THE EFFECT OF TEA ON SURFACE LOSS OF THE DENTAL ENAMEL UNDER EROSIVE CHALLENGE *IN VITRO*

By Maryam Al-Shezawi

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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.

Dedication

This is dedicated to my beloved family

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Abstract

Aim: To investigate the effect of black and green tea on surface loss (progression) of dental enamel under erosive challenge *in vitro.*

Materials and Methods: A total of 150 bovine enamel slabs were subjected to a pH cycling regime with erosive challenges (0.3% citric acid, pH 2.6). The slabs were randomly assigned to 5 treatment groups: black and green tea (≈ 5.0 ppm F), black tea with milk, black tea with citric acid 1.0% pH (3.6) and fluoride-free water (control). Within 28 days of the pH cycling period, the slabs were exposed five times for 2 minutes' periods in erosive challenge citric acid plus three times for 10 minutes in each of 5 treatment groups. Throughout the cycling period the slabs were stored at 37 ºC in artificial saliva in an incubator. The slabs were analysed with the Profilometer to measure the amount of surface loss at days 7, 14, 21 and 28 of the pH cycling period.

Results: Data showed normal distribution by Kolmogrov-Smirnov test and the overall appearance of Q-Q plot for the variables. The results showed that the mean enamel surface loss for each group increased over time.

One-way ANOVA test with Bonferroni correction was used to compare between groups. There was a significant difference in enamel surface loss after 28 days erosive challenge when comparing between the fluoride free-water (control) group with black tea and black tea infusion with additions of milk or citric acid groups ($p<0.05$), but there was no difference in comparison with green tea group.

There was no difference on enamel surface loss between black tea and green tea, and black tea infusion with citric acid groups after 28 days of erosive challenge. However, the black tea with milk group showed a significant less enamel surface loss ($p<0.05$).

Conclusions: All black tea groups with or without additions (citric acid or milk) were significantly beneficial with respect to enamel surface loss under erosive challenge.

Furthermore, black tea infusion with addition of milk provides the greatest protection against tooth surface loss in our model.

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List of Abbreviations

%	Percentage
3D	Three Dimensional
ºC	Degree of Celsius
µm	Micrometre
AFM	Atomic force microscopy
AHDB	Agriculture and Horticulture Development Board
ANOVA	Analysis of Variance
BC	Before Christ
CHX	Chlorhexidine
CPP-ACP	casein phosphopeptide-amorphous calcium phosphate
df	Degree of freedom
DOM	demineralized organic matrix
EDJ	Enamel-dentine junction
EGCG	Epigallocatechin-3-gallate
ESEM	Environmental Scanning Electron Microscope
F	Fluoride
g	Gram
GORD	Gastro-oesophageal reflux disease
H^+	Hydrogen ion
KHN	Knoop Hardness Number
Mg/L	Milligram / litre
ml	Milliliter

mm	Millimetre
mm/min	Millimetres Per Minute
mmol/l	Millimoles per liter
MMPs	Matrix metalloproteinase
nm	nanometres
oz	ounce
p	p-value
pH	Acidity
ppm	Part per million
Q-Q	quantile-quantile
SD	Standard deviation
SEM	Scanning Electron Microscope
Sig	Statistical level
SIMS	Secondary ion-mass spectroscopy
SL	Surface Loss
SP	Scantron ProScan
SPSS	Statistics Package for the Social Sciences
TSL	Tooth surface loss
UK	United Kingdom

1. Introduction

Erosive tooth wear is considered one of the major dental disorders in both adults and children (The Royal College of Surgeons, 2013) and is ranked as the third most important dental condition after dental caries and periodontal disease (O'Toole and Mullan, 2018). Certainly, dental erosion has been seen as great a concern over the last number of years as other dental lesions in the oral cavity (Barbosa et al., 2011). The incidence of dental erosion has increased over recent years according to daily observation among dental practitioners as well as the large number of academic publications on the subject (Lussi and Jaeggi, 2011). A number of studies have supported this fact by increased reporting of dental erosion in both adults and children (Simpson et al., 2001, Taji and Seow, 2010, Lussi and Jaeggi, 2011).

Tea is a commonly consumed beverage worldwide and specifically, it is considered the second most popular drink after water (Lin et al., 2003, Wang and Ho, 2009, Sang et al., 2011). It is considered one of the healthier beverages as it has antioxidant properties.

Previous studies have shown that some natural and chemical products in tea may have anti-erosive effects. Substances such as polyphenol found in green tea act as a matrix metalloproteinase inhibitor reducing the degradation of dentine.

Whilst reviewing the literature it has been found that most of the studies on tea and dental erosion investigate the effect of tea on dentine. There are limited well conducted studies evaluating the effect of tea on tooth surface (enamel) erosion.

2. Literature Review

2.1 Dental Erosion

Dental erosion is one type of tooth wear which is defined as progressive, irreversible loss of dental hard tissue by a chemical process without bacterial involvement (Pindborg, 1970) and with no direct relation with other factors such as mechanical or traumatic factors or with dental caries (The Royal College of Surgeons, 2013). Although the causes of dental erosion are different than other types of tooth wear (attrition and abrasion), the diagnosis of dental erosion can be difficult if the erosion coexists with attrition and/or abrasion (The Royal College of Surgeons, 2013). Dental erosion is called 'near surface demineralisation', which means that along with removal of the surface, acidic solution starts to soften a thin enamel layer by dissolving the mineral crystal within enamel pores. This process helps to distinguish the difference between caries and erosion, in which the dental caries process is the destructive loss of enamel and occurs in both surface and subsurface layers (Lussi and Ganss, 2014). The early recognition of dental erosion and identifying the factors that cause dental erosion, along with preventive measures, can avoid significant damage to the dentition (Abdullah, 2009).

An acidic diet is considered a main cause of dental erosion. Erosion that is caused by drinks that are carbonated and other acidic drinks is not only dependent on their pH but also on other factors that cause or reduce erosion such as their mineral content, titratable acidity and calcium-chelating properties. Supplementation of acidic drinks with compounds such as calcium, phosphate, fluoride, proteins, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) may reduce the erosivity of these drinks (Barbosa et al., 2011).

2.2 Mechanism of Dental Erosion (Chemistry of Dental Erosion)

Oral soft and hard tissues are covered by the acquired salivary pellicle, which is a biofilm, free of bacteria, derived from proteins, glycoproteins, enzyme and mucins. The pellicle is considered an anti-erosive barrier which in turn lubricates the dental surface (Hannig et al., 2005). However, when acid penetrates through the acquired enamel pellicle, the hydrogen ions (H^+) will dissolve the enamel crystals by starting to dissolve the prism sheath, and then the prism core, to form a honeycomb appearance (Meurman and Frank, 1991a). The non-ionized form of the acid can also penetrate the enamel pores. Once within the enamel, these molecules then ionise which, acting as H^+ carriers into enamel pores, then dissolve enamel crystals (Featherstone and Rodgers, 1981). Therefore, the minerals of dental hard tissue (carbonated hydroxyapatite) will dissolve when combined with hydrogen ion (H^+) in acid (Featherstone, 2000). This process depends primarily on the concentration of the non-ionised form of the acid within enamel pores rather than on the buffering properties of the acid itself (Shellis et al., 2013). As H^+ ions from citric acid are released, the citrate ion binds with calcium which is known as calcium chelation (O'Toole and Mullan, 2018) .

With regards to dentine, the presence of matrix metalloproteinase (MMPs) in dentine and saliva, which are defined as host-derived enzymes that expose the organic matrix to break down, cause a removal of the demineralized organic matrix (DOM) by collagenases, which in turn increases the demineralisation process. The presence of MMPs in dentine and saliva accelerate the progression of dentine erosion (Buzalaf et al., 2012b). In contrast, according to Lussi and Ganss (2014), the presence of organic dentine matrix can reduce the demineralisation process because it prevents diffusion of acid deeper into hard tissue and it has sufficient buffering capacity to delay the demineralization.

Picos et al. (2013) described the mechanism of dental erosion as follows: when decalcification occurs from acid and chelating agent, destruction of the saliva organic pellicle protecting the tooth surface occurs first, then solubilisation of the dental structures which may lead to destruction of tooth structure along with mechanical friction or brushing.

2.3 Clinical features and diagnosis of dental erosion

The teeth most significantly affected by dental erosion are maxillary teeth and specifically the incisal, palatal and occlusal surfaces. However, the buccal or labial surfaces are not excluded. The dentine becomes exposed as the enamel layer appears thin or lost, and patients are often concerned about the aesthetic appearance and sensitivity as the teeth become shorter in size (The Royal College of Surgeons, 2013).

Clinically, erosion can be detected early by noticing that the surface of a tooth appears smooth, silky-shiny and sometimes having a dull surface with concavities showing on smooth surfaces (Lussi and Ganss, 2014). When erosion progresses, the cusps, grooves and incisal edges become rounded, and in more advanced stages this leads to loss of occlusal morphology (Taji and Seow, 2010). On smooth surfaces, erosion leads to convex and flattened areas with a dished out smooth appearance seen where the width of the erosion exceeds its depth (Taji and Seow, 2010, Ganss and Lussi, 2014).

Taking a history to distinguish dental erosion from other tooth wear is essential in diagnosing dental erosion (The Royal College of Surgeons, 2013). However, there is no diagnostic device to assess the erosion lesion and therefore it can only be detected clinically, and thus the diagnostic approach is visual diagnosis rather than an instrumental approach (Ganss and Lussi, 2014). The methods of monitoring dental erosion include study cast and photographs which allow visual estimation of wear progression and could help decision making on treatment options (The Royal College of Surgeons, 2013, Ganss and Lussi, 2014). In the worst affected

areas, a silicon putty impression may be a helpful method to assess progression at a subsequent recall appointment, where the putty index is placed over the teeth and any space between the putty index and the tooth surface is evaluated (The Royal College of Surgeons, 2013). Many indices for measuring erosive tooth wear have been proposed which have their root in or are modifications of the indices of Eccles and Smith and Knight (Bardsley, 2008). An accurate index should be used in clinical examination and the dentist must be aware of the diagnostic criteria to maintain good intra-examiner reliability (Lussi and Jäggi, 2008, The Royal College of Surgeons, 2013). Another recent clinical record of dental erosion is the basic erosive wear examination (BEWE) which was developed and recommended in 2008 by Barlett et al. and is a simple scoring system based on a sextant exam of worst wear in the tooth surface (Bartlett et al., 2008, Donovan et al., 2021). The O'Sullivan (2000) was designed an index to measure erosion specifically in children, allowing more specific evaluation of affected tooth surface area and severity of erosion (Salas et al., 2015, Bardsley, 2008).

2.4 The prevalence of dental erosion

Most of studies examining prevalence of erosion are found in children and adolescents rather than in adults. This may be because of easier accessibility of surveying children in schools and other public institutions. The global prevalence of dental erosion in the primary dentition is between 30% and 50%, and for permanent dentition is between 20% and 45% (Schlueter and Luka, 2018).

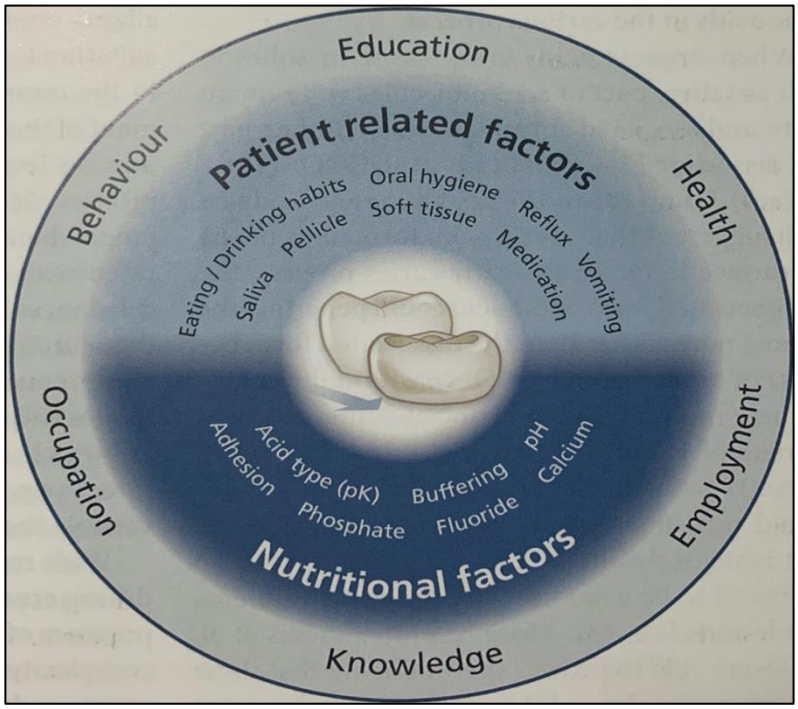
The Child Dental Health survey 2013 in England, Wales and Northern Ireland reported that 33% of 5-year olds had evidence of tooth surface loss (TSL) on one or more buccal surfaces of the primary upper incisors, and 4% overall had tooth surface loss involving dentine or pulp. With regards to older children, for those aged 12 and 15, the dental erosion of the permanent incisors on the buccal surface was found to be 24% and 27% respectively. However, the tooth

surface loss affecting dentine and pulp was consistently low for both ages, reporting only 1% of those aged 15 years having this extent of TSL on the surface of permanent incisors and first permanent molars (Pitts et al., 2015).

2.5 Aetiology of dental erosion

The aetiology of dental erosion can be identified by knowing the behaviour, lifestyle and dietary intake of the patient. The association between erosion and dietary factors is present but weak and needs further research to fully understand the exact relationship between erosion and other co-factors (O'Sullivan and Curzon, 2000b, The Royal College of Surgeons, 2013). The multifactorial process interacts with each other and the interplay of all these factors is important to understand and may explain why some individuals have more erosion than others. Figure 1 illustrates the interaction of these factors for the development of erosive tooth wear (Lussi and Ganss, 2014).

Figure 1: Interactions of the different factors for the development of erosive tooth wear (Lussi and Ganss, 2014)



These factors include intrinsic and extrinsic factors and other modifying risk factors of dental erosion.

2.5.1 **Intrinsic factors**

Gastric acid is the most common source of intrinsic acid inside the mouth and has a pH between 1-1.5. Dental erosion has been observed when chronic direct contact between teeth and gastric acid occurs during vomiting or reflux and can be seen in some conditions such as gastro-oesophageal reflux disease (GORD), anorexia nervosa, bulimia, rumination, alcoholism and pregnancy (Linnett and Seow, 2001, Taji and Seow, 2010, Amaechi, 2015).

GORD is a common occurrence which has been reported worldwide with a prevalence ranging between 9% to 33% in various countries and in the Western World about 7% of adult are affected daily, and one third every few days. However, GORD is considered less common in children (The Royal College of Surgeons, 2013, Dundar and Sengun, 2014). There is a strong association between the development of dental erosion and the prevalence of GORD, especially in adults, and should always be considered as a potential cause of dental erosion with the presence of epigastric pain or indigestion heartburn (Taji and Seow, 2010, The Royal College of Surgeons, 2013).

2.5.2 **Extrinsic factors**

Several extrinsic sources of acid may contribute to the development dental erosion including medication, lifestyle, occupational, environmental and diet (Taji and Seow, 2010).

2.5.2.1 Medication

The frequent consumption of medicine with low pH and high titratable acidity over a long period of time, has the potential to cause dental erosion (Hellwig and Lussi, 2014). Some medications reduce salivary flow and/or buffering capacity of saliva which induce dry mouth, including some tranquilizers, antiemetics, antihistamines and antiparkinsonian medicaments (Taji and Seow, 2010, The Royal College of Surgeons, 2013, Hellwig and Lussi, 2014). In addition, Vitamin C supplements, chewable aspirin tablets and asthmatic medications have been reported to potentially cause dental erosion (Taji and Seow, 2010, Hellwig and Lussi, 2014).

2.5.2.2 Lifestyle

The change in life toward leisure, active lifestyles and some fashion trends have the potential to increase risk of dental erosion (The Royal College of Surgeons, 2013), as well as the change toward the total amount and frequency of the consumption of acidic drinks and food. Paradoxically, healthier lifestyles can also lead to dental erosion. Diets that depend on, for example, lactovegetarian foods may increase the consumption of acidic foods in the diet and have been reported to increase the development of dental erosion. It has also been reported that exercise increases loss of fluid which may cause dehydration and decrease salivary flow. Another example of the effect of exercise is swimming in low pH gas-chlorinated pools which may also increase prevalence of dental erosion (Lussi and Jäggi, 2008). On the other hand, unhealthy lifestyle could also contribute to developing dental erosion. Individuals with increased alcohol consumption are considered an at-risk group for dental erosion, as drinks such as wine have a low pH which may lead to dental erosion, as well as increased consumption of alcohol promoting gastric reflux (Lussi and Jäggi, 2008, Peycheva and Boteva, 2016).

2.5.2.3 Occupational and environmental

Work involving daily exposure to acid may result in developing dental erosion. Factories which produce acidic fumes or aerosols such as sulphuric acid in battery factories and hydrochloric acid in galvanizing factories may lead to increased incidence of erosion where good health and safety processes are not in place (Linnett and Seow, 2001, The Royal College of Surgeons, 2013). There are other occupations that have been shown to increase susceptibility to dental erosion such as those workers in munitions factories and professional wine tasters (Linnett and Seow, 2001).

2.5.2.4 Diet

The consumption of either acidic foods or drinks is the most causative extrinsic factor documented in the literature (O'Sullivan and Curzon, 2000b, Taji and Seow, 2010). Common extrinsic dietary acids include citric acid, malic acid, carbonic acid, ascorbic acid and phosphoric acid (Wang and Lussi, 2010). The erosive potential of different foodstuffs and drinks is not determined by pH only, but is also influenced by mineral content (calcium, phosphate and fluoride), type of acid and buffering capacities (Lussi and Jaeggi, 2011). The duration of the drink's contact with tooth structure, temperature and the frequency of consumption also have an impact on erosive potential (Barbosa et al., 2011).

2.5.3 Dental erosion and drinks

Consumption of acidic drinks have been increasing amongst adolescents and children and this in turn has become the most significant factor reported for causing dental erosion (Shellis et al., 2005, Tahmassebi and BaniHani, 2020). The physical property of the drinks consumed, the time of exposure of the drink on the tooth surface, and the drinking habit have an impact on erosive tooth wear. One of the examples of physical property is the flow rate of drinks around

the mouth which has been shown to influence the erosive tooth wear (Shellis et al., 2005). It has been shown that when increasing the liquid's velocity, it has reduced erosive effect on the tooth surface. Drinking habits such as holding drinks in the mouth and swishing them between the teeth have an association with rapid erosion and for this reason it has been recommended to use a straw when drinking potentially erosive drinks to avoid direct contact and increased time between the erosive liquid and the tooth surface (Shellis et al., 2005).

As described previously, there are different chemical parameters of drinks that can contribute to progression of dental erosion and dissolve the enamel. These include pH, titratable acid content, buffering capacity and calcium, phosphate and fluoride concentration. There is the possibility of reducing erosive potential for some beverages by modifying these factors. For instance in drinks formulations, by using malic acid instead of citric acid, or supplementation of beverages with calcium, phosphate and fluoride the erosive potential can be reduced (Lussi et al., 2004). It has been shown that fluoride alone is unlikely to reduce the effect of erosion, but it can help the reduction of the process with the addition of calcium and phosphate (Lussi et al., 2004).

Solutions with low pH, high titratable acidity and low buffering capacity have more potential to cause dental erosion. However, solutions containing a high concentration of phosphate and calcium cause less enamel dissolution (Zohoori and Duckworth, 2019).

There is correlation between the prevalence of dental erosion and the consumption of carbonated beverages, soft drinks, fresh fruit, alcoholic and other drinks with erosive potential (Lussi et al., 2004). According to the Child Dental Health survey 2013, differences in the prevalence of tooth surface loss amongst children was dependant on the frequency of acidic drinks intake, where it is shown that more wear was found with high-frequency consumption. It has also been shown that children who drank water less than once a day were more likely to have dental erosion (O'Sullivan and Curzon, 2000a). Furthermore, a number of studies

emphasise that the development of dental erosion is associated with an increased frequency of fruity and soft drinks consumption (Millward et al., 1994, Milosevic et al., 1997, Al-Dlaigan et al., 2001b, Dugmore and Rock, 2004).

Soft drinks include bottled water, carbonated drinks, dilutable 100% juice, sport and energy drinks as well as still and juice drinks (British Soft Drinks Association, 2020). According to British Soft Drinks Association in 2020 showed the consumption of soft and fruity drinks in the UK, with about 13,659 million litres of soft drinks being consumed per year. With regard to consumption of fruit-based drinks (100% juice including smoothies and coconut water) about 891 million litres are consumed per year. It is suggested that drinking fruity drinks and smoothies among all are helping to achieve the recommended intake of five portions of fruit and vegetables per day as recommended by the Department of Health to avoid and reduce some chronic conditions. However, they have a high level of sugar and acid which can have an impact on oral health, by causing dental caries and dental erosion (portAli and Tahmassebi, 2014). Other studies also confirm that these drinks can lead to dental erosion (Sukeri S, 2010, Blacker and Chadwick, 2013). In addition, it has been shown that addition of fruit or fruit flavourings to drinks have the equivalent erosive potential to the cola type drinks (O'Toole and Mullan, 2018) .

Tea is a commonly consumed beverage worldwide and specifically, it is considered the second most popular drink after water (Lin et al., 2003, Wang and Ho, 2009, Sang et al., 2011). It is considered one of the healthier beverages as it has antioxidant properties and other benefits which will be discussed below. However, herbal teas which do not use the traditional black tea are considered erosive as they have high amounts of citric acid as they are based on dried fruit. Indeed, it has been found that some herbal teas are more erosive than orange juice (Phelan and Rees, 2003).

2.6 Risk factors of dental erosion

The other predisposing factors that have an impact on developing dental erosion are chemical, biological and behaviour factors. It is very important to understand how their interaction can cause significant damage and comprehensive knowledge of these risk factors is essential to start preventive treatment and minimise the invasive treatment (Lussi et al., 2004, Lussi and Jäggi, 2008).

2.6.1 Chemical factors

The chemical factors that influence the erosive potential in beverages are pH and buffering capacity, type of acid, chelating properties, fluoride concentration, calcium concentration, phosphate concentration and adhesion of the product to the dental surface (Lussi et al., 2004).

Previous research reported that beverages with a low pH (below 4.0) cause dental erosion (Meurman and ten Cate, 1996, Rugg-Gunn et al., 1998). The enamel begins to erode when the critical pH is below 4.5 (Benjakul and Chuenarrom, 2011). The drink's pH is not the primary initiating factor for causing dental erosion. The buffering capacity of drinks or titratable acidity has been shown to play a significant role in causing dental erosion (Owens, 2007). Titratable acid is the total acid level rather than the pH of drinks and it determines the actual hydrogen ion availability for interaction with the tooth surface, which is considered as an important factor for the development of dental erosion (Tahmassebi et al., 2014). It has been proven that the higher the buffering capacity the drinks have, the longer it will take for the saliva to neutralise the acid and then the more apatite that might be dissolved prior to high a pH level being reached and dissolution coming to a halt. (Wang and Lussi, 2010). The intraoral pH which is usually pH 6.8 decreases below pH 5 within 2-3 minutes after drinking acidic drinks. Following this,

the pH intraorally takes about 25 minutes to change the acid environment as further stimulated saliva is needed for neutralisation (Borjian et al., 2010).

The concentration of calcium, phosphate and fluoride ions along with pH value are important factors in dental erosion, in that they determine the degree of saturation with respect to the tooth mineral, which is the driving force for dissolution (Zero and Lussi, 2005, Wang and Lussi, 2010). Solutions supersaturated with respect to enamel and dentine will not dissolve tooth mineral. A low degree of undersaturation with respect to enamel or dentine will lead to an initial surface demineralisation, which is followed by a local pH rise and increased content of mineral in the fluid layer adjacent to the surface. This layer then becomes saturated and does not lead to further demineralisation of the tooth (Zero and Lussi, 2005, Wang and Lussi, 2010). For example, the pH of yoghurts is low (pH ~ 4) but they are not erosive because they have high calcium and phosphate concentrations (Lussi et al., 2004, Wang and Lussi, 2010, Carvalho et al., 2015).

Temperature is one of the factors to be considered when the dietary acid is being consumed (O'Toole and Mullan, 2018). In vitro study has shown that in the erosive drinks, there is a linear relationship between increasing temperature and enamel softening followed by increase tooth surface loss (Barbour et al., 2006). O'Toole and Mullan (2018) reported an example of a patient that had erosive tooth wear after drinking hot tea with lemon flavouring multiple times throughout the day.

2.6.2 **Biological factors**

Biological factors play a significant role in the dental erosion process, including factors such as saliva, acquired pellicle, tooth composition and structure, dental anatomy and occlusion,

anatomy of oral soft tissue in relationship to the teeth and physiological soft tissue movements (Lussi et al., 2004).

Saliva contributes to pellicle formation which is considered an important erosive protective mechanism. The acquired pellicle acts as a diffusion barrier or perm-selective membrane, preventing direct contact between acid solution and tooth surface. This in turn reduces the dissolution rate of dental hard tissue (Buzalaf et al., 2012a). There are other erosion protective functions of saliva which include dilution and clearance of an erosive agent from the mouth, neutralisation and buffering of acid and slowing down of the rate of enamel dissolution through the common ion effect by salivary calcium and phosphate (Zero and Lussi, 2000, Zero and Lussi, 2005). It has been proven that the softened enamel by acid agents can reharden after exposure to saliva or remineralisation solution and that dairy products and fluoride can enhance the re-hardening process (Lussi et al., 2004).

There is association between development of erosion and low salivary flow and/or low buffering capacity, and the testing of the stimulated and unstimulated flow rate and buffering capacity of saliva could provide some information about susceptibility of an individual to tooth surface loss (Buzalaf et al., 2012a). There are some conditions that may reduce salivary flow such as vigorous exercising which may cause dehydration, and some systemic diseases may affect salivary flow such as Sjögren Syndrome, which is a chronic autoimmune disorder (Buzalaf et al., 2012a).

The tooth position and anatomy in the arch, occlusion and soft tissue play a role in the development of dental erosion (Lussi, 2006). It has been shown that the pH on the tooth surface after drinking 1% citric acid recovered above pH 5.5 within 2 minutes from a site adjacent to the palatal surface of the upper central incisor compared to the upper first molar, where the pH recovered to above 5.5 within 4-5 minutes. Furthermore, the soft tissue movement of the tongue and buccal mucosa and swallowing pattern can influence the clearance rate of erosive agent

(Lussi et al., 2004). Nevertheless, it has proven that licking with the tongue exacerbates the loss of enamel under the erosive influence of acidic soft drinks (Seong et al., 2017). Lussi et al. (2004) reported that the tongue can be a contributing factor in erosion caused by vomiting and the physiological tongue movements in relation to the size of the tongue and size of the dental arch may also contribute to some extent to dental erosion. Järvinen et al. (1992) found that when the dietary or gastric acid enters the mouth, the tongue acts as a reservoir for acid, in which the tongue binds the acid, which consequently touches the upper anterior teeth and causing dental erosion. Animal studies supported this evidence whereby the erosion was observed on the lingual surface of rat molars where is the tongue contact with the teeth under beverages influence (Wynn and Meiller, 2001).

2.6.3 **Behavioural factors**

Behavioural factors can influence and play a significant role in development of dental erosion, for example eating and drinking habits, healthier lifestyle which include diets high in acidic fruit and vegetables, excessive consumption of acidic food and drinks, night time baby bottle feeding with acidic beverages and oral hygiene practices (Lussi et al., 2004).

An association between the socioeconomic status and development of dental erosion has been found, in which the socioeconomic status might contribute to different eating, drinking and oral hygiene habits (Carvalho et al., 2014). Alves et al. (2015) reported that dental erosion is more common in deprived communities and the consequence of developing tooth surface loss might be affected by differences in dietary factors and lifestyle amongst socioeconomic strata in developing and developed countries. Following a more healthy lifestyle by drinking juices, and taking sport drinks after exercise could also cause erosive tooth wear (Skalsky Jarkander et al., 2018).

The method of intake of dietary acids and the ways of drinking and keeping the drinks for a long period in the mouth has an influence on the development of dental erosion (Johansson et al., 2002).

Frequent tooth brushing with abrasive oral hygiene products may enhance dental erosion (Lussi et al., 2004). A study showed that patients could benefit from tooth brushing before, rather than after, erosive attack to minimise enamel and dentine wear (Wiegand et al., 2008). However, brushing prior to meals can remove salivary pellicle and the enamel surface will be more susceptible to acid attack during the meal (Kuroiwa et al., 1994).

2.7 Dental complications of dental erosion

Several clinical problems are caused by dental erosion including reduced aesthetics, dentine sensitivity, pulpal inflammation and exposure in immature teeth and difficulty in eating. Severe tooth surface loss can cause enamel fracture and this can progress to shortening of the teeth and loss of occlusal vertical dimension (Linnett and Seow, 2001). Epidemiological studies conducted on over 3000 adults in nine European countries have observed a strong and progressive relationship between dentine hypersensitivity and erosive tooth wear (West et al., 2013).

2.8 Management of dental erosion

Early detection of dental erosion with good follow up and professional advice may avoid any further progression of erosion. Identifying the causative factors and monitoring the erosion is essential prior to beginning with definitive restoration treatment either in children or adult patients (The Royal College of Surgeons, 2013). Furthermore, the patient's compliance toward changing their lifestyle, including the dietary habits that increase susceptibility to dental erosion, may have an impact in preventing further progression of tooth surface loss. Advising

the patient of appropriate prevention measures in order to enhance remineralisation and for reducing progression of dental erosion and elimination of symptoms by the use of fluoride mouth rinses, high fluoride toothpaste or Tooth Mousse ^{R} is important (Amaechi and Higham, 2005, The Royal College of Surgeons, 2013). There is no indication for any restorative treatment in the primary dentition if the child is not having symptoms, however, restoration with composite resin might be considered if a child has sensitivity in small areas of erosion. More severe erosion with symptoms might require coverage with stainless steel crowns or extraction of the tooth. In the mixed dentition, treatment includes long term monitoring or applying composite to eroded teeth in order keep the teeth protected as long as possible. In the permanent dentition, treatment is provided to reduce sensitivity, prevent progression and improve aesthetics. Covering the eroded surface with a composite restoration or veneer or any kind of restoration in order to provide an acceptable appearance, functional outcome and reduce any sensitivity is currently the best option (The Royal College of Surgeons, 2013).

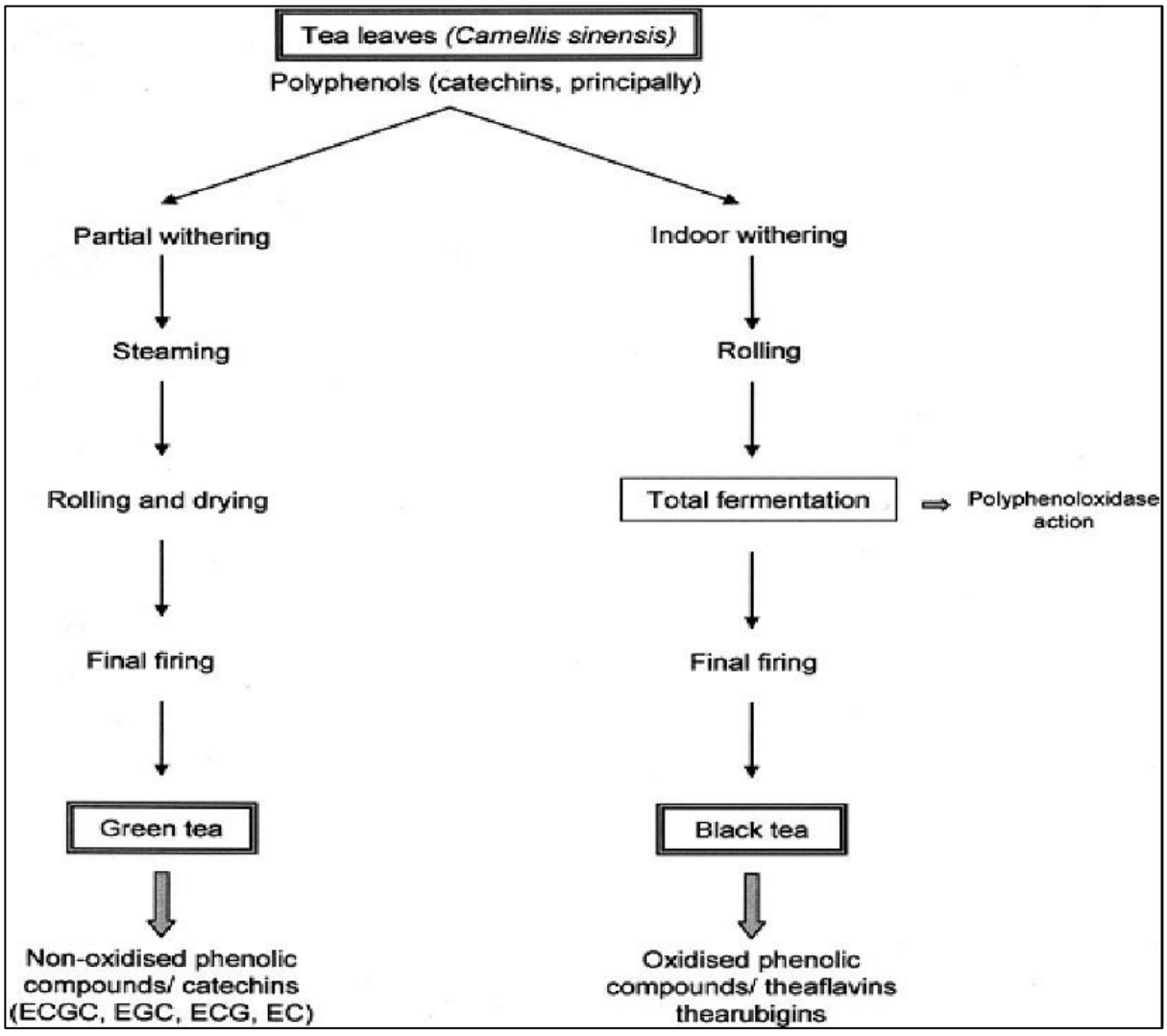
2.9 Tea

2.9.1 History of tea

The most common kinds of tea are produced from the leaves of *camellia sinensis* which are grown in several different countries including China, India, Japan, Sri Lanka, Indonesia and the Central African countries (Sang et al., 2011). The *camellia sinensis* tree historically was introduced by the Chinese emperor Shen Nung (2737 BC), and the drink tea was introduced when humans discovered how to boil water. It then spread worldwide, to Japan and overseas through Dutch traders (Trevisanato and Kim, 2000). Initially the Chinese used tea as a medicine, then as a beverage at the end of 6th century. Tea is categorised into three varieties: black tea (fully fermented), green tea (non-fermented) and oolong tea (partially fermented)

(Lin et al., 2003). Figure 2 shows the difference between black and green tea processing and how its influence on the final polyphenols content (Hayat et al., 2015).

Figure 2: Principal differences between green and black tea processing and its influence on the final polyphenols content



2.9.2 **Components of tea and its health benefits**

The components of tea include polyphenols, alkaloids, amino acids, glucides, proteins, volatile compounds, caffeine, minerals and trace elements (potassium, sodium, calcium, fluoride, chromium, iron, manganese, selenium and aluminium). Green tea has additional components which are considered of interest to human health such as fluoride, caffeine, minerals and trace elements such as chromium and manganese (Reto et al., 2007). The major component of tea is polyphenols (flavonoids) and it has been shown that polyphenols have the potential for disease prevention in conditions such as cardiovascular disease, cancer and aging (Wang and Ho, 2009). In addition, it has been shown to have a good effect in defending against harmful effects from the environment, for instance ultraviolet radiation and pathogens (Wang and Ho, 2009). Polyphenols in tea are also known as catechins. The amount of catechins is different between each type of tea, for instance, 2g of green tea leaves and 200 ml water contains 240-320mg catechins (30-50% epigallocatechin-3-gallate (EGCG)), while oolong tea comprises six different bioactive catechins. However, black tea is considered fully oxidised which leads to conversion of the catechins to theaflavins and thearubigins as a result of fermentation. Other components of black tea are phenolic acids (caffeic, quinic and gallic acid) and theanine (Ruxton, 2013, Passos et al., 2018). These phenolic acids are considered to be strongly antioxidant with radicular scavenging and metal chelating properties, and also possessing anti-inflammatory, antimicrobial, anti-hypertensive, anti-diabetic, anti-tumour, anti-carcinogenic, hepatoprotective, immunoprotective, neuroprotective, anti-atherosclerotics and anti-neoplastic properties (Robbins, 2003). The tannic acid in tea has found to inhibit the growth of Streptococcus mutans (Wu-Yuan et al., 1988).

2.9.3 **Oral health benefits of tea**

Tea has an impact in oral diseases such as dental caries, periodontal disease and tooth loss (National Institute of Dental Research US, 1989). Wu and Wei (2002) reported several studies that have shown the benefits of tea consumption in reducing dental caries in human and experimental animals. It has been proven that the EGCG in green tea, when binding with salivary protein, can be a potent anti-cariogenic reagent (Hara et al., 2012). Various components of black and green tea have properties that suggest anti-cariogenic activity which include a direct bactericidal effect against *Streptococcus mutans* and *Streptococcus sobrinus* by several different stages: prevention of bacterial adherence to teeth, inhibition of glucosyl transferase, limiting the biosynthesis of sticky glucan and inhibition of human and bacterial amaylases (Hamilton-Miller, 2001, Melok et al., 2018). It was found that rinsing by 0.2% of Chinese green tea during brushing, reduces plaque and gingival index significantly (You, 1993). However, a study of Korean adults has shown that drinking less than one cup of green tea was associated with a decreased prevalence of periodontal disease, whereas drinking one or more cups per day could increase the prevalence of moderate and severe periodontitis (Han et al., 2016).

Previous study by Kaneko et al. (1993) found that mouth washing with a dilute catechin solution for 4 weeks regimen, reduced halitosis associated with periodontal disease. Venkateswara et al. (2011) demonstrated that green tea has bactericidal activity against Prevotella and *Porphyromonas gingivalis* and EGCG inhibits growth and adhesion of *Porphyromonas gingivalis* to buccal epithelial cells.

2.9.4 **Health risks of tea consumption**

The presence of tannins in black tea reduces the absorption and inhibits the bioavailability of non-heme iron. Similarly, green tea infusions can also decrease the bioavailability of non-

heme iron from the diet. Thus, it is recommended that patients who suffer from anaemia should avoid tea consumption (Hayat et al., 2015). Furthermore, a systematic review on the effect of black tea drinking on non-heme iron status in the UK concluded that consumption of tea decreased the non-heme iron absorption from the diet and it was also observed that there was statistically significant correlation between tea drinking and poor non-heme iron status amongst preschool children (Nelson and Poulter, 2004).

The presence of trace elements (metal with high relative atomic mass) in tea may have adverse effects on human health – for example, accumulation of aluminium in tea infusions is associated with Alzheimer's disease (Karak and Bhagat, 2010).

Some studies have found that the high consumption of black tea infusions over the long term may lead to exposure to a high amount of fluoride which may result in dental and skeletal fluorosis (Malinowska et al., 2008, Emekli-Alturfan et al., 2009).

Simpson et al. (2001) reported that the excessive consumption of tea may lead to problems of staining of the dentition which is caused by interaction of tea's components with both surface integuments such as acquired salivary pellicle and the mineral crystal of dental enamel, and this can represent a cosmetic problem. It was also reported that in groups at risk of iron deficiency, in young infants and the elderly, the excessive consumption of tea should be avoided to prevent possible effects on intestinal mineral absorption.

2.9.5 **Effectiveness of tea in dental erosion**

Several studies have shown that some natural and chemical products in tea may have anti-erosive effects. This includes substances such as polyphenol that is found in green tea, which acts as a matrix metalloproteinase (MMPs) inhibitor reducing the degradation of dentine

(Mirkarimi and Toomarian, 2012). Specifically, green tea polyphenol (epigallocatechin-3-gallate (EGCG) inhibits the activity against MMP-2 and MMP-9 (Barbosa et al., 2011).

In dentine erosion the collagen matrix, with buffering capacity, acts as barrier against the erosive challenge which stops further mineral loss on dentine. Therefore, with the absence of this organic matrix, the acid can penetrate easily into dentine tissue and cause mineral loss. The matrix metalloproteinases (MMPs) that have been found in saliva and dentine can degrade this matrix which causes dentine erosion. However, the components of tea that have been found such as epigallocatechin, theaflavins, and theaflavins digallate act as inhibitors against collagen degradable enzymes (Passos et al., 2018). Jaâfoura et al. (2014) found that sugar-free black and green teas have anti-erosive effects as they have high pH compared to herbal, fruity, ice and sugary tea.

Reviewing the literature, there are a number of studies that have investigated the effect of tea on prevention of erosion on dentine. Some of these studies investigated the effect of green tea on preventing dentine erosion and showed that green tea can act as a natural inhibitor of MMPs in dentine and saliva and, therefore, can prevent dentine erosion. According to De Moraes et al. (2016) comparing green tea with chlorhexidine (CHX) and distilled water, the study found that green tea is able to reduce the erosion process. Another study showed that supplementation of soft drinks with green tea extract has also beneficial effect in reducing the erosion challenge on dentine (Barbosa et al., 2011).

Oliveira et al. (2017a) evaluated the erosive potential amongst different industrialised teas. The outcome of this study was that all teas such as white tea, green tea with and without flavour (orange with ginger and cranberry), black tea with flavour (lemon and peach) and meta tea with lemon have potentially erosive effects. However, the black tea with lemon had the lowest pH (2.7). Another study analysed the level of pH on the tooth surface after drinking black tea and

considered that black tea is a safe drink as there was only a very small change in pH level on the tooth surface (Simpson et al., 2001).

2.10 Effectiveness of milk in dental erosion

Milk is an important source of several nutrients that contribute to health, and it is considered the only source of nutrients that infants need during the first period of life up to 6 months. Cow's milk is the most widely consumed milk and the UK produced about 15 billion litres of milk during 2019/2020 in which semi-skimmed milk is the largest share of cow's milk volume sales, accounting for about 60.5% of sales (Shahbandeh, 2020, AHDB, 2019).

Milk is considered an excellent source of protein, providing essential amino acids for human, and also contains calcium, phosphate, casein and lipids which were known since early 1945 as factors having anticariogenic properties (Aimutis, 2004, White et al., 2011).

Casein is a phosphoprotein found in cow milk, which comprise 70-80% of the total protein. It binds to amorphous calcium phosphate with its multiple phosphoserine residues and proton-accepting groups that are capable of buffering acids and is also able to inhibit demineralisation and enhance remineralisation of the tooth (Swaisgood, 1996, Vukosavljevic et al., 2014). White (2011) found that casein is an anti-erosive agent which acts by inhibiting the dissolution of hydroxyapatite and also found that these proteins inhibit both surface softening and bulk tissue loss of bovine enamel in an *in vitro* study.

Calcium, phosphate and fluoride are important factors associated with potential erosion and the presence of phosphoproteins and calcium in the composition of cow's milk could prevent enamel dissolution. Drinking milk after an erosive challenge could have a remineralising effect in the tooth enamel (Amoras et al., 2012).

Abd El-Moez et al. (2014) carried out a study aimed at investigating and comparing the possible effect of green tea infusion, milk, and their mixture on the middle one third of the

labial enamel surface using Scanning Electron Microscope (SEM). Teeth were immersed in beverages for 30 hours continuously at room temperature and at 3 hours intervals, the beverages were replaced with new refresh beverages. It was found that green tea infusion had an erosive effect on the labial enamel surface of human anterior teeth while milk had a remineralisation effect and by adding milk to green tea converted the green tea into a repairing effect with the deposition of calcium and phosphate on the enamel surface.

2.11 Effectiveness of lemon in dental erosion

The impact of lemons and other citrus fruit on tooth enamel has been investigated and observed over a long period. Citrus fruit have long been known for their nutritional and health benefits and the reasons for the large consumption because they are relatively cheap, preferred flavours and taste, and they are considered a rich source of vitamin C. Thus, they are produced in wide distribution (Mannerberg, 1962, El-Otmani et al., 2011). Lemon juice is a rich source of citric acid and provide more citric acid per litre than grapefruit juice, orange juice and orange juice squeezed from the fruit, with lemon juice containing up to 6 % citric acid (Penniston et al., 2008, West et al., 2001). Citric acid is a tricarboxylic acid and was first isolated from lemon juice in 1784. In England, it was first commercially produced around 1826 imported from Italian lemons (Max et al., 2010).

The titration curve of lemon juice is very similar to the pure citric acid solution and there is a definite relation between the pH and the amount of acid in the salt or combined form (Sinclair and Eny, 1945).

The citrate ion is a chelating ion that forms a soluble complex with the calcium ion, enhancing more dissolution (Barbour et al., 2003a). Several *in vitro* studies have investigated the enamel dissolution by citric acid, considering several factors such as pH, degree of saturation with respect to hydroxyapatite and time of exposure (Hughes et al., 2000, Eisenburger and Addy,

2001, West et al., 2001, Barbour et al., 2003b). The enamel dissolution and the pH value of citric acid depend on the exposure time, as some studies reported that no enamel dissolution can be detected above pH 3.8 and 4.4 whereas other studies detected the enamel dissolution at pH 6 dependant on the exposure time (Gray, 1962, Rytömaa et al., 1988, Barbour et al., 2003a). Many studies have shown that there is a significant association between dental erosion and increased consumption of acidic food include citric fruit (Allan, 1967, Levine, 1973, Al-Dlaigan et al., 2001a, Milosevic et al., 2004).

Mathew et al. (2018) conducted a study comparing different beverages on the tooth enamel and found that lemon juice had significant tooth tissue reduction compared to green tea and coffee.

2.12 Tooth surface loss evaluation techniques

Barbour and Rees (2004) and Attin and Wegehaupt (2014) describe various assessment techniques for evaluation of tooth surface loss when induced through erosive challenges *in vitro*:

- Surface Profilometry.
- Microhardness (surface hardness).
- Chemical analysis.
- Microscopy techniques (ESEM/SEM).
- Measuring light microscopy.
- Surface 3D focus variation scanning microscopy.
- Attenuated total reflectance infrared spectroscopy.
- Optical specular and diffuse reflection analysis.
- White light interferometry.

- Optical coherence tomography.
- Iodide permeability test.
- Atomic force microscopy (AFM)
- Confocal laser scanning microscopy.
- Nanoindentation.
- Microradiography.
- Confocal laser scanning microscopy
- Secondary ion-mass spectroscopy (SIMS).
- Quantitative light-induced fluorescence.
- Ultrasonic measurement of enamel thickness.

The most commonly used techniques are microhardness and surface profilometry.

2.12.1 **Microhardness**

Microhardness testing has been used by a number of investigators to measure the hardness of enamel and dentine and to determine demineralisation and remineralisation effect (Caldwell et al., 1957, Craig and Peyton, 1958, Koulourides et al., 1965, Koulourides and Staple, 1966). Microhardness measures the enamel surface resistance which is considered a function of the degree of porosity of the superficial enamel layer and indicates mineral loss or gain in the subsurface lesion (Koulourides, 1971). This method is very sensitive to changes in mineral density and can indicate mineral loss or gain which in turn can determine demineralisation and remineralisation effect (Featherstone and Zero, 1992).

The basic method of microhardness is the indentation of a diamond of known geometrical dimension for a given load and duration. For the assessment of eroded tooth surface, Knoop or Vickers diamonds are used on a previously polished surface in order to produce well defined

indentations in which the length of indentations on the surface are measured under a microscope in µm. For precise measurements, the indentation length is required to be about 30-40 µm (Attin and Wegehaupt, 2014).

The main advantages of the microhardness method are relatively low costs, it is a well-established method and that it can be combined with determination of surface loss due to abrasion. The disadvantages of this method are that polished and flat surface are needed and measurement of surface hardness is influenced by non-demineralised deeper layers (Attin and Wegehaupt, 2014).

2.12.2 **Surface Profilometry**

Profilometry was used for the first time in dental research in 1972 for measurement *in vitro* of dentine abrasion by toothpaste (Ashmore et al., 1972). Irreversible loss of tooth surface and surface roughness could be measured using profilometry technique, and also known as surfometry, by scanning the surface sample using either non-contact (laser beam) or a contact stylus (metal or diamond) with diameter of about 2-20 µm at rate of around 10 mm/min (Barbour and Rees, 2004, Attin and Wegehaupt, 2014). In this technique, in order to assess the erosive agent, the enamel surface is divided into two parts: unprotected, which is exposed to the erosive agent and a protected part using nail varnish or adhesive tape, which can then provide a comparison between treated and protected surfaces (Barbour and Rees, 2004, Abdullah, 2009).

In contact profilometry the stylus is loaded with force and penetrates the eroded surface by scanning a complete map of the specimen topography. This can cause damage to the surface and lead to overestimation of early lesion depth (Attin and Wegehaupt, 2014, Joshi et al., 2016). Non-contact profilometry has been used to replace the traditional contact stylus with white light or a laser and has higher resolution compared to the contact profilometry (Abdullah, 2009,

Attin and Wegehaupt, 2014). It generates a surface plan rather than just simple line profiles, allowing better analysis of volumetric loss. However, non-contact profilometry needs careful selection because the reflective and/or translucent surfaces (such as enamel) can lead to inconsistencies when profiled (Paepegaey et al., 2013).

The advantages of profilometry are that it is a simple technique, applicable for measurement in the natural dentition and can be used over a large area of enamel. The disadvantages of this technique are that it is time consuming when completing mapping of surfaces and the stylus could damage the surface (Attin and Wegehaupt, 2014).

2.13 Bovine teeth

Specimens obtained from human teeth are a preferred option in dental research for *in vitro* and *in situ* studies, because they are a more clinically relevant substrate for testing of the study hypothesis (Yassen et al., 2011). However, there are some limitations in using human teeth: for example, they are difficult to obtain in sufficient quantity and with adequate quality because of tooth extraction due to extensively decayed lesions and other defects (Mellberg, 1992). Human teeth are small and have a curved surface which could be a limitation for specific tests that require a flat surface with uniform thickness, and increases in the awareness of the infection hazard and ethical issues (Yassen et al., 2011). Therefore, other alternative substrates have been proposed and used for dental experiments such as bovine, primate, swine, shark teeth and equine teeth (Yassen et al., 2011).

Bovine enamel and dentine demonstrate a similar structural morphology to human in terms of solid, interwoven, rod-like structures extending from the enamel-dentine junction (EDJ) to the surface of the tooth, and the number and density of dentinal tubules and collagen matrix. However, there are differences between bovine and human enamel, such as bovine enamel has thicker crystallites, increased porosity and lower fluoride concentration compared to human

enamel (Laurance-Young et al., 2011). Hence, the demineralisation in bovine teeth progresses three times faster than in human enamel (Attin et al., 2007). However, it was proven by Koulourides (1983) that both human and bovine enamel have a similar response to acidic challenges and remineralisation conditions. Other differences have also been found such as that human enamel has a slightly lower density, lower Vickers hardness and a slightly higher content of calcium and phosphorous compared to bovine enamel (Attin et al., 2007).

Bovine teeth are a preferred substitute because they are attainable in large quantities from abattoirs, do not have carious lesions and other defects and have large flat surfaces which makes them easier to handle and mechanically process (Laurance-Young et al., 2011, Yassen et al., 2011). There are however some concerns in extrapolating findings when using bovine teeth to human teeth as their chemistry and structure are not identical (Yassen et al., 2011).

2.14 The *in vitro* demineralisation/ remineralisation with pH cycling models

A study model is a method that demonstrates some real-world phenomenon of interest, in which the researchers can derive information about this phenomenon (Buzalaf et al., 2010). *In vivo, in vitro* and *in situ* studies have been widely used to study dental caries and tooth surface loss, but it has been found that there are difficulties in measuring tooth surface loss and the inability to achieve very precise tooth wear measurement with *in vivo* studies. Thus, a number of *in vitro* and *in situ* models have been developed and validated to overcome the challenges posed by *in vivo* studies (West et al., 2011).

In vitro studies of remineralisation can be carried out using pH cycling and this can be done manually or under computer control (West et al., 2011). The aim of the cycling erosion model is to investigate the effect of demineralisation and remineralisation that occur during tooth wear (Austin, 2011). Among *in vitro* protocols, pH-cycling models include exposure of enamel or

dentine to a combination of demineralisation and remineralisation which is designed to mimic the dynamic of mineral loss and gain (White, 1995).

The advantages of the application of *in vitro* studies for modelling tooth wear are that they achieve a high degree of standardisation, they can be conducted in a short period of time, can include a large sample size and use of control group, require fewer staff than *in situ* studies, have the ability to control multiple variables (substrate type and nature, acidic challenge and concentration), can obtain the results rapidly with minimal expenditure compared to *in situ* and *in vivo* tooth wear research and are relatively inexpensive (Austin, 2011, West et al., 2011). On the other hand, difficultly in replicating the oral environment with all of the biological variations that influence tooth surface loss make comparisons with *in vivo* problematic (West et al., 2011).

2.15 Research aims and hypothesis

2.15.1 Aims of the study

To investigate the effect of black and green tea on surface loss (progression) of dental enamel under erosive challenge *in vitro.*

2.15.2 Objectives of the study

1- To assess and compare the effect of black and green tea on surface loss (progression) of dental enamel under erosive pH cycling regime *in vitro,* using Surface Profilometry.

2- To assess and compare the effect of black tea with addition of milk or lemon on surface loss (progression) of dental enamel under erosive pH cycling regime *in vitro,* using Surface Profilometry.

2.15.3 The Null Hypotheses for the study

1- There is no difference in the effect of black and green tea on surface loss (progression) of dental enamel under erosive conditions *in vitro.*

2- There is no difference in the effect of black tea with addition of milk or lemon on surface loss (progression) of dental enamel under erosive conditions *in vitro.*

3 Materials and methods

This study was a randomised, single-blinded (examiner) study *in vitro*. The methodology adopted in the present study is described in this chapter including preparation of the enamel samples and the pH cycling with erosive challenge protocol as well as the materials and equipment used.

3.1 Power calculation

Statistical advice was sought, and the sample size was determined to be 30 per group for this pilot study as no relevant effect of size and standard deviation for the primary outcome can be found from high quality publications in the existing literature (Lancaster et al., 2004).

3.2 Materials and Equipment:

The following equipment was used to complete this study:

- Enamel slabs from bovine teeth.
- Impression Compound (green wax, Kerrdental, UK).
- Well Diamond Wire Saw, water-cooled, cutting machine ((Well@Walter EBNER, CH-2400 Le Loche).
- Grinding machine.
- 600, 800, 1000, 1200 and 2000 grade fine grit abrasive paper (3M Company, UK).
- Red ribbon wax (Metrodent, UK).
- Silicone mould compound (Silastic S).
- Cold resin “Stycast 1266” (Hitek Electronic materials, UK).
- Perspex plastic holders.

- Nail polish (Max Factor, UK).
- Methanol
- Plastic containers.
- Artificial saliva chemicals (for night and day saliva).
- Citric acid monohydrate, Analar NormaPur VWR.
- De-ionised water.
- Profilometer SP (ProScan 2000, version 2.1.1.8, Scantron Industrial Products Limited, Somerset, England).
- Semi-skimmed milk (Tesco, Leeds, UK).
- Black tea (Tetley decaffeinated original tea), teabags (Morrisons, Leeds, UK).
- Green tea (Tetley green tea decaffeinated), teabags (Morrisons, Leeds, UK).

3.3 Selection of teeth

Bovine teeth from a local abattoir were used in this study.

3.4 Storage and cleaning of bovine teeth

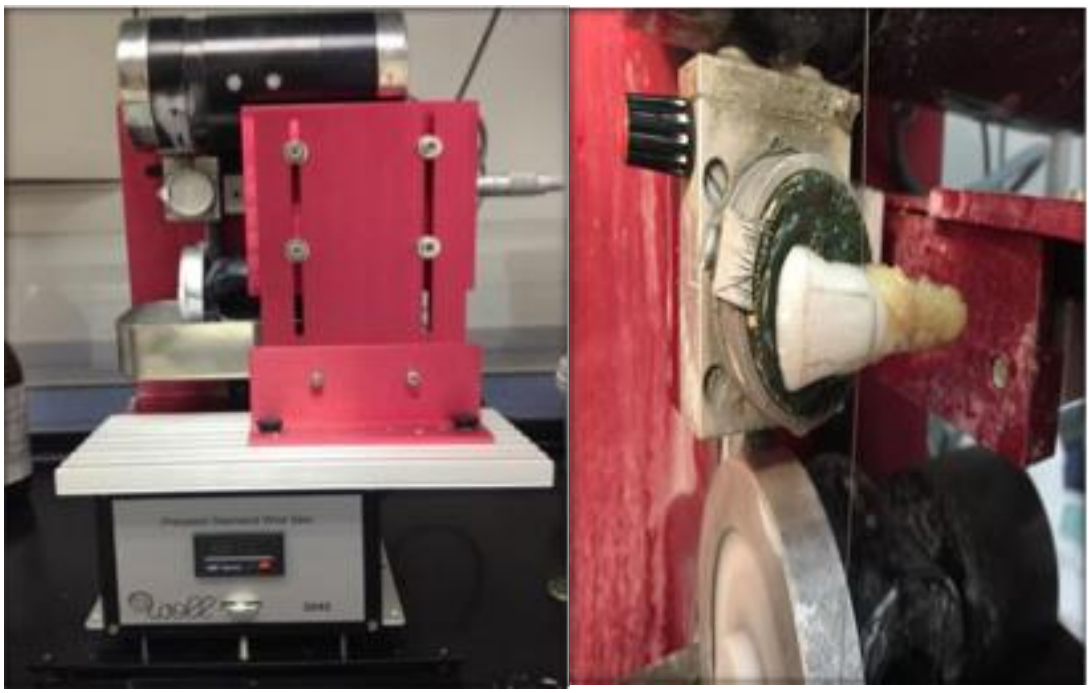
All enamel slabs were selected from bovine incisors. All teeth were stored in de-ionised water and 0.1% thymol (Sigma Aldrich, thymol 98%) at room temperature. Before sectioning, teeth were cleaned using a spoon excavator and a toothbrush with pumice powder and stone to remove all soft tissue remnants. To detect any defects, caries, or cracks, all teeth were screened by trans-illumination and transmitted light using low-power (10 times magnification) microscopy (Leitz, Wetzlar®, Germany). Suitable teeth free from defects were selected for the study.

3.5 Preparation of enamel slabs

3.5.1 Cutting and grinding of the enamel slabs

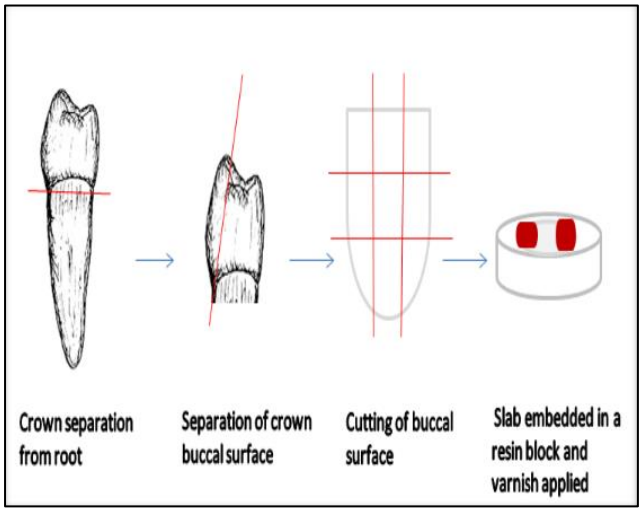
Each tooth was mounted using ‘green stick’ impression compound (Kerr, UK) on plates. The crowns were sectioned using a diamond wire Well Diamond Wire Saw, water-cooled, cutting machine (Figure 3).

Figure 3: Diamond wire machine used for the teeth sectioning (Well® Walter EBNER, CH-2400 Le Loche)



The buccal and palatal surfaces of each crown were separated, and each buccal section was cut into three enamel slabs that were approximately 4 x 3 x 3 mm in size. Figure 4 shows an illustration of enamel slab preparation.

Figure 4: Illustration showing enamel slab preparation



The enamel slabs were polished whilst wet using fine grit abrasive paper (Wet or dry paper, 3M) 1200 grade to remove the outermost remnants of the pellicle and to achieve a flat surface. Care was taken not to fully abrade the enamel. The slabs were then mounted in circular resin blocks of 3 mm thickness and 7.5 mm width to ensure flatness of their surfaces (Figure 5). This was achieved using a rectangular steel block, which had a circular hole of 3 mm depth (Figure 6). A 800-grade fine grit abrasive paper followed by 1200 and 2000 grade was used respectively to grind enamel surfaces after mounting in resin to the same thickness as the hole in the steel block through grinding machine. Care was taken not to fully abrade the enamel. The slabs were then cleaned with methanol to remove any remnants of abrasive paper. Surfaces were then polished with 5µm alumina paste. Thereafter, these slabs were cleaned with de-ionised water and methanol.

3.5.2 **Storage of enamel slabs**

Once the slabs had been prepared, they were kept moist by placing in de-ionised water to prevent dehydration of the slabs.

Figure 5: Enamel slabs in silicon moulds

Figure 6: Rectangular steel block holding prepared slabs

3.6 Test methods

The following tests were used for each enamel slab to ensure eligibility of the enamel slab for inclusion in the study: Knoop Microhardness, Profilometry.

3.6.1 Knoop Microhardness

Each flat enamel slab was tested with the Knoop microhardness machine. Microhardness testing measures the resistance of enamel surfaces to indenter penetration and is a function of the degree of hardness of the dental layer. Microhardness of enamel slabs was assessed using computer aided Duramin indenter machine (Struers A/S, DK 26-10, Denmark) (Figure 7).

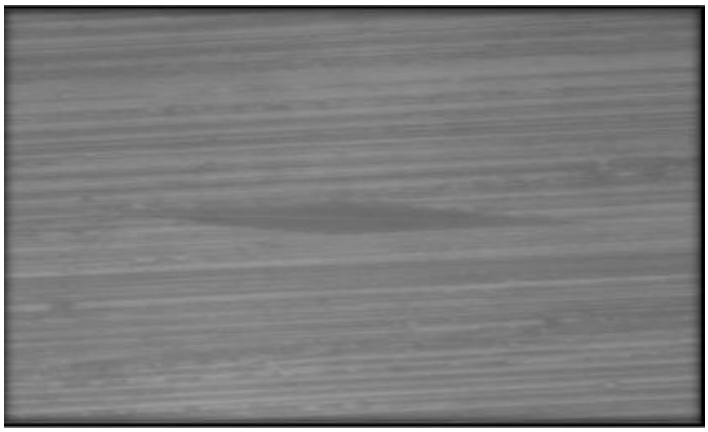
The indentations were made using a Knoop diamond under a 100g load for 15 seconds (Zero *et al.*, 1990). The depth of indenter penetration was measured by means of an image analysis system. The length of the indenter was measured in micrometre using computer software that calculates the indentation length (μm) and microhardness value (KHN) after identifying the border of the indentation. The indents on the slabs were tested as follows: middle, left and right.

2 1 3

Three indentations, spaced 50μm apart, were made for each slab and the mean was determined. The initial surface microhardness of the enamel slabs was measured in order to exclude from the study slabs with very soft enamel or slabs where areas with exposed dentine were present. The length of the enamel indent before any exposure was usually about 60-70μm and the microhardness was 290- 360 KHN (Figure 8). Slabs whose enamel microhardness was not within this normal range were excluded from the study.

Figure 7: Computer-aided Duramin Indenter Machine (Struers A/S, DK 26- 10, Denmark)

Figure 8: Microscopic image of diamond shape indentation on the enamel surface.



3.6.2 **Profilometry**

Baseline measurements of the surface profile of the slabs were obtained using the SP (ProScan 2000, version 2.1.1.8, Scantron Industrial Products Limited, Somerset, England) profilometer to ensure surface flatness.

3.6.2.1 Evaluation of dental erosion with profilometry measurements

A profilometer is a device used to measure the roughness of a surface. It gives the difference between the high and low point of a surface in nanometres (nm). There are two types which are either non–contact or contact profilometers. Baseline measurements of the surface profile of the slabs were obtained using a non-contact profilometry SP (ProScan 2000, version 2.1.1.8, Scantron Industrial Products Limited, Somerset, England. Figure 9) to ensure surface flatness. The measurement was achieved by placing the sample on a key stage on the Scantron ProScan. The sensor used has a working range of 300 µm 5 mm from the surface. The measuring range was set to 150 µm in order to allow readings up to 150 µm up or down from the start point. The resolution of the sensor was 10 nm and the spot size 8 µm. A step size of 0.01 mm was used during scanning. After scanning, the flatness of the surface using cross section views were checked (Figure 10). Slabs that were not flat were ground again, as previously described, and then were checked again. Slabs with exposed dentine after this process were excluded. The enamel slab surfaces were then covered with nail varnish except for a small window of 1x2 mm size in the middle of each slab. At days 7,14, 21 and 28 the nail varnish was removed using methanol and the same procedure of scanning was repeated to check the average depth of surface loss (SL) of the exposed area.

Figure 9: The non-contact surface Profilometer (Scantron Proscan 2000)

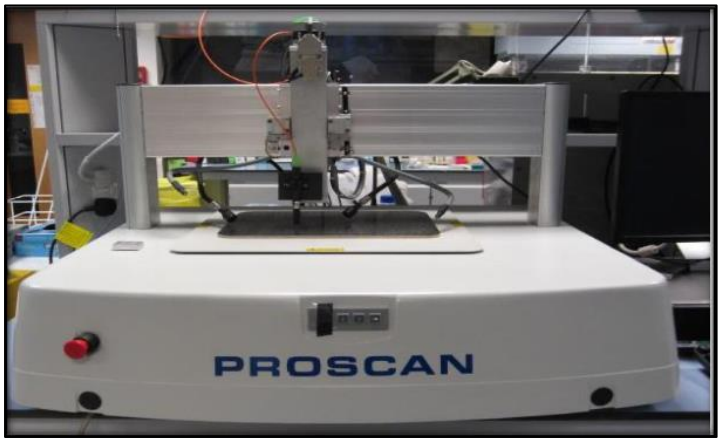
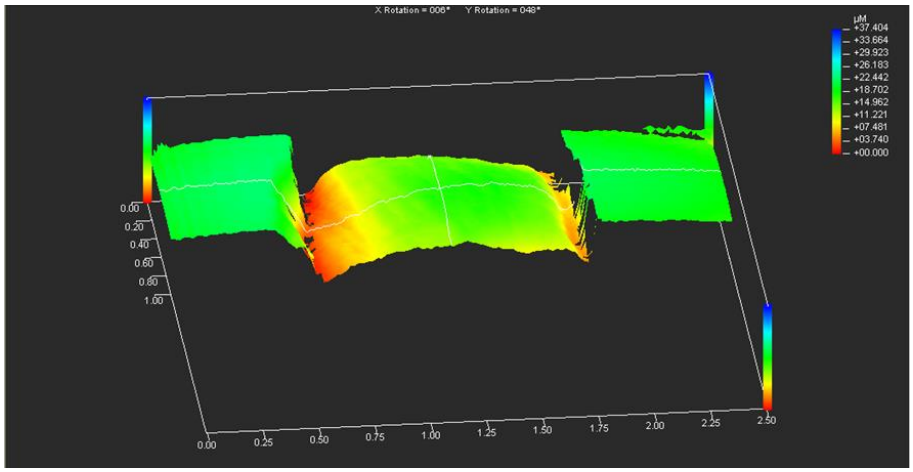


Figure 10: Image of an erosive lesion



3.7 Randomisation and blindness

Enamel slabs were randomly allocated to 5 study groups using a randomisation website (https://www.random.org). The enamel slabs were coded during analysis with surface profilometry. At the end of the study, the code was released to the study investigator thus making the analysis of the enamel slabs blinded.

3.8 Experimental Protocol/Regime

3.8.1 Experimental and control groups

The enamel slabs were randomly assigned to five groups in which were 30 slabs per group.

Groups:

- Fluoride-free de-ionised water (negative control)
- Green tea (≈ 5.0 ppm F) (Tetley green tea decaffeinated)
- Black tea (≈ 5.0 ppm F) (Tetley decaffeinated original tea)
- Black tea (≈ 5.0 ppm F) and semi-skimmed milk (1.8g of fat)
- Black tea (≈ 5.0 ppm F) and citric acid 1.0% (pH 3.6)

3.8.2 Selection of tea and other product

Black and green tea were selected based on the amount of fluoride found in a previous study, which measured the amount of fluoride in different types and brands of tea (Xiarchou, 2016). Black and Green tea with the same amount of fluoride (≈ 5.0) ppm F were selected (Figure 11). In order to mimic the effect of lemon juice with black tea infusion extract, a solution of citric acid (1.0%; pH 3.6) was added into the tea infusion.

Milk was collected from a Leeds supermarket: semi-skimmed fresh bovine milk with 1.8g of fat (Figure 11).

Disposable hot drink paper cups (12oz) (Figure 12) were used for the preparation of the hot tea infusions and de-ionised water (non-fluoride).

Figure 11: Black tea, green tea and semi-skimmed fresh milk

Figure 12: Paper cups (12oz)

3.8.3 **Preparation of black and green tea infusions**

Tea bags were taken out from each box of black and green tea, the content of each bag was removed and weighed on the analytical balance, to determine the mean amount of tea (grams) contained in the bags of each box. Then tea infusions were prepared following the guide (2g of tea per 100ml of freshly boiled de-ionised water) (Xiarchou, 2016).

3.8.4 **Continuous infusion of black and green tea**

The continuous method of preparing each tea infusion was simulated by placing a new randomly selected tea bag from each box of black and green tea in a new disposable hot drinks paper cup (12oz). Then, freshly boiled de-ionised water was added to each cup and at this point the infusions were stirred. The infusion was left to brew for 3 minutes and the tea bags were squeezed and removed from the cups. A sample of 50ml of tea infusion was withdrawn from each cup using a measuring beaker. Each 50ml sample of tea infusion was placed into a dipping container (100ml). All samples of tea infusion were left to cool to a temperature of 65˚C before immersing the enamel slabs.

3.8.5 **Preparation of black tea infusion with the addition of semi-skimmed milk**

A new randomly selected tea bag of black tea was placed in a new disposable hot drinks paper cup (12 oz) and freshly boiled de-ionised water was added, in the same ratio as above. The infusion was left to brew for 3 minutes and the tea bag was squeezed and removed. 10ml of semi-skimmed milk was placed in the cup of freshly prepared black tea infusion. At this point the infusions were stirred and the 50 ml sample from the infusion was withdrawn using a measuring beaker. Each sample of tea infusion with milk 50 ml was poured into a dipping container (100 ml). All samples of tea infusion 50 ml were left to cool to 65˚C temperature before immersing the enamel slabs.

3.8.6 Preparation of black tea infusion with the addition of citric acid (to mimic addition of lemon)

Citric acid 1.0% was prepared by adding three grams of mono-hydrate citric acid to one litre of de-ionised water.

A new randomly selected tea bag of black tea was placed in a new disposable hot drinks paper cup (12oz) and freshly boiled de-ionised water was added. The infusion was left to brew for 3 minutes and the tea bag was squeezed and removed. Citric acid 1.0% pH (3.6) (10ml) was added into a tea infusion and at this point the infusion was stirred and the sample of 50ml from the infusion was withdrawn using a measuring beaker. Each sample of tea infusion with citric acid 50ml was poured into a dipping container (100ml). All samples of tea infusion 50ml were left to cool to 65°C temperature before immersing the enamel slabs.

3.8.7 The pH cycling regime with erosive challenge

A special tray with 30 holes in the resin blocks were used to hold the enamel slabs (Figure 13). Resin blocks were secured in position using adhesive wax. The slabs were dipped in tea infusions and solutions: black tea; black tea and semi-skimmed milk; black tea and citric acid 1.0% (pH 3.6); green tea and fluoride-free water (0 ppm F) for 10 minutes three times daily (Figure 14).

Citric acid was prepared by adding three grams of mono-hydrate citric acid to one litre of de-ionised water. The slabs were immersed in a solution for two minutes five times daily in citric acid 0.3% (pH 2.6) for a period of 28 days. Each group of slabs was immersed at room temperature in fresh 200ml aliquots of citric acid each time. On each occasion, before immersion in citric acid, the slabs were taken out of the artificial saliva and rinsed with de-ionised water (pH 6.85 ±0.05). The slabs were also rinsed in de-ionised water after treatment

before being returned to the artificial saliva, which was changed daily. Day time and night-time saliva were prepared as shown in Tables 1 and 2 respectively.

The artificial saliva composition was based on the electrolyte composition of natural saliva, and it was advised to be used to eliminate any precipitation on the enamel surface (as provided by Dr RP Shellis, Department of Oral and Dental Science, University of Bristol, Bristol, UK).

Figure 13: The enamel slabs mounted in resin blocks, held in a custom made holders and covered with nail varnish except for a small window

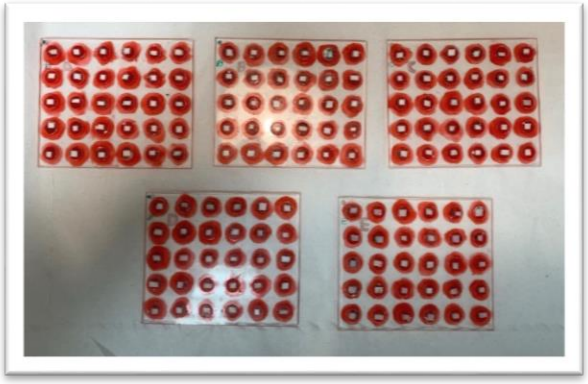


Figure 14: The dipping plastic containers for each group

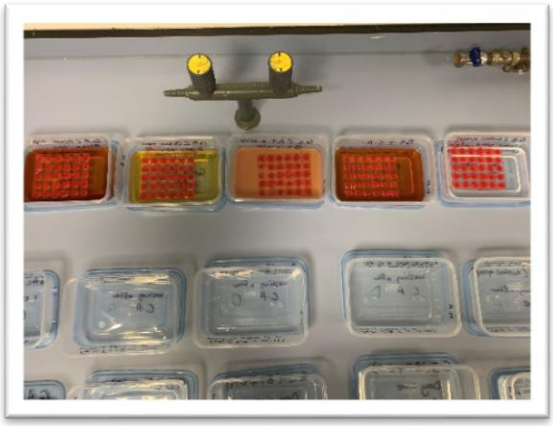


Table 1: Day time artificial saliva

Constituent	Concentration mmol/L
Calcium carbonate	0.7
Magnesium carbonate (hydrated basic)	0.225
Potassium di-hydrogen phosphate	4.0
HEPES buffer (acid form)	20.02
Potassium chloride	31.33

To 900ml of de-ionised water 1.8 ml of 1 mol/L HCl was added followed by the above components and stirred until all had dissolved. The pH was adjusted to 6.8 by adding KOH solution and the solution made up to 1L with de-ionised water. The saliva was kept at room temperature and used within 2-3 days.

Table 2: Night time artificial saliva

Constituent	Concentration mmol/L
Calcium carbonate	0.5
Magnesium carbonate (hydrated basic)	0.225
Potassium di-hydrogen phosphate	0.5
HEPES buffer (acid form)	20.02
Potassium chloride	31.33

The night-time saliva was prepared using the same procedure as above.

Between immersions in citric acid the slabs were left immersed in artificial saliva for 60 minutes to enable remineralisation. The slabs were kept in an incubator at 37.0ºC at all times except while they are being immersed in tea infusions and citric acid. Artificial saliva was changed daily to prevent any contamination or bacterial growth. A 60-minute gap was left between daytime erosive challenges. Before and after dipping in the erosive solutions the slabs were rinsed with de-ionised water.

The slabs were analysed with the scanning profilometer to measure the amount of surface loss at days 7, 14, 21 and 28 of the pH- cycling period.

At the end of the cycling period (day 29), the slabs were rinsed with de-ionised water and air-dried. The nail varnish was then removed using methanol and to ensure that all residues were removed. The slabs were then kept in de-ionised water in micro-centrifuge tubes and left at room temperature.

Figure 15: Flow chart for the pH-cycling regime with erosive challenge for two different experimental groups: black and green tea infusions

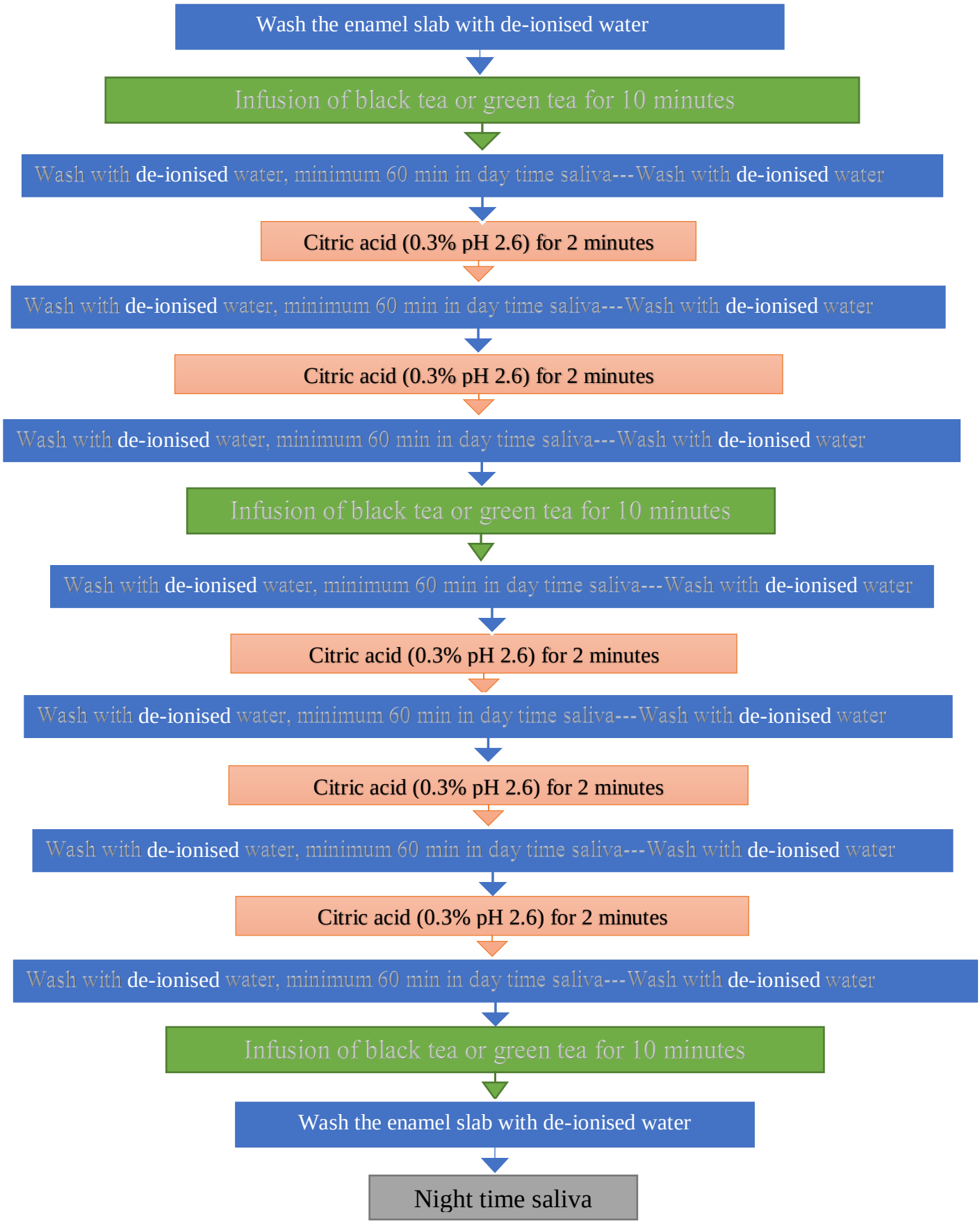


Figure 16: Flow chart for the pH-cycling regime with erosive challenge for two different experimental groups of black tea with additions (semi-skimmed milk or citric acid)

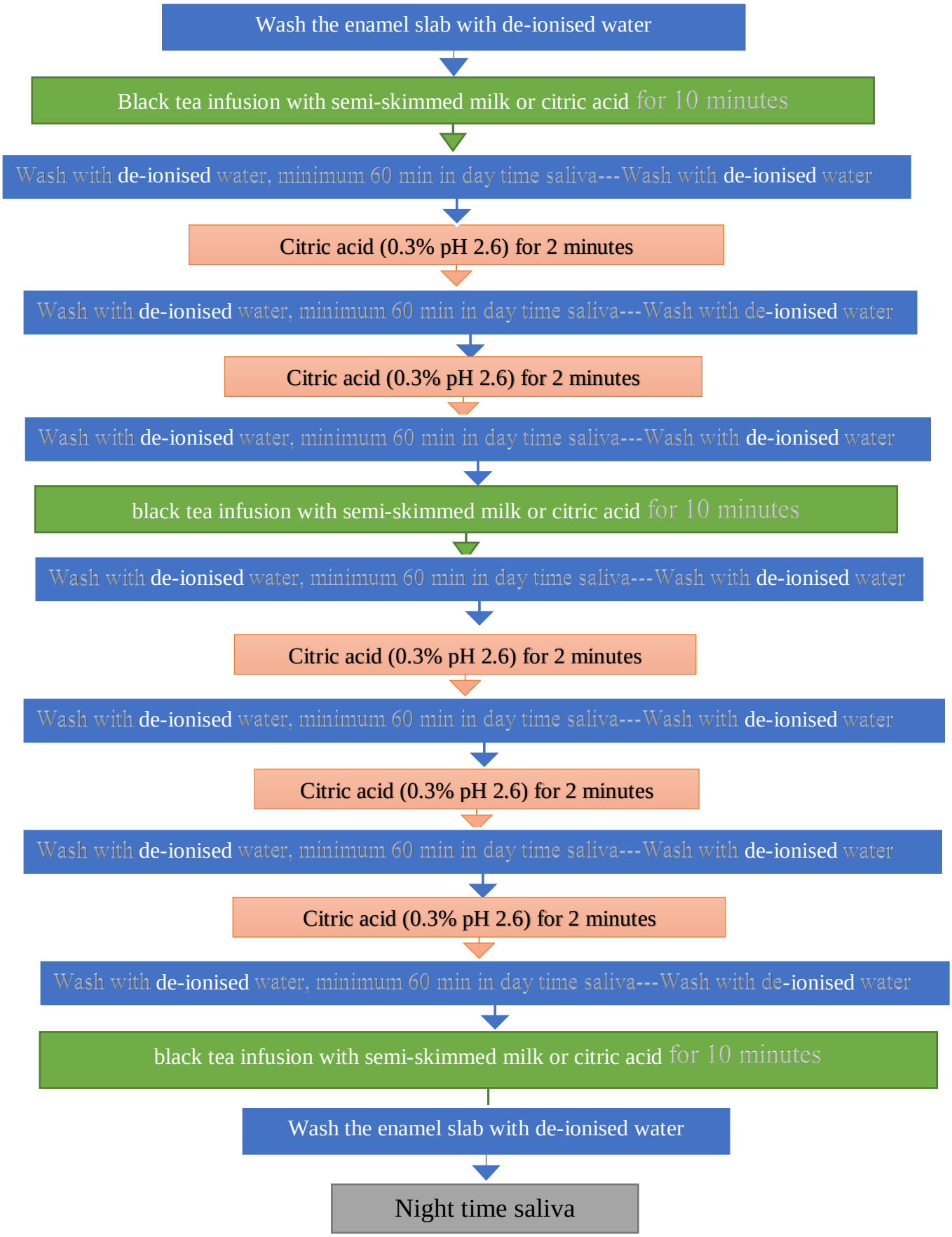
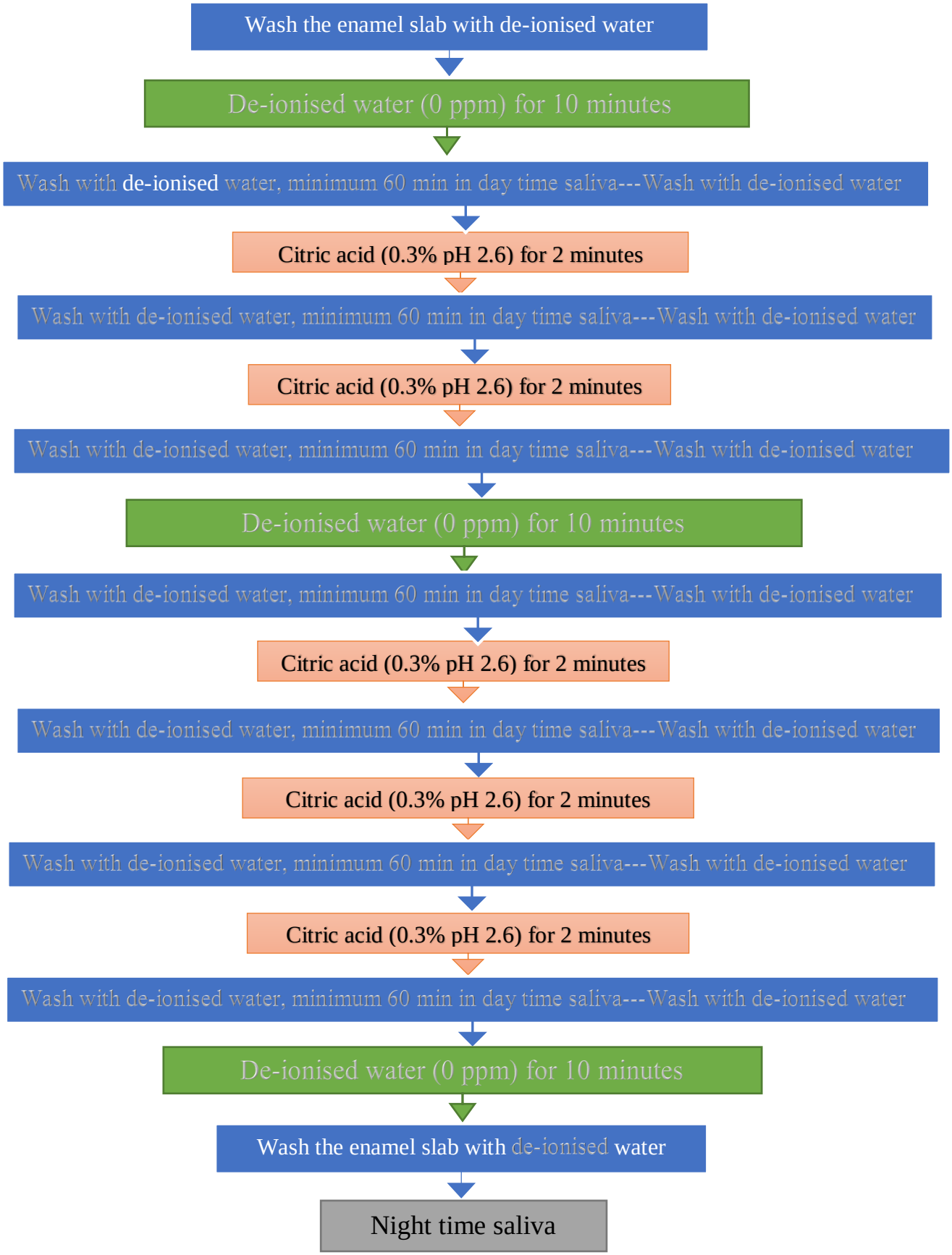


Figure 17: Flow chart for the pH-cycling regime with erosive challenge for control groups 0 ppm F de-ionised water)



3.9 Data collection

At the end of the cycling period at 7, 14, 21 and 28 days, the slabs were rinsed with de-ionised water and air-dried. The nail varnish was then removed using methanol and to ensure that all residues were removed. The nail varnish reapplied to the same window using a magnifier. The slabs were scanned with the profilometer that was set up using the same parameters as for the baseline measurements. The sample was placed on the key stage of the Scantron ProScan and using a 150 mm height of the sensor as standard. The sample rate was set at 300Hz. The step size used was 0.01mm. After scanning the reading was levelled in three points A, B, and C (Figure18). Then 3-point height was selected in the primary plan view and the result was recorded, which can be seen in Figure 19 showing the different surfaces of the scan for example after 7 days.

Figure 18: Image of an erosive lesion, showing the tree points of levelling in the reference areas (A, B and C)

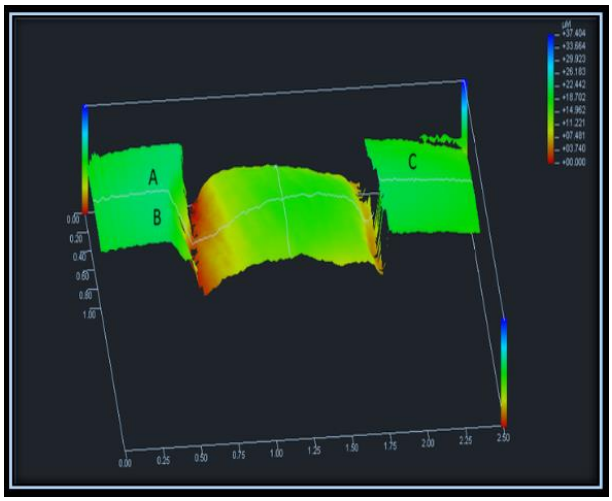
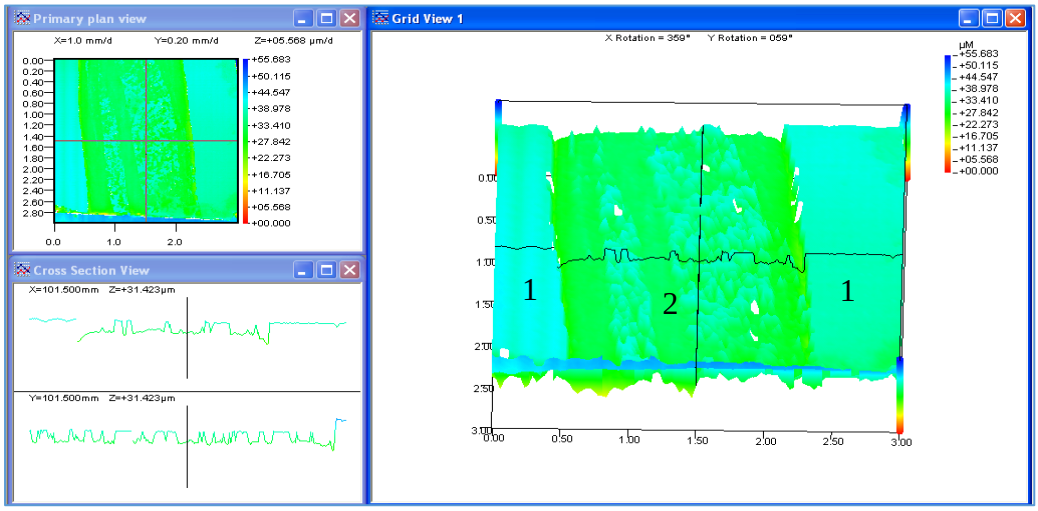


Figure 19: Scan of an enamel slab after 7 days of erosive challenge, showing the un-eroded reference areas (1) and eroded area (2)



3.10 Statistical analysis:

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 27). Descriptive statistics were obtained to measure mean and standard deviation (SD) for continuous data. The assumption of normal distribution of errors were checked for all groups, using Kolmogorov-Smirnov test. Since the data were found to be normally distributed, repeated measure ANOVA test was performed to assess the effect over intervention and time points.

One-way ANOVA test with Bonferroni *post hoc* test was performed to compare the amount of surface loss (SL) between groups under erosive challenges over time.

Significance level was set at 0.05, and 80% power was accepted. A 95% confidence interval was reported.

3.10.1 **Intra-examiner reliability**

Approximately 10% of enamel slabs had the measurement repeated for both Microhardness and Profilometry in order to allow assessment of intra-examiner reliability. In addition, the intra-examiner reproducibility was tested using Intra-class Correlation Coefficient (ICC). It was found to be 0.84 which indicates a good reproducibility (Portney and Watkins, 2009).

4 Results

4.1 Test of normality

Data presented normal distribution by the Kolmogorov-Smirnov test in all groups except one (the fluoride-free water group) after 7 days erosive challenge (Table 3). Since most of the data showed normal distribution, a repeated measure ANOVA test was used to assess the effect over intervention and time points. The overall appearance of Q-Q plot is shown in Figure 20. Most of the quantile points lie along the line which suggests the data that were normally distributed.

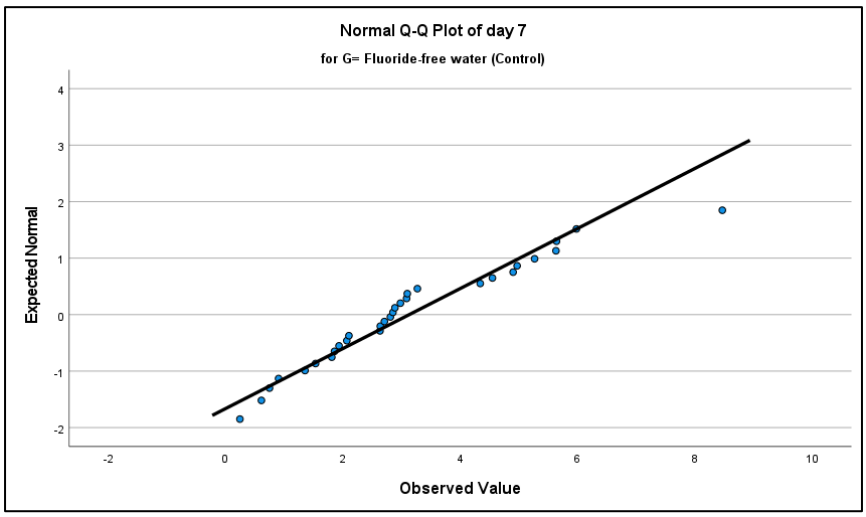
Table 3: Normality test (enamel surface loss - µm) after 7, 14, 21 and 28 days of erosive challenge for all groups

Day	Groups	Kolmogorov-Smirnov		
		Statistic	df	Sig.
Day 7	Fluoride-free water (control)	0.173	30	0.022*
	Green tea	0.132	30	0.195
	Black tea	0.104	30	0.200
	Black tea with milk	0.117	30	0.200
	Black tea with citric acid	0.143	30	0.117
Day 14	Fluoride-free water (control)	0.113	30	0.200
	Green tea	0.078	30	0.200
	Black tea	0.093	30	0.200
	Black tea with milk	0.096	30	0.200
	Black tea with citric acid	0.088	30	0.200
Day 21	Fluoride-free water (control)	0.132	30	0.196
	Green tea	0.085	30	0.200
	Black tea	0.142	30	0.127
	Black tea with milk	0.109	30	0.200
	Black tea with citric acid	0.087	30	0.200
Day 28	Fluoride-free water (control)	0.104	30	0.200
	Green tea	0.139	30	0.147
	Black tea	0.140	30	0.135
	Black tea with milk	0.113	30	0.200
	Black tea with citric acid	0.105	30	0.200

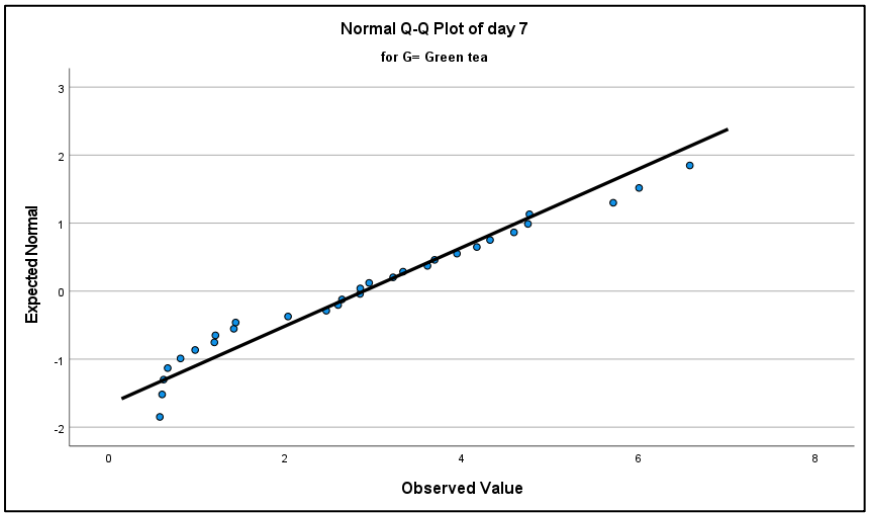
*Significance (Kolmogorov-Smirnov test $p<0.05$)

Figure 20: Normal Q-Q plot test of normality for all groups after 7,14,21 and 28 days of erosive challenge

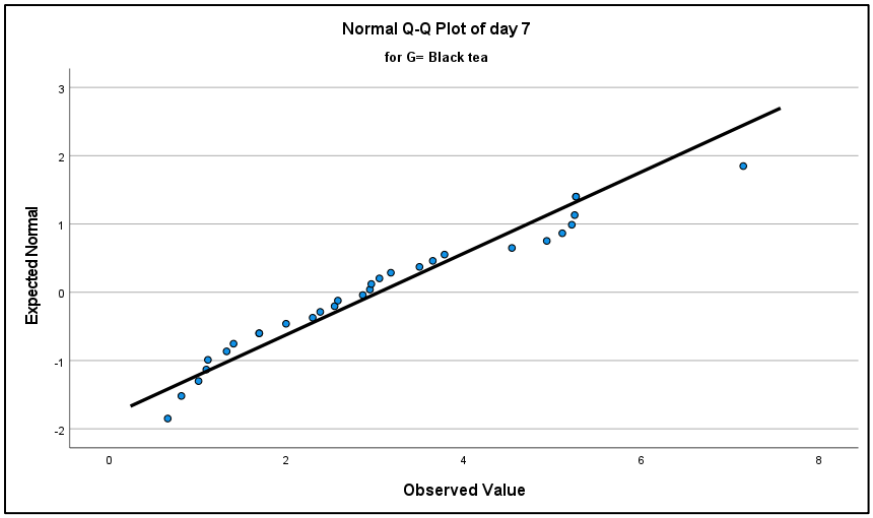
7 Days: Fluoride free water



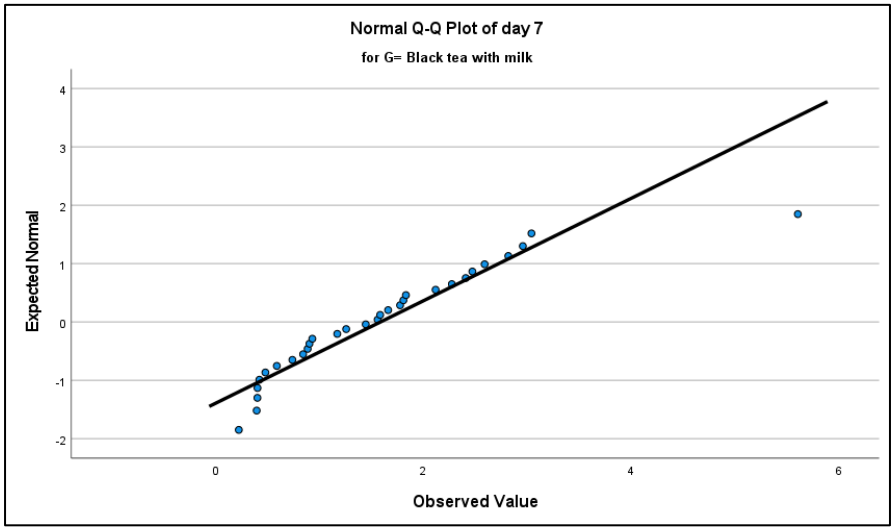
7 Days: Green tea



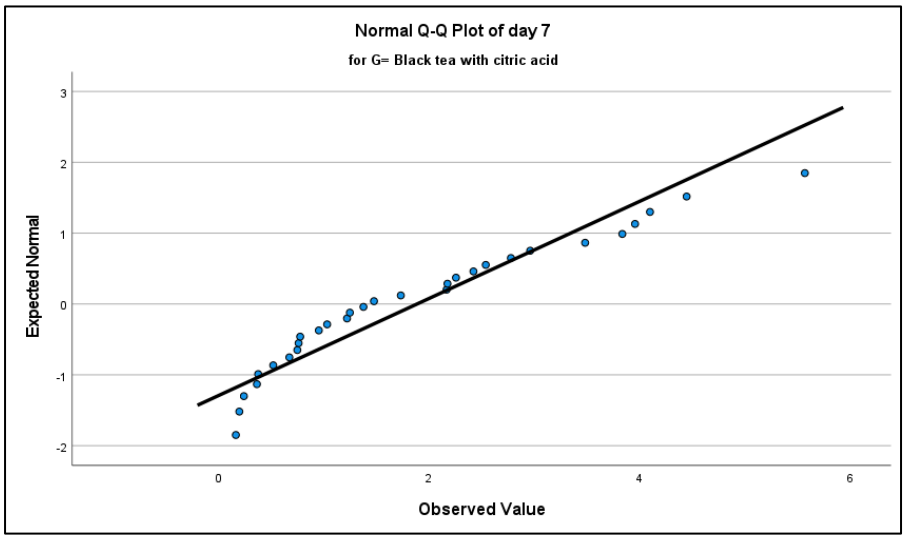
7 Days: Black tea



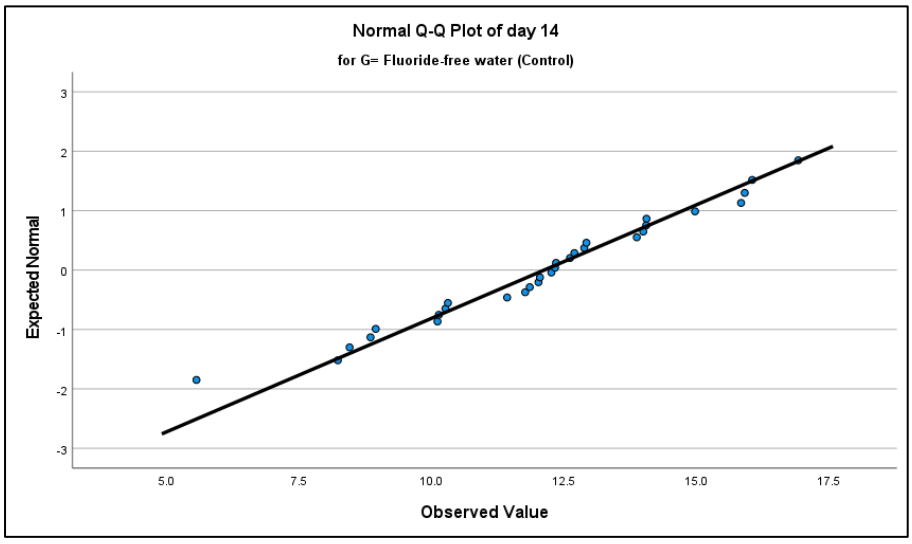
7 Days: Black tea with milk



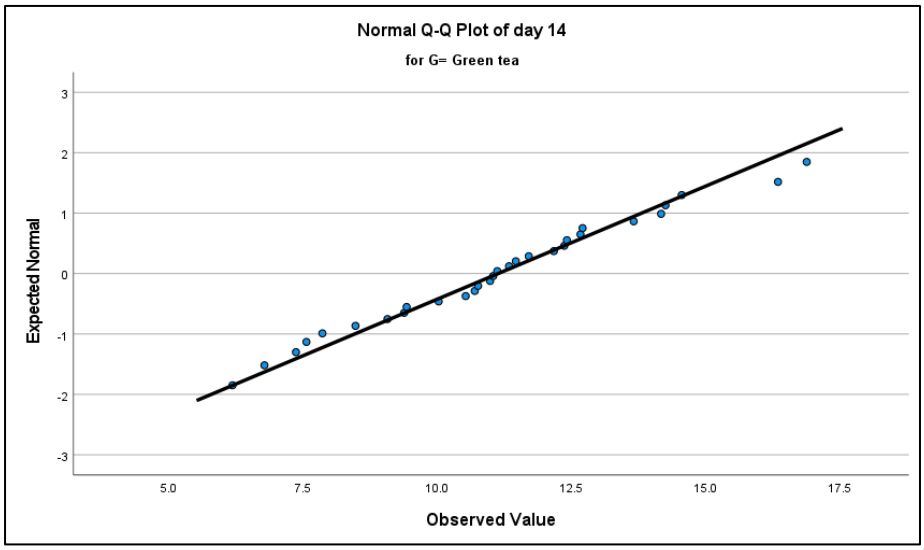
7 Days: Black tea with citric acid



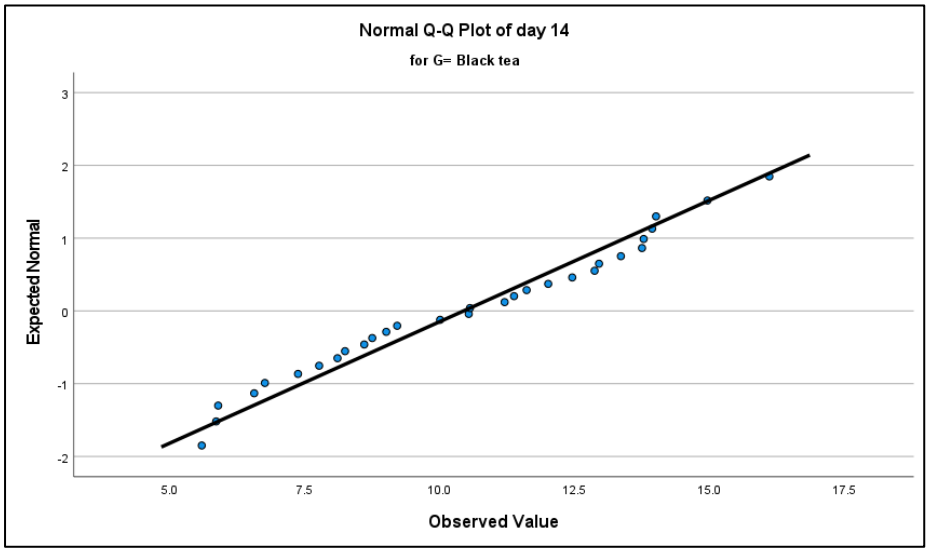
14 Days: Fluoride free water



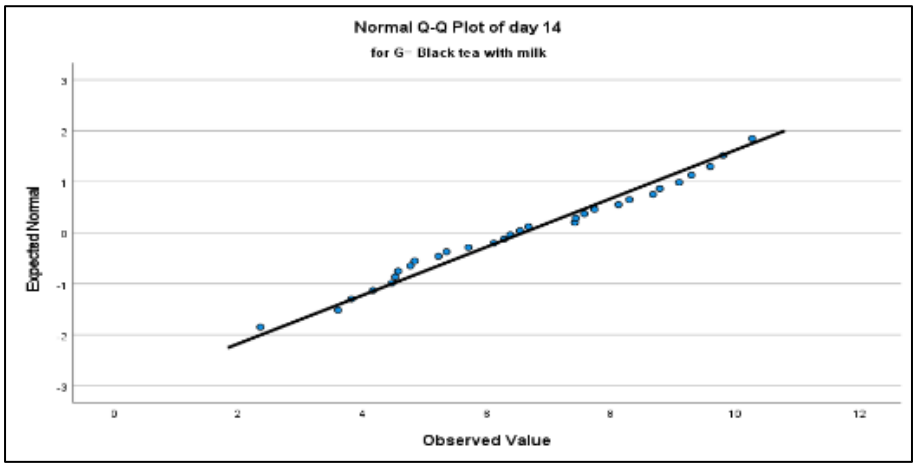
14 Days: Green tea



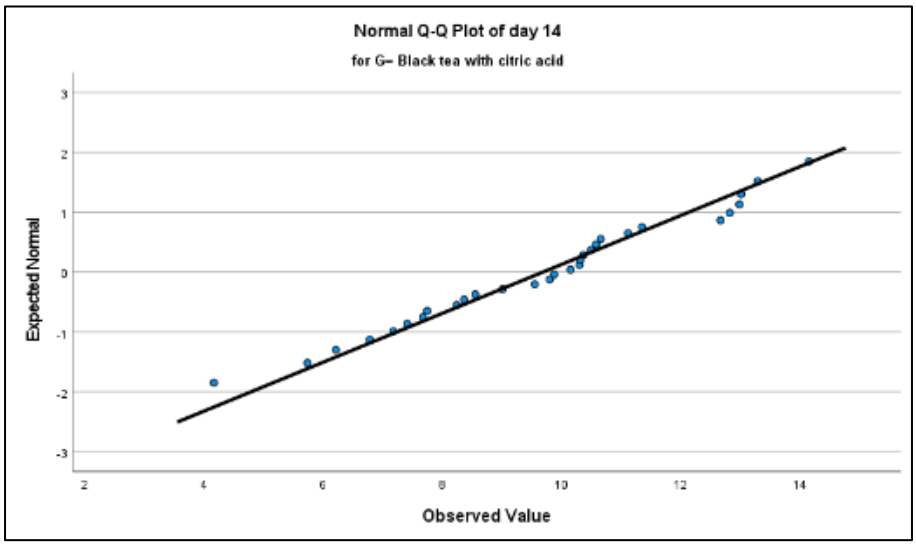
14 Days: Black tea



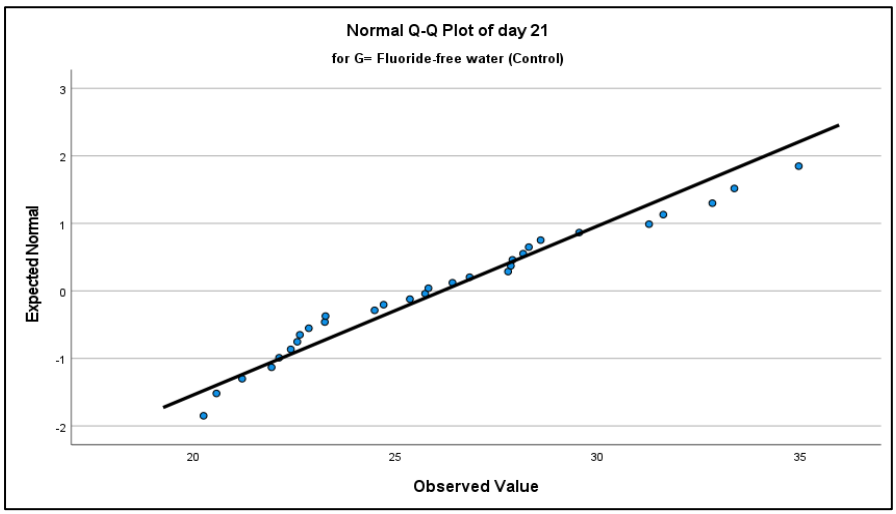
14 Days: Black tea with milk



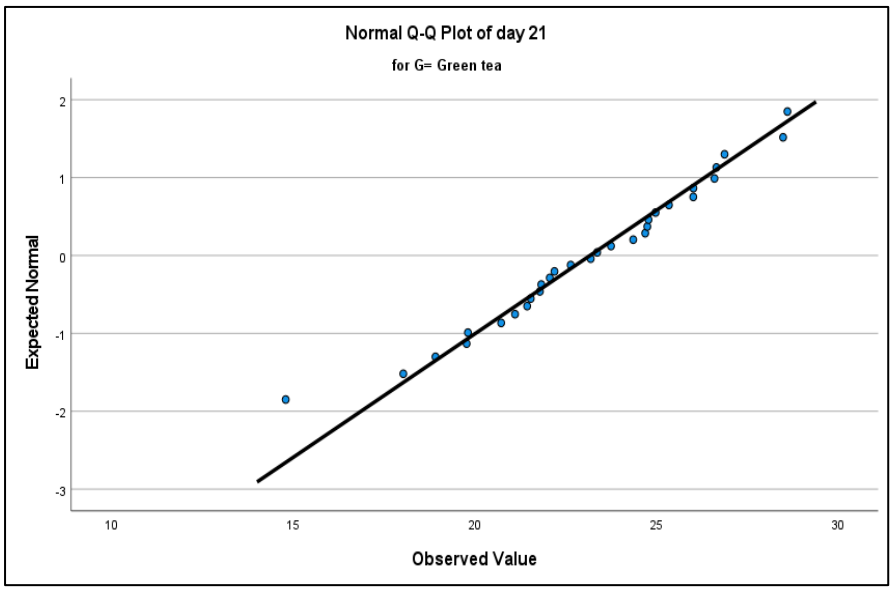
14 Days: Black tea with citric acid



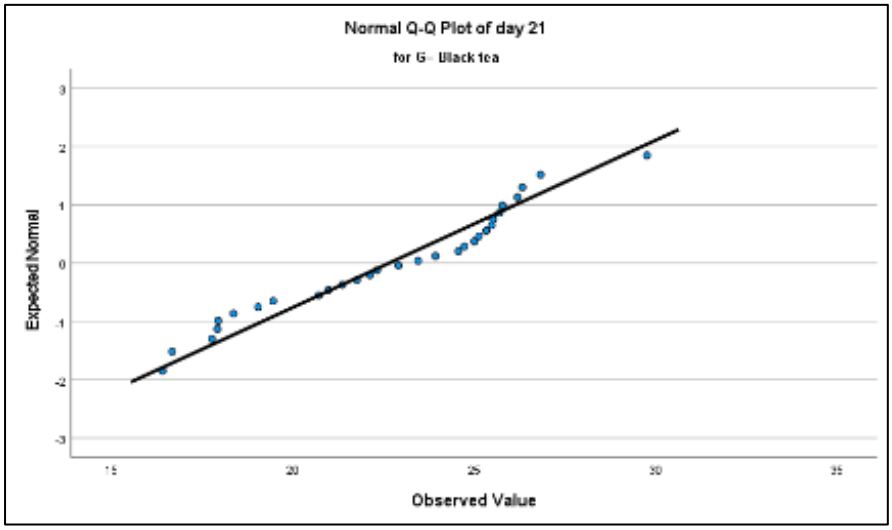
21 Days: Fluoride free water



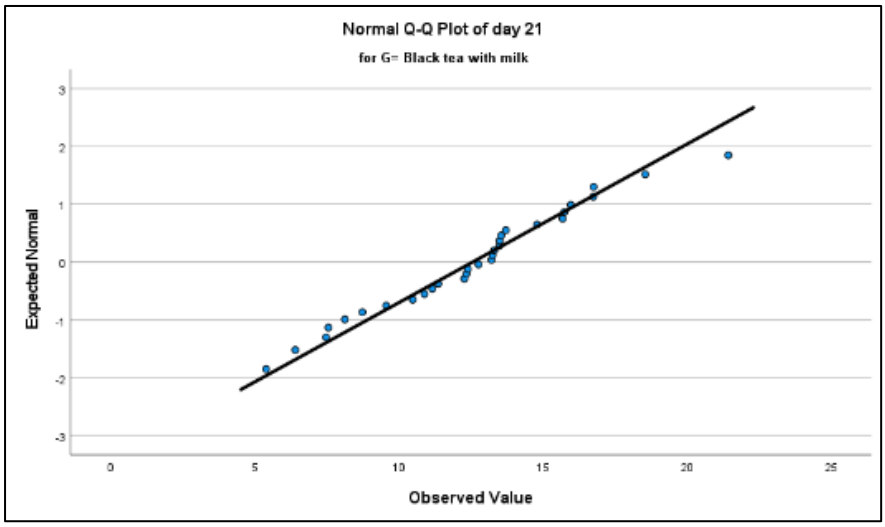
21 Days: Green tea



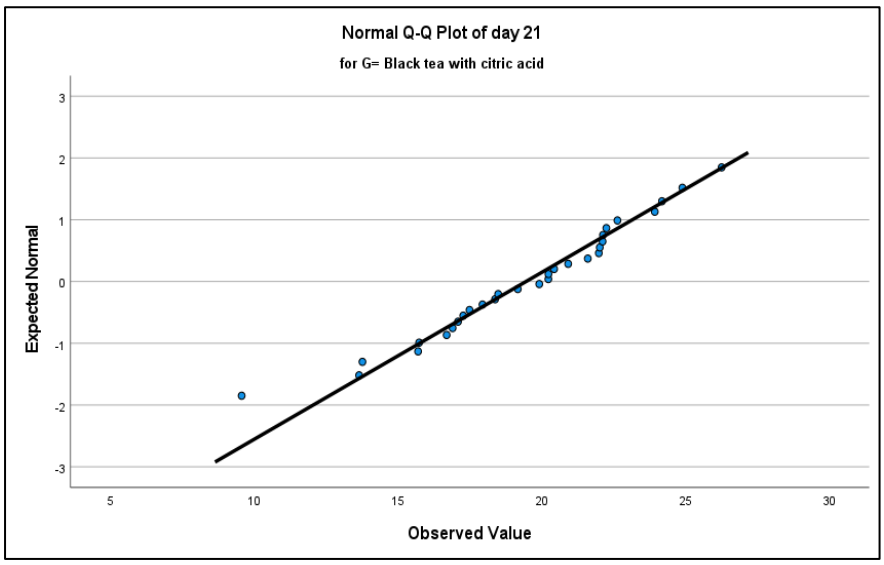
21 Days: Black tea



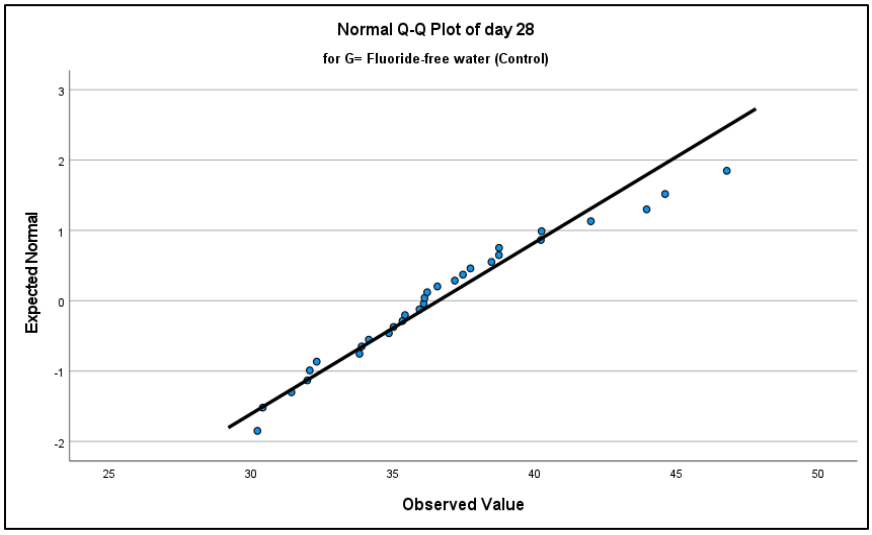
21 Days: Black with milk



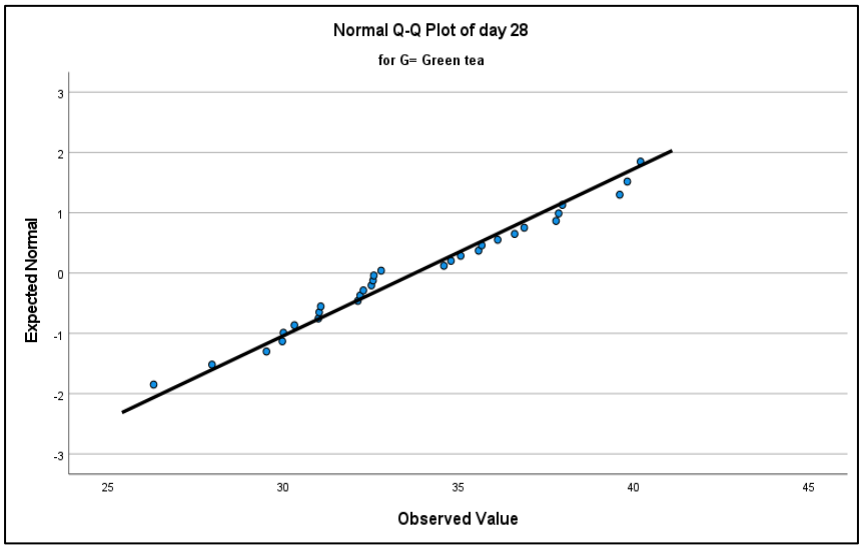
21 Days: Black tea with citric acid



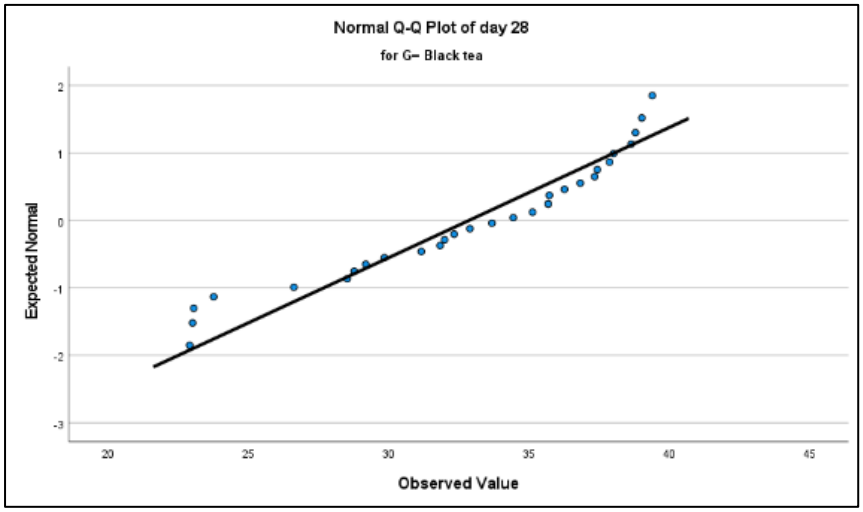
28 Days: Fluoride free water



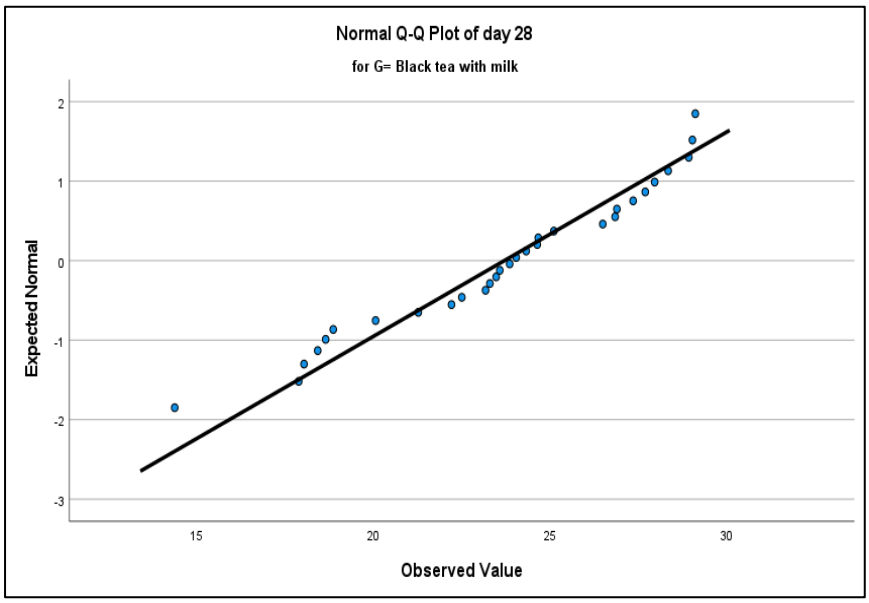
28 Days: Green tea



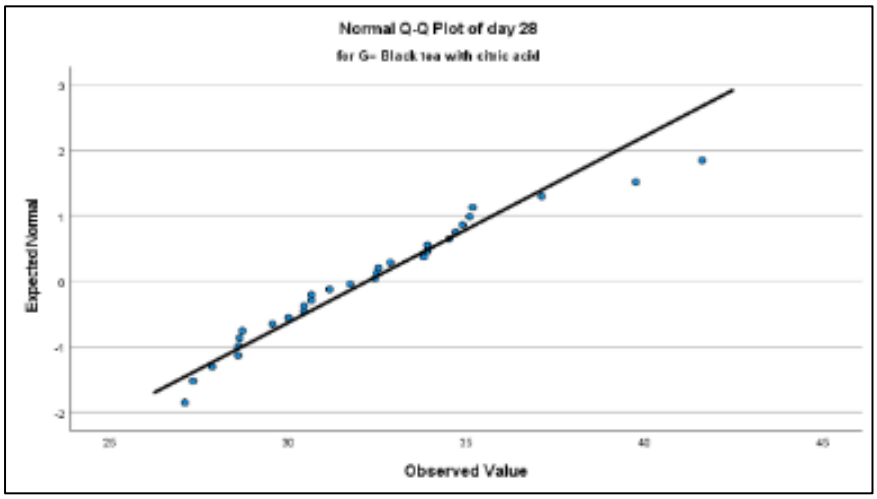
28 Days: Black tea



28 Days: Black tea with milk



28 Days: Black tea with citric acid



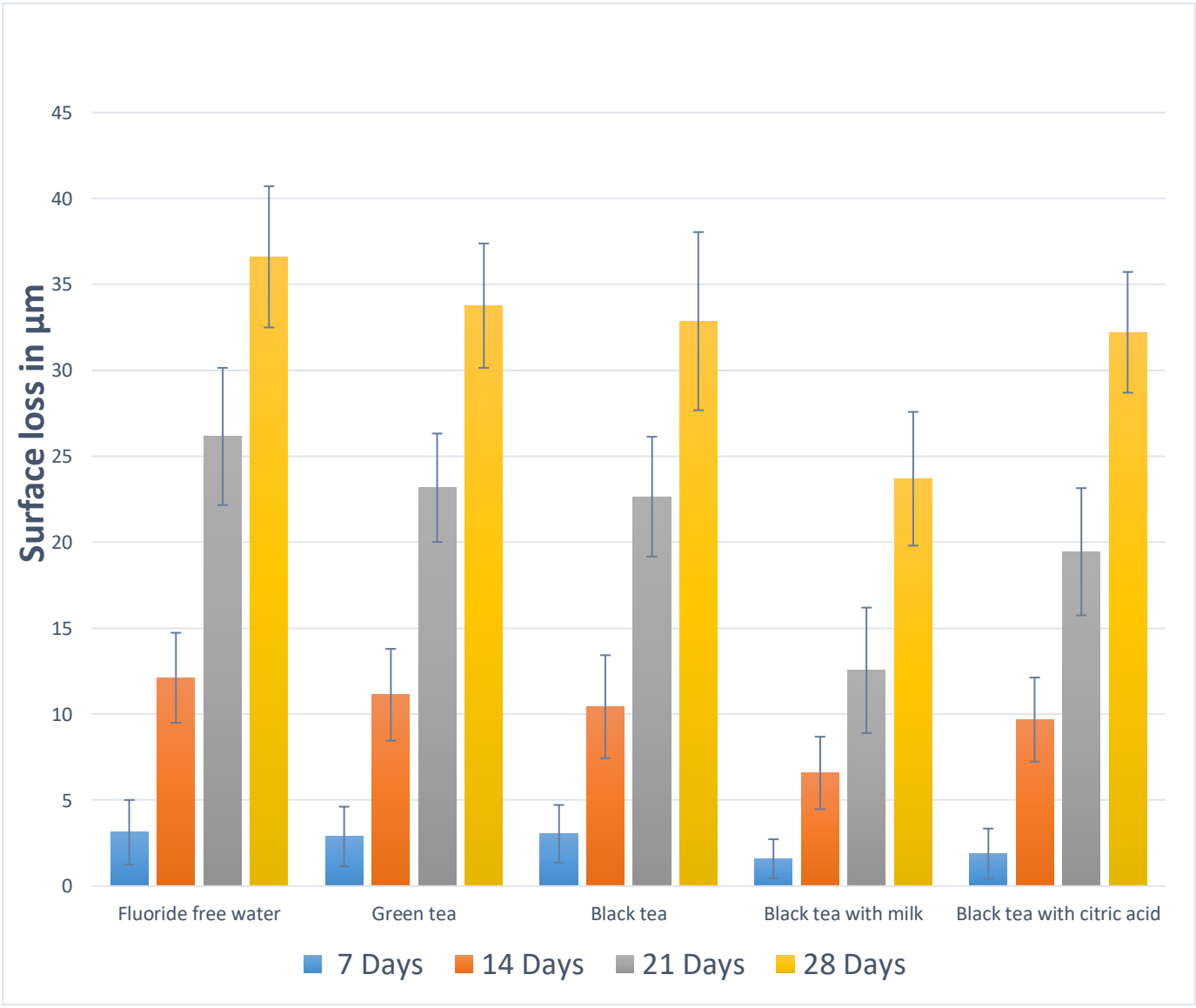
4.2 Descriptive statistics for all groups

Table 4 and Figure 21 show the mean and standard deviation for all groups (fluoride-free water, green tea infusion, black tea infusion, black tea infusion with milk, black tea infusion with citric acid) under erosive challenge. The results showed that the mean of enamel surface loss for each group increased over time. The lowest mean enamel surface loss was seen in black tea infusion with milk group after 7,14,21 and 28 days of erosive challenge while the highest mean enamel surface loss was appeared in the fluoride free-water group.

Table 4: Descriptive statistics (enamel surface loss-µm) for each group after 7, 14, 21 and 28 days erosive challenge (N=30 for each group)

Groups	Day 7		Day 14		Day 21		Day 28	
	Mean SL (µm)	SD	Mean SL (µm)	SD	Mean SL (µm)	SD	Mean SL (µm)	SD
Fluoride free water (control)	3.129	1.88	12.120	2.62	26.159	3.99	36.610	4.10
Green tea	2.887	1.73	11.130	2.67	23.177	3.15	33.767	3.62
Black tea	3.04	1.68	10.440	2.99	22.654	3.49	32.868	5.18
Black tea with milk	1.587	1.14	6.582	2.11	12.555	3.65	23.699	3.89
Black tea with citric acid	1.886	1.46	9.689	2.45	19.452	3.70	32.218	3.51

Figure 21: The effect of different teas on the enamel surface loss after 7, 14, 21 and 28 days erosive challenge



Since the data was normally distributed the repeated measure ANOVA test was performed in the statistical analysis to look at any differences between the groups and across time.

The repeated measure ANOVA revealed a significant difference of enamel surface loss between the five groups over time ($p<0.05$) (Table 5). Table 6 presents the difference within the groups (within subject's test), and show there was a significant time effect, with the mean surface loss of enamel in every group progressing over time. The interaction of time and groups was significant; indicating that the enamel surface loss of the slabs in every group changed in a different way, as illustrated (Figure 22), the lines following similar but not parallel paths. The results showed that by increasing in the number of experimental days, all groups displayed statistically significant surface loss from day 7 to day 28 during the pH cycling period.

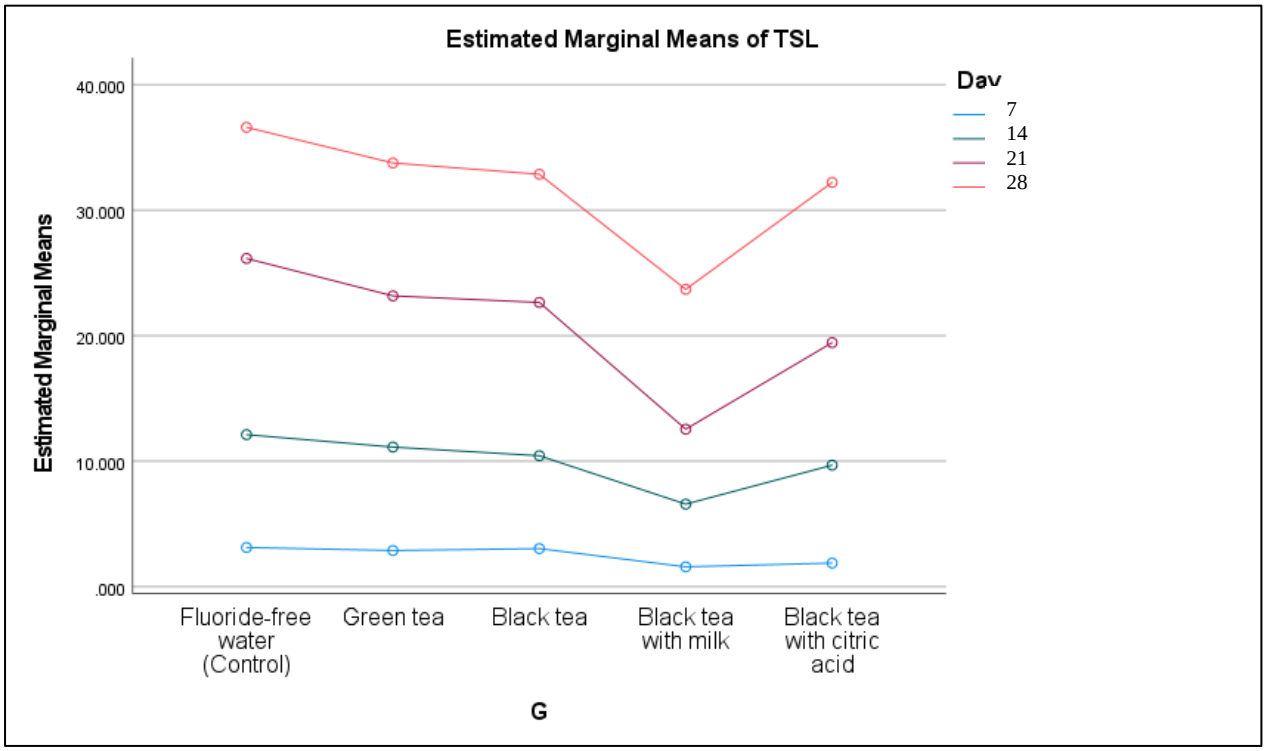
Table 5: Repeated measure ANOVA results between groups for the difference in enamel surface loss over time under erosive challenge

Test of Between-Subjects Effects					
Measure: Tooth surface loss Transformed Variable: Average					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	159070.093	1	159070.093	6686.090	<0.001
G	4855.475	4	1213.869	51.022	<0.001
Error	3449.724	145	23.791		

Table 6: Repeated measure ANOVA results within groups for the difference in enamel surface loss over time under erosive challenge

Test of Within-Subject Effect Measure: Tooth surface loss						
Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Day	Sphericity Assumed	73735.805	3	24578.602	4817.738	0.000
	Greenhouse-Geisser	73735.805	2.362	31217.783	4817.738	<0.001
	Huynh-Feldt	73735.805	2.470	29854.144	4817.738	<0.001
	Lower-bound	73735.805	1.000	73735.805	4817.738	<0.001
Day * G	Sphericity Assumed	1784.732	12	148.728	29.153	<0.001
	Greenhouse-Geisser	1784.732	9.448	188.902	29.153	<0.001
	Huynh-Feldt	1784.732	9.879	180.651	29.153	<0.001
	Lower-bound	1784.732	4.000	446.183	29.153	<0.001
Error(Day)	Sphericity Assumed	2219.235	435	5.102		
	Greenhouse-Geisser	2219.235	342.487	6.480		
	Huynh-Feldt	2219.235	358.131	6.197		
	Lower-bound	2219.235	145.000	15.305		

Figure 22: The interaction of time and groups for difference of enamel surface loss after 7, 14, 21, 28 days under erosive challenge



4.3 Comparison between different groups of tea on the enamel surface loss after 7, 14, 21 and 28 days erosive challenge

One-way ANOVA test and Bonferroni correction were used to compare the amount of surface loss between the five groups after 7, 14, 21, 28 days erosive challenge. The post-hoc test with Bonferroni correction was obtained to identify the difference on enamel surface loss between groups.

4.3.1 After 7 days erosive challenge:

The one-way ANOVA test showed there was a statistically significant difference in the surface loss between the different experimental groups after 7 days of erosive challenge (Table 7).

Table 7: One-way ANOVA test between groups after 7days erosive challenge

		Sum of Squares	df	Mean Square	F	Sig.
7 Days	Between Groups	61.364	4	15.341	5.997	<0.001
	Within Groups	370.915	145	2.558		
	Total	432.279	149			

The results showed that when comparing between the fluoride free-water (control) group with the green infusion and black tea infusion groups, there was no significant difference in enamel surface loss. However, there was a significant difference in comparison with the groups black tea infusion with the addition of milk or citric acid. Comparing the green tea infusion with black tea, black tea infusion with citric acid groups, there was no significant difference in

enamel surface loss. However, in comparison black tea infusion with the addition of milk groups there was a significant difference. In comparison between black tea infusion and black tea infusion with citric acid groups, there was no significant difference in enamel surface loss. However, there was significant difference in comparison between black tea infusion with the addition of milk after 7 days erosive challenge. (Table 8).

Table 8: Multiple Comparison between groups on enamel surface loss after 7 days erosive challenge

Multiple Comparisons Bonferroni							
Dependent Variable	(I) G	(J) G	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
7 Days	Fluoride-free water (Control)	Green tea	0.242	0.41	1.000	-0.935	1.419
		Black tea	0.089	0.41	1.000	-1.089	1.266
		Black tea with milk	1.541*	0.41	0.003*	0.364	2.719
		Black tea with citric acid	1.242*	0.41	0.031*	0.065	2.420
	Green tea	Fluoride-free water (Control)	-0.242	0.41	1.000	-1.419	0.935
		Black tea	-0.153	0.41	1.000	-1.331	1.024
		Black tea with milk	1.299*	0.41	0.020*	0.122	2.476
		Black tea with citric acid	1.000	0.41	0.166	-0.177	2.178
	Black tea	Fluoride-free water (Control)	-0.089	0.41	1.000	-1.266	1.089
		Green tea	0.153	0.41	1.000	-1.024	1.331
		Black tea with milk	1.453*	0.41	0.006*	0.275	2.630
		Black tea with citric acid	1.154	0.41	0.059	-0.023	2.331
	Black tea with milk	Fluoride-free water (Control)	-1.541*	0.41	0.003*	-2.718	-0.364
		Green tea	-1.299*	0.41	0.020*	-2.476	-0.122
		Black tea	-1.453*	0.41	0.006*	-2.630	-0.275
		Black tea with citric acid	-0.299	0.41	1.000	-1.476	0.878
	Black tea with citric acid	Fluoride-free water (Control)	-1.242*	0.41	0.031*	-2.420	-0.065
		Green tea	-1.000	0.41	0.166	-2.178	0.177
		Black tea	-1.154	0.41	0.059	-2.331	0.023
		Black tea with milk	0.299	0.41	1.000	-0.878	1.476

*The mean difference is significant at the 0.05 level.

4.3.2 **After 14 days erosive challenge**

The one-way ANOVA test showed there was a statistically significant difference in surface loss in comparison between the experimental groups after 14 days erosive challenge (Table 9).

Table 9: One-way ANOVA test between groups after 14 days erosive challenge

		Sum of Squares	df	Mean Square	F	Sig.
14 Days	Between Groups	532.263	4	133.066	19.931	<0.001
	Within Groups	968.066	145	6.676		
	Total	1500.329	149			

The results showed that when comparing between the fluoride free-water (Control), green and black tea infusion groups, there was no significant difference in enamel surface loss. However, there was a significant difference in enamel surface loss in comparison with the black tea infusion with the addition of milk or citric acid groups. Comparing the green tea infusion with black tea infusion and black tea infusion with citric acid groups, there was no significant difference in enamel surface loss. However, there was a significant difference found in comparison with group black tea infusion with the addition of milk. In comparison between groups of black tea infusion and black tea infusion with citric acid, there was no significant difference in enamel surface loss. However, there was a significant difference found between black tea infusion and black tea infusion with the addition of milk after 14 days erosive challenge (Table 10).

Table 10: Multiple comparisons between groups on enamel surface loss after 14 days erosive challenge

Multiple Comparisons Bonferroni							
Dependent Variable	(I) G	(J) G	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
14 Days	Fluoride-free water (Control)	Green tea	0.990	0.67	1.000	-0.912	2.892
		Black tea	1.680	0.67	0.129	-0.221	3.582
		Black tea with milk	5.538*	0.67	0.000*	3.636	7.440
		Black tea with citric acid	2.431*	0.67	0.004*	0.530	4.333
	Green tea	Fluoride-free water (Control)	-0.990	0.67	1.000	-2.892	0.912
		Black tea	0.690	0.67	1.000	-1.212	2.592
		Black tea with milk	4.548*	0.67	0.000*	2.646	6.450
		Black tea with citric acid	1.441	0.67	0.325	-0.461	3.342
	Black tea	Fluoride-free water (Control)	-1.680	0.67	0.129	-3.582	0.221
		Green tea	-0.690	0.67	1.000	-2.592	1.212
		Black tea with milk	3.857*	0.67	0.000*	1.956	5.760
		Black tea with citric acid	0.750	0.67	1.000	-1.151	2.652
	Black tea with milk	Fluoride-free water (Control)	-5.538*	0.67	0.000*	-7.440	-3.636
		Green tea	-4.548*	0.67	0.000*	-6.449	-2.646
		Black tea	-3.857*	0.67	0.000*	-5.759	-1.956
		Black tea with citric acid	-3.107*	0.67	0.000*	-5.009	-1.205
	Black tea with citric acid	Fluoride-free water (Control)	-2.431*	0.67	0.004*	-4.333	-0.529
		Green tea	-1.441	0.67	0.325	-3.342	0.461
		Black tea	-0.750	0.67	1.000	-2.652	1.151
		Black tea with milk	3.107*	0.67	0.000*	1.205	5.009

*The mean difference is significant at the 0.05 level.

4.3.3 **After 21 days erosive challenge**

The one-way ANOVA test showed there was a statistically significant difference in surface loss in comparison between the experimental groups after 21 days erosive challenge (Table 11).

Table 11: One-way ANOVA test between groups after 21 days erosive challenge

		Sum of Squares	df	Mean Square	F	Sig.
21 Days	Between Groups	3228.283	4	807.071	62.043	<0.001
	Within Groups	1886.208	145	13.008		
	Total	5114.491	149			

The results showed that when comparing the fluoride free-water (Control) group with other experimental groups, there was a significant difference in enamel surface loss. Comparing the green tea infusion with black tea infusion groups, there was no significant difference in enamel surface loss. However, there was a significant difference found between the green tea infusion and black tea infusion with the addition of milk or citric acid groups. Among black tea infusion and black tea infusion with additions, there was a significant difference in enamel surface loss between black tea infusion and black tea infusion with the addition of milk or citric acid after 21 days erosive challenge (Table 12).

Table 12: Multiple comparisons between groups on enamel surface loss after 21 days erosive challenge

Multiple Comparisons							
Bonferroni							
Dependent Variable	(I) G	(J) G	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
21 Days	Fluoride-free water (Control)	Green tea	2.982*	0.93	0.017*	0.327	5.636
		Black tea	3.505*	0.93	0.002*	0.850	6.159
		Black tea with milk	13.604*	0.93	0.000*	10.950	16.259
		Black tea with citric acid	6.707*	0.93	0.000*	4.052	9.362
	Green tea	Fluoride-free water (Control)	-2.982*	0.93	0.017*	-5.636	-0.327
		Black tea	0.523	0.93	1.000	-2.132	3.178
		Black tea with milk	10.623*	0.93	0.000*	7.968	13.277
		Black tea with citric acid	3.725*	0.93	0.001*	1.071	6.380
	Black tea	Fluoride-free water (Control)	-3.505*	0.93	0.002*	-6.160	-0.850
		Green tea	-0.523	0.93	1.000	-3.178	2.132
		Black tea with milk	10.100*	0.93	0.000*	7.445	12.754
		Black tea with citric acid	3.202*	0.93	0.008*	0.548	5.857
	Black tea with milk	Fluoride-free water (Control)	-13.604*	0.93	0.000*	-16.259	-10.950
		Green tea	-10.623*	0.93	0.000*	-13.277	-7.968
		Black tea	-10.100*	0.93	0.000*	-12.754	-7.445
		Black tea with citric acid	-6.897*	0.93	0.000*	-9.552	-4.243
	Black tea with citric acid	Fluoride-free water (Control)	-6.707*	0.93	0.000*	-9.362	-4.052
		Green tea	-3.726*	0.93	0.001*	-6.380	-1.071
		Black tea	-3.202*	0.93	0.008*	-5.857	-0.548
		Black tea with milk	6.897*	0.93	0.000*	4.243	9.552

* The mean difference is significant at the 0.05 level.

4.3.4 **After 28 days erosive challenge**

The one-way ANOVA test showed there was a statistically significant difference in surface loss in comparison between the experimental groups after 28 days erosive challenge (Table 13).

Table 13: One-way ANOVA test between groups 28 days erosive challenge

		Sum of Squares	df	Mean Square	F	Sig.
28 Days	Between Groups	2818.297	4	704.574	41.806	<0.001
	Within Groups	2443.769	145	16.854		
	Total	5262.066	149			

The results showed that when comparing between the fluoride free-water (Control) group with black tea infusion and black tea infusion with additions of milk or citric acid groups, there was a significant difference in enamel surface loss. However, there was no significant difference in comparison with green tea group. Comparing groups green tea infusion with black tea infusion and black tea infusion with addition of citric acid, there was no significant difference in enamel surface loss. However, there was a significant difference found between groups green tea infusion and black tea infusion with the addition of milk. Among black tea infusion and black tea infusion with additions, there was no significant difference in enamel surface loss in comparison between the black tea infusion and black tea infusion with citric acid groups. However, there was a significant difference between black tea infusion and black tea infusion with the addition of milk 28 days erosive challenge (Table 14).

Table 14: Multiple comparisons between groups on enamel surface loss after 28 days erosive challenge

Multiple Comparisons							
Bonferroni							
Dependent Variable	(I) G	(J) G	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
28 Days	Fluoride-free water (Control)	Green tea	2.843	1.06	0.082	-0.178	5.865
		Black tea	3.743*	1.06	0.006*	0.721	6.764
		Black tea with milk	12.911*	1.06	0.000*	9.890	15.933
		Black tea with citric acid	4.392*	1.06	0.001*	1.371	7.414
	Green tea	Fluoride-free water (Control)	-2.843	1.06	0.082	-5.865	0.178
		Black tea	0.899	1.06	1.000	-2.122	3.921
		Black tea with milk	10.068*	1.06	0.000*	7.046	13.090
		Black tea with citric acid	1.549	1.06	1.000	-1.473	4.570
	Black tea	Fluoride-free water (Control)	-3.743*	1.06	0.006*	-6.764	-0.721
		Green tea	-0.899	1.06	1.000	-3.921	2.122
		Black tea with milk	9.169*	1.06	0.000*	6.147	12.190
		Black tea with citric acid	0.649	1.06	1.000	-2.372	3.671
	Black tea with milk	Fluoride-free water (Control)	-12.911*	1.06	0.000*	-15.933	-9.890
		Green tea	-10.068*	1.06	0.000*	-13.090	-7.046
		Black tea	-9.169*	1.06	0.000*	-12.190	-6.147
		Black tea with citric acid	-8.519*	1.06	0.000*	-11.541	-5.498
	Black tea with citric acid	Fluoride-free water (Control)	-4.392*	1.06	0.001*	-7.414	-1.371
		Green tea	-1.549	1.06	1.000	-4.570	1.473
		Black tea	-0.649	1.06	1.000	-3.671	2.372
		Black tea with milk	8.519*	1.06	0.000*	5.498	11.541

*The mean difference is significant at the 0.05 level.

4.4 Summary of the results

- Repeated measure ANOVA test showed the amount of enamel surface loss for each group was increased over time.
- There was a significant difference in enamel surface loss after 28 days erosive challenge in comparison between the fluoride free-water (control group) with black tea infusion and black tea infusion with additions of milk or citric acid groups. However, there was no significant difference in comparison with green tea infusion group, but the amount of enamel surface loss was lower than in fluoride free water group
- There was no significant difference in enamel surface loss between black tea infusion and green tea infusion groups after 28 days of erosive challenge.
- There was no significant difference in enamel surface loss in comparison between black tea infusion and black tea infusion with citric acid groups after 28 days erosive challenge. However, there was higher significant difference in enamel surface loss in comparison with black tea infusion and milk group. Furthermore, it was higher significant difference in enamel surface loss between black tea infusion with citric acid and black tea infusion with milk groups.

5 Discussion

5.1 Justification of study aim

There are few studies exploring the effect of tea on tooth surface loss (Jaâfoura et al., 2014) and the majority of the *in vitro* studies that investigating the effect of tea, particularly green tea, are studying the effect of dentine erosion. In examining the polyphenol and minerals contents in tea that have anti-erosive effect on dentine erosion, it has been found that polyphenol acts as a matrix metalloproteinase (MMP) inhibitors against dentine erosion (Bassiouny et al., 2008, Kato et al., 2009, Barbosa et al., 2011, Buzalaf et al., 2012b, Mirkarimi and Toomarian, 2012, De Moraes et al., 2016).

Several studies have evaluated the erosive potential of different beverages (Fruit juices, soft drinks, sodas, smoothies, energy drinks, sport drinks, drinking yogurt, lemon tea, tea and coffee) according to distinct parameters such as pH, titratable acidity, buffering capacity and concentration of calcium, phosphate and fluoride (Murrell et al., 2010, Benjakul and Chuenarrom, 2011, portAli and Tahmassebi, 2014, Tahmassebi et al., 2014, Reddy et al., 2016). A many of the herbal teas (lemon tea, raspberry, blackcurrant with ginseng and vanilla, raspberry with cranberry and elderflower, strawberry with loganberry, traditional lemon, camomile, peach and passion fruit) have been found to have erosive effect (Phelan and Rees, 2003), black tea and green tea has been found to have high pH about 5.7 and 6.3 respectively and high amount of fluoride in black tea infusion ranged between 0.57 to 3.72 mg/L compared to herbal (pH 3.2) and fruity teas (pH ranged between 2.98 to 3.95), the fluoride amount for herbal tea ranged between 0.02 to 0.04 mg/L (Emekli-Alturfan et al., 2009, Jaâfoura et al., 2014). The addition of lemon to black tea was shown to have low pH (2.70) and high titratable acidity (1.85 mmol/l in titration to pH 7.0) (Oliveira et al., 2017a).

There are not many well-conducted studies investigating the anti-erosive effect of black tea and green tea on tooth surface loss. Therefore, the aim of this study was to investigate the effect of black and green tea infusion on surface loss (progression) of dental enamel under erosive challenge *in vitro*.

5.2 *In vitro* model

The present study used an *in vitro* pH cycling model to investigate the effectiveness of black tea, green tea and black tea with addition of milk or lemon (citric acid) on the surface loss of dental enamel under erosive challenge.

The key advantage in *in vitro* experimentation is the ability to carry out single variable experiments under highly controlled conditions (White, 1995). In this *in vitro* study we used a multiple exposure acid model, which provided a good understanding of the erosive challenges faced by the dentition under controlled investigation conditions and reducing the experimental time and cost (De Moraes et al., 2016).

In terms of remineralising medium, artificial saliva was used in the present study. It should be remembered that artificial saliva formulation is based on the electrolyte composition of natural saliva but lacks salivary proteins which can result in too high a degree of supersaturation and could thus lead to excessive amounts of calcium phosphate precipitation (Shellis et al., 2011). Another complication is that some proteins in natural saliva, such as statherin, tend to inhibit calcium phosphate precipitation which is difficult to model in artificial saliva, therefore, the remineralisation *in vitro* is likely to be more than *in vivo* (Shellis et al., 2011). This should be taken into account when extrapolating results into the *in vivo* situation.

There are some limitations with *in vitro* models, for instance, difficulty mimicking the complex biological processes associated with tooth surface loss (West et al., 1998). Furthermore, the lack of many significant protective biochemical processes available in the oral environment

that enhance the remineralisation and reduce the demineralisation process such as the salivary flow rate, the salivary composition and the formation of the salivary pellicle on the tooth surface (White, 1992, West et al., 1998).

5.3 Study design

This was an *in vitro* study design to investigate the effect of black and green tea on surface loss (progression) of dental enamel under erosive challenge. Five groups were used in this study: fluoride-free water as control group, green tea, black tea, black tea with milk, black tea with lemon (citric acid). The reason for choosing the milk and lemon as additions in combination with a black tea was because these are the additions that are those normally used by people when they drink black tea and thus, by mimicking the real situation, we can provide more information of any effect of these additions on the enamel surface loss. One hundred and fifty enamel slabs in this study were randomly allocated to 5 groups (30 slabs in each group) by using an online randomisation engine. This sample size was calculated following statistical advice. It was considered a pilot study because no relevant effect of sample size and standard deviation for the primary outcome can be found from high quality publications in the existing literature, and 30 enamel slabs were allocated to the groups as a general rule to calculate the sample size in a pilot study (Lancaster et al., 2004). Two artificial saliva solutions were used in this study: the first saliva solution is supersaturated with respect to hydroxyapatite was used between the acid exposure in pH cycling during the daytime and the second saliva solution is saturated with respect to hydroxyapatite was used to store the slabs in the incubator at night-time. A randomised, single-blinded design was used to prevent the introduction of bias into the study.

5.4 Bovine teeth

As human teeth are limited to obtain for the research purposes, bovine teeth have been the most used substitute for human teeth and their use has dramatically increased in dental research over the past years (Yassen et al., 2011). Moreover, bovine enamel is widely used in analysing the effect of various conditions on dental erosion and gives good reproducibility of outcomes when comparing to human teeth (Oliveira et al., 2017b).

Bovine teeth are easier to obtain with a sufficient number of sound teeth compared with human teeth. In addition, the composition of human teeth is more variable due to genetic influences, environmental conditions (diet, caries lesions, fluoride exposure) and age (dentine sclerosis and post-eruptive maturation) that might influence the condition of the enamel and lead to large variations in their response under acidic challenge or potentially interact with experimental solutions (Buzalaf et al., 2010, Wegehaupt et al., 2011) .

The hardness values of enamel in human and bovine teeth are similar and no difference on surface ultrastructure of bovine and human enamel following erosive challenge (Herkströter et al., 1989, Meurman and Frank, 1991b). Furthermore, the chemical structure of bovine enamel and the response to erosive challenges can be comparable to the human enamel (Davidson et al., 1973). White et al. (2010) found that there was no statistically significant difference between the softening of human and bovine enamel after 60 seconds under citric acid challenge. However, it also reported that there is a lack of consensus in term of rates of demineralisation of human and bovine enamel.

As mentioned above in literature by Laurance-Young et al. (2011) that bovine enamel has an increased porosity and lower fluoride concentration compared to human enamel. Hence, this should be considered in the present study that the bovine enamel might progress the surface loss faster than human enamel.

In the present study, the bovine teeth were used as an alternative to human teeth.

5.5 Enamel slab preparation and storage

The enamel slabs were obtained from a buccal section of bovine teeth incisor in the present study as in this area they have uniform thickness and a flat surface. The enamel slabs were stored in distilled de-ionised water and 0.1% thymol (Sigma Aldrich) in order to inhibit bacterial growth and prevent dehydration of the enamel slabs. Thymol is the recommended storage agent as it shows no change in micro- or nanohardness when stored in this agent for several days (Shellis et al., 2011). The principal mode of action of thymol is the ability to penetrate the cell membrane and subsequently destroy the pathogens that may grow on the teeth (Shapiro and Guggenheim, 1995). Depending on the detrimental effect of thymol on the dentine and enamel permeability, it cannot be assumed that the use of thymol will completely destroy all pathogens that may present on the tooth surface (Preston et al., 2007).

The teeth should be kept moist up to the time of experiment with any chosen disinfected agent to achieve the greatest effect against a prion disease which is transmissible from infected human or bovine tissues (Shellis et al., 2011).

5.6 pH cycling model with erosive challenges *in vitro*

The genesis of modern pH-cycling model was produced by Ten Cate and Duijsters (1982) which involved multiple cycles of the demineralisation and remineralisation process with mineral loss and uptake to mimic the oral environment.

The duration of pH cycling in *in vitro* studies in the literature was for a limited time period, extending over 14 days, and some of these studies investigated the effect of different fluoride concentrations in toothpaste (250 ppm, 500 ppm, 1150 ppm, 1450ppm) and fluoridated milk (2.5 ppm F, 5.0 ppm F, 10ppm F) on tooth surface loss (Magalhães et al., 2014, Albariqi, 2020, Abdullah, 2009). However, the present study extended to a 28-days period of pH cycling to allow sufficient time to investigate effect of tea on the eroded enamel slabs. On the other hand,

Shellis et al. (2011) reported that the duration of pH cycling in tooth surface loss studies does not need to be as long as in caries research because the erosion lesion is a rapidly progressing condition and created early by erosive challenge.

In the present study, the pH cycling regime involved exposing enamel slabs to five erosive challenges for two minutes during the day (0.3% citric acid with pH 2.6). The enamel slabs were dipped in five treatment groups (fluoride free-water, green tea, black tea, black tea with milk and black tea with 0.1% citric acid pH 3.6) for 10 minutes three time daily as the most people drink the tea three times during the day. On each occasion, the slabs were rinsed with de-ionised water before immersion in citric acid, then placed in day-time artificial saliva for 60 minutes and then rinsed again with de-ionised water after immersion in treatment groups. After treatment cycle every day, the slabs were kept in night-time artificial saliva. The day-time artificial saliva was supersaturated with calcium and phosphate ions to promote the remineralisation process. While the night-time artificial saliva was a saturated solution with calcium and phosphate ions to maintain the enamel condition without providing any mineral exchanges.

It is very important to bear in mind that using artificial saliva for *in vitro* study does not simulate most properties of natural saliva. It lacks salivary protein and unable to form the pellicle which acts as a protective surface layer, and also acts as a diffusion barrier and inhibits the contact of acid to the dental surface and consequently, decreases the diffusion of calcium and phosphate ions into the surrounding fluid exposure (Buzalaf et al., 2012a, Batista et al., 2016, Mann et al., 2014).

The pH cycling protocol, which was used in the present study, was developed at the University of Leeds and it has been used in a previous tooth surface loss study by Abdullah (2009) at the Leeds Dental Institute. This protocol is a slightly modified version from Amaechi et al. (1999) who used the method to produce dental erosion in an *in vitro* study.

5.7 Surface Microhardness

The use of microhardness in the present study was to measure the resistance of the enamel surface to the force applied by an indenter and to exclude any enamel slabs with too soft (length of indent bigger than 60-70 microns) enamel (Abdullah, 2009) or slabs with the exposed dentine.

As mentioned above in section 2.11.1, the microhardness is measured with either Knoop or Vicker's hardness which both represent the most common surface hardness techniques in dental research (Lippert and Lynch, 2014). All the enamel slabs were flattened and had polished surfaces which are necessary for the accurate assessment of enamel hardness (Schlueter et al., 2011). Featherstone (1992) reported the recommended load to be applied is between 50-200g. In the present study, a Knoop diamond was used under 100g load for 15 seconds. Davidson et al. (1974) reported the 100g load was necessary to facilitate optical perceptibility.

The main advantages of the microhardness technique are low cost and it can be combined with measurements of abrasive surface loss. However, the main drawback is that the measurements in highly eroded lesions are inaccurate or impossible to achieve as it gives unclear defined indentation boundaries (Joshi et al., 2016).

5.8 Surface Profilometry

In the present study, surface profilometry was used for two purposes: firstly, before the pH cycling experiment, in order to check all the enamel slabs were a flat surface. Barbour and Rees (2004) reported that enamel specimens should be ground flat prior to use to detect minimal loss even below 1 µm. This step was necessary as some slabs required grinding for 2-3 times to ensure the flatness and also it produced less variation among the enamel slabs (Ganss et al., 2000). However, by grinding and polishing the enamel surface, it removed the outermost

layer, possibly the prismatic layer which is hypermineralised by fluoride and saliva during the demineralisation and remineralisation processes, which led to higher dissolution when compared to native enamel and causes more rapid lesion progression (Oliveira et al., 2017b, Elton et al., 2009). Secondly, after pH cycling experiment, surface profilometry quantifies the loss of dental tissue by measuring the step height between unaffected reference areas and an experimental area that is exposed to erosive challenges. Therefore, it gives an indication of progression of tissue loss if the step height is increased in sequential measurements (Ganss et al., 2005, Huysmans et al., 2011).

In the present study, non-contact profilometry was used with white light with vertical range from 300 µm. Schlueter et al. (2011) mentioned that the vertical range for white light non-contact profilometry varies from 300 µm to 10 mm (depending on the sensor selected) and gives better flexibility when analysing very deep erosion, even in pits and curved natural surfaces.

Using non-contact profilometry overcomes the drawbacks of contact profilometry as there is no direct contact of a stylus with the eroded surfaces (Joshi et al., 2016). On the other hand, non-contact profilometry, by using light topography, maps the crater surface and measures the depth of the erosion, but cannot measure demineralised subsurface lesions which means it can only image the actual lesion surface. Thereby, it is likely to under report the true total lesion depth (Elton et al., 2009). Elton et al. (2009) also reported when comparing non-contact profilometry with other methods of assessment of erosive lesion, this under reporting of values did not affect correlation statistics. Therefore, it is as valid as the other methods and can clearly measure the extent of the erosion.

5.9 The effect of black and green tea on dental erosion

Tea is the most popular beverage consumed around the world with two-thirds of the world's population consuming tea (Jaâfoura et al., 2014, Waugh et al., 2017). As mentioned in the literature, several studies reported that polyphenolic compounds present in black and green tea have been proven to be beneficial for oral and general health in the prevention of many debilitating human diseases such as cardiovascular disease, hypertension, diabetes and metabolic disease as well as enhancing innate immunity and may also lead to protect against cancers. It is also shown to prevent dental caries and periodontal disease (Robbins, 2003, Han et al., 2016, Khan and Mukhtar, 2013, Jaâfoura et al., 2014).

Several studies have proven that tea has anti-erosive effect (Brunton and Hussain, 2001, Bassiouny et al., 2008, Jaâfoura et al., 2014). The present study showed that there is no statistical difference between groups black and green tea on enamel surface loss after 7,14, 21 and 28 days erosive challenge, which means that both groups black and green tea have anti-erosive effect on surface loss of enamel. In comparison with water free-fluoride (control group) after 28 days erosive challenge both groups black and green tea showed less tooth surface loss (32.868 µm, 33.767 µm respectively) than fluoride free water (36.610 µm),

Furthermore, the comparison between groups green tea and fluoride free water did not reach statistical significance even it was a trend in the result towards a protective effect. Possibly with the increase of a sample size or a longer period of erosive cycling, this will reach a significance. However, there was a significant difference in enamel surface loss between all black tea groups and control. Barbosa et al. (2011) reported that supplementation of drinks (Coca-Cola,Kuat guarana, Sprite and ligh Coca-Cola) with green tea can reduce erosive potential against dentine in bovine teeth. Moreover, green tea has less weight loss of human teeth structure on exposure of tooth to different beverages (orange juice, Red Bull, Pepsi and lemon juice) (Mathew et al., 2018).

Bassiouny et al. (2008) conducted *in vitro* study to compare both black and green tea with soda and orange juice in order to determine their erosive effect. They found that both black and green tea had low potential for erosion.

The reasons why tea groups showed less tooth surface loss than water free fluoride (control group) might be from several factors. It has been shown that the enamel erosion level caused by erosive drinks is associated with many predisposing factors including pH, titratable acid content, buffering capacity, exposure time, temperature and phosphate, calcium and fluoride concentration (Lussi et al., 2004, Oliveira et al., 2017b).

As mentioned in the literature (Meurman and ten Cate, 1996), the low pH of beverages (below 4.0) causes dental erosion and is potentially a good predictor of dental erosion. The pH of black tea was 4.8 and the pH for green tea was 5.4. Although both black and green tea showed the level of pH below the critical pH of 5.5, which is necessary for demineralisation of enamel (Brunton and Hussain, 2001). Other studies found that enamel begins to erode when the pH value decreases under 4.5 (Phelan and Rees, 2003, Benjakul and Chuenarrom, 2011). Also, Simpson et al. (2001) investigated the composition of black tea in term of its erosive potential and they proved that black tea has low acidity and this can reflect the minimal pH decreases on the dentition after drinking black tea in which the decrease was less than 1 pH unit.

The titratable acidity has been accepted to be a more important indicator than pH value in determining the erosive potential of drinks whereby the acid content of the beverages influences the buffering capacity. Thus, the drinks with low pH such as fruit juices (pH below 4.0) are strongly buffered with a potential erosive capability (Singh and Jindal, 2010).

Tea is known to contain relatively high amounts of fluoride as the tea plant absorbs the fluoride from the soil and accumulates it in its leaves.

Black and green tea in the present study were selected based on the amount of fluoride found in a previous study, which measured the amount of fluoride in different types and brands of tea (Xiarchou, 2016). Black and green tea were selected with the same amount of fluoride (≈ 5.0 ppm F) in tea infusion. Another reason for choosing ≈ 5.0 ppm F is to avoid exceeding the recommended optimal level of daily fluoride intake. Thus, the optimal fluoride exposure level was indicated to be between 0.05 and 0.07 mg F/kg body weight per day. The estimated children's fluoride intake from water 1.0 ppm fluoride and food to be 0.5-1.5 mg per day. As the general rule, the fluoride intake corresponding to consumption of 1.0 ppm water fluoride was about 0.05mg/kg body weight (Warren et al., 2009). Based on milk fluoridation scheme worldwide, the amount of fluoride added to milk was decided about 0.5 and 1.0 mg per day for children, the fluoride concentration in milk typically ranges between 2.5 and 5.0 ppm fluoride (Bánóczy et al., 2013, Magalhães et al., 2014).

In order to further investigate how much of an effect the fluoride in tea has on the surface loss progression an extra group of fluoride free tea may have added to the data. However, as tea is a natural product it proved impossible to find a product which contained 0 ppmF.

The effect of the fluoride in resistance to acid dissolution is not fully understood compared to the understanding of the role of protection of fluoride in the dental caries process (Trevisanato and Kim, 2000, Brunton and Hussain, 2001, Ruan and Wong, 2001, Emekli-Alturfan et al., 2009).

Lussi and Carvalho (2015) described human enamel as being composed of calcium hydroxyapatite. When the fluoride ions enter the enamel crystal, it converts the crystal surface to fluor(hydroxyl)apatite and hence, reducing the solubility of the surface. Therefore, the adsorption of fluoride on the crystal offers direct protection from demineralisation and encourages remineralisation. However, this mechanism is more precise in caries prevention, because in the erosive process there is no sheltered area, as in the subsurface carious lesion.

Thus, any protective mechanism from fluoride within the lesion is limited to the surface or the near surface layer of enamel (Lussi and Carvalho, 2015). Furthermore, Wiegand and Attin (2014) reported that 5.0 ppm fluoride in milk is unable to provide a beneficial effect in reducing tooth surface loss. In order to have a good protection of tooth surface against dental erosion for example when applying conventional fluoride, the calcium fluoride like layer should be dense enough to build up a barrier that hampers the acid contact with underlying enamel and it has the effect against dental erosion (Lussi and Carvalho, 2015). On the other hand, Larsen and Richards (2002), found that in an *in vitro* study the addition of calcium fluoride in which the total fluoride concentrations ranging from 6-15 ppm to carbonated soft drinks reduced erosion by 28% only when the pH of the drinks were above pH 3. Therefore, fluoride alone is unable to prevent dental erosion, there are multifactor reasons.

According to Emekli-Alturfan et al. (2009), Waugh et al. (2017) there are undesirable effects of fluoride on tooth formation and to the health risk with regular tea consumption that causes long-term chronic fluoride intake.

The present study's aim was to explore the anti-erosive effect of both black tea and green tea over time by measuring the tooth surface loss after 7, 14, 21, and 28 days under erosive challenge. There was a significant time effect for both groups green tea infusion and black tea infusion on enamel surface loss. Black tea infusion group showed no significant difference after 7 and 14 days on enamel surface loss compared to water-free fluoride (control group) but by the time after 21 and 28 days showed a significant difference on enamel surface loss. However, green tea group performed differently than the black tea group although they showed anti-erosive effect compared to water free fluoride, in which green tea infusion group showed no significant difference after 7, 14 and 28 days on enamel surface loss. However, it showed a significant difference after 21 days on enamel surface loss. This difference between groups

black and green tea infusions over a period of time might be because the difference was in the pH level in both groups.

As discussed in the literature, natural and chemical products in tea may have anti-erosive effects. This includes substances such as polyphenol that is found in green tea, which acts as a matrix metalloproteinase (MMPs) inhibitor reducing the degradation of dentine. Matrix metalloproteinase (MMPs) are present in both dentine and saliva (Buzalaf et al., 2012b), which are activated during the acid attacks and degrades the collagen from the organic matrix and causes dental erosion. Ozan et al. (2020) have shown that both black and green tea have similar potential in preventing dental erosion as both contain catechins (polyphenol) that naturally act against MMPs, perhaps explaining the results found in this present study. It has been found that black tea had about 10 times lesser polyphenols and about 7 times more fluoride than green tea (Ozan et al., 2020). This also might explain the minimum difference in mean surface loss between black and green tea infusion.

5.10 The effect of black tea with addition of milk on dental erosion

Black tea with milk is a common way of drinking tea especially in the UK. Different additions such as milk, lemon, ginger, fruit and herbs with tea could give good insight when exploring different people's behaviour when they drink tea and aid in evaluating of the effect of different additions with tea.

Milk is a biological solution that contains calcium, phosphate, protein (casein) and lipids, which are proven to enhance remineralisation (Wiegand and Attin, 2014). There are many studies investigating the remineralising effect of milk on dental caries (Reynolds, 1998, Grenby et al., 2001, Walker et al., 2006, Malinowski et al., 2012, Cassiano et al., 2017, Angarita-Díaz et al., 2020, Mahesh et al., 2020), but few studies evaluated the anti-erosive effect of milk (Wiegand and Attin, 2014, Cassiano et al., 2016, White et al., 2011). On the other hand the

anticariogenic effect of milk has been very well investigated and has been attributed to the casein, phosphate and calcium contents in the milk (Vongsawan et al., 2010). Furthermore, the preventive factors to reduce dental erosion are to enhance the acid resistance and remineralisation of the teeth in which calcium, phosphate and fluoride are required (Imfeld, 1996). Cow's milk has more casein, phosphorus and calcium compared to human milk and semi-skimmed milk contains more calcium than whole milk (Hambraeus, 1993, McCance and Widdowson, 2014). It has been proven that supplementing drinks by the addition of calcium and phosphate can help prevent dental erosion (Larsen and Nyvad, 1999). This was mirrored in the present study, where adding milk rich in calcium and phosphate to the black tea infusion showed dramatic decrease in tooth surface loss (23.699 µm) after 28 days under erosive challenge compared to other experimental solutions (fluoride free-water 36.610 µm, green tea 33.767 µm, black tea 32.868 µm and black tea with citric acid 32.218 µm). In addition, it showed higher significant difference on enamel surface loss compared to all other experimental solutions in the current study. This proves the addition of milk generates anti-erosive effect over and above that of black tea on the tooth surface under erosive challenge.

As mentioned above in the literature, tea contains several minerals in which fluoride one of these minerals. It has been proven that black tea contained 7 times more fluoride than green tea (Ozan et al., 2020). In the present study the amount of fluoride in black tea infusion is ≈ 5.0 ppm F. The effect of fluoride on the erosive process has conflicting results when it has been investigated under various conditions (Larsen and Nyvad, 1999). Fluoride integrated into hydroxyapatite reduces the dissolution rate in acids (Shellis et al., 2014). Fluoride ion from solution enters the enamel lattice and interacts with other ions such as calcium and phosphate, it replaces the hydroxyl group and converts the surface composition to fluorohydroxyapatite and fluorapatite which has been proven to have a lower solubility rate than calcium hydroxyapatite (Larsen and Nyvad, 1999, Shellis et al., 2014). This effect of fluoride is

important for remineralisation of caries and erosive lesions (ten Cate and Featherstone, 1991). On the other hand, Wiegand and Attin (2014) analysed the effect of milk (with or without 5.0 ppm F) on dental erosion by involving adult participants who wore an intraoral appliance with bovine teeth. The result showed, when rinsing the oral cavity with milk without fluoride immediately after an erosive attack (erosive soft drink: Sprite, Coca-Cola), it did not reduce dental erosion. The addition of milk with 5.0 ppm fluoride was unable to provide any additional beneficial effect on the erosion process, as the fluoride might partially interacts with calcium or might also interact with milk protein. Therefore, the formation of calcium fluoride on the tooth surface is very limited. Furthermore, there are several studies that showed there was no effect on fluoride quantity or concentration within tea with or without the addition of milk (Cao et al., 2004a, Cao et al., 2004b, Xiarchou, 2016). This suggests there is no direct effect of fluoride in tea on the erosive process, and in the present study, the anti-erosive effect of black tea with the addition of milk might be mainly from the mineral content (calcium and phosphate) of milk. The semi-skimmed milk that was used for the present study contained only 0.021 ppm F (Bussell et al., 2016) and, according to Magalhães et al. (2014), it is expected there is no effect of fluoride doses lower than 2.5ppm in prevention of dental erosion as the erosive challenge is more aggressive than the cariogenic process.

Another factor that might influence on the anti-erosive effect of drinks is the pH. The intraoral pH increased after rinsing with milk under erosive challenge (Lindquist et al., 2011). In the present study, the pH of black tea infusion with milk was 5.6 (which was the highest pH of the experimental solutions) compared to green tea (5.4), black tea (4.8) and black tea with citric acid (4.2). The group black tea infusion with the addition of milk showed less enamel surface loss in comparison to other experimental groups. Therefore, milk might provide an additional beneficial effect to the black tea infusion and might account for the anti-erosive effect of the black tea infusion with milk on the tooth surface. In addition, Barbour et al. (2003a) reported

that the addition of calcium and phosphate to drinks could be sufficient to reduce dental erosion and also by increasing the pH of drinks there is likely to be only a small reduction in enamel dissolution.

Saliva is one of the main biological factors to protect against dental erosion. The present study doesn't include the organic components of natural saliva such as plaque and salivary pellicle which have a protective effect against dental erosion and act as a barrier to prevent direct contact between acid solution and tooth surface (Buzalaf et al., 2012a). In addition, consumption of milk resulting in pellicle formation on teeth involving salivary protein or milk caseins (Johansson, 2002). However, it has been proven that the softened enamel by acid solution can harden after exposure of natural saliva, remineralisation solution (artificial saliva) and with the addition of dairy products (Lussi et al., 2004). Saliva has a reparative role by providing mineral and organic material to fill the defect of erosion process in which remineralisation of softened tissue can occur by the deposition of salivary calcium and phosphate (Gedalia et al., 1991), suggesting the effect of saliva could increase if the food contains a high amount of calcium and phosphate such as that in milk (Cassiano et al., 2016, Gedalia et al., 1991). Available calcium and phosphate in milk have some protective factors to prevent tooth demineralisation and other protective factors referred to as proteose-peptones, glycoproteins, proteoglycans and lactophorins (Grenby et al., 2001).

There was a significant time effect on enamel surface during treatment period of the enamel slabs in black tea infusion with milk as it showed less of enamel surface loss over time (7, 14, 21 and 28 days erosive challenge) compared to other experimental solutions, suggesting that the black tea with the addition of milk is the recommended drink for prevention of dental erosion.

5.11 The effect of black tea with addition of citric acid (lemon) on dental erosion

In the present study, 1.0% citric acid pH (3.6) was used (Abdullah, 2009) to mimic the effect of lemon as an addition to black tea infusion and to identify and compare the difference between this group and black tea group. The addition of citric acid to black tea had no significant effect on the progression of the erosive lesions over and above that of the black tea alone. This indicates that the protective effect of black tea was sufficient to totally counter act any potentially detrimental effect of the citric acid. Previous studies (Abdullah, 2009) proved that citric acid alone resulted in surface loss of enamel. However, in our model this loss was prevented by the addition black tea.

In comparing means in enamel surface loss between groups, black tea infusion with citric acid (32.218 µm) and black tea infusion (32.868 µm) after 28 days erosive challenge, there was no significant difference. However, there was a higher difference in the mean of enamel surface loss in comparison with the group black tea infusion with milk (23.699 µm). Furthermore, there was a significant difference in enamel surface loss when comparing groups black tea infusion with citric acid and black tea infusion with milk, indicating that black tea infusion with citric acid promotes less anti-erosive effect on dental enamel than black tea infusion with milk.

As with the other experimental solutions, black tea with citric acid showed that there was significant time effect on enamel surface loss over time. The black tea infusion with citric acid showed an anti-erosive effect over time (after 7, 14, 21 and 28 days erosive challenge), as it had less surface loss compared to fluoride free water group (36.610 µm). Grando et al. (1996) found that by increasing exposure time from 15 min to 45 min by immersing human deciduous teeth in lemon juice, enamel erosion became more severe. In the present study, the exposure time for black tea infusion with citric acid was 30 minutes per day. Therefore, it performed a less anti-erosive effect than black tea infusion with milk group.

A further study evaluated the dietary behaviour amongst Chinese adults in Hong Kong, and found that many Chinese adults frequently consume lemon tea/water, fruit and fruit juice and they reported significant symptoms that could be associated with dental erosion (Chu et al., 2010). This study result is consistent with our study findings.

When comparing all teas groups, it was shown that there was no significant difference in enamel surface loss between groups black tea infusion with the addition of citric acid, black tea and green tea infusions. Furthermore, all tea groups, whether green tea infusion group or black tea infusion group or black tea with additions of milk or citric acid, showed less enamel surface loss than the fluoride-free water group, which suggests that tea has beneficial effect in preventing dental erosion.

The pH of black tea with the addition of citric acid was measured as 4.2 which was the lowest pH among the groups (Green tea infusion: 5.4, black infusion: 4.8 and black tea infusion with milk: 5.6) As mentioned above in the literature, low pH of beverages (below 4.0) is a significant causative factor in the development of erosion. However, it has been proven that higher fluoride release when the pH is low results in better protection of tooth structure against demineralisation (Gandolfi et al., 2006, Forsten, 1990).

Adding lemon to tea is a traditional cultural practice in many countries. The mineral content such as calcium, iron, potassium, phosphorus, cobalt and zinc are found to be increased with the addition of lemon in all the tea types (herbal and fruit teas) (Gorgulu et al., 2016). This could explain why black tea infusion with citric acid group showed less surface loss of dental enamel than control group (fluoride free water) and promotes a potential anti-erosive effect although it has the lowest pH compared to other experimental groups.

5.12 Suggestions for future research

The results of this research have yielded interesting findings and further questions for future research including:

- The need to investigate the effect of tea on dental erosion as there are limited well conducted studies on tea and its effect on the tooth surface loss. Further research could investigate the most popular brands of tea in the UK and their effects on the dentition and to investigate people's behaviour toward tea.
- The current study was performed under *in vitro* conditions. Artificial saliva was used as an alternative to natural human saliva and the natural protective effects of the oral cavity are lacking in the present study. Therefore, further future *in vivo* and *in situ* studies may yield more clinically relevant results.
- From the results of the present study, black infusion tea with milk promotes anti-erosive effect on tooth surface loss in comparison to other experimental groups. Therefore, additional studies *in situ* are required to confirm its anti-erosive effect on tooth surface loss.
- The additions, the group black tea infusion with addition of milk showed significantly less surface loss. This effect on dental enamel could results from the mineral content of milk such as calcium and phosphate, but this was not proven in the current study. More studies could be suggested to investigate supplement drinks with these minerals to potentially reduce dental erosion.
- Green tea, black tea and black tea with the addition of lemon have been shown in this study to have anti-erosive effect. This has been shown in previous studies, which found that tea has a preventive effect on dental erosion, but there are very limited studies investigating this. Therefore, further studies are needed to investigate more closely the

various elements of tea itself such as the mineral content in tea, titratable acidity, pH, buffering capacity and polyphenols.

- Further studies could include abrasive with erosive challenges in order to investigate further effect of tea on enamel surface loss.

5.13 Null hypotheses outcome

- There is no difference on the effect of black and green tea on surface loss (progression) of dental enamel under erosive conditions *in vitro.* This null hypothesis can be accepted as no significant difference was found on the tooth surface loss between test groups of black tea and green tea after 7, 14, 21 and 28 days erosive challenge.
- There is no difference on the effect of black tea with addition of milk or lemon on surface loss (progression) of dental enamel under erosive conditions *in vitro.* This null hypothesis can be rejected as a significant difference was found on tooth surface loss between test groups black tea with the addition of milk and black tea with the addition of lemon.

6 Conclusions

From the results of this *in vitro* study, it can be concluded that:

- All black tea groups with or without additions (citric acid or milk) were significantly beneficial with respect to enamel surface loss under erosive challenge. Whilst results indicated a similar potential benefit for the green tea group this did not reach a significant in our model.
- Tooth surface loss following exposure to either black or green tea infusions indicated that there was no difference in benefit between the types of tea.
- In our model, the addition of citric acid to black tea infusion resulted in no difference in tooth surface loss. However, the addition of milk to black tea infusion resulted in significantly less tooth surface loss. Therefore, our results would suggest that a black tea infusion with addition of milk provides the greatest protection against tooth surface loss in our model.

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8 Appendices

Appendix 1: Mean of enamel surface loss (µm) for fluoride-free water group after 7, 14, 21, 28 days erosive challenge

Fluoride-free water slabs	Mean after 7 days	Mean after 14 days	Mean after 21 days	Mean after 28 days
7	5.267	14.047	28.592	38.756
8	0.903	8.45	23.277	32.078
14	4.969	12.042	25.362	37.746
15	0.748	8.845	22.42	31.993
17	1.933	8.943	23.26	35.348
20	2.852	15.907	31.269	40.255
25	3.269	10.109	24.49	33.909
27	5.98	16.917	32.834	43.96
31	8.467	15.838	33.376	41.992
34	2.98	12.919	28.299	38.751
37	2.809	12.327	27.787	35.95
41	2.632	10.134	22.864	34.161
45	1.81	12.692	21.945	37.482
48	0.61	11.764	24.712	37.195
56	2.707	12.879	29.544	40.23
64	2.102	8.228	22.132	31.434
69	2.886	13.992	28.16	36.127
81	2.066	12.258	20.584	32.325

83	3.097	10.259	22.644	36.098
85	1.533	11.85	22.58	36.58
87	4.548	10.306	26.414	35.033
107	4.34	14.969	34.967	46.787
117	4.901	11.423	26.838	34.871
128	0.244	12.61	25.819	33.834
132	1.856	12.346	25.739	36.222
133	3.085	14.053	27.896	35.439
135	5.64	16.048	31.621	38.488
136	1.353	5.56	20.265	30.423
149	5.631	13.87	27.855	44.611
150	2.637	12.015	21.218	30.234
Mean of group	3.129	12.120	26.159	36.610
SD	1.88	2.61	3.99	4.10

Appendix 2: Mean of enamel surface loss (µm) for green tea group after 7, 14, 21, 28 days erosive challenge

Green tea slabs	Mean after 7 days	Mean after 14 days	Mean after 21 days	Mean after 28 days
1	1.417	12.169	26.025	36.612
10	1.197	10.024	22.646	35.675
11	0.622	9.07	21.113	30.013
13	4.748	10.758	24.984	37.975
18	1.209	9.381	20.731	31.011
21	2.598	12.363	24.368	32.201
38	0.579	7.859	19.816	30.327
44	4.765	13.656	26.883	39.828
52	0.605	7.561	18.036	27.975
53	4.169	12.704	25.351	29.982
61	0.979	9.427	21.545	29.529
70	3.944	6.779	21.447	32.137
73	3.221	10.981	24.789	37.794
78	3.334	12.665	23.756	37.868
82	4.59	14.248	28.615	39.611
91	1.438	11.461	23.375	31.034
94	0.668	10.527	22.199	32.802
98	2.846	10.696	21.836	35.583

99	3.61	12.412	26.658	36.886
102	5.714	16.343	26.022	32.593
103	2.849	7.366	18.922	26.309
106	6.581	16.878	28.495	40.206
114	0.813	6.184	14.797	32.524
116	2.949	11.334	22.069	31.079
126	2.031	11.039	21.798	34.593
137	2.464	11.702	26.605	36.129
138	4.319	14.55	24.756	34.795
140	6.006	14.166	19.78	32.295
143	2.642	11.115	23.197	35.073
145	3.691	8.475	24.698	32.571
Mean of group	2.887	11.130	23.177	33.767
SD	1.73	2.67	3.15	3.62

Appendix 3: Mean of enamel surface loss (µm) for black tea group after 7, 14, 21, 28 days erosive challenge

Black tea slabs	Mean after 7 days	Mean after 14 days	Mean after 21 days	Mean after 28 days
4	1.994	13.934	26.332	37.879
16	0.66	5.859	22.129	28.778
28	3.046	9.211	20.986	26.626
29	2.578	11.376	25.009	37.442
39	2.294	12.868	23.937	38.799
43	7.148	14.958	20.719	29.191
46	2.859	13.777	25.783	38.018
49	1.691	8.601	19.049	32.901
51	0.814	5.592	16.677	28.532
57	1.691	7.375	17.773	23.769
62	1.007	11.609	25.699	33.685
63	3.175	8.75	18.368	36.272
66	1.113	6.563	25.526	35.692
68	1.095	6.761	19.462	35.732
75	2.541	12.009	25.138	36.833
84	5.108	12.454	25.483	29.854
88	1.402	10.56	22.324	32.34
92	4.54	14.006	26.198	35.692

100	3.78	13.355	24.569	39.026
108	3.648	9.009	23.456	32
110	2.955	5.896	17.945	23.059
112	2.939	8.106	21.368	22.912
113	5.215	8.246	16.412	23.017
115	3.498	12.95	21.773	35.132
119	2.379	11.201	24.733	34.445
121	5.262	13.746	25.333	37.347
123	4.933	10.537	17.925	31.168
125	1.324	7.766	22.917	31.838
129	5.247	16.105	29.767	39.402
134	5.262	10.007	26.836	38.65
Mean of group	3.040	10.440	22.654	32.868
SD	1.68	2.99	3.49	5.18

Appendix 4: Mean of enamel surface loss (µm) for black tea with the addition of milk group after 7, 14, 21, 28 days erosive challenge

Black tea with milk slabs	Mean after 7 days	Mean after 14 days	Mean after 21 days	Mean after 28 days
2	2.275	8.12	13.713	27.958
5	1.585	6.67	15.964	23.471
23	0.48	8.676	13.309	28.34
24	1.662	6.277	11.161	24.32
26	2.819	4.838	8.735	18.859
33	1.809	5.351	13.215	26.889
35	0.904	7.571	16.748	27.692
42	0.74	7.434	13.254	24.663
47	0.396	6.112	8.126	22.207
50	2.119	10.273	15.744	28.924
54	1.258	9.597	14.798	23.853
60	1.833	4.167	5.394	17.881
65	0.404	8.296	15.682	26.839
67	1.777	3.604	16.76	27.349
72	2.591	8.785	13.495	23.171
76	3.042	6.534	10.482	22.499
77	1.561	9.294	12.343	23.573
80	0.843	4.525	21.432	29.027

89	0.422	4.468	7.554	18.419
93	1.446	5.222	12.763	20.058
95	0.932	5.705	7.473	24.038
97	2.959	9.806	12.392	25.104
122	5.607	7.733	9.561	14.371
124	2.473	9.097	13.553	29.109
127	1.172	3.819	6.407	18.032
130	0.224	7.417	18.552	26.489
131	0.403	4.769	12.271	21.266
139	0.59	6.378	10.889	18.644
142	2.409	4.572	13.497	23.293
147	0.887	2.354	11.37	24.635
Mean of group	1.587	6.582	12.555	23.699
SD	1.14	2.11	3.65	3.89

Appendix 5: Mean of enamel surface loss (µm) for black tea with the addition of citric acid group after 7, 14, 21, 28 days erosive challenge

Black tea with citric acid slabs	Mean after 7 days	Mean after 14 days	Mean after 21 days	Mean after 28 days
86	0.166	8.245	18.486	28.647
74	0.366	6.787	17.089	27.879
148	5.571	14.155	22.117	41.622
36	2.54	4.172	9.564	30.658
105	2.176	10.315	24.181	33.918
58	0.521	7.415	21.986	28.606
144	1.033	7.75	19.915	34.707
59	1.222	10.582	22.633	32.54
146	0.762	10.153	19.168	32.874
90	1.733	12.67	22.248	34.903
3	0.378	7.679	18.378	27.11
96	3.484	11.353	24.892	33.814
118	0.954	10.372	16.901	31.169
141	3.958	10.661	13.765	39.76
9	0.674	8.369	17.271	30.456
6	0.242	9.012	22.13	30.019
55	2.168	10.501	17.489	30.66
12	0.777	6.218	15.736	28.725

40	1.478	8.557	20.234	31.749
32	1.378	5.741	13.649	34.522
79	3.837	13.296	20.231	32.485
109	0.199	7.183	16.693	27.343
19	2.423	12.825	21.602	33.91
22	2.779	11.118	17.94	32.441
120	1.249	9.88	22.019	30.447
101	0.75	10.304	20.92	29.569
111	2.962	9.805	15.7	35.182
104	4.1	12.987	26.257	37.121
30	4.448	13.015	23.932	35.097
71	2.257	9.555	20.431	28.614
Mean of group	1.886	9.689	19.452	32.218
SD	1.46	2.45	3.70	3.51

Appendix 6: Intra-class correlation coefficient

Case processing summary

		N	%
Cases	Valid	150	100.0
	Excluded	0	0.0
	Total	150	100.0

Reliability Statistics

Cronbach's Alpha	N of Items
0.839	4

Intra-class Correlation Coefficient

		95% Confidence Interval			F Test with True Value 0		
	Intraclass Correlation	Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	0.566	0.489	0.642	6.223	149	447	0.000
Average Measures	0.839	0.793	0.878	6.223	149	447	0.000