Assessing the *in situ* efficacy of tea tree oil as a topical antiseptic

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Foreword

This report describes results of experiments conducted to examine the antimicrobial activity of tea tree oil and tea tree oil products. Results were obtained using several different European standard methods and as such represent novel findings.

The cost of hospital-acquired infections has been estimated to be in excess of 1 billion dollars for Australia alone. The transfer of pathogenic bacteria via the hands of health care workers is often the cause. Previous research has shown that tea tree oil kills the transient skin microorganisms that are largely responsible for hospital-acquired infections, while having less of an effect against the commensal bacteria of the skin. However, the test method used was simple MIC determination, which cannot take into account the properties of the skin on which these microorganisms reside.

The specific objectives of this study were firstly to examine the antimicrobial properties of tea tree oil-based skin antiseptics by three different methods and then to assess activity using an FDA approved hand wash method. The study showed that the antibacterial effects demonstrated by tea tree oil differed according to the method of assessment that was used. The \textit{ex-vivo} method was a closer approximation to the \textit{in-vivo} situation and it may take into account the skin penetrative properties of tea tree oil that cannot be assessed using traditional methods.

The acceptability of a tea tree oil hand wash has been demonstrated to be high in preliminary studies. If such hand or body washes could be shown to be user friendly and efficacious, they may be considered as alternatives to conventional products in hospitals and other health care settings.

This project was funded from industry revenue 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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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A. baumannii</td>
<td>Acinetobacter baumannii</td>
</tr>
<tr>
<td>AHR</td>
<td>Alcoholic hand rub</td>
</tr>
<tr>
<td>AHSW</td>
<td>Alcoholic hygienic skin wash</td>
</tr>
<tr>
<td>B. cereus</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DCM</td>
<td>Drop counting method</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle’s Balanced Salt Solution</td>
</tr>
<tr>
<td>GA</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>HAI</td>
<td>Hospital acquired infection</td>
</tr>
<tr>
<td>HP</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HSW</td>
<td>Hygienic skin wash</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PVI</td>
<td>Povidone iodine</td>
</tr>
<tr>
<td>QCT-1</td>
<td>Quantitative carrier test 1</td>
</tr>
<tr>
<td>QCT-2</td>
<td>Quantitative carrier test 2</td>
</tr>
<tr>
<td>RF</td>
<td>Reduction factor</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>SDW</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>SH</td>
<td>Sodium hypochlorite</td>
</tr>
<tr>
<td>TTO</td>
<td>Tea tree oil</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume for volume</td>
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</tbody>
</table>
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Executive Summary

Background
The essential oil of *Melaleuca alternifolia*, also known as tea tree oil, has been used as a topical antimicrobial for almost 100 years. Due to its widespread popularity, tea tree oil is currently incorporated into many pharmaceutical and cosmetic products. Tea tree oil exhibits broad-spectrum antibacterial activity when assessed in-vitro. However, there are few reports on the activity of tea tree oil on human skin in controlled studies. Two European standard suspension test methods were used to assess the activity of tea tree oil and tea tree oil products against *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli*. Suspension test EN 1276 is designed for evaluating the activity of antiseptics and disinfectants that are used in domestic and industrial settings. Products eliciting a reduction in bacterial counts of > 5 log₁₀ after 5 minutes are deemed to have passed the test. The second suspension test, the proposed method prEN 12054, is designed for the testing of hygienic and surgical hand rub and hand wash products. To pass this test, products must produce a reduction of 2.52 log₁₀ for a hygienic hand wash and 4.52 log₁₀ for a hygienic hand rub after a 1 minute exposure.

Objectives/Methods
The objectives of this study were to firstly examine the in-vitro antimicrobial and sporicidal properties of tea tree oil-based skin formulations using European standard methods, and then to investigate their activity directly on human skin using an “ex-vivo” test as well as on volunteers in-vivo according to the European standard method. The test products were a 5% tea tree oil hygienic skin wash (HSW), a 5% tea tree oil alcohol hygienic skin wash (AHSW), and a 3% tea tree oil alcohol hand rub (AHR). In most experiments, the activity of solutions containing 1–10% tea tree oil was also assessed for comparison, as was povidone iodine, a commonly useful hospital hand wash.

Results
A reduction in bacterial counts of > 5 log₁₀ after 5 minutes was seen when the AHSW and AHR were tested against all test organisms. However, the HSW produced a > 5 log₁₀ decrease after 5 minutes for *A. baumannii* and *E. coli* only. None of the tea tree oil solutions produced a > 5 log₁₀ decrease against *S. aureus* or *A. baumannii*, whereas solutions of 1, 4, 5, 6, 8 and 10% did against *E. coli*, and solutions of 4, 8 and 10% did against *P. aeruginosa*. Povidone iodine passed the test with all organisms. When the products were tested in the presence of bovine serum albumin to simulate ‘dirty’ conditions, the AHSW and AHR retained their activity against all organisms except *E. coli*. In contrast, most of the tea tree oil solutions that passed the test under clean conditions did not pass when tested under ‘dirty’ conditions. The AHR, AHSW and povidone iodine all passed the proposed method prEN 12054, against all test organisms. The tea tree oil solutions were not tested using this method.

The activity of 5% tea tree oil, HSW and povidone iodine was further assessed using two surface tests. These are also known as capacity tests and are used to imitate microbial attachment to environmental surfaces, including short-term attachment and drying. Activity was assessed with the European standard method using stainless steel carriers (EN 13697, 1997) and the American surface test using glass carriers (ASTM 2111-00, 2000) against *S. aureus*, *A. baumannii*, *E. coli* and *P. aeruginosa*. To pass these tests, products must elicit a ≥ 4 log₁₀ reduction in cell numbers after 5 minutes contact. Povidone iodine (7.5%) passed both the European and American tests against all organisms. With regard to tea tree oil, the only product that passed the European method was 5% TTO in Tween 80 against *E. coli*. Using the American method, only 5% TTO in Tween 80 passed against *A. baumannii*.
and *P. aeruginosa*. Although reductions were less than 4 log₁₀, 5% tea tree oil significantly reduced viability in all of the remaining test organisms, by both methods. With regard to the HSW, a significant reduction in cell viability after 5 minutes was seen for *S. aureus*, *A. baumannii* and *E. coli* when compared to untreated controls using the European standard method. However, no significant reductions in cell numbers were seen for the HSW using the American standard method.

The sporicidal activity of tea tree oil and tea tree oil products was determined against *Bacillus subtilis* ATCC 6633 (as recommended by the European standard method), and *Bacillus cereus* ATCC 13061. This European standard requires a reduction in the number of spores of > 4 log₁₀ within 120 min contact time. The AHR, AHSW and povidone iodine did not pass the standard test and did not have any significant activity against *B. cereus* or *B. subtilis*. Tea tree oil solutions had a degree of sporicidal activity, although it was not great. For *B. cereus*, tea tree oil solutions of 5, 10 and 15% were significantly more active than the sterile distilled water controls after 120, 60 and 30 minutes, respectively. For *B. subtilis*, solutions of 10 and 15% were significantly more active than the sterile distilled water control after 60 and 30 minutes, respectively. The 5% tea tree oil solution did not differ from the control at any time.

The ultimate measure of the efficacy of a hand wash or hand hygiene product is its efficacy in vivo. Therefore, the efficacy of the HSW, AHSW and a solution of 5% TTO in Tween 80 was investigated using the European Standard hand washing test. Tests were performed on two separate occasions, with 13 and 14 study participants, respectively. In the first study, the 5% tea tree oil solution performed significantly better than the soft soap control, whereas the HSW did not. In the second study, the AHSW performed significantly better than the soft soap. When the 5% tea tree oil solution, HSW and AHSW were tested using an “ex-vivo” test, the 5% solution was the most active, followed by the AHSW, the HSW and lastly the soft soap. The ex-vivo test uses excised human skin samples and has been shown to be a reproducible potential substitute for “in-vivo” testing. Data from the “ex-vivo” testing paralleled the hand washing data, also demonstrating that the 5% solution was most active, followed by the AHSW.

**Implications for relevant stakeholders**

This work demonstrated that several, but not all, formulations of tea tree oil tested had antibacterial activity, as assessed with European and American standard methods, when used as hand washes. Generally tea tree oil has a slow mechanism of action compared to many conventional antimicrobials. This means that some formulations may struggle to achieve a substantial reduction in bacterial numbers within the 1 minute timeframe mandated by some of these tests. However, the activity of tea tree oil can be enhanced by the addition of alcohol, and this was evident from the way that both the alcohol-containing skin wash and hand rub products passed the tests. In volunteer hand washing experiments, once again the alcohol-containing products performed well, although simple tea tree solutions were just as effective, further emphasising the importance of formulation. Thus several formulations containing tea tree oil met the requirements of the European standards for antiseptics. These formulations can play a role in reducing hospital-acquired infections, such as those caused by *S. aureus*.

**Recommendations**

Further in-vivo testing as well as clinical trials are encouraged as these products may prove helpful in the hospital and community setting. The industry should be proactive in introducing these products to the healthcare setting.
1. Introduction

1.1. Background

1.1.1. Historical

Handwashing and disinfection are considered the most important steps in preventing the spread of infectious disease. Reviewing the literature over 100 years, the evidence strongly supports a causal relationship between handwashing and infection control (McDonnell et al., 1999; Larson, 2001). As reviewed by Pittet and Boyce (2001), A. Gordon in 1775 in Aberdeen and O. Wendell Holmes in Boston demonstrated the association of epidemic puerperal fever with the hands of health care personnel. A hundred and fifty years ago, I.P. Semmelweis (1818-1865) also associated healthcare personnel hands with puerperal sepsis infections and transmission in the maternity ward of the University of Vienna. However, he was the first to insist that students and doctors wash their hands with a chlorinated lime solution prior to each physical examination. As a result, the maternal mortality dropped dramatically and remained low for years. Semmelweis was the first to show that cleansing hands between patients with an antiseptic agent reduced nosocomial transmission of infectious agents better than washing with only water and soap (Pittet & Boyce, 2001). His data has been the basis for the emphasis on handwashing as a fundamental infection control principle. Nevertheless, the mortality due to life-threatening hospital-acquired infections is still high, and the major mode of transmission is still thought to be transmission of pathogens via the hands, from staff to patient, and from patient to patient (Larson, 1999; Pittet & Boyce, 2001; Naikoba & Hayward, 2001). Despite clear evidence that appropriate handwashing is the leading measure to reduce the transmission of infection, compliance with hand-hygiene practices is still unacceptably low (Naikoba & Hayward, 2001; Pittet, 2001; Karabey et al., 2002). Nosocomial infections still constitute one of the greatest challenges of modern medicine (Pittet & Boyce, 2001).

1.1.2. Hygiene and Nosocomial infections

Nosocomial infections

The microorganisms that normally colonise the hand surfaces represent little risk of infectious disease transmission from health care personnel to immunocompetent patients. The threat of infection comes primarily from transient pathogens which can contaminate hands. In the health care environment, contamination normally occurs by contact with excretions or infectious exudates and is usually transmitted through hand contact (Larson 1988). Over the past century, medical and scientific communities have agreed that skin hygiene, especially of the hands is a primary mechanism to control the spread of infectious agents (Larson 2001; Pittet & Boyce 2001). In March 1996, the Nosocomial Infection National Surveillance Scheme (NINSS) was created in the United Kingdom and based in the Nosocomial Infection Surveillance Unit (NISU). The aim of the scheme is to improve the quality of patient care by reducing infection rates and consequently reducing individual patient costs (NISU website, 2004). However, nosocomial outbreaks still occur (Pitten & Grundling, 2001; Andersen et al., 2002), causing disruption of financial costs and highlighting the need for the development of regulatory processes to ensure safety in the health care industry. According to the Second National Prevalence Survey of Infection in Hospitals, among 157 hospitals throughout England, Wales, Scotland and all Ireland, four groups of infection were identified: urinary tract infection (23.2%), lower-respiratory tract (22.9%), surgical wound (10.7%) and skin infections (9.6%) (Emmerson et al., 1996). A study of wound pathogens including 676 surgery patients over a 6-year period reported the following common pathogens Staphylococcus aureus (28.2%), Pseudomonas aeruginosa (25.2%), Escherichia coli (7.8%), Staphylococcus epidermidis (7.1%) and Enterococcus faecalis (5.6%) (Giacometti et al., 2000).
Recently, concerns have arisen from the widespread prevalence of health-care associated diarrhoea due to *Clostridium difficile* (Riley, 2004) and over reports of *Bacillus anthracis* spores being deliberately sent through the United States postal system in 2001, resulting in five deaths from inhalation (Jernigan et al., 2001; Jernigan et al., 2002). Due to their resistance to dryness, heat, and poor nutrient supply, spores are able to persist in the environment for long periods of time (Hamouda et al., 2002). They are also resistant to ultraviolet and ionising radiation, extremes in pH, temperature, pressure, and many commonly used disinfectants (Gould & Dring, 1975; Gould, 1977; Hamouda et al., 2002).

**Importance of antisepsis and handwashing**

The skin acts as a protective outer covering, prevents loss of interstitial fluids and entry of noxious substances. The skin also carries a rich microbial flora which is inoffensive to the person whose skin it is or to others healthy persons (Larson, 2001). It is especially necessary to keep the skin surface clean to protect a patient against his own flora during a surgical intervention or during injections, blood donation and to protect patients with open wounds against the skin flora of surgeons and other healthcare personnel.

As mentioned previously, J.P. Semmelweis (1818-1865) was the first to demonstrate the efficacy of handwashing in preventing puerperal sepsis and its associated mortality (Pittet & Boyce, 2001). Although it is difficult to demonstrate the effects of handwashing in terms of mortality decrease (Rotter, 1998), there is convincing causal evidence linking hand hygiene and reduced transmission of infections (Reybrouck, 1986; Larson, 1999; Larson, 2001). However, it has been shown that healthcare personnel wash their hands infrequently, with an average compliance usually below 50% (Pittet, 2001; Pittet & Boyce, 2001). Furthermore, handwashing technique has been described as poor, durations of washing are often shorter than recommended (Reybrouck, 1986; Girou & Oppein, 2001; Pittet, 2001) and although many attempts to change this behaviour have been made (Pittet et al., 2000; Girou & Oppein, 2001; Naikoba & Hayward, 2001; Pittet, 2001) they have not always been effective (Handwashing Liaison Group, 1999; Gillespie, 2001).

However, soaps and detergents when routinely applied to the skin during handwashing can also be damaging to the skin leading to a change in microbial flora, an increase in bacterial shedding and thus an increased risk of transmission of microorganisms (Larson, 1999; Larson, 2001). Washing damaged skin is less effective at reducing numbers of microorganisms than washing healthy skin, although the number of bacterial cells shed from damaged skin is usually higher than that of normal skin (Ojäjärvi, 1980). Recently, Larson (2001) highlighted the necessity to carefully reassess handwashing recommendations for healthcare personnel due to the detrimental effects of handwashing on skin that may adversely influence handwashing practices and then result in an increase risk for the transmission of infectious agents.

**1.2. Antiseptics**

Antiseptics and disinfectants are nowadays widely used in hospitals and health care settings, but also most recently by the general public (McDonnell & Russell, 1999). They are essential for the prevention of nosocomial infections.

The efficacy of a biocide has been defined as the capacity to achieve a bacterial reduction factor greater than $5 \log_{10}$ within 5 min when assessed with a suspension test (EN 1276, 1997). The assessment of the antibacterial activity of an antiseptic requires several steps: (i) “in-vitro” and animal studies to evaluate safety and antimicrobial activity of the product, (ii) artificial contamination of skin with microorganisms which are then harvested after antiseptic challenge, (iii) investigation of the efficacy against normal and transient flora, under controlled conditions to isolate the independent effects of the agent and (iv) product testing in the clinical environment to evaluate their effectiveness in reducing nosocomial infections (Larson, 1995).
1.2.1. Synthetic antiseptics

There are many antiseptics commercially available, most of which are synthetic biocides. The most common antiseptics used as handwashing or topical agents are listed below.

1) Alcohols, which have a highly efficient, rapid and wide spectrum of antibacterial activity. They have a low toxicity and are among the safest known antiseptics applied to the skin (Reybrouck, 1986; Russell, 1999). They are widely used for skin antisepsis and hard-surface disinfection (McDonnell & Russell, 1999). They are little influenced by interfering substances such as blood, protein and detergents (Rotter 2001).

2) Chlorhexidine is a cationic biguanide. It is probably the most widely used biocide in antiseptic formulations, but is also used as a disinfectant and preservative (McDonnell & Russell, 1999). Chlorhexidine is a broad spectrum agent, is substantive to the skin and has a low irritancy (McDonnell & Russell, 1999). Studies reported chlorhexidine to be the most persistent agent currently available for handwashing (Bruch, 1981; Ayliffe, 1984). However, its activity is greatly affected by the presence of organic matter (Larson, 1995) and is pH-dependent.

3) Chloroxylenols are synthetic halogen-substituted phenol derivatives that have been used extensively as preservatives, disinfectants and topical antiseptics (Larson & Talbot, 1986). Para-chloro-meta-xylene (PCMX) is the key halophenol used in antiseptic or disinfectant formulations. PCMX has an intermediate rapidity of activity and it has a persistent effect over a few hours (Larson 1995).

4) Triclosan is a synthetic bisphenol antimicrobial agent. It has an immediate broad-spectrum activity and is especially active against Gram-positive bacteria (Russell, 1999). It has an excellent persistent activity on skin and is minimally affected by organic matter (Bruch, 1981; Bartzokas, 1987a).

5) Iodophors are complexes consisting of iodine and a carrier such as polyvinylpyrrolidone (PVP or povidone. The most commonly used iodophor is povidone-iodine (Larson, 1995). The iodophors have several disadvantages, including a decrease in efficacy in the presence of organic matter (Lacey, 1979), a high incidence of dermal irritation, potentially unreliable residual activity and toxicity (Zamora et al., 1985; Larson, 1995).

6) Quaternary ammonium compounds (QACs) are cationic agents. They possess a high antibacterial activity, however, *Ps. aeruginosa* is particularly resistant to the QACs (Russell, 1999) and their activity is greatly affected by organic matter and by pH (Pittet & Boyce, 2001).

A major problem encountered with synthetic antiseptics is that none of them are effective at inactivating spores. The World Health Organization (WHO) recommends the use of sporidical agents such as glutaraldehyde, sodium hypochlorite, hydrogen peroxide, peracetic acid and formaldehyde as effective disinfectants for environmental decontamination against *Bacillus* spores (WHO, 1998). The US Centers for Disease Control and Prevention recommends handwashing with either soap and water or with an antimicrobial agent (Boyce & Pittet, 2002). However, this is a category II recommendation “suggested for implementation and supported by suggestive clinical or epidemiologic studies or a theoretical rationale”. Most compounds used in antiseptic and handwash formulations, such as alcohols, chlorhexidine, hexachlorophene, iodophors, PCMX and Triclosan do not have any significant activity against *Clostridium* spp. and *Bacillus* spp. spores (Gershenfeld, 1962; Russell, 1991; Bettin et al., 1994; Weber et al., 2003). Hence, there is an urgent need for new antiseptic products with sporidical activity to be developed and assessed.

In addition, the continuing emergence of multi-drug resistant organisms in healthcare facilities poses a threat to patients throughout the world (Andersen et al., 2002; Hsueh et al., 2002). Nowadays, in addition to the increasing number of antibiotic-resistant microorganisms, there is also concern over a growing number of these microorganisms which show a lowered susceptibility, or ‘insusceptibility’, to biocides. Such ‘trained’ organisms have been successfully produced in the laboratory (Russell et al., 1998; Suller & Russell, 1999; Tattawasart et al., 1999; Suller & Russell, 2000; Thomas et al., 2000). In practice there are also reports of microorganisms isolated from clinical or industrial areas that show decreased susceptibility to two biocides (Lear et al., 2002). In addition, clinical or industrial isolates as
well as ‘trained’-microorganisms which are less susceptible to biocides, may show a higher resistance to some antibiotics (Heir et al., 1999; Bjorland et al., 2001). Strategies to control the spread of such organisms include reducing misuse of antibiotics and some biocides, and implementing infection control measures (Boyce & Rotter, 2001).

1.2.2. Tea tree oil

The essential oil of *Melaleuca alternifolia*, or tea tree oil (TTO) is obtained by steam distillation of freshly harvested leaves. The composition of TTO has been well characterised and it contains a complex mixture of over 100 terpenes and their related alcohols (Brophy et al., 1989). TTO is a naturally-occurring antimicrobial which has been used medicinally for over 80 years for the treatment of conditions such as vaginal infections (Blackwell, 1990; Pena, 1962) and furunculosis (Feinblatt, 1960). However, it is now being incorporated into a multitude of products including anti-acne formulations, shampoos, antiseptic creams and mouthwashes.

Most antiseptic agents when routinely applied during handwashing can be damaging to the skin, leading to a change in microbial flora, an increase in bacterial shedding and thus an increased risk of transmission of microorganisms (Larson, 1999; Larson, 2001). In contrast, some reports suggest that the repeated use of handwash formulations containing tea tree oil (TTO) does not lead to the dermatological problems associated with other formulations and this information might be used to encourage better compliance by health care staff with handwashing (Carson & Riley, 1995). In addition, Hammer et al. (1996) showed that transient skin organisms were more susceptible to TTO than commensal organisms. This finding has lead to an increased interest in TTO-containing handwash products and is significant since it is now recognised that instead of fighting microorganisms at any cost (in most cases at the cost of skin’s health), skin integrity as well as its resident flora should be protected and considered an ally against infectious diseases (Kownatzki, 2003).

TTO has broad spectrum antimicrobial (Carson & Riley, 1993) and anti-inflammatory (Brand et al., 2001; Koh et al., 2002) activity *in-vitro*. Its antibacterial (Hammer et al., 1996; Carson et al., 2002; Hammer et al., 2003a; Messager et al., 2004a) antifungal (Hammer et al., 2002; Hammer et al., 2003b) and antiviral (Schnitzler et al., 2001) activity has been well-established *in-vitro*. However, TTO has not yet been assessed using European standard methods, which are now widely accepted for the evaluation of disinfectant and antiseptic efficacy. Its potential sporicidal activity also remains unexplored.

1.3. Testing protocols

The purpose of all biocide testing is to categorise minimal efficacy of a product. Nowadays, many testing methods are available including suspension tests, carrier tests, and other practical or “in-use” mimicking tests. It is now widely accepted that the antimicrobial activity of a disinfectant and/or antiseptic is assessed at 3 stages of testing (Reybrouck, 1999). In the first stage of testing, disinfectants and/or antiseptic formulations are assessed to verify whether they possess antimicrobial activity. Suspension tests and carrier tests are mainly used for this preliminary evaluation. After measuring the time-concentration relationship of the disinfectant or antiseptic in a quantitative “in-vitro” test, practical tests are performed under real life conditions (Reybrouck, 1998). These tests are still performed in the laboratory and therefore are better standardised. They aim to verify whether the used dilution still gives the expected results under the “in-use” conditions required. Practical tests are necessary when evaluating antiseptics as suspension tests cannot present the total picture of activity on skin (Reybrouck, 1998). In the final stage, “in-situ” and “in-use” tests are performed in the field to assess whether microbes are still killed by the disinfectant and/or antiseptic after a given period of time.
Many countries already use a test or series of tests, measuring different aspects of disinfection that are not always officially recognised. Some of the most commonly used “in-vitro” and “in-vivo” tests are described here after.

1.3.1. “In-vitro”

“In-vitro” methods are carried out to assess the spectrum of activity and rapidity of action of biocides. Some of these tests, such as minimum inhibitory concentration (MIC) of a product, are often carried out as a preliminary test for the evaluation of its antibacterial properties (Russell, 1998). MICs, performed in either solid or liquid media give some information about a product’s activity, onto which further tests can be based. However, these data do not necessarily correlate with bacterial inactivation. Suller and Russell (1999) noted that in clinical practice, rates of bacterial inactivation are much more relevant than MIC values.

The determination of lethal activity of formulations with antiseptic properties requires other “in-vitro” tests, including suspension, carrier and capacity tests. The main purposes of these tests are: (i) assessment of the intrinsic antibacterial activity of a product in comparison to others, using the results obtained with a collection of potentially pathogenic microorganisms able to infect, colonise or be transmitted by the human skin; (ii) confirmation of the expected susceptibility of a given species and in particular of a given strain under epidemic situations; (iii) surveillance of the evolution of antiseptic resistance in microorganisms (Baquero et al., 1991).

Suspension tests are generally both simple and inexpensive to perform because they do not require expensive laboratory equipment. In essence, in a suspension test, a known volume of bacterial inoculum is mixed with a known volume of biocide at the concentration required. After an appropriate contact time, a sample is taken, the disinfectant activity neutralised and bacterial survival is assessed. These tests are normally well standardised and the best known example is the quantitative European suspension test (EN 1276, 1997). It is widely accepted that the requirement for a test product is to achieve a $\log_{10}$ reduction in bacterial concentration greater or equal to 5 within 5 min. A quantitative suspension test is required in all basic testing for products’ activity evaluation. However, suspension tests may overestimate antiseptic efficacy (Maillard et al., 1998).

Suspension tests have long been preferred to carrier tests because the survival of the microorganisms on the carrier during drying is not constant (Reybrouck, 1998). Carrier tests are the oldest tests. Examples of carrier tests are the European stainless steel carrier test (EN 13697, 2001), and the American glass-carrier test method (American Society for Testing and Materials 2111, 2000). As stated above, the major inconvenience of such tests is that bacterial survival on the carrier is not always constant.

1.3.2. “In-vivo”

In 1985, Selwyn stated that skin disinfection was still the most controversial theme in applied microbiology (Selwyn, 1985). Nowadays, questions about skin hygiene still persist (Larson, 2001; Larson, 2002). When considering skin disinfection, the target organisms as well as the characteristics of the available chemical agents and their possible evaluation in-vivo must be considered. However, although the positive benefits of handwashing are well recognised (Hirschmann et al., 2001), there are no epidemiological data indicating how effective a disinfection procedure has to be in order to prevent hand-transmitted infection (Reybrouck, 1986; Larson & Rotter, 1990; Pittet et al., 2000), even in the controlled environment of a hospital (Bryan et al., 1995). The basic question associated with hand hygiene is not “how clean can hands get?” but rather “how much cleanliness is associated with how much reduction in risk of infection?” (Larson & Rotter, 1990; Larson, 2002). The assessment of the clinical value of disinfecting the hands of healthcare staff and the patient’s operation site is practically impossible. Hence, it has become logical to evaluate the relative merits of alternative methods of skin antisepsis by measuring their ability to reduce the numbers of bacteria, both resident and transient, on the skin.
The main testing protocols for hygienic handwash formulations are the Vienna Model (Rotter & Koller, 1991) and the Birmingham Model described by Ayliffe and colleagues (1978). The Vienna model has been integrated with minor changes in the guidelines of the Austrian (OGHMP) and German (DGHM) societies for hygiene and microbiology for testing procedures of hygienic hand disinfection. The European standard for hygienic handwashing (EN 1499, 1997) has been based on this method. In essence, fingers (up to mid meta carpals) and thumb tips are immersed in a *Escherichia coli* inoculum for 5 sec then air dried for 3 min. Five ml of the product to be tested or the reference product (a non-medicated soap) are applied for 1 min. Recovery of the bacterial cells before and after disinfection is performed as follow: fingertips are rubbed at the bottom of a petri-dish containing 10 ml of broth and an appropriate neutraliser. Log_10 reduction is calculated as the difference between the number of cells prior to and after disinfection. For the formulation to pass the test it has to be significantly more active than the reference product.

A criticism of this test is the use of *E. coli* as the test organism because it dries rapidly on the skin and is not a good representative of organisms commonly causing cross-infection in hospitals (Ayliffe et al., 1990). Ayliffe et al. (1978) recommended the additional use of a Gram-positive coccus. *Staphylococcus aureus* is capable of causing a wide range of skin infections but is also a common transient and occasionally a resident on healthy skin (Noble, 1998). However, because of a case of severe local infection due to *S. aureus* in one subject reported in a past study (Ayliffe et al., 1981), and because *S. aureus* would not be acceptable in industrial laboratories due to potential infection hazards (Ayliffe et al., 1989), *E. coli* was chosen for the European standard method. It is easily identified when mixed with normal resident flora on the skin and is an acceptable non-virulent organism (hazard group 1; i.e. non pathogenic) to be used with volunteers.

In 1997, the European Committee for Standardisation released new standards for the evaluation of antiseptic-containing formulations. Apart from the method for hygienic handwash described previously, the methods published include methods for surgical hand disinfection (EN 12791, 1997) and hygienic hand rub (EN 1500, 1997). The value of such standards is the harmonisation of product testing at least within the European Community, hence enabling direct comparison of results within and between laboratories.

Handwashing and disinfection methods are normally assessed by “in-use” tests in laboratory conditions. Normally using “ideal” conditions with “ideal” populations; i.e. the skin of the subjects is normal: subjects are young, have a low daily handwashing frequency, are healthy and do not have any skin problems. These tests do not give information on the true efficacy of different methods in actual conditions of use (Ojäjärvi, 1991). When the skin is heavily contaminated before disinfection, a high bacterial reduction may be obtained, as most bacteria are not bound to the skin. During practical work we are continuously rubbing our hands against other substances, and possibly causing greater skin binding. It has been shown that bacteria attached superficially to the hands are eradicated more easily than bacteria rubbed into the hands (Bush et al., 1986). The relevance of those tests to practical hand disinfection remains uncertain. Those tests are nevertheless useful for comparing the efficacy of different agents (Ayliffe et al., 1988).

1.3.3. “Ex-vivo”

There is some discord as to the actual antimicrobial effectiveness of commercially available products because there are limited ways to study their efficacy directly on skin. As mentioned above, for ethical reasons, highly infectious agents and new formulations whose safety for humans is not known cannot be tested on volunteers. In addition, handwashing procedures often reflect the handwashing technique rather than product efficacy (Kirita et al., 1993). Furthermore, to evaluate antimicrobial efficacy of a product, the number of transient organisms existing on the hands must be known and not to be confused with the resident flora. Otherwise, the method will only reflect the total reduction of microorganisms from the skin (i.e. resident and transient organisms) as opposed to only transferable ones (transients, possible pathogenic organisms) (McDonnell et al., 1999).
The skin is a dynamic living biomembrane and is the final unit for determining the local and systemic availability of any drug which must pass into and through it (Wester & Maibach, 1992). Cadaver human skin as well as samples excised from living donors is used in hospitals and research laboratories for various reasons (Wester et al., 1998). Furthermore, for nearly four decades, hospitals have banked skin for use as effective temporary covering for burn wounds (May & DeClement, 1980; Bravo et al., 2000; Nanchahal et al., 2001). Human skin has also been used ex-vivo to study the percutaneous absorption of drugs and hazardous chemicals of environmental concern (Roberts et al., 1977; Moss et al., 2000). The most commonly used method involves placing a piece of excised skin in a diffusion chamber (type Frantz diffusion cells), applying a radioactive compound on one side and then evaluating for radioactivity in the recipient compartment in the other side (Bucks et al., 1985).

Development of a new protocol
In 1996, Sattar’s research team in Ontario, Canada, described an “ex-vivo” method using freshly excised skin samples, derived mostly from reduction mammoplasty, for the testing of Herpes virus 2 survival on skin compare to that on inanimate carrier (Graham et al., 1996). The objective of this study was to show the appropriateness of such a method for the study of virus survival directly on human skin ex-vivo. Later, a similar method was developed in our laboratory using freshly excised animal skin (i.e. from euthanised cats and dogs) for the testing of antimicrobial efficacy of antiseptics (Maillard et al., 1998). This method allowed tests to be performed with infectious agents and formulations whose safety for humans is unknown. The “ex-vivo” protocol was then adapted for the use of human skin samples, to investigate the antimicrobial efficacy of formulations with antiseptic properties.

The “ex-vivo” method
In essence, the “ex-vivo” test uses freshly excised as well as frozen human skin samples of about 2 cm², which are maintained in a diffusion cell (JB&DW Jones) containing 1 ml of Phosphate Buffered Saline (PBS) in the recipient compartment to keep the dermis moist (Figure 1). This is the closest representative method for “in-vivo” conditions. The only limitation is that the skin does not have vascularisation going to the dermis or basal layers, but this should have little effect on antiseptic testing, as the stratum corneum is essentially a non-living tissue. This “ex-vivo” test has already been used to investigate bacterial survival on skin and the antibacterial activity of antiseptics on skin (Maillard et al., 1998; Messager et al., 2001; 2004). The method allows for tests to be run
under a controlled environment without the interference of resident microflora and immune properties of the skin, including residual antibiotics. It is a simple, quantitative technique for performing studies on the antimicrobial activity of various topical agents on an actual cutaneous surface.

1.4. Aims & Objectives

The aim of this project is to increase the acceptability of tea tree oil as a naturally-occurring antimicrobial, both nationally and internationally, by producing scientifically valid and publishable data relating to the efficacy of tea tree oil as a topical antiseptic or disinfectant.

Firstly, the antibacterial and sporicidal activity of tea tree oil and tea tree oil-containing products was assessed in-vitro using standard European suspension test methods. The activity of these products on surfaces was then evaluated by another ‘in-vitro’ European test, and an American standard method.

European suspension and surface tests determine whether a preparation possesses antimicrobial properties specific for a defined application (phase 2 / step 1). Once established, the formulation must be tested with methods establishing whether it possesses antimicrobial properties in practice, imitating conditions such as, in this case, a handwashing test (phase 2 / step 2). Hence, the activity of these formulations was examined further by carrying out a handwashing test in-vivo using volunteers. As described above, the ‘ex-vivo’ method gives advantageous benefits over the ‘in-vivo’ method: no volunteers are required, a greater range of pathogenic organisms can be tested and it is cheaper and easier to organise. Thus, the aim of the last part of this study was to find out whether or not the TTO products tested matched European regulations and to compare and validate the “ex-vivo” protocol for the assessment of the antibacterial efficacy of TTO directly applied on human skin.
2. ‘In-vitro’ suspension tests

The results of this chapter are also described in the following publications:


2.1. Background / Objectives

TTTO has been used medicinally for more than 80 years (Carson & Riley, 1993). It has broad-spectrum antimicrobial (Carson & Riley, 1993) and anti-inflammatory (Brand et al., 2001; Koh et al., 2002) activity *in-vitro*. Hammer et al. (1996) showed that transient skin organisms were more susceptible to TTO than commensal organisms (Hammer et al., 1996). This finding supports the use of TTO-containing handwash products since the normal flora of the skin represents one of the natural defenses against colonisation by pathogenic organisms (Noble, 1998). Other reports have suggested that the repeated use of TTO-containing handwash does not lead to the dermatological problems associated with some formulations (Carson & Riley, 1995) and this finding may be used to encourage health care staff to comply with handwashing. Although the antibacterial activity of TTO has been well-established *in-vitro* (Schnitzler et al., 2001; Carson et al., 2002; Hammer et al., 2002; Koh et al., 2002; Hammer et al., 2003a; Hammer et al., 2003b), TTO has not yet been assessed using European standard methods which are now widely accepted for the evaluation of disinfectant and antiseptic efficacy. In this study, we assessed the activity of TTO and TTO-containing formulations according to two European standard suspension methods, EN 1276 and prEN 12054.

2.2. Materials & Methods

2.2.1. Microorganisms

*Staphylococcus aureus* (ATCC 25923), *Acinetobacter baumannii* (NCTC 7844), *Escherichia coli* K12 (NCTC 10538) and *Pseudomonas aeruginosa* (NCTC 6749) stock cultures were made on tryptone soya agar (TSA; Oxoid) plates, stored at 4°C and renewed once a week.

2.2.2. Working cultures of bacteria

Subcultures onto further TSA plates were prepared from the stock cultures and incubated for 18 to 24 h at 37°C. From this second subculture, a third subculture was produced in the same way. As recommended by the European standard method EN 1276 (European Standard EN1276, 1997), the second and/or third subcultures were the working culture(s). To prepare bacterial test suspensions, loopfuls of cells from the working cultures were transferred into 15 mL of diluent, containing 1 g/L tryptone pancreatic digest of casein (Difco) and 8.5 g/L sodium chloride (AnalaR) at pH 7, which was placed in a 100 mL flask with 5 g of glass beads. The flask was shaken for 3 min using a mechanical shaker (VorMix, Scientific Equipment Manufacturers). The suspension was then aspirated from the glass beads and transferred into another tube. Using a nephelometer, the number of cells in the suspension was adjusted with diluent to approximately 1.5 - 5 × 10^8 cfu/mL when performing the EN 1276 suspension test and to approximately 1 - 3 × 10^8 cfu/mL when performing the EN 12054 suspension test. These bacterial suspensions were used within 2 h.

2.2.3. Antiseptic products

**Suspension test EN 1276**

The antimicrobial activity of the following agents was investigated: a hygienic skin wash (HSW) containing 5% TTO (100% HSW; Novasel, Australia), an alcoholic hygienic skin wash (AHSW)
containing 5% TTO and 10% alcohol (100% AHSW; Novasel), an alcoholic hand rub (AHR) containing 3% TTO and 64% alcohol (100% AHR; Novasel), as well as 1%, 2%, 3%, 4%, 5%, 6%, 8% and 10% TTO in sterile distilled water (SDW) with 0.001% Tween 80 (Sigma). TTO was kindly supplied by Australian Plantations Pty Ltd, Wyrallah, NSW, Australia. Batch W\EU04 complied with the international standard for TTO (ISO 4730, 1996). Levels of components were assessed by gas chromatography-mass spectrometry, performed by the Wollongbar Agricultural Institute, Wollongbar, NSW, Australia. Batch W\EU04 contained 38.6% terpinen-4-ol and 3.6% 1,8 cineole. Antimicrobial products were diluted in SDW to the concentration indicated and tested immediately. For comparative purposes, in most experiments the activity of a widely-accepted antiseptic, 7.5% (v/v) povidone iodine (PVI; Orion), was also tested. As the aim was to assess whether or not TTO was the main active component of some of the formulations tested, the efficacy of HSW without TTO (HSW-TTO), AHR without TTO (AHR-TTO) and 0.001% Tween 80 was assessed.

**Suspension test prEN 12054**

As described in the standard method, the formulations being tested, namely 100% HSW, 100% AHSW and, as controls, 5% TTO in 0.001% Tween 80 and 7.5% PVI, were diluted to 55% (v/v) prior to testing. AHR (100%) was used undiluted.

**2.2.4. Neutraliser**

The neutralizing solution used to quench the activity of antiseptics was based on the European Standard EN 1276 and contained: 30g/L Tween 80 (Sigma), 3 g/L lecithin (Sigma), 1 g/L histidine (Sigma), 5 g/L sodium thiosulfate (AnalaR) and 34 g/L potassium dihydrogen phosphate (AnalaR) in tryptone soya broth (TSB; Oxoid). Two controls were required to validate this neutraliser: first, the neutraliser’s toxicity had to be assessed against the test bacteria and, second, its ability to quench the corresponding antimicrobial activity had to be confirmed.

**Antibacterial activity of the neutraliser**

One mL of a working bacterial suspension of *S. aureus*, *A. baumannii*, *E. coli* or *P. aeruginosa* cells was added to 8 mL of neutraliser and 1 mL of SDW. After 5 min contact, this solution was serially diluted in sterile phosphate buffered saline (PBS) (8 g/L NaCl (AnalaR), 0.2 g/L KCl (AnalaR), 1.44 g/L Na2HPO4 (AnalaR) and 0.24 g/L KH2PO4 (AnalaR)). The number of cfu/mL recovered was assessed by the drop counting method (DCM) (Maillard et al., 1998).

**Dilution-neutralization validation**

The ability of the neutraliser to quench the corresponding antiseptic was assessed as follows: 1 mL of SDW was mixed with 1 mL diluent. Eight mL of antiseptic solution was added to this mixture and, after 1 min, 1 mL was transferred into a test tube containing 8 mL of neutraliser. After 5 min, 1 mL of bacterial solution was added to the mixture and left in contact for 30 min. The final mixture was then serially diluted and counted using the DCM.

**2.2.5. Interfering substances**

As recommended by EN 1276, the interfering substance tested was a bovine albumin solution, under clean [0.3 g/L bovine albumin (Sigma)] or dirty [3 g/L bovine albumin] conditions. When the antiseptics were assessed in “perfect” conditions, the interfering substance was replaced by SDW.

**2.2.6. EN 1276 - suspension test**

This quantitative suspension test method was designed for the evaluation of the bactericidal activity of chemical disinfectants and antiseptics used in food, domestic and industrial areas. The requirement of this standard is a minimum reduction by a factor of 10⁵ within 5 min. The antiseptic formulations were assessed also after a 1 min contact time only, to better reflect real life conditions.
One mL of interfering substance (or SDW for perfect conditions) was mixed with 1 mL of bacterial test suspension. After 2 min, 8 mL of one of the product solutions was added to the mixture and shaken. After 1 and 5 min, 1 mL of the test mixture was transferred into a tube containing 8 mL of neutraliser and 1 mL of SDW, and mixed. After 5 min neutralization time, solutions were serially diluted and viable counts performed on TSA plates using the DCM. The DCM was used as log_{10} reductions < 4 cannot be measured with the counting method recommended by the European standard. Plates were incubated for 24 h at 37°C, counted, and then re-incubated for a further 24 h to detect slow growing colonies.

In this study, the aim was to compare the activity of (i) 5% TTO in Tween 80 with that of 7.5% PVI, 100% HSW, 100% AHSW and 100% AHR in perfect, clean and dirty conditions; (ii) the activity of different concentrations of TTO in 0.001% Tween 80; (iii) the activity of 5% TTO in Tween 80 with that of 0.001% Tween 80 alone; (iv) the activity of 100% HSW with that of 100% HSW without TTO and, finally, (v) the activity of 100% AHR with that of 100% AHR without TTO.

2.2.7. prEN 12054 - suspension test

This quantitative suspension test method was designed for testing hygienic and surgical handrub and handwash products. The requirements of this standard are a minimum reduction factor of 2.52 log_{10} cfu/mL for a hygienic handwash and 4.52 log_{10} for an hygienic handrub within 1 min.

One mL of bacterial test suspension was mixed with 9 mL of 55% HSW, 55% AHSW, 2.75% TTO in Tween 80 or 100% AHR in a sterile McCartney bottle. After 1 and 5 min, 1 mL of this mixture was transferred into a bottle containing 8 mL of neutraliser and 1 mL of SDW. After a neutralization time of 1 min, the solutions were serially diluted in PBS and viable counts performed on TSA using the DCM. The appropriate contact time for hygienic handwashes and handrubs should be 1 min. However, in order to assess whether or not a longer contact time would significantly influence the activity of the formulations, a 5 min contact time was also used. All experiments were conducted at room temperature, which was 20°C ± 1°C.

2.2.8. Statistical analysis

Analyses of variance were performed with the Excel® and Prism® software packages. Three to five replicates were performed and P values of < 0.05 were considered significant.

2.3. Results

2.3.1. Antibacterial activity and efficacy of the neutraliser

As shown in Table I, the neutraliser did not significantly reduce (P > 0.05) the concentration of bacterial cells tested. The neutralising solutions effectively quenched (P > 0.05) the appropriate antiseptic at the concentration tested (Table II).

2.3.2. EN 1276 - suspension test

Antibacterial activity of the TTO-containing antiseptics

According to the European standard method used, the test products had to achieve at least a 5 log_{10} reduction in bacterial cell numbers after a 1 or 5 min contact time. Products that complied with the European standard are listed in Table III. After 1 min contact time, the only products that achieved a 5 log_{10} reduction against all four bacteria were 100% AHR (in perfect and clean conditions) and 7.5% PVI (in perfect, clean and dirty conditions). After 5 min contact time, the products that achieved 5 log_{10} reduction against all four bacteria were AHR (in perfect and clean conditions), AHSW (in perfect and clean conditions) and PVI (in perfect, clean and dirty conditions).
It is interesting to note that different concentrations of TTO in Tween 80 (from 4% to 10%) also achieved a 5 \( \log_{10} \) reduction, but only against \textit{E. coli} and \textit{P. aeruginosa}. In addition, \textit{A. baumannii} and \textit{E. coli} were susceptible to a wider range of products and lower concentrations than \textit{S. aureus} and \textit{P. aeruginosa} (concentrations of HSW and AHR ranging from 10% to 75% were also assessed, data not shown).

**5% TTO in 0.001% Tween 80**

When assessed against \textit{S. aureus}, 5% TTO in 0.001% Tween 80 was more active in perfect conditions although the difference was not significant. Also, its activity generally increased after 5 min contact time, however, this was only significant \((P < 0.05)\) when assessed in perfect conditions (Figure 2). When tested against \textit{A. baumannii}, there was no significant difference in activity, regardless of the tests being performed in perfect or clean conditions, after 1 and 5 min contact time. However, TTO was more active when tested in perfect conditions than in dirty conditions, and this difference was significant \((P < 0.05)\) after a 5 min contact time (Figure 3). Regardless of the conditions, TTO was always significantly more active after a 5 min contact time (Figure 3). When assessed against \textit{E. coli}, TTO was as active \((\log_{10} \text{ reduction } \geq 5)\) whether the tests were performed in perfect, clean or dirty conditions after 1 and 5 min contact times (Figure 4). Against \textit{P. aeruginosa}, TTO was more active when assessed in perfect conditions than in clean conditions, however, this was only significant \((P < 0.05)\) after a 5 min contact time. TTO was significantly \((P < 0.05)\) more active in perfect compared to dirty conditions (Figure 5). TTO was also significantly more active \((P < 0.05)\) after 5 min compared to 1 min contact time when assessed in perfect conditions. However, there was no significant difference in the activity of TTO depending upon the contact time when assessed in clean and dirty conditions (Figure 5).

**7.5% PVI**

The different conditions (perfect, clean or dirty) and contact times did not significantly affect the antibacterial activity of PVI (Figures 2-5). PVI (7.5%) was significantly \((P < 0.05)\) more active than 5% TTO against \textit{S. aureus}, \textit{A. baumannii} and \textit{P. aeruginosa} (Figures 2, 3 & 5), while TTO was as active as PVI against \textit{E. coli} (Figure 4).

**Hygienic skin wash (HSW)**

When assessed against \textit{S. aureus}, 100% HSW was generally more active \((P < 0.05)\) in dirty rather than clean conditions, and after a 5 min contact time, however, this was only significant when assessed in dirty conditions (Figure 2). Against \textit{A. baumannii}, conditions did not significantly influence the activity of 100% HSW after a 1 min contact time. HSW (100%) was more active when assessed in perfect and clean conditions after a 5 min contact time, however, this was not significant (Figure 3). There were no significant differences when 100% HSW was assessed in perfect, clean or dirty conditions after a 1 min contact time against \textit{E. coli}. However, after a 5 min contact time, 100% HSW was less active \((P < 0.05)\) in dirty compared to perfect conditions (Figure 4). HSW was significantly \((P < 0.05)\) more active after a 5 rather than 1 min contact time (Figure 4). Surprisingly, when tested against \textit{P. aeruginosa}, 100% HSW was significantly more active \((P < 0.05)\) when assessed in both clean and dirty rather than perfect conditions after a 1 min contact time. However, there was no significant difference in activity between conditions after a 5 min contact time (Figure 5).

TTO (5%) was more active than 100% HSW against \textit{S. aureus} in perfect conditions after 1 and 5 min, but this difference was not significant (Figure 2). After a 1 min contact time, 5% TTO was always significantly \((P < 0.05)\) more active than 100% HSW against \textit{E. coli}, however, after 5 min, there was no significant difference in activity between the two products (Figure 4). In perfect conditions, 5% TTO was significantly \((P < 0.05)\) more active than HSW against \textit{P. aeruginosa} after 1 and 5 min, however, in clean and dirty conditions, 100% HSW was significantly \((P < 0.05)\) more active than 5% TTO after 5 min contact time (Figure 4). Finally, 5% TTO was significantly \((P < 0.05)\) less active than 100% HSW against \textit{A. baumannii}, after 1 and 5 min contact times (Figure 3).
Alcoholic hygienic skin wash (AHSW)
When assessed against *S. aureus*, the different conditions (perfect, clean or dirty) did not affect the efficacy of 100% AHSW and its activity was increased after a 5 min contact time although this was significant (*P* < 0.05) only in perfect and dirty conditions (Figure 2). The conditions (perfect, clean or dirty) and/or the contact time did not significantly influence the activity of 100% AHSW against *A. baumannii* (Figure 3). After 1 min, 100% AHSW was surprisingly more active (*P* < 0.05) when assessed in clean or dirty conditions than in perfect conditions against *E. coli* and *P. aeruginosa* (Figures 4 & 5). Nevertheless, after 5 min, 100% AHSW was more active when assessed in perfect and clean conditions, but this was not significant (Figures 4 & 5).

AHSW (100%) was significantly more active (*P* < 0.05) than 5% TTO, regardless of the contact time and/or the conditions, against *S. aureus* and *A. baumannii* (Figures 2 & 3). After 1 min, 5% TTO was significantly (*P* < 0.05) more active than 100% AHSW in perfect and dirty conditions against *E. coli*, however, in clean conditions, there was no significant difference in activity between the two products (Figure 4). After a 5 min contact time, there was no significant difference in activity between 5% TTO and 100% AHSW regardless of the conditions (Figure 4). When assessed against *P. aeruginosa*, 100% AHSW was significantly more active (*P* < 0.05) than 5% TTO in clean and dirty conditions after 1 min, and regardless of the conditions after 5 min contact time (Figure 5).

Alcoholic handrub (AHR)
The 100% AHR had good activity (> 5 log₁₀ reduction) against *S. aureus*, *A. baumannii*, *E. coli* and *P. aeruginosa* regardless of the conditions and/or contact time (*P* > 0.05) (Figures 2-5). The activity was slightly affected when assessed in dirty conditions against *E. coli* and *P. aeruginosa*, although not significantly (Figures 4 & 5). AHR was significantly (*P* < 0.05) more active than 5% TTO at both contact times and regardless of the test conditions against *S. aureus*, *A. baumannii* and *P. aeruginosa* (Figures 2, 3 & 5). There was no significant (*P* > 0.05) difference in activity between 5% TTO and 100% AHR when tested against *E. coli* (Figure 4).

Antibacterial activity of different concentrations of TTO
The different concentration of TTO did not differ significantly (*P* > 0.05; ANOVA) in their activity against *S. aureus*, regardless of the contact time (Table IV). Activity was always increased after 5 min contact time, but this was only significant (*P* < 0.05) for 2%, 5% and 10% (Table IV). After a 1 min contact time, 1%, 2% and 4% TTO had a comparable activity (*P* > 0.05; ANOVA) against *A. baumannii* although, after 5 min, 1% TTO was significantly less active (*P* < 0.05) than 2%, and 2% TTO had a comparable activity to 4% (Table IV). There was no significant difference in activity between 6%, 8% and 10% TTO (*P* > 0.05; ANOVA), however, 5% was significantly less active than 10% (*P* < 0.05, T-test) against *A. baumannii*. TTO was generally more active after a 5 min contact time than after 1 min but this was only significant (*P* < 0.05) at 1%, 2%, 4%, 5% and 8% (Table IV).

The different concentration and/or contact time did not significantly affect (*P* > 0.05; ANOVA) the activity of TTO against *E. coli* (*P* > 0.05; ANOVA) (Table IV). The activity of TTO against *P. aeruginosa* generally increased significantly (*P* < 0.05) with increasing concentration, although 6%, 8% and 10% TTO had comparable activity (*P* > 0.05; ANOVA) after a 1 min contact time (Table IV). After 5 min, 5%, 6%, 8% and 10% had comparable activity (*P* > 0.05; ANOVA). At all concentrations tested, TTO was generally more active after 5 min, but this was only significant (*P* < 0.05) at 1%, 2%, 4% and 5% (Table IV).

Antibacterial activity of formulations with or without TTO
The 0.001% Tween 80 solution did not have any significant (*P* > 0.05) antibacterial activity against any of the bacteria tested (Figure 6).

Hygienic skin wash
When assessed against *S. aureus*, 100% HSW was more active than 100% HSW-TTO after 1 and 5 min contact time, but this was not statistically significant (*P* > 0.05) (Figure 6). The 100% HSW was more active than 100% HSW-TTO against *A. baumannii*, but this was only significant (*P* < 0.05) after
a 5 min contact time (Figure 6). When assessed against *E. coli* and *P. aeruginosa*, 100% HSW was significantly (*P* < 0.05) more active than 100% HSW-TTO after 1 and 5 min contact times (Figure 6).

**Alcoholic handrub**

The 100% AHR was always significantly (*P* < 0.05) more active than 100% AHR-TTO against *S. aureus* (Figure 6). When assessed against *A. baumannii*, *E. coli* and *P. aeruginosa*, 100% AHR was significantly (*P* < 0.05) more active than 100% AHR-TTO after 1 min contact time only (Figure 6).

**Comparison between bacterial strains**

TTO (5%) was significantly (*P* < 0.05) more active against *E. coli* than against *S. aureus*, *A. baumannii*, *P. aeruginosa* after 1 and 5 min contact times. After a 5 min contact time, 5% TTO was significantly more active (*P* < 0.05) against *A. baumannii* and *P. aeruginosa* than against *S. aureus*. *A. baumannii* was significantly (*P* < 0.05) more susceptible to 100% HSW than *E. coli* which was significantly (*P* < 0.05) more susceptible than *S. aureus* and *P. aeruginosa* after a 1 min contact time. After 5 min, *E. coli* and *A. baumannii* were both significantly (*P* < 0.05) more susceptible to 100% HSW than *S. aureus* and *P. aeruginosa*.

*A. baumannii* was significantly (*P* < 0.05) more susceptible to 100% AHWS than *S. aureus* which was significantly (*P* < 0.05) more susceptible than *E. coli* and *P. aeruginosa* after 1 min. All four bacteria were susceptible to 100% AHWS after a 5 min contact time, and to 100% AHR and 7.5% PVI after 1 and 5 min. *E. coli* and *P. aeruginosa* were generally more susceptible than *S. aureus* and *A. baumannii* to different concentrations of TTO in Tween 80 even if this was not always significant.

### 2.3.2. prEN 12054 - suspension test

To comply with this standard method, a hygienic handwash and a handrub must achieve at least 2.523 log10 and 4.523 log10 reduction in bacterial cells number, respectively, after the chosen contact time. The 100% AHR tested in this study achieved over 4.52 log10 reduction in all bacteria within a 1 min contact time (Figure 7). The AHWS tested at 55% achieved a log10 reduction ≥ than 2.52 against *A. baumannii*, *E. coli* and *P. aeruginosa*, while 4.12% PVI also complied with the standard method by achieving a log10 reduction ≥ 3.5 against all bacteria (Figure 6). After a 1 min contact time, 55% AHWS was always significantly more active than the other skin wash (HSW) regardless of the organism tested. *S. aureus* was always significantly less susceptible than the other bacteria to the formulations tested. The activity of HSW was generally increased with a longer contact time but this was not always significant (*P* > 0.05).

### 2.4. Discussion

When assessed with the suspension test EN 1276, the 100% AHWS and 100% AHR, as well as 7.5% PVI, achieved a log10 reduction ≥ 5 after a 5 min contact time. Their activity was generally not influenced by the presence of interfering substances in suspension. Results obtained with the control antiseptic (PVI) matched those obtained by Hill and Casewell (2000) who reported that 5% PVI induced a 5 log10 reduction in *S. aureus* cell numbers after a 1 min contact time. Results were also similar to those obtained by Maillard *et al.* (1998) who showed a > 5 log10 reduction in *E. coli* and *P. aeruginosa* cell numbers after 1 min challenge with 2% PVI. Economou-Stamatelopoulou and Papavassiliou (1988) also showed that the activity of 5% PVI was not significantly affected by the addition of interfering substances in suspension when assessed against *S. aureus*. “In-vivo” studies have shown 10% PVI to be the most effective agent, with 70% ethyl alcohol, for removing *A. baumannii* (Cardoso *et al.*, 1999) and *S. aureus* (Guilhermetti *et al.*, 2001) from the contaminated hands of volunteers. PVI is generally recommended for use in hospital settings as a 7.5% surgical scrub agent (Hobson *et al.*, 1999) and a 10% hand cleansing agent (Cardoso *et al.*, 1999; Guilhermetti *et al.*, 2001). However, PVI can irritate the skin (Nishioka *et al.*, 2000) and its acceptance amongst healthcare staff varies. With the standard method EN 1276, the AHWS (after a 5 min contact time) and AHR (after a 1 min contact time) were as effective as 7.5% PVI against all bacteria.
The activity of 5% TTO could generally be ranked according to the conditions in which it was tested: perfect ≥ clean ≥ dirty, however, its efficacy was only significantly affected when assessed against *P. aeruginosa* and *A. baumannii*. Walsh et al. (2003) assessed the activity of the essential oils thymol and eugenol using the European suspension test EN 1276. The activity of 0.1% (v/v) eugenol and 1000 µg/ml thymol against *E. coli* was reduced in dirty conditions, however, the efficacy of thymol against *S. aureus* was not. As shown in the present study, the activity of essential oils appears to be affected by the conditions in which they are tested, and depends on the organisms assessed. It was interesting to find that 5% TTO in Tween 80 achieved over a 4 log₁₀ reduction of *P. aeruginosa* cell numbers after 5 min contact time, and 100% AHSW and 100% AHR achieved over a 5 log₁₀ reduction in perfect and clean conditions after 5 min and 1 min, respectively. By comparison, according to the results of Walsh and colleagues (2003), the reduction in *P. aeruginosa* cells achieved by thymol and eugenol was less than 4 log₁₀. The activity of 100% AHSW and 100% AHR against *E. coli* and *P. aeruginosa* was reduced in dirty conditions, although this reduction was not significant. Hammer et al. (1999) demonstrated that no one single interfering substance affected the activity of TTO against the microorganisms tested in their study (Hammer et al., 1999). Our results, showing a different interference level depending upon the formulation and concentration of TTO tested, and the concentration of interfering substances and the organism tested, agree with those of Hammer et al. (1999).

When the formulations that did not contain TTO were assessed, they were generally less active than these containing TTO. Even though it contained 64% alcohol, the AHR was significantly more active than its equivalent without TTO after a 1 min contact time against all bacterial strains, reinforcing the fact that the activity of this formulation is mainly due to the presence of TTO. The AHR passed the standard guidelines even though the concentration of TTO was only 2% in this formulation. The presence of a high concentration of alcohol probably enhanced the activity of the handrub. This was also supported by our results with the AHSW that achieved higher reductions in bacterial numbers than the HSW in both suspension tests although they both contained 5% TTO. Synergism between alcohol and some other active compounds has been reported with chlorhexidine (Sakuragi et al., 1995; Herrera et al., 2003), Triclosan (Messager et al., 2001) and PVI (Jeng & Severin, 1998).

Furthermore, the TTO-containing formulations assessed in this study also performed well according to the draft European standard method prEN 12054 for the assessment of hygienic handrub and handwash solutions. The AHSW successfully passed the European recommendations after a 1 min contact time against *E. coli, A. baumannii* and *P. aeruginosa*. The AHR also matched the European recommendations when tested against all bacterial strains. Previous studies have generally shown that alcoholic handrubs have greater and faster activity than handwash products (Kampf et al., 1999; Marchetti et al., 2003), are more “skin-friendly” and are therefore better tolerated by healthcare staff (Winnefeld et al., 2000; Parienti et al., 2002). The handrub formulation tested in this study passed both European standard methods and the presence of TTO enhanced its activity. Alcohol-based formulations have recently been promoted as being the most important development for improving compliance with hand cleansing in understaffed and overcrowded situations (Pittet & Boyce, 2001; Boyce & Pittet, 2002). In addition, they have been reported to cause less irritation and skin dryness than commonly used soaps and antiseptics (Boyce et al., 2000; Winnefeld et al., 2000, Kampf & Muscatiello, 2003). Thus, our findings regarding the TTO-containing alcoholic handrub are very encouraging and should be investigated further. The activity of the HSW was significantly improved after a 5 min contact time against *E. coli, A. baumannii* and *P. aeruginosa*, however, in real-life conditions, health-care staff are unlikely to wash their hands for longer than 1 min.

Previous studies have emphasised the fact that suspension tests may overestimate the activity of disinfectants and antiseptics (Maillard et al., 1998; Messager et al., 2001), and thus active compounds should be assessed using practical tests. Nonetheless, the AHSW and AHR assessed in this study were shown to comply with the European Standard against all bacteria tested. In addition, concentrations of TTO ≥ 5 % also complied with the EN 1276 by achieving ≥ 5 log₁₀ reduction when tested against *E.
coli and *P. aeruginosa*, and 100% HSW also complied when assessed in perfect conditions against *E. coli* and *A. baumannii*. This indicates that these products have at least met a minimum standard, and it remains for future investigations to evaluate them further.

In conclusion, the formulations tested in this study generally achieved high reductions in bacterial cell numbers. Their efficacy could be ranked as follows: 100% AHR > 100% AHSW > 5% TTO in Tw80 > 100% HSW.

**Table I** Effect of the neutraliser (EN 1276) on bacterial cells number after a 5 min contact time challenge

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; bacterial cell numbers recovered after treatment (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>-0.003 ± 0.072</td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td>+ 0.216 ± 0.137</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>+ 0.270 ± 0.119</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>+ 0.065 ± 0.143</td>
</tr>
</tbody>
</table>

Note: “+” indicates an increase in bacterial concentration.

**Table II** Efficacy of neutraliser in quenching the activity of different antiseptics after 5 min challenge

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; bacterial cell numbers recovered after treatment (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% TTO</td>
</tr>
<tr>
<td></td>
<td>+ Neutraliser (EN 1276)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>- 0.187 ± 0.162</td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td>+ 0.167 ± 0.158</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>+ 0.077 ± 0.020</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>- 0.271 ± 0.220</td>
</tr>
</tbody>
</table>

Note: “+” indicates an increase in bacterial concentration.
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>1 min</th>
<th>5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>/</td>
<td>100% AHSW (P / C / D)</td>
</tr>
<tr>
<td></td>
<td>100% AHR (P / C / D)</td>
<td>100% AHR (P / C / D)</td>
</tr>
<tr>
<td></td>
<td>7.5% PVI (P / C / D)</td>
<td>7.5% PVI (P / C / D)</td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td>100% HSW (C)</td>
<td>100% HSW (P)</td>
</tr>
<tr>
<td></td>
<td>100% AHSW (P / C / D)</td>
<td>100% AHSW (P / C / D)</td>
</tr>
<tr>
<td></td>
<td>100% AHR (P / C / D)</td>
<td>100% AHR (P / C / D)</td>
</tr>
<tr>
<td></td>
<td>7.5% PVI (P / C / D)</td>
<td>7.5% PVI (P / C / D)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>/</td>
<td>1% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>/</td>
<td>4% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>5% TTO (P / C / D)</td>
<td>5% TTO (P / C / D)</td>
</tr>
<tr>
<td></td>
<td>6% TTO (P)</td>
<td>6% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>8% TTO (P)</td>
<td>8% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>10% TTO (P)</td>
<td>10% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>/</td>
<td>100% HSW (P)</td>
</tr>
<tr>
<td></td>
<td>100 AHSW (C)</td>
<td>100% AHSW (P / C)</td>
</tr>
<tr>
<td></td>
<td>100% AHR (P / C)</td>
<td>100% AHR (P / C)</td>
</tr>
<tr>
<td></td>
<td>7.5% PVI (P / C / D)</td>
<td>7.5% PVI (P / C / D)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>/</td>
<td>4% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>6% TTO (P)</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>8% TTO (P)</td>
<td>8% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>10% TTO (P)</td>
<td>10% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>/</td>
<td>100% AHSW (P / C / D)</td>
</tr>
<tr>
<td></td>
<td>100% AHR (P / C)</td>
<td>100% AHR (P / C)</td>
</tr>
<tr>
<td></td>
<td>7.5% PVI (P / C / D)</td>
<td>7.5% PVI (P / C / D)</td>
</tr>
</tbody>
</table>

(P): perfect conditions (C): clean conditions (D): dirty conditions
<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>Contact time</th>
<th>S. aureus</th>
<th>A. baumannii</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% TTO</td>
<td>1 min</td>
<td>0.19 ± 0.36*</td>
<td>0.13 ± 0.39</td>
<td>4.38 ± 1.25</td>
<td>0.22 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>0.92 ± 0.63</td>
<td>0.85 ± 0.41</td>
<td>5.26 ± 0.11</td>
<td>0.93 ± 0.58</td>
</tr>
<tr>
<td>2% TTO</td>
<td>1 min</td>
<td>0.40 ± 0.43</td>
<td>-0.01 ± 0.06</td>
<td>4.60 ± 0.93</td>
<td>0.64 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.39 ± 0.64</td>
<td>1.83 ± 0.39</td>
<td>4.84 ± 0.93</td>
<td>2.24 ± 0.60</td>
</tr>
<tr>
<td>4% TTO</td>
<td>1 min</td>
<td>0.61 ± 0.53</td>
<td>0.53 ± 0.41</td>
<td>4.77 ± 1.05</td>
<td>2.05 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.24 ± 0.74</td>
<td>2.44 ± 0.52</td>
<td>5.26 ± 1.20</td>
<td>5.02 ± 0.91</td>
</tr>
<tr>
<td>5% TTO</td>
<td>1 min</td>
<td>0.88 ± 0.58</td>
<td>0.87 ± 0.43</td>
<td>5.26 ± 1.20</td>
<td>1.76 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.80 ± 0.45</td>
<td>3.81 ± 0.34</td>
<td>5.26 ± 1.20</td>
<td>4.02 ± 0.51</td>
</tr>
<tr>
<td>6% TTO</td>
<td>1 min</td>
<td>0.26 ± 0.41</td>
<td>1.11 ± 1.08</td>
<td>5.26 ± 1.20</td>
<td>5.12 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>0.95 ± 0.71</td>
<td>3.75 ± 1.43</td>
<td>5.26 ± 1.20</td>
<td>4.46 ± 0.57</td>
</tr>
<tr>
<td>8% TTO</td>
<td>1 min</td>
<td>0.32 ± 0.10</td>
<td>1.68 ± 0.94</td>
<td>5.26 ± 1.20</td>
<td>5.12 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.16 ± 1.03</td>
<td>4.72 ± 0.89</td>
<td>5.26 ± 1.20</td>
<td>5.29 ± 0.19</td>
</tr>
<tr>
<td>10% TTO</td>
<td>1 min</td>
<td>0.80 ± 0.38</td>
<td>3.04 ± 1.12</td>
<td>5.26 ± 1.20</td>
<td>5.12 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.89 ± 0.60</td>
<td>4.84 ± 0.68</td>
<td>5.26 ± 1.20</td>
<td>5.29 ± 0.19</td>
</tr>
</tbody>
</table>

*Log\(_{10}\) reductions ± standard deviation.
<table>
<thead>
<tr>
<th></th>
<th>5% TTO</th>
<th>100% HSW</th>
<th>100% AHSW</th>
<th>100% AHR</th>
<th>7.5% PVI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 min</strong> log reduction (cfu/mL) perfect conditions</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
<td><img src="image5.png" alt="Graph" /></td>
</tr>
<tr>
<td><strong>5 min</strong> log reduction (cfu/mL) perfect conditions</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
<td><img src="image5.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

**Figure 2**  
*EN 1276* - Antibacterial activity of 5% TTO, TTO-containing products and 7.5% PVI against *S. aureus* in different test conditions: perfect, clean or dirty conditions (mean ± standard deviation).

<table>
<thead>
<tr>
<th></th>
<th>5% TTO</th>
<th>100% HSW</th>
<th>100% AHSW</th>
<th>100% AHR</th>
<th>7.5% PVI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 min</strong> log reduction (cfu/mL) clean conditions</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
<td><img src="image5.png" alt="Graph" /></td>
</tr>
<tr>
<td><strong>5 min</strong> log reduction (cfu/mL) clean conditions</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
<td><img src="image5.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

**Figure 3**  
*EN 1276* - Antibacterial activity of 5% TTO, TTO-containing products and 7.5% PVI against *A. baumannii* in different test conditions: perfect, clean or dirty conditions (mean ± standard deviation).
Figure 4  
**EN 1276 -** Antibacterial activity of 5% TTO, TTO-containing products and 7.5% PVI against *E. coli* in different test conditions: perfect, clean or dirty conditions (mean ± standard deviation).

Figure 5  
**EN 1276 -** Antibacterial activity of 5% TTO, TTO-containing products and 7.5% PVI against *P. aeruginosa* in different test conditions: perfect, clean or dirty conditions (mean ± standard deviation).
Figure 6  Antibacterial activity of 5% TTO in 0.001% Tween 80, 100% HSW and 100% AHR (perfect conditions) compared with 0.001% Tween 80, 100% HSW-TTO and 100% AHR-TTO (mean ± standard deviation). Note: 0.001% Tween 80 was not tested at 1 min contact time.

Figure 7  EN 12054 - Antibacterial activity of TTO, PVI, HSW, AHSW and AHR against S. aureus, A. baumannii, E. coli and P. aeruginosa, after 1 min and 5 min contact time (mean ± standard deviation).
3. Sporicidal activity of tea tree oil

3.1. Background / Objectives

Recently, plant extracts have been developed and proposed for use as natural antimicrobials (Carson & Riley, 2001; Hsieh et al., 2001). Bacterial spores can be inhibited by extracts of garlic, onions, cinnamon, thyme, origanum, black pepper, clove and pimenta (Al-Khayat & Blank, 1985; Ismaiel & Pierson, 1990a; Ismaiel & Pierson, 1990b) and by aromatic plants such as eucalyptus, chamomile, cedar, savage carrots, artemisia, grapefruit, vervain, orange and rosemary (Chaibi et al., 1997). TTO has broad spectrum antimicrobial (Carson & Riley, 1993) and anti-inflammatory (Brand et al., 2001; Koh et al., 2002) activity in-vitro. However, the potential sporicidal activity of TTO has not yet been explored. The aim of this study was to investigate whether or not TTO has sporicidal activity by carrying out a series of suspension tests based on the European Standard prEN 14347 (European Standard prEN14347, 2002).

3.2. Materials & Methods

3.2.1. Microorganisms

_Bacillus subtilis_ (ATCC 6633) as recommended by the European standard method, and _Bacillus cereus_ (ATCC 13061) strains were maintained on blood agar (BA; Excel laboratory products) plates.

3.2.2. Sporulation method (based on prEN 14347)

**Preparation of Bacillus spores**

BA plates were inoculated from stock _Bacillus_ spp. cultures and incubated overnight at 37°C. Fifty mL of tryptone soya broth medium (TSB; Oxoid) was inoculated with a single colony and incubated overnight with shaking at 37°C. Ten mL of this preculture was transferred into Roux-bottles containing 50 mL of manganese sulfate agar: 15 g/L peptone USP (Difco); 3 g/L yeast extract (Oxoid); 6 g/L sodium chloride (AnalaR); 1 g/L D+ glucose (AnalaR); 0.1 g/L manganese sulfate (AnalaR) and 12 g/L agar (Oxoid). The liquid culture was distributed on the agar surface using sterile glass beads. Agar was then incubated for 1 week at 37°C and stored for 8 weeks at room temperature in a dry place protected from light.

**Harvesting of spores**

Ten mL of sterile distilled water (SDW) was pipetted into the Roux-bottles and the spores washed off with the help of the glass beads. The resulting suspension was transferred into a sterile vial and the same procedure was repeated with another 10 mL of SDW.

**Purification of spore suspension**

The suspension was centrifuged for 30 min at 3000 g, the supernatant was removed and the sediment resuspended in 65% ethanol (BDH). Inactivation of the remaining vegetative cells was achieved by leaving the suspension for 3 h at 20°C. The ethanol was then diluted with SDW to half its starting concentration. Suspensions were centrifuged and washed 5 times with SDW. Spore suspensions were stored for 4 weeks at 4°C until use.

**Purity control of the spore suspension**

Suspensions were stained with the modified “Witrz-Conklin” spore stain (Schaeffer & MacDonald, 1933) after smearing onto a glass slide and fixing with heat. Slides were flooded with 5% aqueous malachite green (Sigma) and intermittently heated with a Bunsen burner flame for approximately 5 min, so that the dye remained hot but not boiling. Slides were rinsed under tap water and then counterstained with 0.5% Safranin-O (Sigma) for 1 min. After drying, slides were examined using a light microscope and oil immersion. The amount of remaining vegetative bacteria did not exceed 20%
per field of view or else suspensions were filtered and washed again. Spore counts were performed by serial dilution using the DCM (Maillard et al., 1998) onto BA plates incubated for 24 h at 37°C. The spore stock suspensions was adjusted to >10⁹ cfu/mL with SDW.

### 3.2.3. Active compounds

The antimicrobial activity of the following agents was investigated: 5%, 10% and 15% TTO in 0.001% Tween 80, 100% AHSW, 100% AHR; 7.5% (v/v) povidone iodine (PVI), 0.05% (v/v) sodium hypochlorite (SH; from a Linley solution containing 14.5 mg/g sodium hypochlorite plus 1 mg/g dichloroisocyanurate; 10 mg/mL available chlorine), 28% (v/v) hydrogen peroxide (HP; Pronalyis) and 3% (v/v) glutaraldehyde (GA; Ted Pella Inc). The batch of TTO used in this study was described previously in Chapter 2. The antimicrobial agents were diluted in SDW to the concentration indicated and tested immediately. Neutraliser was based on the European Standard EN 1276 and contained: 30 g/L Tween 80 (Sigma), 3 g/L lecithin (Sigma), 1 g/L histidine (Sigma), 5 g/L sodium thiosulfate (AnalaR) and 34 g/L potassium dihydrogen phosphate (AnalaR) in tryptone soya broth (TSB; Oxoid).

### 3.2.4. Spore count determination (SDW control) (prEN 14347)

One mL of the original spore suspension was mixed with 9 mL of SDW. After the appropriate contact time, the suspension was mixed and 0.1 mL transferred into a tube containing 10 mL of SDW and shaken. After 30 min, the solution was serially diluted using the DCM and inoculated on glucose yeast extract agar (GYA) that contained 1 g/L amino-acids, without vitamins, obtained by hydrolysis of casein (Sigma); 1 g/L soluble starch (AnalaR); 2.5 g/L glucose (AnalaR); 5 g/L yeast extract; 0.1g/L FeSO₄ (AnalaR); 0.0001 g/L manganese sulfate (AnalaR) and 15g/L agar (Oxoid) at pH 6.8. Plates were incubated at 37°C and counted every day for 4 days.

### 3.2.5. Control of spore solutions with a reference substance

Spore suspensions were tested for sensitivity against a GA standard before starting a test series with a disinfectant according to the European draft standard method EN 14347. The GA standard was prepared from a 25% GA solution as follows:

- *B. cereus*: 5g GA + 0.25g NaHCO₃ + 94.75g SDW = 1.25% pre-concentrated
- *B. subtilis*: 15g GA + 0.75g NaHCO₃ + 84.25g SDW = 3.75% pre-concentrated

Spore suspensions were tested with the GA standard using the suspension test described below.

### 3.2.6. Test procedure for sporicidal activity of product (prEN 14347)

One mL of spore solution (3 × 10⁸ – 1 × 10⁹ spores/mL) was mixed with 1 mL of SDW, then 8 mL of the pre concentrated product solution was added, mixed and kept at 20°C. After 30, 60 and 120 min contact time, the solution was mixed and 0.1 mL transferred into a tube containing 10 mL of neutralizing solution. After 30 min of neutralization, solutions were serially diluted and viable counts were performed on GYA plates using the DCM. Plates were incubated at 37°C for at least 4 days and spores growth recorded every day.

### 3.2.7. Statistical analysis

Analyses of variance were performed with the Excel ® and Prism ® software packages. Three to five replicates were performed and *P* values of < 0.05 were considered significant. In addition to the one-way ANOVA test, each active compound was compared to the SDW control using the Dunnett’s post-test.
3.3. Results

3.3.1. Purity control of spore suspensions
Spore suspensions were examined under a light microscope. *Bacillus* spores appeared as greenish-blue spheres, and vegetative cells stained pink. Suspensions that contained less than 80% spores were discarded.

3.3.2. Testing of spore suspensions against the GA standard and neutralization
The results were in agreement with the reference standard values given by the European standard method (Table V). The neutraliser chosen quenched the activity of the active components tested and did not significantly reduce the concentration of bacterial spores.

3.3.3. Bacillus cereus spores
There was no statistical difference in activity between 100% AHR, 100% AHSW, 7.5% PVI and the SDW control (Figure 8). TTO (5%) was significantly more active ($P < 0.05$) than the SDW control after 120 min contact time. SH (0.05%) and 10% TTO were significantly more active ($P < 0.05$) than the SDW control after at least 60 min contact time (Figure 8). TTO (15%) was significantly more active ($P < 0.05$) than the SDW control after 30 and 120 min contact time and 28% HP was always significantly more active ($P < 0.05$) than the SDW control (Figure 8). After 30 min contact time, 28% HP was significantly more active ($P < 0.05$) than any of the other active compounds. TTO (15%) was significantly more active ($P < 0.05$) than 5% TTO, however there was no significant difference of activity between 10% and 15% TTO. None of the remaining treatments differed significantly from the SDW control.

After 60 min, 28% HP was significantly more active ($P < 0.05$) than all of the other active compounds, followed by 0.05% SH, 15% TTO and 10% TTO which achieved comparable ($P > 0.05$) reductions in spore numbers. None of the remaining treatments differed significantly ($P > 0.05$) from the SDW control. After 120 min, 0.05% SH and 28% HP had comparable high activity ($P > 0.05$) and were significantly ($P < 0.05$) more active than any of the other treatments. The three concentrations of TTO tested achieved comparable results ($P > 0.05$) and were significantly ($P < 0.05$) more active than any of the remaining treatments and the SDW control.

3.3.4. Bacillus subtilis spores
There was no statistical difference in activity between 100% AHR, 100% AHSW, 7.5% PVI, 5% TTO and the SDW control (Figure 9). TTO (10%) and 0.05% SH were significantly more active ($P < 0.05$) than the SDW control after 60 and 120 min contact time (Figure 9). TTO (15%) and 28% HP were significantly more active ($P < 0.05$) than the SDW control after 30, 60 and 120 min contact time (Figure 9).

After 30 min contact time, 28% HP was significantly more active ($P < 0.05$) than any of the other active compounds. There was no statistical difference in activity between the remaining treatments and the SDW control. After 60 min contact time, 28% HP was significantly more active ($P < 0.05$) than 0.05% SH, which was itself significantly more active ($P < 0.05$) than 15% TTO. TTO (10%) and 15% TTO had comparable activity ($P > 0.05$) and were significantly ($P < 0.05$) more active than the remaining of the treatments. After 120 min contact time, 0.05% SH and 28% HP were significantly ($P < 0.05$) more active than 10% TTO and 15% TTO which were also significantly ($P < 0.05$) more active than the remaining of the treatments.
3.3.5. Comparison between spore strains

All TTO-containing formulations were more active against *B. cereus* than against *B. subtilis*, which was significant (*P* < 0.05) for the following treatments and times: 15% TTO after 30 min contact time, 10% TTO after 60 min contact time, 5% TTO and 100% AHR after 120 min contact time.

3.4. Discussion

The control testing carried out with GA was in agreement with the European standard data. *B. cereus* spores were more sensitive to 1% GA than *B. subtilis* spores were to 3% GA, with 4.80 and 2.64 log_{10} reduction within 60 min exposure, respectively. Rubbo and colleagues (1967) showed that 2% GA in water reduced the number of *B. anthracis* spores by 4 log_{10} within 15 min. The relatively low level of sporidical activity of GA against *B. subtilis* was also reported by Dyas & Das (1985) who showed that *B. subtilis* var. *globigii* survived a 2 h exposure to 2% GA, and by Sagripanti & Bonifacino (1996) who found that a 2% GA solution at an ionic strength of 0.05 M sodium bicarbonate killed approximately 99.5% of *B. subtilis* spores after 60 min treatment.

Only HP and SH met the European standards requirements by reducing the number of spores by 4 log_{10} within 120 min contact time, or less. Our results showed that 28% HP killed over 99.99% *Bacillus* spores within 30 min contact time. Other studies showed that 25.8% HP killed 99.999% of *B. subtilis* var. *globigii* spores within 15 min at 24°C (Toledo et al., 1973). Interestingly, after 30 min contact time, 15% TTO was more active against *B. subtilis*, and significantly more active against *B. cereus*, than SH. Our data were in agreement with those of Sagripanti & Bonifacino (1996) showing that SH (at pH12) had little effect against *B. subtilis* after 30 min contact time only.

The TTO-containing solutions tested in our study did not achieve 4 log_{10} reduction as recommended by the European Standard. However, this standard method is designed for the evaluation of minimal requirements for chemical disinfectants and antiseptic products. A previous study showed that a widely used health care personal handwash that contained 1% chloroxylenol failed to reduce the number of *B. subtilis* var. *globigii* spores within 30 min (Sagripanti & Bonifacino, 1996). Another study assessed the activity of two commonly used handwash agents and reported high MICs of 1% for chlorhexidine digluconate and 2.5 to 5% for a PVI soap when assessed against spores of *B. subtilis* var. *globigii* (Penna et al., 2001). In our study, when compared with the control SDW, 5% TTO significantly reduced the number of *B. cereus* spores after 120 min contact time. In addition, 10% TTO significantly reduced the number of *B. cereus* and *B. subtilis* spores after 60 min contact time, and 15% TTO significantly reduced the number of *B. cereus* and *B. subtilis* spores after 30 min contact time.

TTO at a concentration of 5% in Tween 80 was generally more active than AHSW, which also contained 5% TTO. As emphasised by Hammer and colleagues (1999), the pharmaceutical formulation in which TTO is incorporated may influence its overall activity. These findings reinforce the idea that it is necessary to assess final product formulations as well as the active ingredient by itself (Allwood & Shaw, 1987).

There is a lack of clinical data to support the use of antiseptic agents over plain soap and water when sporidical properties are required. However, a new agent composed of ethylenediaminetetraacetic acid, disodium salt (EDTA-2Na), ferric chloride hexahydrate (FeCl3 . 6H2O) and ethanol, called the “ethanol reagent”, was shown to have potent sporidical activity against *B. subtilis* spores in suspension at pH 0.3 (Kida et al., 2003). Its activity at 25°C was greater than that of 0.05% SH. This ethanol reagent had high sporidical activity after a 5 min contact time at 37°C, and it was therefore suggested that it could be used for disinfection of normal skin (Kida et al., 2003). However, “in-vitro” tests cannot predict activity *in-situ* and further studies under practical conditions, as well as data proving the product to be non-toxic and non-irritating are required before it can be used as an antiseptic agent.
The American standard method E 1174-94 (ASTM E 1174-94) assesses the sporicidal activity of handwash products against \textit{B. atrophaeus} (ASTM 1994). Weber \textit{et al}. (2003) used this method to investigate the sporicidal activity of several handwash products against \textit{B. atrophaeus} (Weber \textit{et al}., 2003). Soap and water was generally at least as effective at removing bacterial spores from contaminated hands than a formulation containing 2% chlorhexidine gluconate and chlorine-containing towels. However, the study did not stipulate what reduction in bacterial spore numbers was required, or if it needed to perform significantly better than the control soap to be classified as “effective”. Weber \textit{et al}. (2003) also reported that a waterless wash containing 61% ethyl alcohol was ineffective at removing or inactivating \textit{Bacillus atrophaeus} spores after a 10, 30 or 60 sec wash. Our results also showed that the alcohol-containing formulations AHSW and AHR were not sporicidal, confirming previous studies showing that alcohol is not sporicidal and hence should not be used for hand hygiene or surface decontamination (Russell, 1990; Weber \textit{et al}., 2003).

In conclusion, this study showed that even though the European requirements were not met by formulated TTO products, 5, 10 and 15% TTO in Tween 80 significantly reduced the concentration of bacterial spores when assessed in suspension, and TTO was generally more effective than PVI.

### Table V

<table>
<thead>
<tr>
<th>GA standard</th>
<th>Exposure time</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental values</td>
<td>Standard values</td>
<td>Experimental values</td>
</tr>
<tr>
<td>\textit{B. subtilis} (3% GA)</td>
<td>1.94 ± 0.75*</td>
<td>0.4 - 2.7**</td>
<td>2.64 ± 0.59</td>
</tr>
<tr>
<td>\textit{B. cereus} (1% GA)</td>
<td>3.22 ± 0.45</td>
<td>3.1 - 3.8</td>
<td>4.80 ± 0.40</td>
</tr>
</tbody>
</table>

*Log$_{10}$ reduction ± standard deviation.  
**Log$_{10}$ reduction range
Figure 8  Sporicidal activity of several biocides against *B. cereus* (ATCC 13061) (mean ± standard deviation)

Figure 9  Sporicidal activity of several biocides against *B. subtilis* (ATCC 6633) (mean ± standard deviation)
4. Antibacterial activity of TTO on surfaces

4.1. Background / Objectives

In Chapter 1, “in-vitro” standard suspension methods were used to assess the efficacy of TTO and TTO-containing formulations according to European guidelines (EN 1276, 1997; prEN 12054, 1997). Some of the formulations as well as TTO in Tween 80 passed the suspension European tests, and these products were therefore evaluated further by using capacity tests which are more stringent than suspension test (Maillard et al., 1998). Capacity tests, such as glass-carrier and stainless steel tests are used to imitate microbial attachment to environmental surfaces, including short-term attachment and drying. They are also relatively simple, do not require expensive pieces of material and are reproducible (Bloomfield et al., 1993). The activity of TTO and TTO-containing formulations was assessed according to the European standard method on stainless steel (EN 13697, 1997) and the American surface test on glass-carriers (ASTM 2111-00, 2000) against S. aureus, A. baumannii, E. coli and P. aeruginosa.

4.2. Materials & Methods

4.2.1. Microorganisms

S. aureus (ATCC 25923), A. baumannii (NCTC 7844), E. coli K12 (NCTC 10538) and P. aeruginosa (NCTC 6749) stock cultures were made on tryptone soya agar (TSA; Oxoid) plates, stored at 4°C and renewed once a week. Working cultures of these strains were obtained by a method described in Chapter 2.

4.2.2. Antiseptic products

The antimicrobial activity of the following agents was investigated: 100% HSW, 7.5% (v/v) povidone iodine (PVI) and 5% TTO in 0.001% (v/v) Tween 80. The batch of TTO used in this study was described previously in Chapter 2.

Antimicrobial agents were diluted to the concentration indicated in sterile distilled water (SDW) and tested immediately. The neutralizing solution used to quench the activity of antiseptics was based on the European Standard EN 1276 and is described in Chapter 2.

4.2.3. Stainless steel test (based on European Standard EN 13697 / 2001)

Stainless steel discs of two cm diameter of Grade 2B and with a 1.2 mm gauge were used (Unique Metal Works, Perth, Australia).

Disc preparation
Prior to utilization, the discs were placed in a beaker with 20 mL Linley (Ramprie Laboratories, Western Australia) for 60 min. They were then rinsed twice with SDW for 10 sec and placed in a bath containing 70% (v/v) ethanol (BDH) for 15 min for sterilisation. Lastly, the discs were removed and dried by evaporation in a laminar airflow cabinet.

Antiseptic challenge method
Using sterile forceps, discs were placed in empty, sterile petri-dishes (2 petri-dishes and 8 discs per strain / 4 discs per contact time: 3 test-discs and 1 control-disc) and were appropriately labelled. Each disc was inoculated with 50 µL of bacterial inoculum, and left to dry at 37°C.

Once the inoculum was dry, the discs were allowed to equilibrate at room temperature. A 100 µL volume of antisepetic product (5% TTO in Tween 80, 100% HSW and 7.5% PVI) or SDW (control) was aliquoted onto the appropriate disc so that the dried inocula was totally covered. After 1 min and 5
min contact time the appropriate disc was transferred into a beaker containing 5 g of glass beads (Selby Scientific) and 10 mL of neutraliser, with the inoculated side facing down. Discs were shaken horizontally for 1 min. After 5 min of further neutralisation, the mixture was serially diluted in sterile PBS, and viable counts were performed using the DCM (Maillard et al., 1998). The discs were recovered from the neutralizing solution, rinsed off with 10 mL of SDW and transferred, test side facing up, to a petri-dish that contained 10 mL of TSA. A 100 μL volume of SDW was aliquoted onto the residual desiccated inoculum which was scraped with a pipette tip for 1 min. Ten mL of melted TSA, cooled at 45°C, was poured over the test surface. After the agar had set, plates were incubated at 37°C overnight. The reduction in bacterial number (Log_{10} reduction) after antiseptic challenge was calculated as follow: LR = log_{10} number of cfu in the water control - log_{10} number of cfu in the antiseptic test.

4.2.4. Glass-carrier test (based on the American standard ASTM E2111)

This test was based on the American Standard ASTM E2111, as described by Walsh et al., (1999) and Sattar & Springthorpe (2001). A 50 μL volume of bacterial inoculum was placed on the bottom of a sterile glass bottle (autoclavable McCartney bottle) and left until completely dried (approx. 2h) under a laminar flow cabinet. A 100 μL sample of antiseptic or SDW (control) was added to the dried inoculum and after 1 min and 5 min contact time, 10 mL of neutralising solution and 5 g of sterile glass beads were added into the bottle. The mixture was shaken until the dried cells were resuspended into the solution. After 5 min neutralisation, viable counts were performed using the DCM. The reduction in bacterial number (Log_{10} Reduction) after antiseptic challenge was calculated as described for the stainless steel test method.

4.2.5. Statistical analysis

All tests were repeated 3 to 5 times. Statistical analyses of variance were performed using the Excel ® software and P values of < 0.05 were considered significant.

4.3. Results

4.3.1. Stainless steel test method

Effect of drying step upon bacterial survival

The drying step resulted in a small, yet statistically significant decrease in cell numbers recovered after 1 min: 8.45 log_{10} and 5 min: 8.25 log_{10} contact time with SDW, compared to the original inoculum size of 8.72 log_{10} cfu/mL (Table VI). The number of A. baumannii cells recovered after the drying period was not significantly (P > 0.05) different from that in the original inoculum (Table VI). However, there was a significant (P < 0.05) reduction in E. coli and P. aeruginosa cell numbers due to the drying step after a 1 min and a 5 min contact time, respectively (Table VI).

Antibacterial activity

The only products that complied with the European standard method by achieving ≥ 4 log_{10} reduction in bacterial cell numbers were 5% TTO in Tween 80 against E. coli after 1 min contact time (Figure 10), and 7.5% PVI against all strains after 1 min contact time (Figure 10). Nevertheless, after 1 and 5 min contact time, 5% TTO significantly reduced (P < 0.05) the number of all bacterial strains tested (Figure 10), ie: the number of bacterial cells recovered from the 5% TTO-treated discs was significantly lower than that recovered from the SDW-treated discs (control). The number of bacterial cells recovered after treatment with 100% HSW was always lower than that recovered from the SDW control, although this was only significant when assessed against S. aureus after 5 min contact time (P < 0.05), and when assessed against A. baumannii and E. coli after 1 and 5 min contact time (P < 0.05) (Figure 10).

TTO (5%) was generally more active after 5 min than after 1 min contact time, although this was not significant (P > 0.05). HSW (100%) was generally more active after a 5 min than after a 1 min contact
time, but this was only significant for *E. coli* (Figure 10). The activity of 7.5% PVI was generally not affected by the contact time tested (Figure 10).

TTO (5%) was generally significantly (P < 0.05) less active than 7.5% PVI, apart from when tested against *E. coli* after 1 min and 5 min contact time (P > 0.05) and against *P. aeruginosa* after 5 min contact time (P > 0.05) (Figure 10). TTO (5%) was generally more active than 100% HSW but this was only significant (P < 0.05) against *S. aureus, A. baumannii* and *E. coli* after 1 min contact time and against *P. aeruginosa* after 1 min and 5 min contact time (Figure 10). PVI (7.5%) was significantly (P < 0.05) more active than 100% HSW against *S. aureus*, *A. baumannii* and *P. aeruginosa* after 1 min and 5 min contact time, however, 100% HSW was as active as 7.5% PVI (P > 0.05) against *E. coli* after a 5 min contact time (Figure 10).

### 4.3.2. Glass-carrier test

**Effect of drying step upon bacterial survival**

The number of *S. aureus, P. aeruginosa* and *A. baumannii* cells recovered from the glass-carriers after 1 min and 5 min contact time was not significantly different from that of the original inoculum (Table VI). However, the number of *E. coli* cells recovered from the glass-carriers after 1 min and 5 min was significantly (P < 0.05) lower than that in the original inoculum (Table VI).

**Antibacterial activity**

The only products that achieved ≥ 4 log₁₀ reduction in bacterial cell numbers were 5% TTO in Tween 80 against *A. baumannii* and *P. aeruginosa* after a 5 min contact time (Figure 11), and 7.5% PVI against all strains after a 5 min contact time (Figure 11). When compared with SDW (control), 5% TTO significantly reduced the number of *S. aureus* cells after a 5 min contact time and the number of *A. baumannii, P. aeruginosa* and *E. coli* cells after 1 min and 5 min contact time (Figure 11). The number of bacterial cells recovered from the HSW-treated glass-carriers was always lower than that recovered from the SDW-treated carriers, however this was not significant (Figure 11). PVI (7.5%) achieved a reduction in bacterial cell numbers ≥ 4 log₁₀ when assessed against all strains after a 5 min contact time (Figure 11). The activity of 5% TTO and 100% HSW was increased after a 5 min contact time, although not significantly (Figure 11). However, 7.5% PVI was as active after a 1 min as after a 5 min contact time (P > 0.05). PVI (7.5%) was generally more active than 5% TTO, but this was only significant (P < 0.05) against *S. aureus* and *A. baumannii* after 1 min and 5 min, and was also generally significantly (P < 0.05) more active than 100% HSW against all strains, with the exception of *E. coli* after a 1 min contact time. TTO (5%) was generally more active than HSW, but this was only significant (P < 0.05) against *A. baumannii* and *P. aeruginosa* after 1 min and 5 min contact time.

### 4.3.3. Comparison between surface tests

**Bacterial survival**

*S. aureus* and *P. aeruginosa* survived the drying step significantly better (P < 0.05) on glass-carriers than on stainless steel surfaces. *A. baumannii* survived as well (P > 0.05) on glass-carriers as on stainless steel surfaces and *E. coli* survived as poorly on glass-carriers as on stainless steel surfaces (Table VI).

**Antibacterial activity**

TTO (5%) in Tween 80 and 7.5% PVI were more active when tested on stainless steel surfaces than on glass-carriers against *S. aureus* and *E. coli*, although this was not significant. They were also more active when tested on glass-carriers than on stainless steel surfaces against *A. baumannii* and *P. aeruginosa* although this was also not significant. HSW (100%) was generally more active when tested on stainless steel surfaces than on glass-carriers, but this was only significant (P < 0.05) for *E. coli* after a 5 min contact time.
4.4. Discussion

According to the European standard method (EN 13697), a product can be described as having bactericidal activity on surfaces if it produces a $\geq 4 \log_{10}$ reduction in the number of viable cells within 5 min at 20°C. The American standard method can be adapted to measure up to a $6 \log_{10}$ reduction in cell numbers. However, in this study the glass-carrier method was adapted to match the European standard using stainless steel surfaces.

One of the problems associated with surface testing is the significant loss of bacterial cells due to the drying step (Messager et al., 2001). According to our results, organisms such as *E. coli* and *P. aeruginosa* were affected by the drying step. These findings are in agreement with previous studies by Maillard et al. (1998) and Messager et al. (2001), which highlight the difficulty of testing antimicrobials on surfaces. In one surface test study, *P. aeruginosa* and *S. aureus* lost 1.1 and 0.60 $\log_{10}$ cfu/mL in cell numbers, respectively, after drying for 1h on stainless steel at 37°C (Bloomfield et al., 1993). According to our results, *P. aeruginosa* and *S. aureus* lost 0.91 and 0.39 $\log_{10}$ cfu/mL in cell numbers, respectively, after drying for approx. 1h.

Traore et al. (2002) showed that disinfectants were significantly more active when assessed with the American standard glass-carrier test (QCT-1; ASTM E2111-00) than with the quantitative carrier test on stainless steel surfaces (QCT-2) described by Traore et al. (2002). From our results, there appeared to be a pattern of efficiency depending upon the antiseptic and the microorganism tested. However, even if a product was slightly more active on one surface than on the other, there was generally no statistical difference. This outcome may be partially explained because the same bacterial inoculum / disinfectant ratio was used in our study, thus the surface type was the only difference as opposed to having a higher volume of disinfectant as used in the original QCT-1.

According to our results, TTO was more active against the Gram-negative strains than against *S. aureus*. This may be due in part to the drying step, which is known to significantly affect survival and thus the recovery of *E. coli* and *P. aeruginosa* cells. However, as described in Chapter 2, the efficacy of TTO assessed in suspension using the European standard test (EN 1276, 1997) also showed that TTO was more active against the Gram-negative bacteria than against *S. aureus*, even though this was not always statistically significant.

Although the results were generally not statistically significant, 100% HSW was more active against all strains when tested on stainless steel surfaces, and so was 5% TTO against *S. aureus* and *E. coli*.

Results obtained in this study were compared with that from Chapter 2, in which we assessed the activity of several TTO-containing formulations, with the European standard test EN 1276. This comparison showed that TTO-containing products were generally significantly more active when tested in suspension than on surfaces. A lower reduction of bacterial cell numbers after antiseptic treatment on surfaces as opposed to in suspension tests has been previously reported (Best et al., 1988; Gibson et al., 1995; Van Klingereren et al., 1998; Messager et al., 2001). In laboratory tests, organisms dried onto surfaces clearly represent a significantly greater disinfection challenge than organisms in suspension (Bloomfield et al., 1993). This was confirmed by the high standard deviations observed when antisepsics were assessed on surfaces both in this study and in previous work (Maillard et al., 1998; Messager et al., 2001).

Past studies have shown that the regular decontamination of environmental surfaces in health-care settings helps to reduce the number of outbreaks of infection (Bures et al., 2000; Mayfield et al., 2000). Even though TTO might not be able to compete with existing biocides commonly used for surface decontamination, its long-term presence in the health-care environment does not seem to present the same concerns as other widely used chemicals do. The use of the latter ones may lead to repetitive exposure of microorganisms to low levels of biocide residues (Russell, 2002), and could possibly promote the emergence of biocide-resistant and / or antibiotics-resistant microorganisms.
In conclusion, 5% TTO in Tween 80 generally significantly reduced the number of bacterial cells dried onto surfaces, compared to SDW only. Although the two TTO-containing formulations did not always achieve high reductions in bacterial numbers ($\geq 4 \log_{10}$), 5% TTO proved to be nearly as effective as 7.5% PVI when tested against *E. coli*, *A. baumannii* and *P. aeruginosa* after 5 min contact time on both surfaces. Both surface tests showed comparable results although the organisms tested seemed to be slightly more sensitive to antiseptics when assessed on stainless steel surfaces.

### Table VI
Concentration of bacterial cells recovered after the drying step on stainless steel and glass-carrier surfaces

<table>
<thead>
<tr>
<th></th>
<th>Log$_{10}$ cfu/mL (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td><strong>Stainless steel</strong></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>-0.19 ± 0.23</td>
</tr>
<tr>
<td>5 min</td>
<td>-0.39 ± 0.58</td>
</tr>
<tr>
<td><strong>Glass-carrier</strong></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>0.30 ± 0.27</td>
</tr>
<tr>
<td>5 min</td>
<td>0.32 ± 0.24</td>
</tr>
</tbody>
</table>

Note: “*” indicates an increase in bacterial concentration.
**Figure 10** Antibacterial activity of several antiseptics tested on stainless steel surfaces (mean ± standard deviation).

**Figure 11** Antibacterial activity of several antiseptics tested on glass-carriers (mean ± standard deviation).
5. ‘Ex-vivo’ and ‘in-vivo’ testing

The results of this chapter are also described in the following publication:


5.1. Background / Objectives

The results described in Chapter 2 showed that some TTO-containing formulations, as well as different concentrations of TTO in Tween 80, achieved a ≥ 5 log_{10} reduction in bacterial cell numbers after 1 and/or 5 min contact times using the European standard suspension test EN 1276 and ≥ 4 log_{10} reduction in bacterial cell numbers after 1 and/or 5 min contact times using the European draft-standard suspension test prEN 12054. The European suspension tests determine whether a preparation possesses antimicrobial properties specific for a defined application (phase 2 / step 1). Once established, the formulation should be tested with methods establishing whether it possesses antimicrobial properties in practice, mimicking conditions such as, in this case, a handwashing test (phase 2 / step 2). Therefore, the efficacy of a HSW and an AHSW that both contained 5% TTO, as well as 5% TTO in Tween 80, was investigated in-vivo according to the European handwashing test (European Standard EN1499, 1997). An “ex-vivo” test, which has been shown to be a reproducible potential substitute for “in-vivo” testing (Maillard et al., 1998; Messager et al., 2001) was adapted to imitate the handwashing technique and was also used to assess the activity of TTO. Data obtained ex-vivo were compared to those obtained in-vivo.

5.2. Materials & Methods

5.2.1. Microorganisms

Escherichia coli K12 (NCTC 10538) stock cultures were kept on tryptone soya agar (TSA; Oxoid) plates, stored at 4°C, and renewed once a week.

5.2.2. Antiseptics

The activity of the two following handwash formulations was investigated: 100% HSW and 100% AHSW, as well as 5% TTO in 0.001% Tween 80.

5.2.3. Neutraliser

The neutralising solution used to quench the activity of antiseptics was based on the European Standard EN 1276 and is fully described in Chapter 2. The efficacy of this neutraliser in quenching the activity of TTO, as well as its absence of toxicity against E. coli K12 has been reported in Chapter 2.

5.2.4. Reference product

To compensate for extraneous influences, the results obtained with the products tested were compared with those obtained with a reference handwash (soft soap [SS]) as recommended by the EN 1499. Experiments using the SS were performed under comparable environmental conditions. SS contained linseed oil (100 g/L; Sigma), potassium hydroxide (19 g/L; Sigma) and ethanol (14 g/L; Fisher), with SDW as the balance.

5.2.5. “In-vivo” handwashing test EN 1499

The number of test organisms released from artificially contaminated hands was assessed before and after hygienic handwash. The prevalue is the number of colony forming units (cfu) sampled from the
skin before treatment. The postvalue is the number of cfu sampled from the skin after treatment. The reduction factor (RF) in bacterial cell concentration was calculated as follows: \( \log_{10} RF = \log_{10} \text{prevalue} - \log_{10} \text{postvalue} \), where RF is a measure of the antimicrobial activity of the disinfectant tested. Due to volunteer availability, the handwashing studies were carried out on two separate occasions, referred to as study 1 and 2.

**Subjects**
The necessary precision was achieved by repeating the test on 13 subjects in study 1 (including eight females and five males), and 14 subjects in study 2, consisting of eight females and six males. Volunteer ages ranged from 22 to 52 years old and from 19 to 53 years old in study 1 and 2, respectively. All subjects had short fingernails and intact skin, and were asked not to use any antibacterial soap or toiletries on the hands for 24 h before the test. All volunteers were informed about the procedure and signed a consent form prior to beginning the study. In each study, the products under investigation were tested on each subject.

**Antiseptics**
The following antiseptics were investigated: 5% (v/v) TTO in 0.001% (v/v) Tween 80 and 100% HSW in study 1, and 100% AHSW in study 2. For each antiseptic and the SS the prevalues and postvalues were determined. Volunteers in each study were separated into groups and the order of testing in both studies was determined by a Latin Square Design (Table VII).

**Contamination fluid**
The suspension used for the “in-vivo” test was prepared as described in the European standard EN 1499. *E. coli* K12 was grown overnight in two tubes each containing 5 mL of TSB at 37°C that were then inoculated into two bottles containing 1 L of TSB each and incubated for a further 18-24 h at 37°C. These cultures were then combined and a viable count performed using the DCM. The viable count as well as contamination was then monitored after every four volunteers using the DCM.

**Application of the contaminating fluid**
The subjects’ hands were prepared by washing them for 1 min with SS to remove natural transient microorganisms and then dried on paper towels. The contamination fluid was poured into a container, and the hands were immersed up to the mid meta-carpal for 5 sec, with fingers spread apart in the bacterial suspension. After fingers were removed, the surplus was carefully allowed to drain back into the container. Finally, the hands were allowed to air dry for 3 min, held in the horizontal position with fingers spread out and rotating the fingers to avoid the formation of droplets. The contamination batch was not used for more than 3 h after the first subject’s hands had been contaminated.

**Prevalues**
Immediately after drying, the fingertips and thumb tips were rubbed for 1 min on the base of a petri-dish containing 10 mL of TSB without neutraliser with the aim of assessing the release of the test organism before treatment of the hands (1 petri-dish per hand). Serial dilutions of the sampling fluid were prepared in phosphate buffered saline (PBS): 8 g/L NaCl (AnalaR), 0.2 g/L KCl (AnalaR), 1.44 g/L Na2HPO4 (AnalaR) and 0.24 g/L KH2PO4 (AnalaR) and the number of bacteria released counted by DCM.

**Hygienic handwash procedure**
Straight after prevalue sampling and without recontamination of the hands, the group performed handwashing with either the SS or test antiseptic product using the following method. Five mL of SS or antiseptic product was poured into cupped hands which had been premoistened with tap water, and the volunteers performed the 6 steps of the standard handwashing procedure: palm to palm, right palm over left dorsum, and left palm over right dorsum, palm to palm with fingers interlaced, backs of fingers to opposing palms with fingers interlocked, rotational rubbing of right thumb clasped in left palm and vice-versa, and finally rotational rubbing, backwards and forwards with clasped fingers of right hand in left palm and vice versa. As much warm water as necessary to produce lather was added.
and the handwash procedure continued for 60 sec. Finally, hands were rinsed under tap water for 15 sec from distal to proximal with fingertips upright. To avoid recontamination of the sampling area, the fingers had to remain pointing upwards until postvalue sampling. Wrists and lower arms were dried with paper towels by a helper.

**Postvalues**
Immediately after drying the wrists, the fingertips and thumb tips were rubbed on the base of a petri-dish containing 10 mL of neutraliser for 1 min (1 petri-dish per hand). These sampling fluids were then serially diluted in neutraliser, and viable counts performed by the DCM. After the tests with SS and the antiseptics, the subjects were asked to decontaminate their hands using a hospital grade soap cleanser and then by rubbing with 70% ethanol as the final decontamination step. Colonies were counted after incubation of plates at 37°C for 24 h followed by a further 24 h incubation to detect any slow growing colonies.

5.2.6. Skin samples and the “Ex-vivo” method

**Skin samples**
Skin samples were obtained from breast and abdominal reduction surgery, and were collected from plastic surgery units. Prior to surgery, the donors were thoroughly informed about the research being conducted with the samples (Appendix 1) and had to give their signed consent (Appendix 2). Depending on the availability of the samples, and on the test requirement, tests were performed with either fresh or frozen samples. Fresh samples were stored in Earle’s Balanced Salt Solution (EBSS, Sigma) immediately after surgery and were kept at 4°C and used within 3 days of the surgery. Frozen samples were kept at –20°C. When needed, those samples were left to thaw at room temperature and stored in EBSS prior their utilisation within the same day.

**Sample information**
All donors were healthy and aged between 25 and 62; and only the female gender was represented. Samples were taken from abdominal and breast reduction surgeries (Appendix 3). Prior to excision, patients’ breast skin was generally prepared for surgery using 0.5% chlorhexidine gluconate (w/v) in 70% isopropanol (v/v) or 10% povidone-iodine in aqueous solution. Hence, to remove traces of biocide that may still have been present on the skin after freezing or after storage in the liquid medium, as well as to remove the storage medium itself, samples were rinsed 3 times in SDW prior to use. Information about the donor, such as gender, age, sample origin, race and the date of surgery (and time, for fresh samples) was recorded by the surgeon (Appendix 4).

**Preliminary investigations**
Prior to use, each skin sample was tested to control for the presence of the remaining resident flora, so that they would not be confused with the artificially inoculated bacterial cells, representing transient flora. For each new skin sample, the resident flora were investigated by adding 500 μL of PBS onto the skin surface with a pipette then rinsing the surface by flushing the PBS in and out of the pipette tip. The number of viable cells in the liquid recovered from the skin surface was then enumerated by using the DCM. As a generalisation, the numbers of bacterial cells recovered from the samples was negligible, even from the undiluted solution directly recovered from the samples. The most likely reason for the absence of natural flora was that the skin areas were disinfected with antiseptics, generally chlorhexidine or povidone-iodine prior to surgery.

“**Ex-vivo” method**
The “ex-vivo” model was described in Chapter 1. In essence, skin samples of about 2 cm² were placed onto diffusion cells (Figure 1, Chapter1) containing 1 mL of SDW to keep the dermis moist.
5.2.7. “Ex-vivo” testing to approximate “in-vivo” handwashing test EN 1499

**Bacterial inoculum**
The inocula used for the “ex-vivo” method were prepared as described in Chapter 2.

**Application of the contaminated fluid**
A volume of 20 $\mu$L of bacterial inoculum was placed onto the stratum corneum and left to dry for approximately 3 to 5 min in a laminar flow cabinet at room temperature.

**Prevalue**
A volume of 980 $\mu$L of TSB was added to the dried inoculum and it was resuspended by flushing the liquid in and out of the pipette tip for 1 min. Viable counts were assessed using the DCM.

**Handwash technique mimicking**
Following the prevalue sampling and without recontamination, 40 $\mu$L of antiseptic or reference non-medicated SS solution was added over the bacterial inoculum and rubbed onto the skin for 1 min using a sterile glass rod.

**Postvalue**
Immediately after rubbing, 940 $\mu$L of neutraliser was added onto the skin sample, the surviving bacterial cells were resuspended by flushing the liquid in and out of the pipette tip for 1 min and viable counts were performed using the DCM. Plates were counted after incubation at 37°C for 24 h and reincubated for a further 24 h to detect any slow growing colonies.

5.2.8. Statistical analysis
The “in-vivo” studies 1 and 2 were performed with 13 and 14 volunteers, respectively. The “ex-vivo” experiments were repeated at least seven times, depending on the availability of skin samples. Student’s t-tests and ANOVA tests were conducted (Excel®, Prism®) for results obtained in the handwashing study 1 and in the “ex-vivo” study. Statistical analysis of the “in-vivo” study 2 was done according to the European standard EN 1499 guidelines and is described further below. In all cases, $P$ values of < 0.01 were considered significant when the antiseptics were compared with the SS, as recommended by the European standard method.

5.3. Results

5.3.1. “In-vivo” studies
Both the requirements for acceptance in the EN 1499 were achieved, since all results from the 13 subjects in study 1 and 14 subjects in study 2 were available and the overall mean of the log$_{10}$ prevalues for reference and test procedures was at least 6. In both studies, the reduction in bacterial cell numbers obtained with the antiseptic formulations and the SS was not dependent upon the hand tested ($P > 0.05$; student’s t-test). When comparing male and female volunteers, no statistically significant difference was observed between the reduction in bacterial numbers after handwashing with SS, HSW or AHSW. However, 5% TTO in Tween 80 appeared to be significantly more active against female volunteers than against males, with $4.404 \pm 0.709$ log$_{10}$ and $3.268 \pm 0.025$ log$_{10}$ cfu/mL reductions, respectively. The European standard EN 1499 makes no statement regarding the influence of the volunteers’ gender upon the test results because each subject acts as its own control, performing the test with the reference non-medicated SS as well as with the antiseptic soap under similar environmental conditions (European Standard EN1499, 1997).

**Original bacterial inoculum**
The original inocula contained on average $8.99 \pm 0.32$ and $8.93 \pm 0.15$ log$_{10}$ cfu/mL of *E. coli* cells in study 1 and 2, respectively, which complied with the European standard EN 1499 recommendation of 8.30 to 9.30 log$_{10}$ cfu/mL.
Prevalue
In both studies, the number of bacterial cells recovered from hands during prevalue sampling was not significantly ($P > 0.05$; ANOVA) dependent upon the testing day nor was it dependent upon the product (antiseptic or control) that was tested after prevalue sampling.

Comparison prevalue / postvalue
The number of bacterial cells recovered after treatment (postvalue) with the non-medicated SS or the TTO-containing products was always significantly ($P < 0.05$; student’s t-test) lower than that in the prevalue (prior treatment). The activity of the SS, 5% TTO in Tween 80 and AHSW was not significantly influenced by the testing day ($P > 0.05$). However, HSW assessed in study 1 achieved a slightly higher ($P = 0.049$) reduction in bacterial cell numbers when assessed with Group 2 than with Groups 1 and 3 (Table VII).

Comparison of each antiseptic product with the non-medicated soft soap
The mean $\log_{10}$ RF of each antiseptic was larger than that obtained with the reference SS (Table VIII). The difference in RF was therefore tested for statistical significance.

In study 1, more than one antiseptic was tested in parallel with the non-medicated SS. Hence one-way ANOVA analysis was used to compare all groups together, followed by the Dunnett’s post-test to compare each group with the control soap (Prism ®). TTO (5%) in 0.001% Tween 80 was significantly ($P < 0.01$) more active than the control SS. HSW appeared to be slightly more active than the SS, however, this difference was not statistically significant ($P > 0.05$). Finally, 5% TTO in Tween 80 was significantly ($P < 0.01$, student’s t-test) more active than the HSW.

In study 2, only one formulation was assessed in parallel with the SS, and thus the statistical analysis recommended by the European standard method EN 1499 was followed. The difference in reduction in bacterial cells achieved by the AHSW compared to the SS was assessed using the Wilcoxon matched-pairs signed ranks test (Table IX). The smaller sum of ranks (positive or negative) was compared with tabulated values from the Wilcoxon table, for 14 volunteers ($n = 14$) at level of significance $P = 0.01$ ($= 15$). The smaller calculated sum of ranks was 10 (which is $< 15$) and the AHSW was therefore significantly more effective than the SS.

5.3.2. “Ex-vivo” method
Original bacterial inoculum
Original inocula contained on average $8.57 \pm 0.29 \log_{10} \text{cfu/mL}$ of $E. coli$, within the European standard EN 1499 recommendations of 8.30 to 9.30 $\log_{10} \text{cfu/mL}$.

Comparison prevalue / postvalue
The number of bacterial cells recovered after treatment (postvalue) with the control SS or the TTO-containing products was always significantly ($P < 0.05$; student’s t-test) lower than the prevalue (prior to treatment).

Comparison of each antiseptic product with the non-medicated soft soap
All antiseptic-containing products were compared to each other as a group, and against the control SS. TTO (5%) in Tween 80 and the AHSW were significantly more active ($P < 0.01$) than the SS against $E. coli$ (Table X). The HSW was more active than the SS, although this difference was not significant ($P > 0.05$) (Table X). TTO (5%) was significantly more active ($P < 0.01$) than the HSW and more active than AHSW although this difference was not significant ($P > 0.05$). Finally, the AHSW appeared to be more active than the HSW, although this difference was also not statistically significant ($P > 0.05$) (Table X).
5.3.3. Comparison “in-vivo” with “ex-vivo” results

The results obtained in studies 1 and 2 in-vivo with volunteers were compared with those obtained ex-vivo with skin samples. The formulations tested, as well as the SS, were more active when assessed in-vivo than ex-vivo against E. coli, although only the SS and HSW were significantly more active in-vivo ($P < 0.05$) (Figure 12). There appeared to be a pattern in the comparison of “ex-vivo” and “in-vivo” results. The antiseptics tested appeared to be on average $1.28 \pm 0.06$ times more active in-vivo (from 1.05 times with AHSW to 1.35 times better with HSW) than when assessed ex-vivo.

5.4. Discussion

Our results ex-vivo with skin samples and in-vivo with volunteers showed that 5% TTO in Tween 80 was generally more active than 100% AHSW which was itself more active than the HSW. These findings are similar to the results obtained in-vitro with the European suspension tests described in Chapter 2. Our results showed that TTO has significant activity in-vivo according to European recommendations, when assessed as an active compound by itself or within a formulation, and therefore could be use in healthcare settings to encourage staff compliance with handwashing.

Results obtained ex-vivo in this study were reproducible and even though the antiseptics tested were more active when tested in-vivo, both testing techniques appeared linked by a constant conversion factor. In a previous study that compared results obtained with an adapted version of the “ex-vivo” test with a rubbing effect with that obtained in-vivo with volunteers (EN 1499), Triclosan, para-chloro-meta-xylenol (PCMX), povidone iodine and a SS performed generally 1.62 times better in-vivo than ex-vivo (from 1.13 times with soft soap to 2.29 times better with povidone iodine) (Messager et al., 2004). These results are similar to those from the present study which showed the antiseptics to be on average $1.28 \pm 0.06$ times more active in-vivo than ex-vivo.

The main outcome of the European handwashing standard method is for the formulation tested to be significantly more active than the control soap, and 5% TTO in Tween 80 and the AHSW achieved this result using both “in-vivo” and “ex-vivo” methods. According to the EN standard method, the important conclusion of the test is not the reduction in bacterial cell concentration achieved, because there are no epidemiological data indicating how effective a disinfection procedure has to be in order to prevent hand-transmitted infection (Reybrouck, 1986; Larson & Rotter, 1990; Pittet et al., 2000), even in the controlled environment of a hospital (Bryan, 1995).

In conclusion, we have shown that after passing the European “in-vitro” suspension tests (Chapter 2), 5% TTO in Tween 80, and in formulation, also passed the European “in-vivo” handwashing test. The same conclusions were reached when the products were tested with the adapted “ex-vivo” method. The present study supports the use of the “ex-vivo” model to investigate the efficacy of antiseptics on skin and may help in predicting the outcomes of “in-vivo” studies.

Our findings suggest that TTO-containing handwash formulations may prove useful to help reduce the skin carriage of potentially pathogenic organisms by health-care staff and thus reduce the incidence of nosocomial infections in hospital settings.
### Table VII
“In-vivo” study 1 and 2: Latin square arrangement of the treatments and soft soap control

<table>
<thead>
<tr>
<th>Testing order</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>Soft soap</td>
<td>5% TTO in</td>
<td>100% HSW</td>
</tr>
<tr>
<td>Group 2</td>
<td>5% TTO in</td>
<td>100% HSW</td>
<td>Soft soap</td>
</tr>
<tr>
<td>Group 3</td>
<td>100% HSW</td>
<td>Soft soap</td>
<td>5% TTO in</td>
</tr>
<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>Soft soap</td>
<td>100% AHSW</td>
<td>/</td>
</tr>
<tr>
<td>Group 2</td>
<td>100% AHSW</td>
<td>Soft soap</td>
<td>/</td>
</tr>
</tbody>
</table>

Study 1: Group 1: Volunteers 1 to 4  
Group 2: Volunteers 5 to 8  
Group 3: Volunteers 9 to 13  
Study 2: Group 1: Volunteers 1 to 7  
Group 2: Volunteers 8 to 14

### Table VIII
“In-vivo” study 1 and 2: Log₁₀ reduction in *E. coli* K12 concentration (cfu/mL) after volunteers’ handwashing with soft soap or antiseptic (mean ± standard deviation)

<table>
<thead>
<tr>
<th></th>
<th>log₁₀ reduction in cfu/mL (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td>soft soap</td>
</tr>
<tr>
<td></td>
<td>5% TTO</td>
</tr>
<tr>
<td></td>
<td>100% HSW</td>
</tr>
<tr>
<td>Study 2</td>
<td>soft soap</td>
</tr>
<tr>
<td></td>
<td>100% AHSW</td>
</tr>
<tr>
<td>Subject</td>
<td>Log RF derived from</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td>soft soap</td>
</tr>
<tr>
<td>1</td>
<td>2.394202</td>
</tr>
<tr>
<td>2</td>
<td>2.709113</td>
</tr>
<tr>
<td>3</td>
<td>2.410648</td>
</tr>
<tr>
<td>4</td>
<td>2.453193</td>
</tr>
<tr>
<td>5</td>
<td>3.415063</td>
</tr>
<tr>
<td>6</td>
<td>3.591754</td>
</tr>
<tr>
<td>7</td>
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<td>9</td>
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<td>10</td>
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<td>12</td>
<td>2.340363</td>
</tr>
<tr>
<td>13</td>
<td>2.108633</td>
</tr>
<tr>
<td>14</td>
<td>2.785049</td>
</tr>
</tbody>
</table>

Sum of ranks (+): 10
Sum of ranks (-): 95

Note: Compare smaller sum of ranks with tabulated values from the Wilcoxon table (see below) for n = 14 at level of significance p = 0.01 (=15).
If calculated smaller sum of ranks ≤ 15, then AHSW is significantly more effective than Soft soap.

<table>
<thead>
<tr>
<th>Products</th>
<th>log₁₀ reduction in cfu/mL (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>soft soap</td>
<td>2.126 ± 0.426</td>
</tr>
<tr>
<td>5% TTO</td>
<td>3.257 ± 0.655</td>
</tr>
<tr>
<td>100% HSW</td>
<td>2.365 ± 0.448</td>
</tr>
<tr>
<td>100% AHSW</td>
<td>2.801 ± 0.469</td>
</tr>
</tbody>
</table>
Figure 12  Comparison of the efficacy of the soft soap and TTO-formulations depending upon the test performed (mean ± standard deviation). Data obtained for the soft soap *in-vivo* (study 1 & 2) were compiled.
6. General Discussion

Handwashing is one of the most important measures to reduce the transmission of infection in hospital settings (Pittet, 2003). However, compliance with hand-hygiene guidelines is low (Pittet, 2001; Karabey et al., 2002) and this can be partly attributed to the fact that most recommended handwash agents available in health-care settings can be irritating to the skin when used repetitively (Pittet & Boyce, 2001). The efficacy of TTO has been demonstrated in-vitro and its incorporation into skin products does not appear to cause the dermatological problems commonly reported with other formulations (Carson & Riley, 1995). In this study, we used “in-vitro” methods based on European standards so that our results could be compared with other data in the literature as well as future work.

Some reports suggest that the repeated use of a TTO-containing handwash does not lead to the dermatological problems associated with other formulations (Carson & Riley, 1995) and this finding could be used to encourage health care staff compliance with handwashing. In addition, Hammer et al. (1996) showed that transient skin organisms were more susceptible to TTO than commensal organisms. Hence the formulations assessed in this study could prove useful in healthcare settings where removal of potentially pathogenic organisms is as important as preserving the integrity and resident flora of the skin.

6.1. Antibacterial activity in suspension

6.1.1. Antibacterial activity of TTO

Importance of the formulation

When assessed with the suspension test EN 1276, the AHSW achieved a log_{10} reduction ≥ 5 after a 5 min contact time against *S. aureus, A. baumannii, E. coli K12* and *P. aeruginosa*, and its activity was not significantly influenced by the presence of interfering substances. TTO (5%) in Tween 80 and the HSW also complied with European guidelines when assessed against *E. coli K12*, and against both *E. coli K12* and *A. baumannii*, respectively. An antiseptic must achieved at least a 5 log_{10} reduction in bacterial cell numbers within 5 min to meet EN 1276 requirements. In addition, our results showed that 5% TTO in Tween 80 and AHSW passed the European standard after a 1 min contact time when tested against *E. coli K12*. With the suspension test prEN 12054, the 55% AHSW and TTO in Tween 80 also passed the European recommendations within a 1 min contact time.

It is as important to assess the activity of an active compound as it is to assess the final formulation, as synergy or antagonism can occur between the components of the essential oils and the ingredients of the formulation (Cox et al., 2001). In Chapter 1, the formulations that did not contain TTO were generally less active than those containing TTO. For example, the AHR solution, which contains 64% alcohol, was significantly more active than its equivalent without TTO after a 1 min contact time against all bacteria, reinforcing the fact that the activity of this formulation is mainly due to the presence of TTO.

The AHR formulation passed the standard guidelines (EN 1276, 1997) even though the concentration of TTO was only 2%. The presence of a high concentration of alcohol may have enhanced the activity of the handrub. This explanation is also supported by the AHSW results which achieved higher reductions in bacterial numbers than the HSW in both suspension tests even though they both contained 5% TTO. Synergism between alcohol and some other active compounds has been previously reported with chlorhexidine (Sakuragi et al., 1995; Herrera et al., 2003), Triclosan (Messager et al., 2001) and PVI (Jeng & Severin, 1998).

Following the draft European standard method prEN 12054 for the assessment of hygienic handrub and handwash solutions, the AHSW formulation met the European recommendations after a 1 min contact time against *E. coli, A. baumannii* and *P. aeruginosa*. The AHR also met the European requirements when tested against all bacterial strains.
The handrub formulation (AHR) tested in this study passed both European standard methods and the presence of TTO enhanced its activity. Previous studies have shown that alcoholic handrubs have greater and faster activity than handwash products (Kampf et al., 1999; Marchetti et al., 2003) and are more “skin-friendly” and therefore better tolerated by healthcare staff (Winnefeld et al., 2000; Parienti et al., 2002). Alcohol-based formulations have recently been promoted as being the most important development for improving compliance with hand cleansing in understaffed and overcrowded situations (Pittet & Boyce, 2001; Boyce & Pittet, 2002). Thus, our findings regarding the TTO-containing alcoholic handrub are very encouraging and should be investigated further.

In conclusion, the AHSW and AHR assessed in this study were shown to comply with the European Standard against all bacteria tested. In addition, concentrations of TTO $\geq 5\%$ also complied with the EN 1276 by achieving $\geq 5 \log_{10}$ reduction when tested against *E. coli* and *P. aeruginosa*, and HSW also complied when assessed in perfect conditions against *E. coli* and *A. baumannii*. This indicates that these products have at least met a minimum standard, and it remains for future investigations to evaluate them further.

### 6.1.2. Sporicidal activity

Chemical agents and commercial formulations, such as HP, SH and GA are used extensively in health care settings for disinfection of hard surfaces or surgical instruments. However, they are too corrosive and toxic to be used as topical agents, and thus are not suitable for use as sporicidal agents on the hands of health care workers. TTO is currently used as a popular addition to numerous pharmaceutical and cosmetic products that are available worldwide (Carson & Riley, 1993; Markham, 1999). In addition, an increasing number of patients seem to favour the use of herbal medicines rather than synthetic antibiotics or/and antiseptics, and are therefore more likely to comply with such treatments (Martin & Ernst, 2003).

Recent concerns regarding the emergence of infections from bacterial spores in hospital settings, mainly in the United States, make spores a prime target for eradication. If the efficacy of TTO-containing formulations can be proven *in-vivo*, this may make a contribution towards this objective. Few studies have been conducted assessing the activity of TTO *in-vivo* (Martin & Ernst, 2003). Nevertheless, its efficacy *in-vitro* has now been well documented. The activity of TTO is adversely affected by only a few types of organic matter (Hammer et al., 1999), and as such it is reasonable to assume that it will have activity on human skin *in-vivo*.

In conclusion, this study showed that even though the European requirements were not met by formulated TTO products, 5, 10 and 15% TTO in Tween 80 significantly reduced the concentration of bacterial spores in suspension. In addition, TTO was generally more effective than PVI. Hospital settings have reached a critical point where handwashes may need to possess sporicidal properties and with the current lack of such products our findings appear to be extremely encouraging.

### 6.1.3. Test limitations and conclusion

Previous studies have highlighted the fact that suspension tests may overestimate the activity of disinfectants and antiseptics (Maillard et al., 1998; Messager et al., 2001), and thus active compounds should be assessed using practical tests. Suspension tests are indeed less severe than capacity tests and practical tests (Reybrouck, 1999). The efficacy of handwash formulations may be reduced in real-life conditions because of the presence of organic load, sweat and sebum on the skin. Hammer et al. (1999) demonstrated that no one single interfering substance affected the activity of TTO against the test microorganisms. Our results, showing a different interference level depending upon the formulation and concentration of TTO tested, and the concentration of interfering substances and the organism tested, agree with those of Hammer et al. (1999).
Suspension tests are used to evaluate the activity of a compound under defined conditions. However, they do not demonstrate how the product will perform *in-situ* (Te Giffel *et al*., 1996). In addition, products need to be tested in the context of where they are to be used, e.g. a handwash formulations designed for being used in hospital settings must be assessed *in-vivo* with volunteers. As described by Reybrouck (1999), these latter tests are carried out under practical conditions in the laboratory but simulating field conditions. Such testing may present difficulties when working with spores although the American standard method E 1174-94 (ASTM E 1174-94) does assess the sporicidal activity of handwash products against *B. atrophaeus* (ASTM 1994). Weber *et al.* (2003) used this method to investigate the sporicidal activity of several handwash products against *B. atrophaeus*. Soap and water was generally at least as effective at removing bacterial spores from contaminated hands than a formulation containing 2% chlorhexidine gluconate and chlorine-containing towels. However, the study did not stipulate what reduction in bacterial spore numbers the product being tested needed to achieve, or if it needed to perform significantly better than the control soap to be classified as “effective”.

Further studies could also include the testing of TTO-containing formulations directly on skin by using an “ex-vivo” test using freshly excised human skin. This would allow the testing of any spore-forming bacterial strain as well as any biocide (Maillard *et al*., 1998; Messager *et al*., 2001).

### 6.2. Surface tests

#### 6.2.1. Antibacterial activity of TTO

According to the European standard method (EN 13697), a product can be described as having bactericidal activity on surfaces if it produces a $\geq 4 \log_{10}$ reduction in the number of viable cells within 5 min at 20°C. The glass-carrier method is used to assess biocidal activity under ideal conditions for disinfection, whereas the stainless steel surface test is more stringent with a rougher surface that may protect microorganisms from antiseptic challenge. Both tests can be adapted to imitate ideal conditions, as in Chapter 4, or real life conditions by adding an organic load to the inoculum. The quantitative test on stainless steel surfaces (QCT-2) described by Traore *et al.* (2002) is also more stringent than the American standard glass-carrier test (QCT-1; ASTM E2111-00) because it uses a more realistic ratio of bacterial inoculum to disinfectant per surface area: 10 µL inoculum + 50 µL disinfectant instead of 10 µL inoculum + 1 mL disinfectant in the glass-carrier. We used the ratio proposed by the European standard method: 50 µL inoculum + 100 µL disinfectant for both methods so that the only external influence was the surface tested.

According to our results, organisms such as *E. coli* and *P. aeruginosa* were affected by the drying step. These findings were in agreement with previous studies by Maillard *et al.* (1998) and Messager *et al.* (2001), and this emphasises the difficulty of testing antimicrobials on surfaces. From our results, the Gram-negative test strains appeared to be more sensitive than *S. aureus* to TTO. This may be partly explained by the drying step which significantly affected the survival and thus recovery of *E. coli* and *P. aeruginosa* cells. These results tend to agree with those obtained in Chapter 2 where the efficacy of TTO in suspension was tested using the European standard test (EN 1276, 1997). These results also showed that TTO was more active against Gram-negative bacteria than against *S. aureus*, even though this was not always significant. Hammer and colleagues (1999) also found TTO to be less active against *S. aureus* compared to *E. coli*, with minimum inhibitory concentrations (MICs) of 0.5 % v/v and 0.25 % v/v for each organism, respectively. Cox *et al.* (2000) showed that 0.5% TTO was significantly more active when tested against *E. coli* than against *S. aureus*. Other active compounds such as chlorhexidine, have been shown to be more active against Gram-negative than against Gram-positive bacteria when assessed with the glass-carrier test (Messager *et al*., 2001).

In conclusion, 5% TTO in Tween 80 significantly reduced the number of bacterial cells dried onto surfaces, by comparison with SDW only. In addition, 5% TTO proved to be nearly as effective as 7.5% PVI when tested against *E. coli*, *A. baumannii* and *P. aeruginosa* after 5 min contact time on both surfaces. The efficacy of the antiseptics tested depended on the test microorganism, as well as the
surface they were tested on. However, in the latter case, there was generally no statistical difference between the two surfaces.

6.2.2. Test limitations and conclusion

Surface tests are relatively simple and inexpensive to perform, however, the major problems encountered with glass-carrier tests are (i) the microorganisms have to be removed from the surface to be enumerated, which in itself may induce lethal stresses (Holah et al., 1998) and (ii) the survival of microorganisms on the carrier is not constant and can be difficult to standardise (Reybrouck, 1998). The significant loss of bacterial cells due to the drying step has been reported in other studies (Messager et al., 2001). This decrease in bacterial cell numbers can be influenced by many factors such as the temperature, relative humidity of the air, the drying time and the intrinsic humidity of the carrier itself (Reybrouck, 1999).

In the past few years, there has been an increased concern about the possible link between contaminated surfaces in hospital settings and the rising numbers of nosocomial infections (Malik et al., 2003; Martinez et al., 2003). Recent studies have shown that regular decontamination of environmental surfaces in health-care settings helps to reduce the number of outbreaks of infection (Bures et al., 2000; Mayfield et al., 2000). However the extent to which contaminated surfaces can contribute to the spread of infection is not known (Wenzel et al., 1998; Talon, 1999; Patel, 2004). Uncontrolled routine disinfection of environmental surfaces has been criticised (Dharan et al., 1999) as the repetitive exposure of microorganisms to low levels of biocide residues (Russell, 2002) may result in the possible emergence of biocide and/or antibiotics-resistant organisms. TTO has over 100 different components, and although some antibiotic-resistant organisms have been shown to have a reduced susceptibility to TTO (Gustafson et al., 2001), emergence of TTO-resistant organisms is yet to be demonstrated. Lastly, even though TTO might not be able to compete with existing biocides used for surface decontamination, its long-term presence in the health-care environment does not seem to present the same concerns than other widely used chemicals.

6.3. Comparison of suspension and surface testing

Results obtained on surfaces (Chapter 4) were compared with that obtained in suspension (Chapter 2). The outcome of this comparison showed that TTO-containing products were for the most part significantly more active when tested in suspension than on surfaces.

Other studies have reported lower reductions in bacterial cell numbers after antiseptic treatment on surfaces when compared with suspension tests (Best et al., 1988; Gibson et al., 1995; Van Klingerent al., 1998; Messager et al., 2001). Van Klingerent and colleagues (1998) stated that concentrations of antiseptics that pass the European suspension test (i.e. products that induce $5 \log_{10}$ reduction in 5 min) will usually achieve a RF of $2 - 4 \log_{10}$ less when assessed on surfaces, although simple conversion factors could not be established between both tests. Suspension tests have a number of benefits. They are simple and do not require specialised or expensive pieces of laboratory equipment. They are also repeatable and reproducible (Holah et al., 1998). However, their major problem is that they do not reflect “in-use” conditions (Holah et al., 1998; Van Klingerent al., 1998). Furthermore, suspension test protocols have been shown to give an overestimation of the antibacterial activity of the antiseptic investigated, mainly because in essence, a suspension test involves an intense contact between the antiseptic solution and the bacterial cells (Maillard et al., 1998).

Finally, even though TTO could not be recommended as a highly suitable agent for surface decontamination, surface testing is more stringent than suspension tests hence these results might be useful to predict “in-vivo” data.
6.4. “In-vivo” and “ex-vivo” testing

6.4.1. Antibacterial activity of TTO in-vivo and ex-vivo

In Chapter 2, we showed that 5% TTO in Tween 80 and AHSW passed the European standard EN 1276 after a 1 min contact time when tested against E. coli K12. With the suspension test prEN 12054, AHSW and TTO in Tween 80 also passed the European recommendations within a 1 min contact time, prompting the further testing of these products in-vivo and ex-vivo.

Our results ex-vivo with skin samples and in-vivo with volunteers showed that 5% TTO in Tween 80 was generally more active than 100% AHSW which was itself more active than the HSW. These findings are similar to the results obtained in-vitro with the European suspension tests described in Chapter 2. Both studies showed that it is as important to assess the activity of an active compound as it is to assess the final formulation into which it has been incorporated, as synergy or antagonism can occur between the essential oil and the components of the formulation. This was well illustrated by 5% TTO in Tween 80 being significantly more active than 5% TTO incorporated into different formulations. The presence of 10% alcohol in the AHSW was also significant, as it appeared to enhance the activity of TTO in the formulation compared to TTO only (HSW) when assessed ex-vivo (Chapter 5) and in-vitro (Chapter 2). Other studies have also demonstrated that the combined activity of alcohol with an active compound such as Triclosan (Messager et al., 2001) or chlorhexidine (Larson et al., 1990) was greater than each agent alone.

According to our results, 5% TTO in Tween 80 and the AHSW formulation achieved a significantly higher reduction in bacterial numbers than the reference soap, both in-vivo and ex-vivo, thus meeting the European standard requirements. Indeed, what matters at the end of the test is not the reduction in bacterial cell concentration achieved because as stated previously, there are no epidemiological data indicating how effective a disinfection procedure has to be in order to prevent hand-transmitted infection (Reybrouck, 1986; Larson & Rotter, 1990; Pittet et al., 2000).

6.4.2. “In-vivo” test limitations and use of the “ex-vivo” test

“In-vivo” testing can be limiting because it is expensive, time-consuming, and pathogenic organisms and new formulations whose safety for humans is not known cannot be tested on volunteers. To overcome these problems, other studies have looked for a substitute to human skin and/or an intermediate test between “in-vitro” and “in-vivo” testing, and included the use of pigskin (Bush et al., 1986; McDonnell et al., 1999), monoxenic hairless mice (Barc et al., 1989) and freshly excised samples of euthanased cats and dogs as a substitute to human skin (Maillard et al., 1998). McDonnell et al. (1999) showed that a 60% isopropanol handwash formulation achieved comparable results when assessed on pig skin samples in-vitro and in-vivo with volunteers, resulting in 2.4 ± 0.5 and 2.0 ± 0.5 log10 reductions, respectively. The study by Barc et al. (1989) also presented encouraging results although correlation with “in-vivo” data was dependent upon the formulations tested. Although these studies produced valuable results, it is difficult to rely on animal models since their skin reacts differently to that of humans, and human skin bacteriology is unique. It is also very difficult to induce infections that would resemble to those of humans (Leyden et al., 1979).

There are major differences between human skin in-vivo and ex-vivo, such as moisture level, microbial ecology, surface pH and temperature, and the presence of sebum and sweat. Nevertheless, the “ex-vivo” model appears to be highly suitable for investigating the efficacy of antiseptic agents directly on skin (Maillard et al., 1998; Messager et al., 2001). The main advantages of the “ex-vivo” test are that the experiments are easier and less expensive to perform that those “in-vivo”, parameters such as environment temperature and resident flora can be controlled, pathogenic organisms can be assessed, different handwashing conditions can be simulated and ethical approval is easier to obtain.

Results obtained ex-vivo in this study were reproducible and even though the antiseptics tested were more active when tested in-vivo, both testing techniques appeared to be linked by a constant
conversion factor. According to our results, the antiseptics tested were, on average 1.28 ± 0.06 times more active when tested in-vivo than ex-vivo (from 1.05 times with the AHSW to 1.35 times better with the HSW). The present study also supports the use of the “ex-vivo” model to investigate the efficacy of antiseptics on skin and may help in predicting the outcomes of “in-vivo” studies.

6.5. Conclusion

The use of antiseptics is critical in health-care settings for the prevention of transmission of infections (Pittet & Boyce, 2003). However, resistance to commonly used agents has been reported in the laboratory and this has led to the search for new antiseptic agents (McMurry et al., 1999; Suller et al., 2000; Tattawasart et al., 2000). In addition, epidemiological research has shown that the use of synthetic detergents has increased barrier-dependent skin problems (Kownatzki, 2004). Antiseptics remove adhering bacterial contaminants and surface lipids from the skin and, after multiple applications, the lipid-dependent efficiency is decreased and the active compounds from the antiseptics penetrate the skin deeper (Kownatzki, 2003). Skin lipid removal by antiseptic agents is, amongst others, a big problem associated with hand hygiene. Lipids of plant origin cannot repair a damaged barrier but they are known to improve the feel and overall function of damaged skin (Kownatzki, 2003). Previous research has suggested that the repeated use of a TTO-containing handwash did not lead to the dermatological problems associated with other formulations (Carson & Riley, 1995). TTO is active against a wide range of microorganisms in-vitro (Schnitzler et al., 2001; Hammer et al., 2002; Hammer et al., 2003a). Moreover, one study demonstrated that TTO had greater activity against transient skin-associated bacterial pathogens than against commensal skin flora (Hammer et al., 1996). Hence, it was anticipated that its use as a handwash agent could be useful to remove potential pathogens while preserving the naturally-occurring flora (Hammer et al., 1996).

By investigating further the activity of TTO in-vitro, in-vivo and ex-vivo, this study showed that the TTO-containing formulations may prove useful in hospital settings because of their efficacy in different testing conditions and because TTO formulations are potentially less damaging to the skin, thus reducing transmission of nosocomial infections.

Even though the positive benefits of handwashing are well recognised (Larson, 2001; Aiello & Larson, 2002), there are no epidemiological data indicating how effective a disinfection procedure has to be in order to prevent hand-transmitted infection (Larson & Rotter, 1990; Pittet et al., 2000). Along the same lines, further studies should be carried out to investigate (i) the activity of TTO-containing alcoholic handrub using the European Standard EN 1500 (European Standard EN1500, 1997) for the evaluation of hygienic handrubs; and (ii) the potential sporicidal activity of TTO-containing products in-vivo using the American standard method that investigates the sporicidal activity of handwash products against B. atrophaeus (ASTM, 1994).
7. Appendices

7.1. Appendix 1: Test information sheet

As part of a project entitled: "Assessing the efficacy of Tea Tree Oil hand and body wash as a topical antiseptic"; the antimicrobial efficacy of Tea Tree Oil formulations will be investigated using a newly developed protocol, the ex-vivo test.

In essence, this test requires freshly excised human skin samples. Hence, patients undergoing breast or abdominal reduction will be approached by their surgeon and asked if they would agree for the skin samples removed during surgery to be used for this research study alone instead of being discarded after surgery.

This test was developed so that antiseptic formulations could be directly assessed on human skin. This method imitates the conditions of conducting a test with volunteers, but only using excised skin samples.

The samples would then be used in a microbiological laboratory for the assessment of Tea Tree Oil's efficacy against several strains of bacteria. After testing, the samples will be disposed via autoclaving and then incineration.

Before collection, details about the skin samples such as the gender and race of the donor would be requested for the unique purpose of the tests' results being statistically analysed according to standard methods.

The objective of this work is to generate data that will be published in peer-reviewed medical and scientific journals and to increase the acceptability of Tea Tree Oil as a naturally-occurring antimicrobial both nationally and internationally. Participant's name or other identifying information will not be used.

Your participation in this study does not prejudice any right to compensation, which you may have under statute or common law. The participant is free at any time to withdraw consent to further participation without prejudice in any way. The participant need give no reason nor justification for such a decision.

If you require additional information or if you have some other queries concerning the research, please do not hesitate to contact me.
7.2. Appendix 2: Skin Donor consent form

Consent form

I (the donor) have read the information provided and any questions I have asked have been answered to my satisfaction. I agree to participate in this activity, hence I agree for skin samples excised from my body during breast or abdominal reduction to be used in this research study alone, realising that I may withdraw at any time without reason and without prejudice.

I understand that all information provided is treated as strictly confidential and will not be released by the investigator unless required by the law. I have been advised as to what data are being collected, what the purpose is, and what will be done with the data upon completion of the research.

I agree that data gathered for the study may be published provided my name or other identifying information is not used.

__________________________ __________________________
Donor   Date

The Human Research Ethics Committee at the University of Western Australia requires that all participants are informed that, if they have any complaint regarding the manner, in which a research project is conducted, it may be given to the researcher or, alternatively to the Secretary, Human Research Ethics Committee, Registrar’s Office, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009 (telephone number 9380-3703). All study participants will be provided with a copy of the Information Sheet and Consent Form for their personal records.
### 7.3. Appendix 3: List of skin samples

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*Aa: Antibacterial activity testing  
**HW: Method to imitate the handwashing test*
7.4. Appendix 4: Skin samples details form

Skin Sample Details

Surgery Date: _______________________

Time: _______________________

Donor Gender: _______________________

Age: _______________________

Race: (please tick)

- Aboriginal or Torres Strait Islander
- African or African-American
- Asian
- Caucasian
- Other (Please specify): _______________________

Area of the body from where the sample was taken: _______________________

Use of an antiseptic prior surgery: (Please circle) YES NO

If YES, which one: _______________________

If you have any query or comments please contact Dr Syndie Messager (details as shown above).
8. References


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