The antimicrobial activity of four herbal based toothpastes against specific primary plaque colonizers

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The antimicrobial activity of four herbal based toothpastes against specific primary plaque colonizers

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Keywords

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ABSTRACT

The exact aetiology of periodontitis is unknown but it is believed to result from an infection by a select group of Gram-negative anaerobic bacteria found in dental plaque. However in order for the periodontal pocket to be populated by these suspected periodontopathogens, they require that the local environment be primed for their colonization. This favourable environment results from a series of progressive colonizations by prior groups of bacteria, the process being termed bacterial succession. Essential to bacterial succession is the initial colonization of the tooth surface by bacteria termed "the primary plaque colonizers". These are assumed to be the first species in biofilm formation. Elimination of these species might therefore be beneficial to prevent bacterial succession and later colonization by the suspected periodontopathogens. **Aim:** To determine whether there was any significant difference in the antimicrobial activity of 4 herbal toothpastes against cultures of 3 primary plaque colonizers (Streptococcus mutans, Streptococcus sanguinis and a non-specific *α-heamolytic* streptococcus). **Method:** The study was a laboratory based experiment that used in-vitro diffusion to assess the antimicrobial potential of the various toothpastes. Forty eight cultures were produced and grown overnight in a temperature controlled room. The various zones of inhibition formed around the test toothpastes were then measured after 24hrs of incubation and compared to each other. **Results:** Of the four toothpastes tested, Dentazyme Herbal® showed the greatest ability to inhibit bacterial growth. It was stronger than Colgate Total® (the positive control) for 2 of the 3 species of bacteria tested. Nature Fresh® had the lowest potential for antimicrobial activity and only displayed a moderated inhibitory affect for one of the bacterial species tested. **Conclusion:** The results indicate that there was a significant difference in the ability of the various toothpastes to inhibit the growth of certain bacteria.

DECLARATION

I hereby declare that "The antimicrobial activity of four herbal based toothpastes against specific primary plaque colonizers" is my own work, that it has not been submitted before for any degree or examination in any university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

M. Thabit Peck

Signed:………………………………….

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DEDICATION

To my parents for their constant support, prayers and sacrifice

To my supervisors whose guidance, encouragement, help and support made this project possible

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CHAPTER 1
INTRODUCTION

Periodontal disease is one of the most common causes of tooth loss. It is a condition that affects a considerable portion of the population (Beck and Arbes 2002) and clinically presents in a variety of forms. Although adults make up the majority of those affected, it can and does affect children appearing as either a slowly progressive or, rarely, a rapidly destructive disorder.

The exact aetiology of periodontitis is unknown but it is believed to result from an infection by a select group of Gram-negative anaerobic bacteria found in dental plaque. These include *Actinomyces actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* (Lovegrove 2004; Inagaki *et al*, 2006). However in order for the periodontal pocket to be populated by these suspected periodontopathogens, they require that the local environment be primed for their colonization. This favourable environment results from a series of progressive colonizations by prior groups of bacteria, the process being termed "bacterial succession".

Essential to bacterial succession is the initial colonization of the tooth surface by bacteria termed "the primary plaque colonizers". These include several species of *Streptococci* and *Actinomyces.* These are assumed to be the first species in plaque biofilm formation. Elimination of these species might therefore be beneficial in preventing bacterial succession and later colonization by the suspected periodontopathogens.

Because of the infectious nature of the condition, treatment has always been directed towards the reduction or elimination of the suspected periodontopathogens (van Winkelhoff *et al* 2002; Preshaw *et al* 2004).

Mechanical and surgical debridement has been used as the mainstay of disease management. This approach has never been completely successful and in the past decade, antibiotics and other antimicrobial agents have been used to supplement conventional treatment (Cosyn and Wyn 2006; Greenstein 2006). Antibiotics are used both locally and systemically and both methods are known to significantly influence the microflora associated with periodontally diseased sites.

In order to increase the effectiveness of plaque removal, antimicrobials have been added to toothpastes. The most effective of these being triclosan. It is a chemical that has a wide range of antimicrobial activity and when tested clinically, it improves the condition of patients suffering form gingivitis and periodontitis (Cullinan *et al* 2003). Although it has been touted as a promising agent in the management of periodontal disease, it has recently been shown that triclosan-resistance is starting to emerge. Because of this, the widespread incorporation of triclosan into everyday products may eventually pose a potential public health risk (Yazdankhah *et al* 2006).

In an attempt to find better and more potent antimicrobials, plant and herbal based products have been tested for their potential therapeutic effects (Arora and Kaur 1999; Lai and Roy 2004; Natarajan *et al* 2003; O'Hara *et al* 1998). A number of these agents are effective against oral pathogens and manufacturers have now incorporated them into toothpastes (Wu-Yuan *et al* 1990; Kaim *et al* 1998; Groppo *et al* 2002; Lee *et al* 2004; Uzel *et al* 2005).These anti-microbial properties may therefore have some therapeutic benefit to patients suffering from periodontal disease.

The elimination or reduction of the "primary plaque colonizers" might potentially inhibit the growth of the plaque biofilm and thereby prevent subsequent colonization by the suspected periodontopathogens. This study attempts to investigate the anti-microbial efficacy of four over-the-counter herbal-based toothpastes against specific species of three primary plaque colonizers (*Streptococcus mutans, Streptococcus sanguis and a non specific α-heamolyitic streptococcus termed VS-1*).

CHAPTER 2
LITERATURE REVIEW

2.1. INTRODUCTION

Periodontal disease encompasses a broad spectrum of pathological disorders that affects the supporting structures of the dentition. Clinically it is either limited to inflammation of the soft tissue surrounding the tooth, but it can also appear as a destructive disorder of the alveolar bone and periodontal ligament.

The two most common clinical manifestations of periodontal disease are gingivitis (limited to the gingiva) and periodontitis (involving both the gingiva as well as the periodontal attachment apparatus), both of which are thought to be bacteria related. Gingivitis is commonly controlled with plaque inhibitory measures whereas periodontitis often requires more aggressive treatment options. The uncontrolled progression of periodontitis leads to increased tooth mobility and eventual tooth loss. Recently periodontitis has been linked to systemic conditions such as low preterm birth-weight and cardiovascular disease (Beck and Offenbacher 2001; Yeo *et al* 2005)

The management of periodontitis is not universal or standardized and consequently many treatment options exist. The antimicrobial properties of herbs are well established and their potential clinical benefit in the management of periodontal disease should therefore be explored.

This literature review explores the aetiology and pathogeneses of periodontal disease with specific reference to its associated microbiology and the attempts to manage it using antimicrobial agents.

2.2. PERIODONTITIS

Periodontitis is defined as an inflammatory disease of the periodontium that extends beyond the gingival margin. It results in the progressive destruction of the periodontal ligament and alveolar bone, and may be associated with gingival recession (Beck and Arbes 2002).

The prevalence of the disease is difficult to quantify because no uniform criteria for periodontal research has been established (Borrell and Papapanou 2005; Papapanou and Lindhe 2003). This is further compounded by the fact that the disease classification has repeatedly been revised resulting in different studies using different research criteria (Borrell and Papapanou 2005). As a result, inconsistent data has been produced. This is due mainly to variations in the threshold values used to define the disease (Borrell and Papapanou 2005; Papapanou and Lindhe 2003).

An international workshop was held in 1999 in order to address these contentious issues. The sole purpose of this workshop was to reach consensus on the classification of periodontal diseases and conditions. This workshop, known as the "1999 Workshop for a Classification of Periodontal disease and Conditions" was arranged by the American Academy of Periodontology and included experts from around the world. This meeting lead to the current classification of periodontal-related-diseases (Armitage 2004). Eight major categories of periodontal diseases were listed. These eight categories are as follows (Milward and Chapple 2003):

   I. Gingivitis
  II. Chronic periodontitis
 III. Aggressive periodontitis
 IV. Periodontitis as a manifestation of systemic diseases
  V. Necrotizing periodontal diseases
 VI. Abcesses of the periodontium
VII. Periodontitis associated with endodontic lesions
VIII. Developmental or acquired deformities and conditions.

One of the most significant changes to the previous classification was the discontinuation of terms related to age of presentation and rate of progression of the disease. It was felt that these criteria were ambiguous since it is often impossible to determine when periodontal disease starts or how fast it progresses if previous dental records are not available. The fact that disease progression can either be slow and constant or episodic, and the finding that similar disease presentations are found at most ages, provided additional evidence for removing certain terms (Wiebe and Putnins 2000).

2.3. CLINICAL FEATURES OF PERIODONTITIS

Based on the 1999 classification, periodontitis may present as one of three major clinical forms i.e. chronic, aggressive and necrotizing. All are associated with pathological alveolar bone loss.

Chronic periodontitis is a slowly progressive disease that is characterized by gingival inflammation, bleeding on probing, reduced resistance of the pocket periodontal tissues to probing, loss of clinical attachment and the loss of alveolar bone (Kinane and Lindhe 2003). It may involve both the primary and secondary dentition and can affect any age. However it is most prevalent in adults. (Consensus Report 1999). The condition is subdivided into localized and generalized types depending on the number of sites affected. Localized being up to 30% of the sites, and generalized being more than 30% of sites (Milward and Chapple, 2003). Chronic periodontitis has the following features:

- The amount of destruction of the periodontium is consistent with the presence of local risk factors
- Subgingival calculus being a frequent finding
- Slow to moderate rate of progression
- Modified by systemic disease
- Modified by other factors such as smoking or stress

The severity of the disease can be described for the entire dentition or for individual tooth or sites.

Aggressive periodontitis comprises a group of rare, severe, rapidly progressive forms of periodontitis often characterized by an early age of onset (less than 30 years old) and a distinctive tendency for cases to aggregate in families. It can occur in both the primary and permanent dentition and is clinically associated with a systemically healthy patient that displays rapid loss of alveolar bone and clinical attachment (Milward and Chapple 2003).

Like chronic periodontitis, aggressive periodontitis is subdivided into localized or generalized conditions, each with its own clinical features. Localized aggressive periodontitis is associated with the onset around puberty and commonly involves the first molar and incisor. There is often an associated elevated serum response to serum antibody. Generalized aggressive periodontitis often affects people under the age of 30 years but can be found in older patients. Its diagnosis is based on the involvement of at least three permanent teeth other than first molars and incisors. Another important feature of this condition is the pronounced and episodic loss of clinical attachment and alveolar bone. This characteristic pattern of bone loss is thought to be common to most forms of periodontitis and is consistant Socranky's proposed "Burst Theory". In this hypothesis, Socransky et al (1984) suggests that periodontal destruction occurs in periodic short episodes and not in a time dependant manner.

Necrotizing periodontal disease is the most severe destructive periodontal disorder caused by plaque bacteria (Holmstrup and Westergaard 2003). It is more prevalent amongst children of developing countries and is often seen in the immunocompromised. The condition is acute and the lesions are characterized by rapid attachment loss and open gingival craters. The clinical course of the disease is characterized by intervening periods of acute ulceration. The diagnosis is usually made clinically and the management involves local debridement and supplementary systemic antibiotics.

2.4. THE AETIOLOGY AND PATHOGENESIS OF PERIODONTAL DISEASE

The exact aetiology of periodontal disease is not known but periodontitis and gingivitis appear to be a continuum of the same inflammatory process (Kinane and Attstrom 2005). Most authors regard a consortium of bacteria (and not a single

species) located within subgingival plaque as the main contributing factor to periodontal destruction (Kilian *et al* 2006; Sanz *et al* 2005; Haffajee and Socransky 2005; Kinane and Attstrom 2005). Others consider the biological structure of plaque (i.e. a biofilm), as a more significant factor than individual bacterial species (Marsh 2005). Recently, DNA studies have recognized more than 700 different species of bacteria located in subgingival plaque (Kilian *et al* 2006). Less than half of these have been cultured. Consequently a major limitation exists in our current understanding of the aetiology of the condition.

Animal and epidemiological studies have associated certain species of bacteria with periodontal disease. In 1996, "The World Workshop in Periodontology" identified several of these organisms and classified them as periodontal pathogens. They include a select group of Gram-negative anaerobic bacteria such as *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia* (previously known as *Bacteroides forsythus)* (Lovegrove 2004; Inagaki *et al* 2006).These and other bacteria were implicated, based on their detection by culture and molecular methods, their proportions in plaque, associated immunological responses and the fact that they possess biochemical properties consistent with pathogenicity (Curtis *et al* 2005). They appear in higher numbers in patients with periodontal disease and have rarely been isolated from healthy subjects (Teanpaisan *et al* 1995; van Winkelhoff *et al* 2002; Lovegrove 2004). Even though these bacteria have consistently been found in periodontal diseased sites, not everyone harbouring these organisms develops periodontal disease (Cullinan *et al* 2003). Therefore, the question of whether the presence of specific microorganisms may be the cause or the consequence of disease, remains unanswered (Socransky et al 1997)

2.5. HOST-MICROBIAL INTERACTION

According to Offenbacher (1996), bacterial and endotoxic exposure are not sufficient enough to cause periodontal disease. It is therefore postulated that a major component of both hard and soft tissue destruction is immune modulated (Berglundh and Donati 2005; Salvi and Lang 2005, Madianos 2005).

According to Salvi and Lang (2005), host-mediated tissue destruction is characterized by the expression of endothelial cell and intercellular adhesion molecules together with the production of host-derived inflammatory mediators. These mediators, although meant to protect the host against bacterial infections, are non-specific, and many of the known bacteria associated with periodontal disease have developed mechanisms that protect themselves against them. Failure of the host to eliminate the offending organism results in the adaptive immune response being activated. This is a more specific mechanism of targeting the infectious agent in an attempt to remove the offending organism. It is characterized by the release of a number of specific inflammatory mediators that include cytokines such as interleukin-1 (IL-1) and tumour necrosis factor (TNF)-α. Both of these have been implicated in tissue destruction and bone resorption and are found in high concentrations in diseased periodontal tissues (Shapira *et al* 2005). These and the other cytokines stimulate the uncontrolled production of further mediators that accelerate the inflammatory process and further contribute to the progression of the disease.

Berglundh and Donati (2005) completed a systematic review of the host response in periodontal. From their literature search, it was shown that irrespective of whether periodontitis was chronic or aggressive, the cellular composition was similar. B cells were always found in higher numbers in diseased sites. It was concluded that sufficient evidence exists to suggest that an autoimmune component might play a significant role in the pathogenesis of periodontitis.

Several risk factors for periodontal disease have been identified. These include smoking, diabetes mellitus and stress. Recently, genetic variability has also been studied as a possible risk factor. This is because genetic variation may influence the hosts' inflammatory process and therefore the susceptibility to disease. However according to Shapira *et al* (2005), there is no evidence for a direct interaction between genetic polymorphism and inflammatory responses in periodontal tissues.
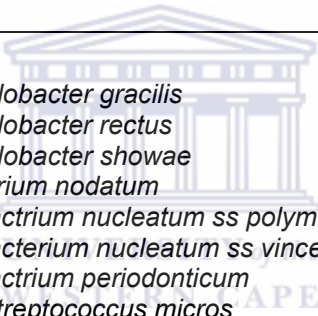
2.6. THE PERIODONTITIS ASSOCIATED BACTERIAL COMPLEXES

In 1998, Socransky and colleagues identified five sets of bacteria that were repeatedly found together in periodontitis. These bacterial complexes were defined based on the analysis of the microbial profiles of over 13,000 plaque samples taken from 185 subjects using DNA probes in checkerboard hybridization assays. They divided the microbiota into groups that appeared to occur together in biofilms of gingival health, gingivitis or periodontitis. These groups of bacteria were categorized and colour coded depending on their sequence of developing on the tooth surface as well as their association with disease severity. The resultant groups/complexes included the yellow, green, purple, orange and red complexes. The constituents of the various complexes are listed in Table 1.

The red complex appears late in bacterial colonization of the periodontal pocket. The appearance of these organisms are preceded by a complex of somewhat less virulent bacteria called the orange complex. The red complex is significant because it represents a portion of the climax community in the biofilm of sites associated with progressing periodontitis. It is thought to be associated with active disease sites and its appearance correlates with the development of clinical signs associated with periodontal disease. Holt and Ebersole (2005) studied the virulence factors associated with the red complex and suggested that *P. gingivalis* benefits from a nutritional interdependence with *T. denticola*. Both these bacteria posses several factors that allow them to be significant in the pathogenesis of periodontal disease. These include;

- they occur concomitantly with clinical signs of periodontal destruction,
- they appear closely linked topologically in the developing biofilm
- they produce a number of outer membrane proteases

How these complexes, as well as how the virulence factors of the individual bacteria contribute to disease progression, remains unclear. However, *P. gingivalis* has consistently been found in higher numbers in patients suffering form periodontal disease. The bacterium also possesses unique factors that contribute to host tissue destruction and its presence is common in patients who are resistant to conventional treatment.

Purple	*Actinomyces odontolyticus* *Veillonella parvula*
Yellow	*Streptococcus gordonii* *Streptococcus intermedius* *Streptococcus mitis* *Streptococcus oralis* *Streptococcus sanguis*
Green	*Actinomyces actinomycetemcomitans* *Capnocytophaga gingivalis* *Capnocytophaga ochracea* *Capnocytophaga sputigena* *Eikeinella corrodens*
Orange	*Campylobacter gracilis* *Campylobacter rectus* *Campylobacter showae* *Eubactrium nodatum* *Fusobactrium nucleatum ss polymorphum* *Fusobacterium nucleatum ss vincentii* *Fusobactrium periodonticum* *Peptostreptococcus micros* *Prevotella intermedia* *Prevotella nigrescens* *Streptococcus constellatus*
Red	*Tannerella forsythia* *Porphyromonis gingivalis* *Treponema denticola*

Table 1: **Bacterial Complexes associated with Periodontal Disease** (Socransky and Haffajee 2005)

2.7. *P. Gingivalis* AS A PERIODONTOPATHOGEN

P. gingivalis is a Gram-negative, anaerobic, non-motile asacchrolytic rod that is commonly found in the biofilm of subgingival plaque associated with periodontal disease. It is a highly virulent organism that is resistant to complement killing and it is often associated with recurrent or persistent periodontal disease. It was identified as a periodontopathogen in 1999 and has been associated with the red complex (Socransky *et al* 1998). Several serotypes of the organism exist. This is based on whether antisera binds to its capsule, fimbria or outer membrane antigens (Table 2).

Strain	Capsular serotype	Fimbrial Serotype	Outer membrane serotype
W50	K1	V	C
W83	K1	V	C
HG184	K2	_	_
A7A1-28(ATCC 52977)	K3	II	B
ATCC 49417	K4	II	_
HG1690	K5	_	_
HG1691	K6	_	_
381	K-neg	I	AD
2561 (ATCC 33277)	K-neg	I	A
FAY19m-1	_	III	_
9-14K1	_	_	_

Table 2: Serotypes of *P. gingivalis* (O'Brien Simpson *et al* 2004)

P. gingivalis has several features that allows it to cause tissue destruction. Holt and Ebersole (2005) listed several of these features which included the production of a variety of cell-surface associated proteolytic enzymes, toxins and hemolysins. These play a significant role in the spread of the organism through tissue and in the evasion of the host defences.

More specifically, the ability of the organism to be an effective periodontopathogen is due to the following identified characteristics;

- *The ability of the bacteria to adhere to various host structures.*

The binding capacity of *P. gingivalis* is a key feature of its virulence. It contributes to the initial attachment of the organism and is important to the coaggregation of multiple species of bacteria within the plaque biofilm. Long, thin, multimetric protein structures known as fimbriae make this binding process possible (Madianos *et al* 2005). These hair-like appendages cover the outer membrane of most strains of *P. gingivalis* and bind to a variety of structures such as epithelial cells, fibroblasts, extracellular matrix proteins and other bacteria. The binding capacity is due to the fact that they stimulate signal transduction and induce cytoskeletal remodeling within cells (Nishihara and Koseki 2004). Besides contributing to co-aggregation, fimbria also allows the bacteria to invade and multiply in host tissues.

P. gingivalis fimbriae are highly antigenic, resulting in high serum IgA and IgG antibody responses in both chronic and aggressive periodontitis (O' Brien Simpson *et al* 2004). The remodelling associated with adhesion also results in the expression of cell surface adhesion molecules which induces the recruitment of leukocytes and other inflammatory mediators to the site (Madianos 2005). These include bone resorbing factors such as interleukin-1α (IL-1α), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α).

- *The P. gingivalis capsule*

A 15nm-thick polysaccharide heteropolymer capsule surrounds the outer membrane of the majority of strains of *P. gingivalis* (O, Brien Simpson *et al* 2005). It is a potent antigen and has the ability to induce a highly invasive spreading ulceration. It provides physical protection to the organism by making it resistant to phagocytosis and the alternative compliment pathway. Long-term exposure to the capsular polysaccharide alters the root surface of the tooth and prevents fibroblast attachment. This may explain why the

bacterium persists in lesions after conventional treatment has been carried out.

- *The ability to produce proteases.*

P. gingivalis produces a number of proteases that degrade host proteins and modulate the host immune response. These macromolecules are non-specific enzymes that contribute nutrients that are necessary for bacterial growth (Holt and Ebersole 2005). They cause collateral damage by degenerating types I and IV collagen (both of which are major components of the periodontal connective tissue) as well as extracellular matrix proteins (e.g. fibrinogen and laminin).

Arginine-gingipain (Rgp) and Lysine-gingipain (Kgp) are trypsin-like proteases that form part of the cysteine protease family. These "gingipains" are found on the outer surface of the organism and are unique in that they have very little structural resemblance to similar studied proteins. As a result they have been classified into a separate protein family i.e. C25 of the cysteine proteases. They are responsible for the release of a large variety of pro-inflammatory factors, including interleukin-six (IL-6), a known bone-resorbing molecule. Arginine-gingipan in particular, induces Ca^{+2} release from infected cells which results in an increased production of IL-6.

- *The production of lipopolysaccharide*

The outer membrane of *P. gingivalis* is made up of lipolysaccharides (LPS). These are amphiphatic molecules that are structurally divided into three parts i.e. the O-polysaccharide (or O-antigen), the core polysaccharide and the lipid A (Madianos *et al* 2005). It induces alveolar bone destruction by stimulating the release of a number of pro-inflammatory cytokines. These include interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon-γ (INF-γ), IL-12, IFN inducible protein-10 (IP-10) and tumour necrosis factor-α (TNF-α) (Holt and Ebersole 2005). Because of this osseodestructive property, it is suggested that in combination with nicotine from smoking, a known risk factor for periodontitis, there is an increased risk for the

potentiation of periodontal disease. Recently it has also been found that LPS induces apoptosis of host cells (Holt and Ebersole 2005).

- *The production of GroEL heat shock protein*

A number of bacteria respond to environmental stresses by synthesizing specific stress proteins, including heat shock proteins. The *P.gingivalis* GroEL heat shock protein is highly antigenic and a number of antibodies directed against this protein has been detected in gingival extracts (O, Brien Simpson *et al* 2004). This protein cross-reacts with heat shock protein 60 (hsp60) and this is thought to induce an autoimmune reaction that is essential in the pathogenesis of arthrosclerosis (Hajishengallis *et al* 2002). However these cross-reactions are non-specific and inconsistent and further research is required to confirm this hypothesis.

P. gingivalis therefore demonstrates multiple components, particularly its cysteine proteases (i.e. gingipains) that alter host cell and tissue functions. This composite of adaptive capabilities make the microorganism a significant component of the climax biofilm linked to periodontal tissue destruction. Data from human epidemiologic studies suggests that *P. gingivalis* may accomplish triggering of disease processes more effectively in conjunction with a microbial consortium of *T. denticola* and *T. forsythia.*

2.8. PLAQUE BIOFILM AND THE ROLE OF THE "INITIAL PLAQUE COLONIZERS" IN THE DEVELOPMENT OF PERIODONTAL DISEASE

Although certain bacteria have been associated with periodontal disease, their role as the sole aetiological agent in the development of the condition has not been established (Marsh 2005). Whether these bacteria are the cause, or are secondary invaders subsequent to the development of periodontal disease, is difficult to determine.

The so-called "periodontopathic" bacteria require specific conditions that favour their growth. These conditions are primed for by other bacteria (known as the early or primary colonizers) that occupy the area prior to the growth of the

periodontopathogens. This process of bacterial colonization is known as "bacterial succession" and is an essential component of plaque formation.

The consequence of this sequential bacterial colonization is the formation of a biofilm that has pathogenic properties which are greater than the sum of its individual components. Because of this, several authors have favoured the hypothesis that biofilm formation and not individual bacterial species, cause periodontal disease (Marsh 2005).

The primary plaque colonizers are essential to plaque formation. This process consists of four distinct stages (Lang *et al* 2003):

- Phase 1 (Molecular adsorption)
- Phase 2 (Bacterial adhesion)
- Phase 3 (Growth of extracellular matrix production and multiplication of adhering bacteria)
- Phase 4 (Sequential adsorption of further bacteria to form a more complex and mature biofilm)
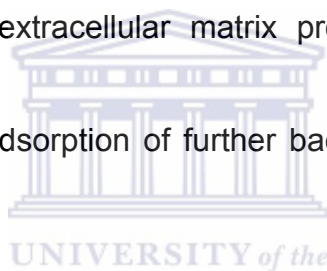
Primary colonization which is the second stage of plaque formation is characterized by bacterial attachment. The bacteria that participate in this process are derived mostly from the Viridans / indifferent group of streptococci which encompass many inconsistent features. They typically inhabit the oropharynx, forming up to 25% of the total cultivable flora and are transmitted via mother to child. Four main groups exist (Samaranayake 1999):

1. *Streptococcus mutans (S. mutans)*
2. *Streptococcus salivarius (S. salivarius)*
3. *Streptococcus milleri (S. milleri)*
4. *Streptococcus oralis (S. oralis)*

These bacteria have unique characteristics that determine the maturation of dental plaque. They use oxygen and lower the reduction-oxygen potential of the environment, which then favours the growth of the more virulent anaerobic species.

Many of the primary colonizers also have the ability to directly modify the characteristics of the local environment. One such species is *S. mutans.*

S. mutans, which is a major aetiological agent in the development of dental caries, has the ability to induce altered gene expression and in so doing, up or down regulate the production of certain proteins (Marsh 2005). This results in an increase in the amount of enzymes available for carbohydrate metabolism as well as the amount of adhesins necessary for subsequent bacterial attachment. This directly affects the ability of subsequent bacteria to colonize the area.

S. mutans also plays an important protective role in biofilm formation. This is characterized by the production of certain proteins, including competence stimulating peptide (CSP). CSP is a specialized protein that's release is stimulated by a local drop in pH. Upon release from the bacteria, it permeates the surrounding area, thereby not only offering protection to the bacterium that has released it, but also to the neighbouring cells.

S. sanguinis, another viridans type streptococcus, is the most prominent organism retrieved from 24 hr old dental plaque. It is a major primary plaque colonizer and has the ability to bind to host tissue. This is likely to play a critical step in bacterial invasion (Haake *et al* 2002). Its binding ability is not only limited to epithelial cells but also allows the bacteria to interact with other colonizing species. This bacterial interaction results in the co-aggregation of a number of different species thereby altering the structure and properties of the plaque biofilm. These types of interactions are thought to be of primary importance in the progressive colonization of the periodontal environment (Haake *et al* 2002). The interactions is that formed between *Streptococcus sanguinis* and *Actinomyces vicosus* is most well known example of this.

The viridans type streptococci therefore play a significant role in the development of the dental plaque. They are the initial colonizers of a process that potentially leads to the development of a biofilm of pathogenic osseodestructive bacteria. It is therefore logical to hypothesize that removal or reduction of the primary plaque colonizers would result in failure or inhibition of a pathogenic biofilm formation. This might have

potential therapeutic applications for the prevention or management of periodontal disease.

2.9. THE MANAGEMENT OF PERIODONTAL DISEASE

The treatment of the infectious component of periodontal disease, and the replacement of damaged or lost periodontal tissues are achieved using different approaches. Mechanical therapies, such as scaling, root planing and surgery, are aimed at improving the clinical condition by effectively lowering the microbial load (and therefore affecting the bioflim) either by physically removing the plaque or by radically altering the subgingival habitat. Antimicrobial approaches that use systemically and locally administered antibiotics target subgingival species residing in the plaque biofilm as well as those species that reside in the adjacent epithelial tissues lining the periodontal pocket.

2.10. THE USE OF TOOTHPASTES IN THE MANAGEMENT OF PERIODONTAL DISEASE

The ability of toothpaste to inhibit plaque formation with subsequent benefit to gingival health was realized more than 20 years ago with the introduction of chlohexidine as an oral antiseptic (Sheen *et al* 2001). Nevertheless most of the chemicals which have demonstrated plaque inhibition in laboratory studies, have not been incorporated into commercially available products. Those that are incorporated into toothpastes and mouthrinses include;

- Cationic antiseptics such as chlorhexidine, hexeditine and cetyl pyrdimium chloride
- Metal salts such as zinc citrate and stannous fluoride
- Essential oils and phenolic derivatives
- Enzymes
- Herbal extracts

Because of its substantivity, toothpastes provide an ideal mechanism for the delivery of an antimicrobial substance to the oral cavity. Certain toothpaste manufactures

have added antiseptics / antimicrobials to their products (Arweiler *et al* 2002). The most common of these agents is triclosan. It has been used since the 1960s in various medicinal products including topical antiseptics, soaps, hand washes and as therapeutic baths for methicillin-resistant-*Staphylococcuss aureus* patients. Triclosan is a non-ionic chemical that has a broad spectrum of antimicrobial activity. It is effective against a wide variety of Gram-negative and Gram-positive bacteria, mycobacteria, spores, strictly anaerobic bacteria and *Candida* (Sheen *et al* 2001). Its antibacterial mechanism of action is derived form the fact that it prevents amino acid uptake from the bacterial cytoplasmic membrane. As a result, membrane disruption occurs followed by leaking of the cellular contents and subsequent cell death (Sheen *et al* 2001).

Triclosan also possesses anti-inflammatory properties (Sheen *et al* 2001). This is due to its ability to inhibit the cycloxygenase/lipoxygenase pathway in arachadonic acid metabolism (Sheen *et al* 2001).

The inclusion of triclosan into toothpastes has been studied extensively. When delivered in this way, the agent seems to bind to oral mucosal and tooth surfaces, and is particularly well retained in plaque (Sheen *et al* 2001). In randomized clinical trials, triclosan based toothpaste was shown to reduce plaque associated with gingival inflammation (Muller 2006). When tested for its clinical benefits in the management of periodontitis, it was shown that the use of a triclosan/copolymer dentifrice was effective in slowing down the progression of periodontal disease (Cullinan *et al* 2003). The Colgate formulation of triclosan and copolymer (*Colgate Total*®) has also been shown to reduce the onset of periodontitis in susceptible adolescents and to reduce the progression of periodontitis in previously "at risk" adults when compared to fluoride only toothpastes (Davies 2004).

Metal salts such as zinc citrate have been added to toothpastes to enhance the antimicrobial activity of these antimicrobial agents (Davies 2004, Moron *et al* 2001).

2.11. THE THERAPEUTIC EFFECT OF HERBS AND SPICES

A herb can be defined as the leafy portion of a plant whereas any other part of the plant, often dried, is referred to as a spice (Hemphill and Cobiac 2006). Both have

been used for thousands of years for culinary use, medicinal purposes and preservatives (Hemphill and Cobiac 2006). It is reported that the Sumerians were using thyme as far back as 5000BCE for its health properties (Hemphill and Cobiac 2006). The Assyrians also developed knowledge about the health benefit of herbs, referring to the use of juniper, saffron and thyme around the time of 1000BCE. The trend of using herbs for their therapeutic properties is currently still practiced and by 1998 more than a third of Americans were using herbal based medication, spending up to $3.5 billion dollars annually on these products (Lee *et al* 2004).

O'Hara (1998) reviewed 12 of the most commonly used medicinal herbs and showed that they had a wide range of pharmacological activity. Patch and Sullivan (2006) showed that garlic had a significant therapeutic effect on patients suffering from cardiovascular disease. From a meta-analysis level III evidence (Australian National Health and Medical Council levels of evidence) was presented showing that the consumption of a half to one clove of garlic (or its equivalent), had the ability to reduce cholesterol levels by up to 9%. It was also shown that garlic had a significant anti-clotting ability.

There is no conclusive evidence indicating that herbs and spices have an anticarcinogenic effect in humans, however there is strong evidence from rodent studies that certain herbs and spices have a chemopreventative effect against the early initiating stages of cancer (Fenech 2006). Rosemary, basil, mint and lemon grass are known to have anticarcinogenic effects in animal models and tumeric has been shown to prevent cancers of the oral cavity, forestomach, liver and colon in mice (Fenech 2006). The cancer protective properties of these herbs are thought to be related to their potent anti-inflammatory qualities (Fenech 2006). It is thought that the water soluble phenolic acids and falvinoids such as caffeic acid and quercetin are significant in this regard since they scavenge for reactive oxygen species that may lead to a state of oxidative stress (Fenech 2006). This oxidative stress has been linked to an increased cancer risk.

Herbs have been studied for their effect on mental health. The use of herbal treatments of anxiety is probably the most common example of a herbal influence on mental health. *Passiflora incarnate,* or passion flower, has been approved for use as a sedative by the German Commission E (an expert committee commissioned by the

German Government in 1978 to evaluate herbal drugs and preparations from medicinal plants). Another herb *Valeriana officianalis* has also been shown to have a sedative effect in humans (Roodenrys 2006). A review of the potential chemical pathway for the action of *Valeriana officianalis* shows that it interacts with the GABA systems in the brain. More recently there has been increased interest in the potential beneficial effects of the Chinese herb *Ginkgo Biloba* on cognitive processes. There exists level I evidence to support the claim that ginkgo can ameliorate cognitive decline in dementia and level II evidence exists showing that the herb can improve some aspects of memory function in human adults (Roodenrys 2006). This cognitive nueroprotective effect is thought to be related to its antioxidant effect. Furthermore extracts from ginkgo are known to affect cerebral circulation, activity in the cholinergic system, and to have anti-oxidant properties.

Herbs and spices also possess antimicrobial properties (Lai *et al* 2004; De *et al* 1999; Arora and Kaur 1999; Harris *et al* 2001). Lai *et al* (2004) showed that commonly used herbs and spices such as garlic, black cumin, cloves, cinnamon, thyme, bay leaves and mustard all possess antimicrobial properties that may be used therapeutically. In certain studies the samples tested showed greater efficacy than synthetic antibiotics when tested against antibiotic resistant organisms (Arora and Kaur 1999). Others such as De *et al* (1999) and Kalemba and Kinicka (2003) have confirmed the antimicrobial properties of several of these plant based products.

2.12. THE EFFECT OF HERBS ON ORAL PATHOGENS

The antimicrobial effect of herbs and spices are well known and several researchers have tested their effectiveness against a variety of oral pathogens (Lee *et al* 2004; Kaim *et al* 1998; Koo *et al* 2000, Kagermeirer-Callaway *et al* 2000, Pistorius *et al* 2003, Sastravaha *et al* 2003). Eucalyptus leaf extract (*Macrocarpals*), and essential oils (derived from plants) such as manuka oil, tea tree oil, peppermint oil and sage oil have demonstrated significant antibacterial activity against periodontopathic and cariogenic bacteria (Nagata *et al* 2006, Takarada *et al* 2004). Tea tree oil in a concentration of greater than 0.5% has antimicrobial activity against a variety of oral bacteria including *Actinomyces spp., Lactobacillus spp., Streptococcus mitis, Streptococcus sanguinis, Prevotella spp, Porphyromonas, Veillonella* and *Fusobacterium* (Hammer *et al* 2003). In 2005, Filoche *et al* showed that there was a

4 to 10 fold reduction in the amount of chlorhexidine used to inhibit a biofilm culture of *Streptococcus mutans* and *Lactobacillus* when it was combined with essential oils such as cinnamon, tea-tree (*Melaleuca alternifola*), manuka (*Leptospermum scoparium*), *Leptospermum morrisonii*, arnica, eucalyptus and grapefruit.

In 1998, Van der Weijden *et al* showed that a herbal mouthrinse containing various abstracts of juniper, nettle and yarrow, had antimicrobial activity against *P. gingivalis*. Bakri and Douglas (2005), tested the effectiveness of garlic (Allium sativum) against *P. gingivalis* and *S. mutans.* They showed that garlic extract killed most of the organisms tested and additionally had the ability to inhibit the trypsin-like and protease activity of *P.gingivalis* by up to 94%.

Propalis, a natural composite balsam produced by honey bees, has shown significant antimicrobial activity against a variety of bacteria, fungi and parasites (Gebara *et al* 2002). When tested against several oral pathogens, Koo *et al* (2000) showed that propalis had considerable antimicrobial activity against all the pathogens tested including *Candida albicans*. Gebara and colleagues (2002) also showed that propalis had significant antimicrobial activity against bacteria such as *P. gingivalis, P. intermedia, P. melaninogenica, A.a, Capnocytophaga* and *F. nucleatum*. Feres *et al* (2005) tested the effectiveness of several herbs and plants against saliva obtained from patients with and without periodontal disease. He concluded that propolis showed significant antimicrobial properties in samples from both periodontally healthy and diseased subjects and suggested that this substance may be used therapeutically in the future to inhibit oral microbial growth.

Groppo *et al* (2002) compared the antimicrobial activity of tea tree oil, garlic, and chlorhexidine solutions against oral microorganisms and concluded that garlic and tea tree oil might be used as an alternative to chlorhexidine.

2.13. HERBAL TOOTHPASTES

Lee *et al* (2004) reviewed the anti-microbial activity of 14 herbal based toothpastes against certain oral pathogens. The majority of the products tested, displayed antimicrobial activity; however the study was only limited to 4 pathogens i.e.

Streptococcus mutans, Streptococcus sanguinis, Actinomyces vicosus and *Candida albicans*.

Netuschil *et al* (2005) tested the efficacy of several toothpastes on plaque and gingivitis. Their study was conducted over a 6 month period, and Bleeding on Probing (BOP) was used as the endpoint. Significant reductions in both the plaque index and BOP were noted for Meliamint$^®$ (WALA Heilmittel, Bad Boll), a fluoride-free herbal based toothpaste that has peppermint, clove, chamomomile, mastic gum, propalis wax and chlorophyll as its main ingredients. When compared to Colgate Total$^®$ (Colgate-Palmolive, Hamburg), (a toothpaste that contains a known antmicrobial, triclosan), Meliamint$^®$ (WALA Heilmittel, Bad Boll), showed a consistently better reduction in the Gingival Index over the 6 month period (Netuschil *et al* 2005). In a recent controlled clinical trial it was shown that a herbal toothpaste had the same clinical benefit effect as a conventional toothpaste containing triclosan and fluoride (Ozaki *et al* 2006). Herbal toothpastes have also been associated with side effects, the most common reported being plasma cell gingivitis (Anil 2007).

Several herbal based dentifrices are now available in South Africa. Many are found in retail stores and are available without prescription. Some of the herbs they contain include Myrrh, Sage, Eucalyptus, Tea tree oil, Mallow, Matricariae and Propalis and have the characteristics listed in Table 3.

Herb	Indication
Myrrh (*Commiphora erythrae*)	*Indicated as a topical treatment of mild inflammatory conditions of the mucous membranes, especially that of the mouth and throat*
Sage (*Salviae trilobae folium*)	*It is often indicated as an antiphlogistic as well as for inflammation of the mouth and throat*
Eucalyptus (*Eucalyptus Globulus*)	*The herb is indicated for bronchitis and inflammation of the throat*
Tea Tree Oil (*Melaleuca leucadendra*)	*The oil possesses antibacterial, anti-inflammatory and anodyne properties. It is indicated for a number of conditions including;* • *Coryza* • *Influenza* • *Cough* • *Asthma* • *Toothache* • *Burns*
Matricariae (*Matricariae recutita*)	*It acts as a spasmolytic, antibacterial and antifungal. It is indicated for GIT complaints such as gastritis, enteriis and colitis. It is also used to manage inflammation of the mucous membranes of the mouth.*

Table 3: Herbs routinely used in herbal medicine (Bisset 2001)

CHAPTER 3
AIMS AND OBJECTIVES

3.1. AIM

The aim of the study was to determine whether there was any significant difference in the antimicrobial activity of 4 locally available, herbal toothpastes against cultures of 3 specific primary plaque colonizers (*Streptococcus mutans* (NCTC 10920)*, Streptococcus sanguinis* (NCTC 1940) and an *α-heamolyitic streptococcus termed VS-1*).

3.2. OBJECTIVES OF THIS STUDY ARE:

- To determine and compare the size of inhibition produced by different toothpastes against *Streptococcus mutans*

- To determine and compare the size of inhibition produced by different toothpastes against *Streptococcus sanguinis*

- To determine and compare the size of inhibition produced by different toothpastes against   *VS-1*

3.3. NULL HYPOTHESES

There is no significant difference in the antimicrobial activity of the four herbal toothpastes tested

CHAPTER 4
MATERIALS AND METHODS

4.1.   STUDY DESIGN:

This study was a laboratory based experiment that used *in-vitro* diffusion to assess the antimicrobial potential of various herb-containing toothpastes.

4.2.   SAMPLE SIZE

A total of 48 cultures were produced. They were subdivided as follows;

Fifteen standardized samples of each toothpaste were set up against each species of bacteria resulting in three distinct groups. This equates to a total of 45 test cultures. The three remaining cultures contained positive and negative controls for each of the three bacterial species.

4.3.   MATERIALS:

1. TOOTHPASTES

Four different over-the-counter, locally available herb-containing toothpastes, were used (Fig 1). These were purchased from a retail store in Cape Town, South Africa. Batch numbers and ingredients are listed as they appear on the container. They included:

A) COLGATE HERBAL ® 100ML (COLGATE-PALMOLIVE COMPANY, SOUTH AFRICA).

Batch number: 7035BR113F

Active Ingredient: Sodium Monoflourophosphate 1.1%

Ingredients: Aqua, Calcium Carbonate, Sorbitol, Sodium Lauryl Sulphate, Sodium Monoflourophosphate, Cellulose gum, Sodium silicate, Aroma, Sodium Bicarbonate, Sodium Saccharin, Eucalyptus Globulus Leaf Oil,

Xanthan Gum, Methylparaben, Commiphora Myrrh Extract, Chamomilla Recutita (Matricaria) Extract, Propylparaben, Metaleuca Alternifolia (Tea Tree) Leaf Oil, Salvia Officinalis (Sage) Oil, Eugenol, CI 74260

B) NATURE FRESH HERBAL (ANTI-MICROBIAL) ® 100ML (NATURE FRESH, CONSTANTIA, SOUTH AFRICA)

Batch number: None stated

Ingredients: Olive leaf, Aloe Ferox bitters, Cloves, Tea Tree, Mint and Aniseed oil with calcium, magnesium, sea salt, zinc and baking soda

C) DENTAZYME HERBAL® 100ML (AMKA PRODUCTS, LONDON)

Batch number: 326664

Ingredients: Sorbital, Hydrated Silica, Water (Aqua), Sodium Lauryl Sulphate, Peg-32, Flavour, Sodium Flouride, Cellulose Gum, Sodium saccharine, Methylparaben, Tea Tree (Melaleuca Alternifolia) leaf oil, CI 19140, CI 42090, Eugenol, Limonene, Linalool

D) AQUAFRESH HERBAL ® 100ML (GLAXOSMITHKLINE, SOUTH AFRICA)

Batch number: 667324

Ingredients: Aqua, Hydrated Silica, Sorbitol, Glycerin, Peg-6, Sodium lauryl sulphate, Aroma, Carrageenan, Xanthan Gum, Sodium Flouride, Sodium Saccharin, Titanium Oxide, Eucalyptus Globulus, Mentha Arvensis, Salvia Officinalis, Anthemis nobilis, CI 77492, CI 74260, CI 73360

E) COLGATE TOTAL ® 100ML (COLGATE-PALMOLIVE, SOUTH AFRICA)

Batch number: 7145ZA10

Active ingredients: Sodium fluoride 0.24%, Triclosan 0.3%

Ingredients: Aqua, Glycerin, Hydrated Silica, Sorbitol, PVM/MA Coplymer, Sodium Lauryl Sulphate, Flavour, Cellulose Gum, Sodium Hydroxide, Sodium Saccharin, Carrageenan (Chondrus Crispus), Sodium Flouride, Triclosan, Limonene, CI 77891

Fig 1: Toothpastes used in the study

2. **MICROORGANISMS**

Three different groups of bacteria were used;

- *Streptococcus mutans* (National Collection of Type Cultures, strain number 10920)

- *Streptococcus sanguinis* (National Collection of Type Cultures, strain number 10904)

- A non specific α-heamolyitic viridans streptococci termed *(VS1)* ＊

The bacteria used in this study were obtained from a standard culture stock that was stored at The Department of Medical Biosciences, University of the Western Cape.

＊ – **The "non specific α-heamolyitic viridans-type streptococci" used in this study is an**
oral bacteria that has not officially been named yet. The term *VS-1* was purely
used to distinguish it from the other species of bacteria in the study and is not
part of the official nomenclature

3) CULTURE MEDIUM

Columbia Blood Agar Base (CM0331) ® (Oxoid, UK) was used as the culture medium for all the bacterial species used in this study. This a multipurpose growth medium used for the cultivation of fastidious organisms (Website). It consists of special peptone 23.0gm/l, starch 1.0gm/l, sodium chloride 5.0gm/l, agar 10gm/l and has a a pH of 7.3 ± 0.2.

4) LABORATORY EQUIPMENT
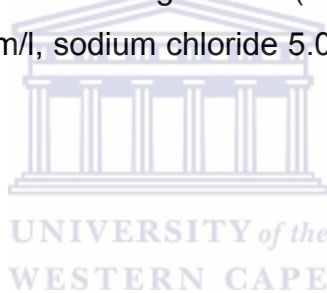
The following laboratory equipment were used (Fig 2):

1. Forty-eight standard 90mm diameter sterile plastic Petri dishes

2. Six standard sterile laboratory test tubes

3. Ten standard McFarland turbidity tubes

4. Sterile loop

5. Sterile forceps

6. Bunsen burner and lighter

7. One hundred sterile 9mm antibiotic disks (Munktell, Germany)

8. One vortex mixer (Heidolph, Germany)

9. One Pipetman®P calibrated pipette (Gilson, France)

The study was carried out in The Department of Medical Biosciences, The University of Western Cape, Cape Town, South Africa, as per prior arrangement.

Fig 2: Laboratory equipment used in the study

4.4. METHODS

The following sequence outlines the experiment that was conducted;

1) PREPARATION OF AGAR PLATES

- 39 Grams of the Columbia Blood Agar Base (CM0331) [®] (Oxoid, UK) was mixed with 1 litre of distilled water

- This was brought to boil in a standard microwave oven

- This mixture was then autoclaved at 120ºC for 15 minutes

- The autoclaved mixture was then left to cool to 50ºC

- 5% defibrinated horse blood was added

- 25ml of this agar was then poured into each Petri dish and allowed to cool to room temperature

2) PREPARATION OF TEST SPECIMENS

Standard 90mm diameter sterile Petri dishes were used to culture each organism. The Mcfarland Scale was used to ensure that each test organism had a similar concentration. This measurement uses a scale that is numbered from 1 to 10 and represents specific concentrations of bacteria/ml. Although not accurate, it is designed to be used as an estimate of the concentration of bacteria used during experimentation (Table 4).

McFarland Scale	No. Bacteria ($\times 10^6$/ml)
1	300
2	600
3	900
4	1200
5	1500

Table-4: McFarland Turbidity Tubes

The McFarland Scale uses standardized tubes that are labelled 1 through 10 and filled with suspensions of Barium salts. Each tube approximates the turbidity of bacterial solutions corresponding to the McFarland Scale number. The advantage of this method is that no incubation time or specialized equipment is needed to estimate bacterial numbers.

Based on a previous pilot study, McFarland Standard 3 was used as the concentration of each organism that was tested in this study. This corresponds to 900×10^6/ml of bacteria.

For each type of organism used (i.e. *Streptococcus mutans, Streptococcus sanguinis, VS-1*), the following sequence was followed:

- **A flame-sterilized loop was used to transfer the bacteria grown on an agar plate to a sterile test tube filled with distilled water**

- **The bacteria was dispersed using a vortex mixer (Hedolph, Germany) for 30 seconds**

- **The tube mixture was then compared against McFarland tube 3 to ensure that the turbidity matched**

- **If the concentration was too low, more bacteria were added until the turbidity approximated that of McFarland tube 3**

- **Using a Pipetman®P pipette (Gilson Inc, France), 0.5ml of the test bacteria was transferred from the test tube to each 90mm diameter agar containing Petri dish**

- **This solution was spread evenly over the agar using a sterile glass spreader so as to ensure that an even lawn of bacterial growth would occur**

- **The inoculated plates were then left for 3 to 5min to allow the moisture from the inoculum to be absorbed into the medium**
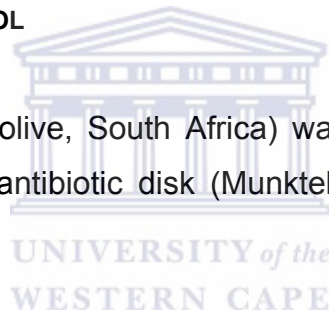
3) PREPARATION OF TEST TOOTHPASTES

All the antibiotic disks (Munktell, Germany) used in the experiment were autoclaved at 121ºC for 15 minutes before being used. For each test toothpaste, and including

the positive control (Colgate Total® (Colgate-Palmolive, South Africa)), the following sequence of steps took place:

- **The toothpaste was squeezed from a freshly opened toothpaste tube into a sterile 90mm Petri dish**

- **Using a flame-sterilized forceps, 9mm sterile antibiotic disks were mixed and soaked into the test toothpaste.**

- **These disks were then removed and placed into another sterile Petri dish.**

- **The second dish which now contained the toothpaste impregnated disks, was labelled with a permanent marker according to the toothpaste used**

- **This labelled dish was then allowed to dry at 37ºC in a temperature controlled room**

4) PREPARATION OF THE CONTROL

Colgate Total® (Colgate-Palmolive, South Africa) was used as the positive control and a standard 9mm sterile antibiotic disk (Munktell, Germany) was used as the negative control.

- The positive control was prepared using the same sequence of steps outlined in the preparation of the test toothpastes.

- The negative control was autoclaved at 121ºC for 15 minutes and stored in a sterile Petri dish at room temperature. These disks had no contact with any of the test toothpastes

## 5) PLACEMENT OF THE TEST TOOTHPASTES ONTO THE PREPARED CULTURES

For each test toothpaste and including the positive control, the following sequence of steps took place:

- Each inoculated plate was visually examined to ensure that the surface was dry and free of moisture

- A toothpaste impregnated test disk was placed onto the surface of the agar using a sterile forceps

- The disks were tapped gently with the forceps to ensure that each disk was in complete contact with the surface of the agar

- All disks were placed approximately the same distance from the edge of the plate and from each other

- Four different test toothpastes were placed onto each test plate

6) PLACEMENT OF THE CONTROLS ONTO THE TEST CULTURE

Three culture plates were used for the controls, one for each bacterial species tested. Two positive controls that consisted of Colgate Total® (Colgate-Palmolive, South Africa) and two negative controls that consisted of 9mm sterile antibiotic disks (Munktell, Germany) were placed onto each of the three culture plates. This was carried out in the same way as the process outlined in the placement of the test toothpastes onto the cultures.
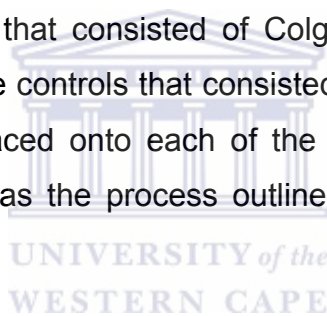
7) INCUBATION

All test plates including the control plates were placed into microaerophilic pots and incubated at 37ºC in a temperature controlled room for 24 hours.

4.5. DATA COLLECTION

After incubation for 24 hours, zones of inhibition were measured around the test disks. This time period was chosen because previous studies have indicated that no significant increase of zone size is observable after 24 hours (Lee 2004). The inhibition zones are circular bacteria-free areas around the test disk on the culture plate. They indicate areas where no bacterial growth has taken place. Viewed against a black background and illuminated with reflected light, each diameter of the zone was measured in millimetres using a venire calliper to the nearest whole millimetre. All measurements were conducted by the same person.

4.6. DATA PROCESSING AND ANALYSIS:

The results were compared and analyzed using SPSS13 for Windows$^{®}$.

For each toothpaste type, the mean and standard deviation of the diameters of zones of inhibition were calculated. The following statistical tests were applied;

- one-way analysis of variance, or ANOVA and

- a multiple comparison Tukey's test to determine the significance of differences among the means at the significance level of *p = 0.5.*

4.7. ETHICS:

The study was purely laboratory based and required no human tissue or sampling. Any significant results will be submitted for publication in a relevant dental or medical journal.

CHAPTER 5
RESULTS

5.1. DESCRIPTIVE ANALYSIS

After 24 hours of incubation, all four test toothpastes displayed observable zones of inhibition (Table 5). The positive control, Colgate Total® (Colgate-Palmolive, South Africa) produced inhibition zones for all three microorganisms cultured (*Strep mutans, Strep sanguinis,* and *VS-1*) (Fig 3), whereas the negative control, a sterile antibiotic disk (Munktell, Germany), produced no observable inhibitory effect for any of the bacteria (Fig 4).
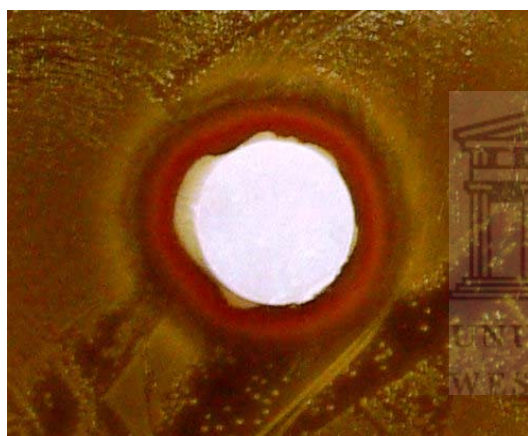
Fig 3: Colgate Total®   **Fig 4:** Sterile Disk

Only three of the test toothpastes i.e. Colgate Herbal® (Colgate-Palmolive Company, South Africa), Aquafresh Herbal® (GlaxoSmithKline, South Africa)   and Dentazyme Herbal® (Amka Products, London), produced zones of inhibition against all the microorganisms (*S.mutans, S. sanguinis* and *VS-1*)*.* Nature Fresh (antimicrobial)® (Nature Fresh, Constantia, South Africa) only showed inhibitory zones for *viridans Streptococci.*

Toothpastes and Controls	S. mutans	S. sanguinis	VS-1
Sterile Disk (Negative Control)	2	2	2
Colgate Total (Positive Control)	1	1	1
Nature Fresh	2	2	1
Colgate Herbal	1	1	1
Dentazyme Herbal	1	1	1
Aquafresh Herbal	1	1	1
1 = inhibition observed			
2 = no inhibition observed			

Table 5: The presence of observable inhibition for the various toothpastes

5.2. STATISTICAL ANALYSIS

All the toothpastes produced different sized inhibition zones (Fig 5). Dentazyme Herbal[®] showed the largest zone of inhibition and Nature Fresh (antimicrobial)[®], the smallest.
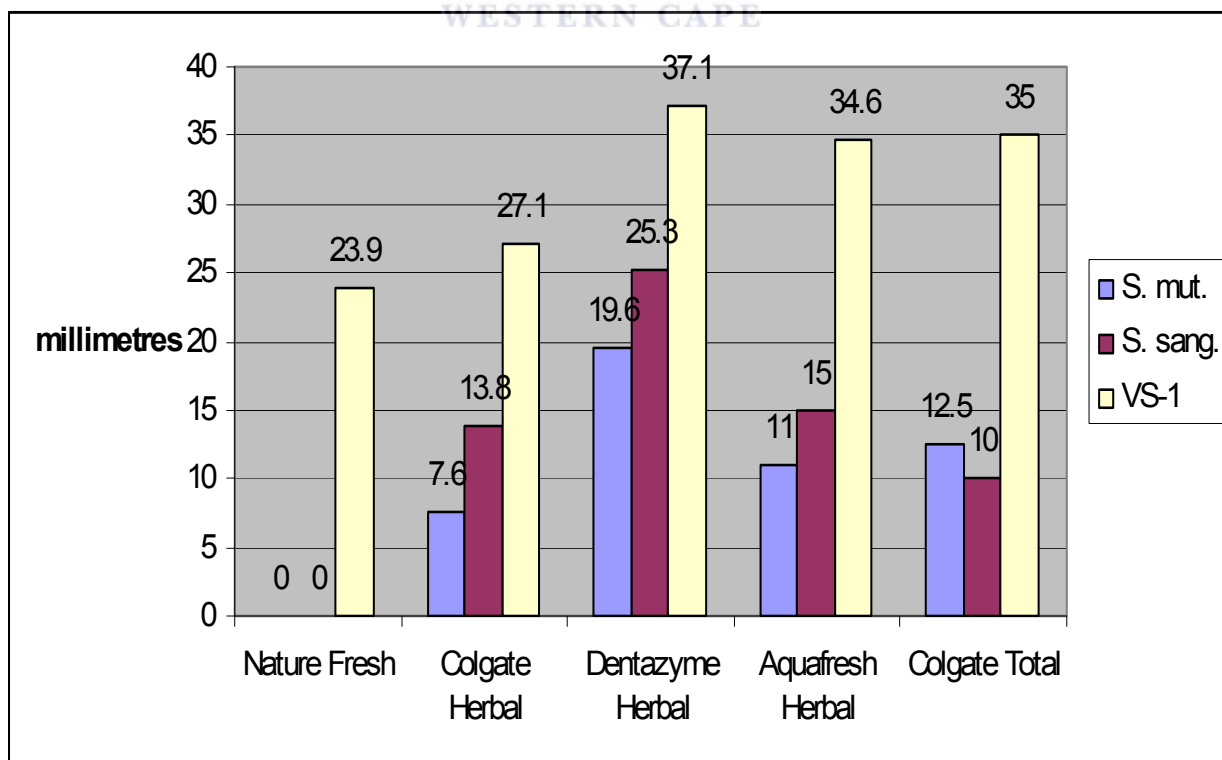
Fig 5 : Mean diameter (in millimetres) of zones of inhibition

Analysis of variance (ANOVA), showed a significant difference in sizes of these zones when the various toothpastes were compared to each other (p<0.05) (Table 6).

Bacteria	Groups	Significance
S. mutans	Between groups	p= 0.000
	Within groups	p= 0.000
S. sanguinis	Between groups	p= 0.000
	Within groups	p= 0.000
VS-1	Between groups	p= 0.000
	Within groups	p= 0.000

Table 6: Analysis Of Variance

Tukey's multiple comparison test was used to determine which of the zone sizes produced by the various toothpastes were statistically different from each other for each of the cultures tested. The following results were obtained:

For *S. mutans:*

	Dentazyme Herbal	Aquafresh Herbal	Nature Fresh	Colgate Herbal	Colgate Total (Control)
Dentazyme Herbal		p = 0.000	p = 0.000	p = 0.000	p = 0.000
Aquafresh Herbal			p = 0.000	p = 0.038	**p = 0.671**
Nature Fresh				p = 0.000	p = 0.000
Colgate Herbal					p = 0.001

Table 7: Tukeys test for *S. mutans*

The differences in inhibition zones produced by the various toothpastes were statically significant except for when Aquafresh Herbal[®] was compared to Colgate Total[®]. Dentazyme Herbal[®] was significantly stronger than any of the other

toothpastes (including the control, Colgate Total®) in its ability to inhibit the growth of *S. mutans.*

For *S. sanguinis:*

	Dentazyme Herbal	Aquafresh Herbal	Nature Fresh	Colgate Herbal	Colgate Total
Dentazyme Herbal		P = 0.000	p = 0.000	p = 0.000	p = 0.000
Aquafresh Herbal			p = 0.000	**p = 0.972**	**p = 0.062**
Nature Fresh				p = 0.000	p = 0.000
Colgate Herbal					**p = 0.232**

Table 8: Tukeys test for *S. sanguinis*

For *S. sanguinis,* the zones of inhibition produced by Aquafresh Herbal®, Colgate Herbal® and Colgate Total®, were not significantly different from each other. Dentazyme Herbal® was significantly stronger than any of the other toothpastes in inhibiting the growth of *S. sanguinis.*

For *VS-1:*

	Dentazyme Herbal	Aquafresh Herbal	Nature Fresh	Colgate Herbal	Colgate Total
Dentazyme Herbal		**p = 0.785**	p = 0.000	p = 0.000	**p = 0.869**
Aquafresh Herbal			p = 0.000	0.006	**p = 1.000**
Nature Fresh				0.639	p = 0.000
Colgate Herbal					p = 0.004

Table 9: Tukeys test for VS-1

Dentazyme Herbal®, Aquafresh Herbal® and Colgate Total® showed no significant difference between the toothpastes in their ability to inhibit the growth of *VS-1.*

Nature Fresh® and Colgate Herbal® were also not significantly different from each other in their ability to inhibit its growth.

CHAPTER 6
DISCUSSION

Periodontal disease is a bacterial related infection that results in the progressive destruction of oral soft and hard tissue (Beck and Arbes 2002). The clinical management of the condition is directed towards the suppression or elimination of the offending bacteria. Not only are these specific bacteria being targeted, but the nature of their growth (a biofilm) has recently also been established as a potential therapeutic target (Marsh 2005). As such, the use of local and systemic antibiotics to treat periodontal disease is increasing and with it, a proportional increase in the amount of resistant oral pathogens is being reported. The safe elimination of plaque associated bacteria without the risk of developing antibiotic resistant strains may therefore offer some clinical benefit in the prevention and management of periodontal disease.

Recently triclosan has been added to toothpaste as an antimicrobial agent (Cullinan *et al* 2003). However because unlike chlorhexidine, it has no ionic attachment to the tooth surface, it requires the presence of a copolymer to adhere to the tissue. This disadvantage together with the emergence of triclosan-resistant bacteria has encouraged researchers to search for alternative antimicrobial sources (Davies 2004).

One of these sources includes the wide variety of herbs and herbal derived products that are currently marketed for therapeutic use. Many of these herbs have established antibiotic properties that have yielded positive results against a host of microrganisms including a variety of oral bacteria.

Relatively few studies in the literature have directly compared the effect of herbal based toothpastes on plaque associated bacteria. Most previous studies have tested individual herbal derivatives on oral pathogens or have used herbal based mouthrinses as their test material (Koo *et al* 2000, Groppo *et al* 2002). The substantivity of toothpaste makes it an ideal mechanism for delivering antimicrobials to the oral cavity, and was therefore chosen as the dentifrice of choice for this study.

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The aim of the present study was to determine whether there was any significant difference in the antimicrobial activity produced by certain herbal based toothpastes. *S. mutans, S. sanguinis* and a non-specific species of α-heamolyitic viridans streptococci (*VS-1*) were selected as the test bacteria because they have been implicated in primary plaque colonization. These bacteria are the pioneer species in plaque formation and their presence alters the structure of the plaque to favour the growth of anaerobic organisms. *S. mutans* modifies gene expression and protein formation whereas *S. sanguinis* is essential for co-aggregation and epithelial cell attachment. Elimination or reduction of these species may therefore prevent the formation of an environment that promotes the growth of the so called "periodontopathogens".

The *in-vitro* diffusion method was used because it displayed a relatively direct means of measuring the antimicrobial strength of each product i.e. the product producing the largest zone of inhibition, had the strongest antimicrobial properties.

The results of the present study indicate that all of the test toothpastes have some degree of antimicrobial ability. Dentazyme Herbal[®] produced the largest zones of inhibition and therefore had the strongest antimicrobial effect, of all the toothpastes tested. This was irrespective of the bacterial culture that was used. It routinely produced stronger inhibition than Colgate Total[®], a toothpaste that contained a known antimicrobial ingredient. The antibacterial properties of Dentazyme Herbal[®] may be attributed to several factors including two significant ingredients that it contains .i.e. tea tree oil and eugenol. Dentazyme Herbal[®] and Colgate Herbal[®] are the only products used in the study that contain both these constituents, both of which have documented anti-bacterial activity. When looking at the results however, it is clearly seen that Dentazyme Herbal[®] has significantly stronger antimicrobial potential as compared to Colgate Herbal[®]. Therefore the antimicrobial effect cannot only be accounted for by the presence of these two ingredients and other contributing factors must be taken into account.

Aquafresh Herbal[®] was as strong as Colgate Total[®] in inhibiting the growth of *S. mutans, S. sanguinis* and *VS-1*. Colgate Herbal[®] however, had a minimal inhibitory effect against most of the bacteria tested, producing inhibition zones that were

consistently smaller than that produced by Dentazyme Herbal®, Aquafresh Herbal® and Colgate Total®.

Nature Fresh® is the only non fluoride containing toothpaste that was used in the study. The product promotes itself as being purely herbal and strongly antimicrobial. In the present study, it was significantly weaker than most of the other toothpastes in its ability to inhibit bacterial growth. It displayed no inhibitory effect for both *S.mutans* and *S. sanguinis* and only produced limited ability to inhibit the growth of *VS-1.* Although limitations are associated with the present study, the effectiveness of Nature Fresh® as a potential therapeutic agent in the prevention or management of periodontal disease therefore needs to be questioned. The toothpaste was also 500% more expensive than any of the other toothpastes used.

Colgate Total® consistently showed the ability to inhibit the growth of all of the bacteria tested. This was similar to the results published by previous studies (Lee e*t al* 2004). This inhibition was likely to be as a consequence of the inclusion of triclosan in the toothpaste. Triclosan had previously shown excellent results in its antimicrobial potential, being effective in both laboratory and clinical based research (Davies 2004). Colgate Total® is currently one of the few toothpastes that contains triclosan and it was therefore chosen as the control for this experiment. The fact that Dentazyme Herbal® displayed a stronger antimicrobial effect when compared to Colgate Total® was an unexpected finding. This result however has therapeutic consequences since previous studies have shown that incorporating antimicrobials into toothpastes may have some clinical benefit in the management of periodontal disease (Cullinan *et al* 2003).

CHAPTER 7
LIMITATIONS

There are some important limitations to the present study.

Although the study shows significant results for the toothpastes tested, these results need to be interpreted with caution.

One such factor is the presence of fluoride, a chemical with known antimicrobial activity. Except Nature Fresh (antimicrobial)®, all the toothpastes tested, contained fluoride. Colgate Total®, Aquafresh Herbal® and Dentazyme Herbal® contained sodium fluoride whereas Colgate Herbal® contained sodium monoflourophosphate. The concentration of fluoride was only indicated on the Colgate® products and was 1.1% for Colgate Herbal® verses 0.3% for Colgate Total®. Whether the difference in the type of fluoride or the variation in concentration affected the antimicrobial activity of the toothpaste, requires further investigation.

Another confounding factor was the variety and concentration of herbs or herbal derivatives contained in the various toothpastes. All of the toothpastes contained more than one herbal derivative but none of the products mentioned the concentration of these ingredients. Although the herbal ingredients may be effective individually, their combination together with the other ingredients such as surfactants, colourants, flavourants and humectants, may affect their ability to inhibit bacterial growth.

Because all the toothpastes were put onto sterile disks and allowed to diffuse into the agar, their relative rates of diffusion must be taken into account. This rate of diffusion, which may be dependent on the individual constituents of the toothpaste, may vary according to manufacturer. This may influence the penetration of the agar and therefore the effectiveness of the antimicrobial activity (Lee *et al* 2004).

Because the study was laboratory based, the substantivity of the toothpastes was not taken into account. The test toothpastes used in the study were used at full concentration and therefore might not have the same effect if used clinically.

CHAPTER 8
CONCLUSIONS

All the herbal toothpastes tested in this study displayed varying degrees of antimicrobial activity against the 3 primary plaque colonizers tested. Their ability to inhibit bacterial growth was significantly different.

Dentazyme Herbal® consistently showed the strongest ability to inhibit bacterial growth for all the bacteria tested. It was stronger than the control toothpaste in most cases. The reason for this particular toothpastes' antimicrobial properties cannot be explained from the present study and further research is required to determine the cause of it antimicrobial effect.

Although the study had significant limitations, the ability of a herbal toothpaste to inhibit the growth of bacteria associated with plaque colonization has several potential therapeutic benefits. It may obviate the development of antibiotic-resistant plaque bacteria or may potentially be used to supplement triclosan in the inhibition of plaque growth. This inhibition of growth of the plaque biofilm may ultimately prevent the development of a biofilm that has the potential to destroy hard and soft tissue.

Further research is required to determine whether the antimicrobial properties of these herbal toothpastes have any clinical relevance.

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