The preventative effects of black and green tea on remineralisation of enamel caries lesions under cariogenic challenge *in vitro*

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

Dedicated to my beloved family

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ABSTRACT

Aim

To investigate the remineralising efficacy of green tea, black tea and black tea plus milk on artificial enamel caries-like lesions under cariogenic challenge *in vitro,* using QLF analysis.

Materials and methods

A total of 150 bovine enamel slabs, with artificial subsurface caries-like lesions, were allocated randomly to five different groups (black tea, green tea, black tea plus milk, 5.0 ppm and 0 ppm F water), with n=30 slabs per group. A 28-day pH-cycling regime was then carried out, in which the enamel samples were subjected to one of the treatment groups, acetic acid and artificial saliva. Throughout the cycling regime, temperature, pH, acid titratability, amount of tea in tea bags and fluoride concentration were analysed. Additionally, tea stains were controlled by polishing the enamel slabs at the end of the cycling period. Quantitative Light–Induced Fluorescence (QLF) system was used to analyse the enamel subsurface lesions under standardised environmental conditions at three different time points (at baseline, following the pH-cycling and after implementing the stain removal method).

Results

All study groups showed regression of early enamel lesions in comparison to baseline, though the extent of this was less in the 0 and 5.0 ppm F water groups compared with the tea groups. Further, Bonferroni's multiple comparison tests were used to determine inter-group differences. In terms of remineralisation efficacy, the results indicated statistically significant differences ($p<0.01$), in favour of the test groups (green tea, black tea and black tea plus milk) over the control groups (5.0 ppm and 0 ppm F water). Another statistical significance was observed in the 5.0 ppm F water group over the 0 ppm F water group ($p<0.05$). However, when comparing between the three tea groups, no significant differences were observed.

Conclusions

Green tea, black tea and black tea plus milk groups demonstrated high efficacy in remineralising artificial enamel caries-like lesions under cariogenic challenge *in vitro.*

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LIST OF ABBREVIATIONS

≈	Approximately equal
µg/ml	Microgram per millilitre
µm	Micrometre
CP	Carbamide Peroxide
DMFS	Decayed, Missing and Filled tooth Surfaces
DMFT	Decayed, Missing and Filled Teeth
DP	Digital Photography
EC	Epicatechin
ECC	Early Childhood Caries
ECG	Epicatechin-3-gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
F	Fluoride
Fe	Iron
g/L	Gram per Litre
GC	Gallocatechin
gm	Gram
HCl	Hydrochloric acid
HIV-1	Human Immunodeficiency Virus type 1
HSV-1	Herpes Simplex Virus type 1
ICC	Intra-class Correlation Coefficient
ISO	International Organisation for Standardisation
IV	Intravenous

k	Wavenumber
KOH	Potassium hydroxide
L	Litre
LDL	Low Density Lipoprotein
Low-Level TISAB	Low-Level Total Ionic Strength Adjustment Buffer
M	Molar
MBC	Minimum Bactericidal Concentration
mg	Milligram
mg/kg	Milligram per kilogram
mg/L	Milligram per litre
mg/ml	Milligram per millilitre
MIC	Minimum Inhibitory Concentration
Micro-CT	Microcomputed Tomography
ml	Millilitre
mL/min	Millilitre per minute
mM	Millimolar
mol/L	Mole per Litre
NaF	Sodium fluoride
NaOCl	Sodium hypochlorite
nm	Nanometre
POs-Ca	Calcium phosphoryl oligosaccharides
ppm	Parts per million
px2	Pixels2
QLF	Quantitative Light-induced Fluorescence

r	Correlation coefficient
RCT(s)	Randomised Controlled Trial(s)
SA	Shatha Alsalem
SD	Standard Deviation
SLR	Single Lens Reflex
TF_3	Theaflavin-3,3′-digallate
TMR	Transverse Microradiography
TPP	Tea Polyphenols
USDA	United States Department of Agriculture
w/v	Weight per volume
WHA	World Health Assembly
WHO	World Health Organisation

INTRODUCTION

Tea has been consumed as a beverage for millennia. The practice, originating in China nearly 5,000 years ago, has gained global popularity through the trading endeavours of the British Empire in the mid-seventeenth century. Black tea can be drunk in a variety of ways; with milk and often sugar in the UK, Ireland and Canada; sweetened or tempered with lemon in other countries. Whereas, green tea is usually taken without additives (Weisburger, 1997).

The degree by which tea is fermented yields the different kinds of tea; black, green, oolong, etc. *Camellia sinensis*, the Latin name of the plant, is an evergreen tree of the Theaceae family. Following harvesting, drying and processing, it is added to boiling water and allowed to steep prior to consumption (Harbowy et al., 1997; Weisburger, 1997).

Tea is known to contain high concentrations of polyphenols (flavonoids). These compounds possess anti-oxidative qualities, that help to promote general health and well-being, as well as exert specific beneficial effects on conditions like obesity, diabetes, cardiovascular disorders, hypercholesterolaemia, renal stones and certain malignancies (Sharma et al., 2007; Ahmed et al., 2017).

Further, few researchers in the field of dentistry have established a link between tea consumption and prevention of dental decay (Hamilton-Miller, 2001; Ferrazzano et al., 2009). However, these analyses have almost exclusively focused on the antibacterial activities of tea polyphenols against the pathogenesis of dental caries. Additionally, these studies usually failed to perform in-depth scrutiny of the effects of other chemical elements in tea such as fluoride. Moreover, the remineralisation potential of tea extracts on artificial enamel lesions has been previously assessed only to a very limited extent, whether by deliberately ignoring that particular question, or inadvertently, by incorporating deficient methodology within research (poor replication of the conditions in the human oral cavity, confounding factors, small sample size, research biases, etc.).

Bearing in mind that a pre-clinical *in vitro* analysis, if conducted properly, could establish a solid base for RCT, therefore, a definitive, all-encompassing answer to the question of the effects of black and green tea on the de-/remineralisation of enamel caries-like lesions is prudent.

This research provides an insight into the cariostatic effects of tea extracts, on the remineralisation potential of early enamel lesions. Tea is a cheap and natural compound, so applying it in the context of a national nutritional programme could provide larger benefits as opposed to other more costly dental health interventions if it is proven to be effective in controlling dental caries. Similarly, decreasing the incidence and prevalence of dental caries could be successfully achieved by adding tea extracts to oral health products, namely, chewing gum, toothpastes, mouthwashes and perhaps dental fillings. In addition, these tea compounds could act as alternative fluoride sources.

1 LITERATURE REVIEW

1.1 The dental carious lesion

Dental caries or tooth decay is a complex microbial disease that appears to be associated with multiple factors. It is described as a progressive degradation of mineralised tooth structure by action of cariogenic acid-producing bacteria on dietary sugars and carbohydrates (Featherstone, 2000).

According to Featherstone (2004), the progression or regression of carious lesions depends primarily upon the interaction between both pernicious and remedial factors. Deleterious factors include specific pathogenic bacteria in dental plaque, found to be associated with dental caries, namely *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus* anaerobic species.

Hicks et al. (2004) stated that the vulnerability level of tooth hydroxyapatite to oral bacteria is influenced by both the physical and chemical properties of enamel, dentine and cementum. It is assumed that those physiochemical features are closely related to the mineral content of the dental tissue as well as the composition of both plaque and saliva. In addition, it has been established that a diet high in fermentable carbohydrates, such as sugar or starch, which produces organic acids after being metabolised by the dental biofilm, could alter the amount, pH and composition of both the plaque and saliva, rendering the teeth susceptibly weak (Touger-Decker and van Loveren, 2003). Moreover, salivary dysfunction and poor oral health care are believed to cause pathological changes.

All of the above-mentioned factors would seem to have the ability to tip the balance towards caries progression or demineralisation (Featherstone, 2004). Remedial factors, on the other hand, which include fluoride treatments, adequate salivary flow, composition and function, favourable clearance rate and buffering capacity, sugar substitutes (e.g., xylitol), antimicrobial agents, healthy diet and limited sugar consumption may promote caries regression or remineralisation (Featherstone, 1999; Selwitz et al., 2007).

1.1.1 The dynamic process of demineralisation and remineralisation

As described in the literature, acids yielded via bacterial fermentation, such as lactic, propionic and acetic, will increase the environmental acidity. As a result of the surrounding acidity, the pH within the dental plaque will fall below the critical value of 5.5, resulting in an efflux of calcium, phosphate, and carbonate ions through the dental enamel, in a process called demineralisation (Featherstone, 1999).

If this distortion is prolonged, a cavity might be created. Whereas, if the pH is raised back to a healthy level, then an influx rather than efflux of calcium, phosphate and fluoride minerals will occur, incorporating fluorapatite crystals within the tooth structure to restore its integrity. This phenomenon is called remineralisation (Aoba, 2004; Featherstone, 2004)

According to Hicks et al. (2004), fluorapatite complexes are generally less soluble and more resistant to acid challenge, when compared with hydroxyapatite crystals. This would help to keep calcium and phosphate ions within both dental plaque and saliva at a supersaturated level, as the former dissolves at a critical pH of 4.5 rather than 5.5 for the latter.

The net equity between demineralisation and remineralisation phenomena, which are closely intertwined and occur continuously at the tooth pellicle interface, dictates if the lesion can be repaired, arrested or reversed.

Biological factors such as salivary function and concentration of aciduric bacteria within the oral cavity, can determine the fate of the caries process. These factors are influenced by the form of carbohydrates, the consumption level and tooth brushing habits (Hicks, 2003).

If the demineralisation process dominates the cycle over the long term, then remineralisation is limited. This will result in reduction of mineral substances in the calcified dental tissues, and unless reversed by remineralisation at a favourable time, could eventually cause cavitation (Aoba, 2004).

1.1.2 Subsurface enamel demineralisation

Dental enamel is one of the most dense biomineralised tissues found in the human body. The basic chemical unit of this highly organised dental structure is the hydroxyapatite crystal, which is formed by the union of calcium and phosphate ions. The inorganic crystals form approximately 96% of the dental enamel tissue, with the organic material and water making up the remaining percentage, of around 4 % (Nanci, 2017).

This complex structure is produced by ameloblasts, which tend to terminate themselves upon enamel maturation, rendering the enamel acellular and non-regenerative. Nevertheless, if the enamel lesion is arrested at an early stage by reversing the acidic damage through the remineralisation process, then this might facilitate a natural physiological repair of the early enamel lesion (Featherstone, 2008).

Macroscopically, the enamel lesion looks conical in shape with its apex directed towards the amelodentinal junction (Kidd and Fejerskov, 2016). Clinically, on the other hand, the lesion appears white or brown in colour, with varying levels of opacity. The later characteristic is attributed to the light scattering parameters of the demineralised enamel (Silverstone, 1973).

In ground section, four zones can be distinguished within the enamel lesion. These areas represent the histological changes occurring at the site of the lesion between the enamel surface and sound enamel, namely the surface zone, the body of the lesion, the dark zone and the translucent zone (Darling,1961; Robinson et al., 2000).

The first layer is the translucent zone, which forms the innermost layer of the lesion. It is characterised by a slight increase in porosity compared to sound enamel structure, with approximately 1-2% of mineral loss. It is believed that this early mineral loss occurs mostly between the enamel rods or prisms at the interprismatic junction (Darling,1961).

Then comes the dark zone, which represents the second layer of the lesion. Unlike the translucent zone, this zone does generally exist, with both large and small pores present, accounting for 5-10 % pore volume. It was reported in the literature that this variation in the size of pores, might either be related to mineral gain or deposition of organic substances (Robinson et al., 2000).

The third layer or the body of the lesion, constitutes the bulk of the lesion. It is characterised by marked demineralisation and significant increase in porosity (between 20-50 %). Another characteristic feature of this region is related to the marked presence and prominence of the striae of Retzius lines, as a result of disturbing the orientation of enamel rods. The last layer, which is called the surface zone, represents the top layer of the lesion. This zone is quite intact during the initial stage of demineralisation, with pore volume almost similar to sound enamel tissue (1-2% pore volume) (Darling,1961; Kidd and Fejerskov, 2004).

As has been previously reported in the literature, the relative immunity of the surface zone could be attributed to deposition of demineralised minerals from sub-adjacent structures, contribution of plaque and salivary fluid at the plaque saliva interface, as well as the availability of fluoride at the surface. Organic substances derived from salivary pellicle in addition to fluoride ions seem to stabilise the

superficial enamel layer, rendering it less susceptible to acid dissolution (Robinson et al., 2000).

1.1.3 The role of saliva in preserving oral and dental health

Saliva is a frothy clear liquid, secreted into the oral cavity after being synthesised in the salivary glands. Water represents more than 99% of the salivary components, with the remaining percentage reserved for electrolytes, mucins, enzymes and antimicrobial substances (about 1 %) (Humphrey and Williamson, 2001).

Approximately 90 % of the total salivary output in the oral cavity is attributed to the major salivary glands, including the paired parotid, the paired submandibular and the sublingual, whereas the minor glands secrete the residual amount, that is equivalent only to 10 % of saliva production (Edgar,1992).

The rate of saliva flow varies between individuals. Hicks et al. (2004), classified the outcome rates into three categories: normal, low and very low (Table 1-1).

Table 1-1: Salivary output (mL/min), adopted from (Hicks et al., 2003).

Salivary flow rates (mL/min)	**Normal**	**Low**	**Very low**
Unstimulated saliva flow rate	0.25 - 0.35	0.10 - 0.25	< 0.10
Stimulated saliva flow rate	1.0 – 3.0	0.7 - 1.0	< 0.7

Diseases such as diabetes mellitus, Sjogren's syndrome, cystic fibrosis, systemic lupus erythematosus and radiation therapy of the head and neck region, may impair the salivary flow (Ciancio,1997; Hicks et al., 2004). Similarly, special factors including aging, being female, polypharmacy as well as medications like antihistamines, diuretics, antidepressants and some Parkinson's drugs, all could predispose to dry mouth conditions (Ciancio,1997).

There exists a considerable body of literature on the vital role the saliva plays to maintain the oral and overall health. Therefore, in certain conditions when saliva flow is impaired, complications such as xerostomia, taste dysfunction, dental caries and oral infections may arise (Hicks et al., 2004).

Mucin which is a gel-like substance, produced by mucus secreting salivary glands, lubricates the oral mucosa and provides a surface coating barrier. The function of

this complex glycoprotein helps to lessen the friction between the intraoral mucous membrane and the surfaces of the teeth (Humphrey and Williamson, 2001). Moreover, the moisturising properties of mucins have a critical role in facilitating the process of food chewing, swallowing and speech mechanism (Tabak, 1990).

Bicarbonate, phosphate and urea are three main elements in saliva, responsible for buffering action. With stimulated saliva, bicarbonate has a key role to play in neutralising acids; whereas during unstimulated salivary phase, phosphate usually takes over, as the buffering action of the former compound diminishes (Humphrey and Williamson, 2001).

In addition, the importance of saliva is much appreciated due to its ability to promote remineralisation and reduce demineralisation, therefore helping to prevent dental cavities as well as aiding to reverse and repair early lesions (Edgar and Michael, 1990). This remineralisation capability is attributed to the high amount of inorganic calcium and phosphate minerals present in saliva in a supersaturated state. Besides, a salivary pellicle which is composed of proteinogenic amino acid-rich proteins, stabilises the hydroxyapatite salts at the tooth saliva interface, permitting alteration of bacterial adhesion and modification of mineral transportation across the enamel, in favour of remineralisation (Perinpanayagam et al., 1995).

With respect to the bioavailability of fluoride in saliva, fluoride has a strong binding affinity towards hydroxyapatite units. This chemical bonding results in the formation of a new apatite structure, namely fluorapatite, that is characterised by its greater potential to withstand acids (Edgar and Michael, 1990).

Antimicrobial activity is one well-established property of saliva. Immunoglobulins like IgG, IgM and most importantly IgA, which is found extensively within the salivary fluid, are both bactericidal and virucidal agents (McNabb and Tomasi, 1981, cited in Humphrey and Williamson, 2001, p.166). Moreover, inhibition of bacterial growth can occur via other salivary proteins such as lysosomes and lactoferrins. The later protein exhibits an additional prevailing antibacterial action, demonstrated by its ability to unite with salivary ferric iron. This iron binding capacity deprives the bacterial cells of an essential nutrient, rendering them unviable due to starvation (Lassiter et al.,1987).

Furthermore, saliva makes a substantial contribution to the sense of taste. This is accomplished when saliva blends chemically with food substances, activates the

taste buds located on the surface of the tongue (Mandel, 1987, cited in Humphrey and Williamson, 2001, p.167).

Finally, yet importantly, chemical digestion of starch and fats are initiated in the oral cavity, by special salivary enzymes known as amylase and lipase, respectively (Humphrey and Williamson, 2001).

1.2 Fluoride

Fluoride is derived from a chemical element called fluorine. This natural element can be found in air, earth and water in varying amounts. Most often, fluorine, due to its high reactivity, is integrated with other minerals to form chemical compounds such as fluorite, fluorapatite and cryolite complexes (Fawell et al., 2006).

Drinking water is the primary source of fluoride in food. With respect to naturally fluoridated water, the variable concentrations of fluoride in different water supplies attribute to the variability in total amount of ingested fluoride. Fluoride is also present in many other beverages and foods, either naturally or added, such as tea, milk, certain types of seafood, herbals, supplements, salts, artificial sweeteners as well as some cereals (Macrae et al.,1993).

In 1945/46, the United States and Canada, implemented community water fluoridation for the first time by adding 1.0 ppm of fluoride artificially to some of their public water supplies in an attempt to reduce caries. The reduction in caries prevalence was almost half (Dean et al., 1950). Ever since then, numerous fluoride-containing products have been developed as a result of the successful outcome achieved in caries prevention, including toothpastes, mouthwashes, gels, sprays and varnishes. Over time, an extensive literature has developed on this established link, in order to identify the exact role fluoride plays in maintaining oral health. There exists an indirect proportional relationship between dental caries and fluoride, that is the prevalence of dental caries tends to decrease with higher fluoride concentration (Featherstone, 1999; Marinho et al., 2016).

Recently, Cochrane Oral Health Research performed an updated review of the 2010 research, to determine the effects of different concentrations of fluoridated toothpastes on the primary and permanent teeth. The evidence suggested that fluoride-containing toothpaste (in the range of 1000-1500 ppm fluoride) had an advantage over fluoride-free toothpaste in terms of caries reduction. Furthermore,

the more the fluoride level in the toothpaste, the less the caries detected (Walsh et al., 2019).

In modern societies, the implementation of multicomponent oral health programmes, which are based primarily on fluoride as a public health tool for the prevention of tooth decay, has resulted in substantial reduction in dental caries. However, in other parts of the world, particularly in areas where people suffer from poverty and health burdens, the prevalence of dental caries is considered to be relatively high (Petersen and Ogawa, 2016). The World Health Organisation (WHO) has adopted a key policy to prevent dental caries. The action plan stated that:

> *"For those countries without access to optimal levels of fluoride, and which have not yet established systematic fluoridation programmes, to consider the development and implementation of fluoridation programmes, giving priority to equitable strategies such as the automatic administration of fluoride, for example, in drinking-water, salt or milk, and to the provision of affordable fluoride toothpaste"* (Petersen, 2008, p.119).

1.2.1 Mechanism of action of fluoride to inhibit dental caries

Fluoride is able to function in a number of ways to prevent, manage and stop early enamel lesions. It is the most commonly applied therapeutic agent in the field of cariology, as it retains special antimicrobial and anticariogenic characteristics (Aoun et al., 2018).

The main role of fluoride is attributed to the demineralisation as well as the remineralisation cycles, that result from the interactions between enamel caries, dental biofilm, oral fluid and fluoride. Ten Cate and Featherstone (1991) suggested that trace doses of fluoride could be sufficient to inhibit demineralisation and promote remineralisation of early enamel lesions, through construction of much stronger fluorapatite units with very low solubility in acids. Unlike enamel, studies on dentine have found that greater amounts of fluoride should be present in the solution in order to be able to cause mineral gain in dentine. This pertains to the fact that dentine is more soluble, and its inorganic crystals have less volume when compared to enamel (ten Cate and Featherstone, 1991).

With regards to the antibacterial action of fluoride, reports from various studies demonstrated that fluoride has the capability to influence the metabolism of acid-producing bacteria by acidifying bacterial cytoplasm, thus intervening with the glycolytic process. It also prevents certain enzymes such as enolase, adenosine

triphosphatase and peroxidase (Marquis, 1995; Featherstone, 1999; Aoun et al., 2018).

As per ten Cate's (1999) paper, even low values of salivary fluoride, could shift the scale away from mineral loss. Nevertheless, he reported that due to the high potential reactivity of fluoride ions, they mostly existed within complexes, thus such low values of fluoride are unsatisfactory in terms of microbial inhibition. Alternatively, Marquis (1995) found that the presence of just 0.1 mM of fluoride under acidogenic challenges, could cause total inhibition of the glycolytic pathway of *Streptococcus mutans* bacteria.

Featherstone (1999) and Aoba (2004) reemphasised the fact that other essential elements such as calcium and phosphate, which come mainly from salivary fluid and plaque, must be present in conjunction with fluoride in order to enable the remineralisation process to occur.

Moreover, few scientific studies have suggested that systemic fluoride may alter tooth morphology, resulting in shallower occlusal surfaces and smaller teeth; rendering teeth less susceptible to plaque retention (Glenn et al., 1984; Burt et al., 1986). However, this theory has yet to be proved, as previous evidence is inconclusive (Limeback, 1999).

1.2.2 Topical versus systemic fluoride administration

Fluoride can be administered topically as well as systemically. However, it should be emphasised that systemic fluorides could also exert some topical effects when they come in to contact with teeth surfaces. This explains why drinking fluoridated water, would influence the teeth topically before being ingested systemically (Hellwig and Lennon, 2004). Conversely, according to Whitford (1994), topical fluoridated dentifrices might also cause some systemic effects if swallowed accidently.

Previously, it was assumed that the systemic ingestion of fluoride during the odontogenesis process, could enhance the adhesion mechanism between both fluoride and enamel in the pre-eruptive phase; creating new crystals that are stronger and more resistant to acid attack (Isaac et al., 1958; Brudevold et al., 1967). While the above principle has initiated water fluoridation, later evidence has proved this incorrect (Fejerskov, 2004). Featherstone (2000) stated that the presence of fluoride during tooth formation is inadequate to protect against caries. Moreover, he concluded that topical fluoride agents are more significant in terms of

caries prevention when compared to other systemic modalities, as the former act primarily on teeth during the post-eruptive phase.

1.2.2.1 Topical fluoride modalities

The role of topical fluoride measures in the promotion of dental health have been studied extensively, particularly with regards to the efficacy of fluoride-containing toothpastes, leading to the nearly universal use of such measures (Marinho, 2014). In this section, summaries of the main results of Cochrane Systematic reviews about the efficacy of various topical fluoride agents are going to be addressed.

A recently updated Cochrane review by Walsh et al. (2019) on *"Fluoride toothpastes of different strengths for preventing tooth decay",* compiled 96 RCTs published between 1955 and 2014, concluded with a high to moderate degree of certainty that:

- Toothpaste with a fluoride concentration of 1000 to 1250 ppm, or, 1450 to 1500 ppm has greater efficacy than non-fluoridated toothpaste.
- 1450 to 1500 ppm fluoride toothpaste has a greater effect on the reduction of the incidence of tooth decay than its lower concentration counterparts.
- Employing a high concentration fluoride toothpaste in children and adolescents (1700 to 2200 ppm and 2400 to 2800 ppm) did not decrease the incidence of tooth decay by a greater amount than 1450 to 1500 ppm fluoride toothpaste.

These results are crucial, especially in younger children, so as to weigh the potential benefits of using certain concentrations of fluoridated toothpaste against the risk of dental fluorosis.

Another updated Cochrane review by Marinho et al. (2016) on *"Fluoride mouthrinses for preventing dental caries in children and adolescents",* scrutinised 37 RCTs which were published between 1965 and 2005. The authors determined, with a moderate degree of evidence that consistent use of fluoride mouthrinse was able to lower the DMFS and the DMFT indices in the permanent dentition by 27% and 23%, respectively.

Further, Marinho et al. (2015) updated the Cochrane review on *"Fluoride gels for preventing tooth decay in children and adolescents"* that was first published in the early 2000s. The review encompassed 28 RCTs published between 1967 and 2005. It showed, with a moderate to low degree of certainty that fluoride gels reduced DMFS by 28% and 20%, in permanent and primary teeth, respectively.

Additionally, a Cochrane review by Marinho et al. (2013) on *"Fluoride varnishes for preventing dental caries in children and adolescents",* looked at 22 RCTs published between 1975 and 2012. There was moderate evidence that fluoride varnishes were able to reduce DMFS in young people by 43% and 37%, in permanent and primary teeth, respectively.

1.2.2.2 Systemic fluoride modalities

There exists a considerable amount of literature on the systemic fluoride systems, including fluoridated water, supplements such as tablets and drops, fluoride added to milk and salt. Besides, beverages such as tea, coffee, drinks containing fluoride naturally or artificially, food prepared with fluoridated water, shellfish and grapes (Harrison, 2005, O'Mullane et al., 2016).

1.2.2.2.1 Fluoride in water

The optimum concentration of fluoride in water based on Dean's research, was determined to be at 1.0 mg/L. It has been suggested that adding fluoride up to this level, could produce a noticeable decrease in caries and at the same time might limit dental fluorosis (Dean et al.,1950).

However, due to the fact that people in cooler environments drink less water than people living in warmer climates, the 1.0 mg/L estimate has been revised to fit within (0.5-1.0 mg/L) range. Consequently, the recommended drinking water fluoride level should be based upon the average weather (WHO, 2011). This should be considered in societies who wish to start water fluoridation programmes, or in other areas that need to adjust the level of fluoride in their water supplies through a procedure called de-fluoridation (O'Mullane et al., 2016).

Nevertheless, in spite of its long-standing history, there has been a lot of debate about adding fluoride to drinking water. This was mainly attributed to health risks and long-term complications, especially when it has been realised that the risks associated with water fluoridation might outweigh its protective anticariogenic action (Horowitz, 2003; Ko and Thiessen, 2015).

A Cochrane review was conducted in 2015, to assess the outcome of natural or artificial water fluoridation in relation to tooth decay as well as dental fluorosis**.** The research analysed a total of 155 papers,19 were about the role of water fluoridation in preventing dental decay and the rest covered dental fluorosis. Among these, only 27% of the studies were performed in areas with optimal water fluoridation, whereas

the majority of the rest were conducted in places with natural fluoride concentrations of up to 5 ppm. Even though the database showed substantial caries reduction with water fluoridation, the review concluded that the current information available about the effectiveness of this measure was unsatisfactory. This is because most of the results were obtained from early studies performed in the 70s, that were judged to be at high risk of bias. Furthermore, it was reported that dental fluorosis and fluoride levels were significantly associated (Iheozor-Ejiofor et al., 2015).

1.2.2.2.2 Fluoride in food

Fluoride can present in many food sources. Table 1-2 shows the average levels of fluoride in various food types based on United States Department of Agriculture (USDA) (Haytowitz, 2005).

Table 1-2: Concentrations of fluoride for various food forms, adopted from (USDA, 2005).

Type of food	Concentration F (ppm)
Black tea	3-5
Shellfish products	2-3
Wine	1-2
Green tea	1.2
Boiled pork or rice	≈0.4
Cheddar cheese	0.35
Apple	0.03
Milk, cream	0.03
Banana, tomato	0.02
Eggs	0.01
Cucumber, onion, celery	0.01

1.2.2.2.3 Salt fluoridation

Fluoridated salt production was started for the first time in the mid-50s, in Switzerland (Marthaler, 2005). In Jamaica, Switzerland and Costa Rica different methods and products have been adopted for salt fluoridation, which resulted in diversity in populations coverage. Some European countries, including France and Germany, added fluoride to their domestic salt only (Cahen et al., 1993; Estupinan-Day, 2001). Whereas, in Switzerland both fluoridated domestic salt and non-fluoridated salt have been accessible since the early 80s (Marthaler, 2005). In the

World Health Assembly (WHA) resolution, the use of fluoridated salt has been advocated in places with no water fluoridation facilities. The optimal concentration of fluoride in salt can only be calculated after considering other variables, such as the daily levels of the total ingested fluoride and the total salt consumption. Based on the former factors, range may vary between 200-400 mg F/kg body weight (WHO, 2006; O'Mullane et al., 2016).

A meta-analysis of nine different research reports was designed to determine the efficacy of salt fluoridation measure in controlling dental decay in children's permanent teeth. The analysis found that salt fluoridation was an effective anticariogenic measure when compared against no fluoride exposure, in children of all age categories (6-8, 9-12 and 13-15 years of age). It has been also suggested that the difference between salt and water fluoridation was insignificant. This conclusion however could not be confirmed as the quality of the scientific evidence included was relatively low (Yengopal et al., 2010).

1.2.2.2.4 Milk fluoridation

Milk and cheese may possess some anticariogenic characteristics, as they are naturally rich with calcium and phosphate minerals. The consumption of such dairy products will promote the precipitation of calcium and phosphate ions within both the saliva and the plaque, thus inhibiting demineralisation and enhancing remineralisation (Herod,1991; Kashket and DePaola, 2002). Tanaka et al. (2010) found that increased yogurt intake (≥4 times per week), was associated with low caries prevalence. One study conducted by Grenby et al. (2001) found that in addition to calcium and phosphorus minerals, milk protein molecules recognised as proteose-peptone fractions 3 and 5, have the ability to bind tightly to enamel crystals. Hence, less enamel demineralisation was noticed under acidogenic challenges.

That said about the anti-cariogenic properties of milk, attention should be brought that milk might also lead to caries initiation (Petti et al., 1997, Johansson et al., 2010). Recently, a systematic review of 139 studies was conducted to assess the impact of changing certain factors on the risk of developing early childhood caries (ECC). The modifiable factors such as: frequency and duration of breast milk feeding, exposure to fluoridated milk or salt and mixing sugar with formula milk, were included. The review concluded based on the best available data that breast feeding for the first two years of life does not raise the risk of ECC, whereas beyond

that age, the risk will be increased. They also found low quality evidence about the protective effects of milk fluoridation on ECC (Moynihan et al., 2019).

In the initial medical and laboratory tests, milk fluoridation involved any of the three inorganic compounds such as sodium fluoride, disodium monofluorophosphate or calcium fluoride. Nowadays, sodium fluoride is the most popular compound added to milk, when considering implementation of such fluoridation programmes (Villa, 2009).

Moreover, an updated Cochrane review of the 2005 analysis, was conducted to assess the cariostatic properties of milk fluoridation in a community-based approach. Only one Randomised Control Trial (RCT) with a high risk of bias was found and analysed before being published. The trial allocated children randomly to two groups. The first group received fluoridated milk at a concentration of 2.5 mg/L, whereas, the comparator group drank non-fluoridated milk. The study found that there was a significant reduction in the caries prevalence for the primary teeth when comparing control versus experimental groups, evidenced by the measure of the dmft index. Nevertheless, the same effects could not be detected when permanent teeth were evaluated. Since, there was only scanty evidence, the 2015 Cochrane update could not make a final decision about the efficacy of this preventive measure in school children (Yeung, 2015).

1.2.3 Fluoride toxicity

This may arise due to ingestion of high levels of fluoride through the excessive consumption of fluoridated oral hygiene products and/or, fluoride-rich foods. Following this, plasma fluoride levels markedly rise within minutes, with peak concentrations occurring within 20-60 minutes. The highest concentrations reached depend on multiple factors, including age, gender, nutritional status, climate, quantity ingested, rate of absorption and the excretory efficacy of the kidneys. The skeletal system is another means of fluoride clearance (Whitford, 1994; Petrone et al., 2013; Ullah et al., 2017).

Hence, maintaining adequate levels of fluoride without exceeding a toxic dose is essential. The American Institute of Medicine (1997) calculated this optimal dose as being between 0.05 and 0.07 mg fluoride/kg of body weight, for children and adults. The highest recommended dose was found to be 0.1 mg/kg body weight. Whitford (1987, p.1057) described a level equivalent to 5 mg/kg as being the probable toxic dose of fluoride, that is *“the minimum dose that could cause toxic signs and symptoms, including death, and that should trigger immediate intervention”.* The

certainly lethal dose was determined by Hodge and Smith (1965) to be between 32 and 64 mg fluoride/kg body weight, for adults. The safely tolerated dose, however, was described as the amount of fluoride that can be taken without causing systemic toxicity and was estimated to range between 8 to 16 mg fluoride/kg according to (Heifetz and Horowitz, 1986).

1.2.3.1 Acute fluoride toxicity

Gastrointestinal disturbance occurs when the fluoride levels ingested are at the lower end of the toxicity spectrum. Higher doses lead to convulsions and muscle spasms (tetany), with decreased cardiac muscle motility arising secondary to associated hypocalcaemia, since fluoride readily binds to calcium in plasma. Further metabolic shifts occur in the form of hyperkalaemia, causing ventricular arrythmias and cardiac arrest. Mortality is mainly due to cardio-pulmonary failure (Shulman and Wells, 1997).

Fluoride toxicity is a medical emergency requiring prompt treatment. A conscious patient may be encouraged to ingest copious amounts of milk. Failing this, vomiting should be induced and encouraged. Induction of vomiting is contraindicated in those with a reduced level of consciousness to avoid aspiration. Pharmaceutical solutions, namely, 1% calcium chloride or calcium gluconate should be administered to slow the rate of fluoride absorption. In severe cases, a gastric lavage is indicated with a solution of calcium or activated charcoal. Calcium and potassium plasma levels must be diligently monitored (Whitford, 2011).

Fluoride toxicity management checklist (Whitford, 2011):

- Monitor and support vital functions.
- Monitor and manage plasma calcium and potassium levels.
- Minimise absorption (milk, vomiting, IV fluids, gastric lavage).
- Correct any acid-base disturbance.

1.2.3.2 Chronic fluoride toxicity

Long-standing intake of high fluoride levels can have a detrimental effect on teeth, bones, kidneys, mental and physical development, reproduction, as well as the central nervous system, to mention a few. Moreover, dental and skeletal tissues have a high affinity for fluoride, which accumulate within them over time (Petrone et al., 2013).

Skeletal fluorosis manifests as high bone mass and density. It also causes muscle/bone pain, stiff joints, reduced mobility, muscle weakness, fatigability and

paraesthesia. Well-established toxicity is associated with osteoporosis, arthritis, spinal cord compression, ligaments ossification and loss of muscle mass. Neurological deficits may also arise (Ullah et al., 2017).

Dental fluorosis results from the intake of high doses of fluoride mostly during the maturation stage of tooth formation and development. Signs of this condition range from mild white hypomineralised opacities to more severe forms of fluorosis, in which the affected enamel appears pitted, rough and heavily discoloured (DenBesten and Li, 2011). Clinical indices have been generated and developed to characterise and describe the appearance of dental fluorosis, pioneered by H. Trendley Dean in 1934 (Rozier, 1994).

1.3 Tea

Tea, which is only exceeded by water, is the world's second most consumed beverage. In actual fact, all tea forms derive from a special plant called *Camellia sinensis*. Although this tea plant was historically produced in the Indian Subcontinent, East and Southeast Asia, it is now cultivated worldwide (Sang et al, 2011).

Different forms of tea are produced through different manufacturing techniques, which are varied based on the level of oxidation or fermentation. Fermentation in the tea production process refers to the browning effect induced by plant-endogenous enzymatic reactions, by a polyphenol oxidase enzyme. Consequently, three main types of tea generally exist, including black tea, oolong tea and green tea which are fully fermented, partially fermented and unfermented, respectively (Harbowy et al., 1997; Sharma et al, 2007).

Green tea is normally made in to two forms namely, white tea (unoxidised tea) and yellow tea or withered tea. It is believed that the withering step in the latter form induces a small amount of oxidation (Bokuchava and Skobeleva, 1980). In oolong and black teas however, the plant leaves are withered for a longer duration until the water content is reduced by at least 50 %, which will in turn lead to accumulation of polyphenols within the leaf structure. An exothermic fermentation reaction will then take place to transfer these basic polyphenols into denser units, giving the black tea its dark intense colour (Harbowy et al., 1997).

1.3.1 Chemistry of tea

The composition of tea has been widely investigated in recent decades, with most chemical analyses performed using either chromatography or capillary electrophoresis (Yashin et al., 2015). It is essential to address each active chemical component thoroughly in order to pursue this investigation.

1.3.1.1 Polyphenols

Tea contains a considerable quantity of polyphenols, which account for approximately one third of its composition (Graham,1992; Harbowy et al., 1997). Most of the chemical and physical characteristics of tea beverages including hue, taste and astringency, are due to the abundant supply of these antioxidant compounds in such products. The dominant groups of polyphenols present in green

tea are referred to as flavonoids (e.g. catechins and flavonols), which are composed of at least 15 carbon pieces (Balentine, 1992). Whereas, in black tea these simple polyphenols will transform chemically into more complex polymers, that are made of 30 carbon molecules or even more (Bailey et al., 1990, Harbowy et al., 1997).

The principal catechins (subgroup of flavonoids) found in green tea, from least to greatest, are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG), respectively (McKay and Blumberg, 2002).

Besides the fermentation of the remaining green tea catechins and flavonols to produce black tea polyphenols, additional types of polyphenols called theaflavins and thearubigins are also produced. It is believed that the latter are responsible for both the colour and aroma of black tea (Harbowy et al., 1997; McKay and Blumberg, 2002; Sang et al., 2011).

1.3.1.2 Caffeine and xanthines

Caffeine is known to be an essential element in tea, owing to its role in improving cognition and enhancing mood (Bokuchava and Skobeleva, 1980; Harbowy et al., 1997).

Hicks et al. (1996), suggested that the brewing process and the type of tea used whether loose or in bag, determine the amount of caffeine produced within tea brews. Moreover, Chin et al. (2008) stated that caffeine concentration, might fall within the range between 14 and 61 mg per average size green or black tea cup. However, there was less than 12 mg of caffeine in decaffeinated teas.

An earlier study by Astill et al. (2001), mentioned multiple aspects that could determine the levels of caffeine and polyphenol ingredients in different black and green tea extracts. The paper concluded that factors such as environmental and processing conditions, preparation techniques, particle size in tea infusions, use of tea bags versus loose leaf tea and finally tea bag materials, may significantly alter the quality as well as the concentration of compounds found in tea beverages.

However, there has been some controversy surrounding the presence of other xanthine derivatives, such as theobromine and theophylline in tea extracts (Hicks et al., 1996; Harbowy et al., 1997; Fernández et al., 2002).

1.3.1.3 Proteins and amino acids

The chemical structure of tea is significantly influenced by its peptides or amino acids content. Protein in tea represents about 6 % dry weight (Harbowy et al.,

1997), whereas, Cabrera et al. (2006) suggested a range of 15-20 % of extract solids. Also, Liang et al. (1990) quantified 18 amino acids in two types of Chinese green tea.

Furthermore, Wang et al. (2010) analysed three types of tea (green, oolong and black) in addition to the Camellia flower itself, using high performance liquid chromatography method, in order to measure the free amino acids. The findings revealed that theanine was the major amino acid present in teas, including the tea flower. Additionally, black tea contained less free amino acids than green tea, owing to the fermentation process.

Therefore, tea withering seems to increase the free amino acids content, whereas fermentation acts oppositely, as some amino acids are utilised during the oxidation process (Roberts and Sanderson, 1966).

1.3.1.4 Enzymes in tea

The major enzymes found in tea plants are peroxidase and polyphenol oxidase, which catalyse simple polyphenols as well as generate theaflavins of black tea during the fermentation process (Parry et al., 1996; Subramanian, 1999).

1.3.1.5 Volatile impurities

Hundreds and various volatile compounds have been identified in *Camellia sinensis* extracts, such as aldehydes, alcohols, esters, lactones, and hydrocarbons. The composition and the concentration of these volatiles determine both the flavour and the aroma of different tea brews (Chacko et al., 2010; Yashin et al., 2015).

1.3.1.6 Carbohydrates in tea polysaccharides and pectins

Analyses have shown that tea extracts contain free sugars, pectins and polysaccharides, together accounting for 11% dry weight according to Harbowy et al. (1997), and 5-7 % dry weight based on Chacko et al. (2010).

Furthermore, it has been stated that the major carbohydrate composition in polysaccharides, might comprise glucose, galactose, fructose, rhamnose, arabinose, galacturonic acid and xylose; depending on different manufacturing techniques. These biological carbohydrates were found to facilitate the enzymatic action during fermentation (Harbowy et al., 1997; Wang et al., 2013; Du et al., 2016).

1.3.1.7 Vitamins and antioxidants

Research on tea has indicated that tea leaves are rich in vitamins and antioxidants such as vitamin C, E (alpha-, beta-, gamma- and delta- tocopherols), also vitamin B2 (riboflavin) (Chacko et al., 2010; Yashin et al., 2015).

1.3.1.8 Fluoride and other elements

Studies have shown that the tea plant is considered to be a good source of many minerals such as potassium, calcium, magnesium, iron, aluminium, phosphorus, zinc and others (Harbowy et al., 1997). More importantly, early evidence from the beginning of the 20th century, showed that the *Camellia sinensis* plant contains substantial amounts of fluoride (Reid, 1936; Waugh et al., 2016).

1.3.2 Bioavailability of fluoride in teas

The presence of fluoride in the leaves, could be explained by the fact that the roots of the tea plant, extract fluoride from the soil and accumulate the majority (nearly 98%) within the leaves during the growth stage (Fung et al., 1999; Lu et al., 2004).

In Fung's et al. (1999) study, fluoride levels in five different soils and tea plants from China, were determined. The analysis concluded that fluoride accumulation was higher in mature compared to immature leaves. The investigators also pointed out other facts that could alter the fluoride levels in such samples; such as the type of soil used for planting, the level of fluoride in soil (total plus extractable), the amounts of extractable aluminium and the level of acidity of soil. This conclusion was in accordance with an earlier study, suggesting that high soil acidity was associated with an increase in water solubility of fluoride and aluminium, thus resulting in higher fluoride uptake by tea plants (Wenzel and Blum, 1992).

Moreover, Lu et al. in 2004, reported that while newly formed leaves might contain between 100-430 mg kg^{-1} of fluoride, the older leaves had fluoride reservoirs ranging from 530-2350 mg kg^{-1}. Accordingly, there exists a direct proportional relationship between fluoride content in leaves and the stage of maturity of such leaves (Emekli-Alturfan et al., 2009).

In recent years, numerous studies have been conducted on fluoride levels in different tea infusions under various conditions. Researchers have identified some factors that could influence fluoride's release and solubility during tea brewing. Tea brewing time and type of tea were amongst the most common attributes. Although

studies have tested different brew times, the general consensus was that the longer the brewing time, the more fluoride was released (Chan and Koh,1996; Fung et al., 1999; Malinowska et al., 2008; Chan et al., 2013).

In a recent study by Miri et al. (2018), the concentration of fluoride in seven different types of tea, including Black tea, Kenya tea, Taksetare tea, Green tea, Ceylon tea, Red tea and White tea, were analysed in duplicate with a fluoride ion selective electrode. It was established that the mean fluoride concentration in black tea was the highest and almost double the amount when compared to green tea. White and red teas, on the other hand, had the least concentrations of fluoride, respectively. Furthermore, there was a major increase in the amount of fluoride with increased brewing time, with Kenya tea showing wide variability in terms of fluoride concentration (3.075 ± 0.696 mg/l, 4.794 ± 1.085 mg/l, and 5.380 ± 0.917 mg/l) measured at different time scales (5, 10 and 15 minutes), respectively. In this experiment, the maximum amounts of fluoride in tea liquors were detected at 5 minutes. There findings are in line with previous reports (Gulati et al., 1995; Zerabruk et al., 2010), but are inconsistent with Koblar et al. (2012), who concluded that the three basic tea types including black, green and oolong, provided almost the same fluoride releasing efficacy when compared with each other.

Likewise, the form of tea (tea bags versus loose tea leaves), is another influencing factor to consider. Generally, tea bags have been known to exhibit higher fluoride content than loose leaf teas (Cao et al., 2006; Emekli-Alturfan et al., 2009). In Cao et al's study (2006), the fluoride levels in different black tea forms (bags, granular and sticks) were measured to be between 1.15–6.01 mg/L, 0.70–2.44 mg/L and 0.95–1.41 mg/L, respectively. They indicated that the main reason was directly related to the maturity level of the black tea extract used in such tea bags. However, their suggestion did not agree with Giljanović et al. (2012), who stated that the larger the surface contact, as in tea bags, the higher the concentration of fluoride.

According to the UK Tea Council (2019), the majority of people in the UK, nearly 96%, prefer to use tea bags to prepare their cup of tea; additionally, almost 98% of them drink their black tea with milk. Earlier in 2004, Cao et al., analysed numerous black tea samples from different geographical regions including the UK, in order to determine the impact of milk or sucrose addition, on the ionic levels of fluoride in black tea brews. Either 50 ml of milk or 30 gm of sucrose was added to the brew. It was deduced that neither milk nor sugar changed the fluoride concentrations in such tea samples. Their conclusion was in harmony with a previous study carried

out by Gulati et al. (1995), only with respect to English style of tea preparation, where milk is only added but not boiled. Though, the latter study established that when boiling milk and tea are mixed together like in Indian style tea, the fluoride concentrations might be lowered. Lung et al. (2008), also tested the addition of sugar to tea. Surprisingly, they found that within few minutes of administration, the levels of fluoride were doubled in tea beverages that contained sugar as opposed to sugar-free teas. However, these results are doubtful because how could sugar increase the fluoride concentration, specially that the type of sugar used, whether fluoridated or not, was not specified.

In addition, filter papers used in tea bags packaging and type of teapots whether (ceramic, glass or pottery), have been suggested in the literature as potential factors that might influence the fluoride release. However, neither tea-filter bags nor tea containers were shown to change the concentrations of fluoride in tea drinks (Cao et al., 2004; Lung et al., 2008).

Furthermore, the round of infusion whether continuous or repeated, might influence fluoride leaching into various tea infusions. Zhu et al. (2013) performed potentiometric analysis, to quantity the amounts of fluoride in different tea brews (black, green and Pu-erh) using continuous versus repeated infusion methods. It was observed that in the continuous infusion technique, the level of fluoride increased with time until plateauing at about 20 minutes. Nevertheless, when doing multiple repeated infusions, the third and second rounds delivered considerably less amounts of fluoride in comparison with the first tea round. This does not align with an earlier study by Fung et al. (1999), who suggested that repeated infusions exhibited higher levels of fluoride when compared to continuous ones.

Last but not least, Waugh et al. (2016), conducted a study in the Republic of Ireland, in which they collected more than 50 commercial black teas to measure their corresponding fluoride contents using fluoridated drinking water versus deionised water. Their research concluded that 59% of tea brews prepared from fluoride-containing tap water, exhibited fluoride concentrations of more than 3.0 mg/L, as opposed to only 22% when deionised water used in such preparations. Hence, in Ireland the use of tap water in tea liquors was found to be associated with higher fluoride concentration, as the current Irish legislation states that fluoridation of water supplies is compulsory in order to control dental decay.

1.3.3 General health benefits of tea

In recent years, tea has gained a growing interest. This has been augmented by the increased awareness of the scientific studies which scrutinised its various health benefits, which range from being cardio-protective, to possessing anti-microbial properties.

Tea's well-known anti-oxidative properties are mainly due to its powerful antioxidant components, namely flavonoids (Rice-Evans, 1999). Van Acker (1996) suggested that such compounds act to neutralise endogenous oxides and hydroxyl radicals. When comparing black and green tea, an article by Leung et al. (2001) concluded that consuming black or green tea provides similar health benefits. The hypothesis behind this was that theaflavins and catechins, which are present in black and green tea, respectively, possess equal potency. Similar studies have also reported that theaflavin in black tea, specifically theaflavin-3,3′-digallate (TF_3), is similar to epigallocatechin gallate (EGCG) found in green tea (Yoshida et al. 1999; Lin et al, 2000). However, the varying concentrations of the diverse antioxidant compounds present in different types of teas provide varying health benefits (Lee et al, 2002).

Graham (1992) and Wang et al. (2000) studied the composition of green and black tea. The results of their studies showed that in black tea, theaflavin levels are low (2-6%) and thearubigens are high (>20%), whereas green tea mostly contained catechins (30-42%). Similarly, Lee et al. (2002) reported that for the same volume, green tea has greater health benefits than black tea. Moreover, numerous studies have pointed out the potential, anti-inflammatory, anti-microbial, anti-hypertensive, anti-carcinogenic, anti-mutagenic and weight loss properties, in addition to the cardio-protective and oral health protective effects of various tea types (Cabrera et al. 2006). These results are in agreement with Reygaert (2017), who concluded in her *"Update on the Health Benefits of Green Tea"* that it was catechins, especially EGCG, that were the main source of these health benefits.

On the other hand, a major Cochrane systematic review Boehm et al. (2009) did not establish a confirmed anti-carcinogenic effect. This review comprised 51 studies with over 1.6 million participants. Twenty-seven case-control studies, 23 cohort studies and one RCT were reviewed. The included studies were attempting to assess the association between drinking green tea and the incidence of certain cancers, including but not limited to, gastrointestinal, breast, prostate, lung, ovarian, urinary bladder, as well as oral malignancies. The sole RCT which was included in

the review suggested there was a reduced risk of developing prostate cancer, but only in individuals who consumed three or more cups of green tea daily. Other results were contradictory, with some studies providing limited to moderate evidence of the reduction in the risk of developing lung, pancreatic, colorectal and urinary bladder cancer, while others showed a positive association between green tea consumption and developing the latter cancer. Thus, the evidence that green tea could reduce the incidence of the aforementioned malignancies, including oral cancer, was found to be inadequate.

Flavonoids in tea have been shown to decrease the risk of developing type II diabetes. Van Dam et al. (2013) conducted one such study, which showed a correlation between consuming flavan-3-ols found in green tea, and a reduced risk of developing type II diabetes as well as cardiovascular disease, through the beneficial effect of reducing LDL-cholesterol, hence achieving cardio-protection to some degree.

Furthermore, it is hypothesised that green tea possesses antiviral characteristics, through the action of the compound polyphenol, as an antioxidant and as an inhibitor of viral enzymes which promote cell damage, as well as preventing viruses from infecting cells directly. Additionally, it has been suggested in the literature that polyphenols might have the ability to impede the action of Human immunodeficiency virus type 1 (HIV-1), Herpes simplex virus (HSV-1), Epstein Barr virus, adenoviruses and more (Friedman, 2007).

1.3.4 Oral health benefits of tea

Koh et al. 2011 discovered that administering EGCG from green tea extract to mice suffering from oral squamous cell carcinoma, led to dose-dependent tumour remission. Theoretically, green tea extract and EGCG may be used in the prevention of cancer growth and metastasis. Unfortunately, in spite of the positive results in many *in vitro* and *in vivo* models, the studies lacked robustness. Hence, green tea consumption to produce anti-oral cancer effects is inconclusive (Boehm et al., 2009).

Periodontitis and gingivitis are other conditions which have been shown to be lessened by green tea consumption. This is probably achieved as a result of its beneficial impact on the periodontium, evidenced by measuring periodontal health parameters such as; gingival bleeding, pockets depth and clinical attachment loss

(Kushiyama et al., 2009; Maruyama et al., 2011). Moreover, EGCG's antimicrobial mechanism of action is manifested through its ability to act on certain pathogens associated with gingivitis, interfering with the fimbriae, thus limiting bacterial adhesion to periodontal tissue. The later compound could also exert an additional antimicrobial action through inhibition of bacterial collagenase enzyme (Kushiyama et al., 2009; Gaur and Agnihotri, 2014).

When examining the effects of green tea on halitosis, Lodhia et al. (2008) showed that green tea powder can halt the formation of sulphur bi-products. Likewise, Zeng et al. 2010 showed that green tea extract could eliminate malodorous sulphurs.

Regarding dental erosion and abrasion, an *ex vivo-in situ* experiment conducted by Magalhaes et al. (2009) in two phases, discovered that using mouthwash with green tea extract defended against dentine erosion and abrasion in a similar manner to rinsing with either fluoride or chlorhexidine mouthwashes. The ex-vivo phase, involved exposing the oral appliances with bovine teeth, to erosive conditions (dipping in Coca-Cola) or erosive along with abrasive conditions (Coca-Cola plus brushing with power toothbrush and fluoride-free toothpaste). Directly following erosion and/or abrasion methods, the participants were asked to wear their appliances and rinse with either a 250ppm fluoride solution, 0.12% chlorhexidine digluconate, 0.61% green tea catechin extract, or de-ionised water. Profilometry was used to measure the dentine wear. The study concluded that when erosion and abrasion process were combined, the tooth wear effects were augmented ($p < 0.001$). Moreover, it was deducted that all the test rinses succeeded to lessen the dentinal wear significantly in comparison to the control group. These results corresponded with Kato et al. (2009), who confirmed the efficacy of green tea in managing erosive and abrasive tooth wear. The major limitation of this study was the small sample size. Further, there was no explanation on how the concentration of green tea mouth rinse was chosen.

A more recent *in vitro* study by Jameel et al. (2016) looked at erosion resulting from exposure to different beverages. The outcomes were quantified using micro-hardness and surface-roughness analysis. The groups were de-ionised distilled water, tap water, coffee, black tea, Coca-Cola and finally orange juice with a particular pH and fluoride content. The investigators concluded that teeth exposed to acidic solutions with high fluoride levels only caused a limited increase in surface-roughness (roughness due to black tea was only 8%, at pH 4.9 and fluoride concentration of 9.38 ppm). On the other hand, acidic solutions without or with low

levels of fluoride, Coca-Cola for instance, induced 37% roughness in the enamel, at pH 2.53 and fluoride level of 0.01 ppm. Another systematic review conducted by Jaâfoura et al. (2014), aimed at establishing associations between different types of tea and dental erosion, showed that erosion was caused by fruity tea, ginger-vanilla tea, iced tea and sugary tea, while unsweetened green and black tea led to no erosion.

With respect to dental caries, it is hypothesised that green tea aids in the prevention of dental caries through the anti-microbial action of polyphenols and tannins, as opposed to cariostasis achieved by fluoride (Onishi et al. 1981). Further, it was shown that plaque formation, oral acidity and cariogenic oral bacteria may be hindered by regular rinsing with black tea solution (Lingstro et al., 2000, cited in Sharma et al., 2007, p.789). Subsequent studies showed that EGCG extract given prior to the introduction of glucose, resulted in less acid production by deactivating the lactate dehydrogenase enzyme, which incidentally, converts pyruvate to lactic acid (Hirasawa et al., 2006). This topic will be addressed in detail in the following section.

1.3.4.1 The antimicrobial effects of tea on dental caries bacteria

Many studies have been published on the antimicrobial properties of tea. It has been shown that the active ingredients in tea can interfere with each pathological stage involved within the pathophysiology of the dental caries process, namely bacterial attachment to susceptible tooth surfaces, glycocalyx synthesis as a result of the interaction between sucrose and glycosyltransferase enzyme, bacterial colonisation as well as acid formation (Hamilton-Miller, 2001; Goenka et al., 2013).

1.3.4.1.1 Studies reporting dental antibacterial implications

Hamilton-Miller (1995) carried out a mini review to assess the antimicrobial characteristics of the *Camellia sinensis* plant. According to the review, catechins found in black tea but in green tea mostly, such as gallocatechin (GC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG), are known to be associated with most of the beneficial cariostatic effects of tea owing to their antimicrobial action against certain cariogenic bacteria. This conclusion was in confirmation with an earlier *in vitro* study published by Sakanaka et al. (1989).

Moreover, subsequent review articles have focused not only on the bacteriostatic, but also the bactericidal properties of these catechins against *Streptococcus mutans* and *Streptococcus sobrinus* species (Hamilton-Miller, 2001; Taylor et al., 2005).

An earlier *in vitro* study by Kubo and Muroi (1993), tested and measured both MIC and MBC of the most common volatile compounds (terpenoids-like products) found in green tea, in *Streptococcus mutans*. It was found that synergism between certain volatile compounds had increased the bactericidal effects significantly, as well as lowered the MIC values, thus indicating their efficacy *in vitro*. Nevertheless, their conclusion needed further experimental verification in order to be validated.

In another *in vitro* study, Huang et al. (2005) declared that polyphenols from tea extracts were effective against acidogenic Lactobacillus bacteria at less than 8.0 mg/ml of MIC. Furthermore, a review paper written by Ferrazzano et al. (2009), stated that the pathogenesis of dental caries could be altered, via the inhibitory mechanism of polyphonic tea on bacterial colonisation and organic acid formation of several *Streptococcus mutans* and *Streptococcus sanguinis* strains.

Moreover, Otake et al. (1991), conducted a study in which they tested a Japanese green tea extract both *in vitro* and *in vivo*. In the *in vitro* experiment, it was concluded that catechins from tea, such as EGC and EGCG, could inhibit the attachment of cariogenic bacteria to saliva-coated hydroxyapatites significantly ($p < 0.01$), at a concentration of 50-100 µg/ml. For the *in vivo* animal model, mice who were specific pathogen-free were tested after being infiltrated with *Streptococcus mutans* bacteria. They found that the numbers of carious lesions were considerably less in a mice group that was exposed to water containing 0.05% tea leaf extract compared to a rat group that did not receive tea extracts. Unfortunately, the study failed to show how the sample size was calculated. In addition, there has been a lack of clarity regarding who measured the caries scores and if calibration was implemented.

In Hamilton-Miller's (2001) review article, the author suggested that when polyphenols were present, bacterial attachment to tooth enamel was prevented, as well as inactivation of both glycosyltransferase and salivary amylase enzymes. However, the quality of evidence was low as the evidence was built on limited *in vivo* experiments and only few clinical trials with many confounding variables.

In an earlier *in vitro* study conducted by Hattori et al. (1990) to evaluate the effects of black and green tea extracts on glucan synthesis, the investigators concluded that among polyphenols, theaflavins extracted from black tea had the strongest effects on glycosyltransferase action, inhibiting the biosynthesis of glucan even at a concentration of just 1 mg/ml; whereas simple green tea catechins exhibited moderate inhibitory effects at 10 mg/ml. These findings complemented the results of

another *in vitro* study, carried out by Hara and Honda (1990). In this experiment, the researchers concluded that catechins from green tea as well as theaflavin derived from black tea, were potential inhibitors of the amylase enzyme, thus preventing decomposition of starch to maltose. Indeed, one of the main shortcomings of the study was that it failed to report how much tea someone should drink to achieve the beneficial effect stated earlier. The results of the previous study were in agreement with the *in vivo* study published by Zhang and Kashket (1998), whom found that using mouth rinse extracted from either black or green tea, resulted in 70% reduction in maltose levels.

Adding to the evidence, a recent study which involved both *in vitro* and *in vivo* testing was conducted by Al-Ezzi et al. (2018), to evaluate the antimicrobial properties of black and green tea extracts with variable concentrations. In the *in vitro* study, twenty bacterial samples were first isolated from 20 volunteers. The total viable count and MBC of *Streptococcus mutans* and *Lactobacillus* bacteria were determined against different concentrations of tea extracts. The findings showed that the total bacterial count was less with all tea samples. However, the biggest reduction was observed at a concentration of 50% green tea extract. Additionally, the MBC of black tea extract was measured to be at 35% whereas green tea showed MBC of 30%. In the further *in vivo* experiment, 30 participants were recruited based on certain inclusion criteria and asked to rinse with 10 ml of 50% green tea extract. The study concluded that the longer the time-lapse since baseline, specifically after 30 minutes, the lower the bacterial count. Nevertheless, this research lacked sample size calculation. In addition, with regards to the *in vivo* trial, multiple confounding factors could be identified such as; the baseline caries level was not considered, there was no clear indication for the use of control groups, and also the use of other topical antibacterial modalities such as chlorohexidine or fluoride mouth rinses the night before or in the early morning before the experiment was disregarded.

In contrast to the above studies, a clinical trial performed by Ooshima et al. (1994) in two stages, to assess the antibacterial activity of oolong tea polyphenols on dental biofilm formation. In this study, 35 human participants were recruited and instructed to avoid all oral hygiene measures for four days. In the first trial, the volunteers were provided with 0.5 mg/ml of oolong tea extract in 0.2% ethanol, to be used between 7-11 times daily. For the second trial which happened one week later, the same participants were provided with just 0.2% ethanol. Based on the results of this study, the oolong tea extract demonstrated significant reduction in plaque

accumulation. However, it failed to show any influences on *Streptococcus mutans* counts. This study exhibited a major limitation in the methodological approach as blinding was not implemented. Furthermore, during the experiment, there were no dietary limitations, and therefore it is assessed to be at high risk of bias.

A subsequent single-blind, crossover randomised control trial was carried out by Neturi et al. (2014), to evaluate the impact of green tea extract on bacterial counts, namely *Streptococcus mutans* using microbiological analysis testing. Thirty patients were allocated randomly to three different groups (2% green tea, 0.12% chlorohexidine or plain water). This was a cross over trial in order to receive all the three interventions, with two wash-out periods of one week each. The study indicated that *Streptococcus mutans* numbers decreased considerably with either green tea or chlorhexidine mouth rinses, whereas plain water rinse showed no differences in bacterial counts. The major limitation of this study was related to the selection process, as both inclusion and exclusion criteria were ill-defined. Additionally, the duration of the trial was short and not sufficient to draw a conclusion, as the analysis was based on a one-time exposure to each mouth rinse.

Tehrani et al. (2011) conducted a double-blind parallel randomised controlled trial, to compare the effects of polyphenolic green tea extract mouth rinse (0.5%) against sodium fluoride rinse (0.05%) on cariogenic bacteria levels (*Streptococcus mutans* and *Lactobacillus*) in school children. Sixty participants were provided with either intervention, to use as per instructions (twice daily for two weeks). The counts of the aforementioned bacteria in the saliva were determined at two points, that is at baseline and end of the trial. It has been concluded from this study that both interventions showed comparable results, as they decreased the salivary bacterial load significantly. The authors claimed that green tea mouth rinse might be superior to sodium fluoride rinse, as the former could cause lesser adverse effects. However, they failed to prove their conclusion about the side effects of sodium fluoride, as none of these effects were reported within the context of the study. The advantages of this trial compared to other trials found in the literature were: the implantation of strict and thorough inclusion criteria, also the children were given the same toothbrush, toothpaste and oral hygiene instructions. Additionally, fluoride analyses of the used mouth washes were performed using fluoride selective electrode. However, the study failed to report the losses of participants and the numbers included in the analysis at the end of the trial.

The previous findings were consistent with another double-blind randomised control trial by Tao et al. (2013), with longer follow up duration of two years. The findings of the trial demonstrated that chewing gum containing polyphenols from tea extracts resulted in significantly less DMFS scores after 2 years, when compared to chewing gum with no tea extracts.

In a recent systematic review, the effectiveness of green tea extracts in caries control was examined. Only five randomised clinical trials which met the inclusion criteria were included. Of these five trials, two assessed Streptococcus mutans and Lactobacillus counts, one study measured only Streptococcus mutans levels, another trial analysed fluoride concentration in the saliva and the last one was about the effects of green tea polyphenols on DMFS indices. The systematic review deduced that despite the fact that green tea and its derivatives have shown positive anti-cariogenic effects on caries progression, little evidence exists on the clinical effectiveness of such measures (Ahmed et al., 2017). Therefore, the authors' conclusion was indecisive.

1.3.4.2 Tea effects on de-/remineralisation of caries lesions

From the dental perspective, it has been established in the literature that tea has a protective role against dental caries, owing to its organic constituents as well as fluoride content (Hamilton-Miller, 2001; Xiao-Yong et al., 2008). However, limited evidence exists about the effects of fluoride in tea, particularly with regards to enamel demineralisation and remineralisation.

1.3.4.2.1 Studies about the anti-caries role of fluoride in tea

Simpson et al. (2001a) led combined *in vivo and in vitro* experiments to explore the interaction between fluoride from tea with salivary pellicle as well as surface enamel. For the *in vivo* section, 11 participants wore intraoral splints with human enamel blocks, and waited for three hours until the formation of the acquired pellicle was evident. Thereafter, other identical enamel slabs (obtained from the same teeth) were incorporated into the appliances on the opposite sides, in order to study the effect of salivary pellicle on fluoride retention. The participants were provided with black tea infusions along with instructions for rinse (three repetitive rinses with five minutes rest period in between), after which the slabs were analysed. The results showed that following the use of black tea mouth rinse, the average percentage of fluoride recovered in the mouth was about 65%, indicating its retention within the saliva and oral fluid. However, there were no statistically

significant differences in fluoride surface retention measurements, whether salivary pellicle was present or not. In the *in vitro* experiment, column chromatography was used to study the direct association between fluoride in tea and powdered enamel. The results demonstrated that the average percentage of fluoride retention within the powdered enamel particles was approximately 66%. Interestingly, the analysis reported that there was a strong relation between brown stains induced by tea solution and fluoride retention at the upper parts of the column.

With regards to the methodology of the previous study, if sample size determination was considered or at least if the duration of the test was prolonged, then probably the evidence would be more reliable. Additionally, the study only focused on the effects resulted from the interaction between fluoride and enamel, excluding the possibility that enamel might have been influenced by other tea components.

Another *in vivo* study by Linke and Legeros (2003), tested the anti-caries effects of black tea extract (containing 4.22 ppm F) against distilled water (with 0.035 ppm F) on caries inhibition in hamsters, consuming either low or high cariogenic food. The study reported 63.7% and 56.6% reduction in caries increments when black tea extract was used by hamsters receiving high and low cariogenic diet, respectively ($p<0.05$). As with some other reports claiming the anti-caries activity of tea (Kavanagh and Renehan, 1998; Touyz and Amsel, 2001), while the claims were based primarily on fluoride's bioavailability in tea, the effects of other components such as polyphenols or tannins were overlooked.

The results of the previous evidence varied from those obtained earlier by Yu et al. (1992), who used both *in vitro* and *in vivo* models to explore the anti-cariogenic potential of tea extract on dental caries in hamsters. Interestingly, the study found that even after the removal of fluoride from tea solutions by electrodialysis, their anti-cariogenic properties remained steady. The authors concluded that the anti-caries characteristics of green tea, were mainly attributed to green tea extracts such as polyphenols rather than fluoride. However, this article was only available as abstract form, therefore, it is difficult to evaluate the methodology of the study.

1.3.4.2.2 Studies about the de-/remineralisation effects of tea

In a different study by Yu et al. (1995), the acid resistance capacity of human enamel treated with various tea extracts (catechin, caffeine, tannin and tocopherol) plus or minus fluoride was tested *in vitro*, utilising three types of tests; namely, Vickers microhardness testing, polarising microscopy and electron micro probe analysis. The results showed that if fluoride was added to either tannin or catechin, the inhibition of calcium dissolution from enamel samples increased from 40% to 90% and 35% to 60%, respectively. These findings seem to be more logical when compared to their previous study, as the protective effect of fluoride on dental caries cannot be disregarded.

Furthermore, Abdulraheam (2011) undertook an *in vitro* study to examine the influence of different tea types on the remineralisation potential of demineralised enamel lesions. A total of 30 extracted intact human premolars were used to create enamel slabs with subsurface carious lesions. These slabs were then distributed evenly into four experimental groups, namely green tea, black tea, Ocimum basilicum and Mentha spicate. In addition, they had positive control (0.05%NaF) and negative control (deionised water) groups. Polarised light microscopy, X40, and chemical analysis of calcium, phosphorus and fluoride were used in this study to evaluate the enamel lesions following seven days of pH-cycling. Microscopically, the enamel slabs in the negative control group showed two distinctive areas of demineralisation, namely the body of the lesion and the advance zone, while the positive control NaF and green tea groups demonstrated total remineralisation. Black tea and Mentha spicate on the other hand, resulted in incomplete remineralisation patterns. The last test group, Ocimum basilicum revealed the least macroscopic changes. With regards to the ion concentration measurements, it was concluded that although the two plants, Mentha spicate and Ocimum basilicum contained more calcium and phosphorous ions than green tea and black tea, the tea groups had higher fluoride content and consequently showed greater remineralisation potential, respectively. A key problem is that although the use of pH-cycling is fully justified in *in vitro* models, the duration of the cycling in this experiment was short compared to other studies. Moreover, sample size determination and random allocation of the enamel slabs were not considered in their methodology.

In another similar *in vitro* study, the remineralisation efficacy of black and green tea on enamel demineralisation was tested against 0.05% NaF and distilled water,

utilising laser-induced fluorescence. In this study, 50 enamel slabs from 200 extracted human premolar teeth, were subjected to pH-cycling for a one-week period. The authors found a statistically significant difference in terms of enamel remineralisation capacity in green tea and sodium fluoride groups, with a mean fluorescence intensity of 414.6 ± 212.5 and 377.4 ± 198.1, respectively. Black tea and distilled water, on the other hand, showed insignificant differences at a mean intensity of 255.6 ± 54.5 and 242 ± 56.3, respectively. In their conclusion, they claimed that remineralisation was evident, though it varied in green tea, sodium fluoride and black tea groups (Babu et al., 2017). However, this research suffers from several pitfalls including lack of sample size determination, absence of randomisation and short pH-cycling. Also, it was mentioned that teeth were collected from patients following their agreement, but neither the ethical approval procedures nor the reasons for extraction of sound teeth were mentioned. Above all, the main weakness of this research was that they failed to explain tea staining effects in relation to fluorescence measurements. Moreover, the determination and standardisation of fluoride concentration in different test groups were neglected. Thus, the evidence is not conclusive.

The microhardness of demineralised human enamel treated with green tea dental toothpaste was investigated *in vitro*. Indrani et al. (2015) reported that the toothpaste containing green tea extract resulted in a remarkably higher Knoop hardness number in the enamel in comparison to the non-green tea containing toothpaste. However, when different strengths (5%, 10% or 15%) of green tea toothpaste were applied to the demineralised enamel surfaces, the results were insignificant. The key limitation in this analysis was related to the small sample size, as there were just four slabs in each group. Additionally, only the ingredients of the green tea toothpaste were mentioned, whereas the components of the alternative toothpaste with no green tea extract were not specified; not to mention, that the parameters that were implemented to create the enamel lesions were completely different than most comparative *in vitro* studies, as they immersed the slabs in 1% citric acid, for 150 seconds and only once.

Li et al. (2004) conducted an earlier *in vitro* analysis with the results deemed inconsistent with the aforementioned papers. The study mainly compared three experimental groups including tea polyphenols, tea polyphenol + fluoride, and fluoride alone against deionised water, on dental enamel slabs utilising microhardness testing. Based on their results, it was implied that polyphenols from

tea extracts exert no remineralisation action on demineralised enamel lesions. Hence, anti-cariogenic properties could only be linked to antibacterial modes of action. Surprisingly, the authors added that when tea polyphenols were combined with fluoride *in vitro*, no additive remineralisation effects were noted. It is difficult to assess this study as the article was written in Chinese, and only the abstract was in English, but their claims seem to be doubtful.

Adding to the controversy, a later *in vitro* study undertook by Rahardjo et al. (2014), to evaluate the remineralisation effects of three test groups; namely milk, milk plus tea and milk plus 0.2% NaF on demineralised enamel lesions (obtained from human premolars), by means of x-ray micro-computed tomography. The test resulted in mean grayscale values of 98.1 ± 24.0 (milk), 92.6 ± 21.4 (milk plus 0.2% NaF), 90.8 ± 19.1 (milk plus tea), and 81.1 ± 20.3 (negative control group). Therefore, the study concluded that the milk alone group had significantly enhanced the remineralisation potential of early enamel lesions, whereas when tea or sodium fluoride was added to the milk, the remineralisation effects were insignificant. In this paper the pH-cycling lasted for just three days, and also the cycling regimen deviated significantly from other well-known protocols. An additional weakness was that the calcium and fluoride concentrations in milk and tea at baseline were not measured. Moreover, the amount of tea or milk used was not specified. Further, micro-computed scanning was the only tool used to assess early enamel lesions, even in irregular surfaces such as pits and fissures. Therefore, the accuracy of the measurements in these regions were limited. In a recent *in vitro* analysis by Rovaris et al. (2018), with a large study sample, to assess the validity of micro-computed scanning in caries detection against histological examination; the authors stated that although micro-computed scanning detected the presence of caries lesions with accuracy value of more than 0.80, the test failed to assess depth of the lesions in outer enamel layers.

1.3.4.2.3 Human trials about the de-/remineralisation effects of tea

In a controlled clinical trial by Doi et al. (2013), the anti-caries activity of chewing gum made of green tea extract and calcium phosphoryl oligosaccharides (POs-Ca) on initial occlusal caries of newly erupted first permanent molars was assessed, using quantitative light-induced fluorescence (QLF). Ninety-two primary school students were assigned to either experimental (green tea extract +POs-Ca) or control (POs-Ca only) groups. The participants chewed two pieces of gum after food (once, for five minutes duration, on daily basis), under their teacher's supervision. The use of chewing gum was extended for a year, and QLF measurements were

obtained biannually. The analysis showed that chewing gum containing green tea extract +POs-Ca resulted in significant reduction in early caries lesions when compared to (POs-Ca only) chewing gum. This trial lacked some important aspects including randomisation and being a blinded study. Additionally, statistical regression models were not considered to control confounding factors such as oral hygiene practice and dietary habits. Moreover, obtaining QLF readings in younger children might present a particular challenge, especially if the first permanent molars are partially erupted. Finally, although the authors claimed that the duration of gum chewing lasted for one year, they failed to take the holidays into account.

In line with the previous study, Suyama et al. (2011) carried out a randomised double-blind crossover study, to assess the efficacy of chewing gum incorporating fluoride from green tea extract on remineralisation and acid resistance capacity of enamel subsurface lesions. In this *in situ* study, a total of 45 human subjects wore intraoral appliances with pairs of demineralised enamel blocks, prepared from intact first premolars extracted during orthodontic treatment. The study consisted of two test phases with a one-week wash out in between. The participants were randomly assigned to either the experimental group (chewing two pieces of gum contained 2.34% green tea extracts and 50 µg F) or the control group (chewing two placebo gum containing no green tea extracts or fluoride). Following cross over, during the second phase of the experiment, they received the other intervention. The lesions were evaluated utilising microradiography along with densitometry. The concentration of fluoride in saliva and enamel was measured using a fluoride selective ion electrode. The study concluded that fluoride containing chewing gum derived from green tea extract resulted in a significant increase in the mineral density and acid resistance property of enamel lesions ($p<0.05$). Unfortunately, there is considerable ambiguity about this study in terms of sample size calculation, type of randomisation and who was blinded to the interventions (patients, health care providers, or data assessors). An additional problem was related to the final analysis which excluded the damaged specimens, even after the original assignment was done. Moreover, the study failed to report any harm, especially that the participants were asked to refrain from using any other fluoride-containing products including toothpastes, during the whole study period which lasted for ten weeks.

1.3.5 Tea consumption and health risks

Consuming tea in excess, can cause detrimental effects, owing to the presence of certain compounds, namely, caffeine, aluminium and polyphenols (Cabrera et al., 2006). Additionally, tea may contain high concentrations of fluoride, thus drinking excessive amounts of tea has been linked to many dental and skeletal side effects (Waugh et al., 2016). Hayat et al. (2015) identified the following to be potential harms of excessive tea consumption:

- Gastrointestinal and hepatic adverse effects (mainly due to TPP).
- Asthma trigger.
- Risk of childhood leukaemia.
- Gestational hypertension.
- Fluorosis.
- Impaired iron absorption.
- Gastrointestinal malignancy.
- Musculoskeletal disorders.
- Neurodegenerative diseases such as Alzheimer's and Parkinson's.

Hepatotoxicity is thought to arise due to the potential of EGCG and its metabolites to instigate hepatic oxidative stress (Mazzanti et al., 2009). Moreover, when consumed excessively, unwanted drug interactions may occur which induce hepatic protein loss through diuresis (Cabrera et al., 2006).

Tea contains a wealth of metal and compounds, including arsenic, aluminium, barium, cobalt, cadmium, copper, nickel, manganese, zinc, strontium and tannins. These are toxic in large quantities. Also, tannins are responsible for reducing the absorption of non-haem iron (Fe) (Nelson and Poulter, 2004; Hayat et al., 2015).

Gardner et al. (2007) advised that the optimal amount of tea consumption to prevent cardiovascular disease was around three cups of tea daily, while one to six cups were needed to achieve anti-oxidation within tissues. However, this study centred around the concentration of polyphenols alone. Concentrations of fluoride and fluorosis risk were overlooked.

Further, the immoderate intake of tea during childhood leads to dental and skeletal fluorosis (Whyte et al., 2008; Waugh et al., 2016). Waugh et al. (2016) examined 54 commercial brands of black tea with regards to their fluoride content. The authors found that 15 brands contained (fluoride concentrations ≥1 ppm / 250 mL cup size). Hence, consuming four cups of tea may surpass the tolerable upper intake level for

fluoride, possibly causing chronic fluoride toxicity. Yi and Cao (2008) considered the risk of fluorosis associated with consuming high amounts of fluoride-rich teas, by analysing a few case reports and *in vivo* studies. They recommended that statutory authorities, e.g., governments should standardise the concentration of fluoride in different tea products.

Moreover, extrinsic dental stain/discolouration is another potential adverse dental effect caused by the integration of tea particles into the enamel crystals and the acquired dental pellicle, as a result of excessive tea drinking. While this has no irreversible effects on teeth either functionally or structurally, it has been suggested that it's cosmetic implications may cause social and psychological stress (Simpson et al., 2001b). An *in vitro* analysis by Lee et al. (2014), stated that staining was minimised significantly when black tea was mixed with milk. This was largely attributed to casein, a protein found in milk.

In summary it is thought that the potential benefits far outweigh the risks when reasonable consumption practices are adopted (Hayat et al., 2015).

1.4 Research models for cariology research

RCTs are the most robust type of scientific studies to utilise when attempting to determine a causal relationship. This is particularly true for dental caries research, since this form of study is able to determine the effectiveness of certain preventive measures or treatments on dental caries. To achieve this, RCTs should be performed properly, with concealment of allocation, using intention to treat analysis, and the application of blinding where feasible. Moreover, the disadvantages of RCTs should be noted and addressed; loss to follow-up, issues with generalisability, and factors such as expense and duration (Xuelian et al., 2016; Hariton and Locascio, 2018).

Various studies that are able to imitate the environment in the oral cavity have been implemented in caries research. These include *in vitro*, *in situ* and animal models. These models supplement RCTs, and have been adopted in both demineralisation and remineralisation studies, as well as in the close scrutiny of how the microbial biofilm affects the teeth. The models possess an array of strengths and weaknesses, ranging from experimental design to cost. (Cochrane et al., 2012a; Xuelian et al., 2016).

1.4.1 *In vitro* design

In vitro studies, where carious lesions are artificially induced in human and bovine dental enamel, are used frequently. Their strengths include a short experiment time, a need for relatively fewer personnel, the capacity to modify experimental factors easily, the ability to identify clinical outcomes precisely in a controlled manner, circumvention of participant compliance factors, as well as their comparatively lower costs (Buzalaf et al., 2010; Xuelian et al., 2016). However, these studies are limited by the fact that they are unable to accurately mimic all the biological mechanisms which occur in the oral cavity, such as bacterial penetration, collagen degradation, tubular occlusion and production of reactionary dentine (Moron et al., 2013; Xuelian et al., 2016). Nevertheless, it is worth mentioning that despite these shortcomings, *in vitro* studies are still widely applied because they allow single-variable studies to be performed under controlled conditions (White, 1995).

Consequently, in spite of improvements in *in situ* and *in vivo* models, properly conducted *in vitro* studies testing demineralisation, remineralisation and fluoridation play significant roles in dental research, where dental caries is the main focus (White,1995).

1.4.1.1 *In vitro* pH cycling

The modern pH-cycling models were first developed by ten Cate and Duijsters (1982). These models involve subjecting substrates, enamel or dentine to a combination of remineralisation and demineralisation. This mimics the physiological processes which occur in the oral cavity, resulting in alterations of pH and mineral situation states. One pH-cycling model involves applying a topical agent to healthy enamel, and subsequently exposing the enamel to an acidic solution in order to measure rates of demineralisation (White, 1995). In other models, demineralised lesions are first created by exposing substrates to acidic gels (e.g. acetate or lactate), or even undersaturated solutions with respect to hydroxyapatite at pH values between 4.4 and 5.0, for any time period between 16h to 28 days. These specific protocols cause distinct enamel lesions to be formed, namely surface-softened lesions (erosion-like lesions), and subsurface lesions (caries-like lesions) (White, 1987; Buzalaf et al., 2010). The benefit of *in vitro* pH-cycling models is they require a smaller sample size and, as previously stated, they can mimic conditions

in the oral cavity, thus achieving reduced variability which is inherent in regular *in vitro* designs (Buzalaf et al., 2010).

Further, researchers have developed models which integrate microsystems, such as *Streptococcus mutans*, within *in vitro* studies (*in vitro* plaque biofilms), and consequently artificially cause caries lesions in order to monitor and evaluate de-/remineralisation (McBain, 2009). Marquezan et al. (2009) concluded that pH-cycling is more effective at mimicking a carious dentine layer, post-caries removal. Whereas the biofilm method is better suited at simulating an infected carious lesion, pre-caries removal.

Stookey (2011) conducted a cross-validation study to evaluate the Featherstone pH-cycling model. Carried out in three independent laboratories, the study concluded that the model was sufficiently robust to be able to differentiate between toothpastes containing different fluoride concentrations.

1.4.1.2 Substrates used for *in vitro* pH cycling

It has been stated that human and bovine dentine are markedly different morphologically and structurally, since bovine dentine contains less tubules per square millimetre (Lopes et al., 2009; Hollanders et al., 2018). Therefore, it is more radio-dense than human dentine (Fonseca et al., 2008). However, the 1994 Clinical Aspects of De-/remineralisation of Teeth Conference, reported that bovine or human enamel were similar and worked best as a substrate for caries investigation (ten Cate and Mundorff-Shrestha, 1995). Bovine enamel is used more frequently due to the lack of human enamel (Tanaka et al, 2008; Laurance-Young et al., 2011). In spite of the fact that bovine enamel differs to that of human in terms of chemical composition (less variable), lower fluoride levels, higher porosity and more rapid lesion progression, it is still an effective alternative to human enamel (Mellberg and Loertscher,1974; Featherstone and Mellberg 1981). Nevertheless, care must be taken when translating results in clinical scenarios (Clasen and Øgaard, 1999).

1.4.1.3 Special factors to consider in pH cycling

When the model is intended to induce caries-like lesions as opposed to erosion-like lesions, it is essential to produce a less demineralised surface layer, by controlling the following factors (Lynch et al., 2007, Buzalaf et al., 2010):

- Mineral levels (calcium, phosphate, fluoride).

- pH.
- Time after initial demineralisation.
- Agitation of the surface layer (less is favourable).
- The degree of porosity.
- Lesion depth.

1.4.2 *In vivo* animal models

Historically, a variety of species have been used in animal caries models, including primates, rats, hamsters and mice, rodents typically being used more commonly (Xuelian et al., 2016). Important considerations such as sufficient animals, animal age, litter source and diet should be taken into account in order to achieve a robust study (Bowen, 2013). Compared with *in vitro* studies, animal models are more robust experimental-caries models. Besides natural saliva, host defences come into play, and the clearance of testing agents occurs per normal physiological processes (Marsh, 1995). On the other hand, animal models are limited by variations in the oral microbiota, plaque formation, salivary composition, structure of the teeth, dental caries patterns, as well as the size of the dentition and oral cavity between animals and humans. Additionally, availability of animals, expense and time are shortcomings inherent to most studies (Stookey et al., 1995; Featherstone, 1996; Xuelian et al., 2016).

1.4.3 *In vivo* experiments in humans

These models are laborious and costly. However, when performed correctly, an *in vivo* model is the most effective model at replicating the conditions in the human oral cavity. The following factors should be addressed with care (Featherstone, 1996):

- Subject selection and numbering.
- Ethics.
- Compliance.
- Control of fluoride use.
- Diet.
- Test duration.

- Methodology.
- Outcome measurement and result interpretation.

1.4.4 In situ Models in humans

These involve blocks or slices of human/bovine enamel/dentine being placed in the oral cavities of human volunteers in special appliances, and leaving them for pre-determined periods of time to evaluate the degrees of de-/remineralisation (Featherstone and Zero, 1992). Hence, volunteers' teeth should not be damaged while simultaneously simulating the natural processes of de-/remineralisation which occur in the human oral cavity (Zero, 1995). The disadvantages of *in situ* models are the expense, time and complicated methodological considerations, such as subject and substrate selection, compliance and sample size (Zero, 1995). These models are designed to preceed human clinical trials and serve as a predictor of the efficacy of the test/treatment in question (Featherstone, 1996).

1.5 Common techniques to evaluate de-/remineralisation

- Quantitative Light-induced Fluorescence (QLF).
- Microhardness.
- Transverse microradiography (TMR).
- Microcomputed tomography (Micro-CT).

1.5.1 Quantitative Light-induced Fluorescence (QLF)

QLF is a highly sensitive, non-destructive, tool which enables the detection and evaluation of caries in smooth surfaces at an early stage of development, both quantitatively and longitudinally, whereas previous methods could only detect the absence, presence and lesion type. It relies on the fact that teeth are naturally fluorescent; areas of high mineralisation are brighter than areas of low mineralisation. This is achieved by making use of the visible light spectrum at certain wavelengths (peak intensity k 370 nm, band pass filter at k 520 nm). The actual process involves capturing an image of the lesion and analysing it using specialised software (Inspektor Research Systems BV, Amsterdam, The Netherlands) (de Josselin et al., 1995; Tranaeus et al., 2001; Pretty et al., 2002; Tranaeus et al., 2002).

This latest version calculates three quantitative parameters (Tranaeus et al., 2001):

- ΔF (Percentage fluorescence loss, in %).
- The Area of the lesion expressed in pixels2 (px2).
- ΔQ (ΔF times the Area, in %).

The robustness of this method has been evaluated to ensure its validity. One such evaluation by Al-Khateeb et al. (1997a), compared both laser-based and light-based techniques with TMR and atomic-absorption spectroscopy. The *in vitro* study reported a highly significant positive association between fluorescence loss and integrated mineral loss by TMR ($p<0.001$, correlation coefficient $r = 0.79$ and 0.84, for the laser-based technique and light-based technique, respectively). Calcium loss was also measured and found to be linked with fluorescence loss ($r = 0.74$). The light-based system was able to detect and evaluate lesions up to 500μm.

Cochrane et al. (2012b) examined the ability of QLF and digital photography (DP) in relation to TMR, to evaluate remineralisation in subsurface lesions. This study determined that both methods yielded similar results to TMR, with a moderate statistically significant positive correlation. Furthermore, the authors concluded that QLF was superior to DP due to the absence of glare in QLF images, advanced quantification software and low variability. The previous results concurred with earlier *in vitro* (Emami et al., 1996) and *in situ* (Al-Khateeb et al., 1997b) experiments. Additionally, the ability of QLF to be repeated and reproduced *in vivo* was examined in the image-capturing phase and the computer quantification analysis phase, with the results showing a high correlation in both phases (the intra-class correlation coefficient (ICC) ranged between 0.93 and 0.99 for intra- and inter-examiner reliability) (Tranaeus et al., 2002).

Like any method, there are factors which limit the robustness of QLF. However, the drawbacks of using QLF as a mineralisation evaluation tool can be simply addressed:

- Plaques, calculus and staining may lead to inaccurate results; tooth cleaning must be carried out (Tranaeus et al., 2001).
- Tooth tissue hydration necessitates using compressed air for 15 seconds prior to imaging (Al-Khateeb et al., 2001; Pretty et al., 2004).
- Ambient light images should be taken under partial-blackout or, failing that, monitoring ambient light levels to ensure they don’t rise above 88 lux (Pretty et al., 2002).

1.5.2 Microhardness

Microhardness measuring records the ability of a substrate to withstand penetration by an indenter (Buzalaf et al., 2010). By measuring surface microhardness or cross-sectional microhardness, an indirect evaluation of de-/remineralisation can be reached. Higher indentation length indicates low mineralisation, whereas lower indentation length represents high mineralisation (Arends and ten Bosch, 1992). Using this technique is disadvantageous in that the surfaces being tested are unnaturally shaped/polished, which does not provide an accurate picture for clinical correlation (Clasen and Øgaard, 1999). Furthermore, the measurement of the integrated demineralisation may be imprecise because the extent of indentation in tissues with mineral loss only permits cross-sectional hardness to be measured at no less than 20 μm distance between two consecutive indentations. Additionally, indentation size is dependent on the organic and water content of tissues, so variation of these might influence results (Buzalaf et al., 2010).

1.5.3 Transverse microradiography (TMR)

Transverse (or contact) microradiography involves slicing the sample to be examined into thin strips (enamel 90 μm, dentine 200 μm). The samples are then irradiated using monochromatic X-rays on a strip of film which are subsequently examined for the degree of X-ray absorption, producing a value for densitometry. Examiners are able to measure lesion depth (Ld) and mineral loss (ΔZ), using Angmar's formula, densitometry is used to calculate the mineral content, expressed in percent volume. Ld and ΔZ fall in remineralisation and increase in demineralisation (Arends and Ten Bosch, 1992).

The drawbacks of TMR include:

- Destruction of samples by cutting
- Changes less than 10μm cannot be measured due to limited densitometer slit width and specimen curvature
- Results may be compromised if the enamel surface contains a disproportionate percentage of ions with a high absorption coefficient, which may be misjudged as remineralisation (Arends and Ten Bosch, 1992).

Despite this, TMR remains the gold standard for measuring mineral uptake/loss, as well as other dynamic changes (White et al., 1992; Fowler et al., 2018).

1.5.4 Microcomputed tomography (Micro-CT)

This is a 3D method which has applications in caries research, including quantifying mineral distribution, as well as de-/remineralisation (Delbem et al., 2009; Park et al., 2011). It allows for accurate measurements of attenuation coefficients, as well as possessing a high sensitivity to dynamic mineral changes over time. Thus, longitudinal evaluation of sound, along with extensively demineralised enamel is possible. In addition, analysis of fluoride, calcium and phosphate content is attainable (Buzalaf et al., 2010). Moreover, this technique permits the use of finer sample slices (Erpaçal et al., 2019). However, this method is demanding in terms of time, expense and expertise. It is also poorly applicable to clinical situations because of the high radiation involved (Mirfendereski and Peters, 2012).

1.6 Research Aims, Objectives, and Hypothesis

1.6.1 Aim

To investigate the preventive effects of black and green tea on enamel de-/remineralisation under cariogenic challenge *in vitro*.

1.6.2 Objectives

1. To assess the remineralising efficacy of black tea; green tea; black tea plus milk, on artificial enamel caries-like lesions under cariogenic challenge *in vitro*, using QLF analysis.
2. To compare the remineralising efficacy of black tea; green tea; black tea plus milk against non-fluoridated (0 ppm F) and fluoridated (5.0 ppm F) water, on artificial enamel caries-like lesions under cariogenic challenge *in vitro,* using QLF analysis.
3. To determine the amount of tea in tea bags, concentration of fluoride, temperature, pH value and titratable acidity of black tea, green tea and black tea plus milk infusions.

1.6.3 Null hypothesis

There is no difference in the effects of black tea, green tea, black tea plus milk, non-fluoridated (0 ppm F) and fluoridated (5.0 ppm F) water, on de-/remineralisation of artificially induced enamel caries-like lesions *in vitro.*

2 MATERIALS AND METHODS

2.1 Synopsis

This *in vitro* investigation was conducted in two stages. The primary stage was designed to investigate the effects of different tea groups on progression/regression of early enamel lesions *in vitro*, using QLF technology. However, one obstacle was encountered in this experiment, the gradual build-up of extrinsic stains on enamel lesions during the pH-cycling, which affected the QLF ability to detect the mineral loss/gain in these lesions. This was overcome by performing several pilot experiments, to explore different methods of stain removal. Finally, the most effective technique, with the least effect on QLF readings was adopted. In the second stage which occurred soon after the completion of the pH-cycling experiment, the original enamel slabs from all experimental and control groups were analysed to obtain QLF readings before being treated equally with the selected stain removal process. Lastly, all the slabs were re-analysed by QLF to obtain the final results.

2.2 Power calculation

Before initiating the experiment, a literature search and a statistical consultation were undertaken to define the sample size. However, due to the lack of similar reliable *in vitro* studies, the sample size was calculated based on the central limit theorem. This theory implies that an adequately large sample (≥ 30), would provide accurate inferences about the population (Browne, 1995; Kwak and Kim, 2017). Hence, a total of 30 enamel slabs were included within each group. Additionally, 25 extra enamel slabs were prepared to compensate for any loss of or damage to the slabs.

2.3 Experimental and control groups

A total of 150 enamel samples were allocated randomly to five different treatment groups (N=30 per group), three times daily for four weeks:

- Fluoride-free water (0 ppm F), negative control.
- Fluoridated water (5.0 ppm F), positive control.
- Black tea (Tetley Decaffeinated ≈ 5.0 ppm F).
- Green tea (Tetley Green Tea Decaffeinated ≈ 5.0 ppm F).

- Black tea plus milk (Tetley Decaffeinated ≈ 5.0 ppm F plus 10 ml semi-skimmed milk).

2.4 Tea bag selection

The types of black and green tea bags were selected based on a previous MSc thesis *"Amount of fluoride in various tea infusions under different influencing preparation factors"* (Xiarchou, August 2016). In Xiarchou's study, fluoride concentrations in a wide range of tea products, commonly available in the UK markets, were determined. Based on the results, the maximum fluoride level in black tea was up to 12.3 ppm, whereas, for green tea the maximum fluoride level was 6.00 ppm. It was also observed that the fluoride levels in both black tea (Tetley Decaffeinated Original Tea) and green tea (Tetley Green Tea Decaffeinated), brewed for a period of 3 minutes, were similar (≈ 5.0 ppm F) (Table 2-1 and 2-2).

Table 2-1: Black tea bags with different fluoride concentrations (ppm), at different brewing times, using continuous infusion (Xiarchou, 2016, p.39).

Time	2 mins	3 mins	5 mins	10 mins	15 mins	30 mins
Brand name						
Tetley Original Tea	4.82	5.35	5.76	5.83	5.89	5.85
Tetley Decaf Original Tea	4.17	4.98	5.23	5.64	5.74	6.12
Twinings English Breakfast	6.18	6.03	6.93	6.95	7.24	7.56
Twining's English Breakfast Decaffeinated	8.60	12.3	11.17	11.40	11.87	12.03
Yorkshire Tea	3.68	3.59	4.35	4.60	4.82	5.12
Yorkshire Tea Decaf	3.77	4.11	4.51	5.54	5.61	5.88
Mean fluoride (ppm)	5.20	6.06	6.33	6.66	6.86	7.09
SD±	1.90	3.18	2.55	2.44	2.58	2.55

Table 2-2: Green tea bags with different fluoride levels (ppm), at different brewing times, using continuous infusion (Xiarchou, 2016, p.40).

Time	2 mins	3 mins	5 mins	10 mins	15 mins	30 mins
Brand name						
Clipper Pure Green Tea	2.79	3.03	3.38	3.45	3.79	3.96
Clipper Decaf Green Tea	1.75	2.23	3.46	3.81	3.92	4.41
Tetley Pure Green Tea	3.03	3.45	3.52	4.03	4.34	4.64
Tetley Green Tea Decaf	3.97	4.98	5.16	5.57	5.65	6.00
Twinings Pure Green Tea	2.00	3.43	3.98	4.12	4.34	4.77
Twinings Decaffeinated Green Tea	2.62	2.79	3.11	3.83	3.92	4.08
Mean fluoride (ppm)	2.69	3.32	3.77	4.14	4.33	4.64
SD±	0.79	0.93	0.74	0.74	0.69	0.73

Further, since previous work has failed to identify whether the anticariogenic activity of tea is attributed purely to its fluoride content or other bioactive ingredients, the decision was to standardise the concentration of fluoride in the tea infusions. Hence, the former tea products, namely, Tetley Decaffeinated Original and Tetley Green Tea Decaffeinated were selected and purchased online (Figure 2-1).

Figure 2-1: Tetley Decaf Original and Tetley Green Tea Decaf – Tesco online groceries.

2.5 Collection of other tea supplies

To prepare the experimental infusions, other products were required:

- Fresh bovine milk (semi-skimmed milk, 1.7%-2.0% fat) was collected daily from a local shop.
- De-ionised water was obtained, using laboratory Water Purelab Option-S system.
- Disposable paper cups (12oz) were provided through an online purchase (Figure 2-2).

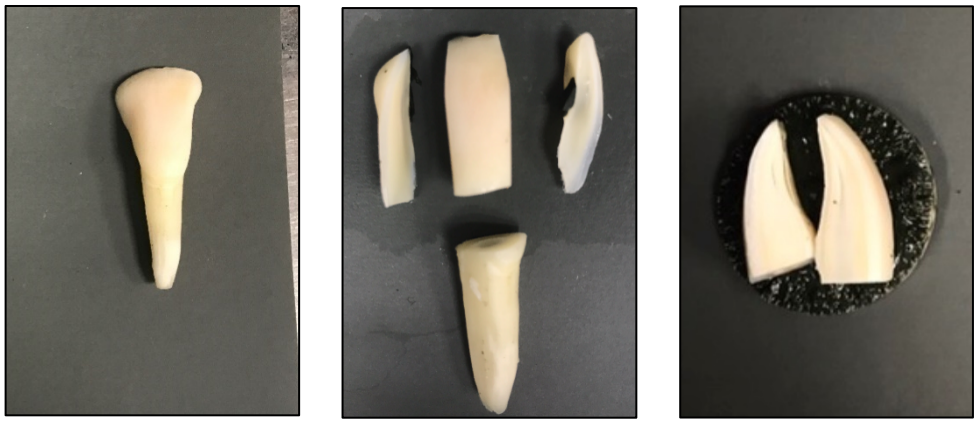
Figure 2-2: Disposable paper cup (12oz) – Amazon online shopping.

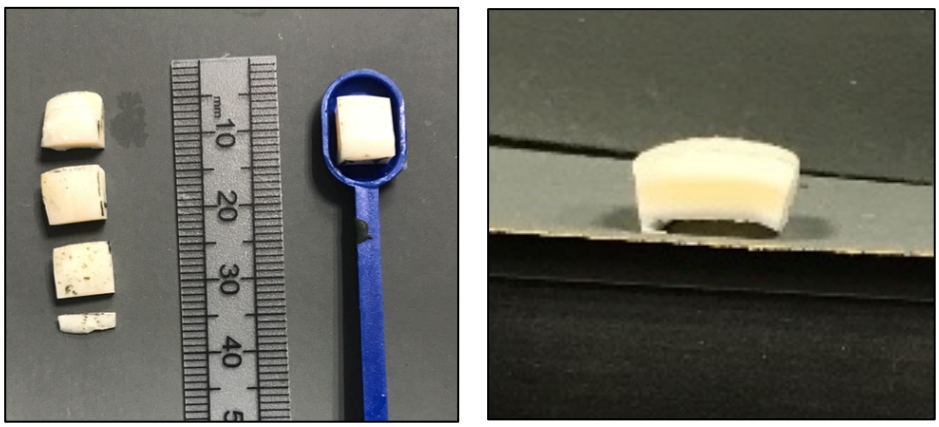
2.6 Preparation of enamel slabs

The enamel samples in this analysis were prepared from bovine incisors. After obtaining clearance from the Food Standards Agency with regards to bovine teeth collection, the teeth were collected from an abattoir and were kept almost immediately in distilled water and 0.1% thymol (Sigma Aldrich), at room temperature. Prior to teeth sectioning, a spoon excavator and a toothbrush with pumice were used to clean the teeth from any debris or soft tissues. Following that, all the teeth were examined thoroughly by trans-illumination and transmitted light utilising low-power microscopy (Leitz, Wetzlar®, Germany), in order to exclude teeth with malformations, such as cracks, caries, etc.

Subsequently, a ‘green stick’ impression compound (Kerr, UK) was used to mount each tooth separately on a different plate. Next, crown sectioning, by means of water cooled, diamond wire saw, cutting machine (Well@Walter EBNER, CH-2400 Le Loche) was performed. Depending on the size of the crown, each buccal surface was cut to obtain 2 to 3 enamel slabs, of about 6 x 5 x 3 mm each (Figure 2-3).

Figure 2-3: Teeth cutting using diamond wire saw machine (Well® Walter EBNER, CH-2400 Le Loche). Notice the final size and the thickness of the enamel slab.

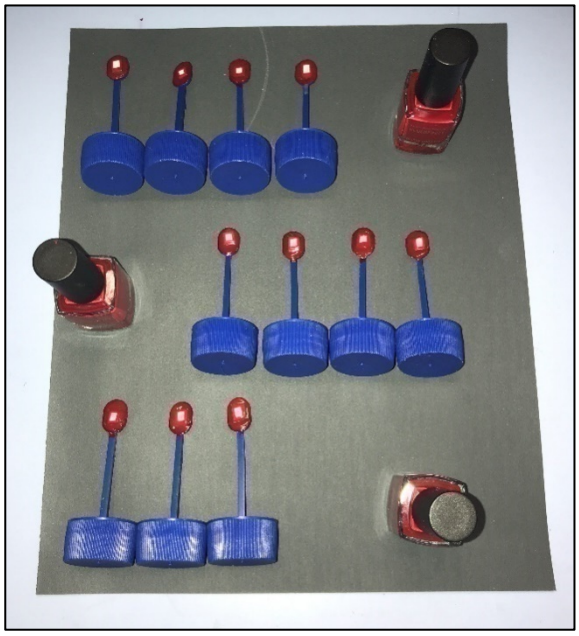




Then, Sticky Wax was used to attach each slab to the plastic rod of the universal tube (Sterilin-type), in order to keep the enamel slab centered within the tube during its immersion in the demineralising acid gel.

The enamel slabs were then coated with two layers of an acid resistant red nail polish (Max Factor “Glossfinity”), with the exception of a small window (≈ 2 x 3 mm) in the middle of each slab which was left unpainted (Figure 2-4). Between the first and the second coats of nail varnish, a period of 24 hours was allowed in order to enable complete drying of the varnish. Thereafter, to avoid enamel dehydration, the slabs were stored at room temperature in tubes containing de-ionised water.

Figure 2-4: Enamel slabs coated with an acid resistant nail varnish around the margins, leaving the centre area unpainted.



2.7 Preparation of enamel subsurface caries-like lesions

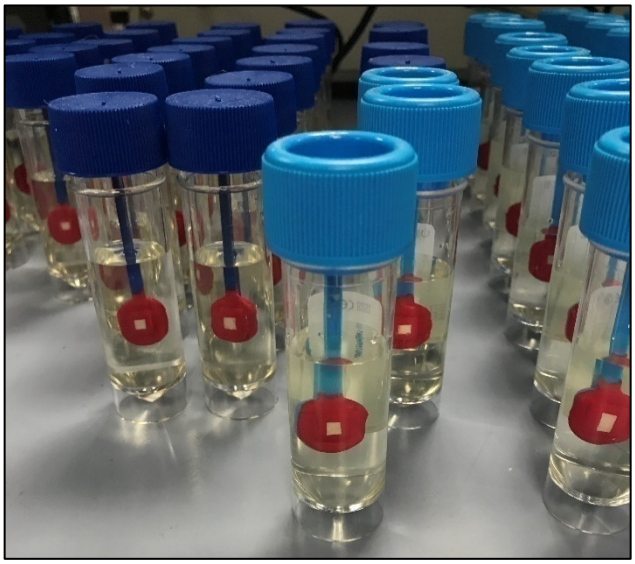
An acidified hydroxyethyl cellulose gel, acid demineralising system, was prepared to create enamel subsurface lesions. This was achieved by the addition of 0.1 M sodium hydroxide (BDH Analar Grade) to 0.1 M lactic acid (Sigma Aldrich D/L GPR 87% Lactic acid) until a pH value of 4.5 was produced. Next, the solution was mixed thoroughly with 6% w/v hydroxyethyl cellulose (Sigma Aldrich) for 60 minutes, until an adequate thickness was achieved, the so called “wallpaper paste” consistency.

At that point, the gel remained for 24 hours to settle down, until it was ready to be added to the universal tubes (Figure 2-5).

Figure 2-5: Demineralising gel (neither thick nor runny).

Then, the enamel slabs were soaked into the acid gel for a period of 10 days, until artificial enamel subsurface lesions were developed (Figure 2-6). Thereafter, the slabs were detached from the gel and rinsed thoroughly with distilled water. Prior to QLF analysis, methanol was used to remove the nail polish, in order to obtain the baseline measures.

Figure 2-6: Enamel slabs immersed in acid gel.



2.8 Quantitative light-induced fluorescence (QLF) method

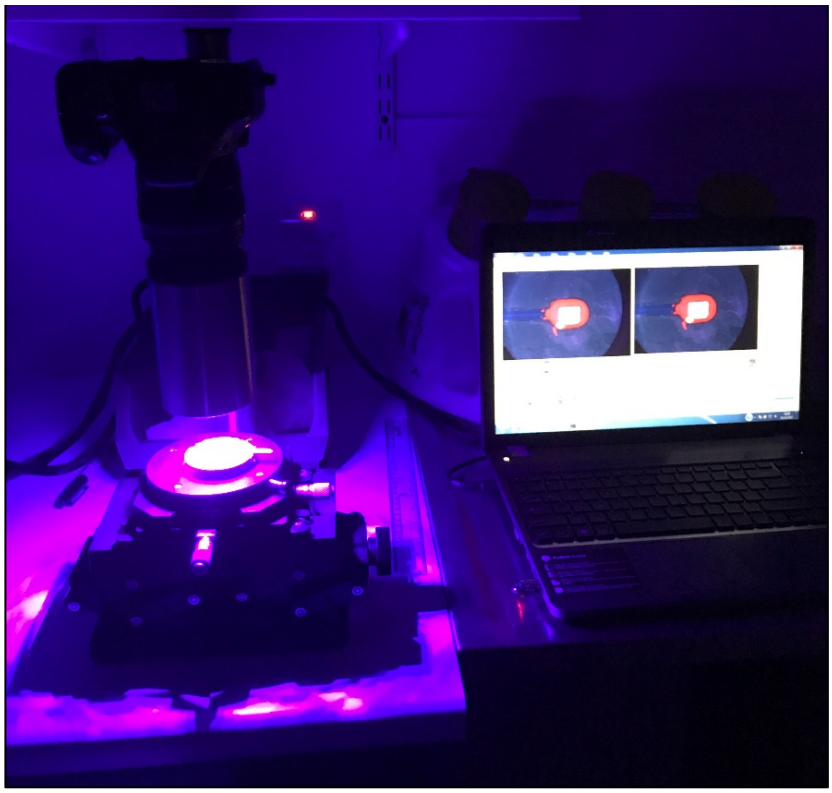
For each enamel slab, the QLF machine (QLF-D Biluminator™ 2 Inspektor Research Systems BV, Amsterdam, The Netherlands), was used to obtain three readings, at three different time points:

- At baseline; after the formation of enamel subsurface lesions.
- Before stain removal; after 28 days of pH-cycling.
- After treatment (at end result); and after implementing the stain removal method.

QLF-D Biluminator™ 2 consists of a Biluminator™, mounted on a Single Lens Reflex (SLR) camera and fitted with a 60 mm macro lens. The Biluminator™ delivers white-light and the filters allow production of QLF™-pictures. Image acquisition of all enamel slabs was performed with a 'Live View'-enabled digital full-sensor SLR camera (model 550D, Canon, Tokyo, Japan) at custom settings: shutter speed of 1/30 s, aperture value of 6.7, and ISO speed of 1600. A personal computer with image-capturing software (C3 version 1.16; Inspektor Research Systems), was used directly to store all the digital pictures. Then, a special analysing software (QA2 version 1.16; Inspektor Research Systems), was utilised to examine the fluorescence images. Capturing of the images and the analyses were carried out by a single trained examiner (SA).

Furthermore, to ensure that the results obtained were reliable, the image acquisition and analysis were performed under standardised environmental conditions. More specifically, prior to imaging with QLF machine, the slabs were dried with compressed air for 15 seconds, then the test was performed in a dark room, under controlled conditions. Also, for all the images, the same camera positions and angles were used. This was possible, as the camera was stabilised by a stand at a recorded level, which also permitted optimum illumination of the enamel surfaces, at all times. Additionally, the camera specimen distance was standardised using the jig thereby controlling specimen stability light intensity and magnification (Figure 2-7).

Figure 2-7: Image acquisition and analysis were always performed in a dark room, with a camera stabilised by a stand at a certain level from an enamel slab.



The investigator outlined the white spot lesion area and drew the boundaries on sound enamel. The fluorescence levels of sound enamel tissue were reconstructed by using the fluorescence radiance of the surrounding sound enamel. Then, the percentage difference between the reconstructed and the original fluorescence levels were measured. More importantly, the same area of interest was used for the baseline and endpoint measurements.

Demineralised areas were manifested as dark spots. The fluorescent radiance of a white spot lesion viewed by QLF was lower than that of sound enamel. The fluorescence loss in the white spot lesion was measured as follows:

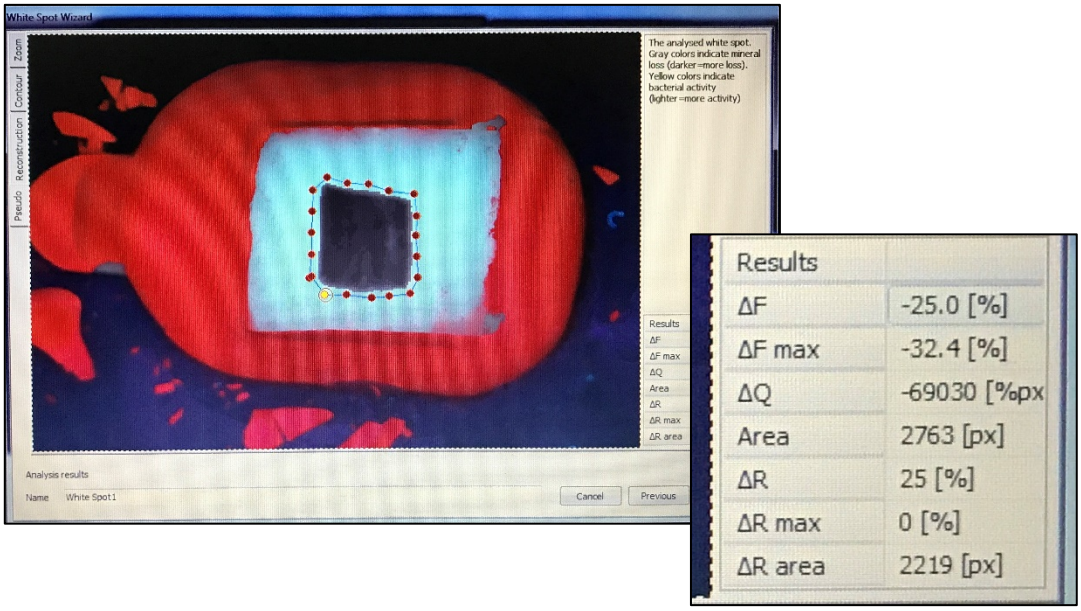
- First, the fluorescent radiance of sound tissue at the lesion site was reconstructed by interpolation from the radiance of the sound tissue surrounding the lesion.
- Then, fluorescence radiance levels less than 95% of reconstructed sound fluorescence radiance levels indicated artificial early caries lesions and were demonstrated as shades of grey where darker grey represents higher fluorescence loss.

- Finally, the difference between the measured values and the reconstructed values provided the net fluorescence loss in the lesion.

For each lesion, three measurements were calculated (Figure 2-8):

1. ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue; related to lesion depth (%).
2. ΔQ: The ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the Area. Related to lesion volume (% px2).
3. Area: The surface area of the lesion expressed in pixels2 (px2).

Figure 2-8: QLF image shows a mark drawn around the demineralised lesion (grey area), with boundaries on sound enamel (red dots). The QLF readings are displayed on the right-hand side of the picture.



2.9 The ΔF range of the enamel lesions

In this experiment, the overall ΔF baseline measurements of the artificial lesions were in the range of -7.00 and -29.00. However, the range of ΔF in which the enamel slabs were selected, was between -11.00 and -26.00; with an average value of -18.5. Any values falling outside that specified range were excluded.

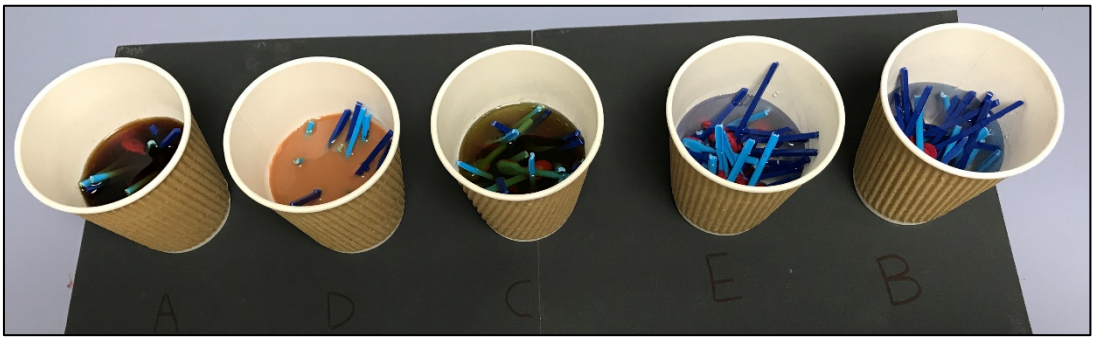
2.10 Randomisation and blindness

Randomisation was implemented, utilising a randomisation plan from http://www.randomization.com website (Appendix 1). All enamel slabs were allocated randomly to five groups. Additionally, when the QLF analysis was performed, the assessor was blinded to the study groups.

2.11 The pH-cycling protocol

In this *in vitro* study, the pH-cycling regimen lasted for 28 days. At the start of the day, distilled water was used to rinse the enamel slabs for 60 seconds. Next, the slabs were dipped in one of the solutions being tested, for 10 minutes duration. This was followed by rinsing the slabs with de-ionised water, placement of the slabs in daytime artificial saliva for 60 minutes, and subsequently immersing them in acetic acid solution for 5 minutes. The pH value of the demineralising solution was 4.8. The previous steps were repeated so that the slabs were exposed to a total of 5 acidogenic challenges each day and dipped 3 times into their corresponding test solutions (Figure 2-9). After the last dipping, the slabs were placed in night-time artificial saliva until the next day.

Figure 2-9: Enamel slabs dipped into black tea, black tea + milk, green tea, 5.0 and 0 ppm F water during pH-cycling, left-to-right.



An incubator was used to store the enamel slabs at 37˚C, except for the time when the slabs were being dipped in demineralisation or study solutions. The artificial saliva was changed once daily, whereas the acetic acid was discarded after each use.

Flow charts for the experimental design and the pH-cycling protocol are illustrated in Figure 2-10 and 2-11.

Figure 2-10: Flow chart for the experimental design (5 exposures to acetic acid, 3 times dipping in one of the study solutions for 28 days).

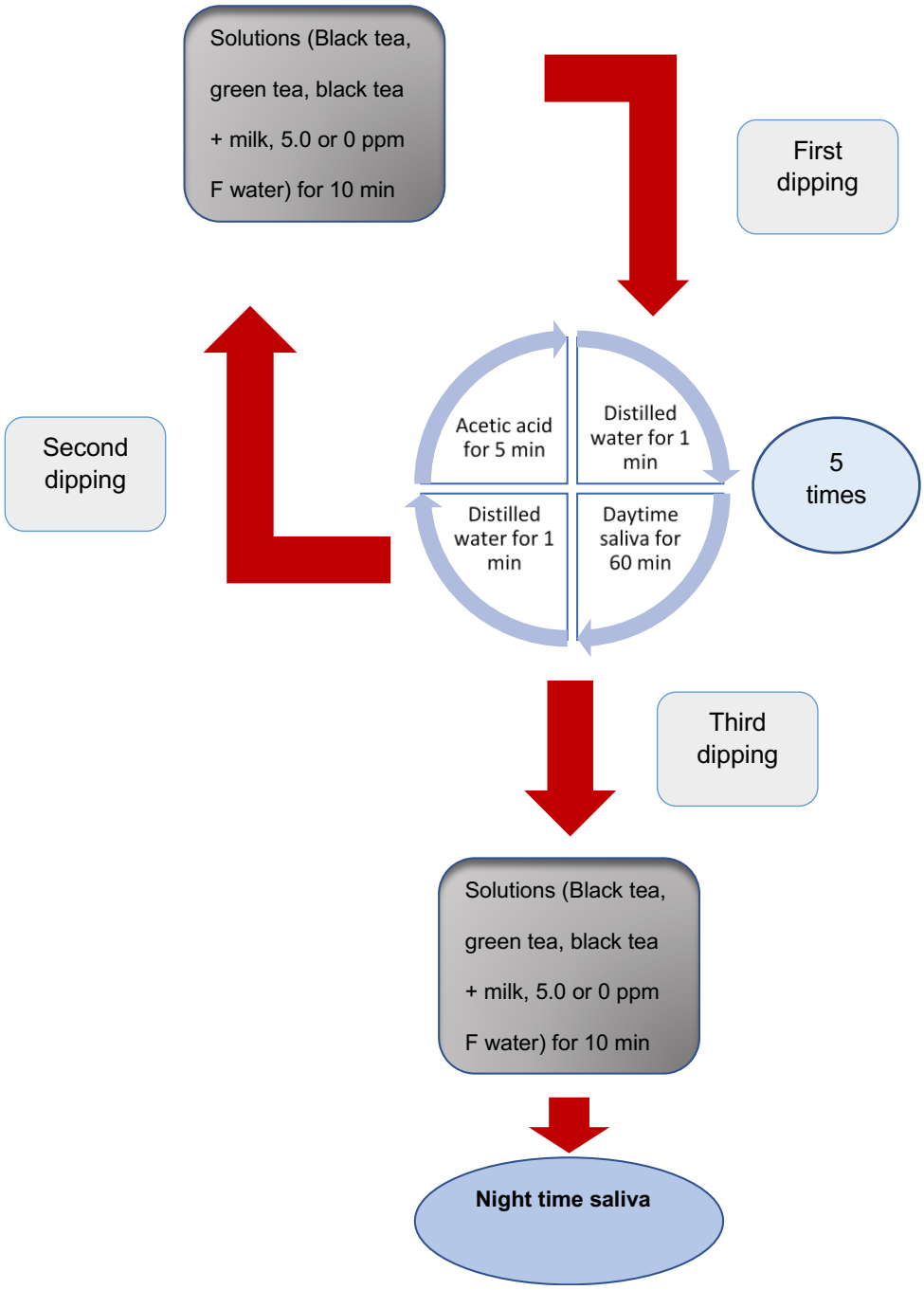
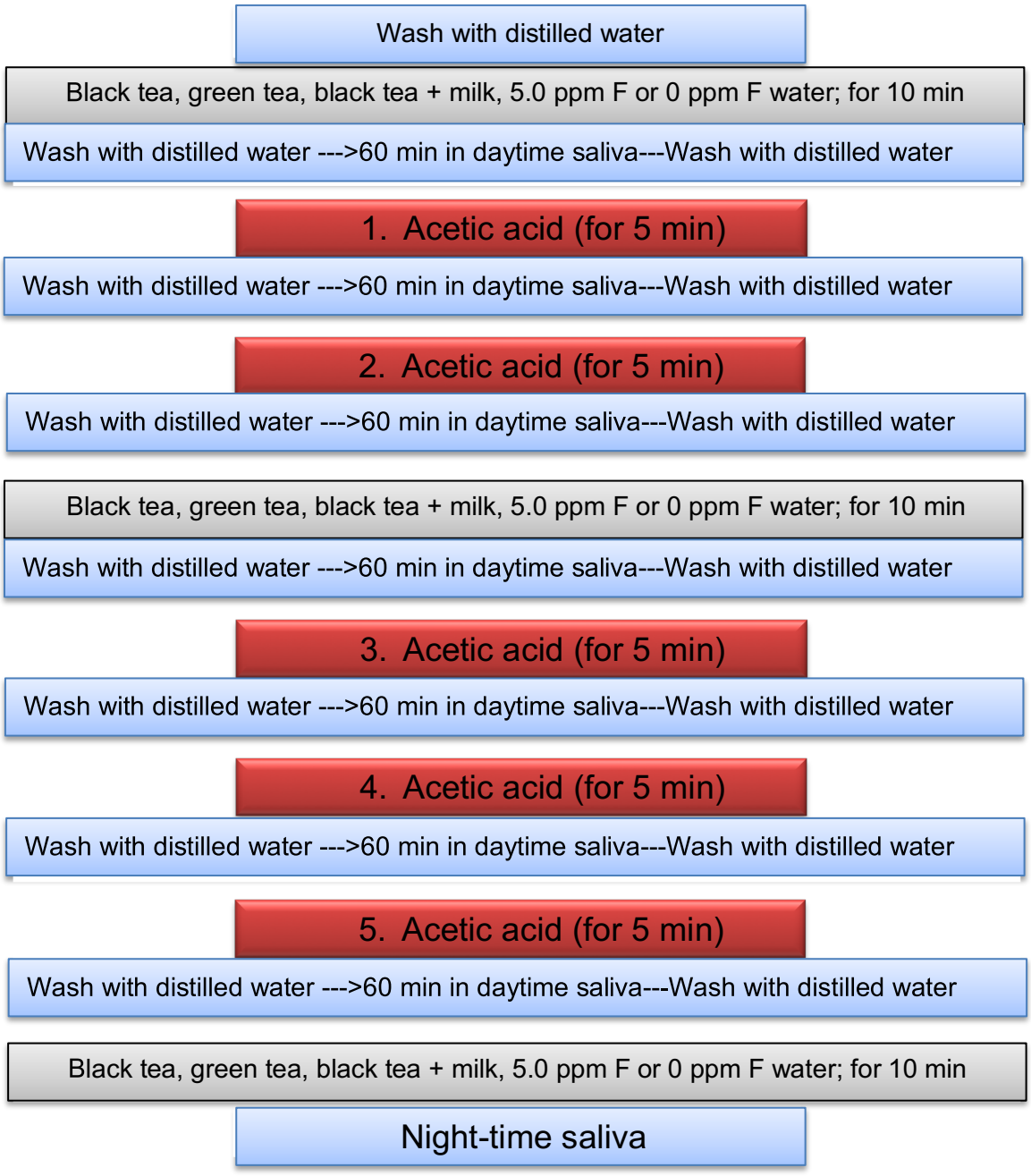


Figure 2-11: Flow chart for the pH-cycling protocol. Five study groups were tested (black tea, green tea, black tea + milk, 5.0 and 0 ppm F containing water).



2.12 Preparations of solutions used in pH-cycling

2.12.1 Artificial saliva

Preparation of artificial saliva was performed as per Dr R. P. Shellis's recommendation, Department of Oral and Dental Science, University of Bristol, Bristol, UK. The electrolyte composition of the artificial saliva mimicked the composition of the natural saliva, to minimise the formation of precipitates on the enamel surface.

During the day, the enamel slabs were immersed in daytime saliva, which was supersaturated with respect to hydroxyapatite, thus allowing remineralisation of enamel slabs; whereas at night the slabs were stored in night-time saliva. The later was a saturated solution with respect to hydroxyapatite, therefore an equilibrium between enamel demineralisation and remineralisation was maintained until the next day.

2.12.1.1 Daytime artificial saliva

The chemical composition of daytime saliva is shown in Table 2-3.

Table 2-3: Daytime saliva formulation.

Salt	Concentration g/L
Calcium carbonate	0.07
Magnesium carbonate (hydrated basic)	0.019
Potassium di-hydrogen phosphate	0.554
HEPES buffer (acid form)	4.77
Potassium chloride	2.24

A shaker was used to dissolve and mix the above chemicals in 900 ml distilled water and 1.8 ml 1 mol/L HCL. Next, KOH solution was added to the mix in order to adjust the pH value to 6.8. This was followed by the addition of de-ionised water, until a total amount of 1L saliva was produced.

2.12.1.2 Night-time artificial saliva

The chemical composition of the night-time saliva is shown in Table 2-4.

Table 2-4: Night-time saliva formulation.

Salt	Concentration g/L
Calcium carbonate	0.05
Magnesium carbonate (hydrated basic)	0.019
Potassium di-hydrogen phosphate	0.068
HEPES buffer (acid form)	4.77
Potassium chloride	2.24

A shaker was used to mix the above components in 900 ml distilled water and 1.4 ml 1 mol/L HCL. Then, KOH solution was added to adjust the pH value to 6.8. This was followed by the addition of de-ionised water up to 1L.

2.12.2 Acetic acid buffer

Acetic acid preparation was performed as per ten Cate et al. (2006). The chemical composition of the acetic acid is shown in Table 2-5.

Table 2-5: Acetic acid formulation.

Contents	Concentration g/L
Calcium chloride	1.665
Potassium di-hydrogen phosphate	1.13
Acetic acid	28.73

A laboratory centrifuge was used to mix the above substances in 1 L distilled water, until complete dissolution was achieved. Then, KOH solution was added to adjust the pH to 4.8, with the aid of a pH probe and pH meter (ORION- model 920A).

2.13 Preparation of green and black tea brews using continuous infusion method

The process was initiated by placing a new tea bag (green or black) in a new disposable paper cup. This was followed by the addition of 100 ml and 150 ml of freshly boiled de-ionised water to the green tea and black tea bags, respectively. These water measurements were based on the guide that for every 2 grams of tea, 100 ml of water is needed (Tiwari et al., 2005). Then, the infusions were stirred and allowed to brew for 3 minutes, after which the tea bags were discarded.

2.14 Preparation of black tea infusions with milk addition

First, the black tea brew was prepared using the same procedure described above, followed by the addition of 10 ml of semi-skimmed bovine milk. Then the mixture was stirred.

2.15 Preparation of 5.0 ppm fluoridated water

First, 2.21gm of sodium fluoride was dissolved in 1 litre of deionised water to produce a stock solution (1000 ppm). Using a 1:10 dilution ratio, serial dilutions were made. Hence, one part of the stock solution was mixed with 9 parts of deionised water, to make a 100 ppm F solution. This was further diluted to produce a 10 ppm F solution.

Finally, a 5.0 ppm F solution was produced by diluting 10 ppm solution with de-ionised water, using 50:50 dilution ratio.

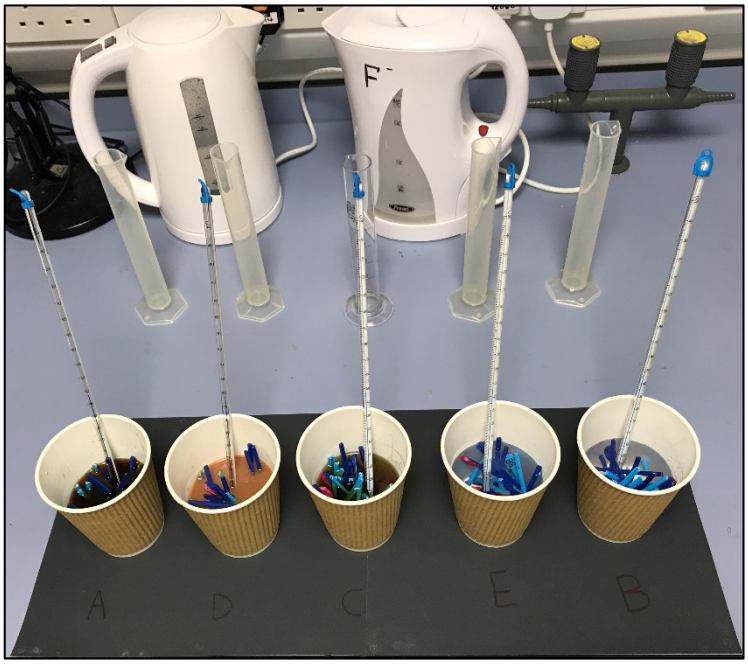
2.16 Physiochemical analyses of tea solutions

During the course of the experiment, multiple physical and chemical parameters of tea were investigated, such as temperature, pH, acid titratability, amount of tea in tea bags and fluoride concentration.

2.16.1 Temperature determination method

Boiled water was used to prepare the study solutions in all groups, including the controls. The temperature of each solution was determined at four specified time points (immediate, 3 minutes of brewing, 0 minutes and 10 minutes of immersing the enamel slabs in study solutions); using glass thermometers.

Figure 2-12: Thermometers used to measure temperature of the solutions during dipping of enamel slabs. Notice two different electric kettles were used to boil fluoride and fluoride-free water.



2.16.2 Method for measuring the amount of tea in tea bags

The amount of tea in each tea bag was measured utilising an analytical balance (A&D instruments, HM-200).

2.16.3 pH determination method

A pH electrode and Orion 920A pH meter were utilised to measure the pH levels in different tea solutions. Two standard buffers of pH 4.0 and 7.0 were used to accurately calibrate the pH apparatus.

Three samples were randomly withdrawn from each batch of teas and prepared using the continuous infusion method, as previously described within this section.

At 3 minutes time point, the tea bags were discarded, and the solutions were left to cool down; as the pH electrode could only tolerate temperatures of 85^{o}C or less.

2.16.4 Method for determination of titratable acidity of tea solutions

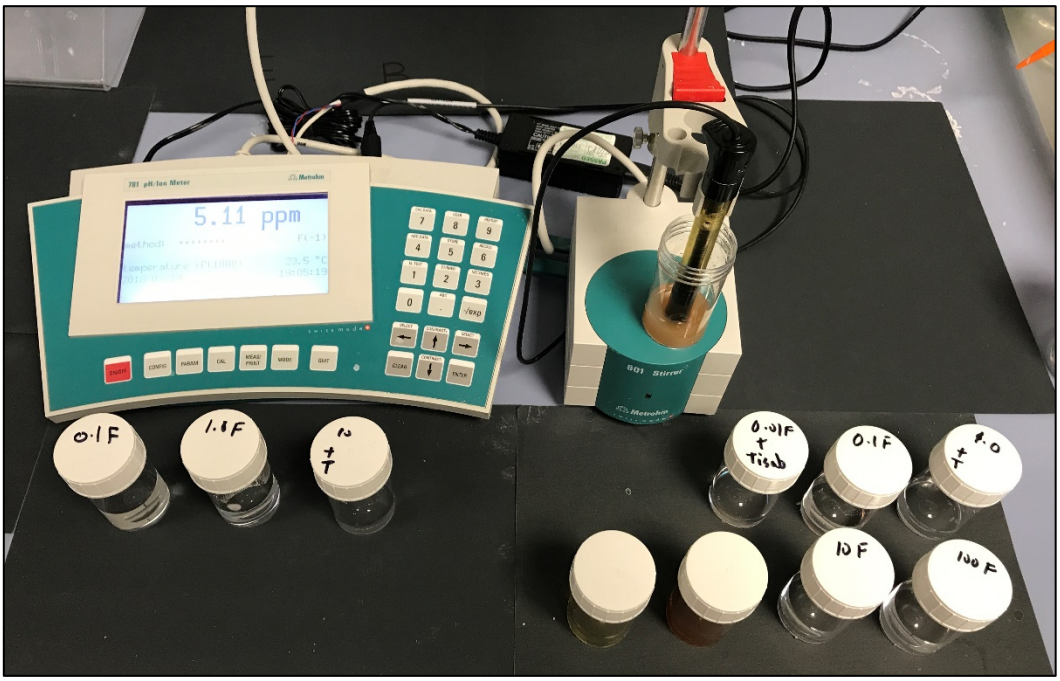
First, baseline pH values of the tea solutions were obtained. 0.01 M potassium hydroxide KOH was then added in 0.1 ml increments to titrate 20 ml of each tea solution, until pH 7.0 was reached. A magnetic stirrer was used to agitate the mixture during the addition process.

2.16.5 Method for direct determination of fluoride concentration in tea

Fluoride ion specific electrode and 781 Ion meter (Metrohm) were used to measure the fluoride content in tea samples. The measurements were obtained in triplicate, with the analyser set to (ppm) mode. Before every analysis, a series of standard fluoride solutions with various fluoride concentrations (0.1, 1.0 and 10.0 ppm F), were prepared in order to calibrate the measuring apparatus. Three samples from each tea batch were randomly selected. Preparation of tea solutions was performed using the continuous method. At 3 minutes time point, a sample of 2 ml was obtained from each brew. Each sample was then dispensed in a Sterilin tube, using Eppendorf pipettes. This was followed by adding an identical amount of Low-Level Total Ionic Strength Adjustment Buffer (Low-Level TISAB) to the tea sample. The samples were then allowed to cool down prior to the analysis (Appendix 2).

For the black tea + milk infusions, the same steps were repeated except that the sample withdrawal was performed after the addition of milk (Figure 2-13).

Figure 2-13: Fluoride analysis of black tea + milk infusion using Fluoride ion specific electrode and 781 Ion meter (Metrohm).



2.16.5.1 Preparation of Low-Level Total Ionic Strength Adjustment Buffer (TISAB)

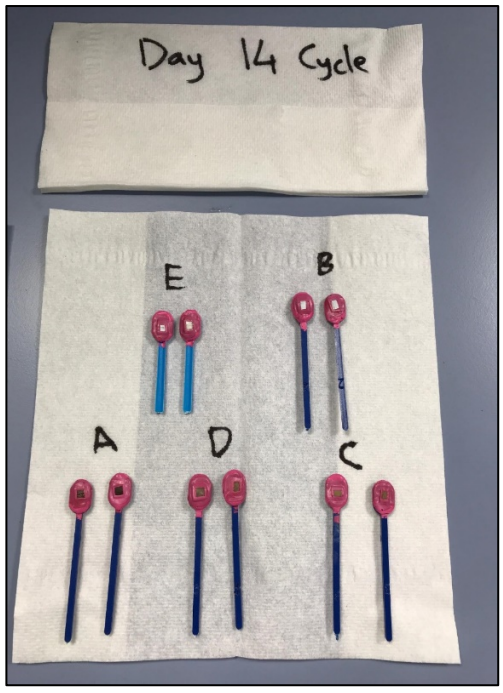
First, 500ml of distilled water was poured into a 1 litre beaker. This was followed by the addition of 57ml of glacial acetic acid and 58 grams of reagent grade sodium chloride. A water bath was used to cool the solution. After which, 5M NaOH was added gradually to the mix, while monitoring the pH simultaneously, until a value of 5.0-5.5 was achieved. A calibrated pH electrode was used for the previous purpose. The solution was left to cool down before being placed in a 1 litre volumetric flask. Finally, distilled water was added up to the flask mark.

2.17 Research obstacle

After fourteen days of conducting the pH-cycling experiment, extensive stains occurred on the enamel slabs in the tea groups (Figure 2-14), namely:

- Green stains in the green tea group.
- Light brown stains in the black tea + milk group.
- Dark brown stains in the black tea group.

Figure 2-14: Build-up of stains in A (black tea), D (black tea + milk) and C (green tea) groups, after two weeks of pH-cycling.



This raised the question of whether the QLF apparatus had the ability to determine the progression/regression of artificial enamel lesions if associated with stains. Hence, an experiment was carried out in order to further explore the issue:

- Three enamel slabs, with subsurface caries lesions, were prepared and analysed by QLF to obtain baseline readings. The slabs were then immersed in freshly prepared solutions (black tea, black tea + milk, and green tea), and kept in an incubator at 37°C, for 24 hours. The staining cycle was repeated twice, until an adequate level of discolouration was observed. Endpoint QLF measurements were obtained at the end of the dipping period.
    - Results: ΔF values of the enamel lesions deteriorated, about 9 times more than their corresponding baseline values.
    - Conclusion: QLF has limited ability to measure de-/remineralisation of early enamel lesions, if the samples are associated with stains.

Based on the above findings, the possible solutions were:

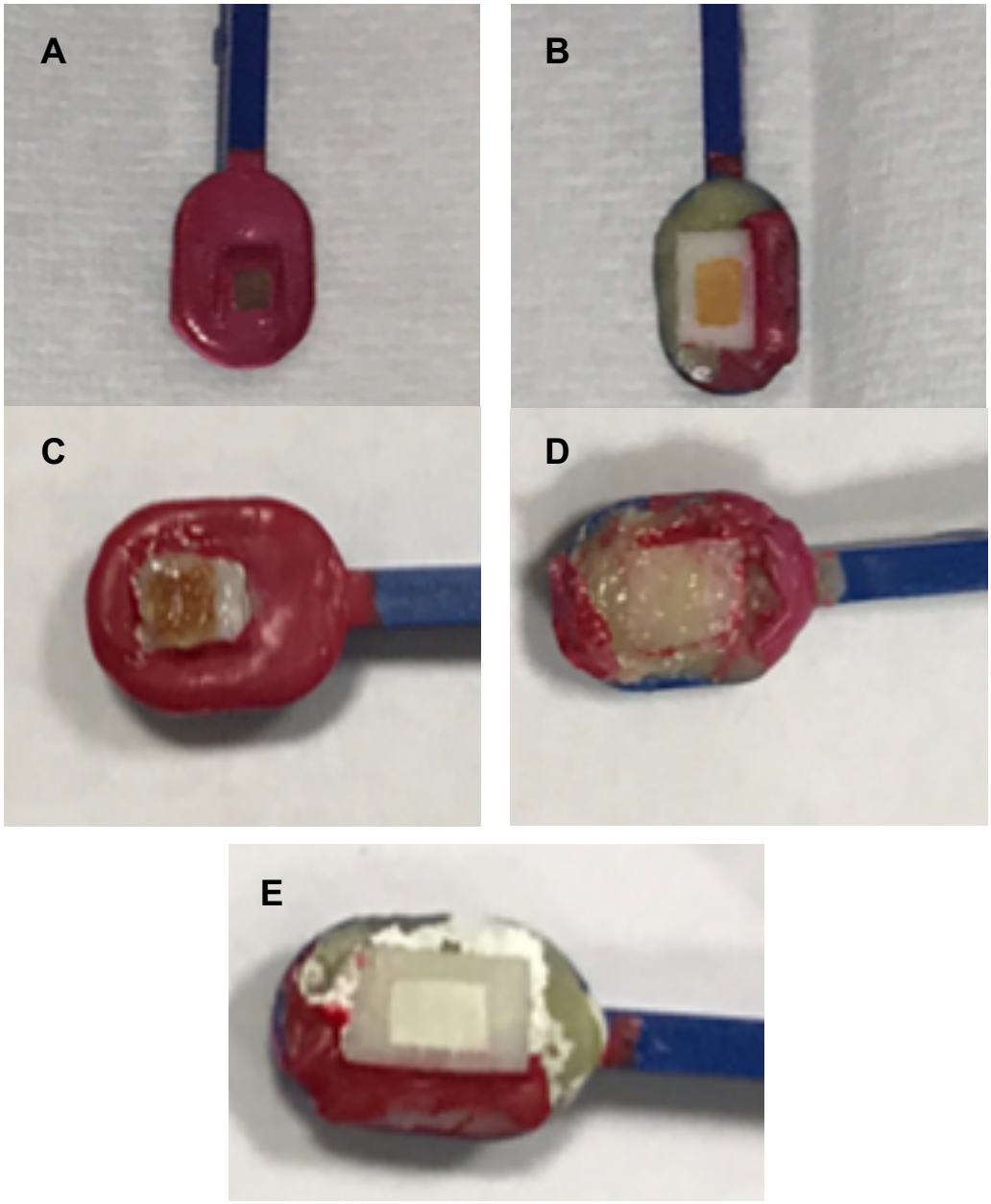
- To contact the manufacturer about the QLF issue.
- To verify whether the above-mentioned effect was related to the acidic nature of tea.
- To try different stain removal methods without altering the microstructure of enamel, in order to enable QLF to read the enamel lesions effectively.

2.18 Development of method for stain removal

First, multiple small experiments were performed to decide on the most effective stain removal technique (Figure 2-15), including:

- Bleaching with 1.0 %, 2.5% or 5.25% sodium hypochlorite solution (NaOCl).
- Bleaching with 10% carbamide peroxide (CP).
- Combined bleaching using NaOCl + CP.
- Mechanical cleaning, using soft rubber cup with extra fine pumice.

Figure 2-15 (A-E): Stain removal methods. (A) Black stains induced by black tea group; (B) residual staining on an enamel slab treated with 5.25% NaOCl for one hour, thus longer treatment duration was needed; (C) residual staining on an enamel slab treated with 10% CP (5 applications × 2 minutes); (D) complete removal of black tea stains using combined bleaching method, 5.25% NaOCl for 30 minutes followed by 10% CP (2 applications × 2 minutes); (E) elimination of black tea stains using polishing procedure for 10 seconds.

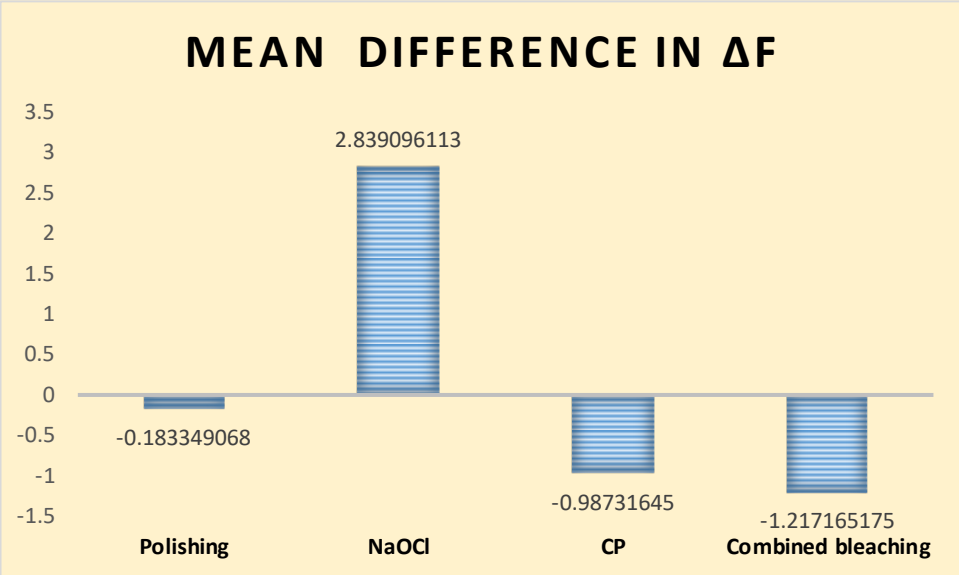


- Conclusion: Combined bleaching, using 5.25% NaOCl and 10% CP, was more effective than either bleaching alone. Additionally, the polishing procedure effectively removed tea stains.

Next, it was prudent to conduct an *in vitro* pilot study to assess the effects of these stain removal protocols on enamel structure, particularly with regards to progression/regression of artificial caries lesions:

- A total of 12 bovine enamel slabs, with subsurface caries lesions, were prepared. Baseline QLF images of enamel lesions were captured and analysed. The slabs were then randomly allocated to four treatment groups (N=3 per group), as follows: 5.25% NaOCl for 30 minutes, 10% CP (2 applications × 2 minutes), combined bleaching (NaOCl + CP) or polishing with soft rubber cup and extra fine pumice grit. At the end of the treatment, the slabs were rinsed with distilled water and cleaned gently, but thoroughly with a soft brush, in order to remove any residual particles. After which, endpoint QLF values were obtained.
    - Results: Figure 2-16 illustrates the mean difference in ΔF in the four treatment groups. In the NaOCl group, the difference was positive, indicating that there was a decrease in the mean fluorescence loss (ΔF) after treatment; whereas the difference was in the negative direction in all other groups. Additionally, the lowest changes in ΔF were seen in the polishing group (-0.183 ± 0.841), while the highest changes were observed in the NaOCl group with a mean difference of (2.840 ± 9.642). Considering the rest of QLF parameters, only minimal changes were observed in the polishing group with mean differences of ΔQ and the Area at just (-1286.323 ± 3463.872) and (-18.333 ± 15.308), respectively. On the other hand, the NaOCl group demonstrated the greatest changes in ΔQ and the Area with mean differences of (14708.7 ± 40838.35) and (-199 ± 241.758), respectively.

Figure 2-16: Means of the difference in ΔF of all treatment groups.



- Conclusion: The polishing group exhibited the least effects on artificial enamel lesions.

Therefore, on combining the results of the above pilot studies, the formal solution was to adopt the polishing technique to overcome the staining issues.

2.19 Polishing protocol

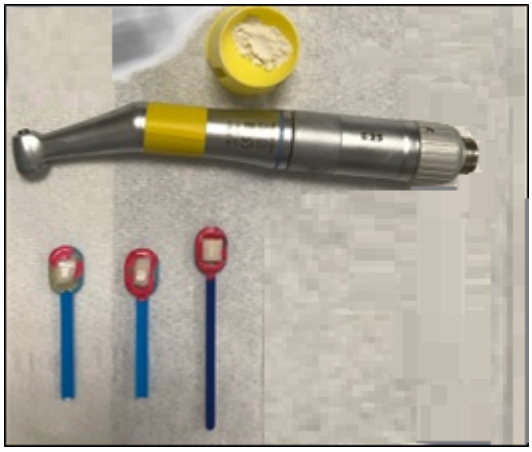
The enamel slabs in all study groups (black tea, black tea + milk, green tea, 0 ppm F and 5.00 ppm F water) were treated similarly. During the process of enamel slab polishing, a timer was set for 10 seconds. The technique was performed using a soft rubber cup, a slow-speed handpiece and extra fine (fluoride-free) pumice powder (Appendix 3).

Only minimal pressure was applied, just sufficient to keep the handpiece spinning. Additionally, the speed of the handpiece was slow and even. This was achieved by applying a controlled pressure on the foot pedal.

The dental instruments that were used in the polishing procedure can be seen in Figure 2-17 and 2-18.

Figure 2-17: Soft rubber cup latch type.

Figure 2-18: Slow-speed handpiece, soft rubber cup and extra-fine pumice grit were used to polish the enamel slabs.



2.20 Action plan outline

The action plan to overcome the staining obstacle was completed in the following sequence:

- The pH-cycling experiment was continued as planned, for its total duration.
- QLF parameters of the enamel slabs, before removal of stains, were obtained for all groups soon after the completion of the cycling experiment.
- The polishing (stain removal) procedure, was implemented in all groups.
- QLF parameters were re-measured, after stain removal, to obtain the end results.

2.21 Training and calibration

Prior to the study, the examiner received intensive guidance by the manufacturer on how to use QLF system (QLF-D Biluminator™ 2), including the software (Inspektor Research Systems BV, Amsterdam, The Netherlands). The investigator was trained and calibrated with regards to acquisition and analysis of QLF images.

Effective assessment of sound enamel, demineralised enamel and the lesion borders were demonstrated. Additionally, the investigator was able to produce comparable QLF pictures and measurements.

2.22 Intra-Examiner Reliability

The Intra-Class Correlation Coefficient (ICC) was calculated in order to assess the intra-examiner reliability. This was achieved by remeasuring the QLF parameters of 20% of the sample.

2.23 Statistical analysis

IBM SPSS Statistics 25.0 was used to perform the statistical analyses. Descriptive statistics, namely, mean, median and standard deviation, were computed to summarise the data.

Normality tests were conducted to assess whether the data were normally distributed. Both Shapiro-Wilk and Kolmogorov-Smirnov tests were used for this purpose.

When data met the assumptions of normality, a paired sample t-test was used to determine if the mean differences at baseline and after treatment were significantly different within the same group. Alternatively, a non-parametric Wilcoxon signed-rank test was carried out when the data were not normally distributed.

When compared between the five groups, based on the normality assumption, either One-way ANOVA (parametric) or Kruskal-Wallis (non-parametric) test was used. Furthermore, pairwise multiple comparison tests were performed in order to evaluate subgroup differences, along with Bonferroni correction. The latter also allowed 95% confidence intervals to be calculated, with the significance level set below 0.05.

Lastly but most importantly, additional statistical analysis was required in order to confirm if any significant relationships that existed at the end of the experiment were due to the effects of the tea solutions rather than the polishing procedure on enamel de-/remineralisation. Hence, based on the normality assumption, either paired sample t-test or Wilcoxon signed-rank test was used to check if the mean differences before and after stain removal were significantly different within each group.

3 RESULTS

3.1 Results of physiochemical analyses

3.1.1 Temperature

As was described in the methods, the temperature of each study solution was determined at four time points, using a glass thermometer. The details can be seen in Appendix 4.

Immediately after pouring the boiled water into the cups, the mean temperature was 83°C ± 2. After 3 minutes of brewing, the mean temperature reduced to 71°C ± 2. The enamel slabs were only dipped in the solutions when the temperature ranged from (50-55) °C, at a mean temperature of 53°C ± 2. At the end of the dipping period, the temperature range was between 40 and 45°C, and the mean temperature was 44°C ± 2.

3.1.2 Amount of tea in tea bags

Table 3-1 summarises the data on the mean amount of tea used in different tea solutions. The mean content of tea in the black tea bags was 3.20 ± 0.05 and 3.20 ± 0.04, for the black tea and black tea plus milk infusions, respectively. For the green tea bags, the mean content was 2.20 ± 0.04 of tea.

Table 3-1: The amount of tea (grams) in each batch.

Group	Batch 1			Batch 2			Batch 3			Mean	SD
Black tea	3.18	3.24	3.22	3.19	3.23	3.18	3.22	3.10	3.11	**3.20**	**0.05**
Black + milk	3.20	3.22	3.24	3.22	3.15	3.23	3.19	3.11	3.23	**3.20**	**0.04**
Green tea	2.17	2.10	2.12	2.20	2.15	2.10	2.21	2.19	2.14	**2.20**	**0.04**

3.1.3 pH of the tea solutions

The minimum and the maximum pH values of the black tea brews, at 3 minutes of brewing, were 4.7 and 5.1, respectively. The green tea infusions produced higher pH values, with the lowest value at 5.5 and the highest at 5.8. This was closely

followed by black tea + milk solutions, in which the range of the pH values was between 5.7 and 6.0 (Table 3-2).

Table 3-2: pH values of the tea solutions (at 3 minutes brewing time).

Group	Batch 1			Batch 2			Batch 3			Mean	SD
Black tea	4.70	5.05	4.80	4.93	5.10	5.0	4.73	5.00	4.85	**4.90**	**0.14**
Black + milk	5.70	5.90	5.85	5.94	5.96	5.78	5.92	5.90	5.86	**5.90**	**0.08**
Green tea	5.50	5.61	5.70	5.68	5.80	5.70	5.53	5.65	5.73	**5.70**	**0.10**

3.1.4 Titratable acidity of the tea solutions

The baseline pH values of the tea solutions are shown in Table 3-3. The total volume of 0.01 M KOH required for neuralisation of 20 ml of each tea solution was determined. For the black tea brew, the titratable acidity value was 0.9 ml; whereas for black tea + milk infusion, less volume of the titration solution (0.5 ml) was sufficient. With respects to green tea, the titratable acidity value was 0.7 ml.

Table 3-3: Titratable acidity (ml) assessment of the tea solutions.

Titratable acidity (ml)	pH values								
	Black tea			Black + milk			Green tea		
Baseline	5.05	5.10	5.00	5.90	5.96	5.90	5.61	5.80	5.53
0.1	5.28	5.32	5.21	6.18	6.20	6.18	5.81	5.99	5.73
0.2	5.51	5.57	5.43	6.40	6.41	6.40	6.01	6.18	5.97
0.3	5.75	5.81	5.68	6.63	6.61	6.63	6.20	6.37	6.20
0.4	5.95	6.04	5.93	6.83	6.85	6.83	6.39	6.57	6.41
0.5	6.17	6.24	6.16	**7.01**	**7.03**	**7.01**	6.58	6.77	6.63
0.6	6.39	6.43	6.41				6.79	6.95	6.85
0.7	6.60	6.65	6.66				**7.03**	**7.14**	**7.07**
0.8	6.81	6.84	6.86						
0.9	**7.01**	**7.04**	**7.06**						

3.1.5 Fluoride concentration in the tea solutions

These measurements were obtained in triplicate, with the analyser set to (ppm) mode. The mean fluoride concentration in black and green tea brews were 5.13 ± 0.09 and 5.06 ± 0.10 ppm F, respectively. With regards to the black tea + milk infusions, a slight increase in the mean fluoride concentration was noticed (5.30 ± 0.15 ppm F) (Table 3-4).

Table 3-4: Fluoride concentration (ppm) in tea solutions.

Group	Batch 1			Batch 2			Batch 3			Mean	SD
Black tea	4.99	5.00	5.22	5.21	5.10	5.20	5.10	5.12	5.20	**5.13**	**0.09**
Black + milk	5.31	5.25	5.62	5.42	5.13	5.27	5.11	5.32	5.27	**5.30**	**0.15**
Green tea	4.91	5.16	5.10	5.15	4.96	5.17	4.99	5.11	5.00	**5.06**	**0.10**

3.2 Quantitative Light-Induced Fluorescence (QLF) results for black tea, black tea with milk, green tea, fluoride-free and fluoride containing water groups

ΔF, ΔQ and the Area constituted the fundamental elements in QLF analysis:

- ΔF stands for the percentage fluorescence loss with respect to the fluorescence of sound tooth structure, which in turn reflects the lesion depth, expressed as a percentage (%).
- Area defines the surface area of the lesion measured in pixels^2 (px^2).
- ΔQ represents (ΔF times the Area), that is the percentage of fluorescence loss with respect to the fluorescence of sound tissue times the Area. This indicates the lesion volume (% px^2).

Throughout this chapter, the main QLF experimental results represent the final results, that is after 28 days of pH-cycling and after implementing the stain removal method. Whereas, the stain removal experimental results refer merely to comparison of results before and after stain removal.

3.2.1 ΔF (main QLF experimental results):

3.2.1.1 The mean fluorescence loss ΔF

First, the values of ΔF at baseline for all groups were compared and analysed, to determine whether significant relationships exist are due to real differences between the groups at the termination of the study project. The normality of the data was checked utilising Shapiro-Wilk test and Kolmogorov-Smirnov test (Table 3-5). The tests showed some p values of less than 0.05, therefore the data was not normally distributed. A non-parametric test, which does not assume normality, was needed to evaluate the baseline slabs.

Table 3-5: Tests of Normality (ΔF baseline).

ΔF Baseline	Group	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.200	.404
	Black+ Milk	.200	.847
	F-Free water	.007*	.033*
	F-water	.200	.716
	Green tea	.200	.601

. This is a lower bound of the true significance.

Non-parametric (Kruskal-Wallis test) was conducted, which showed no statistically significant difference among the distribution of ΔF baseline across all groups (Table 3-6).

Table 3-6: Independent Samples Kruskal-Wallis Test for ΔF baseline.

Null hypothesis	Test	Significance	Decision
The distribution of ΔF Baseline is the same across categories of formulation.	Independent-Samples Kruskal-Wallis Test	.55	Retain the null hypothesis

The significance level is .05.

3.2.1.2 Difference in ΔF within each group

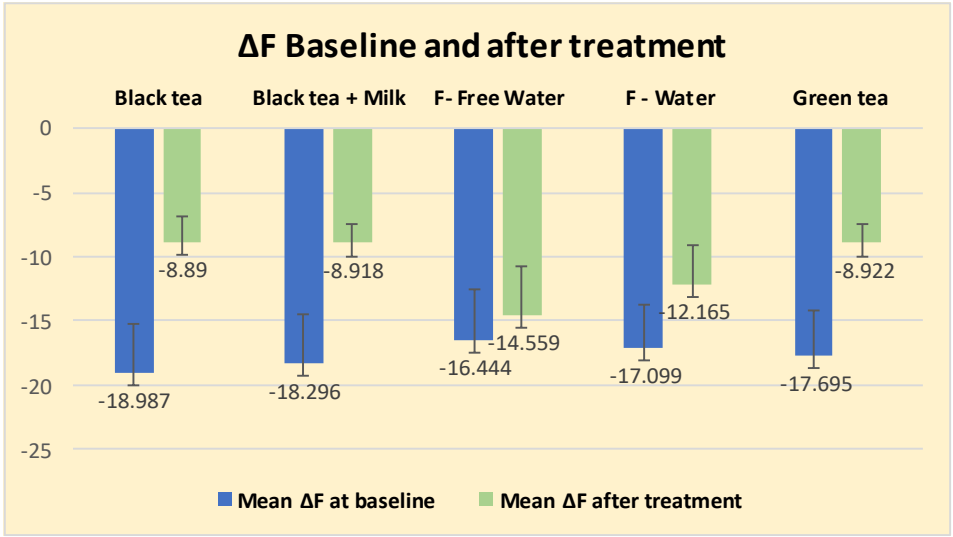
Table 3-7 shows the ΔF mean values at baseline and after treatment. Positive changes in ΔF figures were noticed within all study groups.

Table 3-7: Mean values of ΔF at baseline, ΔF after treatment, ΔF difference and the medians for all the groups.

Group	Mean ΔF at baseline ± SD	Mean ΔF after treatment ± SD	Mean Difference in ΔF at baseline and after treatment ± SD	Median Difference in ΔF at baseline and after treatment
Black tea	-18.987 ± 3.77	- 8.890 ± 2.05	10.097 ± 3.47	10.611
Black tea + Milk	-18.296 ± 3.75	- 8.918 ± 1.47	9.378± 3.37	8.753
F-Free Water	-16.444 ± 3.87	-14.559 ± 3.84	1.885 ± 1.10	1.699
F-Water	-17.099 ± 3.42	- 12.165 ± 3.14	4.935 ± 1.40	4.839
Green tea	-17.695 ± 3.45	- 8.922 ± 1.52	8.773 ± 2.83	7.804

Figure 3-1 illustrates the means of ΔF at baseline and after treatment for all groups along with their standard deviation values.

Figure 3-1: ΔF means at baseline and after treatment.



Shapiro-Wilk and Kolmogorov-Smirnov tests were performed to check if the mean difference in ΔF values meet the assumption of normality (Table 3-8).

Table 3-8: Tests of Normality (Difference in ΔF).

Difference in ΔF (After treatment - baseline)	Group	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.200	.439
	Black+ Milk	.200	.013*
	F- Free water	.200	.088
	F- water	.200	.536
	Green tea	.037*	.018*

. This is a lower bound of the true significance.

Since the data was not normally distributed, a non- parametric (Wilcoxon signed-rank test) was used to determine if the difference in ΔF at baseline and after treatment was significantly different within the same group.

Statistically significant improvements in the ΔF values were noted in all groups when the post treatment figures were compared with those at baseline (Table 3-9).

Table 3-9: Wilcoxon Signed Ranks Test results for ΔF paired differences.

Group	Paired Differences ΔF After Tx – ΔF Baseline						Exact Sig. (2-tailed) *Wilcoxon Signed Ranks Test
	Median	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
Black tea	10.611	10.097	3.47	0.63	8.79	11.39	**0.000***
Black tea + Milk	8.753	9.378	3.37	0.62	8.12	10.64	**0.000***
F-Free Water	1.699	1.885	1.10	0.20	1.48	2.29	**0.000***
F-Water	4.839	4.935	1.40	0.26	4.41	5.46	**0.000***
Green tea	7.804	8.773	2.83	0.52	7.71	9.83	**0.000***

**** Statistically significant***

3.2.1.3 Difference in ΔF between all groups

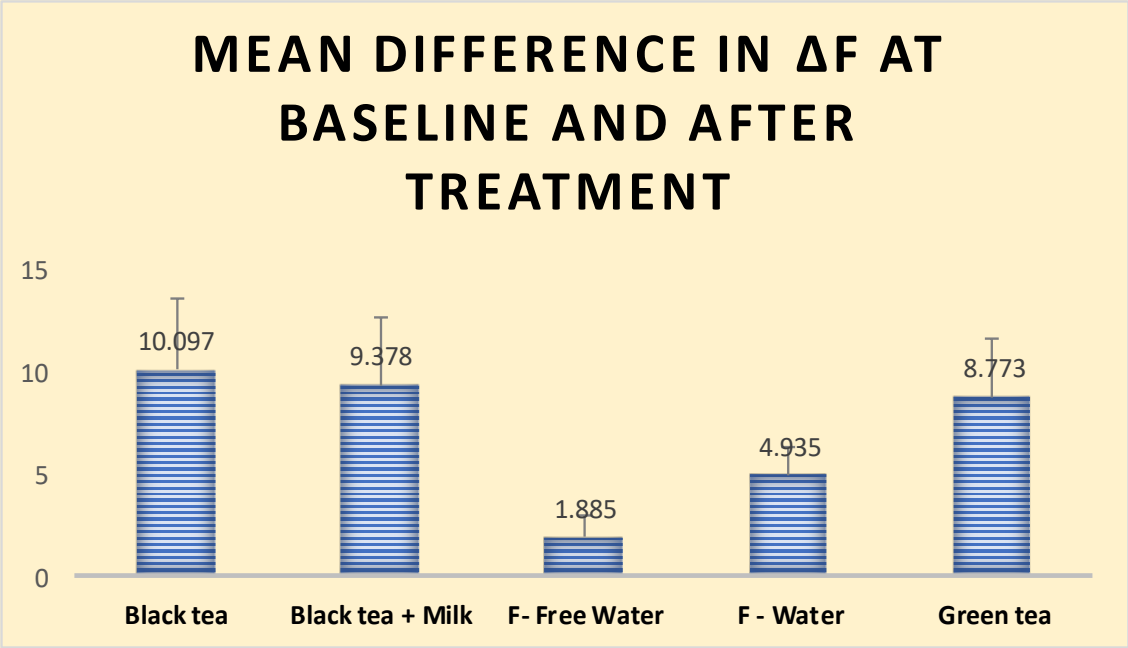
The formula used to obtain the change in ΔF was as follows:

Difference in ΔF = (ΔF after treatment – ΔF at baseline).

There was a reduction in the mean fluorescence loss among all groups, confirmed by the positive figures in the values of ΔF-Difference (Figure 3-2).

The greatest reduction in ΔF was seen in the black tea group (10.097 ± 3.47), next was the black tea + Milk group (9.378 ± 3.37), thereafter came the green tea group with a mean difference of (8.773± 2.83). This was followed by the fluoridated water group at a mean difference of (4.935 ± 1.40). The F-Free water group showed the lowest reduction in the mean ΔF Difference at (1.885 ± 1.10).

Figure 3-2: Mean difference in ΔF values at baseline and after treatment.



As reported above, the data was not normally distributed. Therefore, non-parametric (Independent samples Kruskal Wallis test) was carried out to assess the ΔF differences between the five groups (Table 3-10). The outcome of the test indicated that the mean difference in ΔF was statistically different between the five groups ($p < 0.001$).

Table 3-10: Independent samples Kruskal Wallis test for the ΔF-difference (After-baseline) between all groups.

Null hypothesis	Test	Significance	Decision
The distribution of Difference in ΔF (After-Baseline) is the same across categories of formulation.	Independent-Samples Kruskal-Wallis Test	.000	**Reject the null hypothesis**

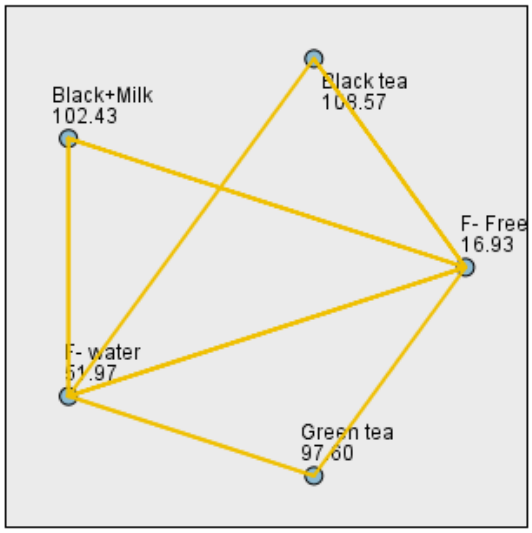
The significance level is .05.

Pairwise multiple comparison tests were performed in order to evaluate subgroup differences, along with Bonferroni correction which was used to compete against the effect of multiple tests (Figure 3-3).

The examination identified a significant difference in favour of the fluoridated water group over the F-Free group ($p< 0.05$). In addition, it showed that the mean difference in ΔF of the black tea, black tea +milk and green tea groups were statistically significantly higher than the mean ΔF difference in the F-Free and the fluoridated water groups ($p< 0.001$). On the other hand, there were no statistical differences between the three tea groups.

Figure 3-3: Pairwise comparisons with Bonferroni correction of the difference in ΔF between all groups

Pairwise Comparisons of Formulation



Each node shows the sample average rank of Formulation.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
F- Free-F- water	-35.033	11.218	-3.123	.002	.018
F- Free-Green tea	-80.667	11.218	-7.191	.000	.000
F- Free-Black+Milk	85.500	11.218	7.622	.000	.000
F- Free-Black tea	91.633	11.218	8.169	.000	.000
F- water-Green tea	-45.633	11.218	-4.068	.000	.000
F- water-Black+Milk	50.467	11.218	4.499	.000	.000
F- water-Black tea	56.600	11.218	5.046	.000	.000
Green tea-Black+Milk	4.833	11.218	.431	.667	1.000
Green tea-Black tea	10.967	11.218	.978	.328	1.000
Black+Milk-Black tea	6.133	11.218	.547	.585	1.000

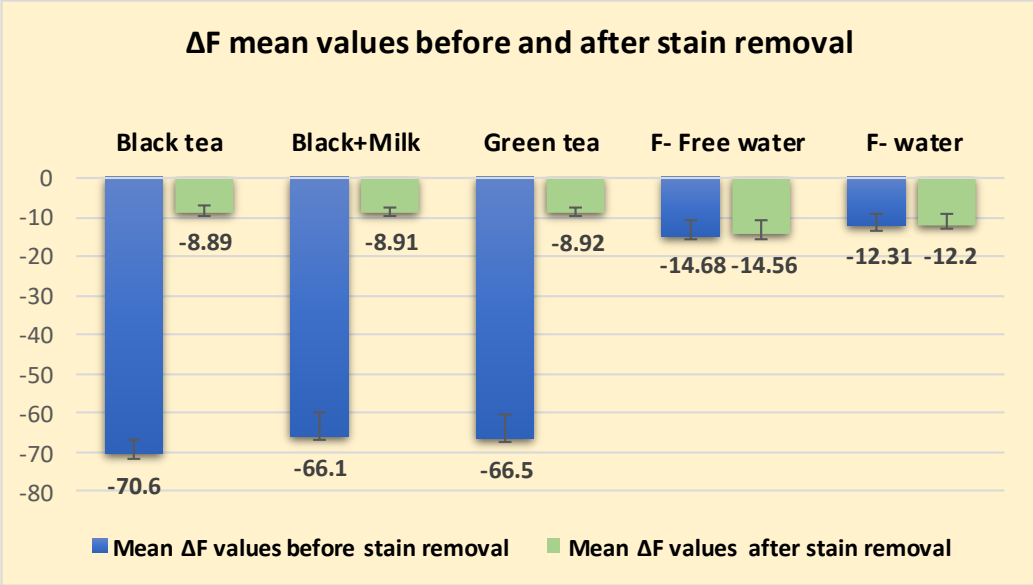
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

3.2.2 ΔF (stain removal experimental results):

3.2.2.1 Difference in ΔF before and after stain removal

Mean ΔF values before and after stain removal are shown in (Figure 3-4). It can be seen from the picture that although there are dramatic changes in the experimental groups that caused staining of the enamel slabs, the controls namely F-Free water and fluoridated water groups show very minimal changes.

Figure 3-4: ΔF means before and after stain removal, along with standard deviation values.



To assess whether the change in ΔF before and after stain removal was significant within the same group, normality tests were first conducted (Table 3-11). The outcome revealed that the data was normally distributed; thus, a Paired T- Test was used.

Table 3-11: Tests of Normality (Difference in ΔF before and after stain removal).

Difference in ΔF (after stain removal - before stain removal)	Group	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.200	.124
	Black+ Milk	.095	.060
	F-Free water	.200	.815
	F-water	.199	.881
	Green tea	.200	.052

. This is a lower bound of the true significance.

The experimental groups, more precisely the black tea, green tea and black tea + milk showed statistically significant decrease in the ΔF values after stain removal ($p<0.001$). Whereas, the F-Free and fluoridated water control groups demonstrated no significant changes, p-values of 0.494 and 0.280, respectively (Table 3-12).

Table 3-12: Paired Sample T-Test for ΔF differences before and after stain removal.

Group	Paired Differences ΔF After stain removal – ΔF Before stain removal					Exact Sig. (2-tailed) *Paired Sample T-Test
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
				Lower	Upper	
Black tea	61.73	4.35	0.79	60.1	63.4	**0.000***
Black tea + Milk	57.20	6.00	1.10	55.1	59.4	**0.000***
Green tea	57.54	7.13	1.30	54.98	60.21	**0.000***
F-Free Water	0.125	1.11	0.18	- 0.24	0.50	**0.494**
F-Water	0.150	0.73	0.13	- 0.13	0.42	**0.280**

**** Statistically significant***

3.2.3 Intra-examiner reproducibility for ΔF

The intra-examiner reproducibility was measured utilising the intra-class correlation coefficient (ICC). A total of 30 enamel slabs, which represented 20% of the sample, were randomly chosen and re-examined. The ICC demonstrated excellent reliability at 0.99.

3.2.4 ΔQ (main QLF experimental results):

3.2.4.1 ΔQ (ΔF times the Area)

It is defined as the percentage fluorescence loss with respect to the fluorescence of sound tissue times the Area and is represented as lesion volume. The ΔQ values at baseline were tested for normality (Table 3-13). Given the normal distribution of the data, one-way ANOVA test was chosen to determine the existence of any statistical differences in ΔQ values at baseline between the enamel subsurface lesions across the five groups. The ANOVA test revealed no significant differences ($p>0.05$) (Table 3-14).

Table 3-13: Tests of Normality (ΔQ baseline).

ΔQ Baseline	Group	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.200	.357
	Black+ Milk	.200	.429
	F-Free water	.060	.092
	F-water	.194	.224
	Green tea	.200	.209

. This is a lower bound of the true significance.

Table 3-14: One-way ANOVA test for ΔQ baseline.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	757766885.330	4	189441721.332	.934	.446
Within Groups	29399809339.187	145	202757305.787		
Total	30157576224.517	149			

3.2.4.2 Difference in ΔQ within each group

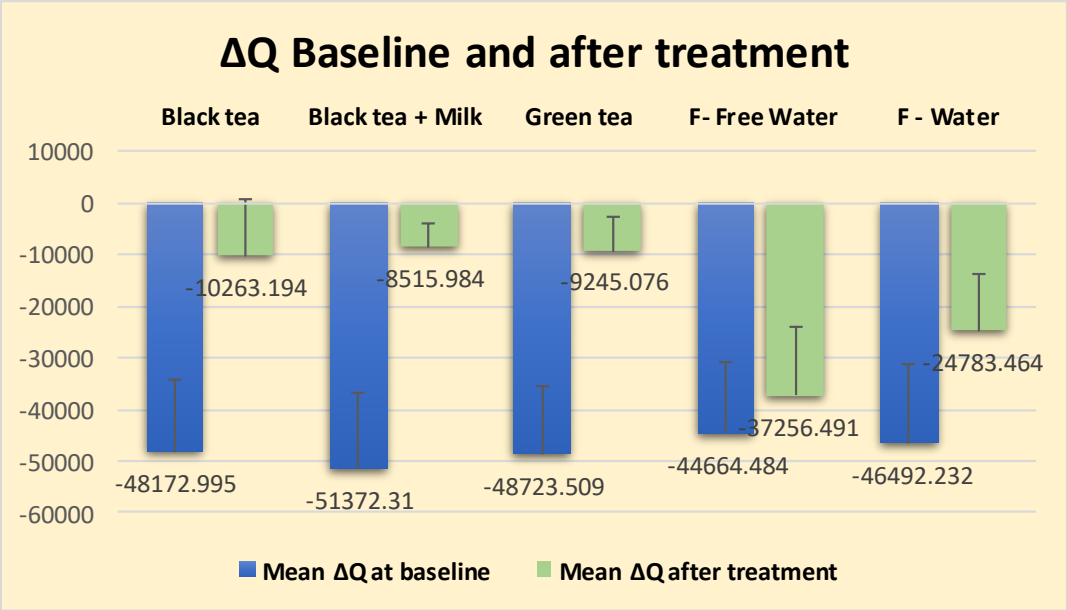
The ΔQ values at baseline and after treatment can be visualised in Table 3-15. Improvements can be seen in all study groups.

Table 3-15: The ΔQ mean values at baseline and after treatment.

Group	Mean ΔQ at baseline ± SD	Mean ΔQ after treatment ± SD	Mean Difference in ΔQ after treatment and at baseline± SD
Black tea	-48172.995 ± 13978.13	-10263.194 ± 11180.71	37909.801 ± 10811.23
Black tea + Milk	-51372.310 ± 14715.90	-8515.984± 4570.32	42856.326 ± 12700.68
Green tea	-48723.509 ± 13277.86	-9245.076 ± 6820.55	39478.433 ± 9455.10
F-Free Water	-44664.484 ± 13898.71	-37256.491 ± 13142.08	7407.995 ± 4145.70
F-Water	-46492.232 ± 15243.53	-24783.464 ± 11256.46	21708.768 ± 7784.48

Figure 3-5 illustrates the change in ΔQ means at baseline and after treatment for all groups and their corresponding standard deviation values.

Figure 3-5: Mean ΔQ values at start and at endpoint for all study groups.



Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the normality of the data (Table 3-16). Accordingly, paired T-test was conducted, and revealed statistically significant improvements in the ΔQ values after the treatment compared with those at baseline in all groups ($p<0.05$) (Table 3-17).

Table 3-16: Tests of Normality (Difference in ΔQ).

Difference in ΔQ (After treatment - baseline)	Formulation	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.200	.767
	Black+ Milk	.200	.972
	F-Free water	.200	.106
	F-water	.200	.459
	Green tea	.200	.311

Table 3-17: Paired sample T-Test for ΔQ values pre and post treatment.

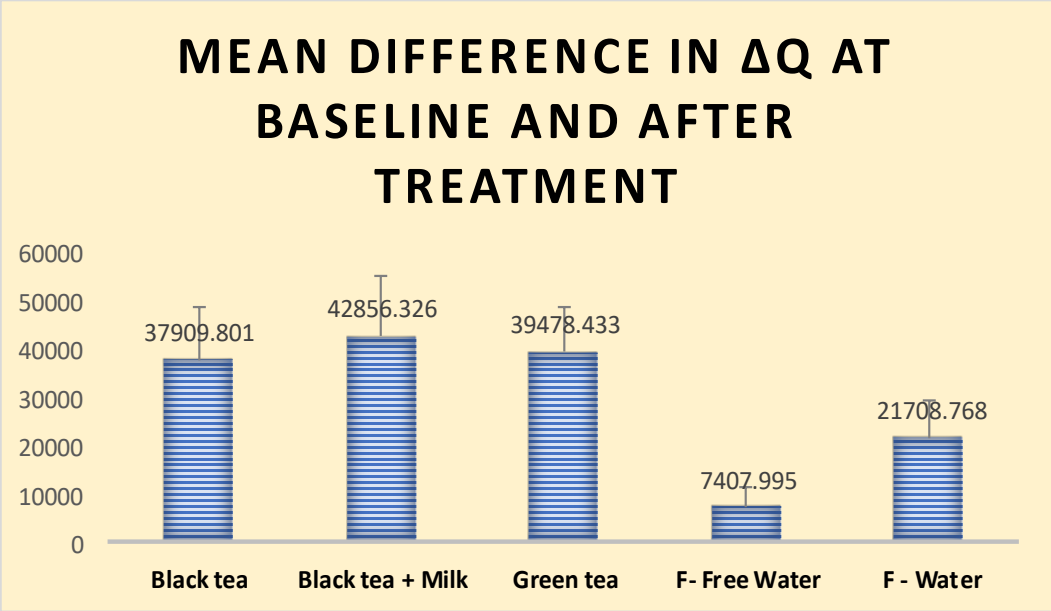
Group	Paired Differences ΔQ After treatment– ΔQ Baseline					Exact Sig. (2-tailed) *Paired Sample T-Test
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
				Lower	Upper	
Black tea	37909.801	10811.231	1973.852	33872.821	41946.781	**0.000***
Black tea + Milk	42856.326	12700.684	2318.817	38113.812	47598.839	**0.000***
Green tea	39478.433	9455.097	1726.257	35947.842	43009.024	**0.000***
F-Free Water	7407.995	4145.699	756.898	5859.965	8956.024	**0.000***
F-Water	21708.768	7784.484	1421.246	18802.994	24615.542	**0.000***

**** Statistically significant.***

3.2.4.3 Difference in ΔQ between all groups

The equation utilised to calculate the change in ΔQ was (Difference in ΔQ = ΔQ after treatment - ΔQ baseline). Figure 3-6 proves that the change in ΔQ was positive among all study groups. The biggest improvement was noticed in the black tea + milk group, with a mean difference of (42856.326 ± 12700.68), followed by green tea, black tea and F-water groups, respectively. On the other hand, the lowermost reduction was found in the F-Free water group at a mean difference of (7407.995 ± 4145.70).

Figure 3-6: Means of ΔQ-differences at and after experiment.



As the data was normally distributed, one-way ANOVA test was chosen in order to evaluate whether the differences in ΔQ were statistically significant between the five groups. The test showed a significant difference ($p<0.001$) (Table 3-18).

Table 3-18: One-way ANOVA for the difference in ΔQ between the groups.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26902572113.843	4	6725643028.461	75.506	**.000***
Within Groups	12915844969.978	145	89074792.896		
Total	39818417083.820	149			

**** Statistically significant.***

Multiple comparison analysis tests with Bonferroni correction method were then utilised (Table 3-19). The outcome revealed that the mean differences in ΔQ of black tea, black tea +milk and green tea groups were significantly higher than the mean differences of both F-Free water and F-water groups ($p<0.001$). However, there were no statistical differences between the black tea, black tea with milk and green tea groups.

Regarding the F-water group, the value of the mean difference in ΔQ was statistically higher than its comparative value in the F-Free water group ($p<0.001$). On the other hand, this was significantly lower than the figures found in the black tea, black tea with milk and green tea groups ($p<0.001$). Finally, the mean difference in ΔQ of the F-Free water group was significantly lower than all other groups.

Table 3-19: Multiple comparisons of the difference in ΔQ at baseline and after treatment between all groups.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Black tea	Black tea +Milk	-4946.525	2436.8667436	.442	-11893.11997	2000.06951
	F-Free	30501.806*	2436.8667436	**.000***	23555.21122	37448.40072
	F-water	16201.033*	2436.8667436	**.000***	9254.43775	23147.62724
	Green tea	-1568.632	2436.8667436	1.000	-8515.22718	5377.96230
Black tea +Milk	Black tea	4946.525	2436.8667436	.442	-2000.06951	11893.11997
	F-Free	35448.331*	2436.8667436	**.000***	28501.73645	42394.92595
	F-water	21147.558*	2436.8667436	**.000***	14200.96298	28094.15247
	Green tea	3377.893	2436.8667436	1.000	-3568.70195	10324.48753
F-Free water	Black tea	-30501.806*	2436.8667436	**.000***	-37448.40072	-23555.21122
	Black tea +Milk	-35448.331*	2436.8667436	**.000***	-42394.92595	-28501.73645
	F-water	-14300.773*	2436.8667436	**.000***	-21247.36822	-7354.17872
	Green tea	-32070.438*	2436.8667436	**.000***	-39017.03316	-25123.84366
F-water	Black tea	-16201.033*	2436.8667436	**.000***	-23147.62724	-9254.43775
	Black tea +Milk	-21147.558*	2436.8667436	**.000***	-28094.15247	-14200.96298
	F-Free	14300.773*	2436.8667436	**.000***	7354.17872	21247.36822
	Green tea	-17769.665*	2436.8667436	**.000***	-24716.25969	-10823.07019
Green tea	Black tea	1568.632	2436.8667436	1.000	-5377.96230	8515.22718
	Black tea +Milk	-3377.893	2436.8667436	1.000	-10324.48753	3568.70195
	F-Free	32070.438*	2436.8667436	**.000***	25123.84366	39017.03316
	F-water	17769.665*	2436.8667436	**.000***	10823.07019	24716.25969

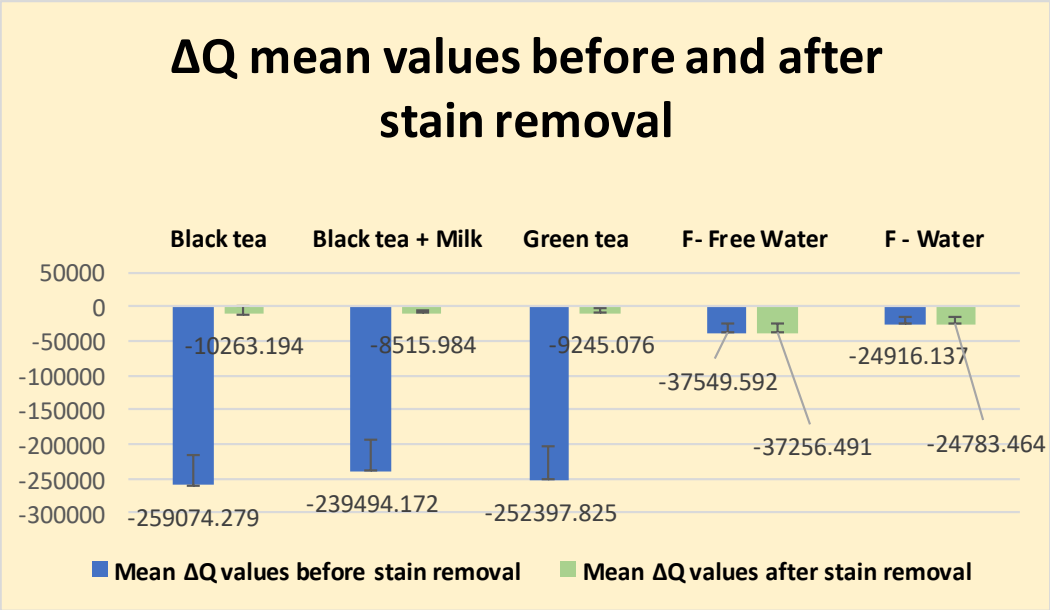
*. The mean difference is significant at the .05 level.

3.2.5 ΔQ (stain removal experimental results):

3.2.5.1 Difference in ΔQ before and after stain removal

The blue and the green bars in figure 3-7 illustrate the mean ΔQ values before and after stain removal, respectively. Control groups (F-Free and F-water) display only minor changes, when compared to the black tea, black tea with milk and green tea groups.

Figure 3-7: ΔQ mean values before and after stain removal.



Although the changes were unnoticeable in the control groups, it was prudent to analyse these data statistically, in order to determine whether or not there were any significant differences. Therefore, normality tests were conducted (Table 3-20), which in turn confirmed the assumption of normality (p>0.05).

Table 3-20: Tests of Normality (Difference in ΔQ before and after stain removal).

Difference in ΔQ (after stain removal – before stain removal)	Group	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.194	.345
	Black+ Milk	.095	.055
	F-Free water	.200	.952
	F-water	.200	.928
	Green tea	.200	.060

Paired T-Test was applied to assess if a significant difference existed between before and after stain removal within each group (Table 3-21). It can be seen that both F-Free and F-water groups demonstrated insignificant changes ($p>0.05$). While this was true for the control groups, the test groups, namely black tea, black tea with milk and green tea showed statistically significant decrease in the ΔQ values after stain removal ($p<0.001$).

Table 3-21: Paired Sample T-Test for ΔQ values before and after stain removal.

Group	Paired Differences ΔQ After stain removal – ΔQ Before stain removal					Exact Sig. (2-tailed) *Paired Sample T-Test
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
				Lower	Upper	
Black tea	248811.085	40312.971	7360.108	233757.975	263864.196	**0.000***
Black tea + Milk	230978.188	43052.067	7860.196	214902.282	247054.094	**0.000***
Green tea	243152.749	45895.114	8379.263	226015.232	260290.267	**0.000***
F-Free Water	293.103	3422.066	624.781	- 984.718	1570.923	.642
F-Water	132.673	2590.841	473.021	-834.763	1100.109	.781

**** Statistically significant.***

3.2.6 Intra-examiner reproducibility for ΔQ

A total of 30 enamel slabs (20%) were randomly selected for re-measurements. The ICC was calculated to assess the intra-observer agreement. An outstanding reliability with ICC value of 0.99 was detected.

3.2.7 Area of the enamel subsurface lesion (main QLF experimental results):

3.2.7.1 The Area

The baseline data of the lesion area was tested for normality. Based on the normality tests, the model was considered to be not normally distributed (Table 3-22).

Table 3-22: Tests of Normality (Area at baseline).

Area Baseline	Formulation	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.082	.019*
	Black+ Milk	.145	.204
	F-Free water	.200	.622
	F-water	.200	.303
	Green tea	.200	.288

. This is a lower bound of the true significance.

A non-parametric (Kruskal-Wallis test) was therefore executed (Table 3-23). The test confirmed that there was no statistically significant difference in the Area values at baseline across the groups (p>0.05).

Table 3-23: Independent samples Kruskal Wallis test for the Area baseline.

Null hypothesis	Test	Significance	Decision
The distribution of Area Baseline is the same across categories of formulation.	Independent-Samples Kruskal-Wallis Test	.369	Retain the null hypothesis

The significance level is .05.

3.2.7.2 Difference in lesion area within each group

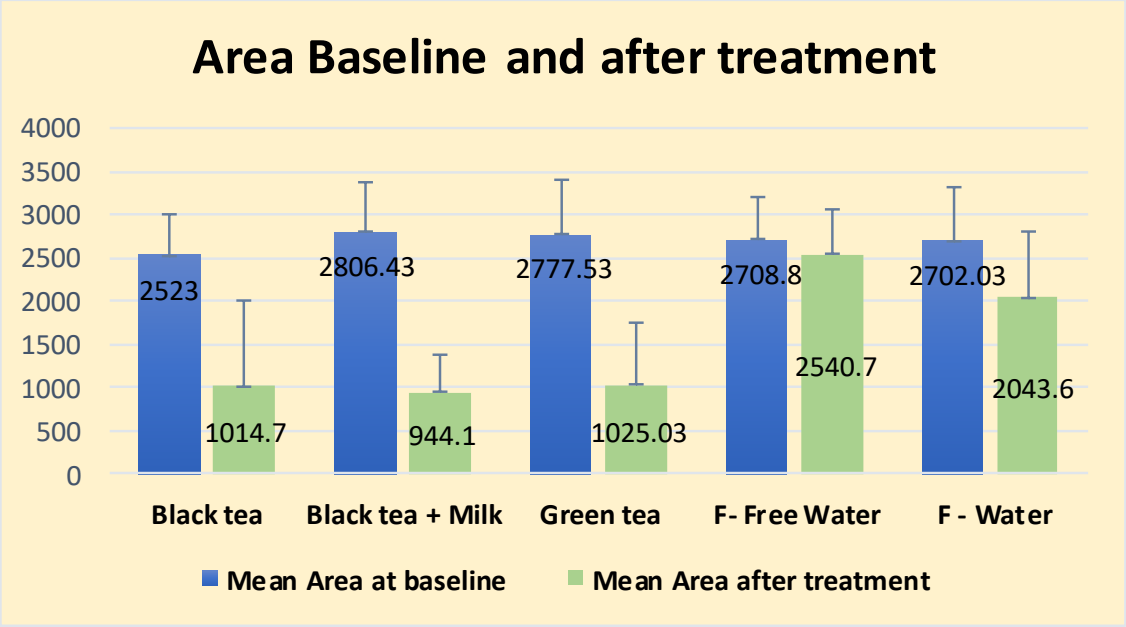
Table 3-24 displays the means of the enamel subsurface lesion area at baseline and after treatment. It can be clearly noticed that there was a reduction in the extent of the white spot lesion area in all study groups.

Table 3-24: The medians and the means of the Area at baseline and after the treatment.

Group	Median	Mean Area at baseline ± SD	Mean Area after treatment ± SD	Mean Difference in Area after treatment and at baseline ± SD
Black tea	-1753.00	2523.00 ± 472.454	1014.70 ± 990.788	-1508.30 ± 882.832
Black tea + Milk	-1754.00	2806.43 ± 561.854	944.10 ± 439.620	-1862.33 ± 554.840
Green tea	-1792.00	2777.53 ± 622.425	1025.03 ± 718.157	-1752.50 ± 704.664
F-Free Water	-176.50	2708.80 ± 511.400	2540.70 ± 514.456	-168.10 ± 125.362
F-Water	-568.50	2702.03 ± 630.109	2043.60 ± 762.668	-658.43 ± 396.762

The bars below represent the means of the Area at baseline and after treatment for all groups. The error bars illustrate the standard deviation figures (Figure 3-8).

Figure 3-8: Area values at baseline and after treatment.



The data was tested for normality, to determine the type of statistical test to be used (Table 3-25). The Wilcoxon Signed Ranks Test was implemented, as the data was not normally distributed.

Table 3-25: Tests of Normality (Difference in Area).

Difference in Area (After treatment - baseline)	Group	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.067	.024*
	Black+ Milk	.176	.452
	F-Free water	.200	.048*
	F-water	.200	.117
	Green tea	.200	.496

. This is a lower bound of the true significance.

The results of the Wilcoxon Signed Ranks test are shown in Table 3-26. Significant improvements in the Area values were detected within each group when comparing the results after treatment to those before ($p<0.05$).

Table 3-26: Wilcoxon Signed Ranks test results for the Area at baseline and after treatment.

Group	Paired Differences Area After Tx – Area Baseline						Exact Sig. (2-tailed) *Wilcoxon Signed Ranks Test
	Median	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
Black tea	-1753.00	-1508.30	882.832	161.182	-1837.95	-1178.65	**0.000***
Black tea + Milk	-1754.00	-1862.33	554.840	101.299	-2069.51	-1655.15	**0.000***
F-Free Water	-176.50	-168.10	125.362	22.888	-214.91	-121.29	**0.000***
F-Water	-568.50	-658.43	396.762	72.439	-806.59	-510.28	**0.000***
Green tea	-1792.00	-1752.50	704.664	128.653	-2015.63	-1489.37	**0.000***

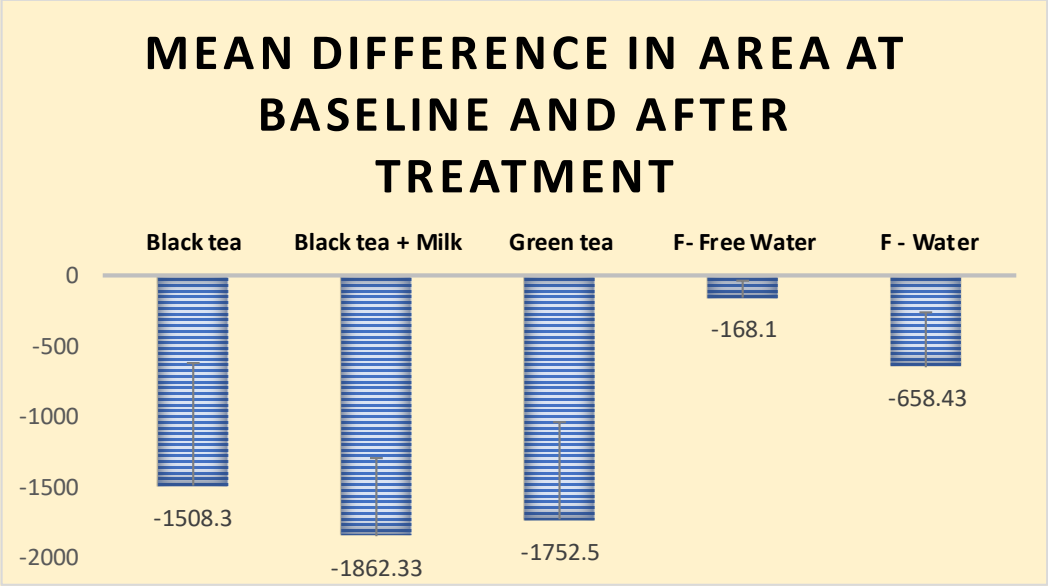
**** Statistically significant.***

3.2.7.3 Difference in lesion area between all groups

The mean difference in Area was computed as follows: Area at endpoint – Area at baseline. The results of the mean differences in enamel lesion area are visible in Figure 3-9. There has been a marked decline in the enamel subsurface area in all tea groups. The largest reduction was noticed in the black tea + milk group (-1862.33 ± 554.840), this was followed closely by the green tea and the black tea groups with a mean difference of (-1752.50 ± 704.664) and (-1508.30 ± 882.832), respectively.

F-water group showed less reduction when compared to the three tea groups. The minimum shrinkage in the size of the lesion area was found in the F-Free water group at a mean difference of (-168.10 ± 125.362).

Figure 3-9: Mean values of the difference in the white spot lesion area for all test groups.



As stated earlier, the data set was not normally distributed. Consequently, the Independent samples Kruskal Wallis test was selected to assess the values of the mean difference in lesion area between the groups (Table 3-27). The test indicated statistically significant difference between the groups ($p<0.001$).

Table 3-27: Independent samples Kruskal Wallis test for the difference in Area between the groups.

Null hypothesis	Test	Significance	Decision
The distribution of Difference in Area is the same across categories of formulation.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis

The significance level is .05.

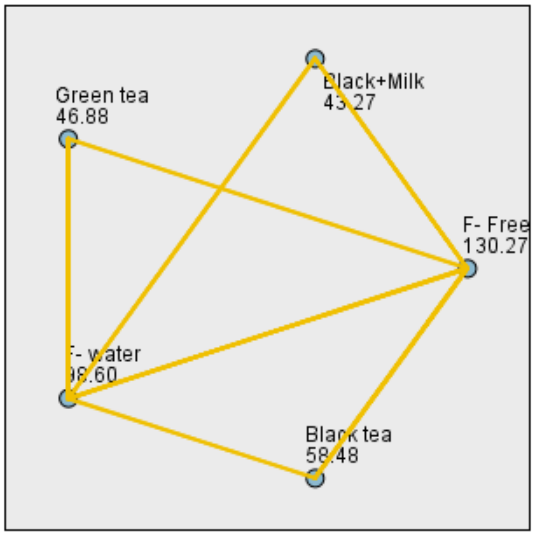
Pairwise comparisons were undertaken between all pairs to determine which means differed. Additionally, significant values were corrected by Bonferroni method (Figure 3-10).

The multiple comparison tests showed that the differences in mean Areas of black tea + milk and green tea groups were statistically significantly higher than the F-Free and F-water groups ($p< 0.001$). Likewise, the difference in the mean Area of black tea was statistically higher than that of either F-Free or F-water group at $p< 0.001$ and $P< 0.01$, respectively.

Moreover, a statistically significant relationship existed between the positive and negative control groups. This was definitely in favour of the F-water group ($p< 0.05$). Lastly, the comparisons showed insignificant differences, when the black tea+ milk, black tea or green tea groups were paired.

Figure 3-10: Pairwise comparisons with Bonferroni correction for the difference in Area between all groups.

Pairwise Comparisons of Formulation



Each node shows the sample average rank of Formulation.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
Black+Milk-Green tea	-3.617	11.218	-.322	.747	1.000
Black+Milk-Black tea	15.217	11.218	1.357	.175	1.000
Black+Milk-F- water	-55.333	11.218	-4.933	.000	.000
Black+Milk-F- Free	-87.000	11.218	-7.756	.000	.000
Green tea-Black tea	11.600	11.218	1.034	.301	1.000
Green tea-F- water	51.717	11.218	4.610	.000	.000
Green tea-F- Free	83.383	11.218	7.433	.000	.000
Black tea-F- water	-40.117	11.218	-3.576	.000	.003
Black tea-F- Free	-71.783	11.218	-6.399	.000	.000
F- water-F- Free	31.667	11.218	2.823	.005	.048

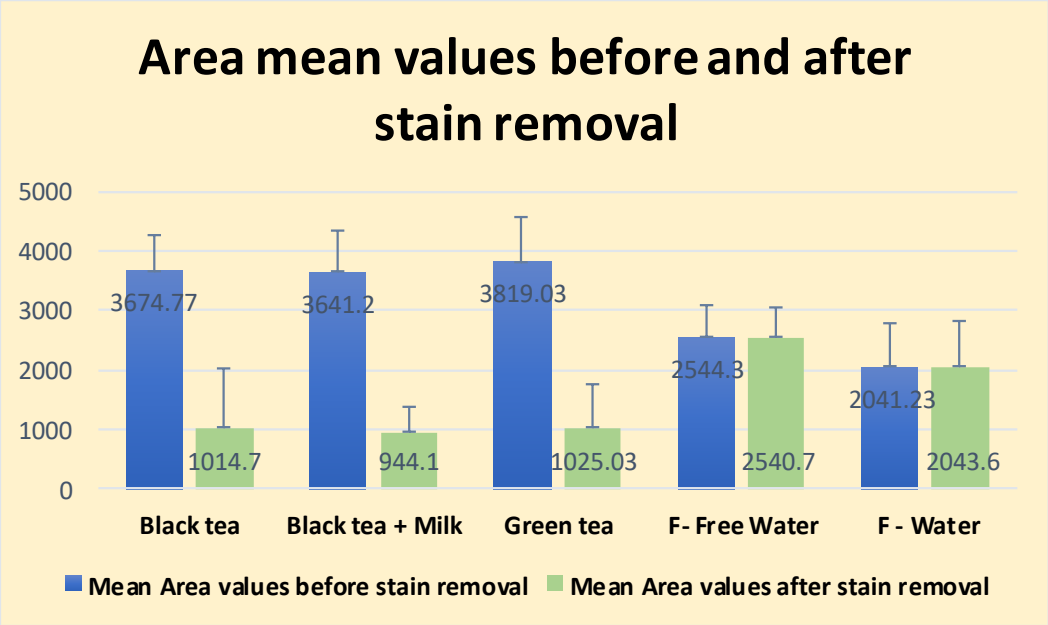
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

3.2.8 Area (the stain removal experimental results):

3.2.8.1 Difference in Area before and after stain removal

Figure 3-11 presents a double bar chart, used to manifest the differences in the enamel subsurface lesion area prior to and post stain removal. The heights of the blue and green bars were nearly similar within the F-Free and F-water groups, whereas with the black tea, black tea with milk and green tea groups, the heights vary considerably.

Figure 3-11: Area mean values before and after stain removal for all groups



Normality tests indicated that the data was not normally distributed (Table 3-28). Hence, a nonparametric Wilcoxon Signed Ranks test was used.

Table 3-28: Tests of Normality (Difference in Area before and after stain removal experiment).

Difference in Area (after stain removal - before stain removal)	Group	Kolmogorov-Smirnov[a]	Shapiro-Wilk
		Significance	Significance
	Black tea	.014*	.033*
	Black+ Milk	.200	.899
	F-Free water	.200	.009*
	F-water	.132	.100
	Green tea	.200	.977

. This is a lower bound of the true significance.

In the control groups, namely F-Free and F-water, no statistical differences were detected between the mean Area values of before and after stain removal ($p>0.05$). Nevertheless, the black tea, black tea+ milk and green tea groups demonstrated statistically significant reduction in the mean area values after stain removal ($p< 0.001$) (Table 3-29).

Table 3-29: Wilcoxon Signed Ranks Test results for the Area values before and after stain removal for all groups.

Group	Paired Differences Area After Stain Removal – Area Before Stain Removal						Exact Sig. (2-tailed) *Wilcoxon Signed Ranks Test
	Median	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
Black tea	-2686.00	-2660.07	898.971	164.129	-2995.75	-2324.39	**0.000***
Black tea + Milk	-2698.00	-2697.10	610.965	111.546	-2925.24	-2468.96	**0.000***
Green tea	-2842.50	-2794.00	874.245	159.615	-3120.45	-2467.55	**0.000***
F-Free Water	-18.50	-3.60	102.689	18.748	-41.94	34.74	**0.886**
F-Water	34.50	2.37	142.205	25.963	-50.73	55.47	**0.491**

**** Statistically significant.***

3.2.9 Intra-examiner reproducibility for Area of the enamel subsurface lesion

The ICC was calculated in order to assess the intra-investigator consistency. 30 enamel slabs were picked randomly to perform a second analysis. This constituted 20% of the total sample. The ICC score was found to be 0.99, which is indicative of high reliability.

3.2.10 Synopsis of statistical analysis results

ΔF outcome: When comparing ΔF mean values at baseline to those after treatment in each group, the analysis showed statistically significant improvements in all study groups. F-Free water group exhibited the smallest amount of reduction in the mean ΔF-Difference. Whereas, the highest recovery in ΔF values was found in the black tea group, closely followed by the black tea + milk, and green tea; thereafter came the F-water group.

Multiple comparisons between the five study groups showed that the mean difference in ΔF values of the black tea, black tea +milk and green tea groups were statistically significantly higher than those of the F-Free and F-water groups. Furthermore, the tests revealed a statistically significant improvement in favour of the F-water group over the F-Free group. However, when compared the three tea groups against each other, the investigation showed insignificant differences.

Finally, the comparison between the ΔF mean values prior to and post stain removal concluded that the changes were not significant in the control groups, neither in the F-Free nor in the F-water. On the other hand, the black tea, green tea and black tea + milk groups displayed statistically significant reduction in the ΔF values after stain removal.

ΔQ outcome: The results indicated that the ΔQ mean values were statistically significantly improved within all groups, when after treatment readings were compared with those at baseline. In contrast to the F-Free water group, which showed the least amount of change in ΔQ, the black tea + milk group demonstrated the greatest level of improvement. This was followed by the green tea and black tea groups with nearly similar results, then finally the F-water group.

Multiple comparisons between the groups revealed that the mean difference in ΔQ was statistically higher in the F-water than the F-Free group. Likewise, the analyses detected statistically significant differences in the mean ΔQ values favouring the experimental groups over the controls. On the other hand, no statistical differences were found between the three tea groups.

Lastly, the comparison between the ΔQ means before and after stain removal confirmed that the changes in both F-Free water and F-water groups were insignificant. Whereas with the black tea, black tea with milk and green tea groups, the results indicated statistically significant reduction in the ΔQ values after stain removal.

The Area outcome: The comparison between the Area mean values at baseline and after treatment showed statistically significant improvements within all groups. While, the least amount of shrinkage was associated with the F-Free water group, the greatest reduction was observed in the black tea + milk group, followed by the green tea, black tea and F-water groups, respectively.

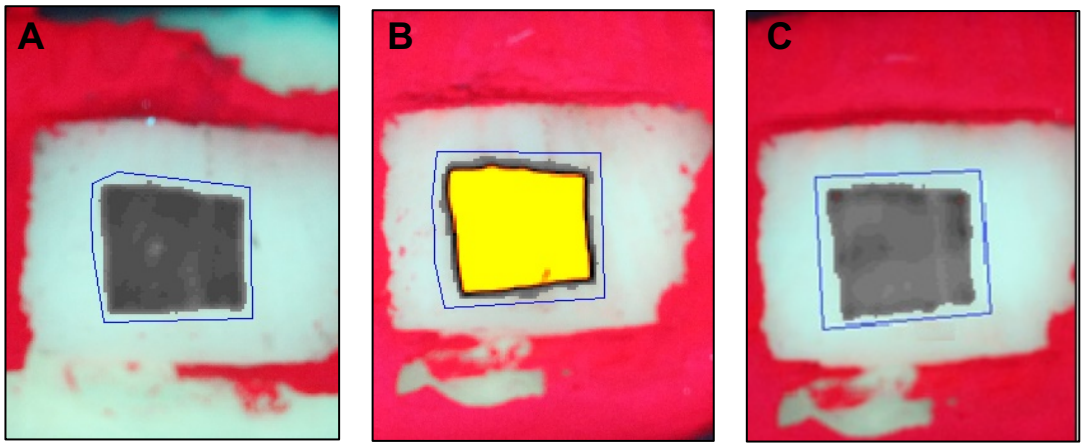
The pairwise comparisons between groups detected a statistically significant difference in favour of the F-water group over the F-Free group. Moreover, there were statistically significant differences favouring the three tea groups over the control groups. However, the statistical evaluation showed no significant differences between the tea groups.

Considering the Area mean values before and after stain removal, the results revealed no significant differences in the F-Free water and F-water groups. Nevertheless, the black tea, black tea+ milk and green tea groups demonstrated statistically significant reduction in the Area mean values after stain removal.

3.3 Quantitative light-induced fluorescence (QLF) images results

Figure 3-12 demonstrates QLF images of one enamel slab from the green tea group at different time points in the experiment.

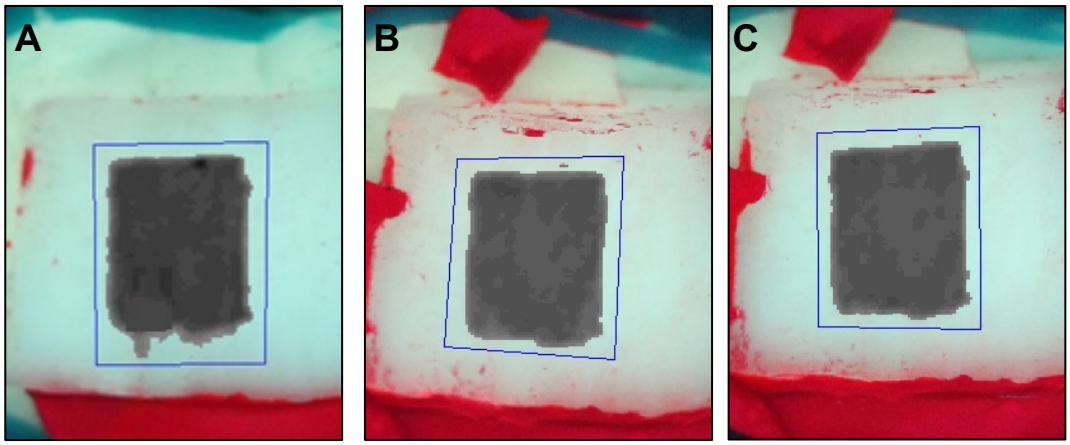
Figure 3-12 (A-C): QLF images of the same enamel slab from green tea group, taken by blue light. (A) At baseline; (B) after pH-cycling; (C) after polishing.



It can be seen from the above left image that the demineralised lesion appears very dark grey in colour at baseline. The yellow shade in the middle picture represents stain formation in the green tea group after 28 days of pH-cycling. The right picture shows the same enamel lesion after stain removal, with lighter grey shades, indicating less mineral loss.

Figure 3-13 shows QLF images from the 5.0 ppm F water group at different time points during the experiment. The baseline image exhibits a dark grey shade at the centre caused by mineral loss, whereas the middle image displays a slightly lighter shade of grey, suggesting some sort of remineralisation. Finally, the post polishing image of the enamel slab is not much different than the image taken at the end of the pH-cycling experiment.

Figure 3-13 (A-C): QLF images of the same enamel slab from 5.0 ppm F water group, taken by blue light. (A) At baseline; (B) after pH-cycling; (C) after polishing.



4 DISCUSSION

The literature search provided limited evidence on the effect of tea on remineralisation of enamel caries lesions. Even those studies that exist, appeared to exhibit poor methodological quality such as lack of sample size determination, absence of standardisation and randomisation, short term pH-cycling protocol, use of inappropriate control groups and many others. But above all, none of these studies to the best of our knowledge, has performed an in-depth analysis of some of the physiochemical characteristics of tea such as temperature, amount of tea, pH, titratable acidity and fluoride level.

Moreover, most of the research in this field has only focused on the phenolic compounds present in tea, such as that of Hamilton-Miller (1995) or Ferrazzano et al. (2009), while fluoride, which presents in significant quantities in tea beverages, has been disregarded. Additionally, most, if not all, have failed to standardise the concentration of fluoride when compared between different tea products, like those carried out by Abdulraheam (2011) and Babu et al. (2017). Consequently, they failed to identify whether the cariostatic effects of tea are due to polyphenols, fluoride or a combination of both. Further, many supportive studies about the remineralisation potential of tea have tended to focus on the green tea products, like Suyama et al. (2011) and Doi et al. (2013), whereas there are limited data about the effect of black tea, particularly when combined with milk.

Hence, the aim of this research was to further extend the current knowledge on the remineralisation effect of green tea, black tea and black tea plus milk on artificially induced enamel caries-like lesions. Additionally, one main objective of this study was to assess the physical and chemical parameters of the tea groups including the amount of tea in tea bags, temperature, pH value, titratable acidity, and fluoride concentration.

4.1 In vitro design

In this experiment, an *in vitro* model has been implemented in order to investigate the effect of black and green tea on de-/remineralisation of artificially induced enamel caries lesions.

This experimental design is increasingly being used in caries research, particularly when testing demineralisation/remineralisation effects of new potential anticariogenic agents (Yu et al., 2017).

When compared to *in situ* and *in vivo* study designs, the *in vitro* studies offer a range of benefits; as they are easily controlled, manageable, more cost-effective and less time-consuming. Additionally, owing to their simplicity, the whole design can easily be reproduced by implementing the same procedures under the same conditions (Salli and Ouwehand, 2015).

Unfortunately, such models also contain some inherent flaws. One obvious flaw is related to the fact that artificial and natural caries lesions exhibit some variations in the morphology as well as the degree of mineral loss or gain. Also, whether a simple or complex design is adopted, the system still fails to reproduce the exact biological phenomenon that occurs in the oral cavity as a result of the interaction between the oral microbiota, the caries lesion and the saliva (White, 1992; Yu et al., 2017).

However, in this experiment, the above-mentioned defects have been minimised by adopting a complex pH-cycling protocol, that lasted for 28 days, to reproduce the natural physiological process by which demineralisation and remineralisation occur in the caries lesion. In addition, artificial day and night time saliva were used in order to simulate the *in vivo* conditions as closely as possible.

4.2 Study design

This *in vitro* experiment, aimed to assess the remineralisation potential of multiple tea groups on artificial enamel caries lesions, utilising QLF as a diagnostic tool. Five study groups were tested, including 3 test groups (green tea, black tea, black tea plus milk), a positive (5.0 ppm F) and a negative (0 ppm F) control groups.

A great advantage of using an additional (5.0 ppm F water) positive control group, besides the negative control group, was the ability to keep the independent variable, namely the fluoride concentration in different solutions at comparable levels (around 5 ppm F), which in turn helped to determine if any of the tea solutions had superior remineralisation potential over 5.0 ppm fluoridated water. Moreover, this was a single-blind, randomised *in vitro* study, in which different strategies including allocation concealment, randomisation and blinding were implemented to reduce the risk of research bias. These are all essential tools to consider when conducting biological experiments (Krithikadatta et al., 2014).

Furthermore, the study was completed in two stages, with the first stage involved subjecting the artificially created enamel lesions to 4 weeks of pH-cycling and experimental solutions. The second stage was designed to deal with the extrinsic

stains developed on the surfaces of the enamel lesions after 28 days of dipping into the experimental solutions, more specifically, black, green and black tea plus milk solutions, as the tea pigments appeared to affect the QLF ability to detect the mineral loss/gain in such lesions.

4.3 Power calculation

Results of previous published studies, on the remineralisation efficacy of tea on enamel subsurface lesions, were deemed either questionable or insufficient to draw conclusions about sample size calculation. Hence, following liaison with a statistician, the sample size was determined in accordance with the central limit theorem. The later states that with a large enough sample size, at least 30 samples per group, the sampling distribution of the means will almost follow a normal distribution pattern; thus, allowing true conclusions to be made about the entire population (Browne, 1995; Kwak and Kim, 2017).

On the basis of the above considerations, a sample size of 150 enamel slabs (n=30 per group), was needed. In this experiment a total of 175 samples were prepared to compensate for any loss or damage to the slabs.

4.4 Standardisation

A key limitation with much of the literature regarding the anticariogenic effect of tea was the lack of standardisation, as most studies failed to take into account the fluoride concentration as a variable. Therefore, it is not yet known whether the cariostatic effect of tea can be attributed mainly to its phenolic compounds, fluoride content or to an additive effect of both compounds.

Zero (1995) stated that a strategy for standardisation must be considered in any research design methodology, to minimise the variation between the study groups. In this experiment, it was decided to standardise the fluoride levels in black tea, black tea + milk and green tea groups. Hence, as was mentioned in the methods, certain black and green tea bags were selected, and brewed for 3 minutes to produce infusions with concentrations of fluoride of ≈ 5.0 ppm F.

Additionally, with regards to black tea + milk group, a standard amount of 10 ml semi-skimmed milk was used when preparing each cup of tea. Further, standardisation was also implemented at another point during the experiment, namely, after the appearance of stains in the tea groups following pH-cycling, not only the stained enamel slabs but also the unstained slabs in the 0 ppm F and 5.0

ppm F water groups, were treated similarly with the designated stain removal method.

4.5 Dental substrates

Several concerns were raised about the use of bovine teeth as an alternative to human teeth in dental caries research. These could be attributed to variations in size, shape, chemical and physical properties. Hence, careful attention must be exercised when analysing and interpreting research data that are based on bovine dental substrates (Yassen et al., 2011).

An earlier paper by Clasen and Øgaard (1999) confirmed the previous findings. The authors also added that when conducting research on anticariogenic treatment modalities, the choice of dental substrate whether bovine or human plays an insignificant role. However, special consideration must be made when implementing such data in clinical practice.

There are however some problems associated with using dental specimens obtained from humans. For example, due to limited availability, it can be hard to collect enough teeth. Also, the majority of extracted teeth are of poor quality, as a result of dental diseases such as caries and/or dental anomalies. Moreover, when compared against bovine teeth, human teeth show greater variation in terms of morphology, thickness and dental structure; not to mention that the tooth surface area is reduced and more curved. Other limitations might be related to ethical considerations and infection risks (Mellberg, 1992; Zero, 1995; Yassen et al., 2011). In the current study, all samples were derived from bovine enamel specimens, owing to their availability, quality and less compositional variability. Furthermore, tooth surface areas were wide and flat; thus, more slabs were obtained from each dental specimen. Buzalaf et al. (2010) pointed that although the porosity of bovine enamel outweighs that of human enamel, allowing rapid changes with respect to progression or regression of artificial enamel caries lesions, these changes are mainly quantitative in nature.

4.6 Preparation and storage of enamel slabs

As stated earlier, the buccal surfaces of bovine teeth are flatter, also, the thickness of their enamel is more even in comparison to human dental substrates. Therefore, during teeth sectioning, the buccal surfaces were targeted as opposed to the lingual surfaces. Moreover, following teeth cutting, it was prudent to keep the enamel

samples in adequate storage media, that would allow protection against bacterial colonization as well as desiccation. Hence, Sigma Aldrich (0.1% thymol) was used along with distilled water to store the slabs.

Shapiro and Guggenheim (1995) and Titley et al. (1998) concluded that thymol could be regarded as an effective storage media, owing to its antibacterial characteristics. This could be attributed to the ability of thymol to penetrate the bacterial cell walls; thus, producing bactericidal actions against the pathogenic bacteria. Further, although previous research indicated that thymol might slightly influence the permeability of dentine, it does not appear to cause any irreversible effects on the enamel structure (Preston et al., 2007; Secilmis et al., 2013).

4.7 Enamel subsurface caries-like lesions

In the literature, many protocols have been adopted in order to create either subsurface or surface lesions. Buffered solutions or acidified gels are two separate systems, identified to be used in pH-cycling models. In either system, the dental substrates are immersed under certain pH scale (from 4.4 to 5.0), and during a particular period of time (between sixteen hours and four weeks). Based on the implemented procedures, different forms of dental lesions are produced, namely erosion-like lesions or caries-like lesions (Buzalaf et al., 2010).

In this experiment, subsurface caries-like lesions were produced by immersing the enamel slabs in an acidified gel (Acidified hydroxyethyl cellulose gel), at a pH value of 4.5, and for a period of 10 days. These standard specifications were followed to create lesions within a certain ΔF range, in order to minimise the baseline differences between the enamel slabs.

Furthermore, the acidified gel system, the one used in this experiment, compared to the buffer solution system allows formation of shallower, smaller and firmer lesions, with less, yet consistent demineralisation depths (Featherstone and Rodgers, 1981; Issa, 2004). Thus, the surface layer of the enamel is maintained, while forming a subsurface caries-like lesion that mimics the natural dental caries process. This can also be attributed to the fact that the acidified gel system is characterised by a slow diffusion rate. Thus, tooth minerals such as calcium, phosphate and fluoride, lost in the demineralisation process, are kept near the surface of the enamel; permitting redeposition of these minerals into the surface layer (Amaechi et al., 1998; Clark, 2001).

4.8 pH cycling

The *in vitro* pH-cycling model was first developed in 1982 by ten Cate and Duijsters. This model was structured to imitate the physiological conditions of mineral loss and gain that occur within the natural caries process. Many previous studies have adopted various experimental protocols with different time durations, varied between 16h to 28 days (Buzalaf et al., 2010; Yu et al., 2017). In the current study, however, the experiment was run for 28 days, allowing time for early enamel lesions to attain sufficient de-/remineralisation effect. Further, the acidogenic challenge in this experiment, was represented by subjecting the enamel lesions to five acetic acid attacks per day, at a pH value of 4.8. Other study solutions namely black tea, green tea, black tea plus milk, 0 and 5.0 ppm F water, acted as potential remineralising agents.

In order to further simulate what happens in the oral cavity, the enamel slabs were immersed in the daytime saliva, which was supersaturated with respect to hydroxyapatite, in between each demineralisation challenge. Additionally, night-time saliva, which is a saturated solution with respect to hydroxyapatite, was used to store the slabs during night-time. The protocol implemented in this study was originally developed in the University of Leeds, UK, and since then has been used, extensively, in *in vitro* caries research, conducted at the Paediatric Dentistry Department.

4.9 Quantitative light-induced fluorescence (QLF) method

In the current study, QLF technology was utilised to enable detection, quantification and monitoring of the de- and remineralisation of artificially induced enamel lesions. This method which is based on the difference in fluorescence intensity between the sound and demineralised enamel; is one of the most widespread methods used for longitudinal evaluation of early enamel lesions in dental research, including *in vitro* pH-cycling, *in vivo* as well as in clinical trials (van der Veen and de Jong, 2000; Lenzi et al., 2016).

In this experiment, QLF parameters were first measured at baseline, that is after the creation of artificial enamel lesions, then after 4 weeks of being subjected to the pH-cycling regime and experimental solutions, and finally at the end of the experiment following stain removal.

The QLF system offers many advantages; as it is non-destructive, highly sensitive, simple, feasible, relatively cheap and cost-effective. Moreover, it allows rapid and accurate detection of the small mineral changes, that occur within the demineralised enamel (Kühnisch and Heinrich-Weltzien, 2004; Pretty, 2006).

As for its effectiveness in early enamel caries detection, QLF has been proved to be a reliable and reproducible technique with high sensitivity and specificity (Shi et al., 2001; Tranaeus et al., 2002). Additionally, the validity of QLF has been widely investigated, with many *in vitro* studies comparing QLF with TMR, digital radiography and histological gold standards, have been reported high correlation coefficient values (Al- Khateeb et al., 1997a; Ko et al., 2015).

In the present study, the intra-examiner reproducibility was determined after repeating the QLF measurements; namely ΔF, ΔQ and the Area, by the same investigator, for 20 % of the sample, one week following the initial measurements. The statistical test detected that the ICC value was 0.99, for all the QLF parameters; which is indicative of an excellent reliability. These findings correlate favourably with Al- Khateeb et al. (1997a) and Tranaeus et al. (2002).

It is important also to highlight that the captured QLF images were saved instantly on a computer, with three digital images taken of each enamel lesion, at three different stages during the course of the experiment; after which all the analyses and reanalyses were performed, with the aim of minimising bias as well as errors in measurement.

However, just like any other analysing tool, QLF has its limitations such as subjectivity. Therefore, in this experiment the examiner received intensive training and calibration with regards to acquisition and analysis of QLF images. Further, the reliability of QLF can be affected by multiple variables, such as presence of dental plaque, calculus and stains, absence of a dentine layer, light and humidity (Tranaeus et al., 2001; Amaechi and Higham, 2002; Pretty et al., 2004). Hence, image acquisition was always performed under standardised environmental conditions, namely in a dark room, and after exposing the enamel slabs to compressed air for fifteen seconds. Additionally, the camera was stabilised by a stand, in the same position and angulation, and at a certain level from the enamel slab, every time a picture was taken.

As to the QLF parameters, in this *in vitro* study, all the three values of ΔF, ΔQ and the Area were assessed and evaluated. Ando et al. (2004) suggested that ΔQ,

which represents the lesion volume, should be considered as the main indicator when evaluating early enamel lesions. This recommendation is based on the fact that ΔQ is the product of ΔF (percentage fluorescence loss) times the Area, also that the changes in ΔF and Area variables might not always be consistent. Alternatively, other authors reported to use ΔF as the primary parameter for assessment of demineralised lesions (Alammari et al., 2013; Lee et al., 2018). However, the ideal cut-off parameter has not been decided on yet, as this will require further evidence.

4.10 Physiochemical analyses of tea solutions

One objective of this research was to determine the temperature, amount of tea in tea bags, concentration of fluoride, pH value and titratable acidity of black tea, green tea and black tea plus milk groups. The purpose of this objective was to perform a general assessment of the physical and chemical characteristics of these test groups, that is to understand the physiological nature of these solutions and to understand what implications these parameters might have on the final outcome.

In this experiment, the water used in the preparation of test and control groups was brought to boil. The enamel slabs were only dipped in the solutions when the temperature ranged from (50-55) °C, in order not to melt the wax that bound the enamel slab to the plastic rod of the universal tube. In a study by Gupta and Sandesh (2012), to determine the amount of fluoride in different tea brews, prepared from tea bags or tea packets containing either leafy or granulated tea, using room temperature or boiled water; the authors found that the mean fluoride concertation of tea brews prepared from boiled water was significantly higher than those made with un-boiled water. However, one downside regarding their study is that the comparisons were made using different forms of tea. Hence, the effect of the available surface area in relation to fluoride release was overlooked. Also, they failed to identify what types of tea were used, as fluoride concentrations in tea infusions may vary considerably depending upon the type used. Moreover, Waugh et al. (2016), suggested that the temperature of the tea infusion may affect its fluoride concentration, but in their study, this was neither explained nor evidenced.

As for the amount of tea measurements, in the current study, the mean weight of tea was 3.20 ± 0.05 and 2.20 ± 0.04 grams in the black and green tea bags, respectively. These measurements were essential to obtain, in order to calculate the required amount of water and to standardise the preparations. In line with Tiwari et

al ’s (2005) study and the International Organisation for Standardisation (ISO) guidelines, the current study implemented a standard tea to water ratio, that is for every 2 grams of tea, 100 ml of water was needed.

Considering fluoride measurements, as mentioned earlier, the target was to use tea solutions with comparable levels of fluoride (≈ 5.0 ppm F). In this experiment, the mean fluoride concentration in the black and green tea brews were 5.13 ± 0.09 and 5.06 ± 0.10 ppm F, respectively. With regards to the black tea + milk infusions, a slight increase in the mean fluoride concentration was noticed following milk addition (5.30 ± 0.15 ppm F). The latter finding is in line with previous results such as those of Cao et al. (2004), and a more recent study by Xiarchou (2016), who stated that adding milk to tea resulted in an increased fluoride concentration, however, this increase was not significant.

Moving on to the pH and titratable acidity parameters, it has been established that the tea solutions used in the current study were acidic in nature. However, the acidity levels varied considerably, especially when compared to the mean pH value of black tea brews which was 4.90 ± 0.14, to green tea brews which had a mean value of 5.70 ± 0.10 or black tea plus milk infusions with a mean pH value of 5.90 ± 0.08. Hence, these results underline that both green tea and black tea plus milk infusions are less acidic than black tea brews. Additionally, the total volume of KOH (base) required for neutralisation of 20 ml of black tea was 0.9 ml; while for green tea and black tea + milk solutions, less volumes of titrant, 0.7 and 0.5 ml, respectively, were needed.

To reflect on these results, although the mean pH value of black tea brews was under the critical pH for demineralisation of dental enamel, such beverage contains considerable amounts of fluoride and polyphenols. Furthermore, the mean pH values of both green tea and black tea plus milk infusions were above the critical pH (> 5.5). These pH values correlate favourably with Xiarchou ’s (2016) findings. Besides, an *in vitro* study conducted by Jameel et al. (2016), to assess the erosive effect of some acidic beverages such as coffee, black tea, Coca-Cola and orange juice on dental enamel concluded that exposure to acidic solutions, containing high levels of fluoride, such as black tea, resulted in significantly less enamel roughness compared to other acidic beverages, that contained no fluoride, like Coca-Cola. Another systematic review by Jaâfoura et al. (2014), looked at the associations between different tea types and dental erosion, indicated that while fruity tea, ginger-vanilla tea, iced tea and sugary tea caused dental erosion, the black and

green tea produced no erosive effects, provided that no sugar was added to the infusions. Unfortunately, their review was based on low-quality evidence.

4.11 Tea-induced staining obstacle

As for the staining issue that has arisen in this study, after four weeks of being subjected to pH-cycling and study solutions, the enamel slabs in the three tea groups developed extensive staining on the surfaces of the lesions. Although the potential for tea to cause stains was considered prior to experimentation, it was thought that exposing the enamel slabs to five acetic acid attacks, during the day, would counteract the tea-induced staining effect.

Moreover, in light of the above consideration, a further test group, namely black tea plus milk was included, in addition to the preselected black and green tea groups. Lee et al. (2014) conducted an *in vitro* investigation to determine if adding milk to tea could inhibit teeth discolouration, utilising VITA Easyshade Compact, spectrophotometer analysing tool. The authors concluded that milk addition to tea resulted in a significant reduction of teeth stains. However, apparently, their approach was insufficient as they only considered a short experimental duration. Hence, in the current study, even black tea plus milk group caused extensive discolouration, after the long duration of the experiment, which lasted for 28 days.

Simpson et al. (2001a) conducted an *in vitro* experiment to assess the affinity of fluoride, present in black tea, to enamel powder by means of column chromatography and fluoride selective electrode methods. The investigation detected a strong association between black tea stains and fluoride retention within the enamel particles evidenced by measuring the fluoride concentration in these pigmented particles, after retrieving them from the upper parts of the columns. The authors also stated that the extrinsic discolouration, caused by excessive tea consumption, occurs as a result of incorporating the tea pigments into the surface of the enamel as well as the acquired dental pellicle. Nevertheless, in the current study, the latter factor was absent owing to the *in vitro* nature.

Simpson et al.'s findings were consistent with Prathap et al. (2013), who concluded that the acquisition of black tea particles, namely tannins by the enamel and pellicle, leads to extrinsic discolouration, as these polyphenolic compounds are naturally brown.

As was stated in the materials and methods chapter, multiple pilot studies were conducted in parallel with the main experiment; for which the first aim was to assess the efficacy of QLF to determine the progression/regression of artificial enamel lesions if associated with stains and the second to explore different techniques of stain removal.

4.12 Method development

The first *in vitro* pilot study was designed to assess the ability of QLF to analyse the demineralised enamel lesions, if accompanied with stains. Initially, bovine teeth were used to create three enamel slabs with subsurface caries lesions; after which baseline QLF measurements were obtained. During the second stage, staining procedures were initiated by subjecting the enamel lesions to black tea, black tea + milk, or green tea; in order to simulate conditions of the main experiment. Then, an incubator was used to store the slabs at a temperature of 37°C, for 24 hours. These procedures were performed twice, in order to allow adequate accumulation of stains. At the end of the experiment, the enamel slabs were analysed by QLF to obtain the endpoint measurements. The results showed that the efficacy of QLF was impaired when assessing demineralised enamel lesions, superimposed with stains; as the instrument was not able to differentiate between demineralisation and staining.

Although, few authors hinted that the presence of stains might be a disadvantage when using QLF to assess the de-/remineralisation of white spot lesions (Tranaeus et al., 2001; Amaechi and Higham, 2002); to date no single study has been conducted to either examine this in depth or to at least develop a solution to the problem.

Further, in the literature, there have been few studies that evaluated the potential use of QLF as an analysing tool to assess and measure teeth staining and whitening (Adeyemi et al., 2006; 2010); but these analyses were done purely on sound rather than demineralised, stained teeth. Therefore, their findings could not be applied directly to this research, as they adopted different aims and approaches.

Several teeth whitening methods have been proposed by many researchers, such as the use of 10% or 33% hydrogen peroxide in multiple cycles, of 150 minutes each; 2g of sodium perborate in cycles over two minutes duration; combination of hydrogen peroxide and sodium perborate or intermittent exposure to 12% NaOCl (Amaechi and Higham, 2002; Adeyemi et al., 2006; 2010; Zimmerli et al., 2010).

Nevertheless, in the present study, it was prudent to come up with an effective technique to eliminate the stains, but at the same time, not to modify the microstructure of the demineralised enamel.

Therefore, the second *in vitro* pilot experiment was carried out to investigate different approaches to remove the stains from the demineralised enamel lesions, including, bleaching with 1.0%, 2.5% or 5.25% NaOCl; bleaching with 10% CP; combined bleaching using NaOCl + CP or mechanical cleaning with soft rubber cup and extra fine, fluoride-free, pumice. Additionally, different time intervals and application regimens were explored, until the stains were removed completely. As demonstrated in the methodology chapter, effective removal of stains was achieved when using either the combined bleaching therapy or the polishing procedure.

Consequently, a third *in vitro* pilot experiment was conducted to investigate the effects of these stain removal procedures on artificial enamel caries lesions, particularly with regards to the measurements of ΔF, ΔQ and the Area. Thus, 12 bovine enamel slabs were immersed in an acidified hydroxyethyl cellulose gel, for a 10 day period, until early caries lesions were produced; after which QLF was used to perform the baseline analysis. Thereafter, the enamel samples were distributed evenly and randomly to four different groups, namely 5.25% NaOCl for 30 minutes, 10% CP (2 applications × 2 minutes), combined bleaching (NaOCl + CP) or polishing with soft rubber cup and extra fine pumice grit (for 10 seconds duration). At the end of the experiment, QLF was used again to obtain the final readings.

As stated in the methods chapter, the biggest changes in ΔF, ΔQ and the Area were associated with NaOCl group, whereas the effects of polishing on demineralised enamel lesions were too small to be of concern. Therefore, the present pilot study successfully concluded that the polishing technique, using a soft rubber cup and extra fine, fluoride-free, pumice grit, for 10 seconds duration, was the procedure of choice to effectively remove the stains from the artificial enamel caries lesions, without interfering with the surface microstructure.

When comparing the findings presented here to the existing literature, they appear to be in good agreement. Research on enamel pre-treatment with NaOCl, prior to phosphoric acid etching, to improve the enamel bond strength has traditionally suggested that the application of NaOCl to the enamel surface, would interfere with the organic content through a process called deproteinisation (Espinosa et al., 2008; Pereira et al., 2013). However, in a more recent *in vitro* study by Pellillo (2015), carried out to investigate the effects of NaOCl on the composition of dental enamel;

the author stated that the mechanism by which NaOCl influences the dental enamel is not only limited to the organic components, but extends to include the inorganic content as well. Further, Abdelmegid (2018) in his *in vitro* investigation, pointed out that enamel deproteinisation with 2.5% NaOCl increases its surface roughness. Sim et al. (2001) conducted a randomised controlled clinical trial to investigate the effects of 5.25% NaOCl irrigation on dentine of single-rooted premolars. The conclusion was that NaOCl resulted in a significant reduction in the modules of elasticity as well as the tensile strength of root dentine. There results were consistent with Slutzky-Goldberg et al. (2004), who also concluded that dentine exposure to 2.5% NaOCl, resulted in a significant reduction in dentine microhardness, and that the effect was greater when 6% NaOCl was used. Thus, the authors recommended to use NaOCl in lower concentrations to avoid interfering with dentine microstructure. However, in the current pilot study, neither the 1% nor the 2.5% NaOCl was effective in stain removal, when used in intermittent cycles over 30 minutes each.

Considering the effect of CP on human dental enamel, an early *in vitro* study by Potočnik et al. (2000), found that 10% CP bleaching gel decreased the concentrations and ratios of calcium and phosphate ions within the dental enamel. Moreover, when the enamel samples were viewed under electron microscopy, local structural alterations in the surfaces of the enamel, which were comparable to those of demineralised enamel lesions, were visible. Additionally, it has been found that the use of 10% CP could also lower the concentration of fluoride in dental enamel and negatively impact the surface microhardness (Oliveira et al., 2005). However, recent research reported that the effect of 10% CP on the surface topography of dental enamel was insignificant, confirmed by electron microscopy (Farawati et al., 2019).

Finally, with respect to the chosen method, that is the polishing technique to overcome the staining issues, although no studies have specifically addressed this in relation to QLF parameters; in the current experiment, the decision to explore this technique was based on the investigator's judgment and best available evidence.

Darby (2012) suggested that the level of abrasivity of the polishing material as well as the size and shape of grits, that is the particles, dictate the effects on teeth surfaces; with the finer, spherical and softer particles, producing less damage to the enamel surface (Sawai et al., 2015). The authors also added that there are other essential factors to consider during the process of teeth polishing; such as the

duration of contact time, speed and pressure applied. Hence, in this experiment, the decision was to use an extra fine pumice grit in order to avoid, or if not possible, to minimise morphological and structural changes to dental enamel. Besides, each enamel slab was polished for 10 seconds, as this was the shortest required contact time to effectively remove the stains. Moreover, the speed of the handpiece was slow and even, and only the least amount of pressure required to keep the handpiece spinning was exerted. Also, the pressure and speed factors were further regulated by applying steady pressure on the foot pedal.

Chowdhary and Mohan (2018) investigated and compared the effects of dental polishing procedures, using rubber cup, air polisher or bristle brush on surface roughness of enamel and cementum, by means of scanning electron microscopy. The authors concluded that the use of rubber cup significantly prevented the increased surface roughness of both enamel and cementum, as opposed to the other two polishing modalities, namely dental air polisher and bristle brush. This is in line with the currently adopted polishing technique, as in this study, soft rubber cups were used to prevent unnecessary damage or changes to the dental enamel.

4.13 The stain removal experiment

As per the successful results obtained from the above pilot experiments, a decision was made to implement these findings and procedures on a large scale.

Hence, the experiment proceeded in the following order; after 28 days of subjecting the enamel slabs to pH-cycling and experimental solutions, QLF analysis was done in order to measure ΔF, ΔQ and the Area parameters, prior to applying the stain removal technique. Thereafter, the polishing (stain removal) method was used to polish the enamel slabs in all the groups, the stained and the unstained enamel lesions. This was done to eliminate any discrepancies between the groups, and furthermore minimise bias. Subsequently, QLF analysis was conducted again to obtain the final readings.

The reason for measuring QLF parameters of the enamel slabs in all study groups, prior and post execution of the stain removal procedure, was to be able to confirm statistically if any significant relationships that existed at the end of the experiment were due to the effects of tea solutions rather than the polishing procedure on enamel de-/remineralisation, or vice versa.

Consequently, in this experiment, when the mean differences in ΔF, ΔQ and the Area, before and after stain removal, for all the groups were compared; the analysis

revealed that there were no statistically significant differences in either control groups, namely 0 ppm and 5.0 ppm F water. Whereas, significant differences were detected within the black tea, green tea, and black tea plus milk groups. These results share a number of similarities with Amaechi and Higham ’s (2002) findings; as the authors stated that lesion staining had enhanced the QLF measurements. Moreover, a recent *in vitro* study by Lee et al. (2019), to assess the effectiveness of a tooth bleaching material on bovine enamel, utilising QLF system, concluded that the fluorescence intensity tends to decrease with enamel staining and increase with enamel bleaching.

To reflect on the current study, the demineralised enamel lesions in the three tea groups were masked with stains, following 28 days of pH-cycling. Owing to this effect, the QLF parameters deteriorated significantly. However, upon elimination of the stains, the enamel lesions regained their fluorescence colour, allowing QLF to accurately assess the remineralisation efficacy of black tea, green tea and black tea plus milk, on artificial enamel caries lesions.

Whereas in the control groups, the enamel lesions maintained their true fluorescence colour, as they had been immersed in transparent solutions. Additionally, even after implementing the polishing protocol in the 0 and 5.0 ppm F water groups, no statistically significant differences were detected between pre and post-stain removal measurements. Thus, the findings of this study indicated that the polishing procedure, as per the developed protocol, did not interfere with enamel de-/remineralisation. Moreover, the present study has managed to find an innovative approach to improve the efficacy of QLF for early enamel caries detection in the presence of stains.

4.14 Remineralisation efficacy of different tea infusions on artificial enamel caries lesions

This *in vitro* experiment was performed in order to evaluate and compare the remineralisation potential of three different tea infusions, in particular, green tea, black tea and black tea plus milk on artificially induced enamel subsurface lesions. Further, two controls, positive (5.0 ppm F) and negative (0 ppm F) water groups were included in this study.

When comparing the QLF parameters, namely ΔF, ΔQ and the Area, at baseline to those after the treatment; the analysis revealed statistically significant remineralisation effects within all study groups. Considering the ΔF outcome, the highest recovery was observed in the black tea group, followed by the black tea plus milk, green tea, and 5.0 ppm F water groups; whereas the lowest remineralisation effect was associated with the 0 ppm F water group. With regards to the other QLF parameters (ΔQ and the Area), the highest improvement was demonstrated in the black tea plus milk group, closely followed by the green and black tea, respectively. Next appeared, the 5.0 and 0 ppm F water groups in descending order.

Although, the control groups (5.0 ppm and 0 ppm F water) demonstrated significant regression in all QLF measurements, within the groups; this was to a significantly lesser extent than the test groups (green tea, black tea, black tea plus milk). A reasonable explanation for this is that the enamel slabs in the control groups, were placed in daytime saliva (for 60 minutes, 7 times a day, during the whole duration of pH-cycling). This daytime saliva was supersaturated with respect to hydroxyapatite, which in turn allowed enamel remineralisation to a certain extent. The latter is in line with Cochrane and Reynolds (2012), who stated that supersaturated saliva could induce enamel remineralisation by allowing diffusion of calcium and phosphate ions into demineralised lesions. Nevertheless, owing to the low concentration and slow diffusion rate of these minerals across the saliva-lesion interface, only the surface of the lesion would have the tendency to remineralise (van der Veen et al., 2007; Philip, 2019).

Further, when comparing the groups against each other in terms of ΔF, ΔQ and the Area, by applying multiple comparison tests along with Bonferroni correction; it was revealed that the remineralising efficacy of green tea, black tea and black tea plus milk groups on artificial enamel lesions were statistically significantly higher than the 5.0 ppm and 0 ppm F water groups ($p<0.01$). Also, as expected, the present study

demonstrated a statistically significant difference in favour of the 5.0 ppm F water group over the 0 ppm F water group ($p<0.05$).

Although, only few researchers have addressed the remineralisation potential of tea products on demineralised enamel lesions, the current findings seem to correlate favourably with previous results such as that of Abdulraheam (2011), who investigated the effects of black and green tea on enamel demineralisation *in vitro*, and concluded that owing to the bioavailability of fluoride, both tea groups demonstrated high remineralisation efficacy; and another review by Ahmed et al. (2017), who deduced that the consumption of green tea compounds may result in significant caries regression, due to their antimicrobial as well as remineralisation properties.

Further, the current study indicated that the remineralisation potential of the three tea groups (black tea, black tea plus milk and green tea), with each containing approximately 5 ppm F, were significantly higher than the positive control (5.0 ppm F water) group ($p<0.01$). In spite that the concentration of fluoride was standardised among the aforementioned solutions, significant differences were found in favour of the tea groups. Therefore, this would appear to indicate that other tea components such as polyphenols, might have played a role by either exerting a direct remineralisation effect or at least potentiating the remineralisation effect of fluoride on demineralised enamel lesions.

Yu et al. (1992) conducted combined *in vivo* and *in vitro* studies to investigate the potential anticariogenic effects of green tea extract in hamsters. The key advantage of their research was that they performed electrodialysis in order to remove the fluoride from tea solutions. Surprisingly, the green tea extract still exerted the same anticariogenic effects on dental caries, even after fluoride elimination. Hence, it was concluded that such effects are most likely correlated with the polyphenolic content in tea.

In contradiction with earlier findings, Yu et al. (1995) tested the effects of different tea extracts, such as catechin and tannin, with or without fluoride, on acid resistance capacity of human enamel *in vitro*. The authors suggested that when either type of polyphenols were combined with fluoride, a significant synergistic effect to inhibit enamel dissolution was produced.

To the best of our knowledge, there have been very few studies in the literature that compared directly between the black tea, green tea and 0.05 % NaF solution. In the

first study, which was a quite recent *in vitro* study by Babu et al. (2017), the remineralisation potential of black and green tea on artificially induced enamel lesions was tested against 0.05% NaF and deionised water, by means of laser-induced fluorescence. The authors concluded that remineralisation of demineralised enamel lesions was noticed, though it was different, in black tea, green tea, and 0.05% NaF groups. However, the remineralisation efficacy of green tea was higher than that of NaF, and that both demonstrated significantly higher remineralisation potential than black tea and deionised water groups. Unlike the present study, they made no attempt to standardise the concentration of fluoride within the test groups, as they not only failed to determine the amount of fluoride in the black and green tea groups, but also, they used an inappropriate control group (0.05 % NaF solution), which contained significantly higher level of fluoride (around 225 ppm F). Additional limitations were related to the lack of sample size calculation and short experimental duration, as their *in vitro* model was based on just 7 days of pH-cycling. Therefore, their conclusion is inconclusive.

In another similar experiment, the effects of polyphenolic green tea extract mouthwash (0.5%) and 0.05 % NaF rinse on cariogenic bacteria in saliva, among school children, were compared in a double-blind randomised controlled trial. The trial concluded that the two interventions, namely green tea and NaF mouthwashes produced equivalent results when compared against each other, as they both led to significant reduction in salivary bacterial counts (Tehrani et al., 2011). In their experiment, again, they failed to take into account the fluoride concentration variable; on the grounds that the fluoride level in the green tea was only 1.4 ppm, whereas the NaF mouth rinse contained 221 ppm F, yet both groups demonstrated comparable findings.

There is still considerable controversy with regards to whether black tea is more efficacious than green tea or vice versa, particularly that the majority of the research on this subject had focussed only on the efficacy of green tea as an anti-caries measure, such as those of Suyama et al. (2011), Indrani et al. (2015) and Ahmed et al. (2017), which were mentioned earlier.

The current study, interestingly, did not detect any significant differences between the black and green tea groups. These findings can be accounted for, in part, by the fact that none of the previous studies had standardised the concentration of fluoride in tea brews.

In this study, however, the fluoride variable was controlled by using tea solutions with comparable levels of fluoride (around 5 ppm). Also, during the course of the experiment, strict criteria, based on preliminary as well as regular measurements of the fluoride concentration in tea groups, were implemented.

Another possible speculation about this finding could be attributed to quality rather than quantity or type of tea polyphenols. In the medical literature, it has been stated that theaflavins in black tea and catechins present in green tea, possess equivalent antioxidant properties, thus producing similar health benefits (Leung et al., 2001; Fuchs et al., 2014). Other studies, however, suggested that green tea extracts may provide better antioxidant characteristics than black tea, owing to their polyphenolic constituents (Lee et al, 2002; Chan et al., 2011).

As far as the dental aspect, Hara and Honda (1990) underlined that catechins and theaflavins derived from green tea and black tea, respectively, demonstrated similar antimicrobial effects on dental caries *in vitro*; as they both successfully inhibited the enzyme amylase, and prevented the decomposition of starch to maltose. Their results were consistent with another *in vivo* study by Zhang and Kashket (1998).

On the other hand, results of another *in vitro and in vivo* experiment by Al-Ezzi et al. (2018), to assess the antimicrobial effects of black and green tea extracts on dental caries bacteria, revealed that green tea extracts resulted in significantly lower bacterial count than black tea extracts. Nevertheless, their approach suffered from several pitfalls such as lack of sample size determination and randomisation, and absence of control groups. Also, in the *in vivo* experiment, the investigators failed to consider the effects of other confounding factors, namely oral hygiene measures, on the cariogenic bacteria.

Further, as put forward by Abdulraheam (2011), although both black and green tea compounds revealed high remineralisation effects on demineralised enamel lesions *in vitro*; the green tea resulted in complete remineralisation as opposed to black tea, which showed incomplete remineralisation patterns, evidenced by the polarised light microscopy and chemical analysis. Again, the main issue with their research was the lack of standardisation, as the concentration of fluoride in black and green tea was considerably different.

Finally, in the present study, although black tea plus milk group demonstrated significant remineralisation efficacy when compared to 5.0 and 0 ppm F water

groups; the analysis revealed that there were no significant differences between the black tea plus milk group and the green or the black tea group.

The reason for the latter finding is still not completely understood but could be interpreted as that addition of milk to black tea infusions resulted in the formation of some calcium fluoride compounds, which in turn slightly limited the bioavailability of free soluble fluoride and calcium ions, compromising the efficacy of milk. Thus, black tea plus milk infusions produced no additive remineralisation effects over black or green tea group.

In the literature, there have been few studies with regards to the effect of adding milk to black tea on remineralisation of demineralised enamel lesions. Rahardjo et al. (2014) conducted an *in vitro* investigation into the remineralisation efficacy of milk, milk plus tea and milk plus 0.2% NaF on artificial enamel caries lesions, utilising micro-computed tomography system. The authors claimed that milk alone group, produced a statistically significant remineralisation effect whereas no significant differences were detected when compared with the other groups, namely milk plus 0.2% NaF and milk plus tea in terms of remineralisation. However, their study suffered many limitations which could have affected the findings such as the duration of the pH-cycling experiment, which lasted only for three days. Further, the adopted cycling protocol differed significantly from other well-established procedures. Another critical flaw of their method was that neither the baseline concentrations of calcium and fluoride were considered, nor the amounts of tea and milk were stated.

Contrary to the findings of Rahardjo et al., Doi et al. (2013) carried out a controlled clinical trial to investigate the anticariogenic properties of chewing gum, incorporating green tea extract and calcium phosphoryl oligosaccharides (POs-Ca) compared to (POs-Ca) alone on early occlusal caries of first permanent molars, by means of QLF. The experiment extended for a year, after which the authors concluded that green tea extract added to POs-Ca, had significantly reduced the early caries lesions compared to POs-Ca alone. Doi et al ’s approach however, lacked essential criteria that need to be at least considered in any effective clinical trial system such as randomisation and blinding. In addition, their results might be limited by the fact that the investigators failed to account for other variables, like oral hygiene practices and dietary habits.

4.15 Suggestions for future research

In this experiment, the enamel slabs were dipped directly into the tea infusions. Perhaps, it would be worthwhile in the future to conduct *in vivo* or *in situ* studies to explore different tea formulations, such as toothpastes or mouthwashes that contain either black or green tea extract.

Moreover, this *in vitro* study indicated that tea solutions are acidic in nature, even after the addition of milk to tea. Hence, it might be interesting to assess the erosive effects of black and green tea on dental enamel.

Furthermore, future studies might consider using different analytical methods when assessing the protective effects of black and green tea on dental enamel, such as scanning electron microscopy, microhardness, TMR or Micro-CT.

Finally, the results obtained from this *in vitro* experiment are very promising, but, before transferring these findings into clinical practice, prospective, well-designed, randomised controlled trials with large sample sizes and long follow-up durations need to be conducted.

4.16 Null hypothesis outcome

- The null hypothesis "There is no difference in the effects of black tea, green tea, black tea plus milk, non-fluoridated (0 ppm F) and fluoridated (5.0 ppm F) water, on de-/remineralisation of artificially induced enamel caries-like lesions *in vitro.*" can be rejected as statistically significant differences, in terms of remineralisation efficacy, were identified favouring the test groups (black tea, green tea, black tea plus milk) over the control groups (0 and 5.0 ppm F water) ($p<0.01$). Additionally, another statistically significant difference in the enamel remineralisation efficacy was detected in favour of the 5.0 ppm F water group over the 0 ppm F water group ($p<0.05$).

CONCLUSIONS

The evidence from this *in vitro* study has concluded the following:

- Compared to baseline, a statistically significant remineralisation effect on early enamel lesions, was detected in all study groups.
- Comparisons between groups revealed that the remineralising efficacy of green tea, black tea and black tea plus milk groups on artificial enamel lesions were statistically significantly higher than those of 5.0 ppm and 0 ppm F water groups ($p<0.01$).
- An additional statistically significant difference, in terms of remineralisation efficacy, was detected in favour of the 5.0 ppm F water group over the 0 ppm F water group ($p<0.05$).
- There was no difference between the remineralising efficacy of green tea, black tea and black tea plus milk groups on artificial enamel lesions.
- Tea consumption may provide considerable anti-cariogenic effects, however owing to its fluoride content, careful attention should be paid not to exceed the recommended daily intake of fluoride.
- Finally, despite a limitation of the QLF method, which had initially affected the instrument's capacity to assess the enamel de-/remineralisation in the presence of staining, the current study has successfully addressed this limitation.

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APPENDICES

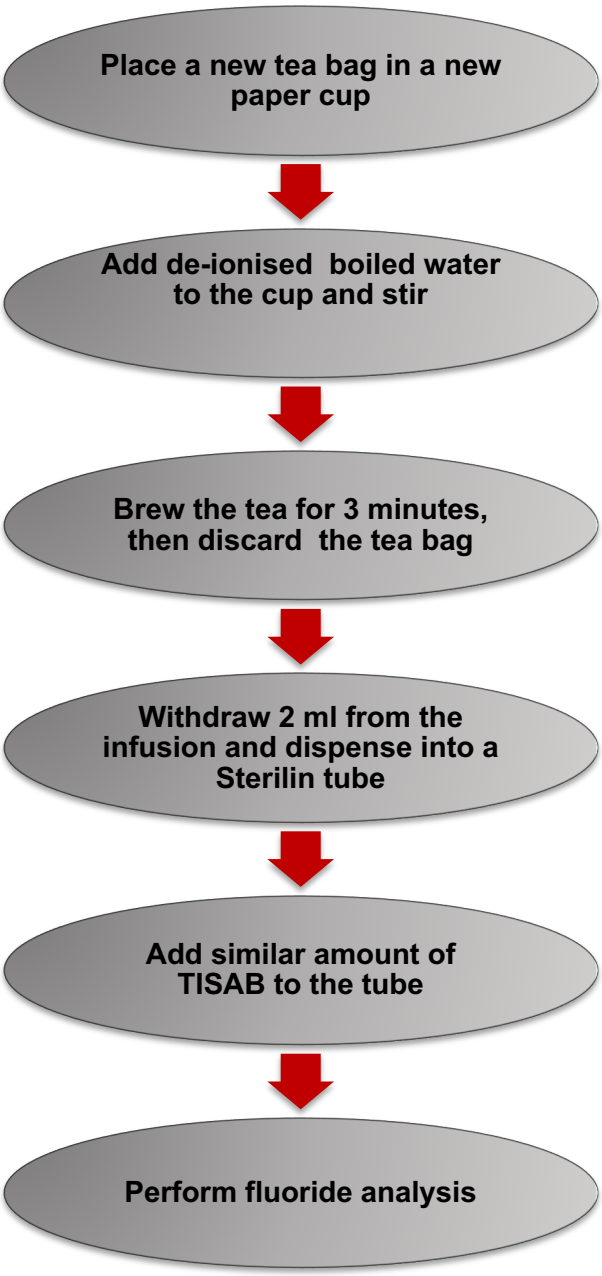
Appendix 1: Randomisation plan.

Available from http://www.randomization.com

8. A	3. B	4. C	1. D	7. E
9. A	6. B	10. C	2. D	11. E
12. A	14. B	25. C	5. D	13. E
27. A	15. B	30. C	18. D	20. E
39. A	16. B	31. C	26. D	21. E
41. A	17. B	32. C	35. D	23. E
44. A	19. B	33. C	45. D	24. E
58. A	22. B	34. C	47. D	28. E
72. A	36. B	40. C	49. D	29. E
75. A	37. B	50. C	57. D	51. E
76. A	38. B	53. C	62. D	60. E
79. A	42. B	56. C	63. D	61. E
81. A	43. B	59. C	65. D	67. E
89. A	46. B	64. C	66. D	69. E
100. A	48. B	74. C	70. D	73. E
103. A	52. B	84. C	77. D	80. E
111. A	54. B	85. C	78. D	82. E
113. A	55. B	87. C	88. D	83. E
115. A	68. B	94. C	93. D	86. E
116. A	71. B	95. C	98. D	92. E
118. A	90. B	97. C	104. D	96. E
121. A	91. B	102. C	109. D	99. E
123. A	108. B	105. C	120. D	101. E
126. A	110. B	107. C	124. D	106. E
136. A	117. B	112. C	128. D	114. E
138. A	119. B	122. C	129. D	130. E
141. A	132. B	125. C	131. D	133. E
142. A	139. B	127. C	134. D	135. E
148. A	147. B	137. C	144. D	140. E
149. A	150. B	143. C	145. D	146. E

To reproduce this plan, use the seed 19420

Appendix 2: Flow chart summarising the steps involved in fluoride analysis of tea brews prepared by continuous infusion method.



Appendix 3: Full certificate of analysis for Kemdent flour of pumice oral extra fine (FFF grade), detailing the sieve analysis and the chemical analysis.

Amorphous Aluminum Silicate

Hardness (Mohs Scale).....6
pH.....7.2
Radioactivity.....0
Loss On Ignition.....5%
Iron as Fe.....No Blue Color
Water Soluble Substance.....15%
Acid Soluble Substance.....2.9%
Softening Point.....900° C

Chemical Analysis

		Typical %
Silicon Dioxide	SiO_2	70.5
Aluminum Oxide	Al_2O_3	13.5
Ferric Oxide	Fe_2O_3	
Ferrous Oxide	FeO	0.1
Moisture	H_2O	3.4
Sodium	Na	1.6
Potassium	K	1.8
Calcium	Ca	0.8
Titanium Oxide	TiO_2	0.2
Sulfur Trioxide	SO_3	0.1
Magnesium Oxide	MgO	0.5

TYPICAL SCREEN ANALYSIS OF GRADES
AVERAGE PERCENT PASSING SIEVES

Size	-325	FFFF	FFF	FF	F	0	0 1/2	0 3/4	1/2	1	1 1/2	2	3	5	7	10	Micron
4																100	4750
8															100	73	2360
10														100	90	38	2000
14												100	100	77	65	7	1400
30												99	21	5	2	4	600
40										100	100	57	7				425
50									100	72	31	11	4				300
60							100	100	75	51	13	6	2				250
80						100	90	77	10	3	4						180
100	100				100	97	75	43	4								150
120					98												125
140				100	93	79	55	9									106
170		100	100	98													90
200	98	99	97	91	65	55	35	4	2								75
325	89	85	76	70													45
Unit wt.	45	45	45	50	55	55	60	55	50	50	47	41	43	43	41	42	Lbs./C.F.
A.P.S.	16	22	33	30	84	95	122	188	277	329	386	503	950	1330			Micron
Sp. Gr.	2.36	2.210	2.175	2.227	2.112	2.187	2.187	2.153	2.037	2.068	2.035	1.866	1.764	1.799	1.605	1.476	Gms./C.C.

Appendix 4: Temperature of the study solutions at different time points.

Time point	Temperature (°C)														
	Black tea			Black + milk			Green tea			5.0 ppm F water			0 ppm F water		
After pouring boiled water in cups	83	81	82	82	84	83	80	82	81	86	83	85	83	84	85
At 3 min of brewing	71	70	72	68 64*	69 65*	69 66*	69	71	70	74	72	73	69	70	71
At 0 min of dipping	55	54	54	50	50	51	52	54	53	55	54	55	54	54	55
At 10 min of dipping	45	44	44	40	41	42	43	44	43	45	44	45	43	44	45

. After milk addition.