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& DEVELOPMENT CORPORATION

# Antimicrobial activity
## of tea tree oil against oral microorganisms

**A report for the Rural Industries Research and Development Corporation**

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# Foreword

The aim of this project was to determine the susceptibility of oral bacteria to tea tree oil, with a view to justifying the use of tea tree oil in oral health.

Tea tree oil has many potential applications in oral healthcare. However, little research has been conducted into the susceptibility of oral bacteria to tea tree oil. Since the market for oral health care products is large, the availability of effective and innovative tea tree oil products may be a welcome addition to the existing range of products.

This report discusses data obtained on the activity of tea tree oil against oral bacteria, including both in vitro susceptibility data and time kill studies. The implications of these data in relation to oral hygiene and oral hygiene products are also discussed.

This project was funded by Australian Bodycare Pty Ltd and matching funds provided by the Federal Government. This report, a new addition to RIRDC's diverse range of over 900 research publications, forms part of our Tea tree oil R&D Program, which aims to support the continued development of a profitable tea tree oil industry.

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**Simon Hearn**
Managing Director
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# Abbreviations

| | |
|---|---|
| BA | blood agar |
| BHIB | brain heart infusion broth |
| cfu | colony forming units |
| d | day |
| g | gram |
| h | hour |
| ISO | International Standards Organisation |
| l | litre |
| MBC | minimum bactericidal concentration |
| MHB | Mueller Hinton broth |
| MIC | minimum inhibitory concentration |
| min | minute |
| *n* | number |
| NCCLS | National Committee for Clinical Laboratory Standards |
| PBS | phosphate buffered saline |
| s | seconds |
| SDW | sterile distilled water |
| SEM | standard error of the mean |
| sp. | species |
| spp. | species (plural) |
| TTO | tea tree oil |
| v/v | volume for volume |
| w/v | weight for volume |

# Contents

# Executive Summary

The human mouth provides a habitat for a diverse range of bacteria, viruses, protozoa and fungi. These micro-organisms colonise essentially every surface in the mouth, including the cheeks, tongue, palate and teeth. Under certain circumstances, these organisms may cause diseases of the oral cavity, although this usually only occurs when there is a break in, or loss of, maintenance of oral hygiene. Loss of oral hygiene may lead relatively quickly to the development of oral diseases or conditions such as gingivitis, halitosis (bad breath), dental plaque, dental calculus, dental caries and periodontitis. More severe outcomes of poor oral hygiene include the loss of teeth and/or bone.

These diseases are avoided in most individuals by the proper maintenance of oral hygiene. This is usually achieved by the regular brushing of teeth, which may be combined with the use of additional oral hygiene products such as mouthwashes. A recent trend has seen the inclusion of antibacterial agents in many oral hygiene products such as toothpastes and mouthwashes, and there may be a role for tea tree oil as one such agent.

The aim of this study was to determine the in vitro susceptibility of a range of oral bacteria to tea tree oil and to use these data to assess the suitability of tea tree oil for use in the oral cavity.

A total of 162 bacterial isolates from the genera *Actinomyces, Branhamella, Capnocytophaga, Clostridium, Eikenella, Fusobacterium, Haemophilus, Lactobacillus, Neisseria, Peptostreptococcus, Porphyromonas, Prevotella, Stomatococcus, Streptococcus* and *Veillonella* were tested for their susceptibility to tea tree oil. All isolates were inhibited and killed by concentrations of tea tree oil ≤ 2%, and in fact most were inhibited and/or killed at concentrations below this. Isolates with the lowest MICs and MBCs were from the genera *Prevotella, Porphyromonas* and *Veillonella* and isolates with the highest MICs and MBCs were from the genera *Streptococcus, Fusobacterium* and *Lactobacillus.*

*Streptococcus mutans* has been strongly associated with the development of dental caries. As such, it was of interest to investigate how quickly cells of *S. mutans* were killed when treated with tea tree oil. Treatment with 4, 2, 1 and 0.5% tea tree oil resulted in a greater than 3 log decrease in viable cells after only 30 seconds treatment. Also, after 5 and 10 minutes, viable cells could not be recovered from these treatments. Less killing was evident when *S. mutans* cells were treated with

0.12 and 0.25% tea tree oil. Time kill studies conducted with an isolate of *Lactobacillus rhamnosus*, a species also associated with dental caries, showed that *L. rhamnosus* was killed rapidly at both 0.5 and 1% tea tree oil, but less rapidly at 0.25%. Studies with these two species showed that tea tree oil exerts a relatively rapid killing effect. This in turn suggests that if tea tree oil was used in a mouthwash formulation it may be effective in reducing the numbers of *S. mutans*, *L. rhamnosus* or other bacteria within the mouth.

The data from this study suggest that tea tree oil may be of use in oral hygiene products. However, the exact role for tea tree oil remains to be elucidated and clinical trials evaluating tea tree oil formulations for a range of oral diseases or conditions are needed to establish this.

# Chapter 1. Introduction

## 1.1 The microbiology of the oral cavity

The oral cavity is perfectly suited to the colonisation, survival and growth of bacteria, yeasts, viruses and occasionally protozoa (Marsh & Martin, 1992). This range of organisms can survive and prosper in the oral cavity for a number of reasons, one of which is the diverse range of microenvironments present. Bacteria are found on the soft interior surfaces of the mouth such as the palate, tongue and cheeks, and on the hard tooth surfaces. Many of the same organisms that are found in instances of oral disease are also found in healthy mouths, and some of these organisms are shown in Table 1.1.

**Table 1.1**. Bacteria associated with oral diseases[1]

| Gram negative | Gram positive |
|---|---|
| *Bacteroides forsythus* | *Actinomyces naeslundii* |
| *Bacteroides* spp. | *Actinomyces viscosus* |
| *Eikenella corrodens* | *Lactobacillus* spp. |
| *Haemophilus actinomycetemcomitans* | *Peptostreptococcus micros* |
| *Neisseria* spp. | *Propionibacterium acnes* |
| *Porphyromonas* spp. | *Streptococcus anginosus* |
| *Prevotella intermedia* | *Streptococcus mutans* |
| *Prevotella* spp. | *Streptococcus sanguis* |
| *Veillonella alcalescens* | *Streptococcus sobrinus* |
| *Veillonella parvula* | |

[1] Data obtained from (Tanner & Stillman, 1993; Eick *et al.*, 1999; Ximinéz-Fyvie *et al.*, 2000; Paster *et al.*, 2001)

### Normal oral flora

Many different species of bacteria have been identified as inhabiting the oral cavity, although the viridans (α-haemolytic) *Streptococcus* species comprise a major portion of the normal oral flora (Marsh & Martin, 1992). The taxonomic groupings of oral streptococci include the mutans, salivarius, anginosus and mitis groups (Marsh & Martin, 1992; Herzberg, 2000). Other bacteria normally found in the oral cavity include *Actinomyces* spp., *Neisseria* spp., *Lactobacillus* spp., *Veillonella* spp., *Porphyromonas* spp. and *Prevotella* spp. These genera overlap to a large extent with the kinds of bacteria seen in dental caries and diseased gums. Some of these bacteria are

strictly anaerobic and do not survive in the presence of oxygen, and some are microaerophilic, meaning that they prefer environments with very low oxygen.

## Dental plaque and dental caries

The term dental plaque refers to the microbial communities found on tooth surfaces, which are embedded in a matrix of polymers of both bacterial and salivary origin (Marsh & Martin, 1992). The terms tartar or calculus refer to calcified plaque. Dental plaque accumulates preferentially in the pits and fissures in teeth, the area between teeth (approximal plaque) and the gingival crevice. The main organisms associated with dental plaque have been reported as *Streptococcus* spp., *Actinomyces* spp. and *Veillonella* spp. (Marsh & Martin, 1992).

Dental caries is described as the localised destruction of the tissues of the tooth by bacterial action. The enamel and dentine of the tooth are destroyed, followed by inflammation of both the pulp and periapical tissues of the tooth (Balakrishnan *et al.*, 2000). The mutans streptococci (in particular *S. mutans* and *S. sobrinus*) play a vital role in dental caries although the prevalence of other bacteria such as *Lactobacillus* spp. also increases dramatically in advanced dental caries (Balakrishnan *et al.*, 2000). The unchecked progression of dental caries can eventually lead to tooth loss.

## Periodontal disease

Periodontal disease refers to both gingivitis and periodontitis, which are oral diseases in which the supporting structures of the teeth such as the gingivae, connective tissues and bone, are attacked.

### *Gingivitis*

Gingivitis is a non-specific inflammatory response of the gingiva or gum to dental plaque (Marsh & Martin, 1992). It is generally regarded as occurring in response to an accumulation of plaque due to poor oral hygiene (Marsh & Martin, 1992) and often settles when adequate dental hygiene is restored. Gingivitis is characterised by redness, swelling and an increased tendency of the gums to bleed upon gentle probing (Kinane, 2001). Changes associated with gingivitis are an increased plaque mass, with a more diverse range of organisms that differs in composition from that of normal healthy gums. In this situation, the normally streptococci-dominated flora is replaced by *Actinomyces* dominated flora (Marsh & Martin, 1992).

### *Periodontitis*

Periodontitis usually follows gingivitis but occurs in only a small subset of gingivitis sufferers (Kinane, 2001). In patients with periodontitis, in addition to the involvement of the gingiviae, patients experience a loss of attachment between the root surface and alveolar bone, with the added

possibility of bone loss (Marsh & Martin, 1992). Periodontitis is often chronic and may eventually lead to the loss of teeth after many years of disease. Bacteria specifically implicated in periodontal disease are *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, *Haemophilus actinomycetemcomitans* and *Treponema denticola* (Kinane, 2001).

### Oral malodour

Evidence suggests that oral malodor, which is also called halitosis or bad breath, originates mostly from the oral cavity and to a much lesser extent from areas of the digestive or respiratory systems (Loesche & Kazor, 2002). This is thought to be because many of the anaerobic bacteria normally inhabiting the oral cavity produce hydrogen sulfide and methyl mercaptane, which are two of the malodourous compounds identified in oral malodour (Rösing *et al.*, 2002).

## 1.2 Products for the maintenance of oral hygiene

Most of the oral diseases or manifestations described above can be treated and/or prevented by simple oral hygiene measures. Many products are available for the maintenance of oral hygiene, including mouthwashes, dental floss, toothpicks, lozenges, oral sprays, dental tape and chewing gum. The aim of using these products is to prevent or reduce the occurrence of oral malodour, gingivitis, and plaque, and, in preventing these oral manifestations, also prevent periodontal diseases and dental caries.

Most maintenance of oral hygiene is achieved by simple toothbrushing. Toothpastes, in combination with toothbrushing act mainly to physically clean the teeth by removing food and stains and to minimise the build-up of plaque (Forward *et al.*, 1997). Although this physical removal by toothbrushing accounts for much of the cleaning of the oral cavity, some may be due to the antibacterial action of oral hygiene products. Mouthwashes in particular often contain an antimicrobial agent to act against the bacteria associated with oral diseases. Antimicrobial compounds that may be found in mouthrinses include chlorhexidine, cetylpyridium chloride, Triclosan, hydrogen peroxide or the essential oil mix of thymol, menthol, eucalyptol and methyl salicylate that is found in Listerine (Wu & Savitt, 2002). Essential oils are also commonly used as flavouring agents in mouthrinses (Forward *et al.*, 1997).

### Evaluating the activity of oral hygiene products in vitro and in vivo

Most, if not all, over-the-counter oral hygiene products that contain antibacterial agents make claims about the effects of the use of the product on oral health. The sorts of claims that oral hygiene products make often relate to the reduction or prevention of gingivitis and build-up of

plaque and tartar. In order to make these claims, in vivo efficacy would have been demonstrated in clinical studies.

The efficacy of oral hygiene products is most often evaluated in large, randomised, blinded and controlled clinical trials. For example, a trial may evaluate the effectiveness of products such as mouthrinses or chewing gums on curing or preventing gingivitis or plaque formation (Addy & Moran, 1997; Coehlo *et al.*, 2000). The activity of oral hygiene products is less commonly investigated in vitro, despite the obvious importance of this as a preliminary step in the overall evaluation of novel products or active ingredients. In vitro activity can be measured by standard antimicrobial activity assays such as those determining minimum inhibitory concentration (MICs) or minimum bacterial concentrations (MBCs). In addition, in vitro activity may be evaluated using time-kill methodology, which assesses the rate at which the agent or formulation exerts the antibacterial action. A subcommittee of the Food and Drug Administration (USA) has recommended that time-kill assays of 30 seconds duration are appropriate for evaluating mouthrinses (Wu & Savitt, 2002). Whilst in vitro assays may not necessarily give an indication of in vivo efficacy, results may provide a basis for the further investigation of an active ingredient.

# Chapter 2. Objectives

The potential usefulness of tea tree oil in oral healthcare has not been explored to any great extent. Determining the antimicrobial activity of tea tree oil against oral microorganisms is an essential first step in this process. Therefore, the aims of this study were to determine the antimicrobial activity of tea tree oil against a range of oral microorganisms and by doing so, evaluate the potential usefulness of tea tree oil as in ingredient in oral healthcare products.

# Chapter 3. Materials and methods

## 3.1 Tea tree oil

*Melaleuca alternifolia* (tea tree) oil was kindly donated by Australian Plantations Pty Ltd., Wyrallah, NSW. Batch 97/1 was used for all studies and had the composition shown in Table 3.1, as determined by gas-chromatography mass spectrometry performed by the Wollongbar Agricultural Institute, Wollongbar, NSW.

**Table 3.1** Composition of *M. alternifolia* oil batch 97/1

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

## 3.2 Microbial isolates

Bacteria were obtained from samples from the oral cavities of volunteers and from existing culture collections. These were the collections of the Microbiology Discipline of The University of Western Australia and the Division of Microbiology and Infectious Diseases at the Western Australian Centre for Pathology and Medical Research (PathCentre). Reference isolates were *Escherichia coli* NCTC 10418, *Veillonella parvula* NCTC 11810, *Haemophilus actinomycetemcomitans* ATCC 43718 and *Lactobacillus casei* var. *rhamnosus* NCTC 10302.

### Isolation and identification of oral bacteria

Bacteria were isolated from the oral cavities of volunteers by rubbing a sterile cotton-tipped swab over the teeth, gums and tongue and then placing the swab into a glass Bijou bottle containing 1 ml of phosphate buffered saline (PBS). The bottle was mixed thoroughly with the use of a vortex mixer to remove bacteria from the swab. The resulting bacterial suspension was diluted 10-fold and aliquots from each dilution were plated onto a range of selective and non-selective media. These included blood agar (BA), lysed blood agar with gentamicin (LBAG), Rogosa agar and Chocolate agar. Media were either obtained pre-prepared from Excel Laboratory Products, Belmont, WA or were obtained as powders and prepared in the laboratory according to the manufacturer's instructions. Inoculated plates were incubated for 3 - 10 days in an anaerobic chamber and colonies were subcultured onto BA for pure cultures.

A range of methods were used to identify isolates to the genus or species level, including colony form and microscopic characteristics, oxygen tolerance, haemolysis, pigment production, Gram stain, catalase, indole and lipase reactions as described in detail by Summanen *et al*. (1993) (Summanen *et al.*, 1993). Biochemical profiles were determined with API 32A strips and antibiotic susceptibilities for identification purposes were determined using Microring AN discs (Medical Wire and Equipment Co (Bath) Ltd., Wiltshire, England).

## 3.3 In vitro susceptibility testing

### Preparation of inocula

Inocula were prepared by growing organisms on BA for 24 - 48 h and suspending colonies in SDW. This suspension was adjusted to 0.5 McFarland using a nephelometer, and then diluted 1 in 10 into the relevant growth medium. This suspension contained approximately $10^6$ cfu/ml and was halved upon inoculation into either the macro- or microdilution assay. Viable counts were performed by serially diluting the inocula ten-fold in SDW and spot inoculating 10 µl amounts onto BA. Viable count plates were then incubated anaerobically until colonies were countable.

### Microdilution assay

Tea tree oil dilutions ranging from 4 - 0.004% were prepared in 100 µl volumes in a 96-well microtitre tray in the relevant growth medium. Todd Hewitt Broth was used for *Streptococcus* spp., de Mann, Rogosa and Sharpe (MRS) broth for *Lactobacillus* spp. and BHIB was used for all other organisms. A final concentration of 0.001% Tween 80 was included in all assays to enhance tea tree oil solubility.

After inoculation, tests were incubated for 24 h under anaerobic conditions except for tests with *Streptococcus* spp. and *Haemophilus actinomycetemcomitans,* which were incubated for 24 h in microaerophilic conditions (5% carbon dioxide). Trays were then subcultured by first mixing the contents of each well, then removing 10 μl aliquots and spot inoculating onto pre-dried BA. Plates were then incubated and growth was recorded. MICs and MBCs were determined as described below.

### Macrodilution assay

Some bacterial isolates did not produce sufficient growth in the microdilution format and susceptibility testing was therefore performed by the broth macrodilution method. This was required for *Porphyromonas endodentalis, Prevotella intermedia* and one isolate of *Actinomyces viscosus.*

Dilutions of tea tree oil were prepared in 1 ml volumes of BHIB supplemented with 5 μg/ml hemin and 1 μg/ml vitamin K (BHIB-HK). After inoculation, final tea tree oil concentrations were 0.5, 0.1, 0.05, and 0.01 (% v/v). Dilutions of tea tree oil were pre-reduced by transferring to the anaerobic chamber for approximately 60 min. Dilutions were removed from the chamber and inoculated with 1 ml volumes, after which dilutions were immediately returned to the anaerobic chamber and incubated for 48 h. After incubation, 5 μl amounts were removed from each dilution and spot-inoculated onto BA. Agar plates were incubated anaerobically for 48 -72 h, colonies were counted and MICs and MBCs determined.

### Criteria for determining MICs and MBCs

MICs were determined as the lowest concentration resulting in the maintenance or reduction of the inoculum and the MBC was determined as the concentration resulting in the death of 99.9% of the inoculum.

## 3.4 Time kill assays

*Streptococcus mutans* (clinical isolate) and *Lactobacillus rhamnosus* NCTC 10302 were used in time kill studies. Inocula were prepared by growing each isolate for 48 - 72 h on BA (*S. mutans*) or Rogosa agar (*L. rhamnosus*). Growth was then suspended in 0.85% saline and adjusted to 0.5 McFarland. The adjusted suspension was diluted 1 in 10 in saline and, after inoculation, the final concentration of organisms was approximately $5 \times 10^6$ cfu/ml. Treatments containing tea tree oil ranging from 4 - 0.12% were prepared in 1 ml volumes of double-strength BHIB (*S. mutans*) or MRS (*L. rhamnosus*) with 0.002% Tween 80. Treatments were transferred to the anaerobic

chamber to pre-reduce for at least 30 min prior to inoculation. Volumes of 1 ml of inocula were added to each treatment at timed intervals and samples were removed at 30 s, 5 and 10 min for viable counts. Bottles were shaken manually before each sample for viable counting was removed. Viable counts were performed by diluting each sample 10-fold in 0.85% saline and spot inoculating duplicate 10 µl aliquots onto pre-dried BA or Rogosa agar. This was performed in the anaerobic chamber. Viable count plates were incubated anaerobically for 48 - 72 h and colonies were then counted. Viable counts were only calculated if each replicate 10 µl spot had one or more colony. As such, the limit of detection for this assay was $1 \times 10^3$ cfu/ml. Assays were repeated at least twice for each tea tree oil concentration and the mean, standard deviation and standard error of the viable count data were calculated.

# Chapter 4. Results

## 4.1 In vitro susceptibility of oral bacteria to tea tree oil

A collection of 162 isolates was obtained and tested for their susceptibility to tea tree oil (Tables 4.1 and 4.2). For all organisms, the range of both MICs and MBCs was from 0.003 to 2%. Isolates with the lowest MICs and MBCs belonged to the genera *Prevotella*, *Porphyromonas* and *Veillonella.* The highest MICs and MBCs were for isolates of *Streptococcus*, *Fusobacterium* and *Lactobacillus*. MICs and MBCs for each isolate were generally equivalent or differed by only one concentration, suggesting bactericidal rather than bacteriostatic activity.

$MIC_{90}$s for genera with more than 10 isolates were 1% for *Actinomyces*, *Lactobacillus* and *Streptococcus* spp. and 0.1% for *Prevotella* spp. $MBC_{90}$s were 1% for *Actinomyces*, 2% for *Lactobacillus* and *Streptococcus* spp. and 0.1% for *Prevotella* spp. In addition, $MIC_{90}$s and $MBC_{90}$s were both 1% for *Streptococcus* isolates belonging to both the anginosus and mitis groups.

**Table 4.1** In vitro susceptibility of oral streptococci (*n* = 79) to tea tree oil

| Organism (*n*) | Group | MIC | | MBC | |
|---|---|---|---|---|---|
| | | Range | 90[1] | Range | 90[1] |
| *S. agalactiae* (1) | (non-viridans) | 0.5 | | 0.5 | |
| *S. bovis* (1) | bovis | 0.5 | | 1 | |
| *S. constellatus* (8) | anginosus | 0.25 - 1 | | 0.25 - 1 | |
| *S. gordonii* (2) | mitis | 0.5 | | 0.5 - 1 | |
| *S. intermedius* (6) | anginosus | 0.12 - 2 | | 0.25 - 2 | |
| *S. mitis* (11) | mitis | 0.25 - 1 | 1 | 0.25 - 1 | 1 |
| *S. mutans* (2) | mutans | 0.25 - 2 | | 0.25 - 2 | |
| *S. oralis* (5) | mitis | 0.25 - 1 | | 0.25 - 1 | |
| *S. parasanguis* (3) | mitis | 0.25 - 0.5 | | 0.25 - 0.5 | |
| *S. salivarius* (2) | salivarius | 0.25 | | 0.25 | |
| *S. sanguis* (19) | mitis | 0.25 - 1 | 1 | 0.25 - 2 | 2 |
| *S. sobrinus* (1) | mutans | 1 | | 2 | |
| *Streptococcus* spp. (18) | (viridans) | 0.25 - 1 | 1 | 0.25 - 2 | 2 |

[1] Percentage tea tree oil inhibitory or bactericidal to 90% of isolates.

**Table 4.2** In vitro susceptibility of non-streptococcal oral bacteria (*n* = 83) to tea tree oil

| Organism (*n*) | MIC | | MBC | |
|---|---|---|---|---|
| | Range | 90[1] | Range | 90[1] |
| *Actinomyces naeslundii* (5) | 0.12 - 1 | | 0.25 - 2 | |
| *A. odontolyticus* (1) | 1 | | 1 | |
| *A. viscosus* (6) | 0.1 - 1 | | 0.1 - 1 | |
| *Actinomyces* sp. (1) | 1 | | 1 | |
| *Branhamella* sp. (1) | 0.06 | | 0.06 | |
| *Capnocytophaga* (3) | 0.03 - 0.06 | | 0.03 - 0.06 | |
| *Clostridium glycolidium* (1) | 0.05 | | 0.1 | |
| *Eikenella* spp. (5) | 0.03 - 0.06 | | 0.03 - 0.06 | |
| *Fusobacterium* spp. (5) | 0.25 - 2 | | 0.25 - 2 | |
| *Haemophilus actinomycetemcomitans* (1) | 0.06 | | 0.06 | |
| *Lactobacillus* spp. (18) | 0.03 - 2 | 1 | 0.06 - 2 | 2 |
| *Neisseria* sp. (1) | 0.25 | | 0.25 | |
| *Peptostreptococcus asaccharolytica* (3) | 0.25 - 0.5 | | 0.5 - 1 | |
| *Porphyromonas endodentalis* (8) | 0.025 - 0.1 | | 0.025 - 0.1 | |
| *Prevotella bivia* (1) | 0.016 | | 0.03 | |
| *Prevotella buccae* (1) | 0.016 | | 0.016 | |
| *Prevotella corporis* (1) | 0.06 | | 0.06 | |
| *Prevotella intermedia* (15) | 0.003 - 0.1 | 0.1 | 0.003 - 0.1 | 0.1 |
| *Stomatococcus* sp. (1) | 0.5 | | 0.5 | |
| *Veillonella parvula* (2) | 0.016 - 0.03 | | 0.03 | |
| *Veillonella* spp. (3) | 0.5 - 1 | | 1 | |

[1] Percentage tea tree oil inhibitory or bactericidal to 90% of isolates.

## 4.2 Time kill studies with oral isolates

Time kill data are shown in Figures 4.1 and 4.2. Treatment of *S. mutans* cells with 4, 2, 1 and 0.5% tea tree oil resulted in similar decreases in viability after 30 s. The numbers of viable organisms recovered from the 2% and 4% treatments after 30 s were $3.25 \times 10^3$ cfu/ml and $3.33 \times 10^3$ cfu/ml, respectively (data not shown in Fig 4.1). Treatment with both 0.5 and 1.0% also resulted in a > 3 log decrease in viability (>99.9% killing) after 30 s and no viable organisms were recovered after 5 or 10 min treatment with 0.5, 1, 2 or 4% tea tree oil. The viable counts from 0.5, 1, 2 and 4% treatments at 30 s were significantly different from controls.

The viable counts of *S. mutans* cells treated with 0.25% tea tree oil differed significantly from controls at 30 s, 5 and 10 min whereas viable counts of cells treated with 0.12% differed significantly from controls at 5 and 10 min only.

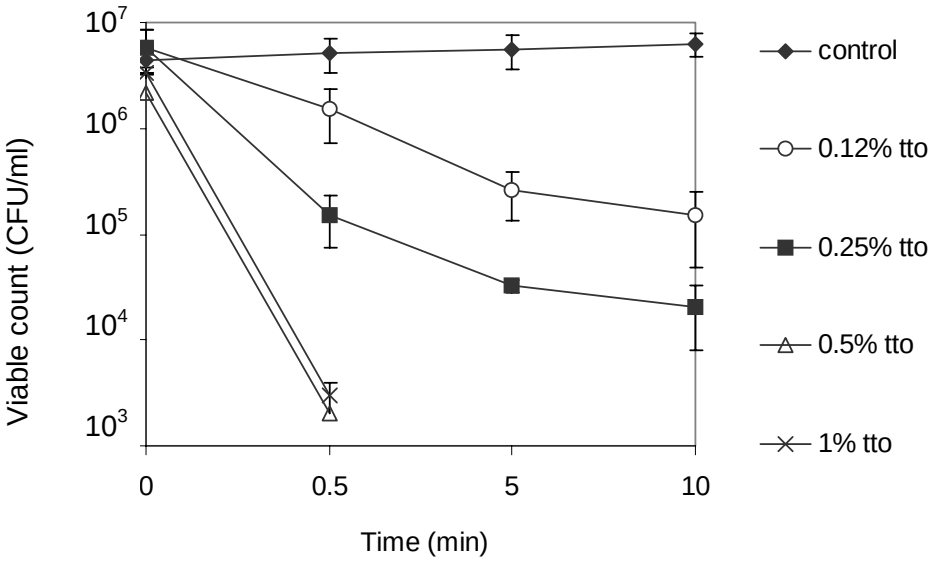


**Fig 4.1** Viability of *Streptococcus mutans* after 30 s, 5 and 10 min treatment with tea tree oil. The MIC and MBC for this isolate was 2.0%

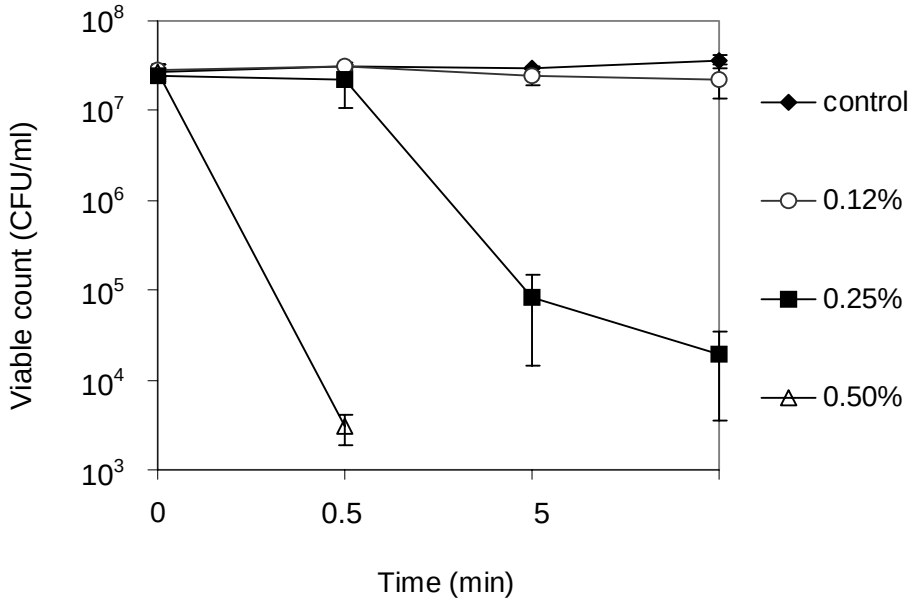


**Fig 4.2** Viability of *Lactobacillus rhamnosus* after 30 s, 5 and 10 min treatment with tea tree oil. For this isolate, the MIC was 0.25% and the MBC was 0.5%

Time kill data for *L. rhamnosus* (Fig 4.2) showed that after 30 s treatment with 1% tea tree oil viable numbers were reduced to below detectable limits. Treatment with 0.5% tea tree oil resulted in a reduction in the numbers of viable organisms by >3 log within 30 s, and at 5 min viable organisms were no longer detectable. Treatment with 0.25% tea tree oil resulted in a modest reduction in viability whereas treatment with 0.12% did not have any significant effects on viability.

# Chapter 5. Discussion

## 5.1 In vitro susceptibility of oral bacteria to tea tree oil

The oral microorganisms tested in this study were all susceptible to tea tree oil. Furthermore, the amounts of tea tree oil required to inhibit or kill isolates were similar to values published previously for a wide range of non-oral bacteria (Carson *et al.*, 1995; Hammer *et al.*, 1996; Banes-Marshall *et al.*, 2001).

Unfortunately, very few studies have extensively investigated the activity of tea tree oil against oral microorganisms. In addition, results from these studies are somewhat limited since none tested a large number of isolates. The earliest published study of the activity of tea tree oil against oral bacteria was by Walsh and Longstaff (1987), who determined MICs of Melasol, a mixture containing 40% tea tree oil, 13% isopropyl alcohol and water, against 12 oral bacteria. They found MICs of 0.02% for isolates of *Haemophilus (Actinobacillus) actinomycetemcomitans, Actinomyces naeslundii, Porphyromonas (Bacteroides) gingivalis, Bacteroides fragilis, Eikenella corrodens, Fusobacterium nucleatum, Peptococcus assacharolyticus* and *Prevotella intermedia.* MICs of 0.04% were found for isolates of *Fusobacterium mortiferum, Streptococcus mutans* and *Streptococcus sanguis* and an MIC of 0.08% Melasol was found for an *Actinomyces viscosus* isolate. Although the values obtained by Walsh and Longstaff are mostly lower than those obtained in the present study, this may be largely attributed to methodological differences. In particular, both the use of the agar dilution method and the alcohol content of the Melasol solution may have contributed to the low values.

A more recent study by Shapiro *et al.* (1994) tested 15 bacterial isolates and reported MICs ranging from 0.11 - 0.6% for isolates of *Haemophilus actinomycetemcomitans, Actinomyces viscosus, Peptostreptococcus anaerobius* and *Porphyromonas gingivalis.* An MIC of > 0.6% was found for an isolate of *F. nucleatum.* MBCs determined after 3 minutes incubation were > 0.6% *Peptostreptococcus anaerobius, Porphyromonas gingivalis, Actinomyces viscosus, Haemophilus actinomycetemcomitans, Streptococcus sanguis* and *Streptococcus sobrinus*. However, when the incubation period was increased to 6 h, MBCs were reduced dramatically to concentrations essentially identical to the MIC for each isolate. Hales *et al.*, (1998) reported MICs and MBCs of 0.5% for a single isolate each of *Streptococcus mutans, Lactobacillus casei, Eikenella corrodens* and *Haemophilus actinomycetemcomitans* and an MIC of 0.5% and an MBC of 1.0% for a single isolate of *Actinomyces viscosus* (Hales, 1998). In general, the values obtained by both Shapiro *et*

*al*. (1994) and Hales *et al*. (1998) were similar to those obtained in the present study, which may be related to the studies using similar microdilution methods.

The most recent study was by Kulik *et al.* (2000) who determined the in vitro susceptibility of nine oral bacterial isolates to tea tree oil. The MIC/MBC values, determined by macrodilution, were 0.26/1.04% (v/v) for *S. mutans*, 0.16/0.42% for *S. sangius* and *S. anginosus*, 0.03/0.05% for *H. actinomycetemcomitans*, 0.21/1.56% for *Lactobacillus salivarius*, 0.13/0.52% for *A. naeslundii*, 0.08/0.17% for *F. nucleatum*, 1.25/2.5% for *P. intermedia* and 0.07/0.07% for *P. gingivalis* (Kulik *et al.*, 2000).

Data from these three studies are for the most part very similar to the data obtained in the present study. However, notably different were the values obtained for *P. intermedia* by Kulik *et al*. (2000) which were considerably higher than those determined in the present study and conversely, the values obtained in the current study for *Fusobacterium* spp. were considerably higher than those found by previous authors (Walsh & Longstaff, 1987; Shapiro *et al.*, 1994). Factors that may have contributed to these divergent results include differences between the types and numbers of bacterial strains tested in each of the studies and the methods used.

Several publications have described susceptibility data for bacteria related to, or similar to those found in the mouth, although they were not obtained specifically from the oral cavity. Carson *et al.* (1996) published MICs for β-haemolytic (pyogenic) streptococci and found MICs ranging from 0.03 - 0.12% and MBCs ranging from 0.03 - 0.25%. Another study found ranges of MICs and MBCs for β-haemolytic streptococci of 0.5 - 2 and 1 - 4% (Banes-Marshall *et al.*, 2001). The susceptibility to tea tree oil of a range of anaerobic and microaerophilic vaginal bacteria, most of which are similar to those found in the oral cavity, has also been determined (Hammer *et al.*, 1999a). MIC ranges were 0.03 - 0.5 for *Bacteroides* spp., 0.03 - 0.25 for *Prevotella* spp., 0.06 - 0.25 for *Fusobacterium* spp., 0.03 - 0.25 for anaerobic Gram-positive cocci and 0.12 - 2% for *Lactobacillus* spp. All of these cited values are generally similar to those found in the present study.

In vitro susceptibility data from this study show that the bacteria found in the oral cavity are susceptible to tea tree oil and, as such, tea tree oil may be a suitable agent for use in the oral cavity.

## 5.2 Time kill studies

Time kill studies with an isolate each of *S. mutans* and *L. rhamnosus* showed that both organisms were killed within a short time period, by concentrations equal to, or less than the MBC.

The significant killing of *S. mutans* that occurred at concentrations of 1/2 and 1/4 of the MBC of tea tree oil was not anticipated. This killing may be a reflection of the different dynamics occurring during the microdilution and time kill assays. For example, it may be that when bacteria are inoculated into the microdilution tray, at concentrations of 0.5% and 1.0% a large proportion of bacteria are killed immediately, but over the total incubation time a small population of bacteria survives, recovers and grows. Given this proposed dynamic, the MIC and MBC values of 2.0% for this *S. mutans* isolate may represent an underestimation of the extent of the activity of tea tree oil. In contrast, the isolate of *L. rhamnosus* was killed at MBC and 1/2 MBC concentrations, which was a more predictable outcome.

Many mouthrinses are recommended to be used twice daily for 30 seconds, with volumes of approximately 20 ml (Pitten & Kramer, 1999; Fine *et al.*, 2000). Thus the time point of 30 sec was chosen in the time kill assay to mimic the elapsed time that mouthwash may be retained in the mouth. Although the time-kill assay showed significant killing of *S. mutans* and *L. rhamnosus* after 30 sec, this may not necessarily reflect what may occur in the oral cavity when rinsing with a tea tree oil mouthwash solution, for several reasons. Firstly, it is recommended that time kill studies for oral products be conducted in the presence of exogenous protein (Wu & Savitt, 2002), which the current studies were not. This is of particular importance since organic matter has been shown to interfere with the activity of tea tree oil (Hammer *et al.*, 1999b). Secondly, the relationship between in vitro and in vivo findings may simply not be a direct correlation, as found previously for the product Lavasept (containing polyhexanide, a compound related to chlorhexidine), which gave promising in vitro results but did not perform well in vivo (Pitten & Kramer, 1999).

Whilst it is feasible that a tea tree oil mouthrinse may reduce numbers of viable bacteria in saliva, which may be as high as $10^8$ microorganisms per ml (Marsh & Martin, 1992), it is perhaps more important that a mouthrinse reduces signs of gingivitis and dental plaque as endpoints of overall efficacy. This is because the organisms that cause plaque and gingivitis are generally not in a free-living or 'planktonic' state within the saliva, but are instead living within biofilm on the teeth. Therefore any antimicrobial mouthrinse needs to be able to affect the organisms contained within these biofilms. Although in vitro models for the investigation of the effects of oral hygiene products on biofilm exist (Shapiro *et al.*, 2002), the efficacy of these products is still more commonly evaluated in clinical studies.

## 5.3 Potential uses of tea tree oil in oral hygiene products

It has been suggested for some time that tea tree oil may be an effective agent for both the treatment and prevention of oral infections or conditions. In 1937, Penfold and Morrison reported that mouth conditions such as thrush, aphthous stomatitis, mouth ulcers, gingivitis and pyorrhoea (periodontitis with pus) had all responded favourably to treatment with tea tree oil (Penfold & Morrison, 1937). Unfortunately, the potential uses of tea tree oil in the oral cavity have not been subsequently researched or documented to any great extent, meaning that scientific reports are few.

Products that already contain, or would be suitable for containing tea tree oil, include toothpastes and mouthrinses. Tea tree oil toothpastes, in particular, are already widely available in supermarket and chemist stores, and the inclusion of tea tree oil in toothpastes is in keeping with the recent trend for toothpastes to contain an active antibacterial agent such as Triclosan, cetylpyridinium chloride or chlorhexidine. Although the principal way that toothpastes aid in cleaning the oral cavity is by mechanical means (in conjunction with toothbrushing), the inclusion of tea tree oil as an antibacterial agent may have additional benefits such as the reduction or prevention of plaque.

Mouthrinses containing tea tree oil are not found as commonly as tea tree oil toothpastes in supermarkets and pharmacies but may be an appropriate product for the treatment, reduction or prevention of halitosis, plaque and gingivitis. One publication has described the efficacy of a tea tree oil mouthwash on plaque formation. In this study, the effects of several mouthwashes, containing either approximately 0.34% tea tree oil, 0.1% chlorhexidine or placebo, on plaque formation and vitality was compared using eight volunteers (Arweiler *et al.*, 2000). On day zero, volunteers had their teeth professionally cleaned, and for the next four days they rinsed twice daily with one of the treatments and did not clean their teeth in any other manner. Teeth were clinically evaluated on days 1, 2, 3 and 4. Each mouthwash was evaluated in this manner, with a wash-out period of 10 days between the end of one treatment and the beginning of the next. The plaque index and plaque vitality from the tea tree oil mouthwash treatment did not differ from placebo mouthwash on any day, whereas the chlorhexidine mouthwash differed significantly on all days. Thus the tea tree oil treatment was considered ineffective at reducing plaque regrowth or the vitality of plaque organisms (Arweiler *et al.*, 2000). The factor that may have contributed to the poor results for the tea tree oil product in this study is the relatively low concentration of tea tree oil used. The small number of volunteers participating in the study may have also made it more likely that the poor performance of tea tree oil occurred by chance.

Two additional studies have been conducted assessing the efficacy of a tea tree oil mouthwash for the treatment of oral candidiasis (Jandourek *et al.*, 1998; Vazquez & Zawawi, 2002). The first study described a case series of 13 patients who used a tea tree oil mouthwash to treat oropharyngeal candidiasis (Jandourek *et al.*, 1998). Patients were HIV positive and had already failed treatment with a 14 day course of oral fluconazole. Treatment was with 15ml of tea tree solution four times a day for up to 28 days. At the end of treatment, of the 12 evaluable patients two were cured, six were improved, four were unchanged and one patient had deteriorated. Overall, eight patients had a clinical response, and seven had a mycological response. The second study was very similar in design, with 12 and 13 evaluable patients in the groups receiving the alcohol-based melaleuca solution or the alcohol-free melaleuca solution, respectively (Vazquez & Zawawi, 2002). After four weeks of therapy, seven patients were cured, eight were improved, six were unchanged and two had deteriorated. The overall clinical response rate was 60%. These two studies illustrate that tea tree oil can be used effectively in the oral cavity and although the focus of the current study is oral bacteria only, the outcomes of these studies with oral candidiasis are still relevant.

Tea tree oil has been shown to have anti-inflammatory effects (Hart *et al.*, 2000; Brand *et al.*, 2001; Koh *et al.*, 2002) and as such may be helpful in the treatment of gingivitis, which is by definition the inflammation of the gingivae. Many oral hygiene products may reduce gingival inflammation indirectly by reducing plaque, whereas tea tree oil has the potential to reduce both gingivitis and plaque mass simultaneously. Unfortunately, it may be very difficult to distinguish these two effects clinically.

The regulatory impediments - if any - to the marketing of oral hygiene products containing tea tree oil remain unknown. In particular, issues of tea tree oil toxicity may need to be addressed. This includes both systemic toxicity if the product is swallowed and toxicity to the oral mucosa. Little is known about adverse reactions to tea tree oil in the oral cavity. In a study assessing an alcohol-based melaleuca solution for oral candidiasis, 8 of 12 patients noted a burning sensation that was mild to moderate. However, this was noted largely during the first week of therapy and gradually decreased thereafter (Vazquez & Zawawi, 2002). In comparison, only 2 of 13 patients receiving the alcohol-free oral solution complained of burning and an additional patient complained of stinging. Coincidentally, an earlier study by the same researchers also found that 8 of 12 patients noted a mild burning sensation after use of the same alcohol-based melaleuca solution, which also decreased after the first week of therapy (Jandourek *et al.*, 1998). Whilst these reactions may be due to the solution coming into contact with oral mucosa that is raw or inflamed due to the candidiasis, the difference in the numbers of reactions between the two groups suggests that the reactions are largely due to the alcohol content of the solution. Similarly, patients using Listerine

(which contains 26.9% alcohol and 0.26% essential oils) have reported reactions comparable to those using the alcohol-based melaleuca solution. They reported an initial burning sensation and bitter taste, both of which decreased over subsequent days (Wu & Savitt, 2002). The possibility of reactions to tea tree oil within the oral cavity or systemic toxicity from ingestion of products requires further investigation.

# Chapter 6. Implications and recommendations

Tea tree oil is a popular 'natural' topical antiseptic with widely varying uses. In addition to tea tree oil being included in oral health care products such as toothpastes and mouthrinses, tea tree oil is often recommended in 'home remedy' recipes for the treatment of bad breath or gingivitis.

The data from this study provide sound support for the use of tea tree oil in oral hygiene products. Tea tree oil is already included in both toothpastes and mouthwashes which are available over-the-counter. In addition to everyday home use, oral tea tree oil solutions could be used in a hospital or hospice setting for patients, in particular the elderly, who are less able to manage their oral health by regular toothbrushing. The ability of tea tree oil oral hygiene products to reduce or prevent plaque, gingivitis or halitosis remains unknown and clinical investigations are required. Issues of mucosal toxicity or systemic toxicity from swallowing any tea tree oil-containing products would need to be addressed.

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