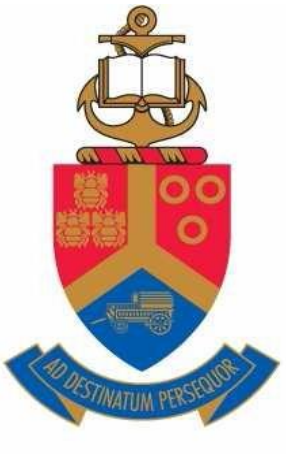

UNIVERSITY OF PRETORIA

Determination of the Cariogenic Potential of Sugar Substitutes

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Dissertation submitted in the fulfilment of the requirement of the degree

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January 2020

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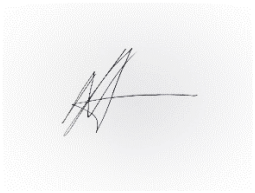
Declaration and conflict of interest

I, Nadine Moelich, declare that this dissertation, entitled:

"Determination of the cariogenic potential of sugar substitutes"

Is my own work and has not been submitted for any degree or examination at any other university. Moreover, all the sources that I have used or quoted have been indicated and acknowledged as complete references.

Furthermore, I declare that no competing interests, either financial or non-financial (e.g. political, personal, religious, academic, ideological, intellectual, commercial, etc.) exist with respect to this research. I do not hold stocks or shares in any organisation that stands to gain or lose financially from the publication of this manuscript. I do not hold any patents, nor am I currently applying for any patents related to the content of the manuscript. No funding has been received from any organisation that holds or has applied for patents related to the content of the manuscript.



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Nadine Moelich
November 2019

Acknowledgements

It is with the highest appreciation that I wish to thank the following people:

- My God: Fil 4:13 “I can do all things through Christ who strengthens me.” Thank you for the strength to persevere. Thank you for the beautiful creation and for the detail hidden therein, waiting to be discovered!

- Dr. Nicoline Potgieter, my supervisor. I cannot find enough words to thank you. Thank you for your motivation and inspiration. Thank you for the many hours of reading, refining and polishing. For your endless advice, help and patience and the energy that you brought to this project. Thank you for challenging me and pushing me. I would not have been able to do this without you. I am forever thankful to you. Thank you for the example you set as a person, and as an academic, your zeal and energy is contagious.

- Dr. Francine Botha, my mentor and saving grace. You are an endless source of knowledge and have an endless amount of experience, thank you for your willingness to share these with me and thank you for the time you invested in me. The hours and late nights/early mornings spent in laboratories and your advice and care have truly touched my heart. Thank you for setting an example of sharing knowledge and helping without expecting anything in return! Thank you for introducing me to knowledgeable, helpful people. Thank you for giving me hope.

- Dr. Lyndy McGaw, for allowing me to work in the Phytomedicine laboratory, and for making me feel at home and welcome.

- Dr James Wesley-Smith and Dr. Chantélle Baker, thank you for allowing me to work at SMU and for introducing me to a whole new world! For the endless hours of explaining and hard work to find the best suitable images which enabled me to finish in time, regardless of electricity cuts and challenges- I thank you!

- Prof. Leanne Sykes, for the effective and efficient language editing of this dissertation and for the positive and helpful commentary. Thank you for your enthusiasm about this project, which gave me the much needed energy to finish in time.

- Prof. PJ Becker, thank you for accommodating me and for helping me with the statistics of this project. Your help, time and patience to find the most suitable formulas and solutions calmed my nerves and gave this project wings!

- Prof. Z Vally, as HOD, thank you for your support and interest in the project! It is reassuring to know that you care and carry our best interests at heart!

- My husband, Danté and children, Pierre and Jean, who supported me through all the difficult and good times, for allowing me the long hours away from home while I was collecting data and for your constant care and support. For tolerating the mess that were sometimes generated by the project and for loving me none the less!

- My parents for their love and endless support and for always believing in me.

Abbreviations

2-AFC	-	2-alternative forced choice
ADI	-	Acceptable daily intake
ANOVA	-	Analysis of variance
BPTS	-	Binding-Protein Transport System
˚C	-	Degrees Celsius
CASO	-	Casein peptone Soy flour peptone
CFU	-	Colony Forming Units
ECC	-	Early Childhood Caries
EPS	-	Exopolymeric substances
EtOH	-	Ethanol
FDA	-	Food and Drug Administration
FE-SEM	-	Field Emission Scanning Electron Microscope
g	-	gram
G6P	-	Glucose-6-Phosphate (G6P)
GTF	-	Glucosyltransferase
h	-	hour
HMDS	-	Hexamethyldisilazane
hrs	-	hours
IAPD	-	International Association for Paediatric Dentistry
K_2HPO_4	-	Potassium phosphate
L	-	Litre
min	-	minutes
mL	-	millilitre
mm	-	millimetre
NaCl	-	Sodium chloride
NCD	-	Non-communicable disease
OsO_4	-	Osmiumtetroxide
PBS	-	Phosphate buffer saline
PCSB	-	Peptone casein soy basal madia

PEP	-	Phospoenolpyruvate
PEP-PTS	-	Phosphoenolpyruvate-Dependant Phosphotransferase System
pH	-	Potential Hydrogen
psi	-	Pounds per square inch
PT	-	Phosphotransferase
SEM	-	Scanning Electron Microscope
S. gordonii	-	*Streptococcus gordonii*
S. mutans	-	*Streptococcus mutans*
S. sobrinus	-	*Streptococcus sobrinus*
Stevia	-	*Stevia rebaudiana Bertoni*
TNTC	-	Too numerous to count
µL	-	microliter

Summary

Early Childhood Caries (ECC) is a burden worldwide which has a negative impact on children's wellbeing and affects aesthetics, speech and mastication. It may lead to loss of space, creating problems that are difficult and expensive to manage in future. The International Association of Paediatric Dentistry (IAPD) declared that more than 600 million children worldwide are affected by ECC.

Caries is a multifactorial disease and frequent excessive sugar consumption is noted as a major risk factor in the development of caries. It also contributes to other non-communicable diseases (NCDs) such as diabetes, obesity and cardiovascular disease. The well-established link between dental caries and dietary sugar, specifically sucrose, can be explained due to the fact that it can be fermented by microorganisms.

The use of sugar substitutes may be justified as an effective way to prevent dental caries by modifying the metabolism of microorganisms which will lead to a reduction in lactic acid production in the mouth. This study explored the cariogenic potential of sugar substitutes. The aim of the study was to determine and compare the cariogenic potential of commercially available sugar substitutes namely: xylitol, erythritol and stevia. The data collected could be useful in finding a suitable substitute for sucrose, one of the main causative factors of ECC.

A total of 52 enamel slabs were prepared from the surfaces of extracted primary teeth and placed in growth media before being inoculated with *Streptococcus mutans (S. mutans)*. The enamel slabs and growth media were used to determine the Colony Forming Units (CFUs) of *S. mutans* after exposure to xylitol, erythritol and stevia and to determine the acid production of *S. mutans* in the presence of these sugar substitutes by measuring the acidity (pH) of the growth media. Biofilm formation in the presence of sucrose, xylitol, erythritol and stevia was confirmed by means of Scanning Electron Microscopy (SEM).

Considering the CFUs, pH and SEM analysis, this study suggests that xylitol, erythritol and stevia are all less cariogenic alternatives to sucrose. Stevia has been shown to have the lowest cariogenic potential, followed by erythritol and then xylitol. These substitutes should however be used with caution as they still produced a drop in pH close to the critical demineralization level.

From the literature studied, it is clear that ECC is a preventable disease. It is the dental professional's duty to raise awareness with parents, caregivers, other health care professionals and all relevant stakeholders. Parents and patients should be educated to limit sugar intake and to substitute sugar with healthier alternatives such as xylitol, erythritol or stevia, which all proved to be less cariogenic than sucrose.

Key words:

Early Childhood Caries (ECC)

Cariogenic potential

Sugar substitutes

Sucrose

Xylitol

Erythritol

Stevia

Colony Forming Units (CFU)

pH

Streptococcus mutans (S. mutans)

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Chapter 1 : Introduction and Literature Review

1.1 Background and motivation for this study

1.1.1 Early Childhood Caries

Early Childhood Caries (ECC), also known as "Nursing Bottle Caries" or "Baby Bottle Tooth Decay", is defined as the presence of one or more primary teeth which are decayed (non-cavitated or cavitated), missing due to caries or filled, in a child under the age of six years.[1] The caries pattern of children in the age range of 12-30 months differs from that of older children. ECC will typically affect the maxillary primary incisors and first primary molars, reflecting their eruption patterns. The longer a tooth has been present in the mouth, the longer it will have been exposed to the aetiological factors associated with dental caries and the more it is affected.[2]

ECC is a disease of serious concern in both local and international public health systems.[3-4] International studies show that ECC is the most prevalent disease in children.[5-7] Tooth decay is five times more prevalent than asthma, seven times more than hay fever and fourteen times more than chronic bronchitis.[8] According to research done by the South African National Children's Oral Health Survey (NCOHS) in 1999/2002, the prevalence of caries in children aged 4-5 years was 51%.[9] This finding confirms that ECC is a serious problem in South-Africa and warrants further research and investigation to prevention strategies.

ECC may lead to detrimental consequences such as pain, difficulty in mastication, infection, malnutrition, disorders of the gastrointestinal system and a low self-esteem in general.[6,10] Caries is expensive and difficult to treat in young patients.[1] Therefore there is a world-wide drive towards research on and prevention of ECC, with the Global summit on ECC hosted by the International Association of Paediatric Dentistry in 2018 as an example,[11] and the IAPD Bangkok Declaration, adopted by the IAPD Bangkok Global Summit on ECC in November 2018, as confirmation.[4]

1.1.2 The aetiology of caries, the caries process and dental biofilm formation

Caries has various causative factors which include microbiological factors and dietary factors with specific reference to fermentable carbohydrates as well as the frequency and mode of ingestion and poor oral hygiene (Figure 1.1).[12]

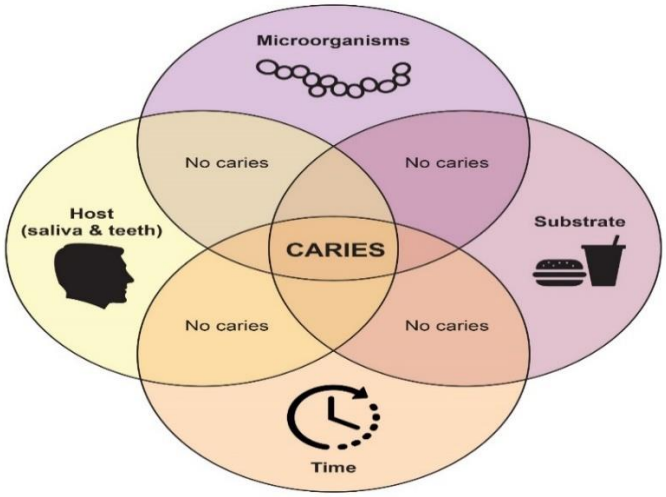

Figure 1.1: Newbrun's Tetrad illustrating the collaborative causative factors of dental decay, adapted from Chockalingam[13]

For bacteria to induce a carious lesion, it must be attached to dental plaque.[14-15] Plaque is defined as bacteria and their metabolic waste products attached to the dental pellicle.[16-17] Plaque adheres to the surface of the tooth in a solid manner preventing salivary flow and muscle-movement from removing it.[17] It is essential to understand the formation of plaque and the adherence of the bacteria, to be able to interfere with this complicated process and ultimately prevent caries formation.

Dental plaque formation starts with pellicle formation. The pellicle first forms on the surface of the tooth, as a thin, membrane-like layer consisting of salivary proteins adsorbing to the surface. Bacteria attach to the pellicle by means of expressing surface adhesins that are able to bind to the acquired salivary pellicle.[18-19] *Streptococcus mutans (S. mutans*) is one particular group of bacteria that expresses a genetically distinct glucosyltransferase (GTF) enzyme. They also produce specific products such as glucan which allows them to adhere to tooth structure

as well as to other micro-organisms to enable the formation of a highly organized micro-colony within an insoluble matrix.[19-20]

The first bacteria that adhere to the pellicle are called primary colonizers. These are predominantly streptococci, which have weak long-range physicochemical interactions between the pellicle and the surface of the cells. This interaction leads to stronger attachments and co-adhesions resulting in the secondary colonizers attaching, multiplying and eventually the formation of dental plaque biofilm.[17,21] A biofilm can thus be defined as a group of microorganisms embedded in a matrix that is attached to the tooth surface.[20,22]

Dental biofilms as a rule are beneficial to the host, because they form part of the resident oral microbiota or oral microflora. A biofilm interacts with the immune system at a level compatible with an individual's health and provides resistance to colonization of pathogenic or exogenous organisms. The balance that exists is termed homeostasis. Dysbiosis occurs if the balance is disturbed and results in a shift in the microflora of the biofilm. Stress factors such as changes in diet, oral hygiene habits or treatment with certain medication that influence the host's salivary flow may result in a shift in the microflora. It may also be due to a change in immunity or immunocompromised states. A shift in the composition of the biofilm can result in an increase in the level of microorganisms which may initiate disease. Dental disease, with specific reference to dental caries is thus mainly caused by both resident bacteria and the reorganization and restructuring of the biofilm, allowing more virulent bacterial species to become dominant.[17,23]

A biofilm is a bacterial ecosystem that has various physiological properties. Under certain conditions such as consumption of high levels of fermentable carbohydrates or the continuous presence of high amounts of sugars, the microbiological and biochemical composition of the biofilm can change. This can lead to an increase in the proportion of pathogenic species, thus transforming a healthy biofilm into a cariogenic biofilm.[24-25]

Bacteria that are attached to the biofilm ferment carbohydrates leading to acid production, such as lactic, acetic, formic and propionic acid. This consequently lowers the pH level in the

mouth and causes demineralization of tooth surfaces (Figure 1.2).[26] If the de-mineralization process progresses for long enough, demineralization will result in cavity formation.[27]

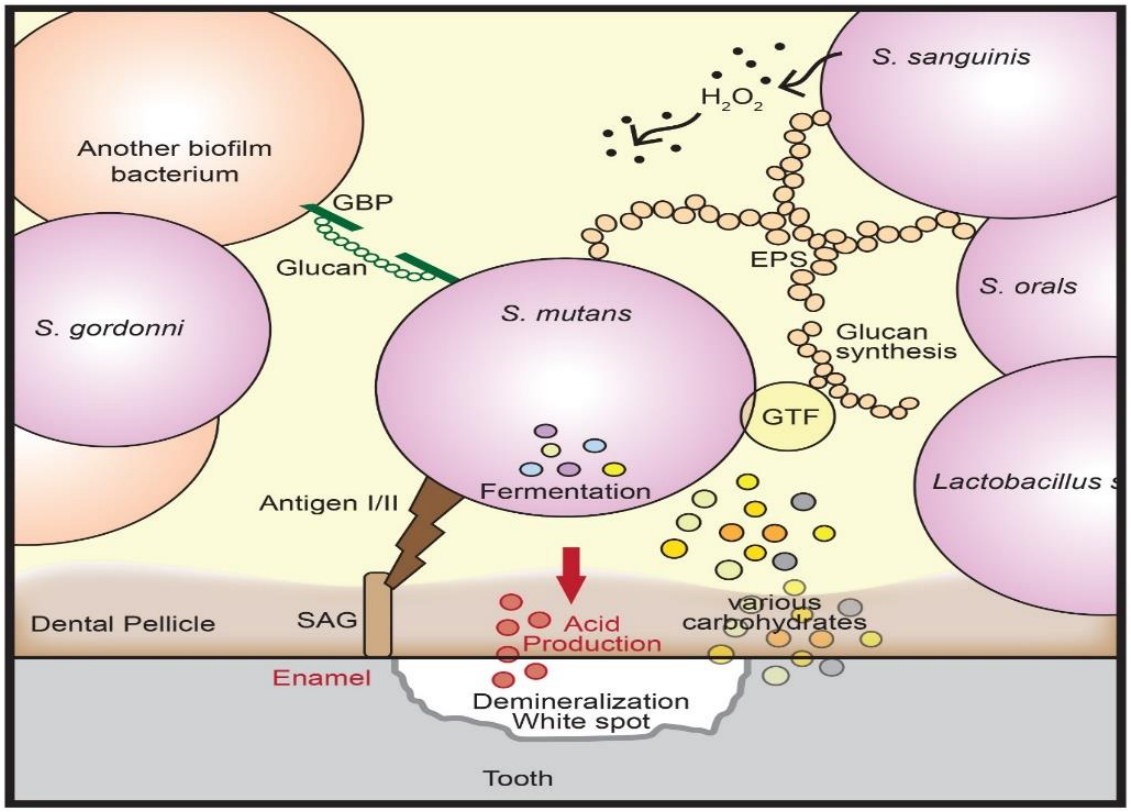

Figure 1.2: The formation of a biofilm and production of acid leading to demineralization and cavitation of tooth structure (adapted from Mikrobewiki[28])

Acid production by bacteria in the mouth is rapid when carbohydrates are available. When the pH level is lower than 5.5, the acidity leads to disintegration of the organic compound of the enamel and dentine with subsequent demineralization and cavity formation. This is why the level is known as “the critical pH level”.[29] Decalcification of the teeth will occur when carbohydrates are introduced frequently to the tooth surface, or mechanically retained for long periods of time. The higher the frequency of exposure, the longer the drop in pH occurs and the more likely the formation of a demineralized or cavitated lesion.[30]

In Figure 1.3, the correlation of pH and time of exposure in the demineralization versus remineralisation threshold is illustrated.

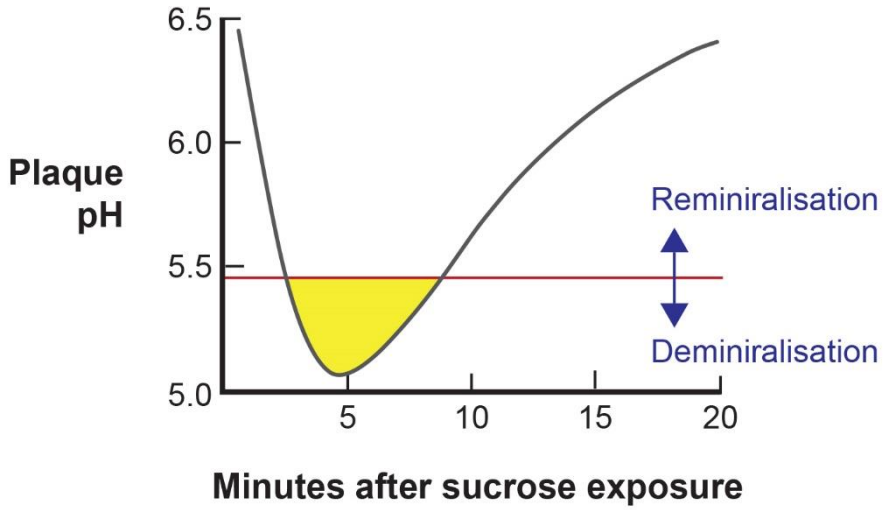

Figure 1.3: Stephan's Curve illustrating the time-related drop in pH levels with sucrose exposure[31]

Children who present with ECC usually consume sugars such as sucrose, fructose and glucose from liquids and infant milk formula at regular intervals.[32] The frequency of exposure to sugars is enhanced by the use of nursing bottles and sippy cups. Children's feeding behaviour due to parental ignorance of this factor, results in an increase in the frequency of exposure, elevating the risk of demineralization with subsequent cavity formation.[32]

1.2 Bacteria in the oral cavity

Microorganisms are classified into major groups namely: algae, protozoa, fungi, bacteria, viruses and organisms in-between bacteria and viruses like ricketsiae and chlamydiae. Bacteria belong to the kingdom of protists, which means that they are unicellular organisms or relatively simple multicellular organisms and lead a parasitic existence. Bacteria are classified according to their phenotypic features, namely morphology, staining properties (Gram-positive or Gram-negative), cultural requirements (aerobic, facultative anaerobic or anaerobic), biochemical reactions (sugar fermentation reactions), antigenic structure (serotypes) and genotypic taxonomy where the genetic characteristic are used to classify a bacterium.[33]

Bacteria are visible with a light microscope, are capable of free growth, contain both RNA and DNA, have rigid cell walls (to protect a fluid protoplast), contain muramic acid in the cell walls, and reproduce essentially by binary fusion. The rigid cell wall determines the shape of the

bacteria (Figure 1.4). Bacteria are classified by shape as either cocci (spherical), bacilli (rod-shaped) or spirochetes (helical). Some bacteria appear with both coccal and bacillary shape and are called pleomorphic. Most bacteria also possess flagella, which helps with movement of the cells toward nutritional and other sources. Cocci however, do not have flagella and are not motile.

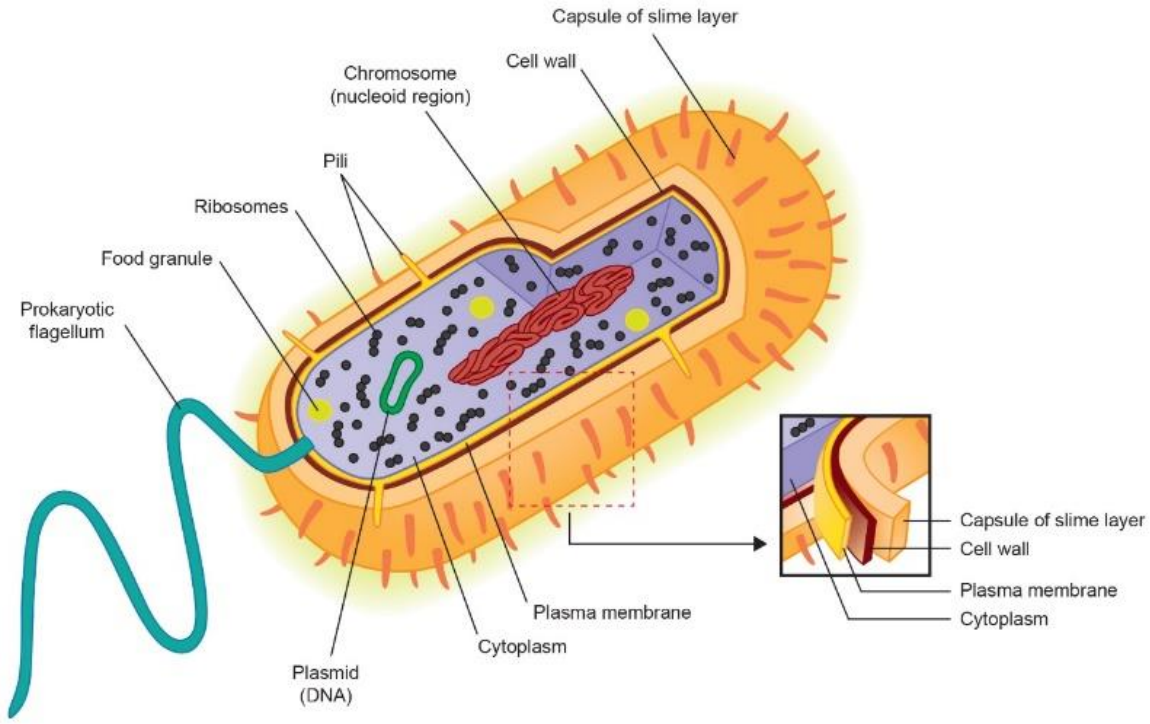

Figure 1.4: An illustration of a bacterial cell

Danish physician, Christian Gram[33] developed a stain used to classify bacteria according to the characteristics of their cell walls as illustrated below (Figure 1.5). A bacterium that stains purple is Gram-positive and one that stains pink is Gram-negative.[33]

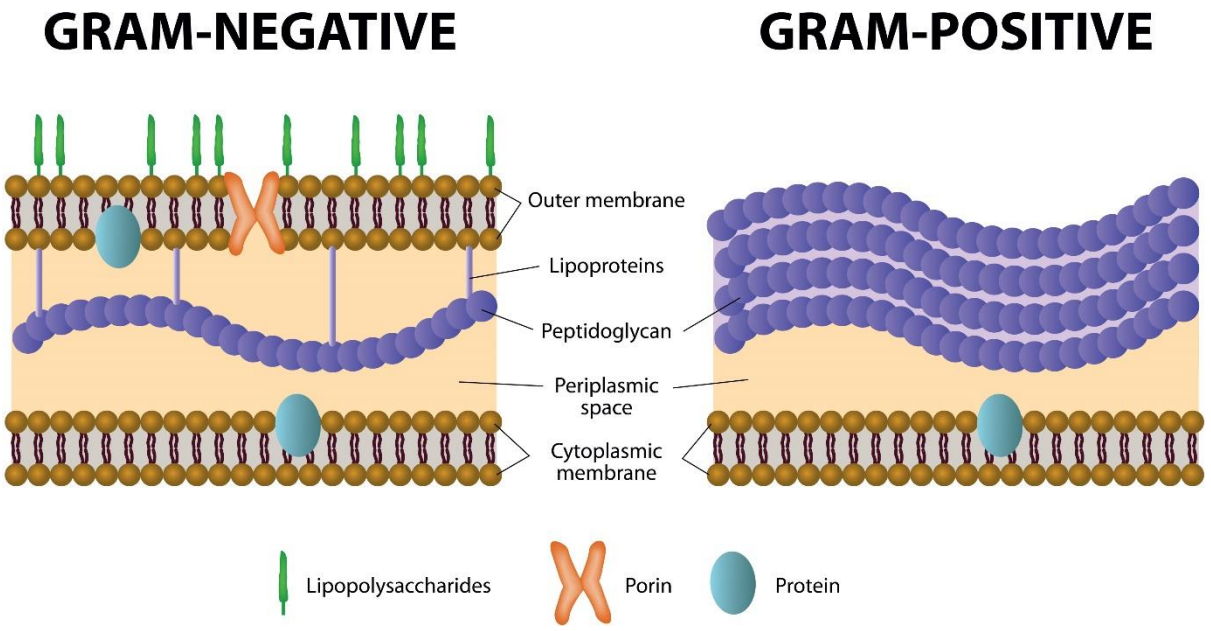

Figure 1.5: Structural features depicting the differences between gram-negative and gram-positive bacterial cell walls

There are more than 700 species of bacteria in the oral cavity.[34] However, many of them have not been cultivated and are still poorly understood.[25] Demonstrating bacterial specificity in dental decay is difficult considering the amount and variation of flora in the mouth. The suspected aetiologic agent in most instances, is *S. mutans* and to a lesser extent *Lactobacilli.*[18]

1.2.1 Streptococcus mutans

S. mutans is a recognized pathogen in humans and is in most instances responsible for the onset, presence and development of dental decay.[18,35] *S. mutans* is a gram positive coccal bacteria and appears in chains due to the replication mode. *S. mutans has* a remarkable ability to adhere to tooth surfaces and form biofilms. They also are acid tolerant, meaning that when exposed to acidic conditions the bacteria still grow and produce even more acid, subsequently lowering the pH level of their surroundings even more. *S. mutans* is thus acidogenic and aciduric.[17] The important structure of *S. mutans* and their role in caries formation is explained below.

The outer surface of *S. mutans* is covered by a polysaccharide coating. This is like a slime layer and is called the glycocalyx. This allows firm adhesion of the bacteria to other structures such as oral mucosa and teeth. *S. mutans* have the ability to produce large quantities of extracellular polysaccharide when exposed to sucrose and other dietary sugars.[33]

An amorphous gelatinous layer, more substantial than the glycocalyx, surrounds the entire bacterium and is called the capsule. It is important because it mediates the adhesion of bacteria to human tissue and is therefore essential for biofilm formation and thus colonization.[33]

When *S. mutans* is acquired at an early age, there is an increase in the caries prevalence in the primary and permanent dentition.[36] The major route of acquisition of *S. mutans* is believed to be the transmission from mother to child, in other words vertical transmission.[36-38] This can occur for example when kissing a baby on its mouth or putting a baby's pacifier in the mother's mouth to "clean" it after it was dropped. This transmission is significant in the development of carious lesions, as the earlier a child is exposed to *S. mutans*, the higher their chances of developing early childhood caries.[36] Although vertical transmission is the most common method of transmission, horizontal transfer is also an important factor to be taken into account. This occurs through saliva sharing with friends at school or playgroups and other family members or caregivers, such as sharing a cold drink.[38]

1.3 The role of sugar and refined carbohydrates in the caries process

When micro-organisms are exposed to a suitable substrate, the caries process can begin. If the host's diet is such that the organisms can benefit from it. Studies have shown that there is a direct correlation between the ingestion of sucrose and the occurrence of dental caries.[17-18,39] Carbohydrates are described as the main causative agent responsible for the biochemical and physiological changes in the dental biofilm. After ingesting glucose, sucrose and fructose the pH level in the biofilm rapidly decreases.[18,25,39-40] The decrease of the pH level can be attributed to the cariogenic organisms' complex ability to ferment carbohydrates and

produce acids.[18,25] An important factor to take into consideration is the frequency of consumption of fermentable carbohydrates.[17,26]

The well-established link between dental caries and dietary sugar, specifically sucrose, can be explained due to the fact that it can be fermented. In addition, the dietary sugar serves as a substrate for extracellular enzymes of plaque bacteria which synthesizes polymers derived from sucrose. These polymers in return are of extreme importance because they mediate attachment to the tooth surface and to other bacterial cells, by doing so, they stabilize the plaque biofilm. The polymers also modulate the plaque permeability and subsequently the acid at the surface of enamel.[6]

The major pathway is described by Loesche[14] as *S. mutans* first forming adhesive colonies adhering to the tooth surface in the presence of sucrose. They do this by virtue of expressing hexotransferase which transforms sucrose to glucans and fructans. These are diffused in the immediate surroundings and also remains associated with the cells to form adhesive colonies. The glucans transformation is seen as a virulent factor of *S. mutans* and *S. sobrinus* and is important primarily for smooth-surface decay.[18]

Oral bacteria possess two types of sugar transport systems: The Phosphoenolpyruvate-Dependant Phosphotransferase System (PEP-PTS) and the Binding-Protein Transport System (BPTS). With the help of these systems fermentable carbohydrates and certain polyols can be utilized by some of the oral bacteria. Glycolysis begins with Glucose-6-Phosphate (G6P) and produce pyruvate which is then transformed to lactate, acetate, ethanol and formate. The glycolytic pathway is shared by most saccharolytic oral bacteria, including *streptococcus actinomyces and lactobacilli* (Figure 1.6).[25]

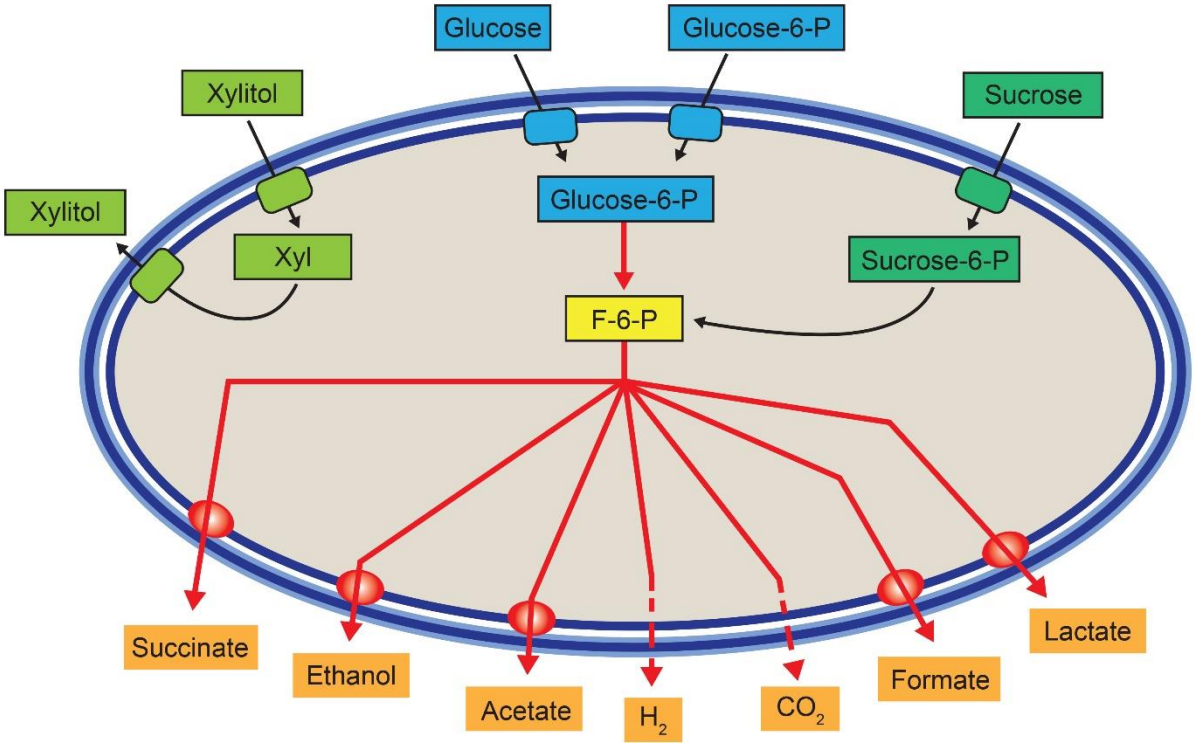

Figure 1.6: Illustration of the glycolytic pathway of saccharolytic oral bacteria in the presence of sucrose, glucose and xylitol

Numerous studies have concluded that sugar is the most important diet-related factor causing dental caries.[27,41-43] Modern day lifestyles exacerbate this problem as more refined carbohydrates are available and easy to access. Economic issues also affect dietary choices, as lower income households tend to consume more refined carbohydrates, because of affordability.[44] The busy lifestyle adopted by many also leads to fewer meals being consumed at home and an increase in consumption of readymade meals and fast foods which are generally higher in sugar content.[44] Furthermore, the human desire for sweet results infants and young children particularly basing their food choices on sweet tastes.[45] Finding healthier, affordable alternatives to sugar could aid in combatting the ECC problem.

1.4 Alternative sweeteners and their cariogenic potential

Literature indicates that cariogenic organisms can be reduced and pH levels in plaque elevated by avoiding snacking of food and drinks that contain fermentable carbohydrates between mealtimes.[17,42,46-48] Consumption of non-fermentable sugar substitutes, such as

aspartame or polyols have also been found to aid in elevating the pH level.[17,49-52] This, in turn, affects the metabolism of microorganisms and could help reduce their production of lactic acid in the mouth. Thus, the use of sugar substitutes may be an effective way to prevent dental caries.[53]

Two categories of sweeteners can be distinguished: non-nutritive high intensity sweeteners and nutritive sweeteners.[54] Sweeteners have also been categorized as artificial or natural: artificial being manufactured from an organic compound and not found in nature and natural sweeteners that are extracted from plants with no chemical modification during this process.[55]

The choice of sugar substitutes used for the purpose of this study was based on sweeteners that are classified as safe for human consumption, occur naturally in plants and are not prepared in an artificial manner. Some artificial sweeteners cause health issues.[54-55] Xylitol, erythritol and stevia were chosen because they have been approved by the Food and Drug Administration(FDA) as being safe for human consumption. They are natural products and are commercially available to the South African consumer.[56]

1.4.1 Polyols

A polyol, in terms of food science, is an organic compound that contains multiple hydroxyl groups. Any molecule containing more than two hydroxyl groups, is considered a polyol. The class of polyols that are obtained by hydrogenation of sugars are termed sugar alcohols. These are often added to foods due to their lower calorie content compared to that of sugar. They are either only partially metabolized or in some cases not metabolized at all by the caries causing microorganisms in the mouth, which also results in reduction of caries incidence. This is of interest to dentists in the field of preventative dentistry.[57]

The more common polyols include maltitol, xylitol, sorbitol, erythritol and isomalt. The amount of dental plaque formed during *in vivo* experiments in individuals that were given erythritol and xylitol in the form of lozenges, were significantly less than in control groups,

who had sugar. The finding was that erythritol and xylitol significantly lowered the quantity of *S. mutans* in plaque and saliva.[56,58] This finding was also substantiated by cultivation experiments *in vitro*.[59, 60]

1.4.2 Xylitol

Xylitol is 'n five-carbon polyol that was discovered in 1891 by Nobel-prize winner Emil Fischer. It is found naturally in various plants and is also commercially prepared by processing plant materials containing the polysaccharide xylan.[49] The human body naturally produces more or less 5-15g xylitol per day during normal carbohydrate metabolism.[61]

Researchers at the University of Turku, Finland, investigated the hypothesis that xylitol as a sugar substitute can reduce the incidence of caries, with positive results.[50] Since then the caries inhibition effect of xylitol has been proven in numerous studies.[50-52,62-63]

Several studies that investigated the anti-cariogenic properties of xylitol include *in vitro* and *in vivo* experiments. All found that not only is xylitol not fermented by dental plaque[64-66] but it also reduced the acid production by microorganisms in dental plaque.[67-69] These findings were supported by other studies that have shown that there was a decrease in *S. mutans* in plaque exposed to xylitol and a decrease in plaque quantity.[69-73] The decrease in *S. mutans* was explained by xylitol's ability to interfere with adhesion mechanisms between the microorganisms and the tooth surface and between microorganisms and microorganisms.[46-50]

Four mechanisms by which xylitol was shown to reduce *S. mutans* levels include:

i) less virulent strains of *S. mutans* formed;[75-79]

ii) plaque acid concentration was neutralized due to the ammonia and amino acid increase after exposure to xylitol;[50,80]

iii) xylitol inhibits cell growth and acid production due to inhibition of glycolytic enzymes[69,75,77] and

iv) *S. mutans* attempts to take up xylitol and incorporate it in a metabolic cycle with futile results.[81-84]

Xylitol is absorbed slowly from the gastrointestinal system due to a lack of specific transport mechanisms in the intestinal mucosa. Only a small percentage of ingested xylitol is absorbed (25%-50%) and enters the hepatic metabolic system, where it is metabolized and then ends up in the normal carbohydrate cycle.[85] The portion that is not absorbed, moves to the distal part of the gastrointestinal tract, where it serves as a substrate for intestinal flora and eventually transforms to short-chain fatty acids and small amounts of gas.[86-88] The production of the fatty acids and gas can lead to borborygmus and diarrhoea, which are dose dependant side-effects of xylitol. Sandler *et al*.[89] found that patients may develop a tolerance to xylitol after repeated exposure. The doses of xylitol could therefore be increased over time without the undesirable gastrointestinal symptoms.[89]

Xylitol is considered a safe product and conventional tests for teratogenicity, embryo toxicity and reproductive toxicity have yielded negative results.[90-91] Due to the gastrointestinal symptoms associated with xylitol, alternative natural sugar substitutes were included in this study to compare the cariogenicity and to find a suitable alternative with no side effects especially for young children and infants.

1.4.3 Erythritol

Erythritol is a four-carbon polyol naturally occurring in various fruit and vegetables. It was first isolated from the algae *Protococcus vulgaris* in 1852 by A Lamy.[92] The production of erythritol began after the realisation that a specific strain of yeast can yield large quantities of erythritol. In addition, erythritol is calorie-free and has a high digestive tolerance compared to other polyols.[93] The commercial production of erythritol started in 1993 and subsequent studies consistently demonstrated its safety.[90,92] This was confirmed by the Joint WHO/FAO Expert committee on Food Safety Authorities and its use in food has been approved in more than 60 countries.[94]

Ninety percent of the erythritol ingested is absorbed by the small intestine, but is not metabolized by the human body. It is removed from the bloodstream via the kidneys and excreted unchanged.[95] The small amount of erythritol not absorbed by the small intestine is excreted unchanged in faeces. It is not fermented like other polyols, does not contribute to energy need of the body and does not have a negative gastric effect.[96]

Erythritol can contribute to the decrease in dental decay as it does not serve as a substrate for *S. mutans*, thus leading to a decrease in plaque formation.[59] *Streptococci* cannot metabolize erythritol and therefore cannot produce glucosyltransferase, which is imperative for the synthesis of glucan plaque material.[97]

1.4.4 Stevia rebaudiana Bertoni

Stevia rebaudiana Bertoni (Stevia) is an indigenous plant from Paraguay, South America. The potently sweet ent-kaurenoid diterpene glycosides stevioside, rebaudioside A and other steviol glycosides are obtained from this plant. It has been known for more than 100 years that this plant possesses this particular sweetness, but it has only been developed commercially in Japan since the 1970's.[98] The interest in these products has grown with the commercial availability of Stevia. It is now often recommended as a healthier alternative to sugar.[53,99]

Stevioside and Rebaudioside A are the two components of interest. Stevioside consists of steviol linked to a glucose and glucose disaccharide. Rebaudioside consists of steviol linked to a glucose and glucose trisacharide. Rebaudioside A is more soluble and therefore tastes better than stevioside. Many other variations of stevioside are available, with the type of steviol-link depending on the plant source. Thus, plants are now being grown specifically to produce more Rebaudioside A.[100]

Many variations in the production and purification process exist due to the variables surrounding these processes. It is therefore not possible to standardise the precise composition of each stevia-product. Toxicology tests are subsequently not precise and

interpretation of the results are not possible. These factors play an important role in the regulatory process regarding stevia as a commercial sweetener.[101-102]

Due to the unregulated processes in the production of stevia, many products tested, showed possible reproductive toxicity effects. Production processes have since been adapted and the safety of the product has now been advocated.[103] Some genotoxicity has been expressed *in vitro*, but it was not expressed *in vivo*. It was found to be safe and it is proven that there were no biochemical, anatomical, pathological or carcinogenic effects.[104] Oral stevioside, when ingested according to the acceptable daily intake (ADI) of 5mg/kg body weight, is safe and not carcinogenic or teratogenic.[102]

Stevia was found to be safe to use in food and beverages by the US Food and Drug Administration (FDA) and the European Food Safety Authority, as well as in Japan, South Korea, China, Brazil and Paraguay.[105] Some studies show that only S. *rebaudiana* has recognized antibiotic properties, but states that its role is still not understood in the prevention of dental caries.[105,106] Giacaman *et al.*[107] showed that stevia appears to induce less demineralization of tooth structure than sucrose and also showed that the biofilms treated with stevia showed less biomass and viable microorganism cells than with the sucrose control. This suggests that stevia does not promote cell proliferation as much as sucrose does. The possible explanation given for these findings is the lack of metabolism of the product by *S. mutans.*

There is evidence that S. *rebaudiana* extracts are also non-acidogenic. The pH level of the biofilm formed after rinsing with a stevia solution as compared to rinsing with a sucrose solution is significantly higher.[108] Despite this, very little research has been done on the cariogenic or anti-cariogenic potential of stevia and further research has been recommended.[107]

1.5 Relative sweetness and concentration of a solution for laboratory studies

Relative sweetness measurements to identify sweeteners that have similar qualities to that of sucrose is usually done in one of three ways: a discrimination test, a rating method or magnitude estimation.[109] Sucrose is the usual standard compound for determining relative sweetness scores. The relative sweetness index of sucrose is usually assigned a score of 100.[110-111]

The discrimination test specifically involves a two-alternative forced choice (2-AFC) method, where a panellist is asked to choose the sweetest sample while comparing the intensity of sweetness between two samples. The results cannot be expressed as a numerical value.[109] With the rating method and magnitude estimation, the intensity of the sensory attributes can be numerically quantified. In a rating method an interval scale and descriptive analysis measures are used.[109]

Many studies have been conducted to compare the sweet concentrations of sweeteners to sucrose.[110,112-114] The potency of various sweeteners can vary with the methodology used to determine sweetness, as well as methods to obtain information. Overall, the results from most studies were similar, allowing a guideline to be formulated.[109,112-114] Sucrose, xylitol and erythritol have approximately the same sweetness and should be used in the same quantities.[109] Manufacturer's instructions for these products are generally given as "One serving xylitol or erythritol substitutes to one serving of sugar." Stevia is 300 times sweeter than sugar.[110] Different sweeteners' sweetness are expressed as a value compared to sucrose as the reference.[110]Based on the above, the following values were proposed:

sucrose : xylitol : erythritol : stevia

1 : 1 : 0.7 : 300

The recommendation according to the manufacturers of commercially available stevia is usually: "One part stevia sweetener in volume is equal in sweetness to 5 parts sugar in volume. Use only the tip of a teaspoon to sweeten beverages or food" (Lifestyle Nutrition Stevia, obtained from Dischem, Glen Austin, South Africa). The potency of stevia as a

sweetener depends on the proportion of stevioside and rebaudioside used in the product which varies according to manufacturer. This affects the intensity of the sweetness which has to be considered when manufacturers recommend which volume should be used.[110]

The products used for this study are readily available in retail stores. The samples were made up according to the manufacturer's instructions, simulating the concentrations that patients will be exposed to.

The method of preparing the solution should be considered when reviewing literature. Concentration is defined as the amount of a given substance in a stated weight or volume of a material.[115] Molar concentration can be used, where the number of gram molecular weight of a substance in a litre of solution is used. In certain instances, the use of molar concentrations have more advantages, especially when comparing thresholds for different substances. Expressing a value in terms of percentage is however not always exact, as there are three different methods to prepare a solution.[115]
According to Pfaffmann *et al*.[115]to make up a 10% sucrose solution, one of three methods can be used:

i) Method 1 (Percentage by weight of solute and solvent): 10g of sucrose dissolved in 90g of distilled water. The total solution regardless of its volume, weighs 100g.
ii) Method 2 (weight added to volume): 10g of sucrose added to 100ml of distilled water. This method should be discouraged, because the total weight of the solution equals 110g and the solution is then a 9.1% solution, not 10% as advocated.
iii) Method 3 (weight by volume): Weigh out 10g of sucrose, dissolve in distilled water, gradually adding water until the total volume of the solution equals 100ml.

For the purpose of this study, the concentration levels of samples calculated were based on weight by volume (method 3) and not molar concentration, as stevia's concentration cannot accurately be expressed.[115]

A detailed description of the preparation of samples will follow in section 3.5.3.

Based on the literature reviewed it is clear that sugar plays an important role in the cariogenic process and it is necessary to further investigate the cariogenic potential of commercially available sugar substitutes in an attempt to lower caries incidence.

This study focussed on determining Colony Forming Units (CFUs) of *S.mutans* in the media exposed to different sweeteners, in order to estimate the growth of viable bacterial cells in a sample. It also focussed on determining the pH in order to establish whether the sugar substitute is incorporated in the microorganism's metabolism and fermented. It lastly focussed on observing the formation and maintenance of a biofilm in the presence of sucrose and the sugar substitutes: xylitol, erythritol and stevia.

Chapter 2 : Aims and Objectives

2.1 Aims

The aim of this study was to determine and compare the cariogenic potential of commercially available sugar substitutes namely: xylitol, erythritol and stevia. Striving to find a suitable substitute for sucrose, one of the main causative factors of Early Childhood Caries.

2.2 Objectives

The objectives of this *in vitro* study were to determine the:

- Colony Forming Units (CFU) of *S. mutans* after exposure to xylitol, erythritol and stevia
- Acid production of *S.mutans* in the presence of xylitol, erythritol and stevia
- Biofilm formation of *S. mutans* on enamel surfaces in the presence of xylitol, erythritol and stevia

2.3 Statistical Null/Zero Hypothesis

There is no difference in the CFU, pH and biofilm formation between the commercially available sugar substitutes (xylitol, erythritol and stevia) and the controls (sugar and quarter strength Ringers solution) in the presence of *S. mutans.*

Chapter 3 : Materials and methods

3.1 Study design

This research project was conducted as a randomized controlled, cross-sectional observational *in vitro* study. Quantitative analysis was used to assess CFUs and pH and biofilm formation was evaluated using descriptive analysis.

3.2 Setting

The teeth used in this study were obtained from the Oral and Dental Hospital of the University of Pretoria. The preparation of the enamel slabs was performed in the Department of Odontology's laboratory in the Oral and Dental Hospital. Further laboratory work was conducted in the Phytomedicine Laboratory of the Department of Paraclinical Science at the Onderstepoort Campus of the University of Pretoria. Scanning Electron Microscopy (SEM) was carried out at Sefako Makgato Health Sciences University, using the Zeiss Supra 55 VP Field Emission variable pressure Scanning Electron Microscope (FE-SEM) as described under laboratory procedures 3.5.6 Permission letters to perform the research at the above mentioned institution were obtained (Appendix A).

3.3 Case selection

Recently extracted human primary teeth were collected from a pool of extracted teeth, as described under ethical considerations 3.4. Only sound buccal surfaces of primary molar teeth were used. Enamel surfaces affected by demineralization, caries or pathology were excluded from this study.

3.3.1 Sample size

A sample size of 52 enamel blocks were used as determined by the statistician. The enamel blocks were numbered and randomly divided into groups using Research Randomizer Software (Research Randomizer version 4.0) (Urbaniak, G.C & Plous S). The samples consisted

of five groups of 10 each (one group per time interval plus an additional group for a 48hrs SEM analysis). Two blocks were left untreated for Scanning Electron Microscopy (SEM) comparison.

The 10 enamel blocks per time interval were further randomly divided into five groups for each treatment (2 blocks each) namely Sucrose (Group A) as the positive control , xylitol (Group B), erythritol (Group C), stevia (Group D) and quarter strength Ringer's solution as the negative control (Group E). The enamel blocks were placed in a numbered well plate as shown in Figure 3.1 below.

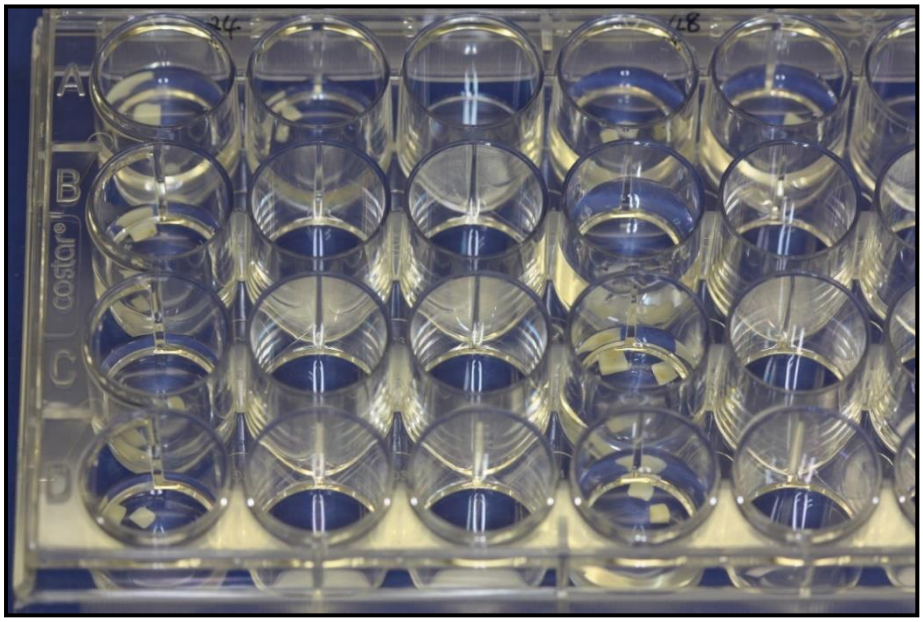

Figure 3.1: Numbered well plates containing enamel blocks

Objective 1, CFU: A total of 960 plates (petri dishes) were prepared (five groups: duplicate dilutions made for each group; eight serial dilutions for each duplicate dilution, plated in triplicate for each of the four time intervals [5x2x8x3x4=960]). The most suitable countable serial dilution plate ranges were selected, counted and recorded (90 plates per time interval; 360 plates recorded in total).

Objective 2, pH: Readings for pH from each well, were done in triplicate, to avoid bias or parallax readings. This enabled 30 readings per time interval. Four time intervals (6h; 12h; 18h; 24h) therefore enabled 120 pH readings in total.

Objective 3, Biofilm formation: All 52 blocks were used for SEM analysis. In total 345 images were taken and analysed for descriptive purposes and to confirm the formation of a biofilm.

3.4 Ethical Considerations

Teeth used for this study were extracted from human patients as part of an individualized comprehensive treatment plan and not for the purpose of this study. All parents/ legal guardians of children who attend the Oral and Dental Hospital of the University of Pretoria are required to complete and sign a patient information leaflet, a treatment plan and consent form before treatment commences. This form also gives them the option to allow extracted teeth to be used for research purposes. Ethical and safety guidelines for the handling and disposal of human teeth and laboratory research were followed strictly.

This study was approved by the Research Committee of the School of Dentistry of the University of Pretoria with the protocol number 2018/17 (Appendix B). Ethical clearance was obtained from the Ethics Committee of the Faculty of Health Sciences of the University of Pretoria with the ethical clearance number 86/2019 (Appendix C).

Bias was avoided by using randomizing software to allocate the prepared enamel blocks to each group of experimental solutions. The microbiologist exposed the blocks to the different media to guarantee that the principal researcher was blinded to the groups while performing all laboratory procedures and data collection. SEM analysis was also performed while blinded to the nature of the groups. The principal researcher was informed which test media was used for the respective groups only after all data was collected.

3.5 Laboratory processes

3.5.1 Obtaining enamel blocks

Immediately after extraction, the teeth were rinsed under running water and brushed with a tooth brush to remove any attached soft tissue. Thereafter the teeth were placed in distilled water in an ultrasonic water bath (Woson®) and sonicated until all loose biological material was removed. The teeth were then stored in sterile distilled water at 4˚C and used within three months after extraction.

Sectioning of the teeth was done with a diamond wafering blade under constant water irrigation in an Isomet 11-1180 low speed saw (Buehler Ltd, Lake Bluff, Illinois, USA). They were initially sectioned horizontally near the cemento-enamel junction to separate the crown and the root of the tooth to enable easier handling (Figure 3.2).

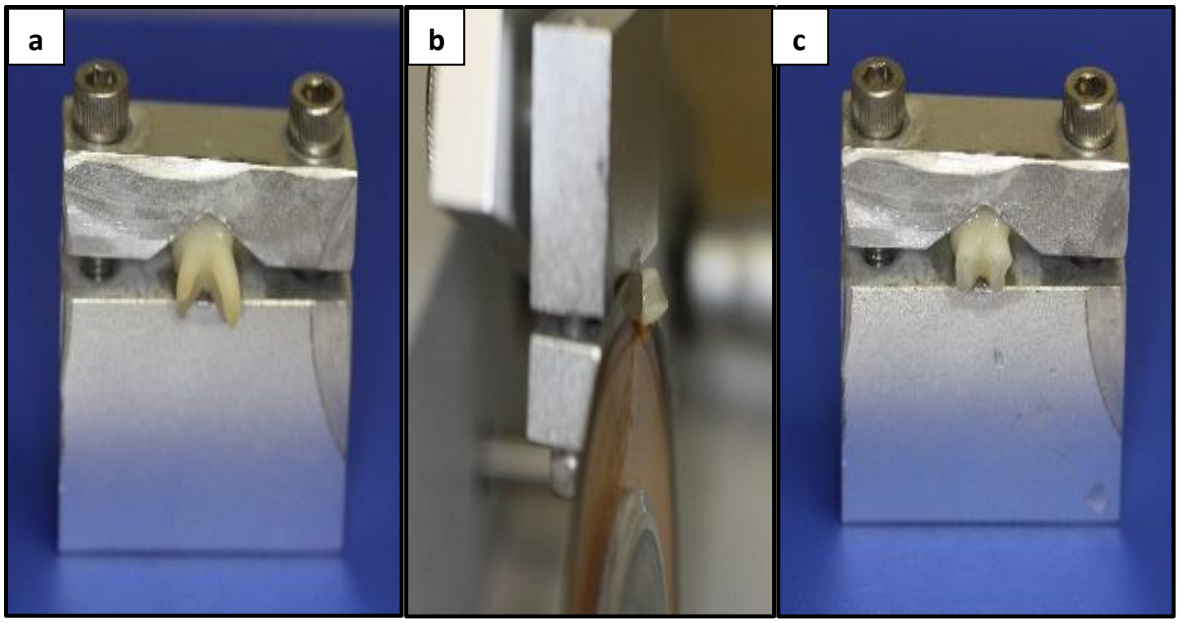

Figure 3.2: Sectioning of primary molars near the cemento-enamel junction with wafering blade: a) A primary tooth firmly secured; b) Wafering blade used to section tooth; c) Crown with severed roots

The remaining crowns were then positioned in such a way to enable severing of the buccal enamel surfaces (Figure 3.3).

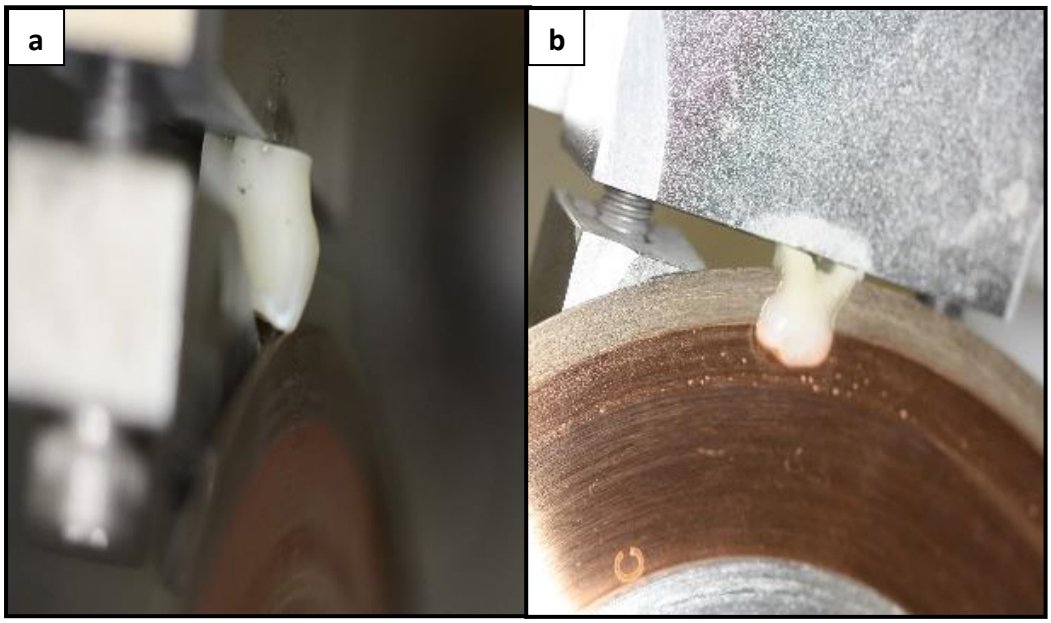

Figure 3.3: Removing intact enamel surface using wafering blade

The enamel was then cut into blocks of 2mm x 2mm in size, under constant water irrigation (Figure 3.4).

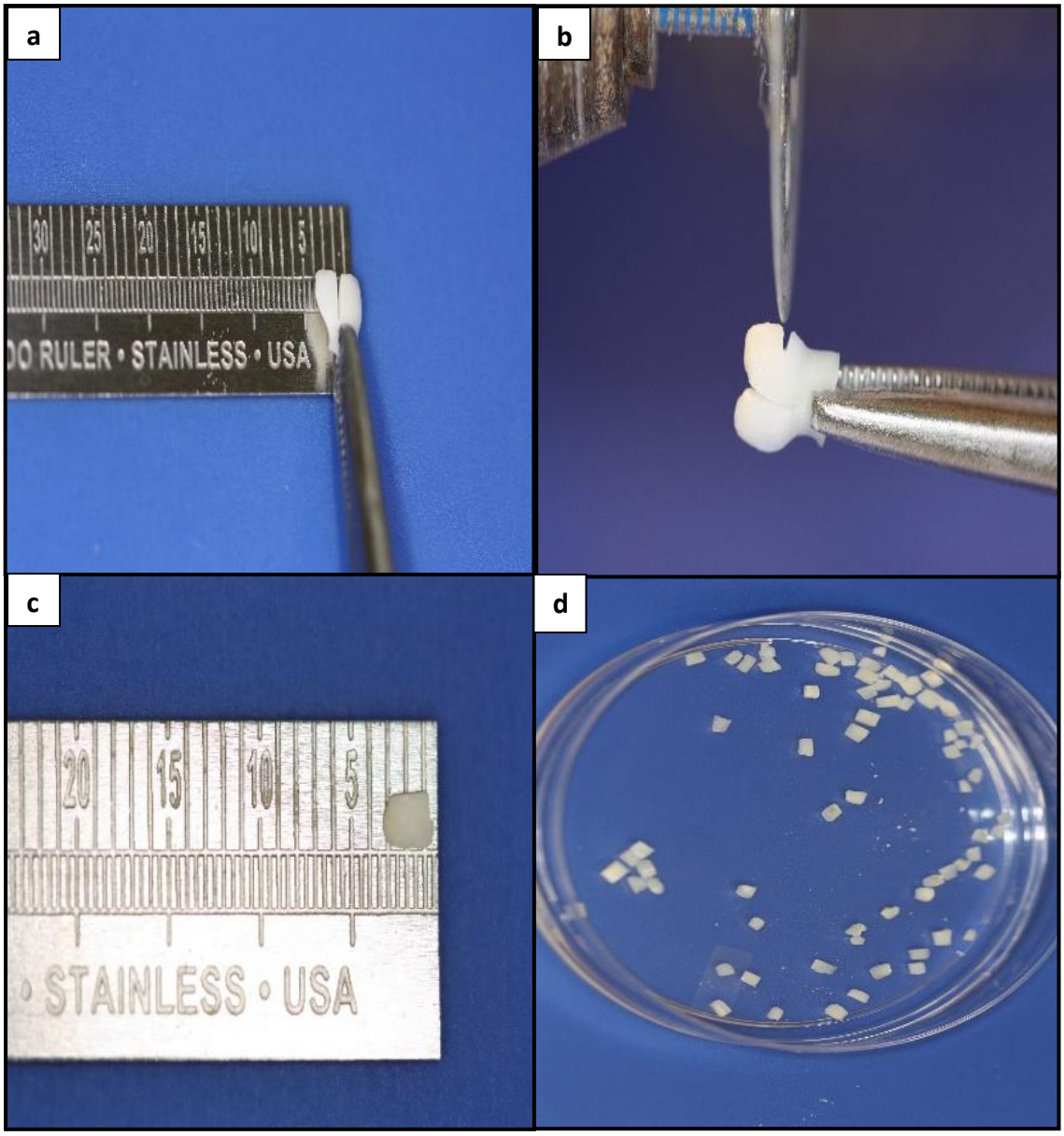

Figure 3.4: Preparation of 2mm enamel blocks: a) Meticulous measurement to ensure blocks of even sizes; b) Cutting of enamel slab into blocks; c) Measurement of blocks after cutting was done; d) Enamel blocks stored in sterile water once cut

The blocks were sterilised in an autoclave at 121˚C for 15 minutes and then stored at 4˚C until laboratory work was conducted.

When the laboratory work commenced, the sterile enamel blocks were placed in individual tissue wells in such a way that the enamel surface faced upward (Figure 3.5).

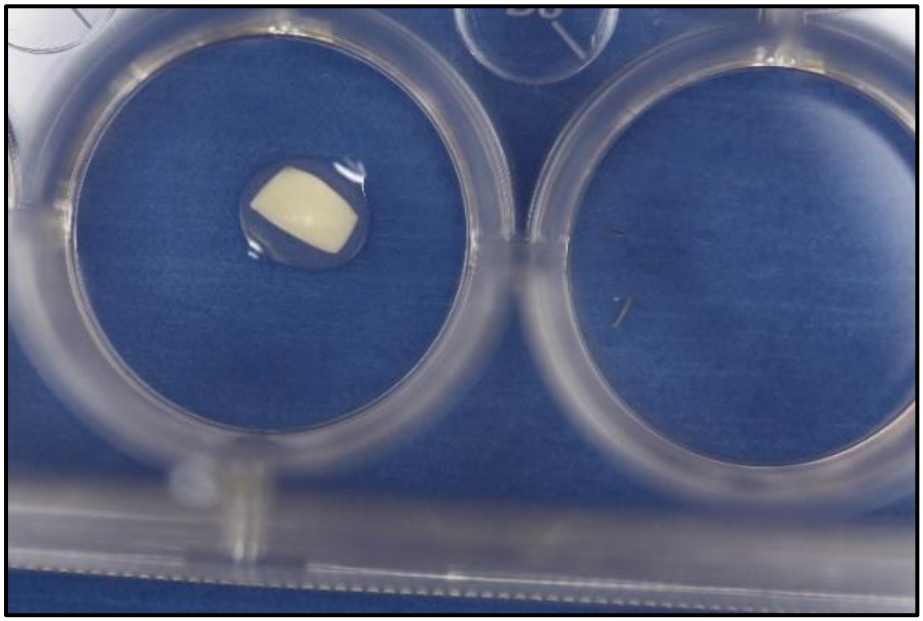

Figure 3.5: Enamel block placed facing upward in tissue well

3.5.3 Exposing enamel blocks to the different media

In this study, the concentration levels of test media was based on weight by volume and not molar concentration.[115] The principle of this method is to precisely weigh the sucrose or sugar substitute, to dissolve it and then to gradually add liquid to the desired volume. The weight of the sucrose and sugar substitutes that were used, were determined according to the relative sweetness and manufacturers' instructions as explained in section 1.5. Solutions were prepared in 20ml sterile water and filtered through 0.22 Millipore filters (Merck SA) to sterilise the solutions before adding them to the Peptone-Casein-Soy-basal medium (PCSB).

The PCSB medium was prepared by dissolving the following in 1L deionized water:

17g Casein Peptone, 3g Soybean Peptone, 5,0g NaCl, 2,5g K_2HPO_4. The medium was autoclaved at 15 psi, 121 ˚C for 30 min on liquid cycle. The medium was stored at 2-8 ˚C after preparation and used within 24hrs.

Treatment A: 5% sucrose: 5 g of sucrose was dissolved in 20mL distilled water, added to PCSB to a total volume of 100mL, and kept at room temperature (25±2˚C) for no longer than 48hrs.[110]

Treatment B: 5% xylitol: 5g of xylitol was dissolved in 20mL distilled water, added to PCSB to a total volume of 100mL, and kept at room temperature (25±2˚C), for no longer than 48hrs[110] (Figure 3.6)**.**

Treatment C: 5% erythritol: 5g erythritol was diluted in 20mL distilled water, added to PCSB to a total volume of 100mL, at room temperature (25±2˚C), kept for no longer than 48hrs[110] (Figure 3.6).

Treatment D: 1g of stevia powder was diluted in 20mL distilled water, added to PCSB to a total volume of 100mL, and kept at room temperature (25±2 ˚C), for no longer than 48hrs[110] (Figure 3.6)**.**

Treatment E: Quarter strength Ringers solution (Merck SA) served as the negative control.

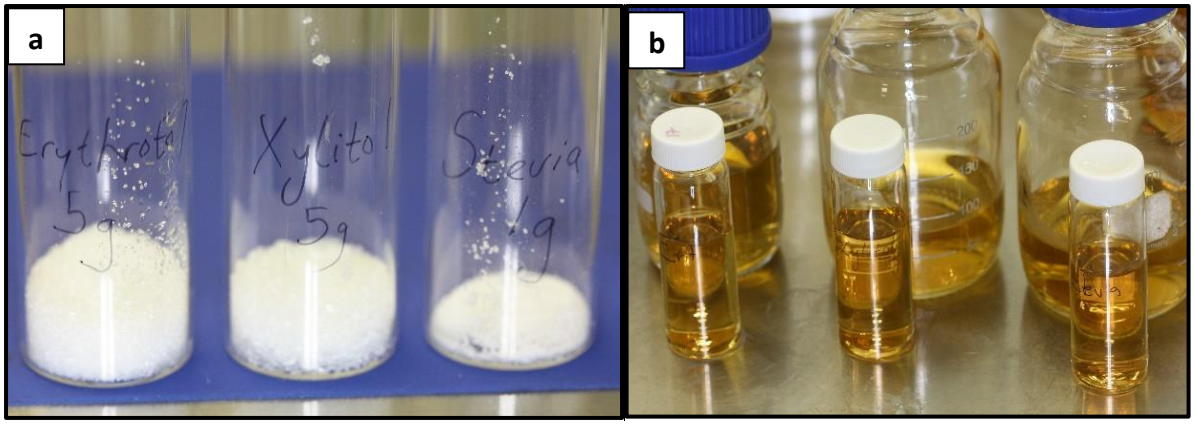

Figure 3.6: Samples weighed and constituted to a 100mL solution

Five different well plates were used, one for each time interval (6hrs, 12hrs, 18hrs, 24hrs and 48hrs) at which tests were conducted and for biofilm analysis and comparison. The tissue wells were numbered per time interval, and marked with A1 and A2 being the positive control, containing sucrose treatment. B1, B2, C1, C2, D1, D2 containing the treatments to be tested and E1 and E2 being the negative control. All the wells except for group E were filled with 2mL PCSB containing the different sweetener treatments. The negative control group E was filled with 2mL sterile Ringers solution (Figure 3.7)**.**

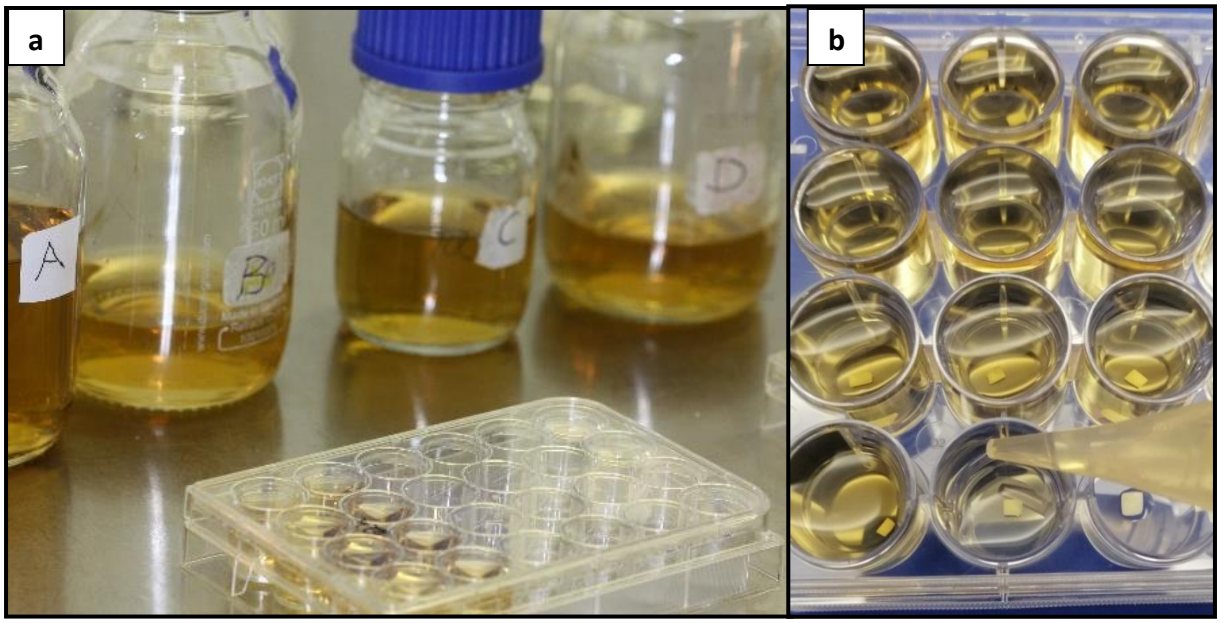

Figure 3.7: Well-plates were filled with media containing different treatments

Once all five well-trays were filled, they were placed in an incubator for 1h at 37˚C to allow for pellicle formation. The wells were then inoculated with *S. mutans* as described under 3.5.4.

The microbiologist grouped the specimens together and provided the principal investigator with the samples at the relevant time intervals. The principal investigator was thus blinded when performing the CFU counts, pH readings and all laboratory procedures and data collection.

3.5.4 Inoculation of the media containing enamel blocks

Before the experiment was conducted, it was imperative to ensure that the *S. mutans* strain used to inoculate the media with was pure. The inoculum was equal to a McFarland standard 1 solution and the viability of the *S. mutans* strain was tested before the experiment was conducted.

The following steps were followed: growth media was prepared; the *S. mutans* strain was grown and viewed under a light microscope; a 1% McFarland solution of the *S. mutans* was

made up; the viability of the S. mutans in the 1% McFarland solution was verified; the experiment was conducted with the different treatments and enamel blocks.

Preparing Agar growth media

Petri dishes were prepared with Agar growth media: growth media made up by meticulously weighing of the ingredients using balance boats and sterile instruments. It consisted of 5g Glucose: 23g Mueler-Hinton Agar: 15g Bacteriological Agar mixed with 1000mL distilled, sterilized water. A heated magnetic stirrer was used to mix the solution until it was completely dissolved and translucent. The solution was autoclaved at 121˚C for 20min and then transferred to the petri dishes under sterile conditions and left to set over night (Figure 3.8).

Figure 3.8: Agar growth media in petri dishes

Test *S. mutans* strain for purity

S. mutans strain ATCC 25175 was grown on growth media in an anaerobe flask with Anaerocult and placed in an incubator at 37˚C for 24hrs (Figure 3.9).

Figure 3.9: Growing of *S. mutans* in an anaerobe flask

The colonies were tested for purity before making up the inoculum. This was done by spreading the culture in a drop of sterile water in a thin film over a microscope glass slide using a sterile inoculating needle. The needle was sterilized between spreads and the process repeated twice more to create three different drops on the slide[116] (Figure 3.10).

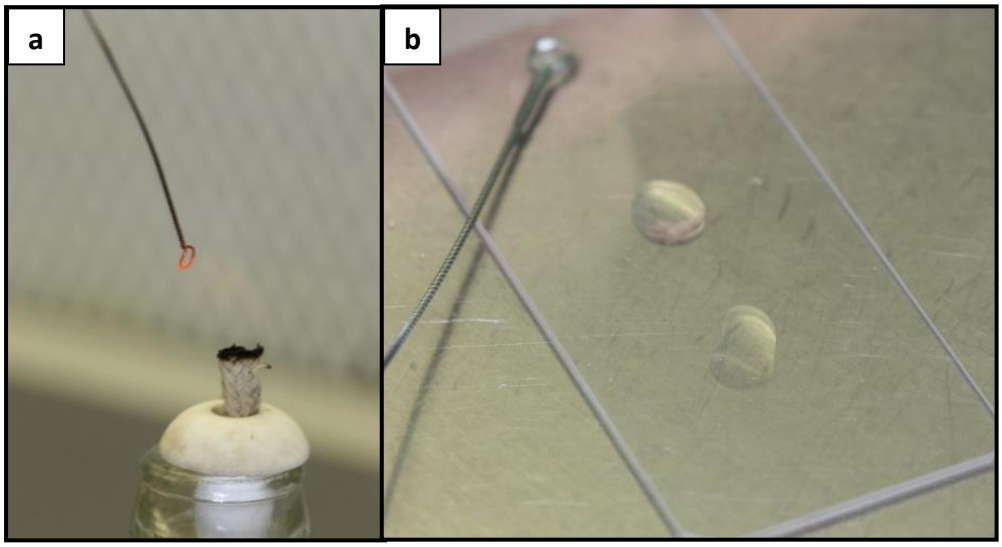

Figure 3.10: Inoculating needle sterilized in flame before being used to transfer culture to the microscope slide

The slide was air dried, and passed through a flame, smear side up, to fix the bacteria. Fixation causes adherence of the specimen to the glass plate and prevents it from being washed off during staining (Figure 3.11).[116]

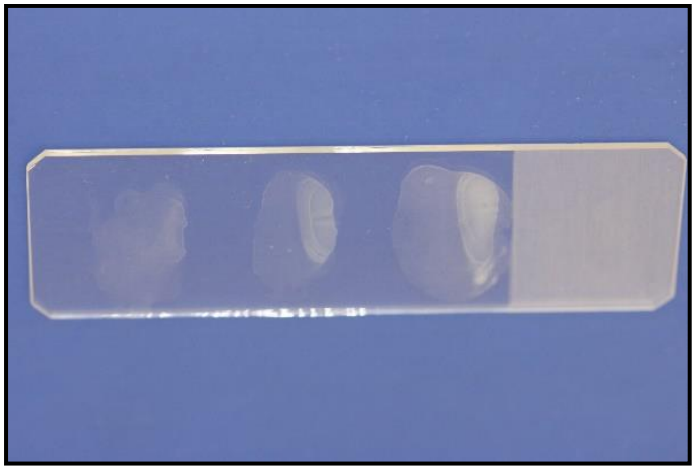

Figure 3.11: Prepared smear fixed to the microscope plate

Gram staining is useful for the identification of bacteria as well as the choice of treatment for bacterial infections.[33] Gram-staining was performed to ensure that a pure bacteria had grown. This was done by dropping Crystal violet on the plate, waiting one minute and then rinsing with water. This was followed by adding a drop of Iodine solution which was left for 1 minute and then rinsed with water. A drop of Safranine was then added, left for 1 minute and rinsed off. The glass plate was air dried and examined under a microscope to determine purity of the bacterial strain (Figure 3.12 a and b).[116]

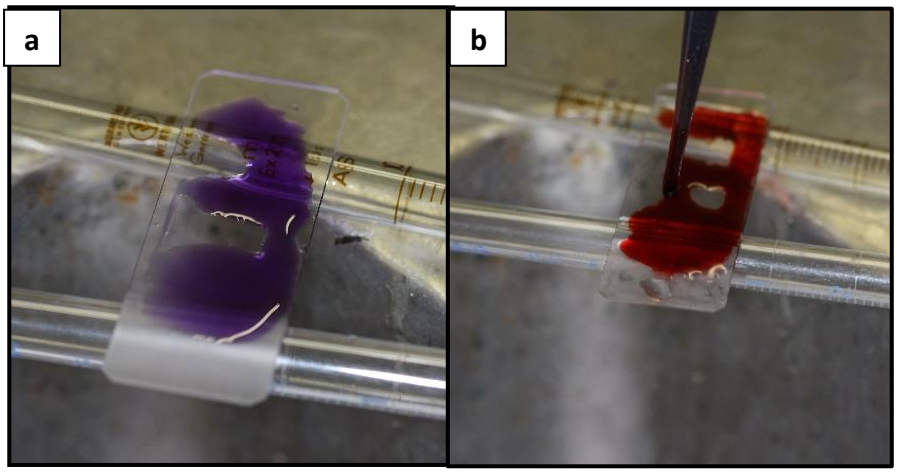

Figure 3.12: Staining process with a) Crystal violet; b) Safranin

The pure colonies that had grown on this medium were identified and confirmed under a light microscope (Figure 3.13).

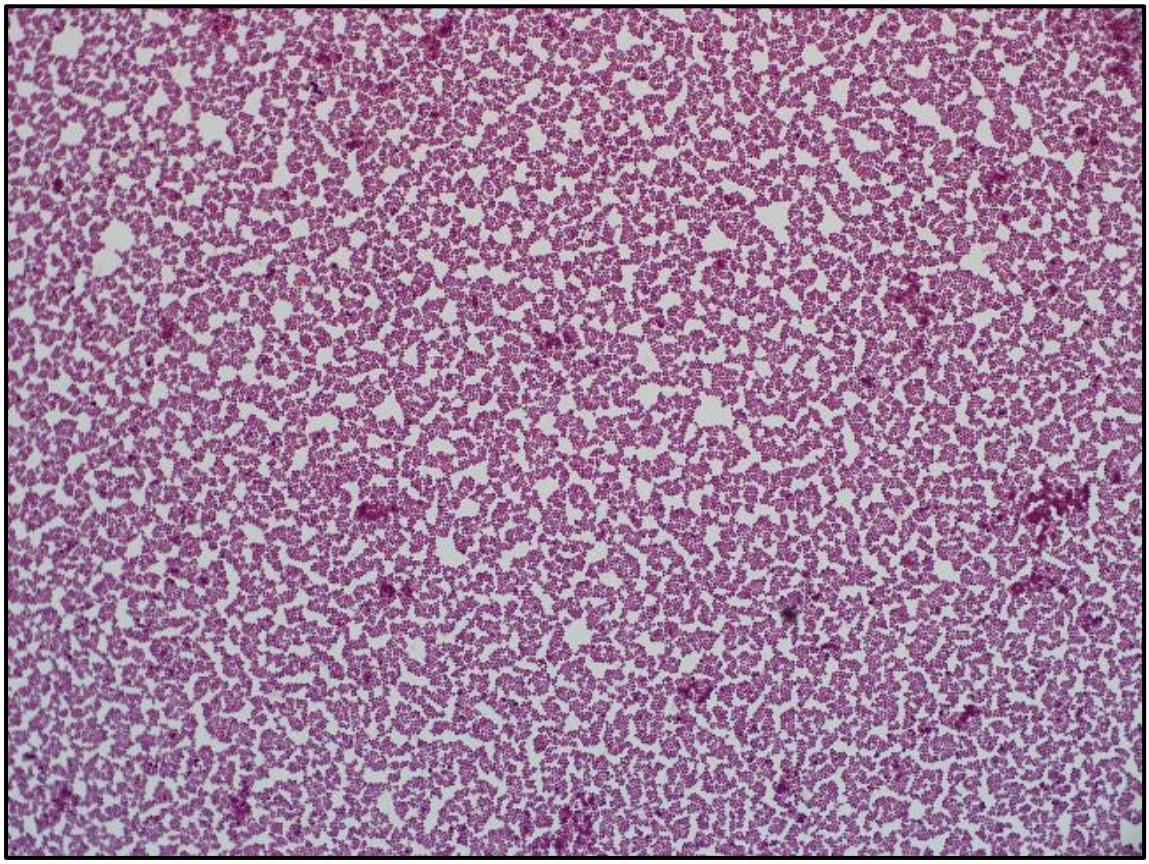

Figure 3.13: A photograph confirming pure *S. mutans* strain as seen under a light microscope

Making up a McFarland Standard 1 suspension of *S. mutans*

McFarland standards are used to standardize microbial testing. Pure colonies were used to make up a 1% McFarland Standard 1 suspension of *S.mutans* that was then used for inoculation. The PCSB media, that does not contain glucose, was used. A sterile inoculation needle was used to transfer bacteria from the agar plate on which it had grown, to the inoculation media. Quarts cuvettes were used in a photospectron meter to determine the exact dilution equal to the MacFarland standard solution. Distilled water was placed in the first cuvette to determine the zero value, followed by the media used to prepare the inoculum in the second cuvette, which gave a reading of 0.010. The McFarland standard solution was placed in the third cuvette with a reading of 0.147. These values were combined to establish a master inoculating solution with the ideal reading 0.157, thus equal to McFarland Standard 1 (Figure 3.14 a and b).

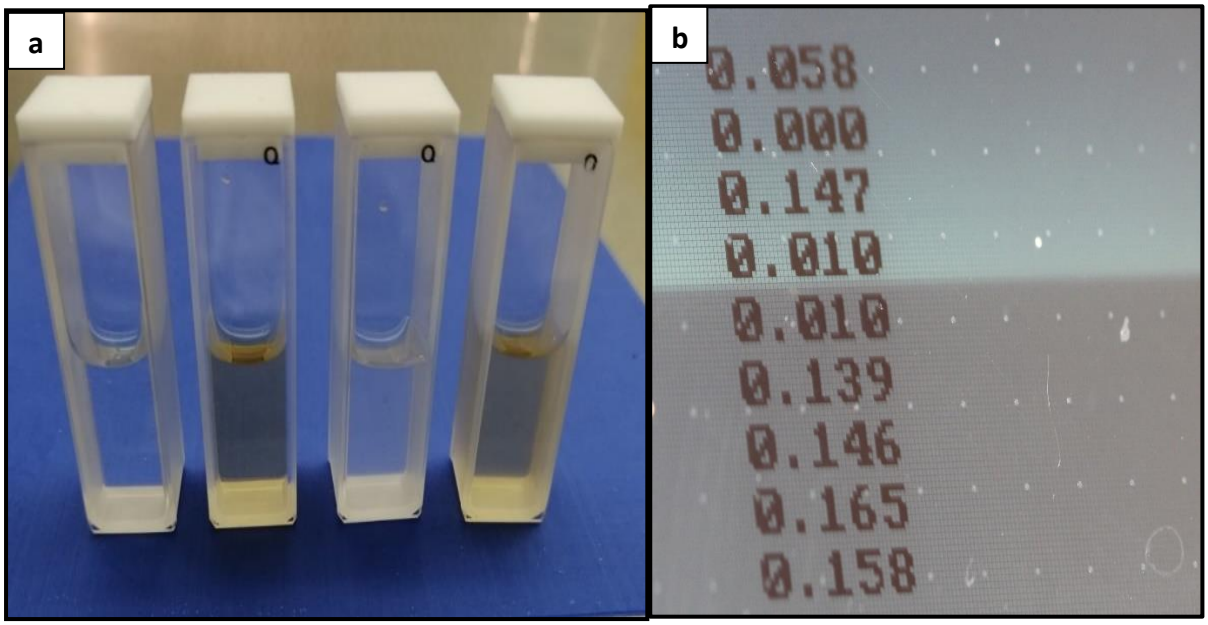

Figure 3.14: a) Quarts cuvets containing distilled water, media, McFarland standard 1 solution and the master solution; b) Readings on the photospectron meter

Serial dilution

Large numbers of bacteria grow and reproduce at a very fast rate. It is not possible to count every cell within a population and therefore a representative sample must be counted and then multiplied to estimate the total number of organisms in the whole specimen.[116] For this study, viable plate counts were carried out by means of serial dilution and the spread plate technique was done. A sample of the suspension contains too many bacterial cells to count if it is plated. To compensate for this problem, a series of dilutions were made and the numbers of resulting colonies were counted (Figure 3.15).

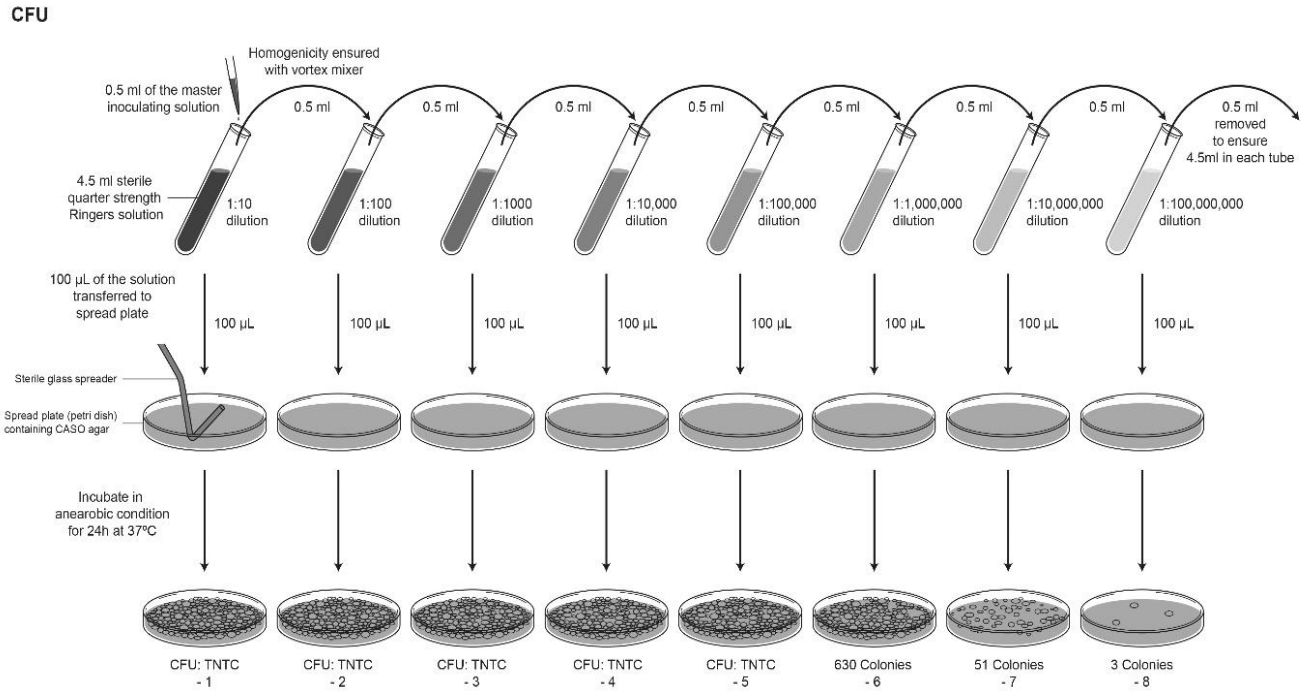

Figure 3.15: Illustration of how serial dilution, the spread plate technique and CFU count was done

The number of colonies were counted and multiplied by the reciprocal of the dilution to estimate the number of bacteria per 1mL in the original culture. The accuracy of this method depends on the cells' ability to grow on the specific medium, the number of cell deaths, the growth phase of the sample population and the homogeneity of the dilution.[116] To ensure that errors were minimized, this procedure was done with the aid of a Vortex mixer to ensure homogeneity. Multiple plates were inoculated and log-phase cultures were used.[33]

Serial dilution was done to ensure that counting would be possible once plated and incubated. For this study, serial dilution was done up to 10^{-8} (Figure 3.16 a and b).

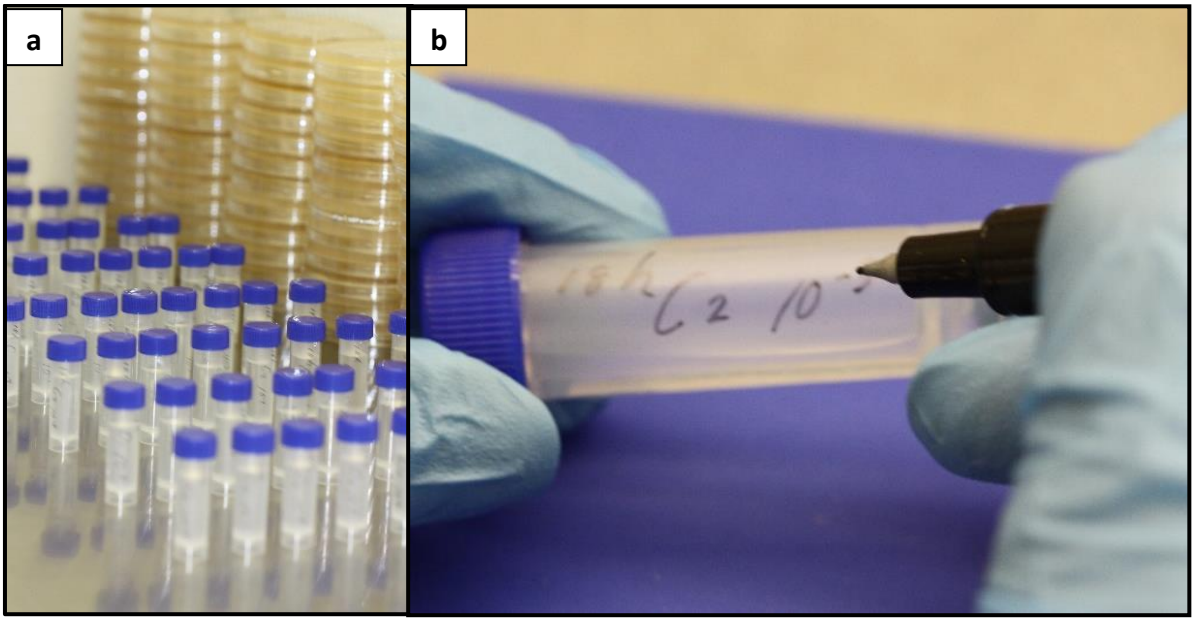

Figure 3.16: a) Serial dilution up to 10^{-8} for all treatments; b) Marked tubes

Explaining the spread plate technique

Automated pipettes were used to transfer 100µL of the mixture in the $^{-1}$ dilution to a correspondingly marked petri dish containing growth media (Figure 3.17).

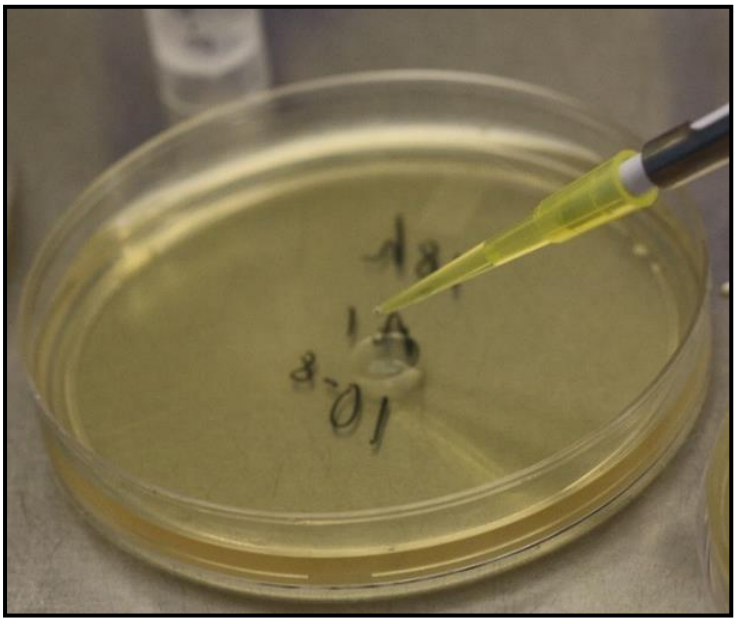

Figure 3.17: Serial diluted solution transferred to petri dish using automated pipettes

A sterile glass spreader was used to spread the mixture thoroughly and evenly. The lid was placed back on the dish and it was stored in an incubator at 37˚C for 24hrs to allow for colony growth. This was done for all serial dilutions (Figure 3.18).[117]

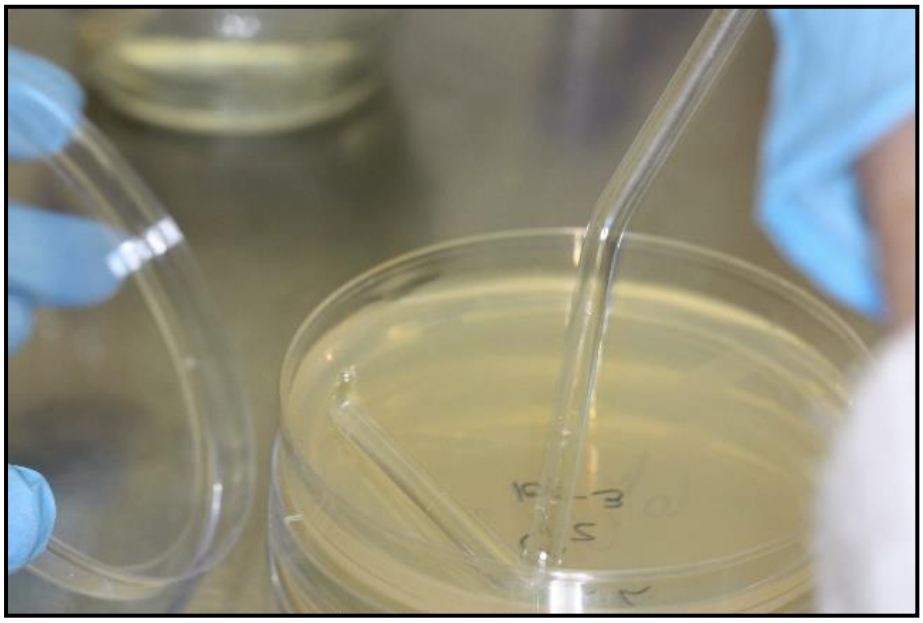

Figure 3.18: The spread plate technique

Viability of McFarland Standard 1 confirmed by means of serial dilution and CFU

The viability of the microorganisms were confirmed by means of serial dilution and the spread plate technique. The CFU for the McFarland standard 1 solution was assessed and the viability of the *S. mutans* strain confirmed (Table 3.1).

Table 3.1 The viability of *S. mutans* strain confirmed by CFU count after serial dilution

Serial Dilution	10^{-6}	10^{-7}	10^{-8}
CFU count	630	51	3
CFU count duplicate	520	88	4

3.5.4 Determination of Colony Forming Units (CFU) for treatments

After allowing pellicle formation on the enamel blocks by exposing the blocks to different solutions for 1h, the 1% McFarland Standard 1 suspension of *S. mutans* was added to the wells containing the blocks. Automated pipettes with sterile tips were used to enable exactly 20µL inoculum to be added to each well of all the well plates (Figure 3.19).

Figure 3.19: Inoculation of wells using automated pipettes

The well plates were incubated anaerobically using Anaerocult A (MerckSA) in a shaker incubator. The procedure was carried out under sterile conditions. Sterility was maintained for the duration of the entire experiment and the experiment was conducted in a sterile biohazard cabinet with positive airflow, using sterile instruments, gloves and masks. To determine the CFUs of the different treatments at different time intervals (6hrs, 12hrs, 18hrs and 24hrs), serial dilution was done as previously described. Duplicate dilutions were used and plated in triplicate to eliminate bias (Figure 3.20).[117]

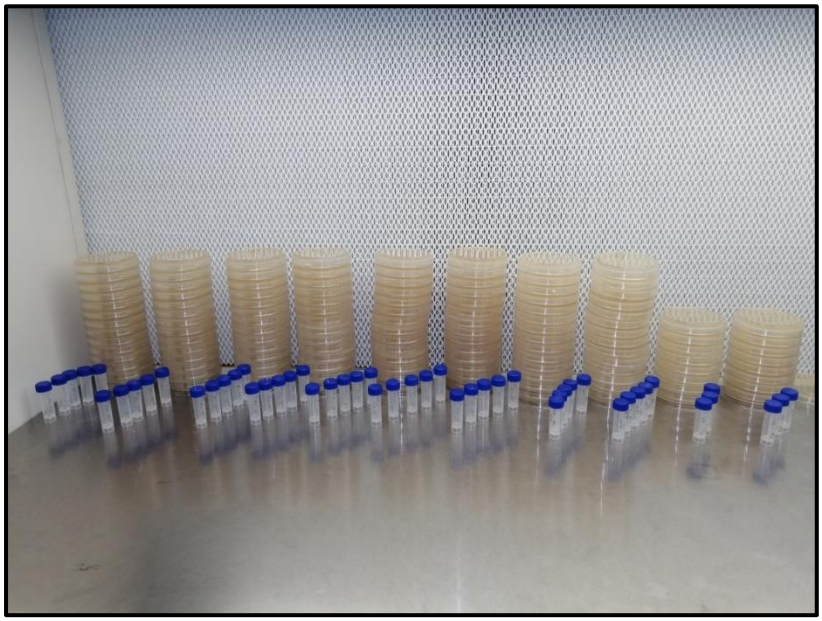

Figure 3.20: Serial diluted samples and petri dishes containing Casein peptone Soy flour peptone (CASO) agar meticulously marked and arranged

The plates were then incubated at 37˚C for 24-48hrs in anaerobic conditions (10% CO_2 using Anaerocult® A) (Figure 3.21).

Figure 3.21: Plates were incubated at 37˚C for 24-48h

Once removed from the incubator, the plates were sorted and CFUs assessed (Figure 3.22).

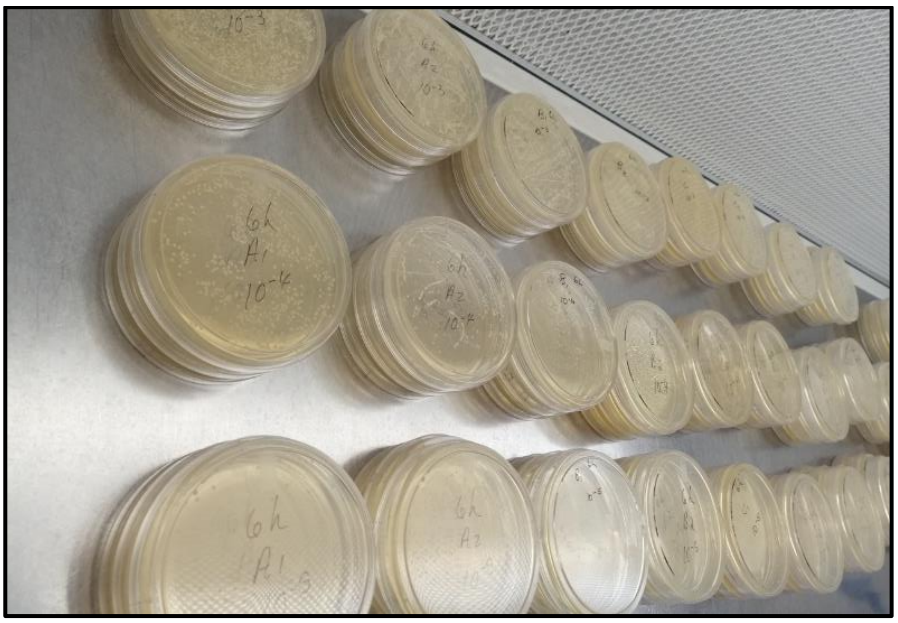

Figure 3.22: Assessment of countable colonies

The colonies that formed within a countable range were counted. By way of explanation, for treatment A, at 18hrs at a serial dilution of 10^{-6}, 31 colonies were counted (Figure 3.23).

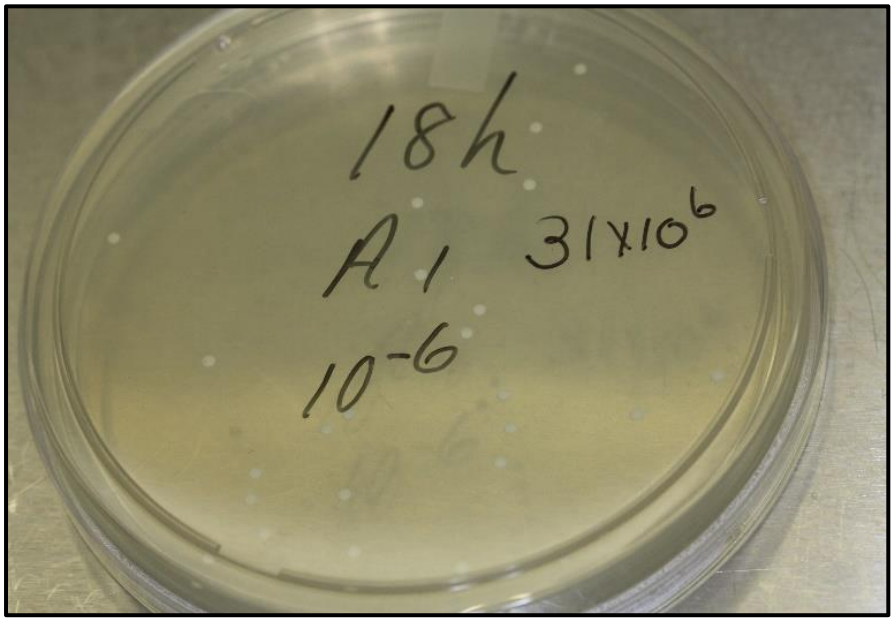

Figure 3.23: Colonies counted at 18hrs at a serial dilution of 10-6

The CFUs were marked by means of a black permanent marker to ensure exact counting (Figure 3.24).

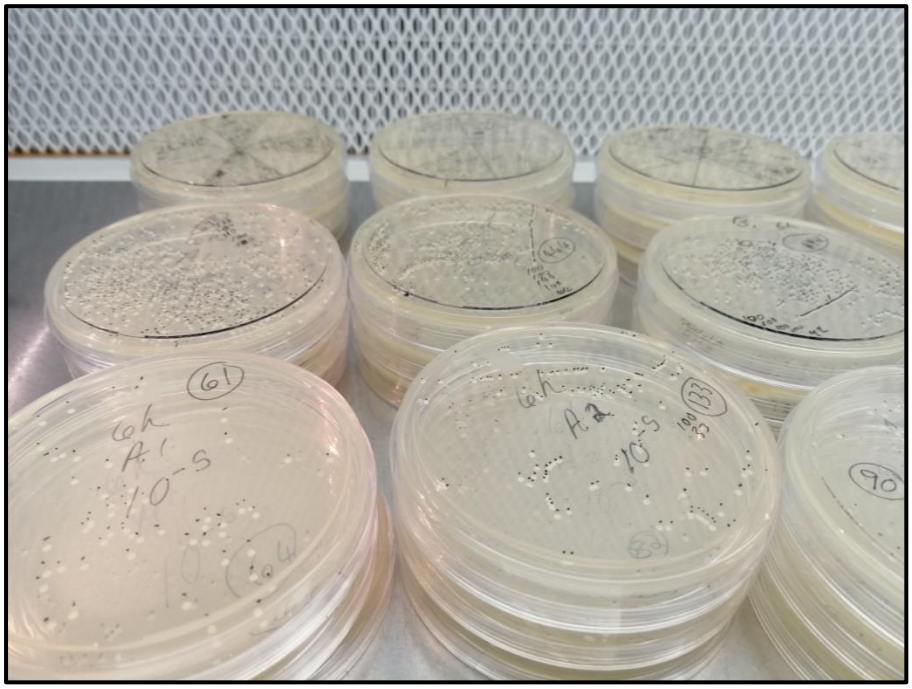

Figure 3.24: A permanent marker was used to ensure exact counting

The samples in the range that were less diluted were too numerous to count (TNTC) (Figure 3.25).

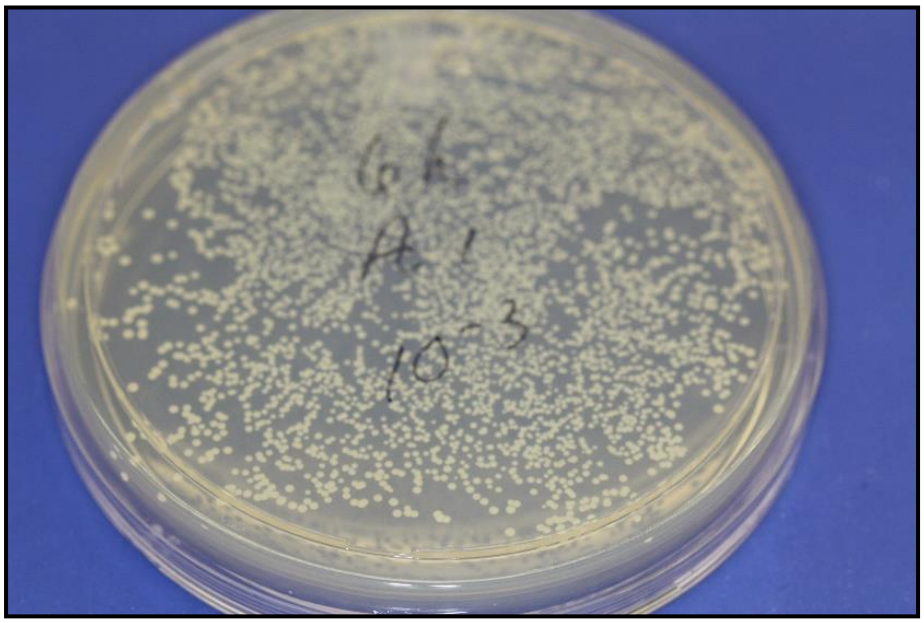

Figure 3.25: Colonies that were too numerous to count (TNTC)

Thus, to determine the colony formation of the same solution, the colonies that were formed in the more diluted ranges were counted. Of the eight available dilutions for each time interval, three representative groups of dilutions that were most suitable for counting for each specimen, were identified and counted. The actual counts were converted to Log-values and compared to ensure that the dilutions were done correctly and to eliminate bias.

An example of how Log-values were calculated:

260 colonies counted in 10^{-6} dilution

260 x 10^{6} =260 000 000 (indicating the amount of organisms in the sample)

Log= 8,4

3.5.5 Determination of pH

At each time interval (6hrs, 12hrs, 18hrs, 24hrs), the enamel blocks were removed from the well plates and the pH of the remaining media was determined in triplicate. The principal investigator, blinded from the groups, determined the pH of each sample using a micro-electrode pH meter (Oakton pH700 pH/mV/ ˚C /F Bench Meter). The pH meter was calibrated before the readings were done, using a standardized pH 4 buffer, pH 7 buffer and pH 10 buffer solution (Oakton, USA) (Figure 3.26 a and b).

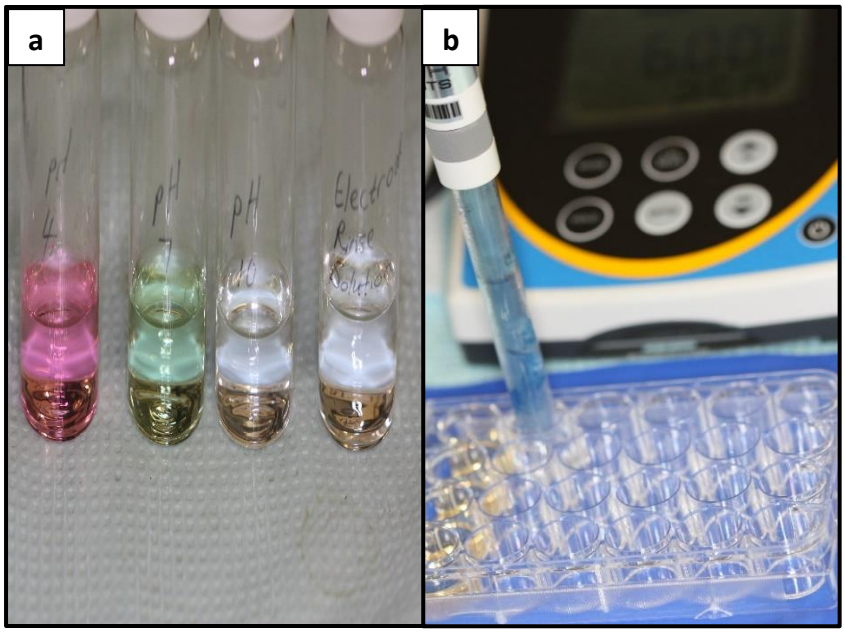

Figure 3.26: a) Buffer solutions for calibration; b) Measuring the pH levels of each well

3.5.6 Scanning Electron Microscope (SEM) analysis

At five time intervals (6hrs, 12hrs, 18hrs, 24hrs and 48hrs), two samples were collected from each group for SEM analysis to determine and describe biofilm formation. Samples were placed in marked, sterile well plates. The samples were prepared according to standard methods for biological materials.[118] Samples were fixed with 1.5mL freshly prepared 0.5% glutaraldehyde in 0.025 M phosphate buffer (pH 7.4), for one hour. The glutaraldehyde was removed with a disposable plastic pasteur pipet and rinsed three times in the same phosphate buffer saline for five minutes each time (Figure 3.27).

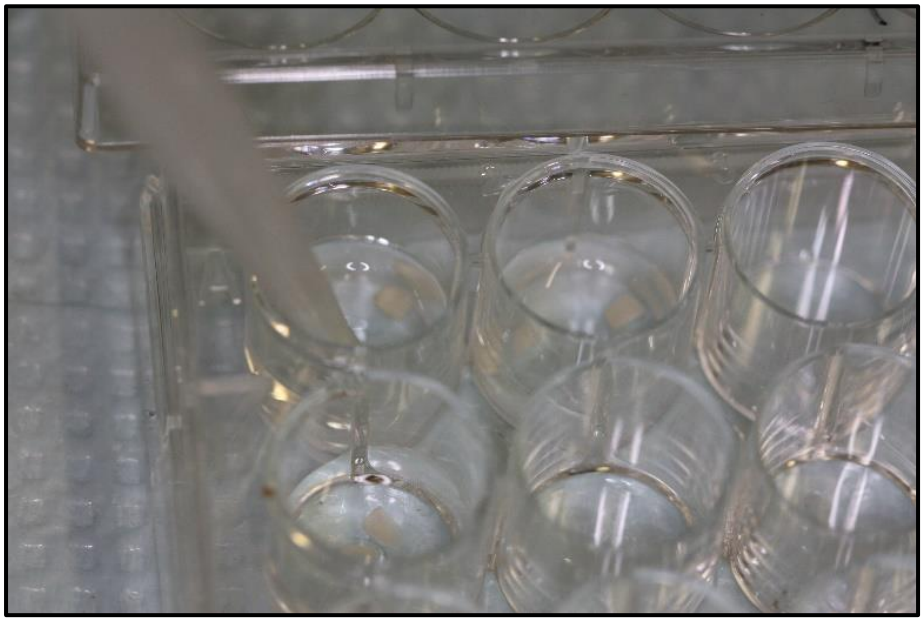

Figure 3.27: Rinsing of samples with phosphate buffer saline

The samples were then transferred to an extraction cabinet and fixed in 0.25% Osmiumtetroxide (OsO_4) (Merck, Darnstadt, Germany) for 30 minutes and again rinsed 3 times in phosphate buffer saline (PBS) for 5 minutes each time. OsO_4 is very poisonous and extreme care was taken not to inhale or expose the skin to this chemical. Special containers for disposable toxic chemicals were used to collect the discarded fluids as well as the used plastic pipettes.

The samples were rinsed for five minutes each time in increasing concentrations of Ethanol (EtOH) (Merck, Darnstadt, Germany), namely 30%, 50%, 70% & 90%. The enamel slabs were then kept in a 96% alcohol solution and transported to the Sefako Makgato University's Scanning Electron Microscopy Unit for further evaluation. The samples now had a grey appearance due to the OsO_4 fixation. The samples were scanned with a light microscope (Zeiss) to confirm that the enamel faced upward before final preparation (Figure 3.28).

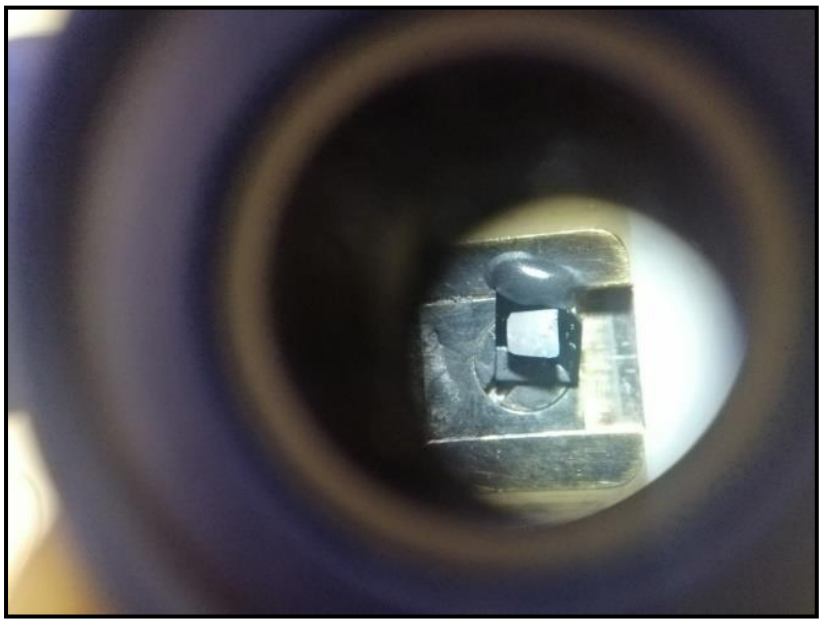

Figure 3.28: Confirmation that enamel faced upward with light microscopy

Once viewed under the microscope, enamel slabs were placed on spotting plates, covered with 100% alcohol and left for 10 minutes. The 100% alcohol was substituted with Hexamethyldisilazane (HMDS), by using disposable pasteur pipettes. The HMDS was left to evaporate. Surface tension was lowered by HMDS and therefore it eliminated the likelihood of distortion of the cells (Figure 3.29).

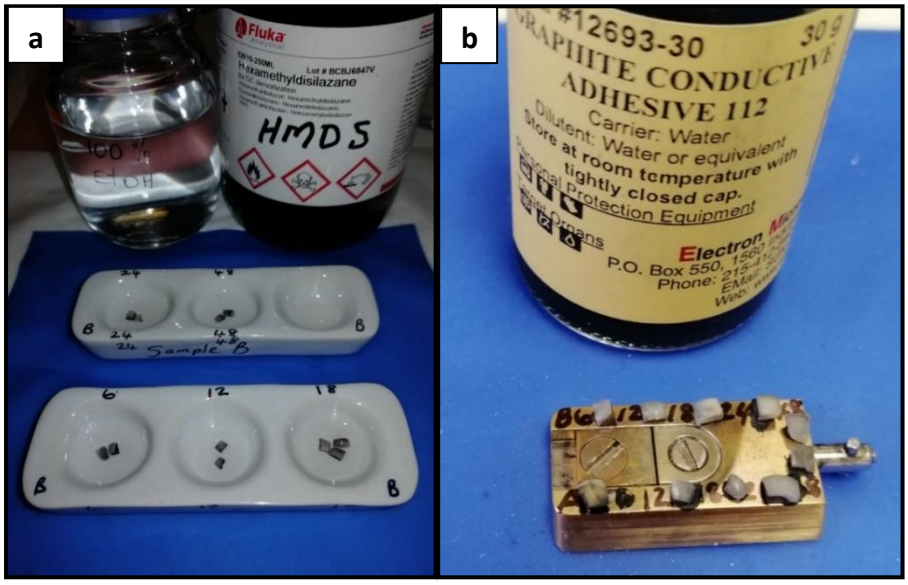

Figure 3.29: a) Enamel slabs on spotting plates treated with 100% Alcohol and HMDS; b) Samples mounted on viewing plate with conductive adhesive

The desiccated enamel slabs were mounted on a FE-SEM viewing plate by means of double sided carbon tape and graphite conductive adhesive 112 (CNTech®) to stabilize the samples (Figure 3.29 b). The viewing plate was then placed in an evacuator to form a vacuum around the sample in a tube before placing it in the preparatory chamber of the FE-SEM (Figure 3.30 a and b).

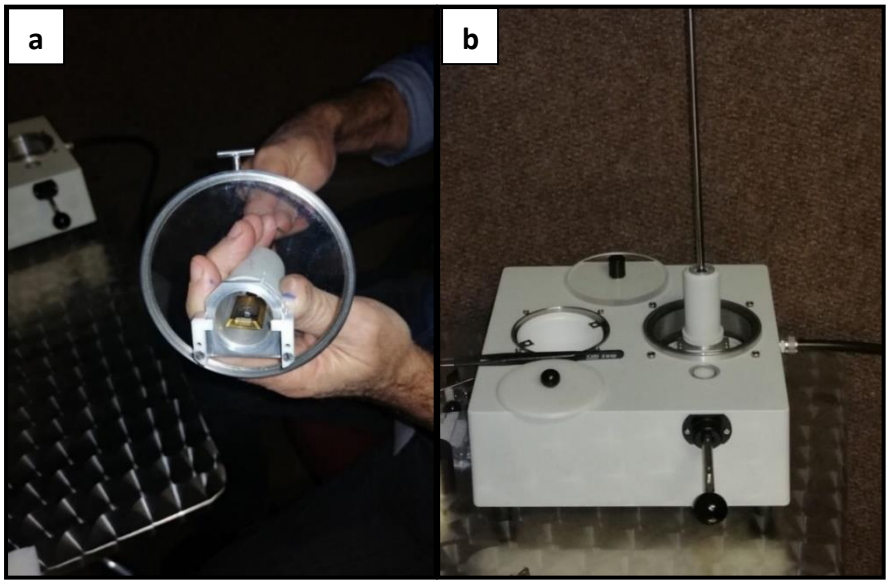

Figure 3.30: a) Viewing plate prepared to be placed in evacuator b) The evacuator creates a vacuum around the viewing plate

Once placed in the preparatory chamber, the samples were coated with platinum. Platinum has a fine grain, which ensured that the sample structure was not distorted when viewed. The carbon tape, conductive adhesive and platinum coating ensured good conduction to prevent splattering of electrons with subsequent distortion of images. Once coated, the viewing plate was transferred to the viewing chamber (Figure 3.31).

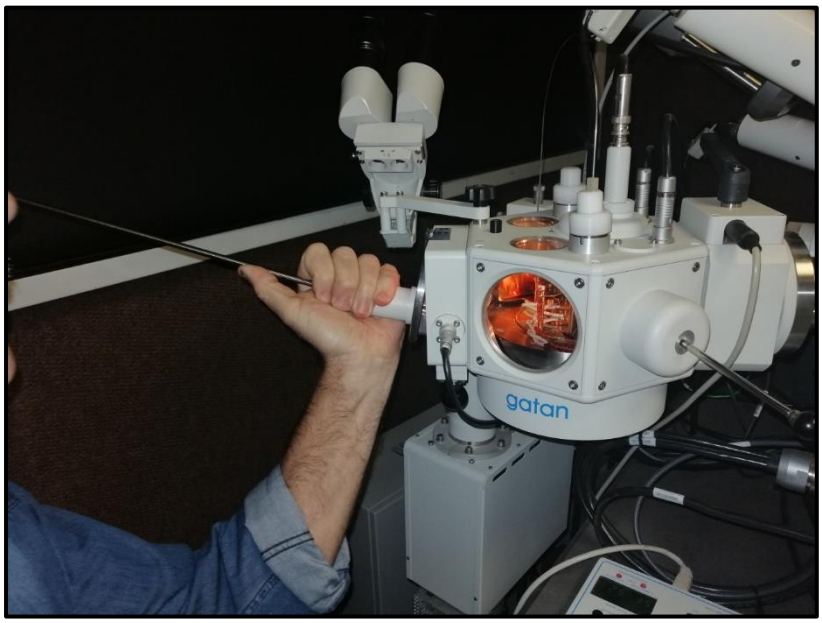

Figure 3.31: Samples placed in the preparatory chamber of the SEM

The samples were viewed with a Zeiss Supra 55 VP FE-SEM at the Sefako Makgato University, South Africa. Images were analysed recording the formation of biofilm. Magnification was set low enough to enable an overall view of each enamel slab and to help with sample orientation. Each enamel slab was viewed at 35 x magnification and thereafter divided into four zones that were viewed at 65 x magnification each. This enabled the viewer to understand the structure and morphology of the enamel. Each zone was viewed and evaluated and all areas on each slab were examined and evaluated at magnifications 1500x, 3000x, and 8000x. Images that were of relevance were photographed and recorded for analysis and future reference as representative images.

3.6 Data collection

The data collected during the laboratory procedures were recorded in a laboratory book and transferred to an excel spreadsheet. This information was shared with the statistician for analysis. The data was interpreted in collaboration with the statistician as reported in chapter four.

3.7 Statistical analysis

The data was statistically managed and analysed using a factorial study design with factors **treatment** (sucrose, xylitol, erythritol and stevia) and **time** (6hrs, 12hrs, 18hrs and 24hrs). The data for CFU counts and pH were respectively analysed using a two-way ANOVA analysis of variance with main factors treatment and time, inclusive of interaction between factors. The significance level was set at ($p < 0.05$).

Chapter 4 : Results

The CFU counts and pH of each group were statistically analysed and compared at different time intervals. The CFU counts and pH were also analysed and compared between different groups at various time intervals. Finally, the CFU counts and pH values of all groups were compared at all time intervals. SEM analysis was done for descriptive and comparative purposes.

4.1 CFU analysis

Values of the mean CFU count for each treatment were determined and compared at 6hrs, 12hrs, 18hrs and 24hrs. The statistical analysis of CFU counts over time for each treatment is summarized below.

4.1.1 Sucrose

The mean CFU count for sucrose at each specific time interval is reported in Table 4.1, whilst Table 4.2 reports the change in CFU counts for sucrose over time.

Table 4.1: Predicted mean CFU count for sucrose over time

Time	Mean	95% confidence interval
6hrs	6.79	(6.70 ; 6.87)
12hrs	8.40	(8.31 ; 8.48)
18hrs	9.38	(9.29 ; 9.46)
24hrs	8.94	(8.86 ; 9.03)

Table 4.2: Change in CFU counts with comparisons over time sucrose

Time contrast	Mean difference	95% confidence interval	P value
12hrs vs 6hrs	1.61*	(1.49 ; 1.73)	0.001
18hrs vs 12hrs	0.98*	(0.86 ; 1.10)	0.001
24hrs vs 18hrs	-0.44*	(-0.56 ; -0.32)	0.001

The mean difference of sucrose treatment compared over time with the superscript (*) were statistically significantly different at $p<0.05$.

4.1.2 Xylitol

The mean CFU count for xylitol at each specific time interval is reported in Table 4.3**,** whilst Table 4.4 reports the change in CFU counts for xylitol over time.

Table 4.3: Predicted mean CFU count for xylitol treatment over time

Time	Mean	95% confidence interval
6hrs	6.76	(6.67 ; 6.84)
12hrs	8.60	(8.51 ; 8.68)
18hrs	9.25	(9.17 ; 9.34)
24hrs	8.87	(8.78 ; 8.95)

Table 4.4: Change in CFUs count with comparisons over time for xylitol treatment

Time contrast	Mean difference	95% confidence interval	P value
12hrs vs 6hrs	1.84*	(1.72 ; 1.96)	0.001
18hrs vs 12hrs	0.66*	(0.54 ; 0.78)	0.001
24hrs vs 18hrs	-0.39*	(-0.51 ; -0.27)	0.001

The mean difference of xylitol treatment compared over time with the superscript (*) were statistically significantly different at $p<0.05$.

4.1.3 Erythritol

The mean CFU count for erythritol at each specific time interval is reported in Table 4.5, whilst Table 4.6 reports the change in CFU counts for erythritol over time.

Table 4.5: Predicted mean CFU count for erythritol treatment over time

Time	Mean	95% confidence interval
6hrs	6.82	(6.73 ; 6.90)
12hrs	8.39	(8.31 ; 8.48)
18hrs	9.37	(9.29 ; 9.46)
24hrs	9.43	(9.35 ; 9.52)

Table 4.6: Change in CFU counts with comparisons over time for erythritol treatment

Time contrast	Mean difference	95% confidence interval	P value
12hrs vs 6hrs	1.58*	(1.46 ; 1.70)	0.001
18hrs vs 12hrs	0.98*	(0.86 ; 1.10)	0.001
24hrs vs 18hrs	0.60	(-0.06 ; 0.18)	0.301

The mean difference of erythritol treatment compared over time with the superscript (*) were statistically significantly different at $p<0.05$.

4.1.4 Stevia

The mean CFU counts for stevia at specific time intervals are reported in Table 4.7 whilst Table 4.8 reports the change in CFU counts for stevia over time.

Table 4.7: Predicted mean CFU counts for stevia treatment over time

Time	Mean	95% confidence interval
6hrs	6.86	(6.78 ; 6.95)
12hrs	7.98	(7.89 ; 8.06)
18hrs	9.19	(9.10 ; 9.27)
24hrs	8.92	(8.83 ; 9.00)

Table 4.8: Change in CFU counts with comparisons over time for stevia treatment

Time contrast	Mean difference	95% confidence interval	P. value
12hrs vs 6hrs	1.12*	(1.00 ; 1.24)	0.001
18hrs vs 12hrs	1.21*	(1.09 ; 1.33)	0.001
24hrs vs 18hrs	-0.27*	(-0.39 ; -0.15)	0.001

The mean difference of stevia treatment compared over time with the superscript (*) were statistically significantly different at $p<0.05$.

4.1.5. CFU comparison of all treatments at specific time intervals

The mean differences between treatments at specific time intervals are reported in Table 4.9 for 6hrs, Table 4.10 for 12hrs, Table 4.11 for 18hrs and Table 4.12 for 24hrs.

Table 4.9: Pairwise comparisons of adjusted predictions at 6hrs

Treatment pair	Mean difference	95% confidence interval	P value
Xylitol vs sucrose	-0.03	(-0.15 ; 0.09)	0.575
Erythritol vs sucrose	0.03	(-0.09 ; 0.15)	0.618
Stevia vs sucrose	0.07	(-0.05 ; 0.19)	0.213
Erytritol vs xylitol	0.06	(-0.06 ; 0.18)	0.296
Stevia vs xylitol	0.11	(-0.01 ; 0.23)	0.080
Stevia vs erythritol	0.04	(-0.8 ; 0.16)	0.442

There was no statistically significant difference between the different treatment groups at the 6hrs time interval ($p>0.05$).

Table 4.10: Pairwise comparisons of adjusted predictions at 12hrs

Treatment pair	Mean difference	95% confidence interval	P value
Xylitol vs sucrose	0.20*	(0.08; 0.32)	0.003
Erythritol vs sucrose	-0.01	(-0.13 ; 0.11)	0.918
Stevia vs sucrose	-0.42*	(-0.54 ; -0.30)	0.001
Erythritol vs xylitol	-0.20*	(-0.32 ; -0.08)	0.003
Stevia vs xylitol	-0.62*	(-0.74 ; -0.50)	0.001
Stevia vs erythritol	-0.41*	(-0.53 ; -0.29)	0.001

The mean difference of treatments compared at 12hrs with the superscript (*) were statistically significantly different at $p<0.05$.

Table 4.11: Pairwise comparisons of adjusted predictions at 18hrs

Treatment pair	Mean difference	95% confidence interval	P value
Xylitol vs sucrose	-0.13*	(-0.25 ; -0.01)	0.042
Erythritol vs sucrose	-0.01	(-0.13 ; 0.11)	0.904
Stevia vs sucrose	-0.19*	(-0.31 ; -0.07)	0.003
Erytritol vs xylitol	0.12	(-0.00 ; 0.24)	0.053
Stevia vs xylitol	-0.07	(-0.19 ; 0.05)	0.245
Stevia vs erythritol	-0.19*	(-0.31 ; -0.67)	0.005

The mean difference of treatments compared at 18hrs with the superscript (*) were statistically significantly different at $p<0.05$.

Table 4.12: Pairwise comparisons of adjusted predictions at 24hrs

Treatment pair	Mean difference	95% confidence interval	P value
Xylitol vs sucrose	-0.08	(-0.20 ; 0.04)	0.188
Erythritol vs sucrose	0.49*	(0.37 ; 0.61)	0.001
Stevia vs sucrose	-0.03	(-0.15 ; 0.09)	0.636
Erytritol vs xylitol	0.57*	(0.45 ; 0.69)	0.001
Stevia vs xylitol	0.05	(-0.07 ; 0.17)	0.385
Stevia vs erythritol	-0.52*	(-0.64 ; -0.40)	0.001

The mean difference of treatments compared at 24hrs with the superscript (*) were statistically significantly different at $p<0.05$.

A summary of the differences in the CFU counts for all treatments at the different time intervals is depicted in the graph below (Figure 4.1).

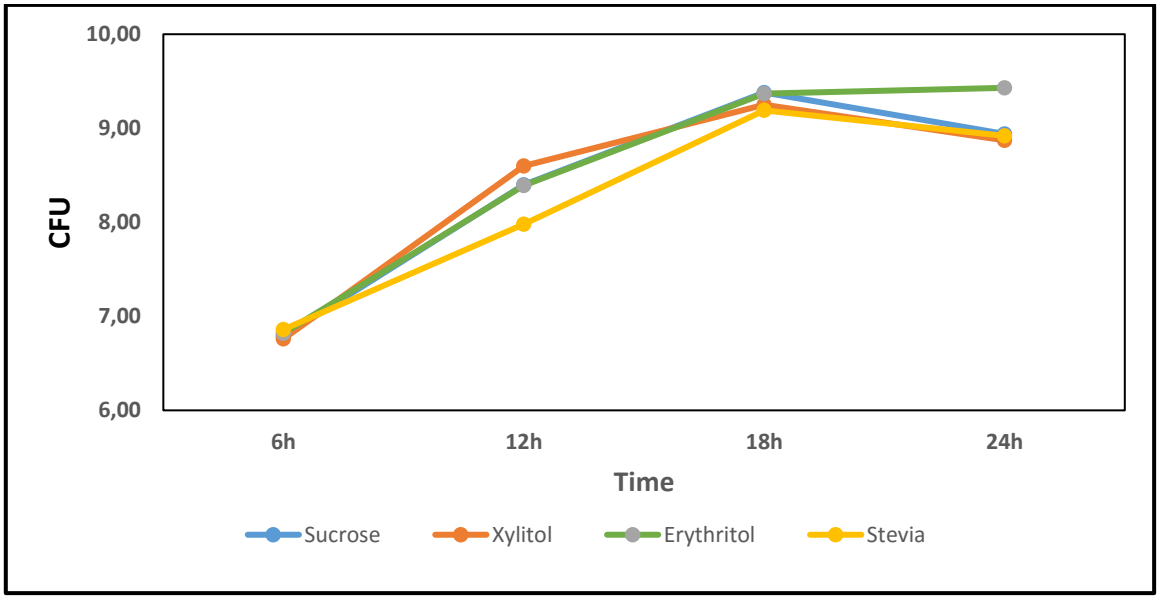

Figure 4.1: Display of marginal mean for mean CFU counts for various treatments over time.

4.2 pH Analysis

Values of the mean pH for each treatment were determined and compared at 6hrs, 12hrs, 18hrs and 24hrs. The statistical analysis of the pH over time for each treatment is summarized below.

4.2.1 Sucrose

The mean pH value for sucrose treatment at specific time intervals is reported in Table 4.13, whilst Table 4.14 reports the change in pH value for sucrose treatment over time.

Table 4.13: Predicted mean pH for sucrose over time

Time	Mean	95% confidence interval
6hrs	6.75	(6.63 ; 6.86)
12hrs	5.16	(5.04 ; 5.28)
18hrs	4.61	(4.49 ; 4.72)
24hrs	4.46	(4.34 ; 4.58)

Table 4.14: Change in pH with comparisons over time for sucrose

Time contrast	Mean difference	95% confidence interval	P value
12hrs vs 6hrs	-1.59*	(-1.75 ; -1.42)	0.001
18hrs vs 12hrs	-0.56*	(-0.72 ; -0.39)	0.001
24hrs vs 18hrs	-0.15	(-0.31 ; 0.18)	0.078

The mean difference in pH of sucrose treatments compared over time with the superscript (*) were statistically significantly different at $p<0.05$.

4.2.2 Xylitol

The mean pH value for xylitol treatment at specific time intervals is reported in Table 4.15, whilst Table 4.16 reports the change in pH values for xylitol treatments over time.

Table 4.15: Predicted mean pH for xylitol over time

Time	Mean	95% confidence interval
6hrs	6.91	(6.80 ; 7.03)
12hrs	5.94	(5.83 ; 6.06)
18hrs	5.80	(5.68 ; 5.91)
24hrs	5.83	(5.72 ; 5.95)

Table 4.16: Change in pH with comparisons over time for xylitol

Time contrast	Mean difference	95% confidence interval	P value
12hrs vs 6hrs	-0.97*	(-1.13 ; -0.81)	0.000
18hrs vs 12hrs	-0.15	(-0.31 ; 0.02)	0.075
24hrs vs 18hrs	0.04	(-0.13 ; 0.20)	0.655

The mean difference in pH of xylitol treatments compared over time with the superscript (*) were statistically significantly different at $p<0.05$.

4.2.3 Erythritol

The mean pH value for erythritol treatment at specific time intervals is reported in Table 4.17, whilst Table 4.18 reports the change in pH values for erythritol treatments over time.

Table 4.17: Predicted mean pH for erythritol treatment over time

Time	Mean	95% confidence interval
6hrs	6.87	(6.75 ; 6.98)
12hrs	6.03	(5.92 ; 6.15)
18hrs	6.02	(5.90 ; 6.13)
24hrs	6.21	(6.09 ; 6.32)

Table 4.18: Change in pH with comparisons over time for erythritol treatment

Time contrast	Mean difference	95% confidence interval	P value
12hrs vs 6hrs	-0.84*	(-1.00 ; -0.67)	0.000
18hrs vs 12hrs	-0.02	(-0.18 ; 0.15)	0.831
24hrs vs 18hrs	0.19*	(0.03 ; 0.35)	0.025

The mean difference in pH of erythritol treatment compared over time with the superscript (*) were statistically significantly different at $p<0.05$.

4.2.4 Stevia

The mean pH value for stevia treatment at specific time intervals is reported in Table 4.19, whilst Table 4.20 reports the change in pH values for stevia treatments over time.

Table 4.19: Predicted mean pH for stevia over time

Time	Mean	95% confidence interval
6hrs	6.84	(6.72 ; 6.95)
12hrs	6.09	(5.97 ; 6.20)
18hrs	6.05	(5.94 ; 6.17)
24hrs	6.28	(6.16 ; 6.39)

Table 4.20: Change in pH with comparisons over time for stevia

Time contrast	Mean difference	95% confidence interval	P value
12hrs vs 6hrs	-0.75*	(-0.91 ; -0.59)	0.000
18hrs vs 12hrs	-0.03*	(-0.20 ; 0.13)	0.670
24hrs vs 18hrs	0.23	(0.06 ; 0.39)	0.009

The mean difference in pH of stevia treatments compared over time with the superscript (*) were statistically significantly different at $p<0.05$.

4.2.5 pH Comparison between treatments at specific time intervals

The mean differences between treatments at specific time intervals are reported in Table 4.21 for 6hrs Table 4.22 for 12 hrs, Table 4.23 for 18hrs and Table 4.24 for 24hrs.

Table 4.21: Pairwise comparisons of adjusted pH predictions at 6hrs

Treatment pair	Mean difference	95% confidence interval	P value
Xylitol vs sucrose	0.17*	(0.00 ; 0.33)	0.046
Erythritol vs sucrose	0.12	(-0.41 ; 0.28)	0.133
Stevia vs sucrose	0.09	(-0.07 ; 0.25)	0.267
Erytritol vs xylitol	-0.04	(-0.21 ; 0.12)	0.567
Stevia vs xylitol	-0.08	(-0.24 ; 0.08)	0.323
Stevia vs erythritol	-0.03	(-0.20 ; 0.13)	0.670

The mean difference in the pH of treatments compared at 6hrs with the superscript (*) were statistically significantly different at $p<0.05$.

Table 4.22: Pairwise comparisons of adjusted pH predictions at 12hrs

Treatment pair	Mean difference	95% confidence interval	P value
Xylitol vs sucrose	0.78*	(0.62 ; 0.94)	0.000
Erythritol vs sucrose	0.87*	(0.71 ; 1.03)	0.000
Stevia vs sucrose	0.93*	(0.76 ; 1.09)	0.000
Erytritol vs xylitol	0.09	(-0.07 ; 0.25)	0.259
Stevia vs xylitol	0.14	(-0.02 ; 0.31)	0.081
Stevia vs erythritol	0.05	(-0.11 ; 0.22)	0.498

The mean difference in the pH of treatments compared at 12hrs with the superscript (*) were statistically significantly different at $p<0.05$.

Table 4.23: Pairwise comparisons of adjusted pH predictions at 18hrs

Treatment pair	Mean difference	95% confidence interval	P value
Xylitol vs sucrose	1.19*	(1.03 ; 1.35)	0.000
Erythritol vs sucrose	1.41*	(1.25 ; 1.57)	0.000
Stevia vs sucrose	1.45*	(1.28 ; 1.61)	0.000
Erytritol vs xylitol	0.22*	(0.57 ; 0.38)	0.011
Stevia vs xylitol	0.26*	(0.09 ; 0.42)	0.004
Stevia vs erythritol	0.37	(-0.13 ; 0.20)	0.640

The mean difference in the pH of treatments compared at 18hrs with the superscript (*) were statistically significantly different at $p<0.05$.

Table 4.24: Pairwise comparisons of adjusted pH predictions at 24hrs

Treatment pair	Mean difference	95% confidence interval	P value
Xylitol vs sucrose	1.37*	(1.21 ; 1.53)	0.000
Erythritol vs sucrose	1.745*	(1.58 ; 1.91)	0.000
Stevia vs sucrose	1.82*	(1.66 ; 1.98)	0.000
Erytritol vs xylitol	0.37*	(0.21 ; 0.54)	0.000
Stevia vs xylitol	0.45*	(0.29 ; 0.61)	0.000
Stevia vs erythritol	0.07	(-0.09 ; 0.24)	0.354

The mean difference in the pH of treatments compared at 24hrs with the superscript (*) were statistically significantly different at $p<0.05$.

The mean pH values for the different treatments compared at the different time intervals are depicted in the graph below (Figure 4.2).

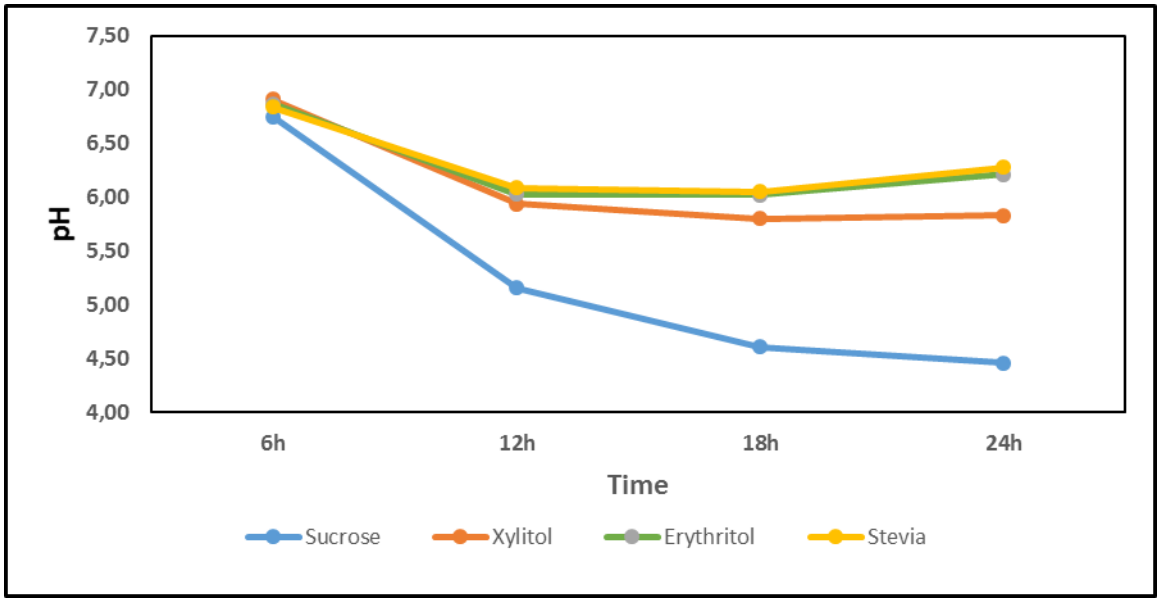

Figure 4.2: Display of marginal means for pH values over treatment and time

4.3 Comparison of CFUs and pH at different time intervals

The CFUs and pH of all treatments were compared at all the time intervals in Table 4.25.

Table 4.25: CFUs and pH of all groups compared at all the time intervals.

Treatment	Mean CFU 6hrs	Mean pH 6hrs	Mean CFU 12hrs	Mean pH 12hrs	Mean CFU 18hrs	Mean pH 18hrs	Mean CFU 24hrs	Mean pH 24hrs
Sucrose	6.79	6.75	8.40	5.16	9.38	4.61	8.94	4.46
Xylitol	6.76	6.91	8.60	5.94	9.25	5.80	8.87	5.83
Erythritol	6.82	6.87	8.39	6.03	9.37	6.02	9.43	6.21
Stevia	6.86	6.84	7.98	6.09	9.19	6.05	8.92	6.28

4.4 Correlation between CFU counts and pH for treatments.

4.4.1 Sucrose

The mean CFU counts (Table 4.1) and mean pH level (Table 4.13) are depicted on a double y-axis graph, showing the correlation between the CFU counts and pH levels for sucrose at different time intervals (Figure 4.3).

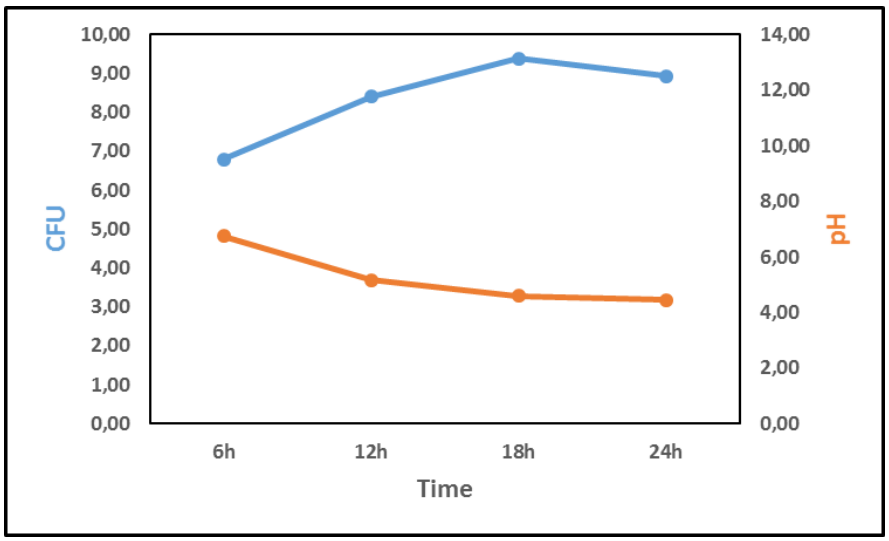

Figure 4.3: The correlation between CFU and pH with sucrose treatment

4.4.2 Xylitol

The mean CFU counts (Table 4.3) and mean pH level (Table 4.15) are depicted on a double y-axis graph, showing the correlation between the CFU counts and pH levels for xylitol at different time intervals (Figure 4.4).

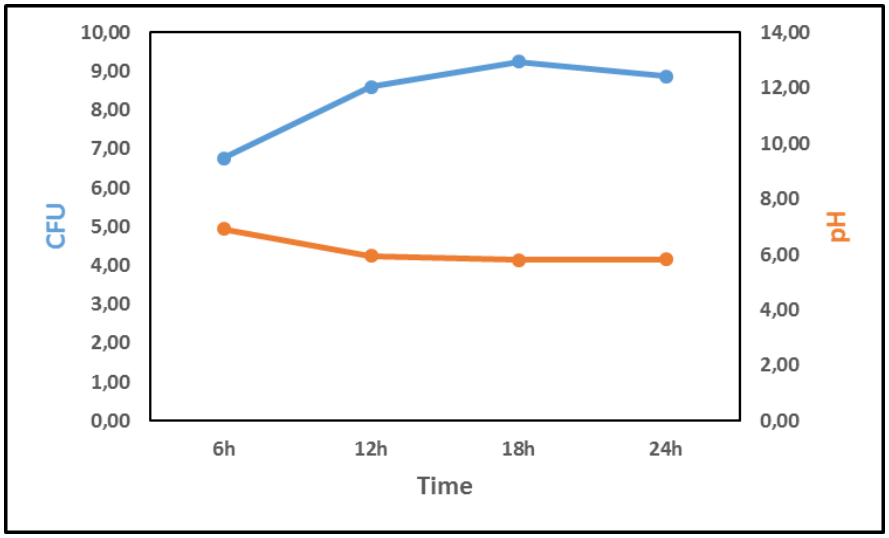

Figure 4.4: The correlation between CFU and pH with xylitol treatment

4.4.3 Erythritol

The mean CFU count (Table 4.5*)* and mean pH level (Table 4.17) are depicted on a double y-axis graph, showing the correlation between the CFU counts and pH levels for erythritol at different time intervals (Figure 4.5).

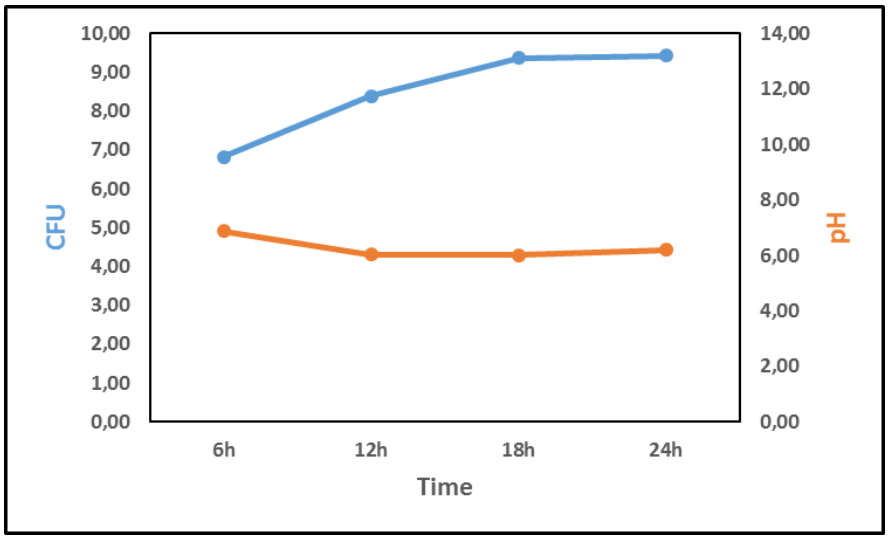

Figure 4.5: The correlation between CFU and pH with erythritol treatment

4.4.4 Stevia

The mean CFU count (Table 4.7) and mean pH level (Table 4.19) are depicted on a double y-axis graph, showing the correlation between the CFU counts and pH levels for stevia at different time intervals (Figure 4.6).

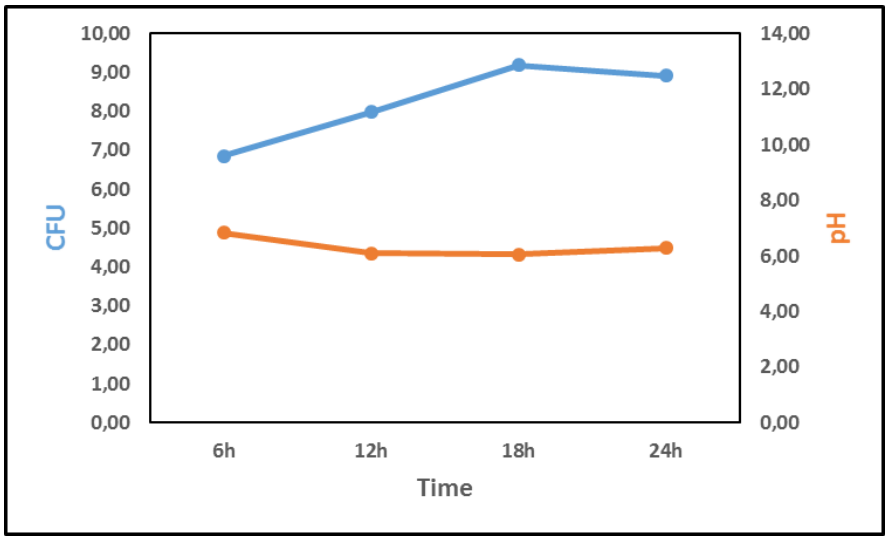

Figure 4.6: The correlation between CFU and pH with stevia treatment

4.5 SEM observation

4.5.1 Untreated Enamel Blocks for comparison:

The porous structure of enamel was visible with small areas covered with visible debris (Figure 4.7 a and b).

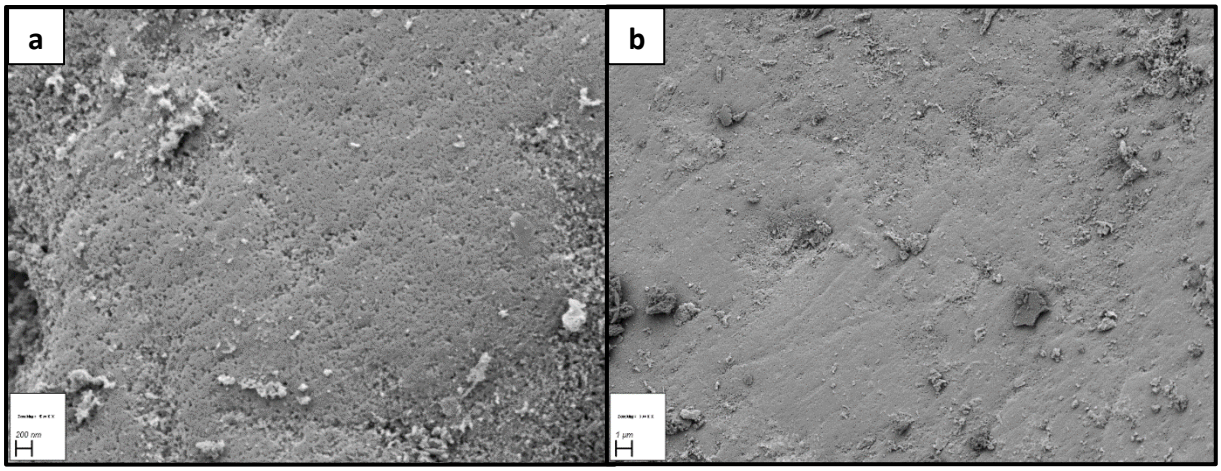

Figure 4.7: a) Porous structure of enamel visible; b) Small debris particles on an untreated enamel slab

4.5.2 Treatment A (Sucrose), positive control:

6hrs:

Enamel was covered by a thin biofilm layer. The appearance of the layer was like that of a very thin, spongy uniform carpet covering the whole of the enamel slab. Bacterial cells were sparse and not easy to observe, although some were found that were clearly visible (Figure 4.8 a and b).

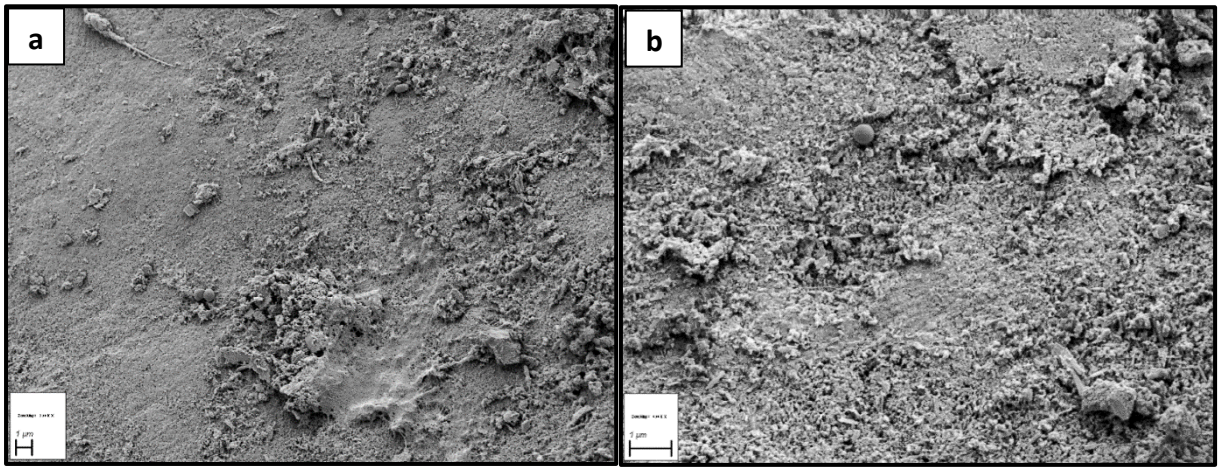

Figure 4.8: a) Deposition of extracellular polysaccharide matrix 6hrs after sucrose exposure. Certain areas seemed darker and more pronounced when viewed at a lower magnification. When zooming in on these areas, it appeared to be deposition of Extracellular Polysaccharide Matrix (EPS), although it created the impression of debris or inorganic structures, arranged in heaps; b) Bacterial cells clearly visible, but sparsely distributed 6 hrs after sucrose exposure

12hrs:

Clusters of *S. Mutans* were now clearly visible and an increase in numbers was noted. The bacterial cells seemed to be gathered in larger quantities around the edges of the enamel slab (Figure 4.9 a and b).

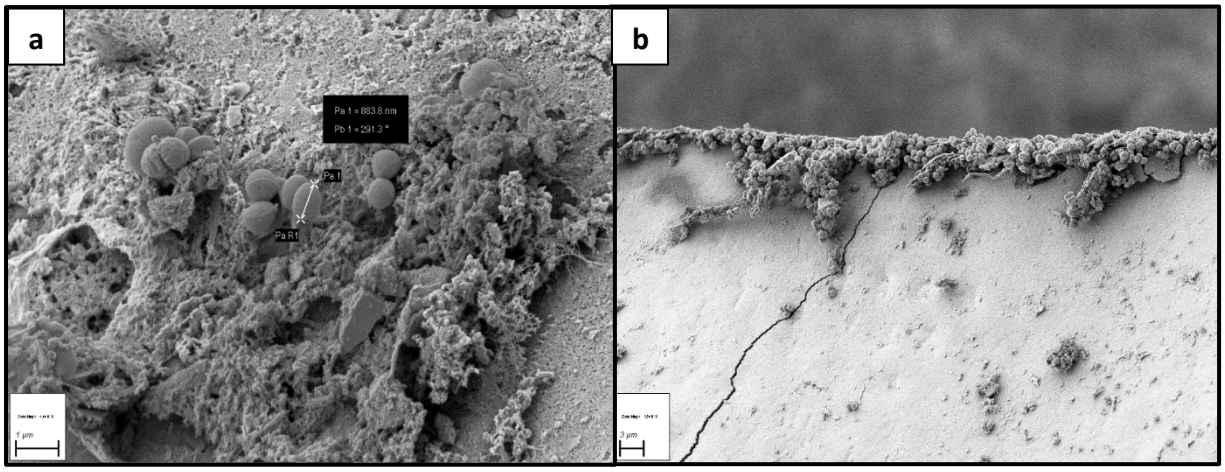

Figure 4.9: a) Clusters of *S. mutans* were found around the rough edges of the enamel slab 12hrs after sucrose exposure; b) Clusters of *S. mutans* grouped together

18hrs:

Bacteria was not found over the entire surface of the slab. Some cells were observed and occured in clusters grouped together (Figure 4.10).

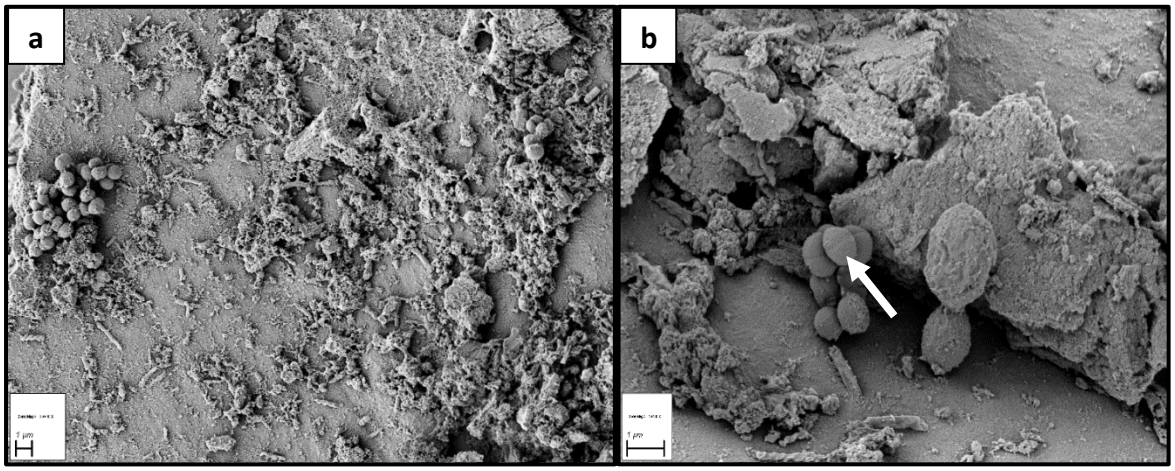

Figure 4.10: a) Clusters of *S. mutans* growing in size 18hrs after sucrose exposure b) Magnification of clusters of *S. mutans* appeared healthy and showed signs of active division as pointed out by the arrow

24hrs:

Large clusters of bacteria were visible covering large areas of the biofilm. Bacteria could be seen over the entire surface of the enamel slab. The biofilm structure seemed to have changed and did not appear as uniform or smooth as it did in earlier time intervals. It had a rougher spongy surface, due to growing biofilm and EPS. It was clear to see that the bacterial cells were actively dividing at this time interval (Figure 4.11a). It was also evident that the bacteria adhered directly to the biofilm and were not only restricted to the rougher areas, edges of the tooth or protected areas, as during earlier time intervals (Figure 4.11b).

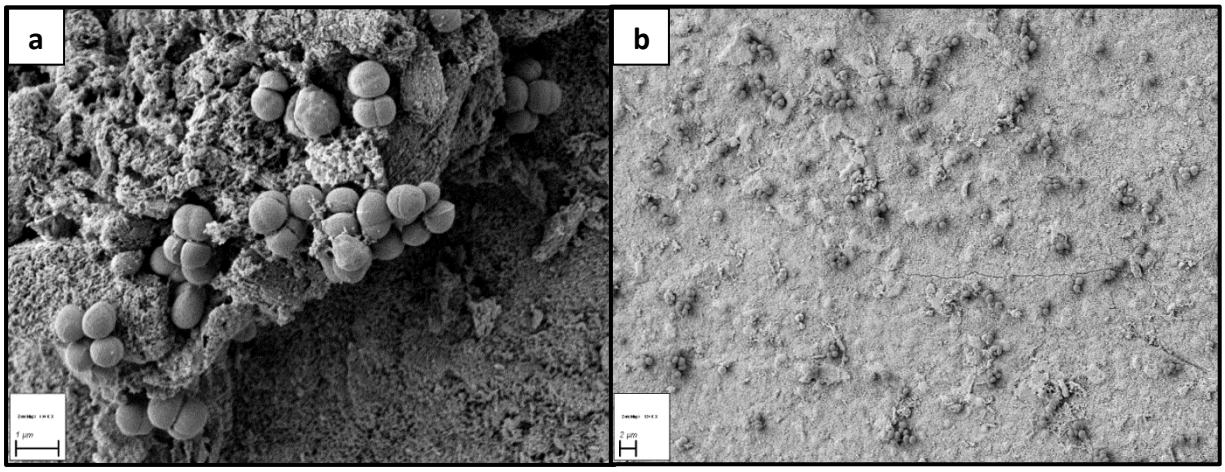

Figure 4.11: a) *S.mutans* actively dividing 24hrs after sucrose exposure; b) Bacterial cells adhering directly to the biofilm on smooth surfaces of enamel at 24hrs after sucrose exposure

48hrs:

Very large clusters of bacterial cells that were anchored to the biofilm were visible and almost covered the enamel slab from wall to wall (Figure 4.12a). A uniform covering of bacterial cells could be seen throughout the sample. Bacteria were still dividing (Figure 4.12b).

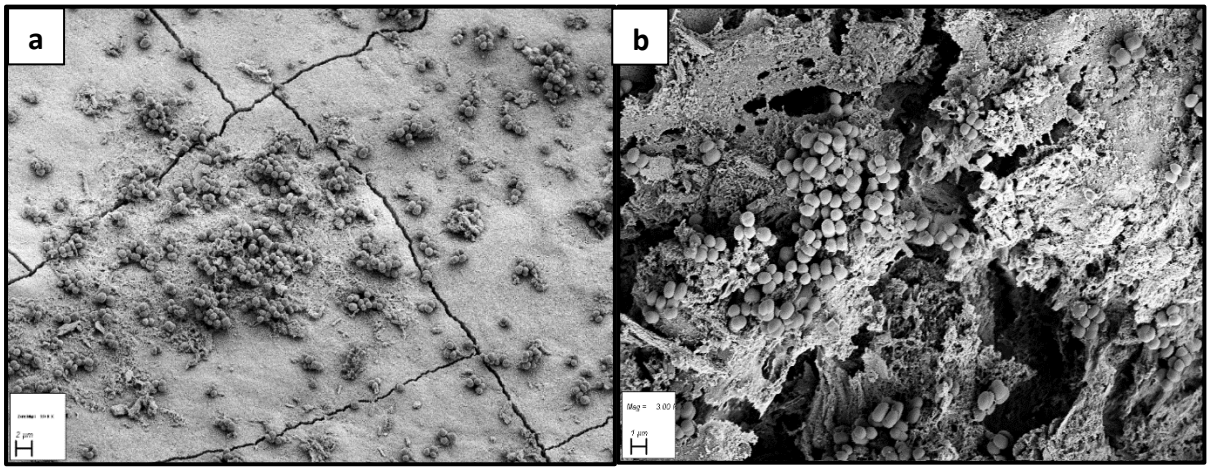

Figure 4.12: a) Viable bacterial cells uniformly spread across the enamel surface seen at 48hrs after sucrose exposure; b) Large clusters of healthy *S. mutans* actively dividing as observed at 48hrs after sucrose exposure

4.5.3 Treatment group B (Xylitol):

6hrs:

A uniform covering of the enamel structure was seen, but very few bacterial cells were observed on the surface (Figure 4.13 a and b).

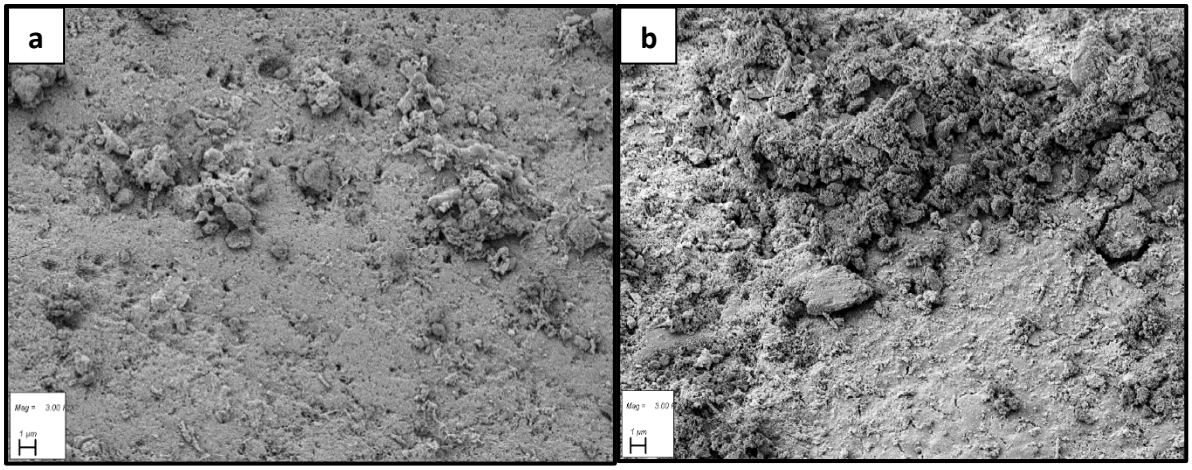

Figure 4.13: a) Uniform distribution of biofilm as observed at 6hrs after xylitol exposure; b) Bacterial cells were scarce on the surface of enamel at 6 hrs

12hrs:

The uniform covering of the enamel surface was still observed with isolated bacterial cells. Some *S. mutans* clusters were observed in cavitated areas only (Figure 4.10 a and b).

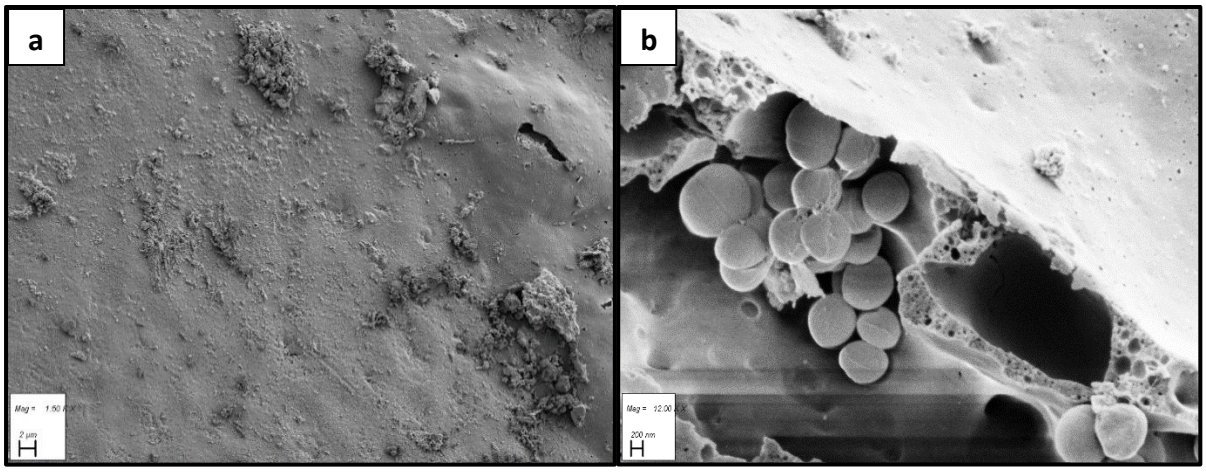

Figure 4.14: a) Bacterial cells sparsely scattered are observed at 12hrs after xylitol exposure; b) Small clusters of *S. mutans* were found mainly in covered and hollow areas at 12hrs

18hrs:

Bacterial cells were only visible in cavitated or cracked areas with not many on the smooth surfaces or areas (Figure 4.15 a and b).

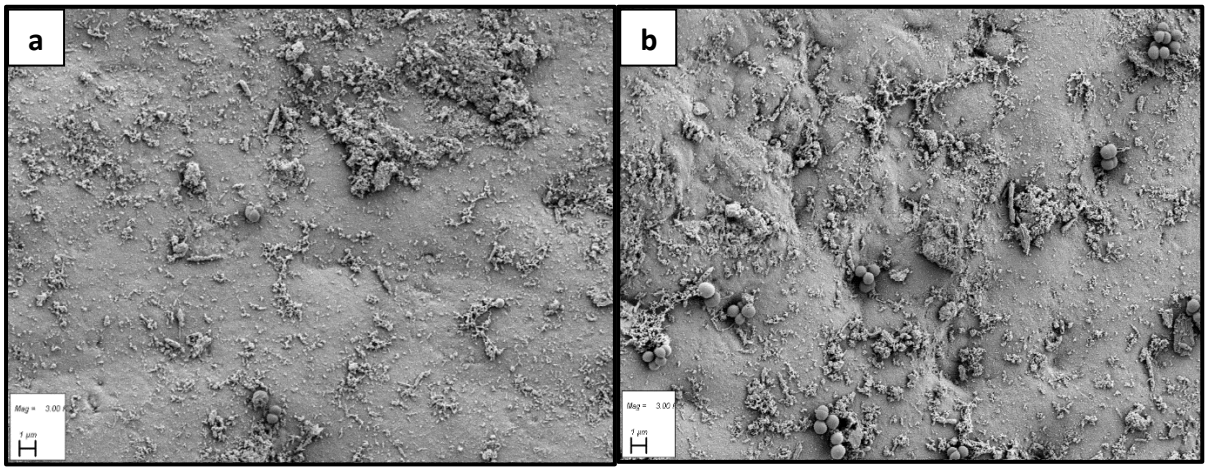

Figure 4.15: a) Sparsely scattered bacterial cells on the surface observed at 18hrs after xylitol exposure; b) Some bacterial cells could be observed in hollowed areas

24hrs:

Bacterial cells were observed, but not in large numbers. A thin biofilm covered the whole of the enamel slab. Debris could be seen (Figure 4.16 a and b).

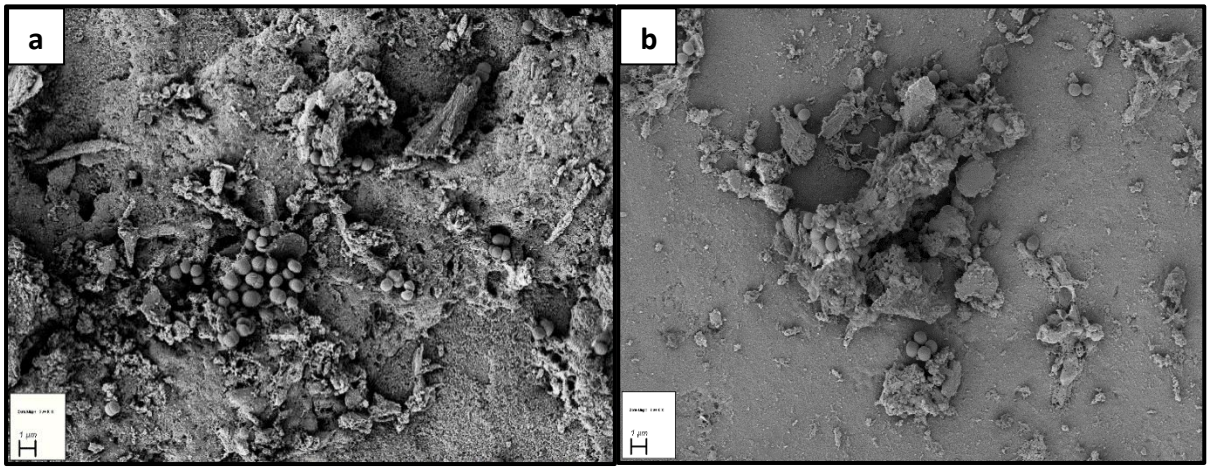

Figure 4.16: a) *S. mutans* observed at 24hrs; b) Bacterial cells lodged between debris on the enamel surface as seen at 24hrs

48hrs:

Bacterial cells were difficult to find, with isolated cells scattered, but none forming clusters. Large areas appeared clear and not occupied by bacteria. Scaffolding of what is supposedly EPS could be seen, but with sparsely distributed bacteria (Figure 4.17 a and b).

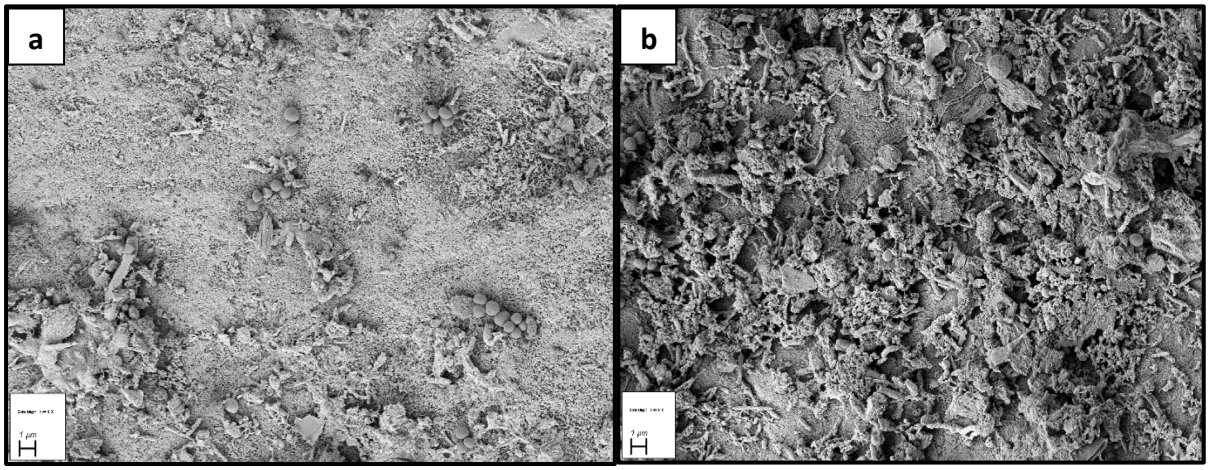

Figure 4.17: a) *S. mutans* seen scattered over large areas, not actively dividing at 48hrs; b) Debris and EPS with sparsely distributed bacterial cells as seen at 48hrs

4.5.4 Treatment C (Erythritol):

6hrs:

Both biofilm and bacteria were observed at this time interval with erythritol treatment (Figure 4.18 a and b).

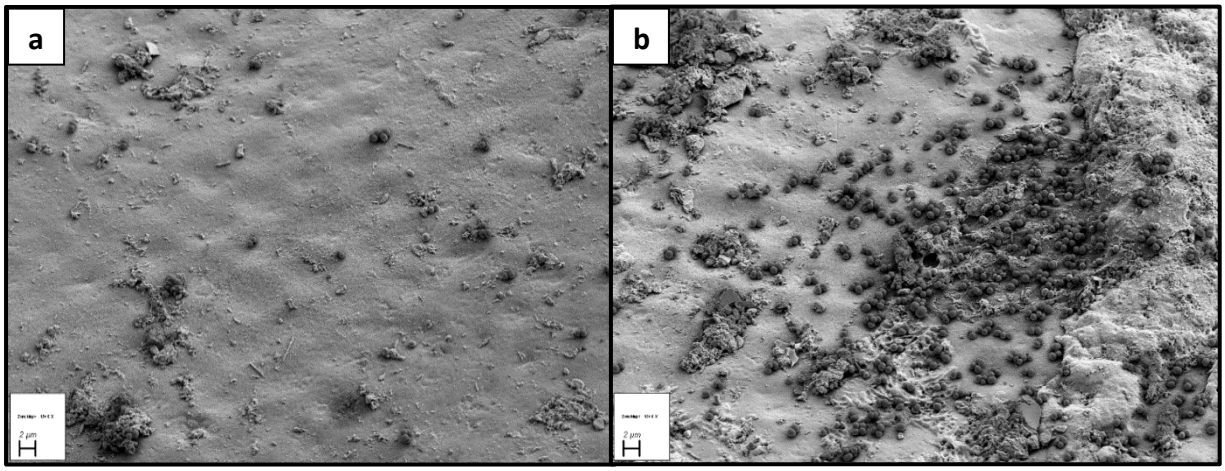

Figure 4.18: a) Biofilm formation with bacterial cells visible at 6hrs; b) Bacterial cells clearly visible at 6hrs

12 hrs:

Indentations on the biofilm were visible on the meshwork (EPS) of the biofilm with scattered clusters of bacteria and debris (Figure 4.19 a and b).

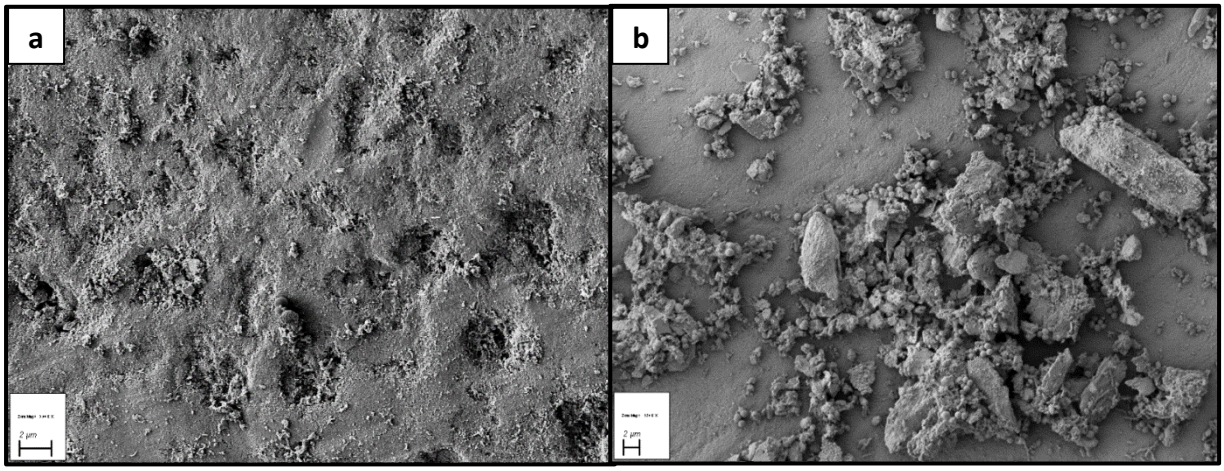

Figure 4.19: a) Biofilm with pronounced indentations seen at 12hrs; b) Bacterial cells and debris visible 12hrs

18hrs:

Fewer indentations were observed in the biofilm than was seen at the previous time intervals. Debris was seen, but only a few bacterial cells were observed. At this stage, the bacteria seemed to be dying (Figure 4.20 a and b).

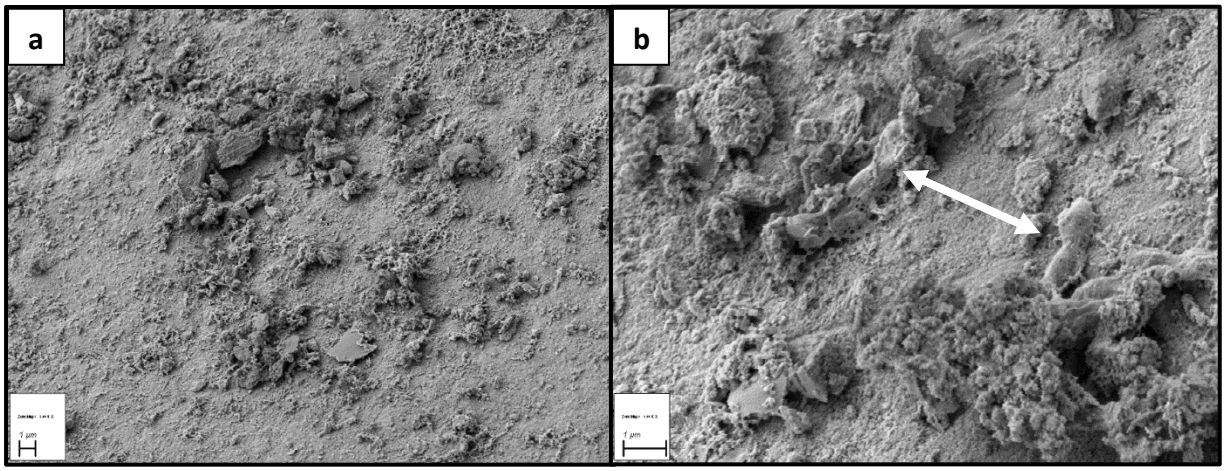

Figure 4.20: a) Very few bacterial cells could be observed at 18hrs; b) *S. mutans* dying, as pointed out, at 18hrs

24hrs:

Dark spots were observed on the surface of the biofilm. Very few live bacterial cells could be seen on the surface. Certain bacterial cells still seemed plump and healthy while others seemed to have died with cob-web-like antennae anchoring them to the surface. The biofilm was clearly visible. Live bacterial colonies were found, mostly only in sheltered areas (Figure 4.21 a and b).

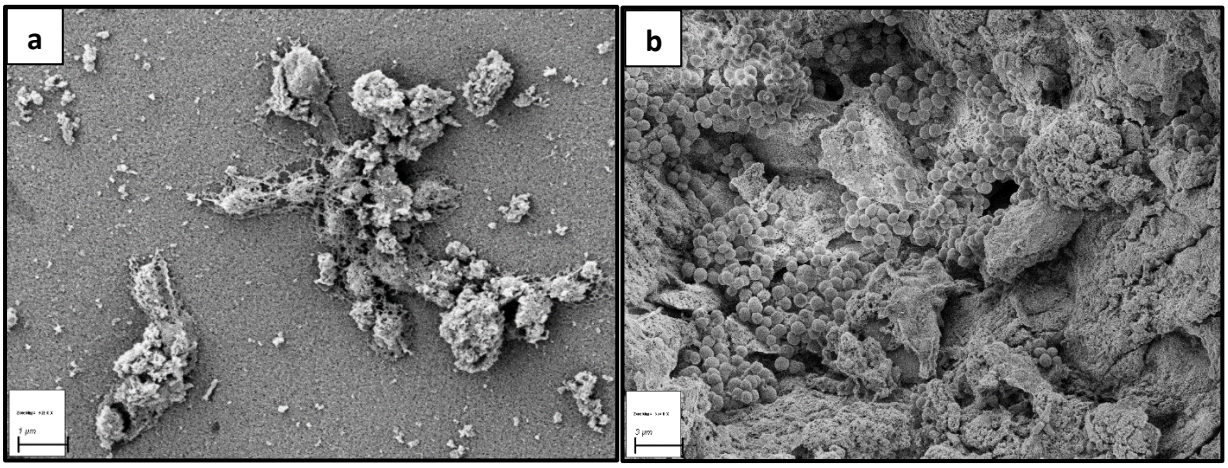

Figure 4.21: a) Dying and dead bacterial cells were observed at 24hrs; b) Live bacterial colonies were seen mostly in sheltered areas

48hrs:

The surface appeared smooth with small areas of debris in clusters. An extensive biofilm with a smooth carpet-like appearance was seen, but only a few isolated bacterial cells were still visible. In many areas it did not appear as if there were any bacterial cells when closely examined. The structure of the biofilm appeared to be porous (Figure 4.22 a and b).

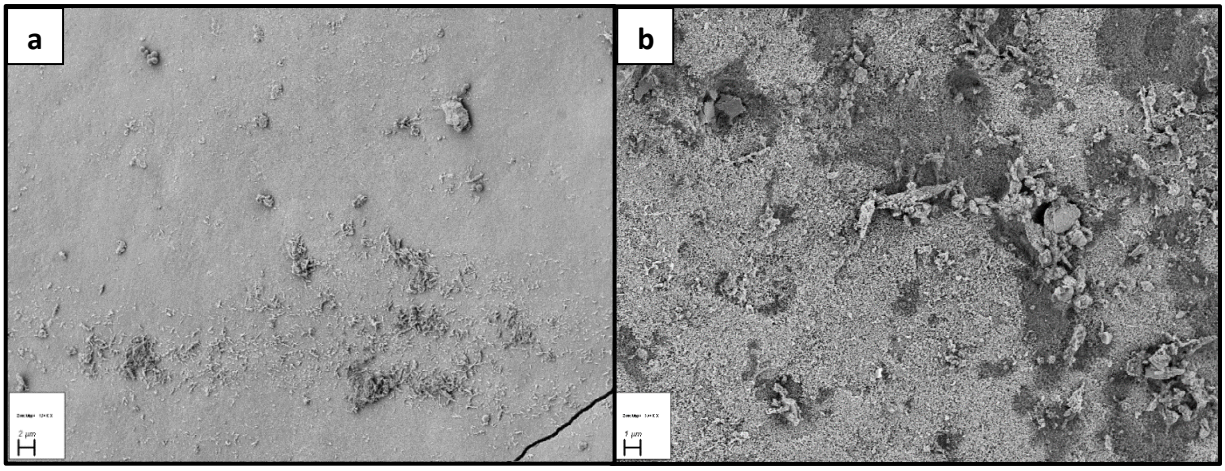

Figure 4.22: a) Very few to no bacterial cells were visible at 48hrs; b) The structure of the biofilm appeared to be porous after 48hrs

4.5.5 Treatment group D (Stevia):

6hrs:

Biofilm growth was visible at the corners of the sample. On higher magnification there were very few bacterial cells detectable in these areas. The biofilm resembled a smooth carpet-like surface covered by heaps of debris. Bacterial cells were difficult to find (Figure 4.23 a and b).

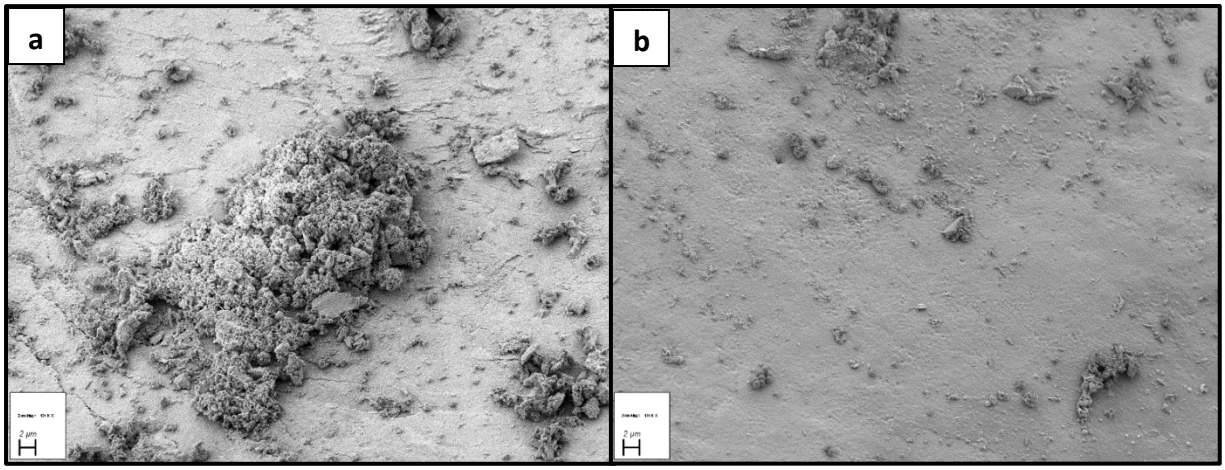

Figure 4.23: a) Biofilm and small heaps of debris visible with sparse amounts of *S.mutans* at 6hrs; b) *S.mutans* sparsely distributed at 6hrs

12hrs:

Very few bacterial cells were visible. The biofilm appeared smooth with rough areas of debris. Under low magnification, dark areas were seen on the surface. When viewed under higher magnification these areas seemed to be rough patches of piled up *debris.* Many surfaces were observed, but no healthy bacterial cells could be found. Certain structures resembled dying bacterial cells (Figure 4.24 a and b).

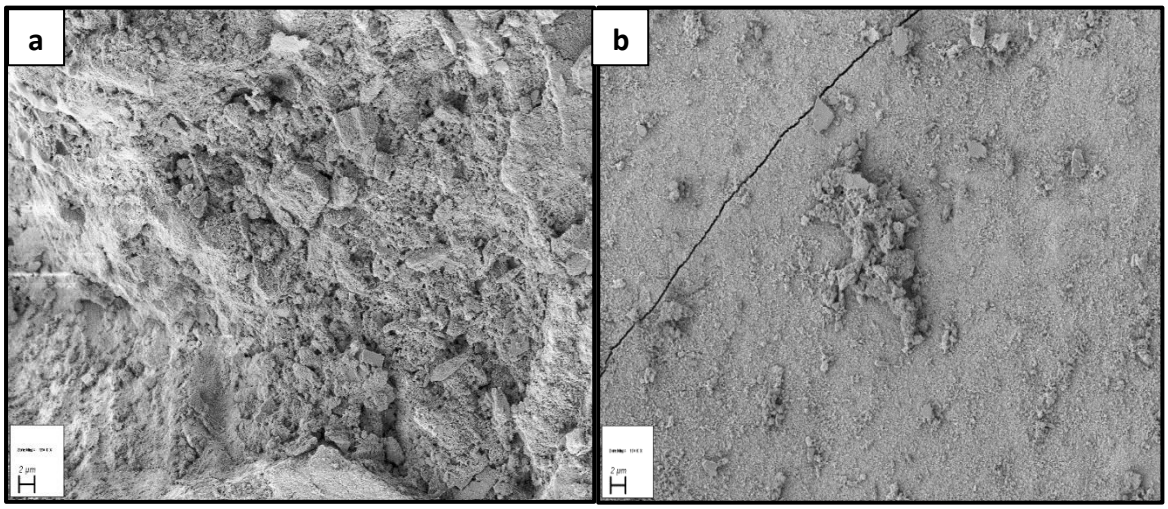

Figure 4.24: a) Biofilm formation at 12hrs with no visible bacterial cells; b) Uniform biofilm with areas of debris and almost no bacterial cells detectable at 12hrs

18hrs:

Only a few bacterial cells were visible. A few individual cells were observed but were not easily found. Less debris was seen and the biofilm carpet seemed to have a smoother appearance. Dying bacterial cells were again observed at this time interval (Figure 4.25 a and b).

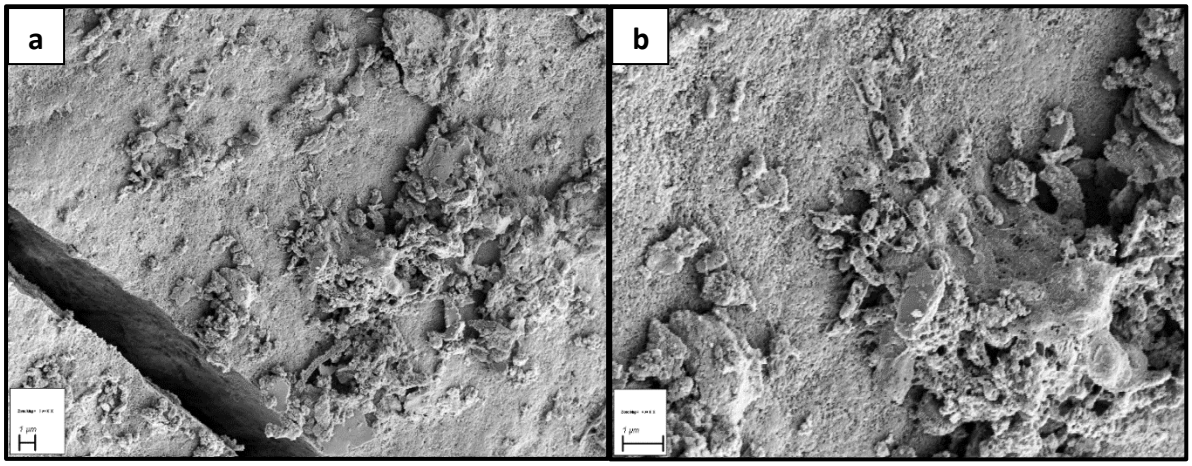

Figure 4.25: a) The biofilm and debris detectable at 18hrs; b) Dying bacterial cells within a cob-web-like structure at 18hrs

24hrs:

The biofilm seemed uniform with fewer rough areas that were covered by debris compared to other time intervals. Single live bacterial cells or small clusters of *S. mutans* were observed. Dead bacterial cells were also observed in some areas (Figure 4.26 a and b).

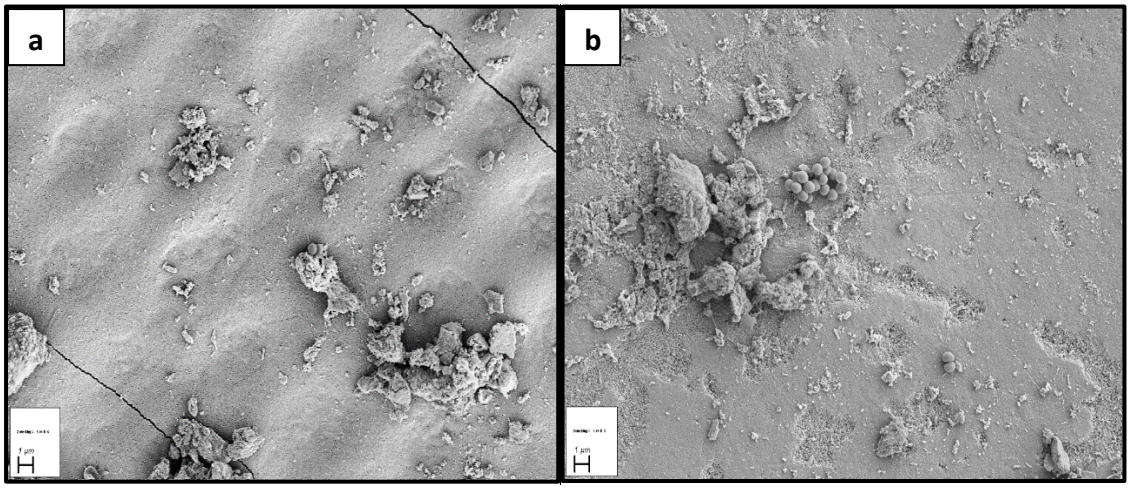

Figure 4.26: a) Smooth appearance of biofilm surface with scanty deposits of debris; b) Individual *S. mutans* sparsely scattered at 24hrs

48hrs:

Altered, lone standing bacterial cells were observed at this time interval. They seemed to be alive but not functioning. These cells did not have a round, plump appearance but were dented and had an irregular shape. Most of the bacterial cells that were seen seemed to have a moss-like covering. Debris was abundant and bacterial cells appeared to be buried in this. Certain areas were completely devoid of any bacteria and a rough film coating was clearly visible (Figure 4.27 a and b).

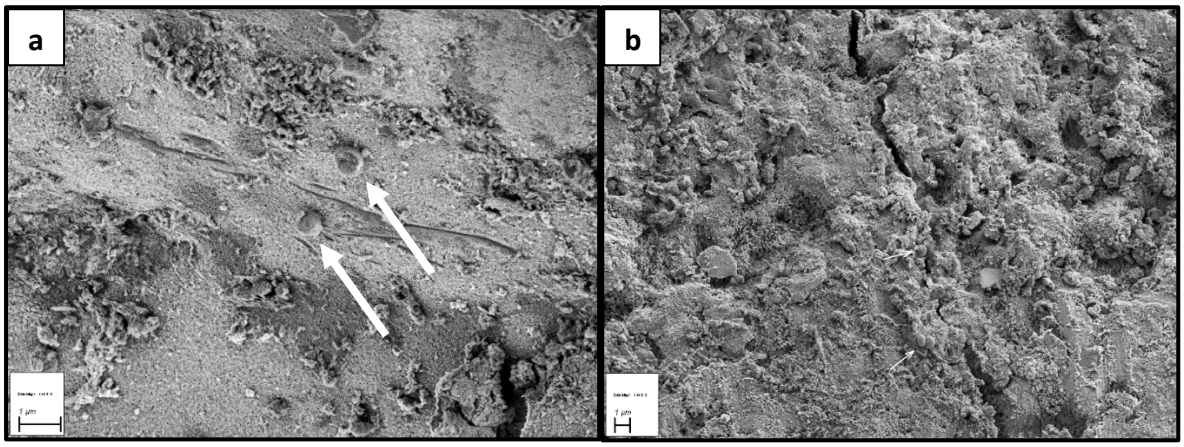

Figure 4.27: a) Lone standing *S. mutans* with unhealthy indented appearance indicated by the arrows; b) A rough film coating was clearly visible 48hrs

4.5.6 Summary of SEM observations

The observations made of the different treatments at 0h and 6hrs under SEM are summarized in Table 4.26.

Table 4.26: Comparison of SEM observations between different treatments at the 0hr and 6hrs time intervals

Time interval	Untreated Enamel slabs	Treatment A Sucrose	Treatment B Xylitol	Treatment C Erythritol	Treatment D Stevia
0hrs	Porous structure of enamel visible. Small areas with visible debris.				
6hrs		Biofilm: Thin covering. Spongy, uniform, carpet. Scattered Debris. Sparse bacterial cells.	Biofilm: Uniform covering of sample. Very few *S. mutans* cells.	Biofilm: uniform covering of. *S. mutans* clearly visible.	Thick biofilm covered the entire sample. Smooth carpet with larger quantities of debris gathered at corners of enamel slab. Very few *S. mutans* cells.

The observations of the different treatments at the 12hrs time interval under the SEM is summarized in Table 4.27 below.

Table 4.27: Comparison of SEM observations between the different treatments at the 12hrs time interval

Time interval	Treatment A Sucrose	Treatment B Xylitol	Treatment C Erythritol	Treatment D Stevia
12hrs	Biofilm appeared rough. Clusters of *S. mutans.* Increase *in S. mutans* numbers. Large quantities of *S. mutans* around edges of the sample.	Uniform covering of biofilm still observed. Sparse sighting of *S. mutans.*	Indentations in the biofilm observed. Pronounced meshwork of biofilm. Debris seen. Clusters of *S.mutans*.	Biofilm appeared smooth with rough patches where debris was gathered. No healthy *S. mutans* cells observed. Certain structures resembled dying bacterial cells.

The observations of the different treatments at the 18hrs time interval under the SEM is summarized in

Table ***4.28*** below.

Table 4.28: Comparison of SEM observations between the different treatments at the 18hrs time interval

Time interval	Treatment A Sucrose	Treatment B Xylitol	Treatment C Erythritol	Treatment D Stevia
18hrs	*S. mutans* did not cover the whole surface. *S. mutans* was found to occur in large clusters.	Biofilm present, but not as thick as at 6 and 12hrs. *S. mutans* mostly visible in cavitated or cracked areas, not on smooth surfaces.	Fewer indentations in biofilm observed. Debris observed. Fewer *S. mutans*. Dying bacteria observed.	Biofilm seemed to have smoother appearance. Less debris observed. Only few live S*. mutans* cells observed, not easily found. Dying *S. mutans* observed again.

The observations of the different treatments at the 24hrs time interval under the SEM is summarized in Table 4.29 below.

Table 4.29: Comparison of SEM observations between the different treatments at the 24hrs time interval

Time interval	Treatment A Sucrose	Treatment B Xylitol	Treatment C Erythritol	Treatment D Stevia
24hrs	Entire area of sample covered by *S. mutans*. Biofilm structure changed, not uniform/smooth anymore, appeared rough. *S. mutans* were clearly actively dividing. *S. mutans* seemed healthy. *S. mutans* adhered directly to biofilm, not restricted to rough areas only.	Biofilm covered whole sample, but appeared thin. Debris observed. *S. mutans* observed in small quantities.	Biofilm clearly visible. Dark spots observed on surface of biofilm. Very few *S. mutans* noted; found almost only in sheltered areas. Certain *S. mutans* cells appeared plump and healthy. Others seemed to have died with cob-web-like antennae anchoring to surface.	Biofilm appeared uniform. Fewer rough areas covered by debris when compared to 6hrs/12hrs/18hrs. Dead *S. mutans* observed.

The observations of the different treatments at the 48hrs time interval under the SEM is summarized in Table 4.30 below.

Table 4.30 Comparison of SEM observations between the different treatments at the 48hrs time interval

Time interval	Treatment A Sucrose	Treatment B Xylitol	Treatment C Erythritol	Treatment D Stevia
48hrs	Very large clusters of S.mutans visible. Uniform covering of S.mutans. S mutans seemed healthy and still dividing.	S. mutans cells difficult to find. S. mutans cells found were scattered, not in cluster formations. Large areas seemed void of both biofilm and bacteria.	Surface appeared porous. Small debris clusters, smoother overall appearance created. S. mutans isolated and very sparse.	Uniform rough biofilm. S. mutans appeared unhealthy and lone standing. Alive but seemed like they were not functioning. Dented appearance with irregular shape. Most of the S. mutans seemed to be coated with a moss-like covering. S. mutans appeared to be buried in debris. Certain areas completely devoid of bacterial cells/residue.

Chapter 5 : Discussion

5.1 Analysis of CFU, pH and SEM images

The CFU, pH and SEM analysis provided a complete picture of the bacterial activity and ultimately the cariogenic potential of the different treatments. Of particular interest in this study, were the mean CFU counts and pH changes over time for each treatment. Values of the mean CFU counts and pH for each treatment were determined and compared at 6hrs, 12hrs, 18hrs and 24hrs.

5.1.1 Sucrose

The CFU counts for sucrose significantly increased between 6hrs and 12hrs and again between 12hrs and 18hrs ($p<0.05$). They significantly decreased from 18hrs to 24hrs ($p<0.05$) (Table 4.2). This increase in CFUs is most likely due to the presence of sucrose that serves as substrate for *S. mutans*, enabling it to flourish and grow. Loesche[18] found similar results in individuals who ingested sucrose frequently and excessively where all had elevated levels of *S. mutans* in their saliva. For microorganisms to grow and flourish, they need to be exposed to a medium that will provide sufficient nutrients and energy.[116] The decrease in CFU counts at the last time interval could be due to nutrients in the media being exhausted by this time. Ccahuana-Vàsquez *et al*.[119] called this "the feast and famine model", where sucrose is depleted after initial exposure, leading to microbial death.

The drop in pH values for sucrose, was statistically significant between 6hrs and 12hrs ($p<0.05$) and also between 12hrs and 18hrs ($p<0.05$). However, there was no significant difference from 18hrs to 24hrs ($p>0.05$) (Table 4.14). The initial drop in pH was also seen in a study by Stephen *et al*.[30] who concluded that the ingestion of sucrose, glucose, fructose, or refined carbohydrates and cooked starches produce a fall in pH value in the mouth as a result of bacterial fermentation of sucrose. Important to note is that the supply of sucrose during this time interval allowed the pH to drop to levels below the critical value of 5.5.[29] The

stabilization of the pH value at 18hrs can be due to the decrease in CFU counts together with the depletion of the substrate, resulting in less acid production. Ccahuana-Vàsquez *et al.*[119] confirmed this with a biofilm model study that showed the biofilm to produce less acid in the absence of sucrose. Similarly Stephen *et al*.[30] found that the lowering of pH in plaque reaches its greatest intensity within the first 30 minutes after ingestion and then remains stable until there is repeated exposure or renewal of the carbohydrate source.[29]

It is important to note that even though the levels were stabilized, the pH stayed below the critical level of 5.5 for the duration of the study. This is probably because this was an in *vitro study*, where the neutralizing and buffering effects of saliva were excluded. In addition, the growing biofilm entraps microorganisms and their waste products, keeping the acid substance close to the surface of the tooth, regardless of saliva activity.[120]

The CFU and pH findings of sucrose were supported by analysing the SEM images. An increase in *S. mutans* was seen on these images over the first two time intervals: At 6hrs, sparsely distributed bacterial cells were observed (Figure 4.8) and at 12hrs clusters of *S. mutans* were found grouped together (Figure 4.9). At 18hrs and 24hrs, there were active bacterial cells grouped together, showing signs of division (Figure 4.10). This confirmed that *S. mutans* was thriving at these time intervals. Even though there was a decrease in the CFUs, the microorganisms managed to survive.
This is because *S. mutans* has the ability to utilize EPS as storage compounds, protecting them from influences such as nutrient depletion and contributing to their colonization.[22]

The clinical relevance of this finding is that patients who brush their teeth only once per day, and specifically only in the mornings have a high risk of developing tooth decay, because there are increased CFUs at 12hrs and 18hrs and along with a stabilized biofilm there will be entrapment of bacteria, nutrients and acids, leading to caries formation while they sleep.[6]

5.1.2 Xylitol

The CFU counts that were compared for xylitol increased significantly between 6hrs and 12hrs and between 12hrs and 18hrs. This then decreased significantly from 18hrs to 24hrs (Table 4.4). The initial increase in CFUs for the first two time intervals was due to *S. mutans* recognizing xylitol as a form of nutrition, as suggested by Ghezelbash *et al.*[121] Sucrose and xylitol are both transported into the microbial metabolism via the phosphotransferase system. Both use the enzymes Phospoenolpyruvate (PEP) and Phosphotransferase (PT) as transport mechanism to enter the bacterial cells.[25] Xylitol is then phosphorylated to xylitol-5-phosphatase and dephosphorylized, after which it is expelled from the microbial cell as xylitol again, thus it is not utilized by the bacterial cells.[121] The drop in CFU counts after 18hrs may be due to the futile energy-consumption, leading to depletion of energy and eventual cell death, when they take up xylitol.[82]

The drop in pH values measured for the xylitol treatment, was statistically significant from 6hrs to 12hrs ($p<0.05$), but the differences in pH values measured from 12hrs to 18hrs and again from 18hrs to 24hrs did not change significantly ($p>0.05$) (Table 4.16).

The initial drop in pH values during the first time interval can be attributed to bulking agents that are often added to the xylitol during production, which might contain refined carbohydrates. Miyasawa *et al*.[122] found that in the presence of xylitol, the lactic acid end-product of the metabolic pathway of bacteria is decreased, but that formic and acetic acids are increased. The statistically significant drop in the pH values in this study are probably due to the formation of formic and acetic acid as metabolic end-products. Drucker *et al*.[66] reported similar findings of a rapid initial drop in pH values when *S. mutans* was exposed to xylitol solutions.

Although there was a significant drop in pH at the first time interval it never dropped below the critical level of 5.5. Stabilization of the pH value from 12hrs to 24hrs can once again be explained by the futile energy consumption of the microorganisms, resulting in decreased metabolism and eventual death. The lack of metabolism by *S. mutans* will lead to less acid production and a decreased rate of glycolysis in the presence of xylitol.[122] The possible

depletion of food source at these time intervals would account for the stabilization of the pH value.[122] A similar pattern was observed by Drucker *et al*.[66] where an initial drop in pH occurred and then stabilized over time.

It is important to note that although many studies have shown that xylitol can lower the pH value in the presence of sucrose, available literature to explain the low pH value produced by xylitol on its own in the presence of *S. mutans,* is limited.[69,122] Furthermore, the xylitol studies by Söderling *et al*.[68] to evaluate salivary pH values, were conducted *in vivo*, with saliva as a pH buffer. A study by Hayes *et al*.[65] supports this view that xylitol given in buffered saline produces a lower drop in pH. They also found that the capacity of *S. mutans* was not reduced, but that acid was again produced in small quantities. The present study was conducted *in vitro* and therefore the buffering capacity of saliva could not be measured.

The CFUs and pH findings were supported by the SEM images. At 6hrs and 12hrs many healthy bacterial cells were observed (Figure 4.13 and Figure 4.14). At 18hrs, live bacterial cells were visible mostly in cavitated areas and not many on smooth surfaces (Figure 4.15). The SEM images at 24hrs (Figure 4.16) showed even fewer *S. mutans* cells that were sparsely distributed. The thin biofilm observed in this SEM image (Figure 4.16), can be attributed to the absence of sucrose, leading to the inability of bacteria to form glucans. The weaker Exopolymeric substances (EPS) lead to weaker biofilm formation and eventually fewer suitable surfaces for the bacteria to adhere to. The survival of *S. mutans* was compromised due to the lack of nutrients and shelter. This was substantiated by Söderling *et al*.[123] who explained that xylitol reduces the virulence of *S. mutans,* with subsequent decrease in the production of adhesive macromolecules which leads to reduced cell-to-cell aggregation and less cluster formation.

Taking into consideration the CFUs, pH and SEM analysis in the presence of xylitol in this study, CFU counts for *S. mutans* increased up to 18hrs with a subsequent acid production close to the critical demineralization pH (5,5). However, the growth of bacteria and production of acid was self-limiting and stabilized thereafter. This study exposed the *S. mutans* to a 5% concentration of xyiltol. De Cock *et al.*[56] found that low concentrations of

xylitol had a weaker effect on bacterial growth and subsequent acid production. Future studies could be useful to investigate which concentrations of xylitol result in the most effective antibacterial action.

5.1.3 Erythritol

The CFU counts for erythritol increased significantly between 6hrs and 12hrs and between the 12hrs and 18hrs time intervals ($p<0.05$). The CFU counts between the 18hrs and 24hrs time interval did not show a significant change ($p>0.05$) (Table 4.6).

The reason for the initial increase in bacterial cells is most likely the same as for other polyols such as xylitol, where the bacteria recognize erythritol as a potential source of nutrients which is taken up into the metabolic cycle, but ends up with futile results. This is substantiated by Ghezelbash *et al*.[121] who found that, as with xylitol, erythritol caused the same inhibition of *S. mutans* growth and biofilm formation. However the exact pathway by which erythritol does this has not yet been described in literature, although it is believed to be similar to that of xylitol.[121] The increase in CFU counts occurred over the first two time intervals, and this was also found by de Cock *et al.*[56] where the growth of *S. mutans* was inhibited during later growth phases.

The drop in pH values measured for erythritol treatment was statistically significant between 6hrs and 12hrs ($p<0.05$). The difference in the pH values between 12hrs and 18hrs did not indicate any statistically significant change ($p>0.05$), but the elevation in pH values between 18hrs and 24hrs was once again statistically significant ($p<0.05$) (Table 4.18).

Being a sugar alcohol, the initial drop in pH value for erythritol at the first time interval is explained in the same manner as for xylitol regarding their influence on the bacteria's metabolic pathway, with a decrease in lactic acid, and an increase in formic and acetic acid.[81] In addition to this, bulking agents present in the product might also serve as substrate for *S. mutans* to utilize as a nutrient source, with subsequent lowering in the pH value due to metabolic waste.

The elevation in the pH value for the 18hrs to 24hrs time interval could be attributed to the decrease in bacterial cells combined with an altered metabolic cycle. A longitudinal study conducted by Runnel *et al*.[124] showed that erythritol produced less acid compared to other polyols over time. Available literature on the metabolic pathway of erythritol is limited. However, a study conducted by Hashino *et al*.[125] investigated the effect of erythritol on *Streptococcus gordonii* and *Porphyromonas gingivalis* and concluded that erythritol decreased the bio volumes of the biofilms and significantly reduced the nucleotide synthesis in the pentose phosphate pathway. This could be the same for *S. mutans*, thus leading to reduced production of metabolic waste products and reduced acidity.

The CFUs and pH findings were supported by the SEM images. At the 6hrs time interval, *S. mutans* was clearly visible and by 12hrs it had formed clusters. These observations correlate with the CFU counts and can be confirmed by De Cock *et al.*[56] where growth was only inhibited during later growth phases in the presence of erythritol. The turning point for the microorganisms was during the 18hrs to 24hrs time interval. The SEM images showed that *S. mutans* cells were under stress with a number of dying bacteria seen at 18hrs and 24hrs. This phenomenon is substantiated by the observations of Perry *et al.*[126] and Leung *et a*l.[127] who described this in terms if an interesting process of quorum sensing (QS) whereby bacteria are considered social organisms and are able to communicate with one another using hormone-like molecules named pheromones. These molecules, allow the bacterial population to initiate adaptive responses and to show altruistic behaviour. When bacterial populations encounter stress situations such as amino starvations, pH changes, oxygen radicals, high temperatures, DNA damage or antibiotics, a death by suicide of a subpopulation of bacteria occurs.

Given the gastric side effects of xylitol, erythritol is a more suitable sugar substitute, as there are no gastric side-effects and the product is excreted unchanged in the urine and faeces, suggesting that it does not have systemic effects.[56] Erythritol can be differentiated from all the other polyols, because it is produced by a natural fermentation process. It can therefore be seen to be a totally natural product which is superior to xylitol as an anticariogenic agent.[56]

Further studies to understand the way in which erythritol is incorporated in the metabolic pathway of bacteria is advised, as the available literature does not give clarity on this process. Lower concentrations of both erythritol and xylitol have a weaker effect on bacterial growth and acid production. It could therefore be useful to consider altering the concentrations of these polyols to gain a better effect.[95]

5.1.4 Stevia

The CFU counts for stevia significantly increased between 6hrs and 12hrs and between the 12hrs and 18hrs time interval ($p<0.05$). A significant decrease was seen between CFU counts measured at the 18hrs and 24hrs time interval ($p<0.05$) (Table 4.8). The reason for the initial increase in CFU count for the first two time intervals is suggested to be due to *S. mutans* recognizing stevia as a source of nutrition and taking it up into its metabolic cycle. Bulking agents in the product that might include carbohydrates providing nutrition to the microorganisms can contribute to their positive growth. At the 18hrs time interval, the CFU count dropped significantly. This may be attributed to the exhaustion of carbohydrate bulking agents and the incapacity of *S. mutans* to metabolize stevia.[107] Vitery *et al.*[128] and Daoud *et al.*[129] also found that stevia extract inhibits growth of *S. mutans*.

The drop in pH values measured for stevia treatment was statistically significant between 6hrs and 12hrs ($p<0.05$). The difference in the pH values measured between 12hrs and 18hrs did not indicate a statistically significant change ($p>0.05$). The elevation in pH values between 18hrs and 24hrs was again statistically significant ($p<0.05$) (Table 4.20).

Initial growth of *S. mutans* and acid production may be due to the other bulking components contained in the commercial form of stevia.[107]

Stabilization of the pH value at the 12hrs to 18hrs time interval and the elevation at the 18hrs to 24hrs time interval, are indicative of depletion of nutrients, leading to microbial starvation and subsequent cell death with less acid production. It may also be that stevia is not taken up in the metabolic pathway of microorganisms and as such, no metabolic end products such as acids are produced. These findings were in line with the findings of Giacaman *et al*.[107] who

confirmed the significantly lower acidogenicity of stevia compared to other commercial sweeteners. The exact mechanism of how stevia affects the metabolism of microorganisms has not yet been described. Gardana *et al*.[130] evaluated the effect of stevia on intestinal flora and suggested that it possesses an inhibitory effect on aerobic bacteria that negatively influences their proliferation. Considering these findings, there could be a link between the metabolism of intestinal flora and oral bacteria with stevia.

The CFUs and pH findings were supported by the SEM images. At 6hrs, a thick biofilm was observed, indicating bacterial activity (Figure 4.23). At 12hrs, heaps of debris were observed, possibly originating from the bacteria (Figure 4.24). At 18hrs and 24hrs, there were dead bacterial cells, confirming the CFU and pH findings that stevia was not a viable source of nutrients for *S. mutans* (Figure 4.25). Quorum sensing and the subsequent altruistic cellular suicide under these stressful conditions, might be another possible explanation for these findings.[126]

5.1.5. CFU and pH comparison of all treatments with each other

The mean CFU counts over time for all treatments are depicted in Figure 4.1. There was no significant difference between the CFU counts for any of the treatments at 6hrs (Table 4.9). The CFU counts for sucrose was statistically higher than those of xylitol and stevia at 12hrs ($p<0.05$), with no significant difference between the CFU counts for sucrose and erythritol ($p>0.05$). The CFU counts for xylitol was statistically higher than those of all the other groups at 12hrs ($p<0.05$). The CFU counts for stevia was statistically lower than those of all the groups (xylitol, erythritol and sucrose) at 12hrs ($p<0.05$) (Table 4.10). The CFU counts for sucrose was statistically higher than those of xylitol and stevia at 18hrs ($p<0.05$). The CFU counts for erythritol was statistically higher than those of stevia at 18hrs ($p<0.05$). There was no significant difference in the CFU counts for sucrose and erythritol, erythritol and xylitol or stevia and xylitol at 12hrs ($p>0.05$) (Table 4.11). The CFU counts for erythritol was statistically higher than the CFU counts for all the other treatments at 24hrs ($p<0.05$). There was no

statistically significant difference between any of the other treatments at 24hrs ($p>0.05$) (Table 4.12).

In summary, the CFU counts for sucrose and erythritol increased over time and stabilized after 18hrs. In contrast, the sugar substitutes xylitol and stevia showed an increase in CFU count followed by a significant decrease after 18hrs.

The mean pH values over time for all treatments are depicted in Figure *4.2*. The pH value for sucrose was statistically lower than that of xylitol ($p<0.05$). When this pH value was compared to the pH values of the other treatments, there were no statistically significant differences (Table 4.21). At 12hrs, the pH value for sucrose was statistically lower than those of xylitol, erythritol and stevia ($p<0.05$). There was no statistically significant difference when the pH values of xylitol, erythritol and stevia were compared to each other ($p>0.05$). At 18hrs, the pH value for sucrose was statistically lower than those of xylitol, erythritol and stevia ($p<0.05$). The pH value of xylitol was statistically lower than those of erythritol and stevia ($p<0.05$). There was no statistically significant difference when the pH value of stevia and erythritol were compared ($p>0.05$) (Table 4.23). At 24hrs, the pH value for sucrose was statistically lower than those of xylitol, erythritol and stevia ($p<0.05$). The pH value measured for xylitol was statistically lower than those of erythritol and stevia ($p<0.05$). There was no statistically significant difference between the pH values for stevia and erythritol at 24hrs ($p>0.05$) (Table 4.24).

In summary, the pH value for sucrose dropped below 5.5 and remained below the critical level throughout the experiment. All the other sugar substitutes that were tested caused an initial drop in pH, which then stabilized. In the case of erythritol and stevia, the pH even elevated after a period of time. Since no sugar substitute that was tested produced a pH lower than 5.5, the findings confirm that the sugar substitutes xylitol, erythritol and stevia may have a lower cariogenic potential compared to sucrose *in vivo*.

A direct correlation between the increase in CFU counts and the decrease in pH can be seen in Figures 4.3-6 for each treatment respectively. *S. mutans* was thriving and multiplying while

fermenting carbohydrates and producing acid. When the CFU counts stabilized or lowered, the pH stabilized or increased accordingly for all treatments. If the CFU counts together with pH values are taken into consideration, stevia has been shown to have the least cariogenic potential of all the sugar substitutes tested, followed by erythritol and then xylitol.

Microorganisms incorporated into a biofilm become more harmful. Thus, finding a way to prevent initial biofilm formation in the first place, would aid in preventing the harmful effects of the bacteria. Considering that caries is a multifactorial disease, a holistic approach is needed to counter its development. Strategies that have been proposed in the prevention of dental disease include: inhibiting acid production (this might be achieved by using fluoride containing products which inhibit key enzymes involved in glycolysis); avoiding snacking on fermentable carbohydrates between meals; and replacing sugar with sugar substitutes such as xylitol, erythritol and stevia in order to prevent repeated episodes of low pH. Another strategy may be to try and stimulate salivary flow after meals and snacks.

The products used in this study have been advocated as safe for human consumption by the FDA and are widely used. Some studies however, contradict this finding and further research is suggested, particularly regarding the production process and safety of stevia.[55,100]

5.3 Limitations of this study:

This in vitro study did not fully mimic the *in vivo* environment which is influenced by buffering of saliva, variable salivary flow rates, different quantities of bacterial material on teeth and the variety of bacteria capable of producing acid or alkaline substances. In addition, only one concentration of each of the treatments was tested.

Chapter 6 : Conclusions and Recommendations

6.1 Conclusions

This study generated valuable information regarding the effect of xylitol, erythritol and stevia on the growth of *S. mutans* and the pH changes noted with their use. Within the limitations of this study, the statistically significant results suggest that the sugar substitutes that were evaluated may serve as suitable substitutes for sucrose.

It is however, essential to note that the possibility of caries formation by sugar substitutes cannot be excluded completely as a drop in pH was still noted with all sugar substitutes. Biofilm formation of *S. mutans* on enamel surfaces was evaluated and the SEM images confirmed the findings associated with CFUs and pH.

With regards to the CFUs, Acid production and Biofilm formation, this study suggests that compared to sucrose, stevia poses the least cariogenic potential, followed by erythritol and then xylitol. Xylitol, erythritol and stevia are all less cariogenic alternatives to sucrose, but should still be used with caution.

Reducing the consumption of sugar and foods rich in refined carbohydrates and replacing these with natural sugar substitutes, might provide a significant benefit in decreasing or preventing caries incidence, especially that of ECC.

6.2 Recommendations:

Further research is recommended to compare the pH of sugar substitutes *in vivo,* to investigate the influence that different concentrations and frequencies of consumption will have on the cariogenic potential of sugar substitutes and to understand the metabolic process of *S. mutans* for erythritol and stevia more fully.

Chapter 7 : References

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UNIVERSITY OF PRETORIA

Appendix A : Permission to use the FE-SEM at SMU

SEFAKO MAKGATHO
HEALTH SCIENCES UNIVERSITY

ELECTRON MICROSCOPE UNIT

01 April 2019

To Whom it may Concern,

SCANNING ELECTRON MICROSCOPY FOR RESEARCH STUDY: DR N MOELICH (REG NO: 95016172)

We hereby confirm that the study for Dr N Moelich entitled: *'Determination of the Cariogenic Potential of Sugar Substitutes'* has been discussed with the Electron Microscope Unit.

Field Emission Scanning Electron Microscopy (FE-SEM):

25 samples @ R420.00 per hour

Total estimated cost = R10 500

The costing includes preparation, coating, viewing and imaging analysis of samples as required.

With best wishes and kind regards

PROF CHANTÉLLE BAKER
DIRECTOR: ELECTRON MICROSCOPE UNIT

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UNIVERSITY OF PRETORIA

Appendix B : University of Pretoria RESCOM approval certificate

Chairperson:
Prof LM Sykes
Members:
Prof SM Dawjee
Dr P Brandt
Prof A Bhayat
Dr S Naidoo
Dr T Madiba
Dr C Davidson
Secretary:
Ms C Swart

RESCOM

School of Dentistry
Faculty of Health Sciences

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Prof JG White

2018-11-19

CEO/Chair of the School of Dentistry.

Dear Professor

PROTOCOL APPROVAL: DENT 2018/17

Name: Drs N Moelich and N Potgieter

Title: "Determination of the Cariogenic Potential of Sugar Substitutes"

The protocol attached hereto was evaluated by the Research Committee of the School of Dentistry. The Research Committee recommends the approval of the title and the protocol. Submission to REC will be accompanied by an additional clearance certificate from a registered statistician.

Yours sincerely

PROF L SYKES

CHAIRPERSON: RESEARCH COMMITTEE

Protocol approved/not approved

PROF JG White

Acting CEO/ CHAIR OF THE SCHOOL OF DENTISTRY

Appendix C : Univeristy of Pretoria Ethical clearance certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Exoires 03/14/2020.

28 March 2019

Approval Certificate
New Application

Ethics Reference No.: 86/2019
Title: Determination of the Cariogenic Potential of Sugar Substitutes

Dear Dr N Moelich

The **New Application** as supported by documents received between 2019-02-26 and 2019-03-27 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-03-27.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-03-28.
- Please remember to use your protocol number (86/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

UNIVERSITY OF PRETORIA

Appendix D : Letter of statistical support

Date: 16 / 10 /2019

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that,

Name(s): DR NADINE MOEDICH

from the University of PRETORIA

discussed with me the study titled Determination of the cariogenic potential of sugar substitutes

I hereby confirm that I am aware of the project and also undertake to assist, if possible, with the Statistical analysis of the data generated from the project.

The analytical tool(s) that will be used is (are) Descriptive statistics, two-way ANOVA (factor TREATMENT at 4 levels and TIME at 4 levels) with interaction. Comparison of means (predicted) and contrasts between levels of factor were assessed using the mergeus command following ANOVA.

to achieve the objective(s) of the study. PS. I did data analysis as the consulting statistician was not available any longer.

Name: PJ Becker **(Tel:** 012-319-2203)

Signature _____

Research Office,

Faculty of Health Sciences, UP

BIOSTATISTICS
Faculty of Health Sciences
Research Office
2019 -10- 16
UNIVERSITY OF PRETORIA