The Effect of a Homoeopathic and Herbal Mouthwash Formulation on the Growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*

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Homoeopathy

by

Samba Adelaide De Oliveira

(Student number: 820413284)

Supervisor: _____ Date: _____

Dr T. Tsele-Tebakang M-Tech Hom (UJ)

Co-supervisor: _____ Date: _____

Vusi Ntema M-Tech. Bio-med (UJ)

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DECLARATION

I, Samba A. De Oliveira declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Technology at the University of Johannesburg. It has not been submitted before for any degree or examination in any other institution to obtain a research diploma or degree.

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(Signature of Candidate)

_____ Day of _____2016

ABSTRACT

There are a few hundred different types of bacterial species within the oral cavity which amount to more than several 100 millions in number within dental plaque. Some bacteria are commensal and others play a key role in the progression of periodontal disease. Periodontal disease can manifest as gingivitis which is seen as infection of the gingival tissue, or periodontitis which results in loss of collagen attachment of tooth to bone and loss of bone tissue. *Streptococcus salivarius* is the principal commensal bacterium in the oral cavity of humans. *Fusobacterium nucleatum* is commonly found in the dental plaque of humans and is frequently associated with periodontal disease. The mouthwash formulation is sea salt based together with homoeopathic remedies and herbal extracts. It is indicated for the treatment of sensitive irritated gums, halitosis, sore throat and mouth ulcers.

The aim of this study was to determine the *in vitro* effect of a homoeopathic and herbal mouthwash formulation on *Streptococcus salivarius* and *Fusobacterium nucleatum*. This study forms part of a three part *in vitro* study to determine the effect of the mouth wash formulation. The effect was evaluated by measuring zones of inhibition around discs impregnated with the mouthwash solution, saline solution and distilled water.

Lypholized ATCC 25586 *Fusobacterium nucleatum* and ATCC 13419 *Streptococcus salivarius* were obtained from Quantum Biotechnologies. *Fusobacterium nucleatum* was cultured on chocolate blood agar and nutrient agar and incubated at 35 ^{o}C in 5-7% CO_2 for 48 hours. *Streptococcus salivarius* was cultured on Tryptic Soy Agar (TSA) agar with 5% sheep blood and incubated at 35 ^{o}C in 5-7% CO_2 for 48 hours. Agar plates were refrigerated at 2-8 ^{o}C for two weeks and used as stock cultures. In the Kirby-bauer disk diffusion method, agar plates incubated with *Fusobacterium nucleatum* and *Streptococcus salivarius* respectively were streaked with the mouth wash solution, and the control substances distilled water and saline solution respectively. The agar plates were incubated at 35 ^{o}C in 5-7% CO_2 for 48 hours. In Adaptation experiment one, a five times, and two times strength mouth wash solution was prepared and tested following the Kirby-bauer streaking method. In the Adaptation experiment two, a volume of 100 µl of one time, two times and five times strength mouth wash solution was mixed with 10 µl cultures of *Streptococcus salivarius* and *Fusobacterium nucleatum* in a 1ml eppendorf tube. The mixture was incubated for 5 minutes at

room temperature and streaked on TSA agar with 5% sheep blood and nutrient agar plates respectively, agar plates were incubated at 35 ^{o}C in 5-7% CO_2 for 48 hours.

Results showed no zones of inhibition in the Kirby-bauer disk diffusion method, Adaptation experiment one and Adaptation experiment two. The results showed that the homoeopathic and herbal mouth wash formulation had no significant effect on *Streptococcus salivarius* and *Fusobacterium nucleatum* respectively.

DEDICATION

I dedicate this work to my husband and family for their never ending support and belief throughout my studies.

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I would like to thank the following people for their assistance

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LIST OF ABREVIATIONS

ANOVA	-	Analysis of Variance
TSA	-	Trypticase Soy Agar
ATCC	-	American Type Culture Collection
CASO	-	Casein-peptone Soymeal-peptone broth

CHAPTER ONE

INTRODUCTION

1.1 Problem statement

There are a few hundred different types of bacterial species within the oral cavity which amount to more than several 100 millions in number within dental plaque. Some bacteria are commensal and others play a key role in the progression of periodontal disease (Belda-Ferre *et. al*., 2012). Periodontal disease can manifest as gingivitis which is seen as infection of the gingival tissue, or periodontitis which results in loss of collagen attachment of tooth to bone and loss of bone tissue (NIH, 2012). *Streptococcus salivarius* is the principal commensal bacterium in the oral cavity of humans. It is abundant in number within the oral cavity but benign in the dental plaque (Burton *et al*., 2006a). *Fusobacterium nucleatum* is commonly found in the dental plaque of humans and is frequently associated with periodontal disease. Antibiotics are commonly prescribed as an adjunct to oral cleaning, scaling and root planing to treat periodontal disease (Wang, 2010). Antibiotics pose the risk of producing resistant strains of bacteria and causing an imbalance of the flora residing within the body (Liebana *et al*., 2004). The mouthwash formulation used in this study was sea salt based together with homoeopathic remedies and herbal extracts. It contains no preservatives, colourants, artificial ingredients, alcohol or sugar (IVOhealth, 2010). It is indicated for the treatment of sensitive irritated gums, halitosis, sore throat and mouth ulcers.

1.2 Aim of the study

The aim of the study was to determine the effect of the mouthwash formulation on the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum* bacteria using the Kirby-Bauer disk diffusion susceptibility test method and a Dilution method. The Dilution method was used to determine the effect of diluting the mouthwash formulation to one time, two time and five time concentrations on *Streptococcus salivarius* and *Fusobacterium nucleatum*. The effect was determined by measuring a zone of inhibition in millimeters (mm).

1.3 Importance of the study

The results of the study could provide scientific validation of the effect of the mouthwash formulation on *Streptococcus salivarius* and *Fusobacterium nucleatum* respectively. The results of

the study will allow for well informed decision making in the use of the mouthwash formulation for the treatment of sensitive gums, halitosis, bleeding gums, sore throat and mouth ulcers.

1.4 Hypothesis

It is hypothesized that the mouthwash formulation containing sea salt (119.5g), *Arnica montana 30cH, Echinacea purpurea 30cH, Staphysagria 30cH, Phosphorus 30 cH, Hepar sulphuris calcarea 30 cH,* Cinnamon (0.27g) and Liquorice (0.27g) will have an effect on *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*.

1.5 Null hypothesis

The null hypothesis is that the mouthwash formulation containing sea salt (119.5g), *Arnica montana 30cH, Echinacea purpurea 30cH, Staphysagria 30cH, Phosphorus 30 cH, Hepar sulphuris calcarea 30 cH,* Cinnamon (0.27g) and Liquorice extract (0.27g) will have no effect on *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The flora of the oral cavity contains 500 to 650 different bacterial species (Landers, 2013). Some species are commensal bacteria and some play a role in the development of oral diseases (Belda-Ferre *et al*., 2012). Poor oral health is a public problem with periodontal diseases such as gingivitis and chronic periodontitis being amongst the most commonly occurring human infections (Cullinan *et al*., 2009).

Oral diseases initiate with the growth of dental plaque on the tooth surface and accumulation of bacteria on the dental plaque (Marsh, 2006). In periodontal disease the areas at or below the gingival margin become infected causing an inflammatory response of the gums and surrounding connective tissues (Palombo, 2011). This can manifest as gingivitis or periodontitis. Gingivitis presents as bleeding of the gingival tissue, halitosis, sensitive irritated gums, sore throat and mouth ulcers, while periodontitis results in loss of collagen attachment of tooth to bone and loss of bone tissue (Palombo, 2011).

The increase in periodontal disease incidence coupled with increased resistance of pathogenic bacteria to currently used antibiotics poses the need for alternative prevention and treatment to periodontal disease (Bradia and Zidan, 2004). Mouthwashes together with other good oral hygiene measures such as teeth brushing twice a day and flossing daily is the first line in the treatment and prevention of gum diseases (NIH, 2012). In addition to good oral hygiene root planing and scaling are additional first line treatments of periodontal disease before it becomes severe (WebMD, 2013).

Commonly used antibiotics to treat periodontal disease include Tetracyclines, Metronidazole, Amoxicillin, Ciprofloxacin, Erythromycin and Azithromycin. These are usually prescribed as a single antibiotic therapy (Heitz-Mayfield, 2009) or in combination (Wang, 2010). Antibiotic therapy however has side effects such as nausea, diarrhoea, and vomiting and oral and vaginal thrush. Other symptoms include colitis, headache, jaundice, abdominal cramps, hives and anaphylaxis due to an allergic sensitivity to the antibiotic (Prakasam *et al*., 2012).

The homoeopathic and herbal mouth wash formulation is not meant to replace antibiotics but rather conventional mouthwash antiseptics such as Clorhexidine (NIH, 2012).

Streptococcus salivarius is a numerically abundant, non-disease causing microorganism and the principal commensal bacterium of the oral cavity in healthy humans (Kazor *et al*., 2003). *Fusobacterium nucleatum* plays an important role in the progression of periodontal disease as it is one of the key organisms in dental plaque (Zilm and Rogers, 2007).

2.2 Periodontal Pathology

Periodontal disease affects one or more periodontal tissues i.e alveolar bone, periodontal ligaments, cementum and gingiva. The most common are plaque induced inflammatory conditions considered as gingivitis and periodontitis (Armitage, 2004a).

Gingivitis and periodontitis are inflammatory conditions which arise from infection of the gingival tissue. Gingivitis is a reversible inflammatory condition whereas periodontitis is non-reversible, destructive and results in loss of connective tissue attachment to bone (Tatakis and Kumar, 2015). Plaque formation begins with adhesion of bacteria onto the tooth surface followed by passive auto-aggregation between same species of bacteria and co-aggregation between different species of bacteria. The attached bacteria multiply and secrete an extracellular matrix which results in a mixed bacterial population biofilm. The biofilm provides a protective barrier against antimicrobial agents. With this barrier the infective bacteria can thus develop antibiotic resistance to antimicrobial agents such as Tetracycline (Kolenbrander *et al*., 2002).

In the early stages gingivitis presents as oedema of the gingival tissue, increase in gingival fluid flow, accumulation of leukocytes and loss of connective tissue. With breakdown of connective tissue, periodontal pockets form in the connective tissue. There is increased permeability of the epithelial tissue lining the pockets within the connective tissue which allows continuous infiltration of bacterial products thus increasing the inflammatory response. As the latter increases alveolar bone loss follows (Ohlrich *et al*., 2009).

Periodontal disease is divided into four stages; stages 1-3 indicate gingivitis in levels of increased severity and stage 4 indicates established periodontitis.

Stage 1: Initial lesion

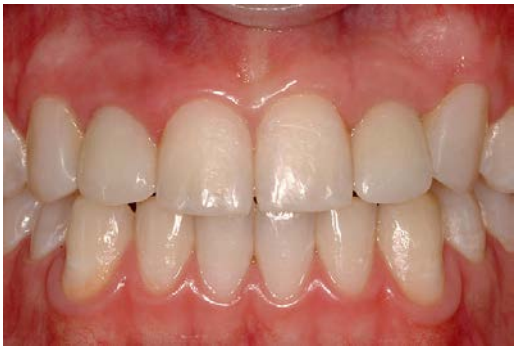

Figure 2.1 Subclinical initial lesion of gingivitis (Ganz, 2015)

The initial lesion reflects enhanced levels of bacterial activity of host physiological response mechanisms normally operative within gingival tissues (Wolf and Hassell, 2006). The initial lesion occurs within the first 4 days of gingival tissue being subject to plaque accumulation (Ohlrich *et al*., 2009). The initial lesion shows increased capillary permeability with increased number of neutrophils migrating from dilated gingival capillary plexus into the junctional epithelium. There is decreased connective tissue integrity, macrophage infiltration and increased numbers of lymphocytes in the affected area (Stephen, 2014). There is a loss of perivascular collagen due to degrading enzymes released by leukocytes which cause degrading of collagen and other connective tissue fibers surrounding blood vessels in the area. The gingiva then takes a bright red bulbous appearance due to oedema building up in the infected area (Figure 2.1) (Armitage, 2004b).

Stage 2: Early lesion

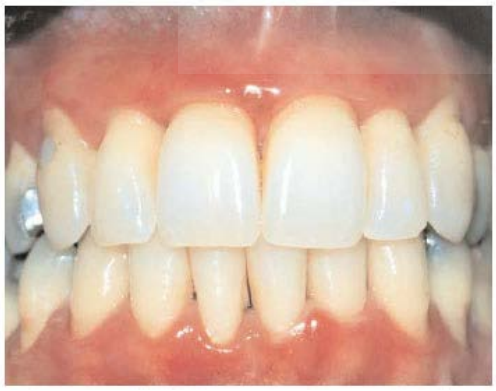

Figure 2.2 Early lesion of gingivitis (Motta *et al*., 2006)

The early lesion involves the inflammatory changes that occur after plaque accumulation has begun from days 4-7 (Figure 2.2) (Ohlrich *et al*., 2009). This stage occurs after one week of plaque accumulation (Geminiani, 2013). This stage is characterized by increased numbers of mature lymphocytes, immunoblast cells and fibroblast cells infiltrating the area (Silva *et al.*, 2007). The junctional epithelium may become infiltrated with enough neutrophils and monocyte cells (Bassam, 2013).

Stage 3: Established lesion

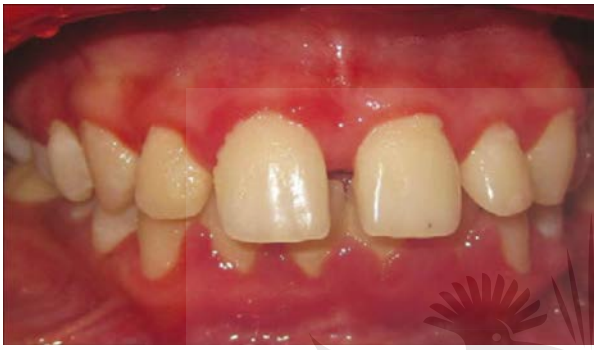

Figure 2.3 Established lesion of gingivitis (Janam *et al*., 2012)

The established lesion is distinguished by an overwhelming presence of plasma cells (Kawai *et al.*, 2006). This stage occurs 14-21 days after plaque accumulation (Hanes and Krishna, 2010). The lesion displays an inflammatory reaction with increased leukocyte infiltration confined to the area near the base of the gingival crevice (Bassam, 2013). Most of the plasma cells produce IgG antibodies (Geminiani, 2013).

The junctional epithelium is destroyed no longer attached to the epithelium against the tooth surface (Bassam, 2013). The junctional epithelium responds by proliferating and growing into adjacent spaces in an apical direction (Figure 2.3) (Ohlrich *et al*., 2009). The gingiva can be moderately or severely inflamed at this stage (Hanes and Krishna, 2010).

Stage 4: Advanced lesion

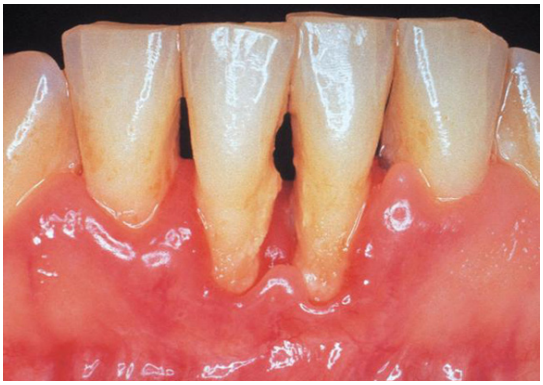

Figure 2.4 Periodontitis (Colgate, 2015)

The advanced lesion presents as periodontitis due to soft tissue destruction and bone resorption (Kawai *et al.,* 2006). The lesion spreads apically and laterally deep into the connective tissue (Figure 2.4). Plasma cells are the main cell types at this stage (Bassam, 2013). Alveolar bone and connective tissue fibers are destroyed (Hanes and Krishna, 2010). The features of the advanced lesion are periodontal pocket formation, gingival ulceration and suppuration, destruction of alveolar bone and periodontal ligament and tooth loss. At this stage there is irreversible damage to periodontal tissues (Hasan and Palmer, 2014).

2.3 Bacteria of the Oral Cavity

The flora of the oral cavity contains 500 to 650 different bacterial species. An estimated 20 billion oral microbes are found within the oral cavity. In a 24-hour period, an average of 100 billion microbes is swallowed (Landers, 2013). Some bacterial species are commensal and some play a role in the development of dental caries and periodontitis (Belda-Ferre *et. al*., 2012). Maintenance of oral health includes brushing of teeth at least twice daily, flossing and regular oral cleaning by a dentist or oral hygienist (NIH, 2012).

2.3.1 *Streptococcus salivarius*

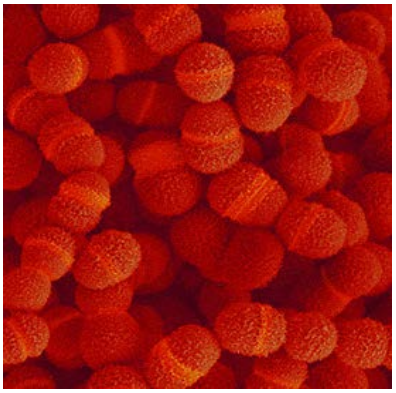

Figure 2.5 *Streptococcus salivarius* (Kunkel, 2009)

2.3.1.1 Morphology

Streptococcus salivarius is a facultative anaerobic gram positive cocci bacterium with a single plasma membrane followed by a periplasmic space and a peptidoglycan layer called murein (Figure 2.5). The latter gives the bacterium rigidity and allows it to survive in media with osmotic pressure less than that of its cytoplasm. The murein layer is the target layer for the action of antibiotic treatment (Silhavy *et al*., 2010).

2.3.1.2 Mode of action

Streptococcus salivarius colonizes the oral cavity, digestive tract and upper respiratory tract of humans a few hours after birth (Kaci *et. al*., 2011). *Streptococcus salivarius* is one of the earliest colonizers of epithelial surfaces in the human oral cavity and nasopharynx and is an uncommon cause of invasive infection (Wilson *et. al*., 2011). It is also abundant on the dorsum of the tongue and oropharyngeal mucosa of humans (Tagg, 2004). It has a protective action against pathogens that cause throat infections, otitis media and halitosis (Tagg and Dierksen, 2003). It displays protective effects against pathogens involved in the development of tooth decay and periodontal disease (Wilson *et al*., 2011). *Streptococcus salivarius* within the oral cavity is generally associated with good health and several strains have been developed as oral probiotics (Heng *et al*., 2011). Their probiotic property prevents colonization and proliferation of pathogenic bacteria within the oral cavity (Wilson *et al*., 2011). *Streptococcus salivarius* has been shown to inhibit harmful upper respiratory tract bacteria such as *Streptococcus. pyogene* and *Streptococcus pneumoniae* in addition

to decreasing halitosis (Wescombe *et al.,* 2011). Additionally it inhibits organisms such as *Haemophilus influenza* and *Streptococcus sanginosus* which are potential pathogens in the ear and oral cavity of humans causing acute otitis media, halitosis and tonsillitis (Di Pierro *et al.,* 2012). *Streptococcus salivarius* has inhibitory effects against pathogenic bacteria attributed to the production of antimicrobial peptides namely Salivaricin A2 and Salivaricin B (Cosseau *et al.,* 2008). Salivaricin A2 has a bacteriostatic mode of action and Salivaricin B has a bacteriocidal mode of action (Burton *et al.,* 2006a). The antimicrobial peptides show inhibitory properties against gram positive bacteria that are implicated in causing halitosis (Burton *et al.,* 2006b). Some studies have shown that *Streptococcus salivarius* modulates the host immune system by down regulating the inflammatory response of the host and stimulating beneficial inflammatory pathways in periodontal disease (Cosseau *et al.,* 2008).

2.3.2 *Fusobacterium nucleatum*

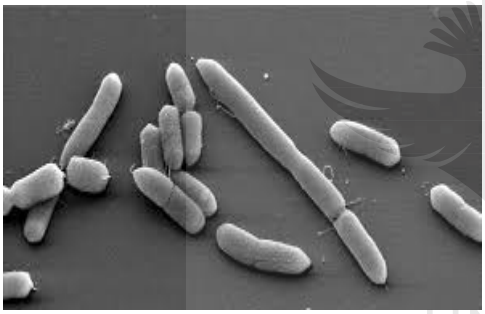

Figure 2.6 *Fusobacterium nucleatum* (Pati *et al.,* 2011)

2.3.2.1 Morphology

Fusobacterium nucleatum is a rod shaped non-motile, non-spore forming gram negative anaerobic bacterium (Signat *et al.,* 2011) associated with periodontal disease (Figure 2.6) (Diaz *et al.,* 2002). It contains an outer membrane with a periplasmic space composed of peptidoglycan between its inner and outer membrane layers. The inner layer is composed of a symmetrical phospholipid bilayer and the outer layer composed of an asymmetrical membrane containing phospholipids, lipoppolysaccharides, lipoproteins and proteins. The inner and outer membrane forms a capsule surrounding the bacterium which is essential for its pathogenic characteristic (Bolstad *et al.,* 1996).

2.3.2.2 Pathogenesis

Fusobacterium. nucleatum is commonly found in the oral cavity of humans in healthy or diseased individuals (Kapatral *et al.,* 2002). *Fusobacterium nucleatum* is one of the microorganisms which form a key component of dental plaque due to its ability to co-aggregate with other species in the oral cavity. It is one of the first gram-negative species to become established in dental plaque biofilm (Signat *et al*., 2011). *Fusobacterium nucleatum* is considered as an intermediate colonizer facilitating the attachment of commensal bacteria that colonize dental plaque with pathogenic bacteria (Kolenbrander *et al*., 2002). It has the ability to protect other pathogenic bacteria species such as *Porphyromonas gingivalis* allowing it to flourish in dental plaque (Diaz *et al.,* 2002). *Fusobacterium nucleatum* reacts on the early stages inflammatory response during periodontal disease (Han *et al.,* 2000). In the initial stages of periodontal disease aerobic streptococcal species of bacteria and other pathogenic bacteria adhere to and colonize the tooth enamel. The colonization allows a platform for *Fusobacterium nucleatum* batcteria to co-aggregate with early dental colonizers and in turn allow late pathogenic colonizers to form a dental plaque biofilm thus leading to tooth decay (Kapatral *et al.,* 2002). *Fusobacterium nucleatum* is considered to play an important role in the progression of periodontal disease (Zilm and Rogers, 2007) it increases in proportion as plaque forms and is closely related to gingivitis and periodontitis at active site of inflammation. It does so by stimulating pro-inflammatory cytokines and interleukin-8 (IL-8) which favours the progression of inflammation therefore causing further tissue destruction (Han *et al.,* 2000). It is the most common bacteria with an oral etiology cultivated from other sites of infection (Bolstad *et al.*, 1996). It has been cultivated from other sites of infections such as skin ulcers, peritonsilar abscesses, septic arthritis and endocarditis (Kapatral *et al.,* 2002).

2.4 Diagnosis of Periodontal Disease

Clinical diagnosis of periodontal disease is based on visual and radiographic assessments of periodontal tissues and measurements of the spaces between tooth and gingiva (Armitage, 2004b). The space between gingival and tooth are normally 1-3mm in depth in healthy individuals. There is absence of bleeding from the gingival crevice on gentle probing with presence of a small amount of interstitial fluid (Highfield, 2009). During clinical examination, pocket depths and connective tissue are measured at four to six locations around every tooth, the amount of supragingival plaque is

measured, dental calculus, gingival bleeding and exudates are recorded and used to diagnose existing disease, determine prognosis and monitor disease progression (Pihlstrom *et al*., 2005).

Additionally the Journal of Periodontology (2003:1237-1247) states that to arrive at a clinical diagnosis of periodontal disease there is a reliance on presence or absence of clinical signs of inflammation i.e. loss of attachment of tooth to bone, patient medical and dental history and the presence or absence of pain, ulceration and amount of observable plaque in addition to assessment of the subgingival microflora.

2.5 Treatment of Periodontal Disease

Oral hygiene is essential for maintaining oral health and reduces plaque buildup on teeth surface. Plaque is a sticky colourless deposit of bacteria that is constantly forming on tooth surface. Buildup of plaque is the primary factor of gum disease; it begins forming 12 hours after brushing the teeth therefore teeth have to be brushed twice daily and flossed daily (Kenny and Roger, 2014). The American Dental Association (2016) states that teeth should be cleaned professionally every six months as well as following a well balanced diet.

Root planing and scaling are the most common non-surgical methods used to treat moderate periodontal disease such as periodontitis before surgery is employed. The procedure involves cleaning between the gums and teeth down into the roots. This is done when the gums have started to recede or there is plaque accumulation (NIH, 2013). The procedures involve removing plaque from deep periodontal pockets and smoothing the tooth root surface to remove bacterial toxins (Perio, 2015). Subgingival scaling involves removing plaque from the tooth around and below the gingival tissue. Root planning involves scraping and smoothing the root of the tooth. It decreases inflammation of the gingival tissue and prevents bacteria from adhering to the tooth surface (NIH, 2013).

Scaling and root planing carries the risk of introducing opportunistic pathogenic bacteria into the blood stream during the procedure, therefore, antibiotics are administered prior to and post procedure (NIH, 2013). There is a risk of contamination on treated areas from untreated sites carrying opportunistic pathogens (Cionca *et al*., 2009). There is also a risk of increased hypersensitivity of teeth after therapy by 54-55% (Von Troil *et al*., 2002).

Antibiotics are commonly prescribed as an adjunct to oral cleaning, scaling and root planing to treat periodontal disease (Wang, 2010). Antibiotics kill bacteria in gingival plaque thus reducing the amount of plaque production, which in turn reverses gum disease. Antibiotics can be applied topically on the gingival surface, ingested orally as pills and capsules, used as a mouthwash or inserted directly into the pockets of advanced gingival diseased tissues (Prakasam *et al*., 2012). Periodontal infections are a combination of a variety of bacteria which are aerobic, anaerobic and both gram positive and gram negative bacteria (Prakasam *et al.*, 2012). Antibiotic therapy used to treat periodontal disease is therefore usually prescribed serially (Heitz-Mayfield, 2009) or in combination such as Metronidazole-Ciproflaxin and Metronidazole-Amoxicillin (Wang, 2010). The most common used antibiotics to treat periodontal disease are listed below.

2.5.1 Tetracyclines

Tetracyclines are a group of broadspectrum antibiotics that have an effective bacteriostatic action against a number of oral gram-negative and gram-positive species of bacteria (Jorgensen and Slots, 2000). They have an anti-collagenase effect which inhibits tissue destruction and aids in bone resorption (Sapadin and Fleischmajer, 2006), arrests bone loss and suppresses microbial levels in conjunction to scaling and root planning (Pejcic *et al.*, 2010). The oral recommended dose for tetracycline therapy is 250 mg-500 mg four times daily (Prakasam *et al.*, 2012). Adverse effects of tetracycline include transient retardation of bone growth, photosensitivity, and permanent discoloration of developing teeth, teratogenesis, as well as hepatic and renal toxicity in susceptible individuals (Kumar *et al.*, 2012).

2.5.2 Metronidazole

Metronidazole is a nitroimidazole compound with a broad spectrum of activity (Pejcic *et al*., 2010). It has a specific bactericidal action on gram negative anaerobes and gram positive bacteria and aggressive periodontitis (Walker *et al.*, 2004). It is most effective against obligate anaerobic gram negative bacteria such as the *fusobacterium* species (Kumar *et al*., 2012). Metronidazole is administered orally at 250 mg three times a day for 14 days (Kumar *et al.*, 2012). Clinically it is used in the treatment of refractory periodontitis when used in combination with Amoxicillin, root planning, scaling and oral hygiene practice (Jorgensen and Slots, 2000). It is also used in treatment of acute necrotizing ulcerative gingivitis. Adverse effects include nausea and vomiting when alcohol

is ingested concomitantly with the tablets. It inhibits Warfarin metabolism in the liver and prolongs prothrombin bleeding time. It is also contra-indicated in patients taking lithium therapy (Kumar *et al.,* 2012).

2.5.3 Amoxicillin

Amoxicillin is a broad spectrum bactericidal drug that inhibits the synthesis of bacterial cell walls and results in cellular disruption due to high osmotic pressure (Tripathi, 2005). It is often combined with Clavulanate which inhibits beta-lactamases produced by some oral pathogenic bacteria. It is stable in a highly acid environment and over 90% of the administered dose is absorbed (Kumar *et al.,* 2012). It is used in combination with Metronidazole for treatment of chronic and aggressive periodontitis (Jorgensen and Slots, 2000). In addition Amoxicillin in doses of 250mg-500mg three times daily may be effective in treating periodontitis resistant to treatment (Rams and Slots, 1992). Except for allergic reactions toxicity is extremely low and it is one of the safer known drugs (Kumar *et al.,* 2012).

2.5.4 Ciproflaxin

Ciproflaxin is a quinolone drug that is active against gram negative anaerobic bacteria including many periodontal pathogens. It does not suppress *streptococcus* species, which are associated with promoting a healthy oral environment (Prakasam *et al*., 2012). The recommended dosage is 500mg-750mg twice daily for 7–10 days (Kumar *et al*., 2012). Adverse effects include nausea and vomiting, oral candidiasis, headache, restlessness, hypersensitivity, hyperpigmentation and photosensitivity (Tripathi, 2005).

2.5.5 Erythromycin

Erythromycin belongs to the macrolide group of antibiotics. It has a bactericidal action and acts by inhibition of protein synthesis of bacteria (Wolff, 2012). Erythromycin has been shown to be active against most strains of gram positive and gram negative bacteria which cause upper respiratory tract and lower respiratory tract infections, skin and skin structure infections of mild to moderate severity. Side effects of erythromycin use include nausea, vomiting, abdominal pain, anorexia, hepatic dysfunction with or without jaundice and mild diarrhea. Onset of pseudomembranous colitis symptoms may occur during or after antibiotic use. Prolonged or repeated use of erythromycin may

result in an overgrowth of non-susceptible bacteria or fungi. The therapeutic dose of erythromycin is 250mg every 6 hours taken one hour before meals (NIH, 2008).

2.5.6 Azithromycin

Azithromycin is an antibiotic from the macrolide group; it has a bacteriostatic activity by blocking bacterial proteins synthesis (Aurer and Plancak, 2004). It is a broad spectrum antibiotic and has an effect on gram-negative bacteria, including enteric bacteria. Azithromycin penetrates phagocyte cells which are released onto inflammatory sites (Prakasam *et al.,* 2012). Periodontal inflamed tissues show higher concentrations of administered Azithromycin compared to healthy periodontal tissues (Wang, 2010). Clinical administration of Azythromycin is 1 tablet once a day for three days (Wang, 2010). Azithromycin is indicated for the treatment of advanced chronic or aggressive periodontitis (Aurer and Plancak, 2004). Adverse effects of Azythromycin use include diarrhea, flatulence, nausea, gastric pain or discomfort deafness, vertigo, headaches, dermatitis and vomiting (NIH, 2014).

2.6 Antibiotic resistance

Antibiotic therapy however has side effects such as nausea, diarrhoea, and vomiting and oral and vaginal thrush. Other symptoms include colitis, headache, jaundice, abdominal cramps, hives and anaphylaxis due to an allergic sensitivity to the antibiotic (Prakasam *et al*., 2012). Inappropriate antibiotic usage poses unnecessary expenses and contributes to antibiotic resistant bacteria which are reported with increased frequency (Cosseau *et al.*, 2008). Periodontal infections may deplete the levels of commensal bacteria which favour the balance of the oral ecosystem. This causes opportunistic bacteria to increase in number within the oral cavity (Burton *et al.,* 2006b).

2.7 Homoeopathy

2.7.1 Definition

Homoeopathy is a complementary and alternative medicine founded by a German physician Dr Fredrich Samuel Hahnemann (1755-1843) (Loudon, 2006). It has the basic principle stating “Let likes be cured by likes” (*simila similibus curentur*) (Ernst, 2000), which implies that substances which produce certain symptoms in healthy individuals in crude form can cure the same symptoms

when taken in homoeopathic form in a sick individual who presents with a disease picture similar to the disease producing substance (De Schepper, 2010). Homoeopathic remedies produce an artificial medicinal disease similar and stronger than the natural disease. The artificial medicinal disease induced by a homoeopathic remedy acts on an energetic plane. Homoeopathic remedies aim to direct and regulate the body's self-regulatory mechanisms (Mathiessen, 2011). Health and healing in homoeopathy is based on the energy force within the body termed by Samuel Hahnemann as the Vital Force or life force which animates the body and maintains harmony within the body. Homoeopathy is based on a paradigm that the vital force brings about cure through stimulation by the homoeopathic remedy (De Scheeper, 2010).

Homoeopathic remedies must be administered first in minute or infinitesimal doses when the natural disease is similar to the symptom picture of the artificial medicinal disease (De Schepper, 2010). The preparation of the homoeopathic medicines involves serial dilutions to the extent that no molecules of the original substance remain (Shang *et al.*, 2005). The highly diluted substances have no toxic effect on the body (Schmukler, 2006).

2.7.2 Potentisation

Potentisation is a process by which the medicinal properties latent in a crude substance become activated. This is done by trituration of dry substances and by means of succussion of liquids between dilutions (Vickers and Zollman, 1999). Trituration is a mechanical process whereby a crude substance insoluble in liquid is ground in a solid vehicle. The trituration ratio is one part substance plus 9 parts or 99 parts lactose powder (Banerjee, 2006). Succession and trituration add kinetic energy to a solution increasing its therapeutic action (Roy, 1999). Potentisation is achieved by using different scales of dilutions to achieve solubility of substances. The scales of dilution are the decimal scale denoted as X (10) and the centesimal scale denoted as C (100) (Barnejee, 2006).

The decimal scale is a dilution of 1:10 ratio achieved by taking one part of the mother tincture and diluting it in 9 parts alcohol. The liquid is succussed ten times and the resulting liquid is 1X potency. This is the first stage of dilution with X referring to 1:9 dilution ratio. To make a 2X potency one part of 1X potency is diluted and succussed in 9 parts alcohol (Banerjee, 2006). Higher dilutions are made using the lower potencies.

The centesimal scale is a dilution of 1:100 ratio where one part mother tincture is diluted in 99 parts alcohol. The liquid is succussed 100 times and resulting liquid is 1C potency which denotes 1:100 dilution ratios. To make 2C potency one part of 1C is diluted and succussed in 99 parts alcohol (Ezine, 2009).

2.7.3 Complex remedies

Complex remedies are a preparation containing a mixture of two or more homoeopathic remedies in one container. The individual remedies used are those known to be the most effective in relieving symptoms of a condition. The combined remedies used are in their lowest potencies (Hpathy, 2006).

2.8 Mouthwash Formulation

The mouthwash formulation consists of a sea salt base medicated with homoeopathic remedies and herbal extracts. It contains no preservatives, colourants, artificial ingredients, alcohol or sugar. It is indicated for the treatment of sensitive irritated gums, halitosis, sore throat and mouth ulcers (IVOhealth, 2010). The mouth wash formulation contains the following ingredients: Sea salt (119.5g), *Arnica montana 30cH*, *Echinacea purpurea 30cH*, *Staphysagria 30cH*, *Phosphorus 30 cH*, *Hepar sulphuris calcarea 30 cH*, Cinnamon (0.27g) and Liquorice (0.27g).

2.8.1 Sea salt

Figure 2.7 Sea salt (Barron, 2006)

Salt water as a mouthwash temporarily alkalinizes or increases the pH in the oral cavity. This inhibits bacterial proliferation, as most bacterial species prefer acidic environments for growth. Sea salt alleviates symptoms of teething, toothache and aphthous ulcers in the mouth (Morris, 2013).

2.8.2 *Arnica montana*

Figure 2.8 *Arnica montana* (Smith, 2012)

Botanical name denoted *Arnica montana* belongs to the Compositae family. Its synonyms are *Crysanthemum latifolium*, Leopard's bane, Mountain arnica, Mountain tobacco. The entire fresh plant is used to make the homoeopathic remedy (Banerjee, 2006).

Arnica montana is a perennial herb, with a slender blackish rhizome from which numerous roots are given off. It has a rough pubescent stem; the leaves are long crowded at the base and pubescent with smaller leaves. The flowers are large orange-yellow in colour, solitary and have an aromatic smell and a herby taste. The flowers blossom in July and August (Banerjee, 2006).

The homoeopathic indication for this remedy is for foetid breath, pain and inflammation associated with toothache where the root of the tooth has been damaged after dental filling (Vermeulen, 2011).

2.8.3 *Echinacea purpurea*

Figure 2.9 *Echinacea purpurea* (Marcus, 2003)

Botanical name denoted *Echinacea purpurea* belongs to the Asteraceae family. Its synonyms are purple cone flower, black samson, comb flower, hedgehog, scurvy root. Parts used are the entire fresh plant, root or part above ground (Wildflower, 2015).

Echinacea purpurea is a perrenial plant with smooth stems and long lasting lavender flowers. The plant has rough scattered leaves that become small toward the top of the stem (Ehrlich, 2013). It has single flowers on top the stems, purplish-brown in colour with spiny centers and drooping lavender rays. Purple petals surround a spiny brownish central disc (Huntley *et al*., 2005).

The homoeopathic indication for *Echinacea purpura* is for canker sores in the mouth and gingiva that recedes and bleeds easily (Vermeulen, 2011).

2.8.4 *Delphenium staphysagria*

Figure 2.10 *Delphenium staphysagria* (Basset, n.d)

Botanical name denoted *Delphenium staphysagria* belongs to the Ranunculaceae family. Its synonyms are lakspur, lice-bane, stavesacre (Cretanflora, 2011).

The seeds of the plant are used to make the homoeopathic remedy. *Delphinium staphysagria* is a mauve-blue to plain blue flowers, grows to 1m in height, native to Asia and southern regions of Europe. It has a stout stem, hairy bienneal plant with large palmate leaves (Bown, 2001).

The homoeopathic indication for this remedy is for gingiva prone to haemorrhage; teeth are black and crumbling with spongy gums (Boericke, 2002).

2.8.5 *Phosphorus*

Phosphorus has a symbol denoted P its atomic number is 15 on the periodic table. It has a melting point of 44.2 °C with atomic mass of 30. 973762 $g.mol^{-1}$ (Lenntech, 2015).

Phosphorus is a multivalent non-metal of the nitrogen group that comes in the form of a red amorphous powder and a white/yellow crystalline form (Banerjee, 2006). It also appears in a red and black variety. White phosphorus is manufactured industrially, glows in the dark, is spontaneously flammable when exposed to air and is poisonous. Red phosphorus varies in colour from orange to purple. Black phosphorus is made under high pressure, looks like graphite and conducts electricity (Lenntech, 2015).

The homoeopathic indication for phosphorus is for profuse or persistent bleeding after dental procedures. The gums are swollen, bleed easily and are ulcerated (Boericke, 2002).

2.8.6 *Hepar suphuris calcarea*

Symbol CaS with synonyms as calcium sulphide of Hahnemann, sulphur of lime (Jones, 2014).

Hepar sulphuris calcarea is an impure sulphide of calcium prepared from burning the white interior of oyster shells with pure sulphur (Jones, 2014).

As a homoeopathic remedy *Hepar sulphuris calcarea* has the ability to fight infections with a muco-purulent component and abscess formation. There is a characteristic hypersensitivity to pain, specifically with the gingiva being sensitive to touch with easy bleeding (Vermeulen, 2011).

2.8.7 Cinnamon

Figure 2.11 *Cinamomum zeyllanicum* (Russo, 2015)

Botanical name denoted *cinamomum zeyllanicum* belongs to the Lauraceae family. Its synonyms are cassia, canel, canella, ceylon cinamon. The parts used are the bark of trees from the genus cinnamomum (Maguelone and Bell, 2009).

Cinnamon comes in different varieties namely chinese cinnamon which is medium to light reddish brown, hardy and woody in texture and thicker as all the layers of the bark are used. Ceylon cinnamon has a lighter brown colour as only the thin inner bark is used, it is finer and less dense (Rudrappa, 2015). In its crude form cinnamon has anti-oxidant, antiseptic and anti-inflammatory properties and acts as a local anaesthetic (Rudrappa, 2013). A daily dose of no more than 4g is recommended for usage (Ferris, 2010).

The homoeopathic indication for cinnamon is for offensive taste in the mouth, rawness and soreness of the mouth which returns after rinsing with cold water, toothache and sore throat (Vermeulen, 2011).

2.8.8 Liquorice

Figure 2.12 Liquorice plant (NIH, 2006)

Botanical name denoted as *Glycyrrhiza glabra* it belongs to the Fabaceae family. Its synonyms are Glycyrrhiza glandulifera, sweet wood, licorice. The parts used are the root of the plant (NIH, 2006).

Glycyrrhiza glabra is an herbaceous perrenial plant with pinnate leaves. The flowers are long purple to pale whitish blue. It has an oblong pod for a fruit with stoloniferous roots. The root is soft and fibrous with a bright yellow interior (Geetha and Roy, 2012). The root has an anti-inflammatory action by causing macrophages to release fewer pro-inflamatory cytokines (Bodet *et al*., 2008). The bioactive ingredients found in the roots of *Glycyrrhiza glabra* and *Glycyrrhiza uralensis* liquorice species have been found to have the potential to be used as a natural modality to cure periodontal disease (Messier *et al.,* 2012). A daily therapeutic dosage of 0.3g or less is recommended for long term consumption of liquorice extract (NYU, 2014).

The homoeopathic indication for this remedy is redness of the oral mucosa with a burning sensation, soreness of the throat with dry cough (Nayak *et al*., 2010). It is indicated for swelling and soreness of gums; on inner side of lower jaw (Vermeulen, 2011).

2.9 Related studies

2.9.1 Inhibition of oral organisms by the mouthwash formulation

A study done at the University of Pretoria tested the effects of the inhibition potential of the homoeopathic and herbal mouthwash formulation containing the following ingredients: Sea salt (119.5g), *Arnica montana 30cH, Echinacea purpurea 30cH, Staphysagria 30cH, Phosphorus 30 cH, Hepar sulphuris calcarea 30 cH,* Cinnamon (0.27g) and Liquorice (0.27g). The study was done on *Streptococcus mutans, Porphyromonas gingivalis, Actinobacillis actinomycetemcomitans, Prevotella intermedia, Tannnerella forsythensis, Eikenella corrodens and Candida albicans in vitro*. The tube dilution method was used with increasing concentrations of the mouthwash formulation (0-5% m/v) prepared in Casein-peptone Soymeal-peptone (CASO) broth. The series of increasing concentration dilutions were inoculated with standardized suspensions of the microorganisms. All tubes were incubated at 37 ^{o}C for 48 hours in anaerobic conditions created with Anaerocult A obtained from Merck. After 48 hours growth in the different tubes in terms of turbidity was measured with a spectrophotometer. Results showed no inhibition of growth of any of the tested microorganisms (Molobela and Botha, 2006).

2.9.2 *In vitro* evaluation of antibacterial activity of ethanolic root extract of *Glycyrrhiza glabra* on oral microbiomes

This study investigated the antibacterial activity of *Glycyrrhiza glabra* extract on *Streptococcus mutans* (*S.mutans)*, *Streptococcus sanguinis* (*S. sanguinis*), *Streptococcus salivarius* (*Streptococcus salivarius*), *Streptococcus mitis* (*S. mitis*) and *Lactobacillus acidophilus* (*L. acidophilus*) *in vtro*. The disc diffusion method was used with concentrations of 125 µg, 250 µg and 500 µg respectively inoculated onto filter papers. Plates were incubated at 37 ^{o}C overnight and zones of inhibition were measured. Results showed that the extract was effective against *S.salivarius*, *S. mutans*, *S. mitis*, *L. acidophilus* and *S. sanguinis* respectively with clear zones of inhibition around filter papers inoculated with extract of *Glycyrrhiza glabra* (Geetha and Roy, 2012). The results obtained from this study could be due to the extract containing active ingredients which have antimicrobial properties.

CHAPTER THREE

METHODOLOGY

3.1 Materials

Refer to Appendix C for materials and equipment used in the study.

3.2 Research design

This quantitative *in vitro* control study was conducted at the Water and Health Research Centre at the University of Johannesburg, Doornfontein campus, under the supervision of qualified laboratory technicians with relevant permission granted (Appendix A).

3.3 Research Procedure

3.3.1 Stock cultures

Lyophilized *Fusobacterium nucleatum (*ATCC 25586) and *Streptococcus salivarius (*ATCC 13419) were obtained from Quantum Biotechnologies (Randburg, South Africa). *Fusobacterium nucleatum* and *Streptococcus salivarius* were reconstituted by culturing on selective growth media. *Fusobacterium nucleatum* was cultured on chocolate blood agar and nutrient agar and incubated at 35 °C in 5% to 7% CO_2 for 48 hours. *Streptococcus salivarius* was cultured on trypticase soy agar (TSA) with 5% sheep blood and incubated at 35 °C in 5 to 7% CO_2 for 48 hours. TSA agar with 5% sheep blood was obtained prepared from Quantum Biotechnologies (Randburg, South Africa). The TSA with 5% sheep blood agar plates, chocolate blood agar and nutrient agar plates (Appendix D and E) that had growth were refrigerated at 2-8 °C for 2 weeks and used as stock cultures.

3.3.2 Sub-cultures

For experimental test, fresh culture was prepared by sub-culturing *Fusobacterium nucleatum* and *Streptococcus salivarius* from stock culture into fresh media plates. *Fusobacterium nucleatum* and *Streptococcus salivarius* were sub-cultured on chocolate blood agar, nutrient agar and TSA with 5% sheep blood agar respectively. The plates were incubated at 35 °C in 5 to 7% CO_2 for 48 hours.

3.3.3 Mouth wash formulation and saline preparation

The mouth wash formulation was prepared as described by manufacture instructions. Briefly, the mouth wash solution was prepared by adding 1 scoop which was equivalent to 62,5 grams when measured, in 125 ml sterile warm distilled water. Saline was prepared by adding 9 grams of table salt to 1L of sterile distilled water and shaken until the granules dissolved. Saline solution was used to compare the anti-bacterial inactivity from the mouth wash formulation. Sterile distilled water was used as a negative control.

3.3.4 Disk diffusion method

The antimicrobial activity of the mouth wash formulation was determined by using the disc diffusion procedure recommended by the Clinical and Laboratory Standards Institute (Wikler *et al*., 2006). Using a sterile swab TSA agar with 5% sheep blood and chocolate blood agar plates were streaked respectively with a fresh culture of *Streptococcus salivarius* and *Fusobacterium nucleatum* that had been adjusted to a turbidity of 0.5 McFarland standards.

To obtain uniform growth, the agar plates were streaked with a sterile swab in one direction and then the agar plates were rotated by 90° and streaked again in that direction. The rotation was repeated three times. After streaking, the plates were allowed to dry for approximately 5 minutes. Sterile discs were placed one at a time on the streaked plates using a flame-sterilized forceps. The discs were impregnated with the mouthwash solution (10 µl), distilled water (10 µl) and saline (10 µl).

The agar plates were inverted and incubated at 35°C in 5% to 7% CO_2 for 48 hours. Each agar plate was numbered before incubation to allow identification of the contents progress.

After 48 hours incubation the TSA agar with 5% sheep blood and chocolate blood agar were checked for a zone of inhibition around each of the impregnated discs. This zone marks the effective inhibition of microbial growth. The diameter of each zone was measured in millimeters (mm) and recorded to determine the extent of antimicrobial activity. The experiments were done in triplicates and repeated three times.

3.3.5 Adaptation experiment one

A five and two times strength mouthwash solution was prepared. The five times and two times strength mouthwash solutions were tested following the method described in section 3.3.4. The same controls, which are saline and distilled water, were included in the experiments. The experiments were done in triplicates and repeated three times. For this adaptation experiment *Fusobacterium nucleatum* was cultured and grown on chocolate agar and *Streptococcus salivarius* was grown on TSA 5% sheep blood agar.

3.3.6 Adaptation experiment two

A volume of 100 µl (one time, two time and *five* time strength) mouthwash formulation was mixed with 10 µl fresh cultures of *Streptococcus salivarius* and *Fusobacterium nucleatum* in 1 ml eppendorf tube and incubated for 5 minutes at room temperature. *Streptococcus salivarius* and *Fusobacterium nucleatum* were adjusted to a turbidity of 0.5 McFarland standards. In parallel a 10 µl *Streptococcus salivarius* and *Fusobacterium nucleatum* that were adjusted to 0.5 McFarland standards were incubated in 100 µl saline and distilled water for 5 min at room temperature. After incubation the mixtures were streaked on TSA agar with 5% sheep blood and nutrient agar plates for *Streptococcus salivarius* and *Fusobacterium nucleatum* culturing respectively. The TSA agar with 5% sheep blood and nutrient agar plate were streaked with a sterile swab in one direction and then the agar plates were rotated by 90° and streaked again in that direction. The rotation was repeated three times. After streaking, the plates were allowed to dry for approximately 5 minutes. The agar plates were inverted and incubated at 35°C in 5% to 7% CO_2 for 48 hours. Each agar plate was numbered before incubation to allow identification of the contents progress.

After 48 hours incubation the TSA agar with 5% sheep blood and nutrient agar was checked for a zone of inhibition around each of the impregnated discs. This zone marks the effective inhibition of microbial growth. The diameter of each zone was measured in millimeters (mm) and recorded to determine the extent of antimicrobial activity. The experiments were done in triplicates and repeated three times.

3.4 Data Collection

After a total of 48 hours of incubation (disk diffusion and the two adaptation experiments), agar plates were checked for a zone of inhibition around each of the impregnated discs. This zone marks the effective inhibition of microbial growth. The diameter of each zone was measured in millimetres (mm) and recorded and assessed to determine the extent of antibacterial inhibition.

3.5 Data analysis

It was anticipated that the ANOVA Statistical method was to be used to analyse the data gathered (Devey, 2013). The ANOVA method was not used because there was no effect of the mouth wash on the bacteria.

3.6 Validity and Reliability

The growth media used with the Kirby-Bauer disk diffusion test was TSA agar with 5% sheep blood and chocolate blood agar. Using this agar type ensures that zones of inhibition can be reproduced from the same organism. The concentration of the cultures will be standardized using McFarland standards.

All experiments were done following aseptic techniques and each experiment was done in triplicate. Positive, negative and sterility testing controls were included in all the experiments.

3.7 Ethical considerations

Laboratory safety procedures were carried out. Safety glasses and gloves were worn; hands and utensils were washed before and after the experiments. A laboratory technician supervised the experimental procedures and oversaw that the researcher conducted herself in an appropriate manner at all times.

Ethics clearance was obtained to conduct this study. Ethics clearance number: AEC01-108-2014.

CHAPTER 4

RESULTS

4.1 Introduction

This study aimed to investigate the antimicrobial efficacy of a homoeopathic and herbal mouthwash formulation against organisms *Streptococcus salivarius* and *Fusobacterium nucleatum.* The primary objective of the study was to expose pathogenic *Fusobacterium nucleatum* microorganism and commensal microorganism *Streptococcus salivarius* to a homoeopathic and herbal mouthwash formulation, saline solution and distilled water to determine their effect *in vitro* on the above mentioned microorganisms. It was hypothesized that the homoeopathic and herbal mouthwash formulation would inhibit the growth production *in vitro* of *Streptococcus salivarius* and *Fusobacterium nucleatum*. This could be illustrated by exposing clear zones of inhibition in experimental plates. The disc diffusion method and two adaptation disc diffusion experiments were done to test the stated hypothesis of this study.

Thirty samples of *Streptococcus salivarius and Fusobacterium nucleatum* were prepared for this study. The study was conducted over a period of three weeks at the University of Johannesburg, Doornfontein campus, Water and Health Research Centre.

The first results that will be presented in this chapter are for the homoeopathic and herbal mouthwash formulation as prepared according to manufacture instructions. The initial experiment was followed by two experiments where the strength of the mouthwash formulation was increased and tested against the selected organisms.

4.1.1 Experiment one: Kirby-bauer disk diffusion method

The antimicrobial activity of the mouthwash formulation, saline solution and distilled water was determined by using the disc diffusion procedure. The mouthwash was prepared as per manufactures instructions. *Fusobacterium nucleatum* and *Streptococcus salivarius* cultures were adjusted to 0.5 McFarland standard before being used in the experiment. The experiments were done in triplicates and repeated three times. As shown in Figure 4.1 all the plates for all the solutions tested (mouthwash formulation, saline solution and distilled water) had good growth for *Streptococcus*

salivarius and *Fusobacterium nucleatum*. This indicated that the three solutions tested at the specific concentration do not have antimicrobial capabilities for *Streptococcus salivarius* and *Fusobacterium nucleatum* at a concentration of 0.5 McFarland standard.

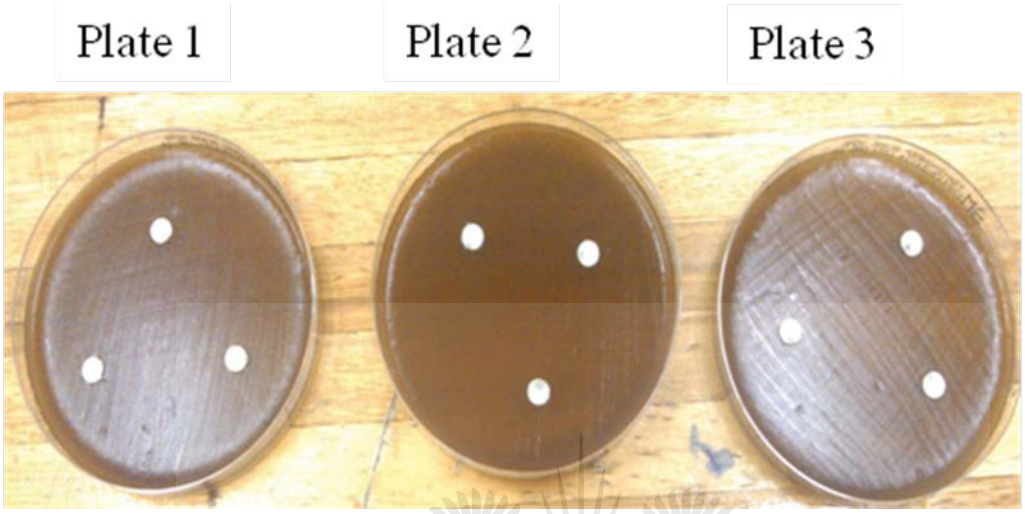

Figure 4.1 *Fusobacterium nucleatum* on chocolate blood agar for experiment one. From left to right: plate 1 (mouthwash formulation), 2 (saline) and 3 (distilled water)

4.1.2 Adaptation experiment one

Due to the results of the first experiment, an increased strength (two times and five times strength) of mouthwash solution was tested with *Streptococcus salivarius* and *Fusobacterium nucleatum* at a concentration of 0.5 McFarland standard. The aim of this experiment was to determine whether increasing the strength of the mouthwash solution will have an effect on the inhibition of *Streptococcus salivarius* and *Fusobacterium nucleatum.* The same controls, which are saline and distilled water, were included in the experiments. The experiments were done in triplicates and repeated three times. The increasing strength of mouthwash solutions did not have any effect on the good growth of *Streptococcus salivarius* and *Fusobacterium nucleatum* (Figure 4.2). The results for saline and distilled water were still consistent with the first experiment as expected. There were no zones of inhibition across all experimental plates (Appendix F).

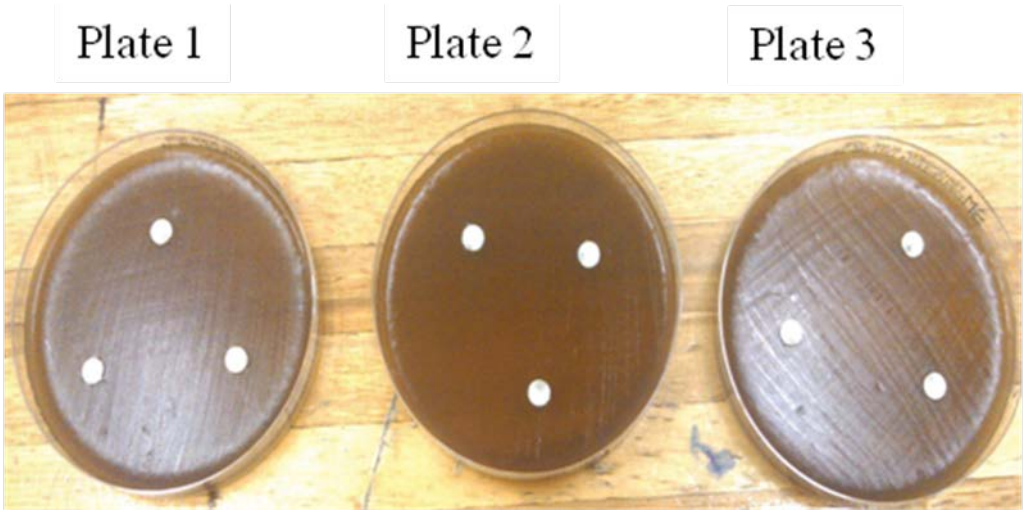

Figure 4.2 *Fusobacterium nucleatum* on chocolate agar. From left to right: Plate 1 (one time), Plate 2 (two times) and Plate 3 (five times) strength mouthwash solution

4.1.3 Adaptation experiment two

According to the manufacturer instructions the mouthwash is to be gargled on the mouth and throat for 30 seconds then expelled. With the current experiment the mouthwash exposure time was increased to 5 minutes. A volume of 100 µl (one time, two time and five time strength) mouthwash solutions was mixed with 10 µl fresh cultures of *S.salivarius* and *Fusobacterium nucleatum* in a 1 ml eppendorf tube. *Streptococcus salivarius* and *Fusobacterium nucleatum* were adjusted to a turbidity of 0.5 McFarland standards. The mixture was incubated for 5 minutes and streaked on TSA agar with 5% sheep blood and Nutrient agar plates for *Streptococcus salivarius* and *Fusobacterium nucleatum* culturing respectively. The aim of this experiment was to determine the effect of incubating *Streptococcus salivarius* and *Fusobacterium nucleatum* cultures for 5 minutes as compared to the 2 minutes described by the mouthwash manufacture. The bacteria were immersed in the mouthwash solution therefore, allowing direct contact with the active ingredients of the mouthwash solution. Incubating *S.salivarius* and *Fusobacterium nucleatum* cultures for 5 minutes in different concentration of the mouthwash solution did not have any effect on the growth of the two bacteria as shown by good growth of chocolate blood agar and TSA agar in (Figure 4.3). The same results were found for saline and distilled water (Appendix F).

Figure 4.3 *Streptococcus salivarius* one time strength

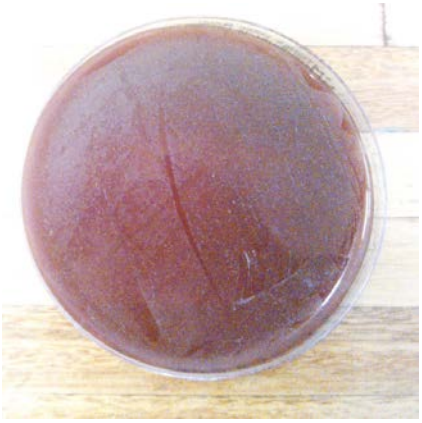

Figure 4.4 *Streptococcus salivarius* two time strength

Figure 4.5 *Streptococcus salivarius* five time strength

Figure 4.6 *Fusobacterium nucleatum* one time strength

Figure 4.7 *Fusobacterium nucleatum* two time strength

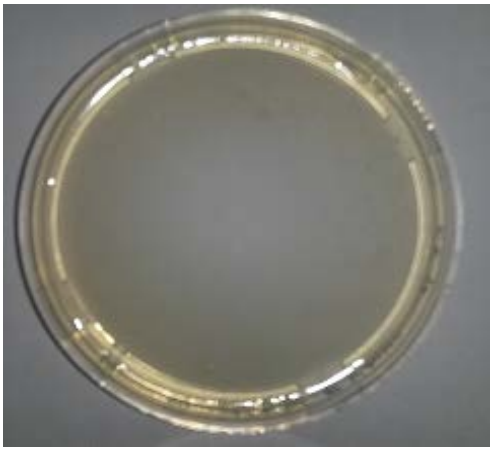

Figure 4.8 *Fusobacterium nucleatum* five time strength

From the results it was evident that there was no inhibitory effect of any of the test substances (mouthwash, saline and distilled water) on *Streptococcus salivarius* and *Fusobacterium nucleatum* using different susceptibility methods, increasing concentrations and longer incubation times. The mouthwash formulation showed no effect on the growth of the two bacteria.

CHAPTER 5

DISCUSSION

5.1 Introduction

This study aimed to determine the in vitro effect of a homoeopathic and herbal mouth wash formulation on the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*. *Streptococcus salivarius* is considered as a commensal bacterium within the oral cavity. It has a protective and probiotic action against opportunistic pathogenic bacteria. As a bacterium that is beneficial to maintaining oral health; oral rinses should therefore not aim to kill the bacteria. The tested homoeopathic and herbal mouthwash did not have an effect on the growth of *Streptococcus salivarius in vitro*. The discussion that follows is focused on possible factors affecting the *in vitro* results obtained for *Fusobacterium nucleatum* as it is the bacterium associated with periodontal disease.

5.2 Result summary

Results showed that the homoeopathic and herbal mouthwash formulation containing salt (119.5g), *Arnica montana 30cH, Echinacea purpurea 30cH, Staphysagria 30cH, Phosphorus 30 cH, Hepar sulphuris calcarea 30 cH*, Cinnamon (0.27g) and Liquorice (0.27g) had no effect on *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*. Comparisons between different concentrations of the mouthwash showed no significant difference in results.

5.3 Possible factors contributing to the results of the experiment

The Kirby-bauer disk diffusion method is not always reliable at determining the antimicrobial activity of natural antimicrobials such as plant extracts (Klarcnick *et al*., 2010). This is due to factors such as the polarity of natural compounds. Compounds with less polarity diffuse slower than more polar ones (Moreno *et al*., 2006). The extract of liquorice is reasonably polar (Texeira and Vicente, 2013) whilst cinnamon is non-polar (Yiu *et al*., 2008). The Kirby-bauer disk diffusion method is designed to test allopathic medicines therefore; this method may not be sensitive enough to test homoeopathic preparations due to the extreme dilutions of homoeopathic medicines.

Compared to allopathic mouthwashes such as chlorhexidine which have a quantifiable concentration of active ingredients, the homoeopathic remedies in the tested mouthwash are diluted to the point where they cannot the quantified. The homoeopathic remedies are diluted to the centesimal scale i.e 1:99, a diution at a concentration above 12 centecimal scale has no molecule of the original substance (Kayne, 2006). The only measurable active ingredient in the mouthwash would be liquorice, cinnamon (provided these are extracts and not artificial flavourants) and sea salt. Mouthwashes such as chlorhexidine have active ingredients with a concentration ranging from 0.12% alcohol based or 0.1-0.2% non-alcohol based. These concentrations have an antibacterial effect against pathogenic bacteria in the oral cavity when gargled for 30 seconds or 60 seconds. (Balagopal and Arjunkumar, 2013).

The saline control group showed no effect on the growth of *Fusobacterium nucleatum*. The saline solution was prepared as 9 grams in 1 liter of distilled water. This preparation pertains to normal saline or physiological saline; it is an isotonic solution that is also used for aseptic purposes. Normally this saline would inhibit growth of a bacterium as there is no nutritional value in the solution but, because it is isotonic there is therefore no net movement across the cell membrane of the tested microorganisms. This concentration of saline prevents lysis of cells making them more viable for growth. The pH range of normal saline is 6.5 at a temperature of 25 ^{o}C. *Fusobacterium nucleatum* can grow at a pH range of 5.0-7.0 (Comolli *et al*., 2015). *Fusobacterium nucleatum* can therefore grow in normal saline concentration.

Fusobacterium nucleatum is a gram negative bacterium surrounded by a double membrane as a structural envelope. Substances move across the double layered membrane through channels called porins. The outer membrane of gram negative bacteria function as a complex permeability barrier protecting the bacterium in hostile environments, substances such as antibiotics do not move freely across the cell membrane of the bacteria due to their polarity. Hydrophilic solutes usually diffuse freely across the membranes (Kleivdal *et al*., 2001). The homoeopathic remedies in the mouthwash are diluted beyond Avagrado's constant which indicates that after dilution; homoeopathic remedies do not contain physical molecules of the solute within the solvent. The latter indicates that there are no molecules of the active substance present in the solvent to diffuse across the bacterial cell membrane.

The manufacturer does not specify whether the Cinnamon and Liquorice in the mouthwash formulation are extracts or artificial flavourants. As artificial flavourants cinnamon and liquorice have no antimicrobial properties; artificial cinnamon can lead to contact stomatitis (Georgakopoulou, 2010). When combined with homoeopathic remedies artificial flavourants can antidote homoeopathic remedies thus affecting their curative properties.

The homoeopathic remedies were impregnated into a salt medium. Salt has not been established as a suitable substance for impregnating homoeopathic remedies. Homoeopathic remedies are impregnated onto vehicles such as pillules or granules made from lactose sugar or sucrose, powders made from lactose sugar and alcohol or purified water (Banerjee, 2006).

Homoeopathic remedies work on the vital force which acts as the energy maintains the functions of the body in harmony (Gunavante, 2000). The remedies stimulate the body's ability to heal itself. The illness is seen as a disturbance of the vital force within the organism (Hpathy, 2006).

In vitro studies are not able to reproduce the action of the vital force nor does it reflect the mental, emotional and physical characteristics of the body.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Periodontal disease is influenced by multiple factors which might have a systemic or hereditary component which an individual is susceptible to in addition to bacterial etiology. Conventional therapy alone may not be able to arrest the progression of periodontal disease (Kumar *et al.*, 2012).

The homoeopathic and herbal mouth wash formulation is indicated for the treatment of sensitive irritated gums, halitosis, sore throat and mouth ulcers (IVOhealth, 2010).

The research study aimed to determine the effect of the mouth wash formulation on the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*. Using the Kirby-bauer disk diffusion method and the two adaptation methods (Chapter 3) it was determined that the mouth wash formulation displayed no action against the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*.

The results recorded in Chapter 4 supports the null hypothesis that the mouth wash formulation has no effect on the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro* and rejects the hypothesis that states the mouth wash formulation has an effect on the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*.

6.2 Recommendations

- *In vivo* studies should be used to assess the effect of the mouth wash formulation. The study should be able to determine the effect of the mouth wash formulation as a first line treatment for sensitive gums, halitosis, bleeding gums, sore throat and mouth ulcers.
- Lower potencies of the homoeopathic remedies should be tested in isolation which may demonstrate the precise action of the remedies.

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APPENDIX A

WATER AND HEALTH RESEARCH CENTRE
FACULTY OF HEALTH SCIENCES

Water and Health Research Centre
PO Box 17011
Doornfontein
2028
14 May 2014

To: Whom it may concern

Re: Access to Water and Health Research Centre laboratories

This serves to confirm that Ms Samba A. De Oliveira (Student no. 820413284) has been allowed access to use the facilities and equipment at the Water and Health Research Centre to complete her laboratory work for the project titled "An *in vitro* study of the effect of Ora Salts™ Mouthwash on the growth of Bacteria *Streptococcus salivarius and Fusobacterium nucleatum*". Mr Vusi Ntema (Co-supervisor) will train Ms Samba De Oliveira on the proper use of the methods and equipment and oversee her work done in the laboratory.

Yours sincerely,

Dr TG Barnard
Director: Water and Health Research Centre
Faculty of Health Sciences
Tel 072 579 5748
Fax 011 559 6342 Email tgbarnard@uj.ac.za

APPENDIX B

McFarland Standard

There are various ways of estimating the population of microbes in suspension cultures. One of the easiest methods is through visual comparison with a stable and known standard--the McFarland Turbidity Standard.

McFarland Turbidity Standards are a set of tubes with increasing concentration of barium sulfate suspension. The cloudiness or turbidity created by barium sulfate's white precipitates is used as a point of comparison of bacterial suspensions to known bacteria's turbidity. Unlike culture broths which may change appearance through time, barium sulfate solutions are un-reactive, non-changing and stable for a span of at least 6 months. Hence, it can serve as a long-term ruler for turbidity. Known strains of bacteria have already been measured using McFarland standards and had their population determined relative to it. With such knowledge, comparison with those known bacteria is possible even with samples in other laboratories.

APPENDIX C

Materials

The following materials and equipment will be used in the study:

- Refrigerator
- Autoclave
- Incubator
- Marker pen
- Disinfectant
- Plate spreaders
- Petri dishes
- Mueller Hilton Agar
- Blank cartridge discs
- McFarland Standard 0.5
- *Streptococcus salivarius* (ATCC 13419)
- *Fusobacterium nucleatum* (ATCC 25586)

APPENDIX D

Nutrient agar

Nutrient agar is a simple basal medium used to grow common pathogens. The agar consists of peptone which helps control the pH of the agar; distilled water; beef extract which provides carbohydrates; nitrogen and vitamins; agar used as a solidifying agent and 0.5% sodium chloride.

The agar is prepared by mixing powdered agar with distilled water stirring constantly with a sterile rod over a flame. Mixture is boiled for 1 minute then removed from heat. It is cooled to a temperature of 50 ^{o}C then autoclaved at 121 ^{o}C for 15 minutes. Melted agar is poured into individual sterile plates. The agar plate is covered immediately with a lid and left to set and cool on a counter at room temperature. Agar plates are stored upside down to prevent condensation form dripping onto the agar surface. The plates are stored in a fridge at 4 ^{o}C.

APPENDIX E

Chocolate blood agar

Chocolate blood agar is a non-selective enriched growth medium used to grow fastidious bacteria such as streptococci. Red blood cells are lysed giving the medium a chocolate brown colour. The lysed red blood cells release haemoglobin, hemin (Factor X), and coenzyme Nicotinamide adenine dinucleotide (NAD or V Factor) utilized as nutrients for bacterial growth.

The agar plates are prepared by sterilizing nutrient agar by autoclave at 121 ^{o}C then cooled to 80 ^{o}C. Sheep blood is lysed by heating slowly in a water bath at a temperature of 50 ^{o}C. The lysed sheep blood is poured onto the nutrient plates allowed solidify and condensation to dry at room temperature. Plates are stored upside down in sterile plastic bags and stored at 4 ^{o}C.

APPENDIX F

Recording Zone Diameters Data: *Streptococcus salivarius*

		Day 1	Day 2	Day 3
		Millimetres (mm)	Millimetres (mm)	Millimetres (mm)
Mouthwash formulation				
Dilution	1X	0 mm	0 mm	0 mm
	2X	0 mm	0 mm	0 mm
	5X	0 mm	0 mm	0 mm
Kirby-Bauer Disk diffusion	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
Saline Solution				
Dilution	1X	0 mm	0 mm	0 mm
	2X	0 mm	0 mm	0 mm
	5X	0 mm	0 mm	0 mm
Kirby-Bauer Disk diffusion				
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
Distilled water				
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm

Recording Zone Diameters Data: *Fusobacterium nucleatum*

		Day 1	Day 2	Day 3
		Millimetres (mm)	Millimetres (mm)	Millimetres (mm)
Mouthwash formulation				
Dilution	1X	0 mm	0 mm	0 mm
	2X	0 mm	0 mm	0 mm
	5X	0 mm	0 mm	0 mm
Kirby-Bauer Disk diffusion				
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
Saline Solution				
Dilution	1X	0 mm	0 mm	0 mm
	2X	0 mm	0 mm	0 mm
	5X	0 mm	0 mm	0 mm
Kirby-Bauer Disk diffusion				
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
Distilled water				
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm
	10µL	0 mm	0 mm	0 mm

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