would be of great interest to investigate the effects of tea tree oil on cell-surface hydrophobicity, adherence to eukaryotic cells or artificial surfaces and susceptibility to phagocytosis. Several different plant extracts alter the cell surface hydrophobicity of *Helicobacter pylori* (Annuk *et al.*, 1999), *E. coli*, *Acinetobacter baumannii* (Turi *et al.*, 1997) and *Streptococcus mutans* (Nostro *et al.*, 2004). The investigation of *S. aureus* VFs other than extracellular proteins may provide interesting results, as seen when the effects of tea tree oil on biofilm formation were investigated.

4.2 Implications for the use of tea tree oil in treating or preventing *S. aureus* infections

The in vitro activity of tea tree oil against *S. aureus*, including methicillin-resistant isolates, has been the subject of many publications and is indisputable (Carson *et al.*, 1995a; Carson *et al.*, 1995b; Carson *et al.*, 2002). These previous in vitro findings have collectively suggested that tea tree oil could be useful for eradicating *S. aureus* carriage or treating infection.

Two studies have investigated this very possibility, both assessing the effectiveness of tea tree oil products in the eradication of MRSA carriage (Caelli *et al.*, 2000; Dryden *et al.*, 2004). Data from the first study showed that tea tree oil formulated into nasal ointment and skin wash compared favourably to mupirocin nasal ointment and Triclosan skin wash for the decolonisation of MRSA carriage (Caelli *et al.*, 2000). Similarly, the second study indicated similar clearance rates for both the tea tree oil regimen and standard treatment (Dryden *et al.*, 2004). These clinical outcomes may have occurred by any number of mechanisms. Firstly, the topically applied tea tree oil may have had a direct killing effect against the target organisms. However, it has been postulated that antimicrobial agents are often present at only sub-inhibitory levels, and cannot therefore produce killing effects. This means that sub-inhibitory levels of tea tree oil may somehow responsible for eradicating *S. aureus* carriage or infection. Sub-inhibitory tea tree oil may have any number of effects against the infecting organism, host cells, and any interactions between the two. Since data from this study suggest that extracellular VFs such as coagulase, protease and toxins are not reduced in the presence of tea tree oil, a reduction in the levels of these proteins is not likely to be the mechanisms by which

tea tree oil is acting. One possibility is that the tea tree oil alters cells of *S. aureus* in such a way as to render them susceptible to the effects of the host immune system. This mechanism and others require investigation.

Chapter 5: Conclusions and recommendations

Data from this study showed that levels of extracellular proteases and toxins do not appear to be specifically inhibited by sub-inhibitory tea tree oil. Instead, reductions were for the most part a function of a decrease in cell density. In contrast, investigation of the protein coagulase indicated that levels were either unaffected or increased in the presence of tea tree oil. Lastly, for some isolates, the formation of biofilm was significantly reduced in the presence of sub-inhibitory tea tree oil, but for other isolates no such effect was seen. This study has demonstrated that although the presence of tea tree oil does affect the production of VFs by *S. aureus* in vitro, it does not necessarily cause a reduction. Exactly how these in vitro findings may relate to in vivo effects remains unclear.

Given that the expression of VFs has such a central role in bacterial pathogenicity and disease, it would have been a positive outcome to find that their production was significantly reduced or even completely suppressed by the presence of low levels of tea tree oil. Even though this result was not found, several other interesting effects were observed. The increase in coagulase levels in the presence of tea tree oil could not readily be explained, and further work to investigate this phenomenon is warranted. Also, the range of effects of tea tree oil on biofilm formation was intriguing and also requires further investigation. Much of the work in the present study was focused on investigating the production of extracellular proteins. Further studies investigating characteristics that are either distantly or unrelated to the production of extracellular proteins, such as the susceptibility of cells to phagocytosis or changes in adherence characteristics, may reveal more promising results.

Although these in vitro studies are both informative and revealing, the effectiveness of tea tree oil in preventing, treating and eradicating *S. aureus* colonisation or infection will ultimately be determined by clinical studies. More of these studies are urgently required and continue be a priority for stakeholders in the tea tree oil industry.

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