INFLUENCE OF CHEWING GUM CONTAINING NATURAL HOST PROTEINS WITH ANTIMICROBIAL PROPERTIES ON SALIVA IN SUBJECTS WITH HYPOSALIVATION

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A research report submitted to the Faculty of Health Sciences, School of Pathology, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science in Dentistry, 2014.

DECLARATION

I, Thanusha Devi Pillay, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Dentistry in the branch of Maxillo-facial and Oral surgery at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

Ethics clearance by the Committee for Research on Human Subjects (Medical) was granted for this study and the clearance certificate number is M120282. (Appendix A)

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DEDICATION

To my husband Clinton and our precious son Matthew

ABSTRACT

Biotène® products have been developed with the intention of preventing tooth decay, plaque accumulation and oral infections in individuals with xerostomia (dry mouth). Not much is known about the effect of Biotène® chewing gums. Biotène® chewing gum contains host proteins. Due to these contents the manufacturer claims that Biotène® chewing gum is an "enzyme gum" that "boosts and strengthens the mouths natural defences". The aim of this study was to investigate the effect of Biotène® chewing gum on saliva flow rates, saliva buffering capacity, plaque index, as well as salivary *Streptococcus mutans* and Lactobacilli counts, in healthy subjects with hyposalivation.

One hundred and nine subjects with an age range of 18 to 23 years were screened for hyposalivation. Hyposalivation is a reduced salivary flow rate in a subject based on examination of the subject. Thirteen healthy subjects, who initially presented with hyposalivation, were included in the study. A baseline laboratory analysis of saliva was performed. Saliva was collected at rest and with masticatory stimulation, and measured. Resting saliva is saliva produced without any stimulation and can be obtained by allowing the subject to passively drool into a sputum jar. Stimulated saliva is produced as a result of stimulation of the salivary glands and may be obtained by allowing subject to chew inert rubber tubing while expectorating into a sputum jar. Buffering capacity was performed on both the saliva samples. Plaque index and DMFT was measured. Bacterial counts such as *S. mutans* and Lactobacilli counts were performed on the stimulated saliva.

Subjects were given rubber tubing, xylitol chewing gum or Biotène® chewing gum to use for 2 weeks. A rubber tubing phase was introduced into the study to eliminate the effect of masticatory stimulation, which any chewing gum can provide. A xylitol-containing chewing gum (xylitol) phase was also introduced into the study in order to eliminate the effect of xylitol, as Biotène® chewing gum contains xylitol.

A second laboratory analysis of saliva was performed. After a two weeks wash out period the second test product was given and the same procedure was repeated with the third product.

The results showed that two weeks use of Biotène® chewing gum had no significant effect on the resting and stimulated saliva flows. It did not increase the buffering capacity of either the resting or stimulated saliva samples. Although it did not reduce the plaque index and *S. mutans* counts, it significantly reduced the Lactobacilli counts. Xylitol chewing gum, which was used as a control to eliminate the xylitol effect from the Biotène® chewing gum, significantly increased the stimulated saliva, reduced the plaque index and the salivary Lactobacilli count. Biotène® chewing gum which contains host proteins has no beneficial effects regarding saliva flow rate or against dental plaque and therefore against dental caries.

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NOMENCLATURE AND ABBREVIATIONS

BHT	Butylated hydroxytoluene
C. albicans	*Candida albicans*
CMC	Carboxymethylcellulose
CFU	Colony forming units
DMFT	Delayed Missing Filled Teeth
G	Grams
HIV	Human Immunodeficiency Virus
$OSCN^-$	Hypothiocyanite ions
HOSCN	Hypothiocyanous acid
µl	Microlitres
mg	Milligram
ml	Millilitres
min	Minutes
MBA	Mutans Bacitracin Agar
N	Number of samples
%	Percentage
PBS	Phosphate buffered saline
PI	Plaque index
SD	Standard Deviation
S. mutans	*Streptococcus mutans*
RA	Rogosa Agar

PREFACE

Many studies have investigated the effects of Biotène® products on hyposalivation. However not much is known about the effect of Biotène® chewing gums alone. Thus the purpose of this study was to investigate the effect of Biotène® chewing gum, which contains host proteins, on certain salivary parameters. The results of this study will establish whether Biotène® chewing gum improves certain salivary parameters which are responsible for the development of dental caries in hyposalivators.

1 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Saliva has many important functions in the oral cavity. Lack of saliva results in xerostomia (dry mouth) which may become extremely debilitating for patients as it leads to difficulties in speech, swallowing and taste. Lack of saliva may result in increased susceptibility to dental caries as saliva has many functions which prevent dental caries. These include its mechanical washing action, buffering capacity and antimicrobial functions. Xerostomia may be a result of certain medications, systemic conditions, cancer therapy to the head and neck regions, or dehydration. But xerostomia or hyposalivation may also affect normal, healthy individuals.[1, 2]

Salivary stimulants and salivary substitutes are commonly used for the treatment of xerostomia. Chewing gum offers masticatory as well as gustatory stimulation of the salivary glands. Biotène® products have been developed with the intention of supplementing the natural saliva with enzymes and proteins. This chewing gum contains lactoperoxidase and glucose oxidase as well as natural sugar alcohols, including xylitol according to the manufacturers packaging. No studies have focused specifically on Biotène® chewing gum. This study was undertaken in order to investigate the effect of Biotène® chewing gum on saliva flow rate, buffering capacity of saliva, plaque index and salivary levels of *Streptococcus mutans* and Lactobacilli, in healthy subjects with hyposalivation.

1.2 LITERATURE REVIEW

1.2.1 Saliva

Saliva is primarily composed of water, proteins and electrolytes.[3] It plays an important role in lubrication of the oral cavity, speech, swallowing and taste. Saliva contains mucin, which aids in the production of the food bolus, as well as lingual lipase and amylase which initiates the breakdown of fats and carbohydrates respectively. The mechanical washing action of saliva is important in removing food debris and unattached oral microorganisms. Saliva has a high buffering capacity which neutralises acids produced by bacteria on tooth surfaces. It is supersaturated with phosphate and calcium ions that aid in the remineralisation of teeth, as well as potassium bicarbonate which aids in the creation of a neutral pH. In addition it has advanced antimicrobial functions as it contains immunoglobulin A, histatins, lysozyme, lactoperoxidase, lactoferrin, agglutinins and defensins. Lysozymes hydrolyse the bacterial cell wall polysaccharides, which results in lysis of the cell. The lactoperoxidase system protects mucosal cells from the toxicity of hydrogen peroxide which is produced by oral bacteria.[4] Lactoferrin is an iron-binding glycoprotein which exhibits bacteriostatic and bactericidal activity against oral bacteria.[5]

Dental caries is an irreversible, infectious disease of the teeth which is characterised by demineralisation of the inorganic portion and destruction of the organic portion of the tooth which eventually leads to cavity formation.[6] It is a multifactorial disease which is dependent on a susceptible host, a host with a diet that is rich in fermentable carbohydrates, the presence of cariogenic bacteria and extended periods of time in which plaque is in contact with tooth surfaces. Risk factors for dental caries include poor oral hygiene, high levels of cariogenic bacteria, low fluoride levels in the water, low saliva flow rates and frequent exposure to fermentable carbohydrates.

Dental plaque is a colonisation of endogenous microorganisms on the tooth surface that causes tooth dissolution.[7] Endogenous micro-organisms which are capable of adhering to the salivary pellicle which has formed on the tooth surface, adhere to the tooth surface via the pellicle and also aid in the subsequent aggregation of other micro-organisms that were not capable of initial aggregation. Micro-organisms found in dental plaque include Streptococci, Staphylococcus, Actinomyces, Veillonella, Propionibact, Prevotella, Neisseria, Lactobacilli, Fusobact and Rothia.

S. mutans bind to the tooth pellicle via adhesins. The *S. mutans* then secrete glucosyl transferases which aid in the accumulation of more *S. mutans*. *S. mutans* are one of the initial colonisers of the tooth pellicle but they initially only comprise 1% of the colonising population.[8] *S. mutans* then metabolises fermentable carbohydrates and releases lactic acid which causes enamel demineralisation. Furthermore, this acidic environment causes aciduric organisms such as *S. mutans* and Lactobacilli to flourish. *S. mutans* are implicated in dental caries as *S. mutans* are aciduric and acidogenic, they rapidly metabolise sugar and there is a correlation between salivary counts of *S. mutans* and the prevelance of caries. Lactobacilli are implicated in dental caries as they are also aciduric and acidogenic, they are high in numbers in most carious lesions, their numbers in plaque and saliva increase with an increase in caries activity and they produce lactic acid below a pH of 5.0.

The oral fluids buffer the plaque on the tooth within 30-60 minutes with phosphates and bicarbonates. This results in remineralisation of the tooth enamel. However if there are repeated fluctuations in the pH and if the acid production outweighs the buffering effect of the saliva, the aciduric micro-organisms are allowed to multiply, produce even more acid and cause sufficient tooth demineralisation that will result in a dental cavity. Thus saliva is important in prevention of dental caries as a decrease in saliva allows cariogenic microorganisms to flourish.

A decrease in saliva may also result in difficulties with speaking, chewing, swallowing and tasting. Furthermore patients with hyposalivation may present with increased risk of dental caries [9], oral candidiasis[10], periodontal disease and poor retention of dentures.[11]

1.2.2 Xerostomia

Xerostomia can be defined as "a subjective sensation of a dry mouth that is frequently, but not always, associated with salivary gland hypofunction".[12] Symptoms of xerostomia start when there is a decrease of 45% in normal salivary flow.[13] A patient is considered to have reduced salivary flow if the unstimulated salivary flow is <0.1 ml/min measured for 5 to 15 minutes or if the chewing-stimulated salivary flow is < 0.7 ml/min measured for 5 minutes.[14]

1.2.2.1 Xerostomia and gender

Several studies have shown that xerostomia is more commonly found amongst females. A study conducted by Nederfors *et al* (1997) using a questionnaire on 4200 randomly selected individuals aged 20-80 years, found a prevalence of xerostomia in 21.3% of men and 27.3% of women.[15] Billings *et al* (1996*)* administered an oral health questionnaire and oral examination on 710 American adults with ages ranging from 19-88 years. Eighteen percent of males and 24% of females from this sample suffered from xerostomia.[16] Similarly a study in Sweden also showed 15% of men and 22% of women had an unstimulated saliva flow below 0.1ml/min.[2]

1.2.2.2 Xerostomia and medication

Xerostomia may be caused most frequently by dehydration, certain medications, diseases of the salivary glands, anxiety and radiation therapy to the head and neck. Ionising radiation can cause atrophy of the secretory components of both major and minor salivary glands resulting in xerostomia.[17]

There are certain drugs that may be commonly associated with xerostomia. These drugs include bronchodilators, antiparkinsonian drugs, tricyclic antidepressants, antipsychotics, decongestants, antihistamines, mydriatic eye drops, antihypertensives, drugs for urinary incontinence, drugs for irritable bowel and diverticular diseases, cytotoxics, antiepileptics and diuretics.[18, 19] People on xerogenic drugs produce significantly lower unstimulated and stimulated salivary flow rates than individuals not taking these drugs.[2] Many studies have shown that the incidence of xerostomia may be directly proportionate to the amount of these drugs used by the patient.[20, 21]

The level of radiation that is required to destroy malignant cells ranges from 40-70 Gy, yet salivary gland tissue may be permanently damaged when exposed to radiation dosages which are greater than 30 Gy.[22] Addington-Hall and McCarthy (1995) found that xerostomia was present in 30% of patients dying from cancer.[23] Davies (2000) reported the prevalence of xerostomia as more than 30% in a mixed group of cancer patients and 77% in cancer patients admitted to a hospice.[24] In a subsequent study they reported that in patients receiving chemotherapy for advanced cancer, the degree of xerostomia was proportionate to the number of chemotherapeutic agents used.[20]

1.2.2.3 Xerostomia and systemic illnesses

There are several systemic disorders which are also associated with salivary gland hypofunction. These disorders include Sjögren′s syndrome, diabetes mellitus, sarcoidosis, human immunodeficiency virus, primary biliary cirrhosis, systemic lupus erythematosus, rheumatoid arthritis, depression, and cystic fibrosis. [25] Up to 42% of patients with rheumatoid arthritis suffer with xerostomia[26] and patients with type I diabetes also have symptoms of dry mouth.[27] Furthermore up to 43% of diabetic patients complained of dry mouth.[28]

Sjögren′s syndrome is a chronic, inflammatory autoimmune disorder that is characterised by a lymphocytic infiltration of the salivary and lacrimal glands most commonly. This results in xerostomia and xerophthalmia. There are two forms of the disease, primary Sjögren′s syndrome and secondary Sjögren's syndrome. In primary Sjögren′s syndrome the xerostomia and keratoconjunctivitis sicca occur as an isolated clinical entity whereas in secondary Sjögren′s syndrome, the xerostomia and keratoconjunctivitis sicca occur together with another autoimmune disease. The estimated prevalence of Sjögren′s syndrome in the population is 0.6%, with the highest prevalence in the fourth or fifth decade of life. [25] Sjögren′s syndrome predominantly occurs in women over the fourth decade.[29]

1.2.3 Treatment of Xerostomia

Due to the diminished saliva output, patients who suffer from xerostomia are at higher risk for dental caries and other oral infections. Thus they should be advised of a good oral hygiene regimen and the importance of regular dental visits. These patients should be advised to take frequent sips of water and to avoid caffeine and alcohol in order to prevent dehydration.

A humidifier may also be used at night, when the xerostomia tends to worsen. Treatments for xerostomia may be divided into saliva substitutes and saliva stimulants.

1.2.3.1 Salivary substitutes

The most widely used saliva substitute is water, but milk also provides properties suitable for a salivary substitute. Milk provides excellent lubrication, and contains calcium and phosphate which aids in the buffering of acids as well as the remineralisation of teeth.[30] Saliva substitute most commonly refers to artificial saliva. Artificial saliva may contain carboxymethylcellulose (CMC), glycerate polymer gel base, natural mucins or a mucopolysaccharide and may be presented as a rinse, gel or spray.

Artificial salivas may provide excellent relief of dry mouth but artificial saliva is not tolerated by patients and does not last[19] and therefore, patients prefer saliva stimulants to saliva substitutes.[31]

1.2.3.2 Saliva stimulants

Various saliva stimulants are presently available for the treatment of xerostomia. These include sugar free chewing gums, organic acids and parasympathatomimetics. Organic acids such as malic acid, ascorbic acid and citric acid will increase salivation but these acids also result in demineralisation of teeth. Therefore long-term use is not recommended for the treatment of xerostomia. Parasympathatomimetics are used in severe cases of xerostomia in order to increase saliva production. Pilocarpine is a nonspecific, muscarinic agonist which results in the parasympathetic stimulation of the exocrine glands in order to increase serous secretions.

Pilocarpine is most commonly used in patients with Sjögren′s syndrome as well as patients that have received radiation therapy, in order to increase saliva flow. Nyárády *et al* (2006) conducted a prospective randomised study in order to assess the effectiveness of orally administered pilocarpine (Salagen®) during and after radiotherapy to the head and neck.[32] This study found that patients who received 5mg of pilocarpine orally three times a day, from the beginning of radiotherapy as well as patients who only commenced this treatment 6 weeks after start of radiotherapy, showed a significant increase in saliva production and decrease in symptoms related to xerostomia. Similarly Zimmerman *et al* (1997) also found that pilocarpine administered during radiation therapy increased saliva production and decreased the symptoms of xerostomia.[33]

However Gornitsky *et al* (2004) found no significant increase in saliva production in patients treated with pilocarpine during radiation therapy as compared to a control group.[34]

Furthermore, although pilocarpine may increase saliva flow, it is also associated with many parasympathetic side-effects such as gastro-intestinal disturbances, excessive sweating, increased pancreatic secretion, rhinitis, urinary disturbances, vasodilation and headaches. These side-effects have been shown to decrease patient compliance.[35] Due to its parasympathetic effects, pilocarpine is contraindicated in patients with uncontrolled asthma, gastric ulcers, narrow-angle glaucoma, hypertension and patients on β-blockers.

Cevimeline (Evoxac®) is another drug that is used to treat xerostomia in patients with Sjögren′s syndrome. Cevimeline, unlike pilocarpine, has specific affinity for receptor types that are not present in respiratory or cardiac tissue. Amifostine (intravenous), which is a thiol drug, is also used for the treatment of moderate to severe xerostomia in patients undergoing postoperative radiation treatment for head and neck cancer. Although Amifostine has the potential to reduce xerostomia during and post radiation treatment, a significant proportion of patients continue to experience xerostomia.[36]Although these parasympathatomimetic drugs may stimulate saliva flow and decrease the symptoms of xerostomia, their effects are not long lasting.

1.2.3.3 Acupuncture

Acupuncture involves the insertion of tiny needles at specific points, with the intent to prevent or cure diseases and symptoms.[37] Braga *et al* (2011) have shown that patients who received acupuncture treatments before and during the entire period of radiation therapy for head and neck cancer showed significant increase in saliva flow rates and decrease in xerostomia- related symptoms compared with patients in the control group, who did not receive acupuncture treatment.[38] Furthermore, the effects of acupuncture on the secretion of saliva can be maintained for up to 6 months and with additional therapy, this improvement on saliva secretion may be maintained for up to 3 years.[39, 40]

1.2.4 Mouthrinses, Gels and Chewing gums

Salivary glands can be stimulated by mechanical and gustatory stimuli, to increase the secretory capacity, for which gums and sucking tablets have been developed. They may contain antimicrobial compounds or enzymes and mouth wetting agents. Citric acid and vitamin C are also added into sucking tablets, which stimulates saliva flow.[4] Decreased mastication results in an increased atrophy of the salivary glands.[41] Therefore the salivary glands must be stimulated by masticatory or gustatory stimuli in order to prevent atrophy and to maintain saliva flow.

Sugar free chewing gum provides both masticatory and gustatory stimuli. Risheim and Arneberg (1993) found, in a study conducted on rheumatic patients, that sugar free chewing gum increases saliva flow by stimulating taste receptors.[42] Abelson *et al* (1989) found that 85% of saliva flow is related to gustatory stimulation and 15% is related to masticatory stimulation.[43] Therefore flavoured chewing gums stimulate saliva flow to a greater degree than unflavoured chewing gums.[44] Davies (2000) conducted a prospective randomised study on patients with advanced cancer. It was concluded that although both artificial saliva and chewing gum relieved xerostomia in these patients, more patients preferred chewing gum to artificial saliva.[24]

Xylitol is a natural sugar alcohol that is frequently added to chewing gums. *S. mutans* are unable to utilise xylitol resulting in less acid production and thus a decrease in plaque acidogenicity.[45] Furthermore xylitol also affects the adhesiveness of *S. mutans* to tooth surfaces.[46] It has been found that 6g/day of xylitol is required to affect the oral ecology.[47] Similarly, Autio (2002) and Caglar *et al* (2007) have shown the levels of *S. mutans* to decrease in response to xylitol-containing chewing gum.[48, 49] However, Twetman and Stecksén-Blicks (2003) found that although chewing xylitol containing gum decreased the

lactic acid concentration in supragingival plaque by 22%, in caries active children, as compared to chewing a control gum, the levels of *S. mutans* remained unaffected by both chewing gums. This could be due to the high counts of *S. mutans* due to the carious lesions.[50]

1.3 Biotène® products

Biotène® products including chewing gum, toothpaste, gel, spray and mouthrinse are available in South Africa through GlaxoSmithKline. These products contain three primary enzymes, glucose oxidase, lactoperoxidase, and lysozyme which can replenish the salivary antibacterial properties. They also contain fluoride, calcium and xylitol. These products are claimed to fight cavities, periodontal disease and oral infections caused by dry mouth, according to the manufacturers packaging.

The anti-microbial protein, lactoperoxidase, present in the Biotène® has been well researched. Lactoperoxidase system generated hypothiocyanite ions ($OSCN^-$) and hypothiocyanous acid (HOSCN) are inhibitory against a number of oral bacteria including mutans Streptococci.[51] In vitro studies with Biotène® dry mouth oral rinse have shown an antibacterial effect against *S. mutans* and Lactobacilli but not against *C. albicans*.[4] A study conducted in elderly, institutionalised individuals with xerostomia with Biotène® mouthwash, Biotène® Oralbalance gel and Biotène® toothpaste also showed no effect on *C. albicans* counts as well as on dry mouth sensation.[52] However, some studies have shown that Biotène® has no effect on oral bacteria. Lenander-Lumikari *et al* (1993) as well as Kirstilä *et al* (1994) found that Biotène® toothpaste did not induce antibacterial effects against total streptococci, *S. mutans*, Lactobacilli or the total anaerobic flora.[51, 53]

In addition, Kirstilä *et al* (1994) also found that plaque pH, acidogenicity and lactic acid production were unaffected by a 2 week daily use of Biotène®.[53] In a subsequent study Kirstilä *et al* (1996) showed that a 4 week Biotène® toothpaste and Biotène® mouthwash

regimen on 20 patients who suffered from chronic dry mouth symptoms resulted in a significant increase in the concentration of salivary hypothiocyanite and relieved dry mouth symptoms in xerostomic patients but once again there were no significant changes in the salivary microflora.[54] Even in young individuals with a high caries activity, use of Biotène® mouthrinse has shown no effect on the *S. mutans* counts.[55]

Although the above mentioned studies have shown that Biotène® has no effect on *S. mutans* counts, other studies have found Biotène® products to have many beneficial effects in the treatment of xerostomia.[56, 57, 58, 59, 60] Nagy *et al* (2007) conducted a randomised, double-blind, placebo-controlled clinical study on Biotène® products among 37 patients who had developed pronounced oral mucositis and xerostomia following radiation therapy. The results showed that there was a significant reduction in the counts of disease associated commensal oral aerobic and anaerobic bacteria. Furthermore there was a reduction in the counts of opportunistic candida species that are associated with radiation therapy. Most patients in the Biotène® group showed a 50-100% improvement in whole resting saliva.[56] Similarly, in the oral cavities of children, a significant reduction in *S. mutans* and Lactobacilli counts were found when they used Biotène® toothpaste. Furthermore the test group showed a significant increase in the levels of thiocyanate ions ($OSCN^-$) during the experimental as well as the washout periods, compared to the control group.[59]

The hypothiocyanite ion is an important antimicrobial agent which is generated by the peroxidase system. $OSCN^-$ has been shown to inhibit acid production by dental plaque [61], glucose uptake by cariogenic bacteria [62] and also inhibit the initial phases of dental caries.[63] In a double-blind crossover study of the Biotène® Oralbalance gel dry-mouth system and the BioXtra dry-mouth system in patients with post-radiotherapy xerostomia it was found that both systems were effective in alleviating symptoms of xerostomia. Both systems contain the hypothiocyanite ion.

The BioXtra system was superior in the alleviation of certain symptoms when compared to the Biotène® Oralbalance system. Furthermore, patients preferred the BioXtra system. Possible reasons for this could be because the viscosity of the BioXtra gel was 23 Pa.s which resulted in a longer retention time as compared to the Biotène® Oralbalance gel which has a dynamic viscosity of 16.8 Pa.s.[57] The BioXtra gel is formulated with minimal sweetening which may be better tolerated by patients and BioXtra also contains more peptides and immunoglobulins than Biotène® Oralbalance.

1.3.1 Biotène® chewing gums

The Biotène® range also includes Biotène® chewing gum which can be used during the course of the day in order to provide relief of oral dryness, reduction of odour-causing bacteria as well as stimulate saliva flow according to the manufacturer. Biotène® chewing gum contains Maltitol , Sorbitol , Gum Base , BHT , Xylitol , Artificial flavour , Titanium Dioxide , Lecithin , Resinous Glaze , Acesulfame K , Potassium Thiocyanate , Lactoperoxidase , Glucose Oxidase , Bees Wax and Carnauba Wax according to the manufacturers packaging.

Biotène® chewing gum in combination with other Biotène® products has been used to alleviate oral discomfort due to xerostomia. Improvement in oral discomfort and intraoral dryness following a two month treatment with the Biotène® system, composed of toothpaste, mouthwash and chewing gum, as well as Oralbalance gel have been reported in xerostomic patients receiving radiation therapy for head and neck cancer.[64] However, the authors did not evaluate the effect on the oral microflora.

Hyposalivation may occur in normal healthy individuals.[1, 2] The effect of hyposalivation may not be drastic and the patient may experience subtle long term discomfort and changes. In this case simple measures can provide comfort in their daily life and prevent long term changes.

Ultimately, the only Biotène® product that has not been subjected to extensive research is the chewing gum. Chewing gum might be more beneficial regarding saliva flow rate, acid clearance, accumulation of plaque and reduction of cariogenic bacteria as chewing gum stimulates saliva flow by offering mechanical stimulation of the salivary glands. In addition it is also convenient to chew gums during the course of the day without requiring water or a sink facility rather than using a mouthrinse or a gel.

1.4 AIM

This study investigated the effect of Biotène® chewing gum on the saliva flow rate, buffering capacity of saliva, plaque index and salivary levels of *Streptococcus mutans* and Lactobacillus in healthy subjects with hyposalivation.

1.5 OBJECTIVES

- To establish prevalence of hyposalivators in young adults
- To study the effect of Biotène® chewing gum on the saliva production by hyposalivators
- To examine the effect of Biotène® chewing gum on the buffering capacity and plaque index
- To investigate the effect of Biotène® chewing gum on the salivary counts of *Streptococcus mutans* and Lactobacilli.

2 MATERIALS AND METHODS

2.1 Study population

Students enrolled for the Bachelor of Dental Sciences at The University of The Witwatersrand were approached. Ethical clearance from The Human Research Ethics Committee was obtained. (Appendix A) The study was explained to all students and those that agreed to take part signed the consent form (Appendix B). One hundred and nine dental students aged between 18 and 23 years were screened for their resting saliva secretion. Resting saliva secretion was obtained by asking students to sit quietly, without talking, and to collect saliva into a sterile sputum jar for 5 minutes. The jars were then taken to the lab and the saliva volume in each jar was measured using a graduated pipette and recorded. Students with a resting saliva flow rate of less than 0.3ml/min were considered having hyposalivation and included in the study. Hyposalivation in young, healthy individuals is rare as seen from the small sample size obtained thus a resting saliva flow rate below 0.3 ml/min, instead of below 0.1 ml/min, was used as a selection criteria as this enabled a larger sample size. The design of the study is included in appendix C (page 61).

2.2 Baseline analysis

At baseline tests, resting and stimulated saliva samples were collected which were also used to measure buffering capacity of saliva. Stimulated saliva was also used to measure bacterial counts such as *S. mutans* and Lactobacilli. In addition, an oral examination was performed to determine decayed, missing, filled teeth index (DMFT) and plaque index (PI).

2.2.1 Collection of saliva

Each student was given a number in order to maintain confidentiality. A baseline resting saliva flow rate and stimulated saliva flow rate was taken for each subject. Resting saliva flow rate was obtained by allowing subjects to sit quietly without talking and expectorate

saliva into a sterile sputum jar for 5 minutes. The volume of saliva was measured using a graduated pipette and recorded. The volume of saliva was then divided by 5 in order to obtain saliva flow rate/min and was recorded as the resting saliva flow rate before the use of the test products which were either xylitol chewing gum or Biotène® chewing gum or the use of the saliva stimulant which was the inert rubber tubing.

The stimulated saliva flow rate was obtained by asking subjects to chew a sterile piece of rubber tubing while continuously collecting saliva into a sterile sputum jar for 5 minutes. The volume of saliva was then divided by 5 in order to obtain saliva flow rate/min and was noted as the stimulated saliva flow rate before the use of the test products or the saliva stimulant. One ml of stimulated saliva was also taken to conduct the buffering capacity test (section 2.2.2). The stimulated saliva was plated onto culture media for *S. mutans* and Lactobacilli counts (section 2.2.4).

2.2.2 Buffering capacity of saliva

One ml of each of the resting and stimulated saliva sample was transferred into sterile tubes Three ml of 0.005mol/L hydrochloric acid was then added into both the tubes. The two sample tubes were then closed, shaken and left open in order to allow carbon dioxide to escape. After 10 minutes the pH of the resting and stimulated saliva samples were measured using a pH meter.[65]

2.2.3 Oral examination and determination of "Decayed, missing, filled teeth" index

A Decayed, Missing, Filled, Teeth index (DMFT) was completed on each subject. A DMFT score was calculated for each subject in order to ensure that the subjects were similar regarding teeth affected by dental caries. This allowed a form of standardisation as the oral physiology of each individual is different.

The DMFT index was calculated in the following way:

1. The number of teeth with existing dental decay was counted.
2. The number of missing teeth was counted. (excluding third molars)
3. The number of teeth with fillings was counted. This included teeth that had composite fillings, amalgam fillings, crowns or veneers.
4. The total number of decayed, missing and filled teeth was then divided by the total number of teeth, in order to obtain a DMFT ratio for each subject.
5. The ratio was then multiplied by 100 in order to determine the percentage of teeth that were affected by dental caries.

An O'Leary plaque index was then completed on each subject in order to record plaque on tooth surfaces.[66] Disclosing solution was first applied to all supragingival tooth surfaces using a large cotton pellet. Each subject was then asked to rinse his/her mouth in order to remove excess disclosing solution. Each tooth surface, except for the occlusal surface was examined for the presence of stained deposits at the dentogingival junction. If there was a stained deposit present on a tooth surface, it was recorded on the appropriate box in the plaque index form. The plaque index was then calculated by dividing the number of tooth surfaces with stained deposits by the total number of tooth surfaces scored. This number was then multiplied by 100 in order to obtain a percentage.

2.2.4 *S. mutans* and Lactobacillus counts

Two Mutans Bacitracin Agar (MBA) plates were used for each subject to obtain *S. mutans* counts. Tenfold dilution (1:10) was prepared from the stimulated saliva by adding 0.1 ml into 0.9 ml phosphate buffered saline (PBS) which was further diluted by adding 0.1 ml of 1:10 dilution into 0.9 ml (1:100).

One hundred microlitres of each dilution was transferred on each of the MBA plates and evenly spread onto the surface using a sterile glass rod. Agar plates were incubated at 37°C for 48 hours under CO_2.

Two Rogosa Agar (RA) plates were used for each subject to obtain Lactobacillus counts. One hundred microlitres of stimulated saliva was transferred on to the RA plate surface (concentration) and spread with a sterile glass rod. In addition 0.1 ml was transferred into a 0.9 ml PBS solution (1:10 dilution) from which 0.1 ml was spread on a second RA plate. Agar plates were incubated at 37°C for 48 hours under CO_2. After incubation, the number of *S. mutans* colonies on the MBA plates (dark blue, rough colonies) and the number of Lactobacilli colonies on the RA plates (creamy white colonies) were counted. The number of *S. mutans* and Lactobacilli present in the saliva were calculated by multiplying the number of colonies on the plates by the dilution factor of the plate and 10 because only 0.1 ml was tested. The counts were expressed per ml of saliva.

2.3 Saliva stimulant or test products

Biotène® chewing gum, xylitol containing chewing gum (Stimorol®) and an inert rubber tubing was used in this study. Biotène® chewing gum also contains xylitol, therefore xylitol gums were included to eliminate the effect of xylitol from the Biotène® chewing gum. Rubber tubing was used as a control to eliminate the chewing effect from each of the products. Following the baseline saliva analysis (section 2.2) the subjects were firstly given xylitol chewing gum and instructed to chew one piece of chewing gum for 10 min after meals or approximately every 2 hours. A total of 5 pieces of gum were chewed each day for 2 weeks and the endpoint saliva analysis was then repeated (same as baseline analysis described in section 2.2) at the end of the 2-week period. After a wash off period (rest) of two weeks, the study was repeated with a repeat baseline saliva analysis. Instead of xylitol chewing gum, the subjects were asked to chew sterile pieces of inert rubber tubing.

The chewing instructions were the same as for the xylitol chewing gum. The end point saliva analysis was done after 2 weeks, in the same way as described in section 2.2. After a wash off period (rest) of two weeks, the study was repeated with a repeat baseline saliva analysis. Instead of the sterile pieces of inert rubber tubing, the subjects were now asked to chew Biotène® chewing gum. The chewing instructions were the same as for the xylitol chewing gum. The end point saliva analysis was done after 2 weeks.

2.4 End point saliva analysis

Following 2 weeks of xylitol chewing gum, rubber tubing, or Biotène® chewing gum usage, an end point saliva analysis was conducted, same as described in the section 2.2 baseline analyses. Subjects were asked to bring back empty packaging of xylitol chewing gum, rubber tubing or Biotène® chewing gum to ensure that all products were finished and to ensure the subject compliance.

2.5 Ethical considerations

The study protocol was approved by the Human Research Ethics Committee of the University of the Witwatersrand. Informed verbal consent was sought from participants. The following information was given to ensure that participants have information needed to make an informed choice; a complete description of the aims of the study, potential risks and benefits.

2.6 Statistical analysis

The STATA program was used to analyse the data. A two-tailed Wilcoxon signed-rank test was performed to establish if there was a difference between before and after values within a specific treatment option such as use of Xylitol chewing gum, Biotène® chewing gum and inert rubber tubing. The chosen significance levels of the tests i.e the p-value was 0.05.

A two-tailed Kruskal-Wallis equality-of-populations test was performed to establish if there was a difference between the three treatment modalities.

3 RESULTS

3.1 Hyposalivation

One hundred and nine healthy subjects, 41(37.6%) male and 68(62.4%) females, were screened for hyposalivation. Only 18 subjects were found to have less than or equal to 0.3 ml/min resting saliva and therefore they were considered hyposalivating subjects and included in the study. Of these 18 students only 13 agreed to participate in this study. In this small group of students that were screened, the prevalence of hyposalivation was 16.5%. (TABLE 1)

TABLE 1: Saliva production by subjects screened for hyposalivation

SUBJECT NUMBER	GENDER	SALIVA FLOW (ml/min)
1	F	0.6
2	F	0.4
3	M	0.65
4	F	0.65
5	F	**0.3**
6	F	0.65
7	F	0.6
8	F	0.47
9	F	**0.25**
10	F	0.9
11	F	**0.3**
12	M	0.9
13	M	0.4
14	F	0.4
15	F	0.57
16	M	0.6
17	M	0.9
18	F	1.4
19	M	0.9
20	F	1.0
21	M	1.2
22	M	1.5
23	F	0.65
24	M	1.5
25	F	1.6
26	F	0.35
27	M	**0.3**
28	F	0.8
29	F	0.45
30	F	0.45
31	M	0.35
32	M	0.7
33	M	1.2
34	M	1.0
35	M	0.35
36	F	0.45
37	F	**0.03**
38	F	0.65
39	F	1.4
40	M	0.53
41	F	0.54
42	F	1.5
43	M	0.5
44	M	**0.1**
45	F	1.2
46	F	2.8
47	F	**0.1**
48	M	1.5
49	M	1.1
50	F	0.34
51	F	**0.1**
52	F	1.0
53	F	1.14
54	F	**0.32**
55	F	1.1
56	F	0.9
57	F	0.8
58	F	0.8
59	M	0.4
60	F	0.46
61	M	0.5
62	F	1.1

SUBJECT NUMBER	GENDER	SALIVA FLOW (ml/min)
63	M	0.4
64	F	**0.3**
65	M	1.0
66	M	0.5
67	F	1.6
68	F	0.4
69	M	0.4
70	M	0.5
71	M	**0.3**
72	F	0.4
73	F	0.7
74	F	**0.2**
75	F	**0.24**
76	F	**0.24**
77	M	0.5
78	M	0.6
79	F	0.4
80	M	0.5
81	M	0.5
82	M	0.5
83	F	0.46
84	F	0.5
85	F	**0.3**
86	F	0.46
87	F	0.6
88	F	0.6
89	M	0.46
90	F	0.5
91	M	0.5
92	M	0.6
93	M	0.6
94	F	**0.24**
95	M	0.6
96	F	0.5
97	M	0.5
98	F	0.4
99	F	**0.16**
100	F	0.6
101	F	**0.26**
102	F	0.4
103	F	0.44
104	M	0.46
105	M	1.0
106	F	0.4
107	F	0.5
108	F	0.5
109	F	0.48
	Hyposalivating females=22% Hyposalivating males=7.3%	hyposalivating subjects=16.5%

F= Females
M=Male

Students with saliva flow less than or equals to 0.3 ml/min.

3.2 Saliva flow

Table 2 and figures 1 and 2 shows the resting and stimulated saliva flow rates of the hyposalivating subjects. Flow rate is expressed as millilitres of saliva per minute. The resting saliva sample was obtained while the subjects sat quietly while spitting into a sputum jar. The stimulated saliva sample was obtained while the subjects chewed a piece of inert rubber tubing while spitting into a sputum jar. Values were recorded before each treatment modality was started and also after 2 weeks use of each treatment modality and the control (rubber tubing, xylitol chewing gum or Biotène® chewing gum).

The mean resting saliva flow rates increased slightly after use of all three of the treatment modalities, with the greatest increase recorded after treatment with the xylitol chewing gum (Table 2, Figure 1). However none of the increases in the resting saliva flow rates among the three treatment modalities were considered statistically significant as none of the p-values were <0.05. It was seen that the mean stimulated saliva flow rate decreased after treatment with the rubber tubing. But this was not statistically significant.

The mean stimulated saliva flow rates increased following the use of both xylitol chewing gum and Biotène® chewing gum (Table 2, Figure 2). However, only the increase in the stimulated saliva flow rate after the xylitol chewing gum was considered statistically significant ($p=0.05$). The mean stimulated saliva flow rate decreased after treatment with the rubber tubing.

TABLE 2: Resting saliva flow and stimulated saliva flow of hyposalivating subjects before and after the use of rubber tubing (control), xylitol chewing gum and Biotène® chewing gum.

Subjects	Resting saliva flow (ml/min)						Stimulated saliva flow (ml/min)					
	Rubber tubing		xylitol chewing gum		Biotène® chewing gum		Rubber tubing		xylitol chewing gum		Biotène® chewing gum	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	1.0	0.9	0.55	0.3	0.8	1.0	1.94	1.14	1.32	0.8	1.66	2.0
2	0.6	0.9	0.2	1.3	0.7	1.12	1.6	1.54	1.82	2.14	1.28	1.7
3	0.4	0.34	0.55	0.3	0.3	0.4	0.84	0.62	0.77	0.9	0.74	0.7
4	0.3	0.3	0.3	0.7	1.1	0.8	1.24	1.14	0.74	1.0	1.5	1.4
5	0.5	0.9	0.4	0.3	0.46	0.4	1.04	0.94	0.64	0.54	0.68	0.74
6	0.6	0.4	0.4	0.4	0.42	0.3	0.84	0.84	0.44	0.84	1.1	0.62
7	0.64	0.64	0.4	0.5	0.7	0.62	0.82	0.74	0.64	0.84	0.34	0.88
8	0.4	0.4	0.4	0.9	0.5	0.42	0.56	0.7	0.64	0.94	0.3	0.68
9	0.8	1.1	0.5	0.8	0.82	1.4	0.9	1.4	0.8	1.2	1.22	1.74
10	0.2	0.24	0.3	0.1	0.1	0.2	0.58	0.3	0.4	0.4	0.38	0.3
11	0.76	0.72	0.5	1.3	0.6	0.8	0.82	0.9	0.84	1.4	0.64	1.38
12	0.14	0.38	0.1	0.1	0.1	0.22	0.32	0.7	0.26	0.56	0.4	0.42
13	0.7	0.74	0.4	0.54	0.7	0.5	1.0	0.9	0.84	0.7	0.86	0.68
Mean	**0.54**	**0.61**	**0.38**	**0.58**	**0.56**	**0.63**	**0.96**	**0.91**	**0.78**	**0.94**	**0.85**	**1.02**
SD	**0.24**	**0.28**	**0.13**	**0.4**	**0.29**	**0.37**	**0.43**	**0.33**	**0.4**	**0.45**	**0.46**	**0.55**
P value	**0.3067**		**0.1714**		**0.3449**		**0.4625**		**0.0589**		**0.1961**	

Figure 1: **Effect of the rubber tubing, xylitol chewing gum and Biotène® chewing gum on the resting saliva flow of subjects with hyposalivation.**

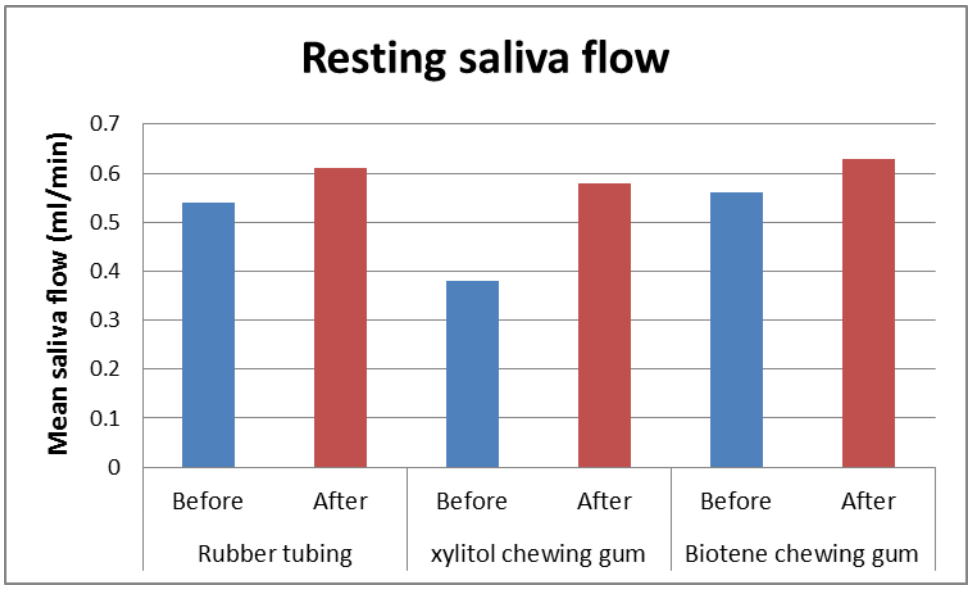
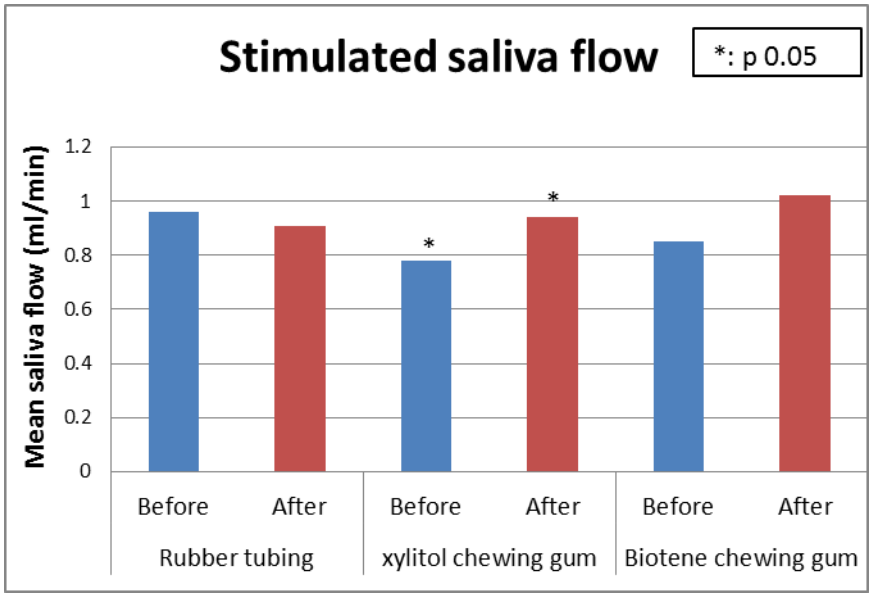


Figure 2: **Effect of the rubber tubing, xylitol chewing gum and Biotène® chewing gum on the stimulated saliva flow of subjects with hyposalivation.**



3.3 Buffering capacity

Table 3 shows the resting buffering capacity of the saliva and the stimulated buffering capacity of the saliva in the thirteen subjects. The resting buffering capacity was obtained from the resting saliva sample in each subject whereas the stimulated buffering capacity was obtained from the stimulated saliva sample in each subject. Both resting and stimulated buffering capacities were recorded for each subject before and after each treatment modality.

The mean resting buffering capacity was shown to increase after treatment with rubber tubing but decrease after treatment with xylitol chewing gum and also Biotène® chewing gum (Figure 3). However, none of these changes were statistically significant as the p-values were > 0.05.

The mean stimulated buffering capacity of the saliva was again shown to increase after treatment with rubber tubing but decrease after treatment with xylitol chewing gum and Biotène® chewing gum (Figure 4). None of these changes were said to be statistically significant as none of the p-values were < 0.05.

TABLE 3: Buffering capacity of resting and stimulated saliva in subjects with hyposalivation before and after the use of rubber tubing (control), xylitol chewing gum and Biotène® chewing gum.

Subjects	Buffering capacity of resting saliva (pH)						Buffering capacity of stimulated saliva (pH)					
	Rubber tubing		xylitol chewing gum		Biotène® chewing gum		Rubber tubing		xylitol chewing gum		Biotène® chewing gum	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	4.08	3.67	3.26	3.94	3.62	4.91	5.81	4.94	4.82	4.75	5.59	6.15
2	3.85	4.21	3.94	3.23	3.85	4.01	3.90	4.91	4.20	3.31	4.00	4.48
3	4.16	4.14	3.60	3.36	4.52	3.55	3.60	4.70	3.10	3.35	4.85	4.65
4	5.62	5.09	3.34	3.43	4.44	5.75	4.92	6.02	3.58	3.66	5.15	6.00
5	2.96	2.71	2.94	2.86	5.35	4.03	3.15	3.09	3.48	3.11	5.83	4.91
6	3.81	3.52	2.94	3.18	3.42	2.93	3.41	3.83	2.97	3.35	4.32	3.12
7	4.26	3.92	3.63	3.31	5.28	4.91	5.24	4.33	3.63	4.45	5.81	5.80
8	2.94	5.24	2.88	3.44	4.79	3.91	3.04	3.99	2.83	3.10	4.58	3.26
9	3.64	5.24	5.45	4.92	5.58	4.82	3.59	5.27	4.88	5.24	5.75	4.76
10	3.69	3.74	2.83	3.04	3.14	3.00	3.45	3.54	2.81	2.69	3.36	2.85
11	4.77	4.66	4.65	4.47	4.86	3.99	4.55	4.33	4.87	4.03	4.57	4.20
12	4.33	4.25	4.27	3.80	3.62	4.63	4.77	4.62	3.84	4.93	4.42	4.64
13	4.64	3.93	3.75	4.19	3.75	4.52	5.05	3.67	4.53	3.37	3.96	4.31
Mean	**4.06**	**4.18**	**3.65**	**3.63**	**4.32**	**4.23**	**4.19**	**4.40**	**3.81**	**3.80**	**4.78**	**4.54**
SD	**0.72**	**0.74**	**0.78**	**0.6**	**0.81**	**0.80**	**0.90**	**0.79**	**0.78**	**0.80**	**0.80**	**1.05**
P value	**0.4631**		**0.8339**		**0.7532**		**0.3822**		**0.9721**		**0.2787**	

Figure 3: **Effect of the rubber tubing, xylitol chewing gum and Biotène® chewing gum on the buffering capacity of resting saliva in subjects with hyposalivation.**

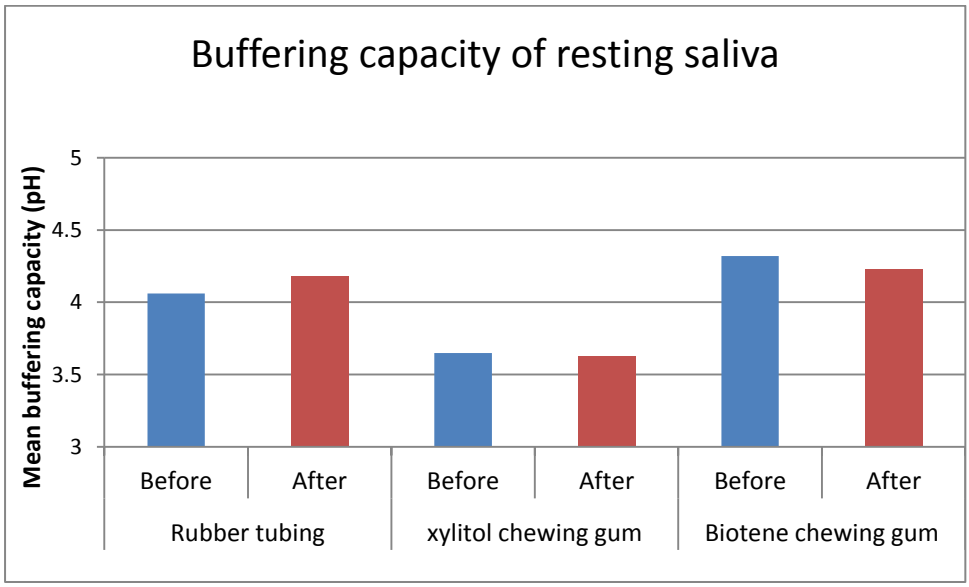
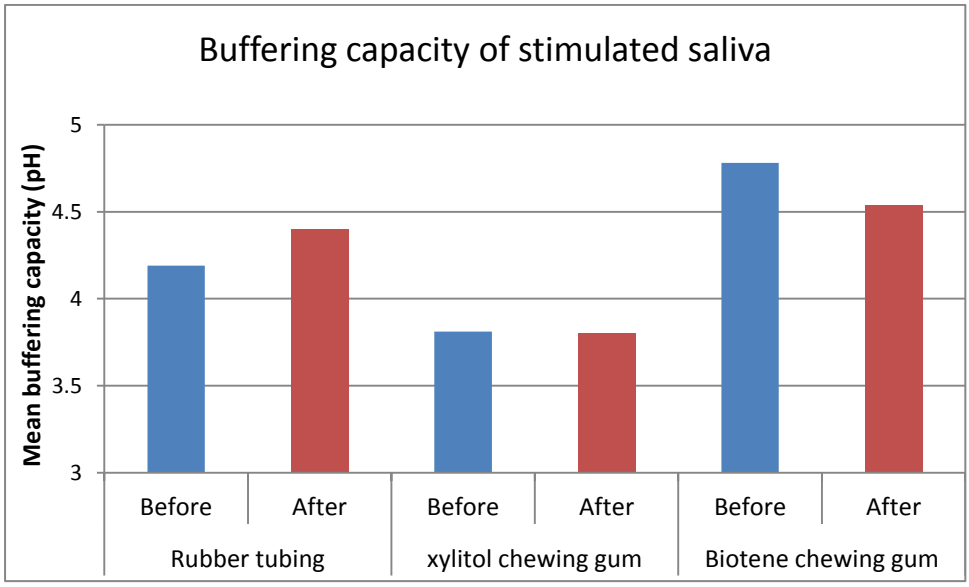


Figure 4: **Effect of the rubber tubing, xylitol chewing gum and Biotène® chewing gum on the buffering capacity of stimulated saliva in subjects with hyposalivation.**



3.4 DMFT and Plaque index

Table 4 shows the plaque indices of the study subjects. A plaque index was recorded before and after use of each treatment modality. The plaque index was expressed as a percentage. The mean plaque index decreased after treatment with the rubber tubing and the xylitol chewing gum but remained unchanged after treatment with the Biotène® chewing gum. However, only the plaque index decrease following xylitol chewing gum treatment was considered statistically significant with a p-value of 0.0019 (Table 4 and Figure 5).

Table 5 shows the total number of teeth present in each of the subjects as well as their DMFT scores. The DMFT scores were used to ensure that the subjects were standardised and that they did not have too many teeth that were affected by dental caries. The DMFT ratio describes how much of each subject's dentition has been affected by dental caries. The DMFT percentage provides the percent of decayed, missing and filled teeth present in each subject. The DMFT ratio ranged from 0 to 0.32. Therefore the DMFT ranged from zero teeth being affected by dental caries to 32% of teeth being affected by dental caries. There was a mean DMFT ratio of 0.08 with a mean DMFT percentage of 8.1%. Therefore there was a mean of 8.1% of teeth that were affected by dental caries.

TABLE 4: Plaque index in subjects before and after the use of rubber tubing (control), xylitol chewing gum and Biotène® chewing gum.

Subjects	Plaque Index (%)					
	Rubber tubing		xylitol chewing gum		Biotène® chewing gum	
	Before	After	Before	After	Before	After
1	66.9	75.8	70.5	46.4	53.5	53.5
2	25	46	74.2	49.2	41.4	31.25
3	66.6	30.8	88.3	33.3	30	43.3
4	57.1	66.9	78.5	56.2	26.7	31.2
5	53.5	58	84.8	66.9	44.6	18.75
6	19.7	8.3	67.7	50	8.3	10.4
7	52.6	44.6	83.9	84.8	45.5	53.5
8	55	25	73.9	30	23.9	21.8
9	23	44.2	73	14	38.4	37.5
10	26.6	29.1	66.6	29	34	46.6
11	33	30.3	100	21.4	10.7	12.5
12	28.5	22.3	59.8	44.6	31	32.1
13	62.5	16.9	92.8	66	17.8	15.1
Mean	**43.8**	**38.3**	**78**	**45.5**	**31.2**	**31.3**
SD	**18.0**	**19.9**	**11.4**	**20.0**	**13.7**	**15.0**
P value	**0.5067**		**0.0019**		**0.6245**	

Figure 5: Effect of the rubber tubing, xylitol chewing gum and Biotène® chewing gum on the plaque index of subjects with hyposalivation.

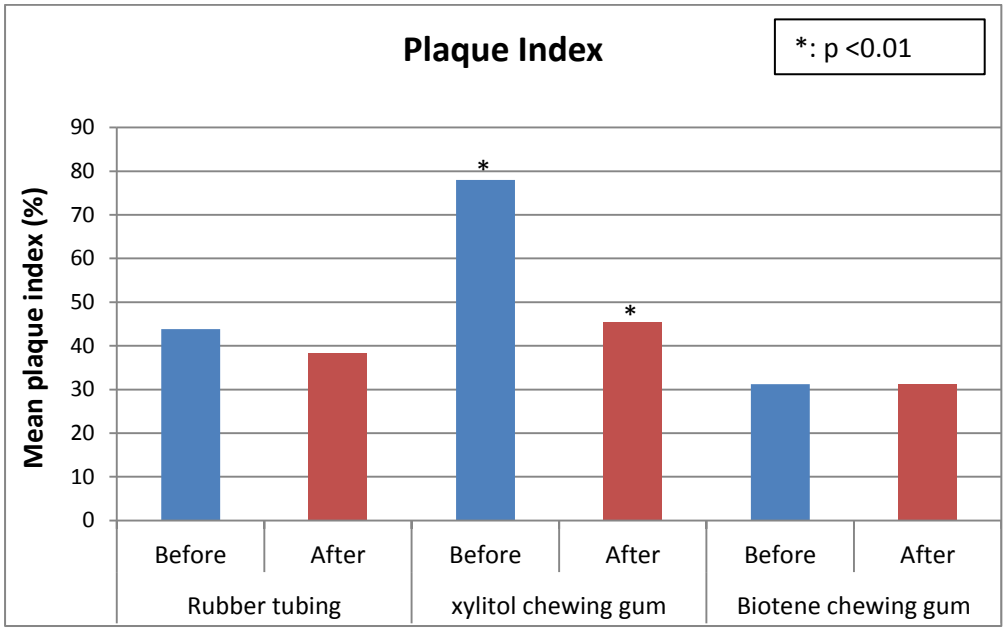


TABLE 5: DMFT results of hyposalivating subjects. (n=13)

SUBJECT NUMBER	TOTAL NUMBER OF TEETH PRESENT	DMFT RATIO	DMFT PERCENTAGE (%)
1	28	0	0
2	28	0.32	32
3	28	0	0
4	28	0.04	4
5	28	0.04	4
6	24	0.04	4
7	28	0	0
8	28	0.21	21
9	26	0.12	12
10	28	0.07	7
11	24	0.17	17
12	28	0	0
13	28	0.04	4
MEAN	27.2	0.08	8.1
SD	1.5	0.1	9.8

3.5 *S. mutans* and Lactobacilli counts

Table 6 and Figure 6 show the quantity of *S. mutans* and Lactobacilli present in the saliva of the thirteen subjects. These values were expressed as colony forming units per millilitre of saliva and were obtained before and after each treatment modality for each subject. The mean *S. mutans* counts decreased following treatment with rubber tubing and xylitol chewing gum. The decrease in *S. mutans* count was only statistically significant following rubber tubing treatment ($p=0.0015$). The mean *S. mutans* count following treatment with Biotène® chewing gum was shown to increase but this was not statistically significant.

The mean Lactobacilli count increased following treatment with rubber tubing but this was not statistically significant. The mean Lactobacilli count was shown to decrease following treatments of xylitol chewing gum and Biotène® chewing gum. These decreases in Lactobacilli counts were considered statistically significant ($p<0.05$). (Table 6, Figure 7)

TABLE 6: *S. mutans* and Lactobacilli counts in hyposalivating subjects before and after the use of rubber tubing (control), xylitol chewing gum and Biotène® chewing gum.

Subjects	*S. mutans* count (CFU/ml)						Lactobacilli count (CFU/ml)					
	Rubber tubing		xylitol chewing gum		Biotène® chewing gum		Rubber tubing		xylitol chewing gum		Biotène® chewing gum	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	1.5×10^5	6.2×10^4	5.1×10^4	5.0×10^5	4.2×10^4	5.0×10^4	0	0	1.3×10^2	0	0	1.5×10^2
2	Overgrowth	9.5×10^3	1.1×10^5	5.6×10^5	1.5×10^5	5.8×10^5	0	0	0	0	0	0
3	1.5×10^5	7.1×10^5	3.5×10^5	1.9×10^6	2.2×10^5	1.9×10^5	5.0×10^1	0	1.6×10^2	4.5×10^2	3.0×10^1	0
4	2.3×10^5	3.8×10^4	3.6×10^5	9.2×10^4	9.0×10^3	1.4×10^5	1.4×10^4	3.2×10^3	1.3×10^4	1.5×10^4	5.2×10^3	4.2×10^3
5	6.5×10^4	1.6×10^4	3.1×10^6	1.5×10^5	2.3×10^4	2.3×10^3	0	1.0×10^1	0	0	4.0×10^1	1.0×10^1
6	8.5×10^3	2.1×10^6	6.6×10^5	4.1×10^5	4.7×10^5	2.0×10^5	0	1.5×10^5	2.5×10^4	2.8×10^3	1.1×10^3	3.5×10^2
7	1.5×10^5	1.2×10^5	2.5×10^5	2.4×10^5	4.2×10^5	4.2×10^5	0	1.0×10^1	1.6×10^2	0	1.0×10^1	2.7×10^2
8	7.1×10^4	8.6×10^3	2.8×10^4	3.0×10^3	9.3×10^3	1.6×10^4	0	0	0	0	0	0
9	7.4×10^4	8.6×10^4	2.3×10^4	4.5×10^4	2.4×10^4	2.3×10^5	1.8×10^2	1.4×10^3	1.6×10^3	9.3×10^2	7.0×10^1	9.5×10^2
10	1.4×10^5	5.3×10^5	1.9×10^6	5.1×10^5	8.1×10^5	2.2×10^6	6.2×10^4	1.0×10^4	2.8×10^4	TNTC	9.8×10^4	TNTC
11	1.5×10^6	5.7×10^5	1.1×10^6	2.6×10^5	4.3×10^5	2.6×10^4	9.5×10^3	7.7×10^3	5.3×10^3	8.2×10^2	1.3×10^4	9.6×10^3
12	2.1×10^5	7.4×10^5	8.4×10^5	3.0×10^5	2.7×10^5	2.1×10^5	5.0×10^1	9.5×10^3	1.5×10^2	2.6×10^2	1.4×10^3	8.5×10^1
13	2.4×10^6	4.8×10^5	1.7×10^5	3.3×10^5	4.8×10^5	2.1×10^5	1.8×10^3	3.6×10^3	4.3×10^3	1.9×10^3	1.4×10^4	2.2×10^3
Mean	**7.7×10^5**	**5.9×10^5**	**9.0×10^5**	**4.8×10^5**	**2.4×10^5**	**5.8×10^5**	**1.7×10^4**	**4.1×10^4**	**9.8×10^3**	**4.4×10^3**	**2.7×10^4**	**2.8×10^3**
SD	**152500**	**117000**	**348000**	**300000**	**220500**	**198250**	**50**	**1425**	**160**	**445**	**70**	**265**
P value	**0.0015**		**0.3824**		**1.0000**		**0.6981**		**0.0057**		**0.0058**	

TNTC-too numerous to count or number OVERGROWTH- plate had overgrowth of gram –ve organisms obscuring *S. mutans*

Figure 6: **Effect of the rubber tubing, xylitol chewing gum and Biotène® chewing gum on the *S. mutans* counts of subjects with hyposalivation.**

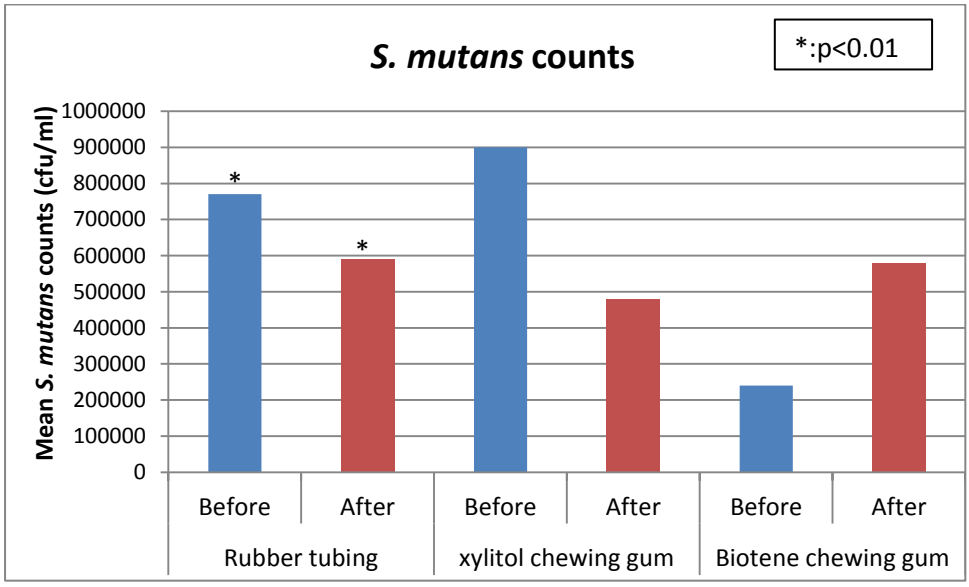
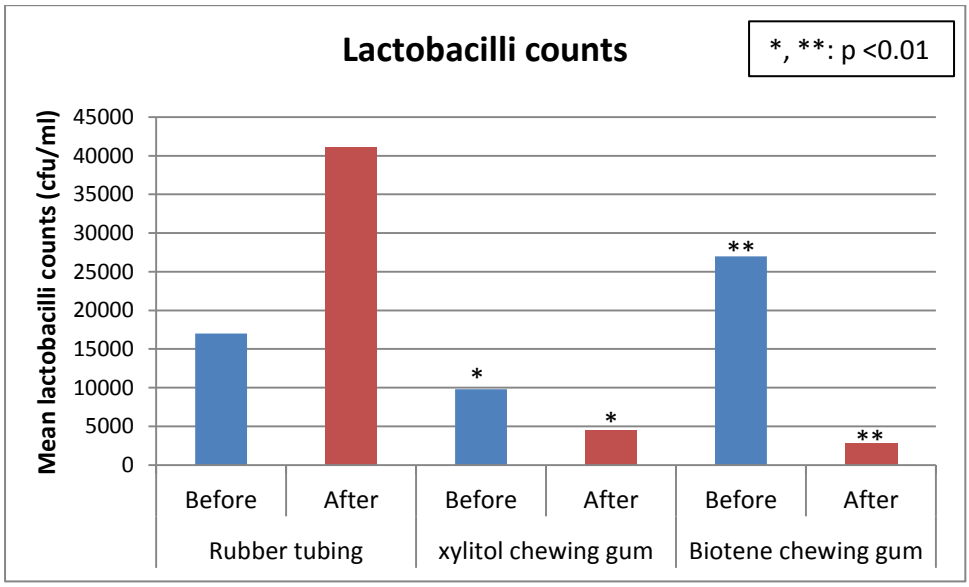


Figure 7: **Effect of the rubber tubing, xylitol chewing gum and Biotène® chewing gum on the Lactobacilli counts of subjects with hyposalivation.**



3.6 Summary results of test parameters after the use of test products.

Table 7 shows a comparison of each treatment modality within each different parameter. The p-values for each parameter have been noted as well as the difference in the mean before and after values which are represented as a percentage.

The changes in resting saliva flow were not statistically significant with all treatment modalities ($p>0.05$). The changes in stimulated saliva flow following use of rubber tubing and Biotène® were not statistically significant ($p>0.05$) but was statistically significant ($p=0.05$) following the use of xylitol chewing gum. The changes in resting and stimulated buffering capacity following use of all three products were not statistically significant ($p>0.05$). The changes noted in the plaque index following the uses of rubber tubing and Biotène® chewing gum were not statistically significant ($p>0.05$), however the change in plaque index following the use of xylitol chewing gum was statistically significant ($p<0.01$). The change in *S. mutans* count was statistically significant ($p<0.05$) following the use of rubber tubing but not significant ($p>0.05$) following use of xylitol chewing gum or Biotène® chewing gum. The change in Lactobacilli count was not statistically significant ($p>0.05$) following use of rubber tubing but was statistically significant ($p<0.05$) following use of xylitol chewing gum and Biotène® chewing gum.

Table 7: Summary results of test parameters after the use of test products.

Tests	% Increase (+) or (-) decrease (p value)		
	Rubber tubing	xylitol chewing gum	Biotène® chewing gum
	Before and After compared	Before and After compared	Before and After compared
Resting saliva flow	+13.0 (0.3)	+52.6 (0.17)	+12.5 (0.34)
Stimulated saliva flow	-5.2 (0.46)	+20.5 (0.05)	+20.0 (0.19)
Buffering capacity of saliva at rest	+3.0 (0.46)	-0.5 (0.83)	-2.1 (0.75)
Buffering capacity of saliva after stimulation	+5.0 (0.38)	-0.3 (0.97)	-5.0 (0.27)
Plaque index	-5.5 (0.5)	-32.5 (<0.01)	+0.1 (0.62)
***S. mutans* counts**	-23.4 (<0.01)	-46.7 (0.38)	+141.7 (1.0)
Lactobacilli counts	+141.2 (0.7)	-55.1 (<0.01)	-89.6 (<0.01)

Saliva flow and buffering capacity should increase. Plaque index and bacterial counts should decrease.

4 DISCUSSION

4.1 Saliva production by subjects screened for hyposalivation

109 subjects were screened for hyposalivation. We found that 16.5% of these subjects were hyposalivators with a resting saliva flow rate that was less than or equal to 0.3ml/min. Our results are thus in agreement with Yamamoto *et al* (2011) who found that hyposalivation does occur in healthy, young individuals.[1]

Our results also showed that 7.3% of males and 22% of females were hyposalivators (resting saliva flow rate less than or equal to 0.3ml/min). Thus our study showed that hyposalivation was much more prevalent amongst females than men, which is in agreement with other studies.[2, 16] It is rare for hyposalivation to be present amongst young, healthy individuals. A possible explanation for the presence of hyposalivation amongst this group of subjects may be due to stress.

4.2 Resting and stimulated saliva flow rates

Saliva is important for the maintenance of good oral health as it provides immunological protection, aids in the production of the food bolus, provides lubrication and also provides an ion reservoir which contributes to the remineralisation of teeth. Furthermore a constant flow of saliva is required to eliminate bacteria, plaque and food debris. Saliva can be collected at rest or with stimulations. Resting saliva flow rate can be defined as the flow of saliva which occurs in the absence of any physiological or oral stimulation whereas the stimulated saliva flow rate is defined as the flow of saliva which occurs in the presence of oral or physiological stimulation.

Our results showed that there were increases in resting saliva flow rates following the use of rubber tubing (13%), xylitol containing chewing gum (52.6%) and Biotène® chewing gum (12.5%). This suggests that if the salivary glands are constantly stimulated due to

masticatory stimulation, which was provided by all three test products, there will be an increase in saliva flow rate. The greatest increase in resting saliva flow rate was noted following the use of xylitol containing chewing gum (52.6%), which suggests that xylitol provides better gustatory stimulation compared to the inert control or enzyme containing Biotène®. Although all the test products increased the resting saliva flow, there was no statistical significance in any of the groups.

Various saliva substitutes and stimulants have been developed in order to alleviate the debilitating effects of dry mouth. Chewing gums have proven to be a popular treatment alternative amongst hyposalivators and offer masticatory stimulation as well as gustatory stimulation.[24] However if the hyposalivation has occurred due to damage to the salivary glands, this regime may not provide benefit. These products can provide benefit if the hyposalivation has occurred due to other reasons such as use of medication or due to dehydration.

In our results there were increases in stimulated saliva flow rates following use of xylitol chewing gum (20.5%) as well as following the use of Biotène® chewing gum (20%). The increase following use of xylitol chewing gum was statistically significant with a p-value of 0.05. These results are not unexpected as chewing gums have been shown to offer gustatory stimulation of the salivary glands.[24] However, a decrease of 5.2% was noted in the stimulated saliva flow rate following use of the rubber tubing which cannot be explained.

The xylitol chewing gum was shown to be most effective in increasing both resting and stimulated saliva flow rates. A possible explanation for this may be that xylitol chewing gum offers a better taste than rubber tubing or Biotène® chewing gum. Sugar free chewing gum increases saliva flow by stimulating taste receptors.[42]

Furthermore, Dawes and Kubleniec (2004) found that a twofold increase in saliva flow rate occurs when the sweeteners and flavour is at a maximum and this increase is then maintained for as long as the gum continues to be chewed.[67] Thus xylitol chewing gum produced the best result in increasing resting and stimulated saliva flow rates possibly due to a more acceptable gustatory stimulus.

4.3 Buffering capacity of resting and stimulated saliva

The buffering capacity of saliva is very important in the maintenance of a normal salivary pH of about 6.6 in resting saliva and 7.4 for stimulated saliva.[68] The mechanism of buffering capacity is dependent on the concentration of bicarbonate ions present in the saliva. Due to repeated acid exposure in the oral cavity, there is an increase in hydrogen ions which results in a decrease in the pH of saliva. Demineralisation of enamel occurs after the pH of saliva drops below 5.5. Carbonic anhydrase is an enzyme which catalyses the reaction between the free hydrogen ions and the bicarbonate ions, which are present in the saliva, resulting in the production of carbon dioxide gas and water, which is in turn expelled from the oral cavity. Therefore if there are more bicarbonate ions present in the saliva, more free hydrogen ions will be bound and the pH of saliva will return to normal faster resulting in less damage to the enamel. The buffering capacity of saliva has been shown to have a positive correlation with salivary flow rate [69], thus any factor that decreases saliva flow rate will also decrease its buffering capacity.[70]

Our results showed a positive correlation between saliva flow rate and buffering capacity following the use of rubber tubing. They however failed to show a positive correlation between saliva flow rate and buffering capacity for xylitol chewing gum and Biotène® chewing gum which is contrary to previous findings.[71]

Fenoll-Palomares *et al* (2004) found, in an observational prospective study on 159 volunteers, that there was a positive correlation between saliva flow rate and bicarbonate concentration.[71]

4.4 Plaque index and DMFT score

A plaque index is calculated in order to measure the state of oral hygiene of an individual, as it is able to assess the level of plaque accumulation on tooth surfaces. The plaque index is often used by dental practitioners for dental education purposes and also to monitor oral hygiene progress of patients. The O'Leary plaque index is commonly used and was also used in our study.[66]

Our results showed a decrease in plaque index to have occurred after use of rubber tubing (5.5%) and xylitol chewing gum (32.5%). The decrease in plaque index following the use of xylitol chewing gum was statistically significant ($p=0.0019$). Xylitol chewing gum has been shown to significantly decrease the plaque index in children.[72, 73] Increased saliva flow often eliminates bacteria, plaque and food debris. Thus from our results it can also be seen that there is a positive correlation between saliva flow and a decreased plaque index, as xylitol chewing gum was shown to be most effective in increasing saliva flow (by 53%) as well as decreasing plaque index by 32.5%.

Biotène® chewing gum had no effect on the plaque index which shows that although this gum had antimicrobial enzymes, it did not reduce the amount of plaque. Biotène® chewing gum also contains xylitol. One possible explanation for this could be that the levels of xylitol in the Biotène® chewing gum may not be as high as that in the xylitol chewing gum.

The DMFT ratios are an indication of how many teeth in the subjects mouths have been affected by dental caries. A higher DMFT score would indicate that more teeth in the subject's mouth had been affected by dental caries.

Plaque contains large amounts of cariogenic bacteria such as *S. mutans.* Thus it follows that if there is a high plaque index, there will be more cariogenic bacteria in contact with tooth surfaces for a longer period of time, which may result in cavity formation and thus a higher DMFT score. The DMFT ratio was taken in order to standardise the subjects. The mean DMFT ratio was very good at a value of 0.1 which indicates that the subjects had relatively few teeth that were affected by dental caries.

4.5 Salivary *S. mutans* and Lactobacilli counts

Streptococcus mutans is a gram-positive, facultatively anaerobic bacteria that is found in the oral cavity and that has been shown to contribute to dental caries. *S. mutans* is an early coloniser of the tooth surface which grows and metabolises carbohydrate, thus allowing other organisms to colonise the tooth surface and eventually form dental plaque. In addition, *S. mutans* metabolises sucrose to form lactic acid which results in demineralisation of enamel. Many treatments, such as Biotène®, have been developed in order to decrease *S. mutans,* thus decreasing their deleterious effect on mineralised structures in the oral cavity.

Our results showed that there was a decrease in the *S. mutans* count following the use of rubber tubing (23.4% based on mean) and xylitol chewing gum (46.7% based on mean). But only the decrease in *S. mutans* count following rubber tubing use was statistically significant ($p=0.0015$). These results suggest that chewing on anything that provides masticatory stimulation might be sufficient to decrease the *S. mutans* counts. The effect of xylitol on the *S. mutans* count is controversial. Autio (2002) showed that xylitol decreases salivary *S. mutans* counts.[48] However, Twetman and Stecksén-Blicks (2003) found that xylitol did not decrease salivary *S. mutans* counts but this result may have been due to the low quantity of xylitol that was used per day.[50] Twetman and Stecksén-Blicks (2003) only administered 5g/day of xylitol, but 6g/day of xylitol is required to affect the oral ecology.[47]

Thus the quantity of xylitol in the chewing gum is also an important factor in reduction of *S. mutans*. There was possibly a larger quantity of xylitol in the xylitol gum than the Biotène® chewing gum. This could have been the reason why a reduction in the *S. mutans* count was recorded following the use of xylitol gum but not following the use of Biotène® chewing gum.

The exact amount of xylitol present in both xylitol chewing gum and Biotène® chewing gum is, unfortunately, unknown as the manufacturers do not make this information available. Our results also showed increase in *S. mutans* count due to Biotène® chewing gum by 141.7% (not statistically significant) which is contrary to what has been expected of this chewing gum. Biotène® chewing gum contains glucose oxidase and lactoperoxidase which inhibit *S. mutans*. Thus the *S. mutans* count should have decreased.

Lactoperoxidase has been shown to inhibit a number of oral bacteria including *S. mutans*[51], yet Biotène® chewing gum in our study resulted in an increase in *S. mutans* count. In lab conditions Biotène® was shown to be superior to Zendium toothpaste, which is another product that has been designed with the same purpose as Biotène® and that also contains lactoperoxidase , in inhibiting the growth of *S. mutans* and Lactobacilli[74], yet *in vivo* studies showed that Biotène® toothpaste, containing the peroxidase system components, did not show any antibacterial effects against total streptococci, *S. mutans*, Lactobacilli or the total anaerobic flora.[51, 53] Kocak *et al* (2009) also found that Biotène® mouth rinse which contained glucose oxidase, Lactoperoxidase, and Lysozyme had no effects on salivary *S. mutans* levels.[55]All these previous studies usually focused on Biotène® toothpaste, gel, mouth rinse, or a combination of toothpaste, mouth rinse, gel and chewing gum, with none of the studies exclusively utilising Biotène® chewing gum.

Lactobacilli are gram positive, facultatively anaerobic bacteria that are found in the oral cavity and have been implicated in the progression of dental caries. Lactobacilli produce lactic acid from sugars. This lactic acid causes demineralisation of dental enamel. Treatments have also been developed to decrease Lactobacilli counts, thus preventing excessive lactic acid production and the subsequent demineralisation of dental enamel.

In our study Lactobacilli counts were shown to increase following rubber tubing use (141.2%). There were decreases in Lactobacilli counts following use of xylitol chewing gum and Biotène® chewing gum by 55.1% and 89.6% respectively. Both decreases were statistically significant with p-values <0.01. This suggests that xylitol which is present in both the products has antibacterial effects against Lactobacilli. In addition, Biotène® has additional antibacterial effects against Lactobacilli because the reduction was greater by 34.5% compared to the xylitol gum.

Caglar *et al* (2007) found that there was no significant decrease in salivary Lactobacilli counts following use of 6g/day of xylitol chewing gum for a period of 3 weeks.[49] However our results show a decrease in Lactobacilli count of 55.1% following use of xylitol chewing gum which possibly contains a large quantity of xylitol. This decrease is in agreement with findings by Mäkinen *et al* (2008) who found that the levels of salivary Lactobacilli were significantly decreased following the use of xylitol containing chewing gums.[75]Although the 2 weeks use of Biotène® toothpaste which contained a lactoperoxidase system, showed no notable changes in the Lactobacilli counts[53], our results showed a decrease in counts which is in agreement with Jyoti *et al* (2009) who found that salivary Lactobacilli counts were significantly reduced following use of lactoperoxidase containing Biotène® toothpaste.[59]

4.6 Summary results of test parameters after the use of test products.

There were no statistically significant changes in resting saliva flow rate following rubber tubing, xylitol chewing gum or Biotène® chewing gum. The small increases in resting saliva flow following the use of rubber tubing, xylitol and Biotène® chewing gum could be simply attributed to a masticatory stimulus as the increases were very similar. Therefore the Biotène® chewing gum did not result in any greater benefit in terms of resting saliva flow as compared to any inert masticatory stimulus.

Stimulated saliva flow was not significantly affected by rubber tubing or by Biotène® chewing gum but was significantly affected by xylitol chewing gum ($p=0.05$). Again xylitol chewing gum has been shown to offer the greatest increase in stimulated saliva flow, when compared to rubber tubing or Biotène® chewing gum.

There was no effect on the buffering capacity by either chewing or the stimulants such as xylitol or enzymes which is surprising because stimulated saliva usually contains 7 times more bicarbonate compared to the resting saliva.[76]

Xylitol significantly decreased the plaque index (by 32.5%) which can be explained by the increase in the saliva flow by 53%. A constant flow of saliva eliminates bacteria, plaque and food debris which reduces plaque development. Rubber tubing resulted in a decrease in plaque index (5.5%) as rubber tubing offered masticatory stimuli thus increased saliva flow slightly (13%) which in turn decreased retention of plaque on tooth surfaces. Xylitol chewing gum also offered a masticatory stimulus but in addition contained xylitol, which has been shown to significantly decrease plaque index in children.[72, 73] Thus xylitol chewing gum was shown to be the most effective in decreasing plaque index. However Biotène® chewing gum has not been proven to affect plaque index.

Rubber tubing resulted in a 23.4% decrease in *S. mutans* count which was statistically significant (p=0.0015). This could have been due to some missing data due to overgrowth of gram negative organisms in this group. This occurs in cases where the individual was carrying gram negative bacilli (gut flora) in their saliva. This situation would have occurred if the individual had extremely poor oral hygiene or was immunocompromised. Even if xylitol chewing gum resulted in a 46.7% decrease in *S. mutans* count it was not significant because the results varied tremendously with a high standard deviation. In addition, a larger sample size is required to achieve a meaningful result.

Biotène® chewing gum should have resulted in the greatest decrease in *S. mutans* count as it provides a masticatory stimulus, xylitol as well as host proteins but instead it resulted in a great increase in *S. mutans* count. It is possible that the quantity of xylitol is not substantial enough to affect the *S. mutans* counts. Also it may be possible that one of the components in the Biotène® chewing gum renderes the host proteins ineffective. Another possibility is a poor, cariogenic diet which may have been adopted by the subjects during the use of Biotène® chewing gum.

Rubber tubing which offered only masticatory stimulation increased Lactobacilli counts (141.2%) which were possibly due to dietary influences in the subjects during the rubber tubing phase of the study. The decrease in Lactobacilli count following the use of xylitol chewing gum may be attributed to xylitol which has been shown to decrease salivary Lactobacilli.[75] Biotène® was shown to be more effective than xylitol chewing gum in reducing Lactobacilli counts. This was possibly due to the presence of host proteins in the Biotène® chewing gum. Thus the only parameter where Biotène® chewing gum was shown to be effective in was in Lactobacilli count reduction.

5 CONCLUSION

The prevalence of hyposalivation among the study population was 16.5%. Chewing gum containing natural host proteins, Biotène®, did not increase resting and stimulated saliva significantly in hyposalivating subjects. Biotène® had no effect on the buffering capacity of resting and stimulated saliva. In addition, Biotène® did not reduce the plaque index and salivary *Streptococcus mutans* counts. However, it reduced the salivary Lactobacilli counts significantly. Xylitol chewing gum which was used as a second control to eliminate the effect of xylitol from the Biotène® showed significant increase in the stimulated saliva, reduced plaque index and salivary Lactobacilli. These results have shown that host protein containing chewing gum, Biotène® has no additional benefits. However, chewing gums containing a substantial amount of xylitol (offering more than 6g/day) are beneficial in the prevention of dental plaque and hence dental caries. Xylitol containing chewing gums together with other oral hygiene products may provide additional benefits.

6 LIMITATIONS

- The greatest limitation to this study was the small sample size. When the sample size is small, the statistical tests may have inadequate power to detect a particular effect. Our study population required healthy, young (18-23yrs old) individuals who had reduced saliva flow rates. However, after screening 109 students, only 18 met the criteria because although hyposalivation is present among young individuals, it is rare. Of these 18, only 13 agreed to participate in the study.
- Another limitation was subject compliance. Although we asked students to bring back empty boxes of the treatment modalities, compliance was not guaranteed. Furthermore the diet of the students may have been very different in each phase of the study such as being more cariogenic during stressful periods such as exams. Thus this lack of a standardised diet during all treatment modalities may have resulted in some inaccuracies.

7 APPENDICES

APPENDIX A- Ethics clearance certificate granted by the Committee for Research on Human Subjects (Medical)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Thanusha D Pillay

CLEARANCE CERTIFICATE	**M120282**
PROJECT	Influence of Chewing Gum Contaiing Natural Host Proteins with Antimicrobial Effects on Saliva in Subjects with Hyposalivation
INVESTIGATORS	Dr Thanusha D Pillay.
DEPARTMENT	Clinical Microbiology & Infectious Diseases
DATE CONSIDERED	24/02/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 04/05/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Mrudula Patel

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX B- Consent form for subjects

For verbal consent

Good Day,

How are you?

I am Dr Thanusha Pillay a postgraduate student at University of The Witwatersrand. In order to fulfil my M Sc dent degree requirements, I am conducting a research project on Biotène® chewing gum and dental caries susceptibility. Dental caries or tooth decay is a major problem worldwide including South Africa. Secretion of saliva in our mouth protects us from caries. Some people have low saliva flow in their mouth which makes them vulnerable to caries. Many oral hygiene products are commercially available which improves saliva flow and oral health. Biotène® chewing gum is one of the products that can be used to improve saliva flow. I would like to study the effect of Biotène® chewing gum on the production of saliva and oral health status.

In order to do my study, I would like to measure your plaque index where you will rinse your mouth with a colour solution given to you which will stain plaque on your tooth and I will count the stained areas. This solution is perfectly safe to use and dentists use it on their patients regularly. You will be asked to collect saliva for 10 minutes at resting, 10 minutes while chewing rubber tubing given to you and 10 minutes while putting drops of diluted citric acid which is lemon juice on your tongue. You will be given toothpaste and a tooth brush to use while participating in this study. You will be asked to chew 5 pieces of Biotène® chewing gum a day for two weeks which will be given to you. After two weeks the plaque index and saliva test will be repeated. These tests will take 40 minutes of your time at the beginning and at the end of the study participation. I know as a student your time is precious, we can do these tests at your convenience such as lunch time or after hours. The samples will be processed in the laboratory.

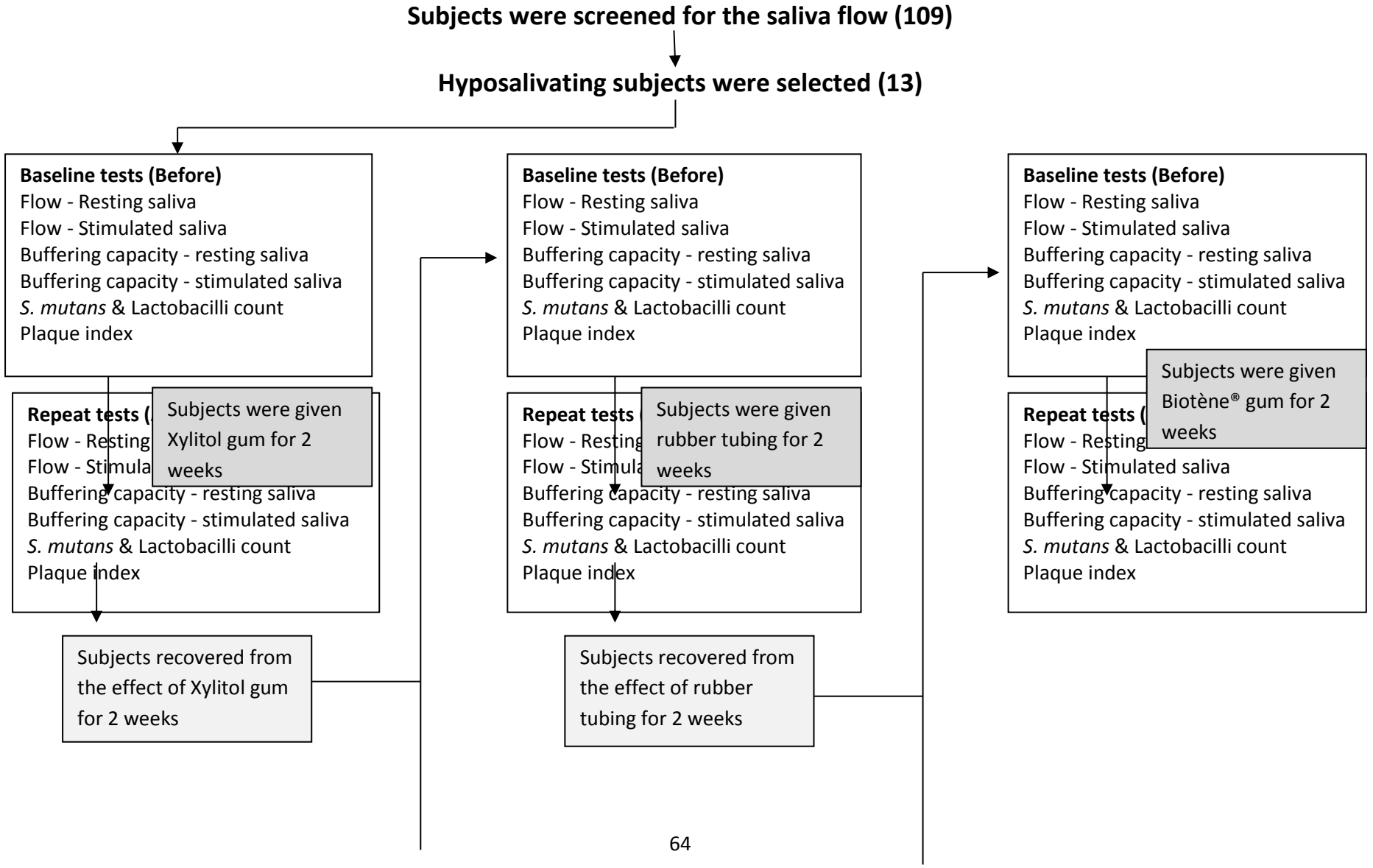
These tests will cause no harm to you. Whether you decide on participating or not, is entirely up to you. Your decision will not affect you in any way. If you agree to participate, you may withdraw from the study at any time without affecting you in any way. If you wish, I will disclose the results to you and if required advise you on corrective measures. We all will benefit from the knowledge achieved from this study.

Your sample will be given a number and will be processed under a number. Your name will not appear anywhere on the results or on any publications. This study has been through University ethics committee. Should you have any problems please contact Prof P. Cleaton-Jones at 011 717-1234

Patient's name:	Investigator's name:
Date:	Date:
Signature:	Signature:

Tel. No: 082 705 8992

APPENDIX C- Flow diagram showing study design



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