THE EFFECT OF FLUORIDATED MILK ON SURFACE LOSS OF DENTAL HARD TISSUE UNDER EROSIVE / ABRASIVE CONDITIONS *IN VITRO*

Ahmed Ali M Albariqi

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Leeds Dental Institute

Division of Child Dental Health

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

Dedication

This thesis is dedicated to my beloved family

My mum, and dad (late) for their patience, guidance and supplications

My wife (Miad) for her endless love, encouragement and care

My brothers and sisters for their support

My dear daughter (Jood) for all the

moments I took away from them

to fulfil my dream.

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Abstract

Aim: To investigate the effect of fluoridated milk 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive/ erosive and abrasive challenges after 28 days *in vitro*, using Surface Profilometry. **Methods:** A total of 90 bovine enamel slabs were subjected to a pH cycling model with erosive (0. 3% citric acid, pH 2.6)/ erosive and abrasive challenges (0.3% Citric acid pH 2.6 + automated toothbrushing). The slabs were randomly assigned to 6 treatment groups within two phases. *Phase A- Erosive challenge*: (1) 0 ppm F milk, (2) 2.5 ppm F milk, (3) 5.0 ppm F milk. *Phase B- Erosive and abrasive challenges*: (4) 0 ppm F milk, (5) 2.5 ppm F milk, (6) 5.0 ppm F milk. The enamel slabs underwent a 28 day pH cycling regimen where the slabs were subjected to one of the concentrations of fluoridated milk for 5 minutes twice daily, followed by 10 minutes in a milk in one of the concentrations of fluoride/saliva slurry twice daily. During pH cycling period the slabs were exposed to erosive challenge five times daily for 2 minutes periods. In Phase B, the abrasive challenge was carried out with an automated toothbrushing machine (15 strokes, for 2 minutes/twice daily). Throughout the cycling period the slabs were stored at 37 ºC in artificial saliva, in an incubator. The slabs were then analysed with the profilometer to measure the amount of surface loss. **Results**: After 28 days, data analysis was carried out using one way ANOVA with Bonferroni correction to compare between groups (0, 2.5 and 5 ppm F milk) under erosive challenge and, separately, between

groups (0, 2.5 and 5.0 F milk) under erosive and abrasive challenges. There was a statistically significant difference in enamel surface loss ($P < 0.05$) between the groups in both phases. In addition, Independent t-test was performed to compare the amount of surface loss between groups with the same concentrations of fluoridated milk, with and without abrasive challenge after 28 days of erosive pH cycling. At each concentration (0, 2.5 and 5.0 ppm F milk), there was a significant reduction on enamel surface loss after 28 days when compared each fluoridated milk concentration under erosive challenge with similar fluoridated milk concentration under erosive and abrasive challenges. **Conclusion**: Addition of 2.5 and 5.0 ppm F to milk was shown to be effective in reduction of tooth surface loss under erosive/ erosive and abrasive challenges with the most reduction demonstrated with the 5.0 ppm F milk in both phases. The combination of erosive and abrasive challenges caused more enamel surface loss compared with erosive challenge alone.

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LIST OF ABBREVIATIONS

%	Percentage
µm	Micrometre
ACP	Amorphous calcium phosphate
ANOVA	Analysis of Variance
BSPD	British Society of Paediatric Dentistry
Ca	Calcium
CPP	Casein phosphopeptide
CPP-ACPF	Casein phosphopeptide -amorphous calcium phosphate fluoride
CPP-ACP	Casein phosphopeptide-amorphous calcium phosphate
DEJ	Dentine- enamel junction
dmfs	Decayed, Missing and Filled Surfaces (for primary teeth)
e.g.	Example
EAPD	European Academy of Paediatric Dentistry

TSL	Tooth Surface Loss
F	Fluoride
g	Gram
HA	Hydroxyapatite
ICC	Intra-class Correlation Coefficient
Kg	Kilogram
L	Litre
mg	Milligram
min	Minutes
ml or mL	Millilitre
mm	Millimetre
Mol	Mole
N or n	Number
Na	Sodium
ºC	Degree of Celsius
p	p-value

pH	Acidity
PO_4	Phosphate
ppm	Part per million
s	Second
RDA	Relative dentine abrasivity
µm	micrometer
KHN	Knoop hardness number
SD	Standard deviation
SE	Standard error
SEM	Scanning Electron Microscopy
TMR	Transverse Microradiography
Sig	Statistical level
SMH	Surface Microhardness
SPSS	Statistics Package for the Social Sciences
vs	Versus
w/v	Weight per volume
wt	Weight

1.0 Introduction

Tooth surface wear is defined as the irreversible, non-traumatic loss of dental hard tissues due to processes classified as erosion, abrasion as well as attrition (Ganss, 2006). Erosion is defined as chemical dissolution by extrinsic or intrinsic acids. Abrasion is the mechanical wear of a tooth as a result of interaction with foreign objects other than tooth to tooth contact. Whittaker (2000) upholds the theory that the human teeth were designed to wear (even extensively) and that a certain level of tooth wear optimised the functional abilities of human teeth.

The greater effect of fluoride on reduction of tooth surface loss by enhancing remineralisation and reducing demineralization has been reported in many published studies in literature (Abdullah, 2009; Magalhaes *et al*., 2014).

There are different methods of fluoride delivery reported in literature, fluoridated milk being one of them. Several epidemiological studies have been carried out to evaluate the effectiveness of fluoridated milk against dental caries and tooth surface wear. Studies have reported the significant effect of fluoridated milk on caries prevention (Stephen *et al*., 1984; Riley *et al*., 2005; Banoczy *et al*., 2013) and also on tooth wear reduction (Magalhaes *et al.,* 2014; Casssiano *et al.,* 2016).

However, there are a limited number of studies in the literature on the preventive effect of fluoridated milk on erosion and abrasion, and looking at the plethora of studies and opinions, it is clear that the debate on this area has not yet been established. Therefore, the aim of this *in vitro* study was to investigate the

effectiveness of fluoridated milk (2.5 and 5.0 ppm F) on tooth surface loss erosive/ erosive and abrasive conditions using a pH cycling regime.

2.0 LITERATURE REVIEW

2.1 Dental erosion

As described above, dental erosion is defined as the irreversible loss of dental hard tissue by a chemical process without bacterial involvement (Seow, 2001). Erosion is known as the most common and important aetiological factor that can lead to tooth wear (Nunn, 2000). Ten Cate *et al*. (2008) defined dental erosion as the complete loss (dissolution) of minerals of apatite crystals in enamel and dentine as a result of the chemical action of acids which are not formed by the oral flora. The complete loss of minerals is preceded by demineralisation, which as partial loss of the tooth minerals, leads to a softening of the tooth structure (enamel and dentine). Dental erosion is multifactorial, and, it is essential that this condition be recognised in its early stages. Furthermore, identifying and analysing the potential risk factors is fundamental to establishing and implementing the preventive measurements beforehand.

2.2 Aetiology

Components of erosion, abrasion and attrition are usually co-diagnosed as contributing to the overall clinical picture of tooth surface loss (Smith and Knight, 1984; Addy and Shellis, 2006). This multifactorial process is further adjusted by risk factors which are intrinsic, extrinsic, idiopathic or a combination of these (Lussi and Ganss, 2014). Identifying the main aetiological factors is important

prior to formulating a treatment plan. If left undetermined, the success of any treatment may be compromised.

2.2.1 Intrinsic factors

The occurrence of dental erosion can be seen on those who have any of the behaviours or disorders known to promote or attract acid from the gastrointestinal tract. Such conditions are:

- **2.2.1.1 Gastroesophageal Reflux (GORD)**

GORD or in some literature known as GERD (Gastroesophageal reflux) is the chronic involuntary muscle-relaxing of the lower oesophageal sphincter which allows refluxed acid to move upward through the oesophagus into the oral cavity which may go on to cause dental erosion. GORD is a relatively common condition worldwide with prevalence rates ranging from 9% to 33% in various countries (Locke, 2019). GORD is less common in children (Dundar and Sengun, 2014).

- **2.2.1.2 Rumination disorder**

Defined as chronic effortless regurgitation from the stomach contents of most meals into the mouth following ingestion due to the involuntary contraction of the abdominal muscles. It can be seen in children with psychosocial stressors such as abuse or childhood neglect. Rumination disorder is found to be associated with dental erosion (Javier *et al*., 2017). Bartlett and Coward, (2001) found that intrinsic erosion happens from the regurgitation of gastric contents mainly from

hydrochloric acid. Pure gastric juice has been found to contain a mean pH of 2.92 and mean titratable acidity 0.68 ml and therefore gastric contents have erosive potential.

- **2.2.1.3 Other medical conditions**

Any conditions that lead to spontaneous or self-induced vomiting may impact oral health, mainly causing dental erosion due to gastric acid (Uhlen *et al.*, 2014), such as Bulimia nervosa, or morning sickness during pregnancy. Women in the early stages of pregnancy may have vomiting and nausea which leads to a drop in salivary pH and increases vulnerability to dental erosion (Lopez *et al*., 2011).

2.2.2 Extrinsic factors

- **2.2.2.1 Dietary products (Foods and drinks)**

Dietary products with a high acidity such as: citrus fruits, foods containing vinegar, pickled foods, fruit juices, fruit flavoured waters and smoothies, are most widely recognised as proposed sources of extrinsic acid that may lead to dental erosion. A number of studies demonstrate the association between the acidic content of foods and drinks and dental erosion (Milosevic, 2004; Taji and Seow, 2010). The degree of erosive attack from these erosive products is affected by several factors such as: titratable acidity, the presence of calcium, phosphate, pH value, and fluoride content of the acidic foodstuff and drink (Lussi and Jaeggi, 2006).

Hara *et al*. (2006) found that after the acid intake, the tooth surface pH was under pH 5.5 for a shorter period than predicted. This highlights that the erosive consequences of dietary acids are changed by intra-oral biological factors like: the effect of salivary neutralisation, role of the salivary pellicle and oral clearance. Products such as fruit juices, smoothies and some alcoholic drinks have erosive potentials, whether or not that translates into erosion is difficult to forecast with any degree of certainty.

Therefore, there is no clear-cut critical pH for dental erosion. Even a low pH acid attack may not develop dental erosion if the chemical and biological factors specified above are strong enough to prohibit an erosive lesion developing *in vivo* (Lussi and Jaeggi, 2006).

- **2.2.2.2 Medication**

Any medication or oral hygiene product known to have a low pH level or high titratable acidity may have erosive potential, especially with these in regular and prolonged contact with the surfaces of the teeth. Many medications are prepared using acidic contents and some medications induced a dry mouth or vomiting. An example of medication-induced erosive potential is chewable salivary substitutes tablets which may have a low pH level and high titratable acidity and the patient should be aware of this potential side effect of the medication (Lussi and Hellwig, 2014). In addition, vitamin C supplements found to be a potential cause of dental erosion (Bahal and Djemal, 2014).

- **2.2.2.3 Environmental**

Environmental factors can also contribute in developing extrinsic erosion. Any occupational field known to involve a daily exposure to acids means that the people in such positions are at a greater risk of developing dental erosion. For example, battery, fertiliser, and galvanising factory workers who are exposed to acid fumes from batteries (Isaksson *et al*., 2014). Wiegand and Attain, (2007) reported that professionals who work as wine testers are at greater risk of having dental erosion as a result of increased acid-tooth contact.

- **2.2.2.4 Lifestyle**

Lifestyle may also contribute to increase the risk of developing dental erosion, such as the time and frequency of foods and beverage consumption, as well as oral hygiene practices (Peycheva and Boteva, 2014). Zero *et al*. (1996) stated that some prolonged dental habits may also be detrimental to enamel surface loss such as overzealous tooth brushing. Another example of lifestyle a factor affecting dental erosion is high alcohol consumption which may result in erosion from both intrinsic and extrinsic sources. As increasing alcohol intake leads to increased acid exposure to the teeth and promotes gastric reflux (Peycheva and Boteva, 2014). Isaksson *et al*. (2014) mentioned that swimming in low pH gas-chlorinated pools frequently may increase the risk of experiencing some degree of dental erosion.

2.2.3 Idiopathic dental erosion

Idiopathic dental erosion may occur due to acid contact from undefined origin where neither the patient history taking nor any tests are capable of giving an etiological clarification for the tooth surface loss. From the literature, it seems the idiopathic dental erosion mentioned in many case reports is a result of multifactorial aetiology that has not been illustrated (Gupta *et al*., 2009).

2.3 Risk factors for dental erosion

Moreover, dental erosion can be a result of other factors. There are a number of chemical, behavioural and biological factors known to interact with tooth surface that may influence dental erosion development. This damage can be more significant if the interaction goes on for a longer-term. However, the effect can be different from one individual to another, even though the exposure to the acid attack is at the same level in their diet. (Lussi, 2006; Lussi and Jaeggi, 2008).

2.3.1 Chemical factors

A number of *in vitro* and *in situ* studies have reported that not only does the pH level of the foods and beverages determine the erosive potential, but there are also other chemical factors known to impact food and drink's erosion capabilities. For example: buffering capacity, acid type, product's adhesion to tooth surface, fluoride, phosphate and calcium concentration, temperature and products' chelating properties (Barlett, 2005; Lussi and Jaeggi, 2008).

Based on the data available on beverages and simple acid solutions, the erosion threshold is reported to be around the pH level of 5.0 – 5.5. The long-time exposure to the acidic drink and the intake of large volumes can play a crucial role in tooth surface loss (McNally *et al*., 2006).

In addition, it has been proven that the buffering capacity has a real impact on progression of dental erosion. When the buffering capacity is at a high level, it will result in a long time for saliva to reach its neutral pH level. Furthermore, the erosive attack will be at a higher degree when the solution is on contact with the tooth surface and not replaced by saliva which can neutralise acids (Lussi, 2006; Taji and Seow, 2010). Borjian *et al*. (2010) reported that the intra-oral natural pH is 6.8, but it decreases to below 5 within 3 minutes after dinking an acidic drink, a large amount of stimulated saliva is needed for neutralisation.

Temperature may also affect the erosive potential of foods and beverages. Hankermeyer *et al.* (2002) reported that with rising temperature, there is a decrease in the pH level of weak acid solutions. This dissociation of the acid happens because of the influence of thermodynamics.

Amaechi *et al*. (1999) examined the effect of temperature and exposure time on the development of enamel erosion on bovine and human teeth *in vitro*. Samples were exposed to orange juice at different length of times and different temperatures. It was concluded that the degree of erosion was increased as exposure time increased, and the enamel erosion was typically less within a lower

temperature. West *et al.* (2000) reported that raising the temperature from 5℃ into 60℃ significantly increased enamel and dentine surface loss. And, an increase in temperature of 25℃ resulted in a tooth surface loss of approximately 5πm.

The calcium, phosphate and fluoride contents of foods and beverages are essential elements as they can affect the concentration gradient in regard to the tooth surface's environment. Evidence in the literature found that the tooth surface loss is reduced when calcium and phosphate are added to juice with a low pH level (West *et al*., 2003; Attin *et al*., 2003). However, no such link between phosphate concentration and the erosive potential of beverages was reported by Hemingway *et al*. (2006).

Moreover, Luci *et al*. (2006) reported that the daily use of fluoride shows some protective impacts from dental erosion as the remineralisation and the demineralisation rotations help in the production of fluor-hydroxyapatite or fluorapatite. Both of these chemicals are known for having a lower solubility level than the hydroxyapatite.

2.3.2 Behavioural factors

Behavioural factors, namely excessive use of tooth bleaching products, high consumption of citrus fruit, illegal designer drugs, and overzealous oral hygiene practice, are also known to play a crucial role in regard to tooth surface loss development (Salas *et al.*, 2017).

Behaviour can also be significantly affected by the individual's socioeconomic status. The socioeconomic status is found to have had an impact on the development of dental erosion (El-Aidi *et al*., 2010). However, there are contradictory results regarding the association between socioeconomic status and tooth surface loss as some studies have reported a higher prevalence of dental erosion among families with low income, other studies have found higher prevalence of tooth wear in wealthy families, or from highly educated families (Alves *et al*., 2015).

2.3.3 Biological factors:

Biological factors are also reported to have significant impact on tooth surface loss. For example, Saliva, Pellicle, tooth structure and position in relation to oral tissues (Lussi, 2006).

Saliva is known as one of the most protective factors from the development of dental erosion. It contributes to neutralisation, remineralisation and dilution of the acid, as well as pellicle formation which covers the surface of the tooth as a protective membrane (Hara and Zero 2016). Pandey *et al*. (2015) reported that the average unstimulated salivary flow rate is between 0.3-0.4 mL/min with daily production between 0.5 to 1.5 litres and if it is below 0.1 mL/min then it is considered as evidence of hyposalivation. Literature has reported a positive link between the occurrence of tooth wear and patients with a low salivary flow rate. Low salivary flow rate can be associated with some conditions like diabetes

mellitus, having radiation therapy in head and neck region (Hellwig and Lussi, 2014; Hara and Zero, 2014).

Saliva is also reported to be pivotal in the formation of the acquired dental pellicle, which acts as a dispersal barrier or a perm-selective-membrane preventing any direct contact between the tooth surface and acid. The barrier is an organic film, free of bacteria and formed by adsorption of lipids, proteins and peptides, which are initiated immediately once the enamel is in contact with saliva (Carvalho *et al*., 2016). Lussi, (2006) found the pellicle's protection level is regulated by its density, maturation time and configuration. Hanning and Joiner (2006) reported that the thickness of pellicle varies between individuals and differs within dental arches, the thinnest pellicle formed on the lingual surface of the upper anterior teeth, the thickest formed at the lingual surface of lower posterior teeth. Buzalaf *et al*. (2012) stated that saliva has a repetitive effect on early eroded enamel as it contains fluoride, calcium and phosphate.

The occlusion, quality, position and anatomy of dental and soft tissues may impact the tooth wear development. Tooth anatomy and location in the arch in relation to the way of swallowing and drinking may also impact the vulnerability of teeth to erosion (Lussi *et al.,* 2006).

2.4 Mechanism of Dental Erosion

Enamel is the hard protective layer coating the tooth. Erosion influences the outer surface of enamel or dentine. It is believed that dental erosion arises when the

tooth's minerals are dissolved. The enamel surface in the oral condition is coated by a pellicle which covers the tooth surface and helps to prevent tooth wear development (Hannig *et al*., 2005).

The chelating capacity of acid will start dissolving the crystal when the erosive solutions diffuse through the pellicle layer. After that these solutions communicate with the mineral stage of the tooth, which is a carbonated and calcium deficient hydroxyapatite.

A honeycomb appearance is created after the prism sheath area and prism core are dissolved (Meurman and Frank, 1991). Featherstone and Rodgers, (1981) mentioned that the unionised pattern of acid will then diffuse into the interprismatic regions of dental enamel and dissolve mineral in the subsurface areas. This will lead to an outflow of calcium and phosphate (tooth mineral ions) leading to a local pH rise in the tooth structure (Lussi and Hellwig, 2001). In the absence of any chelating agents or new acids this process will stop (Zero and Lussi, 2005).

Featherstone and Lussi, (2006) found that the hydrogen ions in acids or anions (chelating substances) that combine to calcium in enamel are behind the chemistry of dental erosion. The hydrogen ions bond with either the carbonate or the phosphate ions of enamel crystals that dissolve them.

Acids, for example, citric acid, have two chemical mechanisms in dental erosion. Preparing citric acid in water can produce H+ ions, unseparated acid molecules in addition to anions like citrate. The amount of each of these elements is determined by the acid dissociation constant and the pH of the solution. The H+ ion performs as explained above, the first chemical mechanism. The second chemical mechanism occurs through the citrate anion which binds to calcium and eliminates it from the crystal surface (Featherstone, 2000).

Importantly, the remineralisation process can occur following erosion if there is no direct etching on the tooth surface. Availability of saliva or remineralisation solution for sufficient time may result in the regaining of mineral (Koulourides 1968; Collys *et al*., 1993; Eisenburger *et al*., 2001). Nevertheless, once there has been the occurrence of dental erosion, the tissue loss cannot be returned to its original form.

2.5 Prevalence of dental erosion

The Office of National Statistics, (1994) based on a Children's Dental Health Survey of 1993 was the first to assess the prevalence of dental erosion in the United Kingdom. The survey found that the prevalence of dental erosion was found in more than half of children aged 5 to 6 years old, with 25% of these children having dentine involvement. Regarding permanent dentition, the survey also found 25% of children aged 11 years and older showed some degree of dental erosion with 2% having dentine wear (Office of National Statistics, 1994).

It was highlighted by the Children's Dental Health Survey of 2013 that 33% of all five year old children in the UK presented with some degree of tooth surface loss (TSL) in one or more of the buccal surfaces of their primary upper incisors, with 4% showing overall TSL with dentine or pulp involvement. However, the most reported TSL was located on lingual surfaces of the teeth (57%) of five year old children in the UK, with 16% showing TSL had progressed to dentine or pulp. For those aged 12 years old in the UK, the survey found that 25% show TSL on molars, with buccal and lingual surfaces of permanent incisors showing 24% and 38% TSL respectively. Moreover, the survey found that the proportion of children with any occlusal TSL at age 15 years was higher than those at age 12 years of age (31% compared to 25%) (Office of National Statistics, 2015).

A further study has assessed the prevalence of TSL in Saudi Arabia amongst a sample of 3–5 year old preschool children. The study found that among the 388 children examined, 61% exhibited TSL, with 4% showing severe erosion (Al-Dlaigan *et al*., 2017).

Numerous studies have been carried out to assess the prevalence of dental erosion in both primary and permanent dentition in different countries. However, there is no comprehensive knowledge on the prevalence of this condition on a global level. Therefore, a recent review study on the global prevalence of erosive tooth wear was carried out by Schlueter and Luka (2018). They found with regard to primary teeth there was a wide span in the prevalence even within one country.

For example, Australia had a rate of 0-33%, Great Britain 28%-50%, Saudi Arabia 31-61% and Brazil 1%-62%. The estimated mean of all gathered data showed that the global prevalence of erosion is between 30% - 50%. They reported that erosion was only confined to enamel in more than 80% of cases, dentine progression in 21% to 48% and pulpal involvement was rare with prevalence of less than 1% (Schlueter and Luka, 2018).

Some studies have mentioned that the erosion prevalence is age dependent, and there is an increase in the prevalence of erosion over time (Schlueter and Luka, 2018).

Furthermore, no explicit statement with regard to the association between gender and dental erosion has been reported. Some studies have stated that there is no association, whereas others have observed that females showed higher prevalence of dental erosion than males and a few studies have reported males are more affected by erosion than females (Schlueter and Luka, 2018).

2.6 Abrasion

Once an acid-induced softening happens then abrasion and erosion have been shown to work synergistically (Jaeggi and Lussi, 1999; Attin *et al*., 2004). The main factors regarding the development of abrasion are the frequency, duration and force of brushing and the relative dentin abrasiveness of the toothpaste.

Addy and Hunter, (2003) reported that tooth brushing is considered the most common cause of dental abrasion in Western populations. Force, frequency and duration of tooth brushing and the relative dentin abrasiveness of the toothpaste are the most common factors related to development of dental abrasion. However, previous literature has shown that the amount of tooth surface wear is negligible if standard toothpaste with normal tooth brushing duration and force was used (Addy and Hunter, 2003).

Toothbrushing is known as one of the most common and recommended methods to maintain good oral hygiene. However, several studies reported that both toothpastes and toothbrushes played a significant role in developing tooth wear by removing the demineralised tooth surface layer (Hunter *et al*., 2002; Bartlett and Shah, 2006) especially when an acid challenge was present, as it can soften the hard tissue, making it more vulnerable to abrasion (Voronets and Lussi, 2010). This can lead to significant tooth wear, loss of tooth's form and function, dentin hypersensitivity and costly restorative treatment afterwards (West *et al*., 2013). There are some factors that may contribute in the severity of tooth surface loss. For example, brushing duration, frequency and force, slurry viscosity, abrasive concentration and type, toothbrush type, toothbrush filament stiffness, acid challenge time and duration, and severity of acid attack (Lippert, 2017). Wiegand and Schlueter, (2014) found that the toothpaste abrasiveness is the most important factor that causes abrasion with the toothbrush acting as the carrier.

The role of filament stiffness of the toothbrush is still unclear, it can be controlled by filaments diameter, length and modulus of elasticity. Assuming that elasticity and lengths of filaments are constant, filament stiffness will be affected only by their diameter. Commonly, we have three types of toothbrushes which are hard (large filament diameter), medium (most common) and soft (less filament diameter) (Wiegand *et al*., 2008). Some studies have reported that hard toothbrushes cause more surface wear compared to the softer ones. However, other studies reported that soft toothbrushes can accelerate the surface wear due to their greater ability to carry abrasive particles across the tooth surface which can lead to more surface wear when using them (Wiegand *et al*., 2008; Wiegand *et al*., 2009; Bizhang *et al*., 2016).

Wiegand *et al*. (2008) investigated the effect of toothpaste slurry abrasiveness and toothbrush filament stiffness on abrasion of eroded enamel, in an *in vitro* study. Seventy two enamel slabs were eroded by using hydrochloric acid pH 2.6 for 15 seconds and they were assigned to nine groups. All samples were brushed with 40 strokes by using automated toothbrushing machine with different toothbrush filament stiffness (diameter) (0.15, 0.20, and 0.25 mm). And, three experimental slurries were used with each toothbrush type, making it a total of nine groups. After sixty cycles of erosion/abrasion, the enamel surface loss was measured by surface profilometry. They reported that the amount of enamel surface loss was affected mainly by the abrasiveness of the toothpaste slurry and

increased along with higher relative enamel abrasiveness (REA) value. At a lesser degree, the enamel surface loss was also influenced by filament diameter.

2.7 Diagnosis and clinical features

Early signs of eroded tooth surface in the anterior teeth are shown by the translucency of the incisal edges as the enamel or dentine become thinner and in the posterior teeth, one can see cupped out lesions on the occlusal surfaces. In advanced erosion cases, there are other morphological changes which can lead to developing enamel concavity and further surface flattening. The entire occlusal morphology may entirely disappear (Lussi, 2006) and may result in symptoms such as sensitivity or pulp involvement (Smith, 1991).

Lussi *et al*. (2006) demonstrated the early signs of enamel surface wear of anterior teeth include perikymata deficiency on enamel surface, which may result in the silky, shiny appearance as the enamel wears thin.

2.8 Dental complications of tooth wear

Several clinical issues have been reported in literature as a result of tooth surface loss such as aesthetics, progression of tooth wear may lead to shortening of the teeth and change in occlusal vertical dimension (Linnett and Seow, 2001). It may also result in dentin exposure which may cause tooth sensitivity to temperature. In children with rapid loss of immature teeth structure from erosion, pulp exposure may occur as a result (Linnett and Seow, 2001).

2.9 Management

To prevent tooth wear, early diagnosis and identification of causative factors are essential. A detailed and thorough oral examination is required in order to determine the source of acid, either extrinsic or intrinsic, and specific and tailored made preventative advice for each individual patient is required. Such preventive measures should consist of dietary advice and analysis, reducing exposure to extrinsic and intrinsic sources of acid, changes in behaviour, lifestyle and improving oral hygiene (O'Sullivan and Milosevic, 2007).

Patient's awareness should be increased toward habits that may exacerbate the effects of tooth wear, for example, regular acid intake as a last thing at night or holding acid drinks in mouth for a while before ingestion. Liaising with a medical physician is important in order to manage suspected vomiting or presence of any sign or symptoms of GORD in a patient (O'Sullivan and Milosevic 2007; Bartlet, 2005).

Fluoride products are often recommended for a patient diagnosed with tooth surface wear. Resin-based adhesives have also been recommended in some cases. However, the adhesive restorations may only result in short term protection against erosive wear (Sundaram *et al*., 2004).

Furthermore, if the patient has functional or aesthetic issues then the interventional restorative dental procedure is indicated. But it should be as minimally destructive as possible by using adhesive techniques such as composite

restorations (O'Sullivan and Milosevic, 2007) or resin-bonded metal alloys (Chana *et al*., 2000).

Finally, conventional indirect restorative techniques, for example: full coverage metal, metal-ceramic or all-ceramic crowns may be considered as the final course of action.

2.10 Erosive tooth wear evaluation techniques

Several techniques have been used to assess tooth surface loss. Barbour and Rees, (2004) reported the following quantitative methods for measuring tooth surface loss when induced through erosive challenges:

a) Microhardness (surface hardness).
b) Chemical analysis.
c) Micro CT.
d) Quantitative light-induced fluorescence microradiography.
e) Surface Profilometry.
f) Micro-radiography.
g) Microscopy techniques (ESEM/SEM).
h) Atomic force microscopy.
i) Confocal laser scanning microscopy.
j) Secondary ion-mass spectroscopy.

2.10.1 Microhardness

There are two types of microhardness tests; Surface microhardness (SMH) and cross-sectional microhardness (CSMH). They are used to measure the tooth

surface resistance to penetration force and a function to porosity's degree of the superficial enamel layer that may illustrate mineral gain or loss in the subsurface lesion (Koulourides, 1971).

The length of indentations is calculated microscopically by using a Knoop or Vickers diamond placed on the sample with a defined load for a specific duration to make indentations in the tooth surface. The length of indentations is calculated microscopically in πm (Ten Bosch and Angmar-Mansson, 1991).

In surface microhardness (SMH), the load with a diamond indenter is applied perpendicularly to the polished tooth surface and can give qualitative information on mineral changes when utilising in the demineralisation/remineralisation assessment. The sample surface should be flat. While, in CSMH the load is applied parallel to the polished surface and it shows the ability to determine quantitatively the mineral profile and the mineral gain or loss (Aends and Ten Bosch, 1992).

Some research has used microhardness technique to measure the amount of tooth surface loss caused by erosive/abrasive challenges (Jaeggi and Lussi, 1999; Joiner *et al*., 2004). They measured the depth of indentation before and after abrasion. However, they were not able to measure the amount of tooth surface loss by the erosive attack as acids caused surface loss in the body of indentation not solely from its surroundings.

2.10.2 Surface Profilometry

One of the common laboratory techniques for determining the sample surface loss is surface Profilometry. The enamel surface loss results are accurate, quick, highly reproducible and easy to obtain. However, the sample surfaces have to be polished and flat before the measurements (Barbour and Rees, 2004).

The surface Profilometry has the advantage that during scanning there is no danger of scratching or physical contact with the eroded/abraded enamel surface. Also, it can provide accurate measurements even if the sample is placed at an angle, as it can be levelled in a horizontal axis. Hence, the findings are accurate and highly reproducible. It takes only a few minutes for each scan and data can be analysed at any time as it can be easily saved.

It uses a small metal style (20 mm diameter) that scans for the acquisition, graphical display, evaluation and recording of surface profiles across the enamel surface at a rate of around 10 mm/min. A non-contact Profilometry has been developed to determine the tooth surface loss. The traditional contact stylus is replaced with a light or laser in this method, and interferometry is utilised to construct a surface map.

2.11 Fluoride

Fluoride is a chemical compound which comes from a naturally occurring element known as Fluorine (F^-) which is part of the halogen group in the periodic table. F^- has a high reactivity level and is always found to be combined with other

elements to form F^- salts. F^- is widely available in nature as a mineral salt of cryolite, apatite and fluorspar with a high affinity to mineralised tissue (Ullah and Zafar, 2015). Also, Fluorine is found in soils, rocks and sea. F^- concentration in a substance is presented as the number of parts per million (ppm). 1 mg of F^- in on litre of water is equivalent to 1.0 ppm. Tooth tissues (enamel, dentine, and cementum) are highly mineralised tissues which occur as apatite. In presence of F^-, the apatite mineral will incorporate with it to form fluorapatite (Tenuta *et al*., 2008; Kanduti *et al*., 2016).

2.11.1 Methods of fluoride delivery

There are different methods of fluoride delivery reported in literature. This was one of the main factors behind the success of fluoride. The delivery methods of fluoride have been classified systemically such as: water, milk, salt and supplements and topical such as: toothpaste, mouth rinses, gels and varnish.

- **2.11.1.1 Systemic fluoride**

A) Water fluoridation

Fluoridated water is made by adding fluoride to communal drinking water to the suggested level for dental health. Several studies and reviews have investigated the effects of fluoridated water and dental caries. They reported that water fluoridation was effective at reducing dental caries and increase remineralisation process (McDonagh *et al*., 2000; National Health and Medical Research Council report, 2007).

B) Salt fluoridation

This method of delivery is recommended by the World Health Organization (WHO) and is utilised in over 30 countries worldwide. The recommended dose is 250mg of F per Kg salt (Espelid, 2009).

C) Milk fluoridation

Many studies have investigated the effect of fluoridated milk on dental caries and dental erosion. Torress *et al*. (2016) reported that fluoridated milk is effective in reducing dental caries. Magalhaes *et al*. (2014) assess the effectiveness of different concentrations of fluoridated milk on erosion. They found that fluoride concentration in milk is negatively associated with tooth surface loss. This topic is reviewed in section (2.13).

- **2.11.1.2 Topical fluoride**

A) Fluoride gel, mouth rinses and varnishes

It has been reported in literature that the higher fluoride concentration in these products can enhance the resistance of dental tissues against erosive potentials (Amaechi and Higham, 2005; Lussi *et al.*, 2019).

B) Fluoride toothpaste

Fluoride toothpaste was introduced in the early 1970s. And since that time a significant reduction in worldwide dental caries levels has been reported. Evidence in literature has reported the efficiency of fluoridated milk in decreasing dental erosion development (Ren *et al*., 2011; Hooper *et al*., 2014). In summary,

all fluoride-containing products show evidence of increasing remineralisation and reducing demineralisation processes. However, there are some recommendations to avoid any issues that may occur. For example, the ingestion should be minimised, the delivery method should be cost-effective, and combination methods are also suggested only for people at high risk of dental caries (Morinho *et al*., 2004).

C) Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP)

The beneficial dental effects of milk and milk products have been studied for many years. Benefits have been attributed to high concentrations of calcium and phosphate that present in milk, which help to prevent the dilution of tooth enamel (Birkhed *et al*., 1993) along with the presence of casein, which is a multiphosphorolyted protein that can stabilise milk phosphate and calcium ions in a colloidal state in micelles (Monyhan, 2000).

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is a technology of calcium phosphate remineralisation and it is postulated that CPP stabilises calcium and phosphate in high concentration as well as fluoride ions, by binding to the pellicle and plaque at the tooth surface. This allows the calcium and phosphate ions to be bioavailable to diffuse into the enamel subsurface lesion and promote remineralisation (Reybolds, 2009). Beerens *et al.* (2010) stated that CPP-ACP promotes enamel lesion remineralisation by maintaining a supersaturated state of the enamel minerals calcium and phosphate in plaque,

inhibiting bacterial adhesion and delaying formation of biofilm in addition to acting as a buffering agent to prevent the reduction of pH in the oral micro-environment.

Several studies have examined the effect of CPP-ACP on dental erosion. Ramalingam *et al*. (2005) reported that the addition of CPP-ACP to a sport drink (Powerade) resulted in a significant reduction in the erosive potential of the beverage. They found that 0.125% w/v of CPP-ACP reduced the depth of enamel surface loss more than that obtained with de-ionised water *in vitro*.

Another experimental study by Ranjitkar *et al*. (2009) examined the protective ability of CPP-ACP on dental erosion under a severe erosive challenge. Thirty-six human enamel specimens were divided into 3 groups. 1)- CPP-ACP paste, 2)- non CPP-ACP paste with the same formula. 3)- control group (no paste). The specimens were subjected to 10.000 wear cycles of 100 N in the presence of hydrochloric acid pH 1.2 using a tooth wear machine. The machine stopped after every 2 minutes. Then, treatment was applied for 5 minutes on groups 1 and 2. No treatment (paste) was applied on group 3. They reported that CPP-ACP paste, and non CPP-ACP paste significantly reduced the enamel wear compared to the control group. Furthermore, the mean wear rate of CPP-ACP paste was significantly lower than that in the non CPP-ACP paste group. Similar results were reported by Sirinvasan *et al*. (2010). They examined the effectiveness of pastes containing CPP-ACP and CPP-ACP with 900 ppm F on human enamel

eroded by a soft drink *in situ*. Forty-five enamel specimens were eroded in a soft drink for 8 minutes then attached to an intraoral appliance worn by 5 participants. The specimens were subjected to three different groups. 1)- CPP-ACP, 2)- CPP-ACP with 900 ppm F, 3)- Saliva (control). They found that CPP-ACP and CPP-ACP with 900 ppm F significantly remineralised the softened enamel. Furthermore, a higher remineralisation potential was reported with CPP-ACP combined with fluoride compared to CPP-ACP. They concluded that there is a synergistic effect for CPP-ACP with a fluoride addition on the remineralisation of eroded enamel.

2.11.2 Fluoride concentration in teeth

The Enamel has the highest concentration of fluoride at its surface however it drops within the outer 100µm. After this point, the fluoride concentration remains even up to the enamel dentine junction. However, dentine is reported to have a higher concentration of fluoride and increases deeper into the tooth. There is a continuous accumulation of fluoride at the dentine-pulp surface as the dentine formation continues throughout life (Malinwoski, 2010).

2.11.3 Fluoride absorption, distribution and elimination

Dental fluorosis only happens when teeth are developing. So, ingestion of fluoride is critical for infants. Evidence in literature has noted that human, as well as other mammalian milk, has low fluoride content and there is a poor

transportation of fluoride from plasma to milk (Spak *et al*., 1982; Campus *et al*., 2014).

There is a rapid absorption of fluoride into blood plasma in the stomach after ingestion, the absorption mainly occurs in the stomach without the need of an enzyme. The rate of absorption is greatly determined by the contents and composition of the stomach. The kidneys form the major removal route of fluoride if there is a high absorption rate. But, a very small amount of fluoride, about 10% of the total ingested each day, is excreted through faecal matter (Matinez-Mier, 2012). The distribution of fluoride throughout the body is done by plasma, mainly as ionic fluoride. The concentration of plasma fluoride is varied over the day as it depends on fluoride intake. Due to bone remodelling, there is an increase in plasma fluoride level as age increases.

The distribution of fluoride starts from plasma and reaches all the body tissues and organs, although the kidneys generally have a higher fluoride concentration than plasma. The central nervous system only has a 20% concentration of fluoride present in the plasma (Spak *et al*., 1986; Sener *et al*., 2007).

2.11.4 Optimal exposure to fluoride

All sources of fluoride available are important to consider, including dental products and natural sources. Moreover, the impact of fluoride must be taken into account over all age groups including those living in fluoridated versus non-

fluoridated areas. Fluoride exposure is exhibited in milligrams per kilogram body weight (Sener *et al.*, 2007).

In the past, the estimated appropriate daily exposure to fluoride was around 1.0 to 1.5 mg which is equal to 0.05 mg F/kg body weight daily. Recently, the optimal fluoride exposure level was indicated to be between 0.05 and 0.07 mg F/kg body weight per day. For infants, the exposure must not exceed 0.1 mg F/kg body weight (Warren *et al*., 2007; Viswanathan *et al.*, 2008). Monitoring the exposure to fluoride, especially in children, is critical as it forms one of the appropriate preventative mechanisms (Buzalaf and Levy, 2011; Buzalaf, 2017).

Fejerskov *et al*. (1996) noted that excessive intake of fluoride may cause dental fluorosis in children. Since fluoride is toxic, appropriate level of fluoride must be taken for its health benefits and to reduce risks of any harmful effects.

2.11.5 Fluoride toxicity

Although fluoride-containing products have been shown to result in a significant reduction of tooth caries and tooth wear; it is also important to remember that it is a toxic substance that can have adverse effects. So, it is important to use it in proper ways to enhance oral health. Fluoride toxicity can be divided into acute and chronic.

- **2.11.5.1 Acute fluoride toxicity**

A number of studies have reviewed the toxic dose of fluoride. Driebach, (1980) mentioned that it is 6-9 mg of F/Kg. However, due to uncertainty of the dose that

was ingested, the estimation of the fluoride toxic dose has been estimated differently. Some studies state that 5–10grams of sodium fluoride ingested at one time for an adult with a 70Kg weight is a certainly lethal dose (CDL) if they do not receive immediate treatment. Whereas other studies have reported that it is 32-64mg F/Kg. It is believed that a quarter of the CLD i.e. 8-16 mg/Kg of fluoride is considered as a safely tolerated dose (STD), which is the amount of fluoride that can be ingested without causing any symptoms of acute systemic toxicity (Hiefetz and Horowitz, 1986; Whitford, 1990; Whitford, 2011). Whitford, (1987) reported the probably toxic dose (PTD) which is known as the minimum dose that results in signs and symptoms of toxicity was 5 mg F/Kg. Table 1 shows a common sign and symptoms of acute fluoride toxicity (Heifetz and Horowitz, 1986).

Table 1: Common signs and symptoms of acute fluoride toxicity

Low Dosages	High Dosages
Nausea Vomiting Hypersalivation Abdominal pain Diarrhoea	Convulsions Cardiac arrhythmias Comatose

2.11.5.2 Chronic fluoride toxicity

Chronic fluoride toxicity can occur with frequent exposure to high levels of fluoride over a prolonged time. An example of the common effect is dental, skeletal fluorosis and kidney damage.

Table 2: Toxic effect of chronic excessive fluoride uptake (Heifetz and Horowitz, 1986)

Effect	Dosage	Duration
Dental fluorosis	>2 times optimal	Until 5 years of age (excluding third molars)
Skeletal fluorosis	10-25 mg/d	10-20 years
Kidney damage	5-10 mg/kg	6-12 months

2.12 The *in vitro* demineralisation/remineralisation models (pH cycling)

The pH cycling model is an *in vitro* model that contains a process of alternating demineralisation and remineralisation, making the tooth surface loss process better simulated. This model has become popular and the method of choice for many tooth wear and caries researchers. Also, it has been widely used to assess the dynamics of enamel demineralisation and the remineralisation of preventive agents (White *et al*., 1995; Featherstone, 1996; ten Cate *et al*., 2006).

Several studies have investigated the effect of fluoride on the enamel demineralisation and remineralisation process and reported enamel resistance to form a lesion as the tooth-bound fluoride content increased (Borsboom *et al*., 1985; Takagi *et al*., 2000; Soi *et al*., 2013).

Among *in vitro* studies, pH cycling regimes include exposure of dental tissue (enamel or dentine) to a combination of demineralisation and remineralisation processes. Such experiments help to mimic the dynamics of the loss and gain of minerals (White, 1995).

Other advantages include achievability of a high degree of standardisation, ability to control numerous variables such as acidic challenge and concentration, and results which can be obtained relatively faster with minimal expenditure required as compared with *in vivo* and *in situ* tooth wear studies (Austin, 2011). The pH cycling model advantages have helped researchers to understand the tooth wear process as well as the possible mechanisms in which fluoride enables its remineralisation effect. These models have been employed widely in profile studies as well as low-cost testing of new and recently marketed substances. Also, they have helped facilitate the acquisition of sufficient quantitative data that can be used by investigators to confidently design appropriate clinical trials (Zero, 1995).

2.13 Milk fluoridation

The idea of adding fluoride to milk was proposed in the 1950s in several different countries: Japan (1952), Switzerland (1953) and the United States of America (1955). The amount of fluoride added to milk depends on the background of fluoride exposure and the age of the children: commonly in the range 0.5 to 1.0 mg per day. An advantage of this method is that the actual amount of fluoride can be delivered under controlled conditions (Banoczy *et al*., 2013). Several epidemiological studies have been carried out to evaluate the effectiveness of fluoridated milk against dental caries and tooth surface wear. Studies have reported the significant effect of fluoridated milk on caries prevention (Riley *et al*., 2005; Banoczy *et al*., 2013) and also on tooth wear reduction (Magalhaes *et al*., 2014; Casssiano *et al*., 2016).

Early studies proved that adding fluoride to milk does not alter its taste or other characteristics for example, absorption. However, adding fluoride to milk is considered advantageous especially as an infant food or for those who need it most and agree to receive it. Regarding the patho-mechanism of fluoride, it is proven that fluoride can increase the degree of the remineralisation process and decrease the degree of demineralisation. Moreover, 30-60 minutes after the ingestion of fluoridated milk, both the levels of fluoride in dental plaque and saliva are shown to increase and there is also an increase in fluoride

concentrations in salivary secretions after the absorption of ingested fluoride (Banoczy *et al*., 2013).

2.13.1 Bioavailability

Adding fluoride to the milk has raised questions concerning its bioavailability and its interactions in the oral cavity. However, previous experimental studies reported satisfactory results, for example Spak *et al*. (1982) reported that 72% of all fluoride in the milk and 65% of all fluoride in the diluted water formula were absorbed. Most of the fluoride that has been added to milk forms a soluble complex with the protein fraction of milk, from which the fluoride can be liberated in its ionic form so that it is bioavailable (Bancozy *et al*., 2013).

2.13.2 Effectiveness of milk and fluoridated milk

There are a number of studies reported in literature on the effectiveness of fluoridated milk in preventing dental caries. Malinowski *et al*. (2012) conducted a study using varying concentrations (0, 2.5 and 5.0 ppm F) of Fluoridated milk on the prevention of demineralisation with a cariogenic challenge. They found that the extent of enamel softness was reduced with increasing the fluoride concentration in milk; 5.0 ppm F showed the lowest extent of enamel softening compared with other groups. The fluoride concentration in the milk showed a dose-dependency effect and even at low concentration it can promote enamel remineralisation, with a trend of increasing the remineralisation process with increasing fluoride concentration in milk.

Fluoride is considered one of the best preventive strategies to control tooth erosion. Magalhaes *et al.* (2014) conducted a study to assess the effect of milk containing different fluoride concentrations. Twelve dentine slabs and twelve enamel slabs were randomly distributed into seven groups: 1)- 0 ppm F milk before erosion; 2)- 0 ppm F milk after erosion; 3)- 2.5 ppm F milk after erosion; 4)- 5.0 ppm F milk after erosion; 5)- 10.0 ppm F milk; 6)- NaF, (0.05% F (positive control) after erosion); or 7)- 0.9% NaCl (negative control) after erosion. The slabs were immersed in a soft drink (coca-cola, pH 2.6) four times daily for 90 seconds each for five days using a pH cycling model. They reported that compared to the negative control group, rinsing with milk before erosive attack had a significant effect in reducing tooth wear (24% and 67% reduction in enamel and dentine, respectively) and only for dentine if milk was applied after the erosive challenge. Rinsing with milk without fluoride and sodium fluoride solutions after erosive challenge showed no significant reduction in the degree of enamel erosion. However, rinsing with fluoridated milk can reduce the tooth wear by a 36% and 44% reduction in enamel and dentine, respectively compared with the negative control. In addition, they reported that fluoride concentration showed dose-dependency as it was negatively correlated with tooth loss for both enamel and dentine.

Cassiano *et al*. (2016) carried out a study aimed to analyse the protective ability of milk against both enamel and dentine erosion considering three factors: 1- time

of application (before or after erosive challenge), 2- presence of different concentrations of fluoridated milk, 3- type of milk being used (bovine whole or fat-free). Fifteen bovine enamel specimens per group and twelve root dentine specimens per group were subjected to the following treatment; 0.9% NaCl solution (negative control), whole milk containing (0, 2.5, 5.0, and 10 ppm F milk), fat-free milk with (0, 2.5, 5.0 and 10.0 ppm F milk) and 0.05% NaF solution (positive control). The specimens were submitted to an erosive demineralisation regime four times a day for five days. 0.1% citric acid (pH 2.5) for 90 seconds was used for the demineralisation process. They reported that for enamel, compared with the negative control, treatment with whole milk containing 10 ppm F before the application of the erosive challenge was the most protective treatment. Other treatments did not differ from the negative control except the group treated with fat-free milk containing 2.5 ppm F before the erosive challenge. For dentine, compared with enamel, adding fluoride to milk showed a better protective effect against erosion. Groups treated with whole milk (all concentration) either before or after the erosive challenge showed a significant difference from the negative control (except the group treated with 2.5 ppm F before application of erosive challenge). Whole milk without fluoride (applied before the erosive challenge), fat-free milk with 10 ppm F and the positive control (before and after the erosive challenge) reported significant difference compared with the negative control. Furthermore, the application of milk before the erosive challenge was more effective in reducing dentine erosion

compared with the application after the erosive challenge, but only in the absence of fluoride. This might be due to the presence of protein contents rather than fat.

Lindquist *et al*. (2011) carried out a study to examine the ability of different neutralising products on raising the intra-oral low pH after erosive attack. Eleven adult participants were requested to rinse with a hydrochloric acid (as a gastric acid stimulation) for 60 seconds. The pH was measured intra-orally at four sites (buccal, mesial, distal, and the dorsum of the tongue) for up to 30 minutes. After rinsing with the erosive potential (HCL), the following products were used: antacid tablet, Arabic gum lozenge, mineral water, milk and tap water (positive control). The negative control was considered as no product use. All five tests were applied for two minutes following erosive challenge. All the tested products were showed to cause a rapid increase of intra-oral pH after the erosive challenge compared with the negative control. Even though this study examined milk without the addition of fluoride, milk showed a positive result in regard to intra-oral acid neutralisation.

Moreover, Wiegand *et al*. (2008) examined the effects of milk, water and fluoride rinsing on surface re-hardening of acid softened enamel, *in situ* study. Ten participants were given intra-oral appliances with bovine enamel samples attached. Specimens demineralisation process was performed extra-orally using soft drink (Coca-cola company, pH: 2.9) for two minutes. Thereafter, the participants were asked to rinse with SnF_2, milk or water for 60 seconds. At each

test, one enamel sample was covered with a tape during intraoral rinsing which served as a control. After rinsing both test and control enamel samples were exposed to oral cavity for four hours. After analysis, they reported that rinsing with milk, water, or 250 ppm fluoride after erosive challenge had a significant effect on surface re-hardening. The highest finding was after rinsing with 250 ppm F followed by milk and then water. However, they did not achieve the baseline values. They found that their study was in line with previous study carried out by Gedialia *et al*. (1991) who reported that rinsing with milk can enhance the surface re-hardening of demineralised enamel. Also, caries research studies reported that rinsing with milk and fluoridated milk are effective in reducing enamel acid solubility and may increase remineralisation of artificial caries-like lesions.

Nahas *et al*. (2011) studied the prevalence and associated factors of dental erosion in a total of 232 participants aged 2-20 years old who had attended dental treatment at a private dental centre in Sao Paolo, Brazil. The participants' parents answered a questionnaire containing information about participant identification, oral hygiene habits (OHI), tooth grinding, dietary habits, and any associated gastric disorder if present. They reported that dental erosion was associated with some factors like frequent consumption of soft drink and candies. However, they found that participants who frequently drank milk had a 60% less chance of developing dental erosion.

Another study by Arnold *et al.* (2014) was carried out to investigate the impact of fluoridated milk on root dentin remineralisation. In this *in vitro* study, thirty premolar teeth were divided into six groups. Using a diamond bur, the cervical root cementum was removed. The dentin surface was subjected to a demineralisation process using 1.6% hydroxyethyl cellulose acidified with acetic acid at (pH 4.7) for three days. After demineralisation lesion, the teeth were incubated at 37ºC for seven days in one of the following six agents: 1- artificial saliva, 2- artificial saliva with 10 ppm F milk, 3- milk, 4- milk with 2.5 ppm F, 5- milk with 10 ppm F, 6- Sodium Chloride. They reported that the depth of the lesions was decreased with increasing the fluoride concentration and concluded that fluoridated milk showed a definite effect on root dentin remineralisation.

Abdul-Manaf *et al*. (2011) studied the effect of diet on developing tooth erosion among 150 undergraduate students at the University of Kebangsaan Malaysia, a cross-sectional study with basic erosive tooth wear examination was used for assessment of dental erosion development. They found 68% of participants had dental erosion. However, there was a significant negative association between having dental erosion and milk consumption ($p= 0.004$). They reported that only 13% of those who had a high frequency of milk consumption showed dental erosion compared with 87% in the low consumption group. In the same manner, Salas *et al*. (2014) assessed the influence of diet on erosion prevalence in children and adolescents. They reviewed all published studies up to May 2014, which had

addressed this influence. Thirteen studies fulfilled their criteria. They found that higher consumption of snacks/sweets, carbonated drinks and acidic drinks were associated with the increased occurrence of tooth erosion. While, a greater consumption of milk or yoghurt showed a protective effect against developing tooth erosion. This indicates the importance of diet especially milk and milk products with regards to preventing or reducing the development and progression of dental erosion.

However, Wiegand and Attin (2014) carried out a randomized *in situ* study aimed to analyse the protective ability of milk with and without adding a 5.0 ppm F and CPP-ACP pastes with and without adding a 900 ppm F on dental erosion. Fifteen healthy adult participants with a mean age of 33.2 +/- 7.6 years were given intraoral appliances with enamel and dentin specimens. The enamel and dentin specimens were extra-orally eroded using a soft drink (Coca cola, pH 2.7), six times and for 90 seconds a day and brushed twice for 30 seconds a day using fluoride-free toothpaste as a negative control. The study was seven phase (five days each) crossover design. The test products were; milk, milk with 5.0 ppm F (2xday, each 100 ml/120 seconds), CPP-ACP paste, CPP-ACP with 900 ppm F (180 seconds/day), $SnCl_2$/AmF/NaF mouth rinses as a positive control for 30 seconds/day, fluoridated toothpaste (1250 ppm F) and non-fluoridated toothpaste as a negative control. The test products were applied intra-orally, immediately and after application of an erosive attack. Using a profilometry machine the tissue

loss was assessed after each five day period. They reported that only fluoridate toothpaste and $SnCl_2$/AmF/NaF mouth rinses showed a significant reduction in tooth surface loss. They claimed that milk and CPP-ACP were not effective because they might be mechanically dissolved or scattered by toothbrushing or acid attack.

As there are a limited number of studies in the literature on the preventive effect of fluoridated milk on erosion and abrasion, and looking at the plethora of studies and opinions, it is clear that the debate on this area has not yet been established. Therefore, the aims of this study were as follows:

2.14 Research aims and hypothesis

2.14.1 Aims of the study:

1- To investigate the effect of fluoridated milk 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive condition after 28 days *in vitro*.

2- To investigate the effect of fluoridated milk 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive and abrasive conditions after 28 days *in vitro*.

2.14.2 Objectives of the study:

1- To assess and compare the effect of fluoridated milk 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive pH cycling regime after 28 days *in vitro*, using Surface Profilometry.

2- To assess and compare the effect of fluoridated milk 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive pH cycling regime and abrasive conditions after 28 days *in vitro*, using Surface Profilometry.

2.15 The Null Hypotheses for the study:

1- There is no difference in the effect of fluoridated milk 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive condition after 28 days *in vitro.*

2- There is no difference in the effect of fluoridated milk 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive and abrasive conditions after 28 days *in vitro.*

3. Materials and methods:

This was a randomised, single-blinded (examiner) study *in vitro*. This study comprised two phases: phase A- erosive challenge and phase B- erosive and abrasive challenges.

The methodology adopted in the present study is described in this chapter including preparation of the enamel samples and the pH cycling protocol as well as the materials and equipment used.

3.1 Power calculation:

Statistical advice was sought and the sample size was calculated by using data from a previous study (Magalhaes *et al*., 2014), the estimated enamel loss difference between 0 ppm F (3.63 +/- 0.04) and 5.0 ppm F (2.81+/-0.27) is 0.82 (=3.63-2.81), resulting that the minimum group sample size of 3 slabs per group was needed to achieve more than 90% power at 0.05 significance level. The total sample size needed would be minimal N=18 slabs (3 per group). With enough resources (bovine teeth), it was decided to increase the sample size to 15 slabs/group, resulting in 90 slabs. A total of 3-4 slabs were obtained from each bovine tooth, requiring 30-35 bovine teeth in order to obtain 90 slabs.

3.2 Materials and Equipment:

- 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).
- Fluoride-free toothpaste (AloeDent, Holland and Barrett, UK).
- Medium toothbrushes (Basic, Sainsbury's, UK).
- Plastic containers.
- Toothbrush Simulator machine (ZM, 2016, By SD Mechatronic GmbH, Feldkirchen-Westerham Germany).
- Artificial saliva chemicals (for night and day saliva).
- Citric acid monohydrate, Analar NormaPur VWR.
- Distilled water.

- Profilometer SP (ProScan 2000, version 2.1.1.8, Scantron Industrial Products Limited, Somerset, England).
- Semi-skimmed milk (Tesco, Leeds, UK).

3.3 Experimental groups:

The enamel slabs were randomly assigned to two phases, each phase containing three groups.

A) Phase A- Erosive challenge:

1. 0 ppm F milk (control group)
2. 2.5 ppm F milk
3. 5.0 ppm F milk

B) Phase B- Erosive and Abrasive challenges:

4. 0 ppm F milk (control group)
5. 2.5 ppm F milk
6. 5.0 ppm F milk

3.4 Preparation of enamel slabs:

All enamel slabs used in the present study were obtained from bovine incisors. All teeth were stored immediately in distilled water and 0.1% thymol (Sigma Aldrich) at room temperature. Before their sectioning, the teeth were cleaned using a spoon excavator and a toothbrush with pumice powder and stone to

remove all soft tissue remnants. Suitable teeth were selected for the study. Each tooth was mounted using ‘green stick’ impression compound (Kerr, UK) on plates. The crowns were sectioned using water-cooled, diamond wire saw, cutting machine (Well@Walter EBNER, CH-2400 Le Loche, Figure 1). 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 2 shows an illustration of enamel slab preparation.

Figure 1: (a - b): Diamond wire saw apparatus used for the teeth sectioning (Well® Walter EBNER, CH-2400 Le Loche).

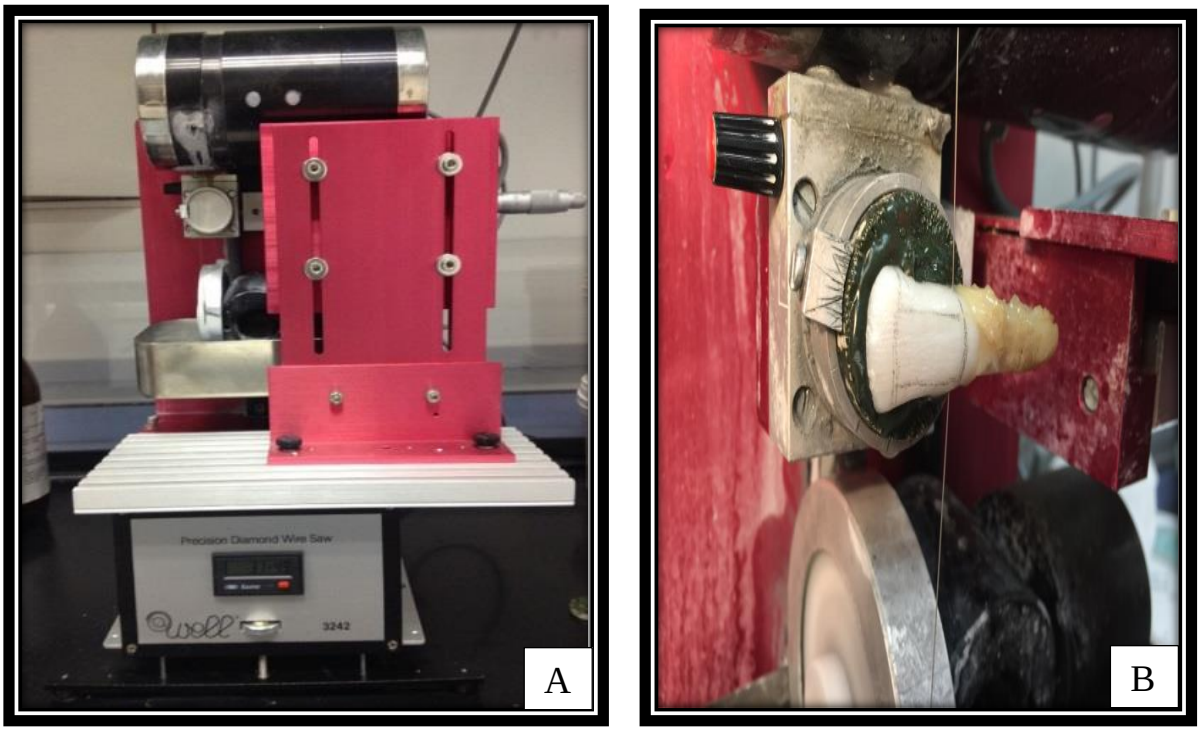
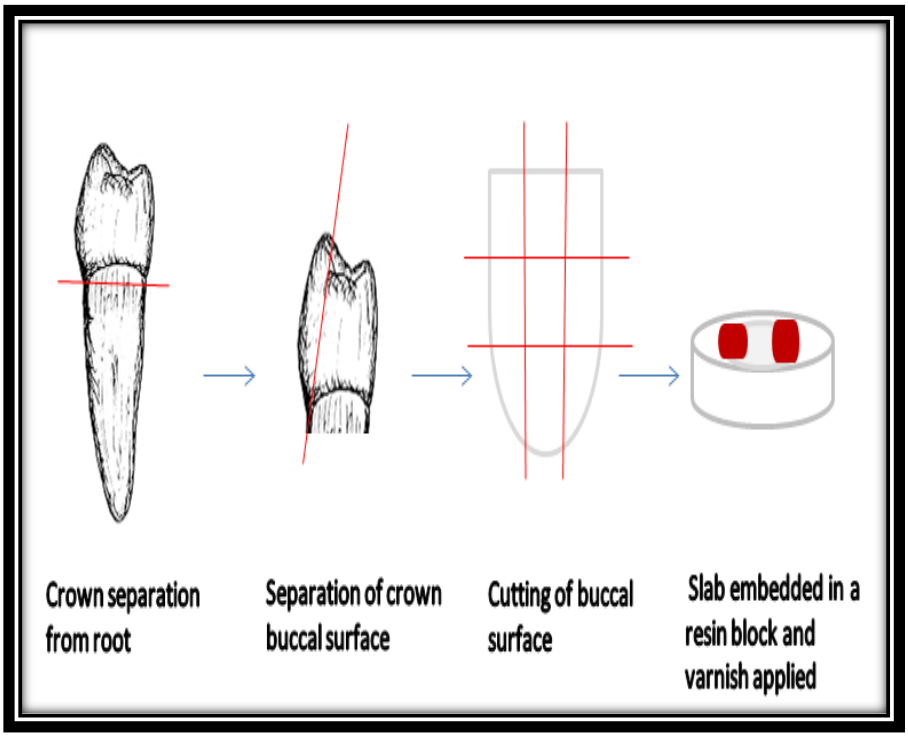


Figure 2: Illustration showing enamel slab preparation.



The prepared slabs were placed in silicone moulds and then embedded in clear resin (Stycast; Hitek Electronic materials, Scunthrope, UK) and left for 24 hours to dry in order to form circular resin blocks of 3 mm thickness (Figure 3).

To ensure the flatness of slabs the blocks were placed in rectangular steel blocks, which had circular holes of 3 mm depth (Figure 4). 600 grade fine grit abrasive paper (Wet or Dry paper, 3M) followed by 800, 1200 and 2000 grade were 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 Storage of enamel slabs

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

Figure 3: Enamel slabs in silicon moulds.

Figure 4: 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 to be included in the study.

3.6.1 Knoop Microhardness

Each flat enamel slabs were then 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 porosity of the superficial enamel layer. Microhardness of enamel slabs was assessed using computer-aided Duramin indenter machine (Struers A/S, DK 26-10, Denmark) (Figure 5).

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.

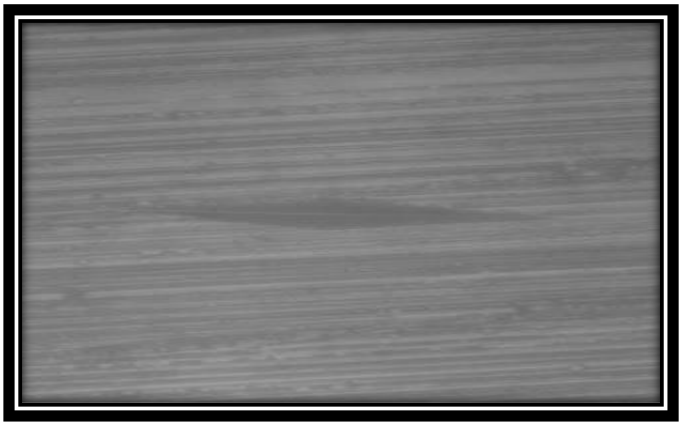
2 1 3

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 were 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 6). Slabs whose enamel microhardness was not within this normal range were excluded from the study.

Figure 5: Computer-aided Duramin Indenter Machine (Struers A/S, DK 26-10, Denmark).

Figure 6: Microscopic image of diamond shape indentation on the enamel surface at baseline.



3.6.2 Surface Profilometry

To ensure the flatness of the enamel slabs baseline measurements of the surface profile of the slabs were assessed using a surface profilometer (ProScan 2000, version 2.1.1.8, Scantron Industrial Products Limited, Somerset, England. Figure

7). The average height to the average depth should be in range of (Rz) ±1μm. The measurement was achieved by placing the sample on the key stage of the Scantron ProScan and using a 150mm height of the sensor as standard. The sensor used has a working range of 300μm 5mm from the surface. Sample rate/frequency was set at 300Hz to give a minimum intensity of 5% of reflected light to analyse. The step size used was 0.01mm. After scanning, the flatness was checked. Slabs which were not flat were repolished (reground if needed) as described before and then were checked again with Microhardness and ProScan if the rescan was within the measurements then the slabs are considered a flat and assumed to be zero. Figure 8 shows an example of flat surface for one of the slabs. The enamel slab's surfaces were covered with nail varnish **(**Glossfinity nail varnish, MaxFactor®, England, UK) except for a small window approximately 2x3mm size in the middle of each slab that was left exposed. A special tray with holes was used to hold the slabs of each group (Figure 9). Resin blocks were secured in position using adhesive wax. After 7, 14, 21 and 28 days, the nail varnish was removed using acetone and the same procedure of scanning was repeated to check the average depth surface loss (SL) of the exposed area compared to unexposed reference surfaces.

Figure 7: The surface Profilometry (Scantron Proscan 2000)

Figure 8: Flat surface profile analysis

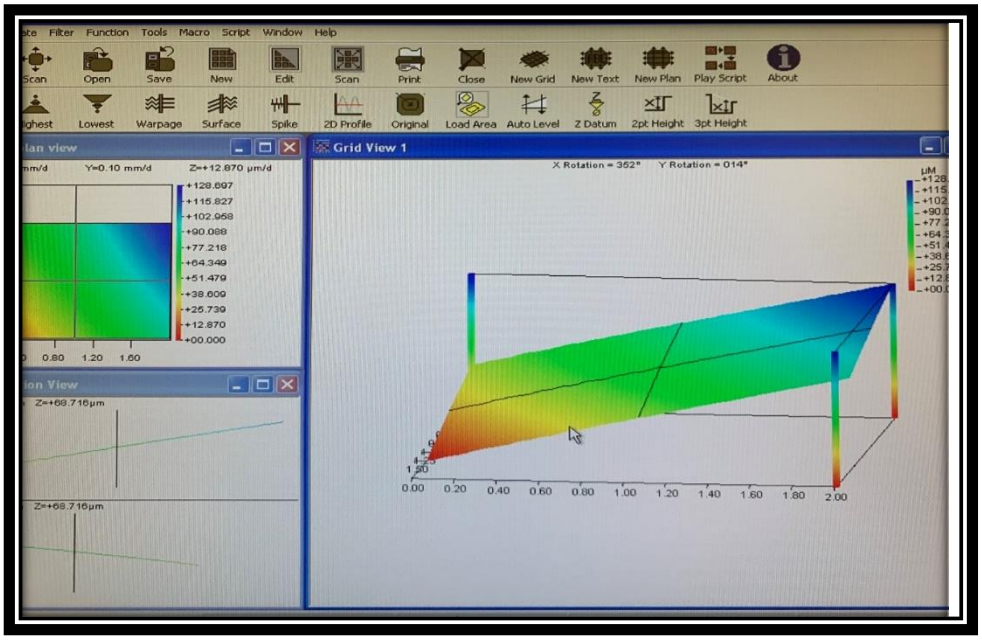
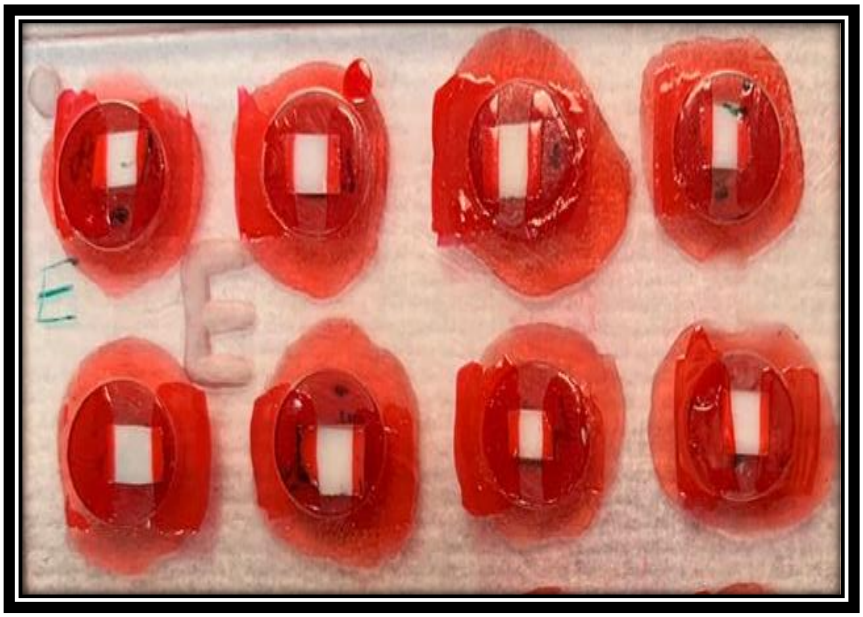


Figure 9: Slabs after covered with nail varnish except for a small window.



3.7 Randomisation and blindness:

Enamel slabs were randomly allocated to 6 study groups using a randomisation website (https://www.randomizer.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. This makes the analysis blinded.

3.8 Study groups:

The enamel slabs were divided into 6 treatment groups.

Phase A- Erosive challenge:

Group (1) 0 ppm F milk (control group)

Group (2) 2.5 ppm F milk

Group (3) 5.0 ppm F milk

Phase B- Erosive and Abrasive challenges:

Group (4) 0 ppm F milk (control group)

Group (5) 2.5 ppm F milk

Group (6) 5.0 ppm F milk

3.9 pH cycling regime with erosive challenge.

The slabs (Phase A and Phase B) were dipped in fluoridated milk for 5 minutes twice daily in one of the concentrations of F milk (0 (control), 2.5 and 5.0 ppm F). Then the enamel slabs were dipped for 10 minutes in a milk slurry of 1 part of milk and 3 parts of artificial saliva (Figure 11).

The slabs were immersed in a solution for 2 minutes five times daily in 0.3% citric acid (pH 2.6) for a period of 28 days. Citric acid was prepared by adding three grams of monohydrate citric acid to one litre of de-ionised water. Each group of slabs was immersed at room temperature in fresh 200 ml 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. The slabs were also rinsed in de-ionised water after treatment before they were returned to the artificial saliva, which was changed twice daily.

Two artificial saliva solutions were used in this study. The first solution was used for day time during the pH cycling, between the acid exposures. The second solution was used to store the slabs during the night. The day saliva was a

supersaturated solution that allowed remineralisation of enamel slabs, the night saliva was a saturated solution that maintained the enamel condition and did not provide any minerals exchange. The artificial saliva composition was based on the electrolyte composition of natural saliva and it was advised to be used in order 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).

3.9.1 The preparation of day time saliva

The formulation of day time saliva is shown in Table 3.

Table 3: Day time artificial saliva

Constituent	Concentration g/L
Calcium carbonate	0.07
Magnesium carbonate (hydrated basic)	0.019
Potassium di-hydrogen phosphate	0.544
HEPES buffer (acid form)	4.77
Potassium chloride	2.24

The above components were added in about 900 mL of distilled water. Addition of 1.8 ml 1mol/L HCL followed and the solution was stirred until all components

had dissolved. The pH was adjusted to 6.8 by adding KOH solution. The saliva was kept at room temperature and used it within 2-3 days.

3.9.2 The preparation of night time saliva

The formulation of night time saliva is shown in Table 4.

Table 4: Night time artificial saliva

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

Again using 900 mL distilled water 1.4 mL 1 mol/L HCL and above components were stirred using a shaker until it all dissolves. The pH was adjusted to 6.8 by adding KOH solution. The saliva was kept at room temperature and used it within 2-3 days.

Between immersions in citric acid the slabs were left immersed in artificial saliva for minimum 60 minutes to enable remineralisation. The slabs were kept in an incubator at 37.0^{o}C at all times except when they are immersed in citric acid.

Artificial saliva was changed daily to prevent any contamination and/or bacterial growth. A minimum 60-minutes gap was left between daytime erosive challenges. Before and after dipping in the erosive solutions the slabs were rinsed with de-ionised water.

3.10 The pH cycling regime with erosive and abrasive challenges (brushing).

For the erosive challenge, a similar procedure as has been previously described in section 3.9.

3.10.1 Abrasive (Brushing) Challenge

The enamel slabs (Phase B/groups 4, 5, and 6) were brushed twice per day after treatments with fluoridated milk.

The toothbrush (medium) coarse bristles were used for brushing (Sainsbury's, Sainsbury’s supermarket Ltd, London, UK). Fluoride-free toothpaste slurry (AloeDent, Holland and Barrett Company, UK) was applied to the enamel slabs for 2 minutes during which time a 200g weight was applied for 15 strokes using a brushing machine (toothbrush Simulator (ZM, 2016, By SD Mechatronic GmbH, Feldkirchen-Westerham Germany). The intervals between brushing and dipping in citric acid was a minimum of 60 minutes (Figure 12).

3.10.2 Toothpaste slurry:

Toothpaste slurries were prepared by mixing the fluoride-free toothpaste with artificial day time saliva in a volume ratio 1:4 (toothpaste: saliva) by weight, using a WhirliMixer® (Fisons) for 1 minute (Figure 10).

Figure 10: WhirliMixer® (Fisons)

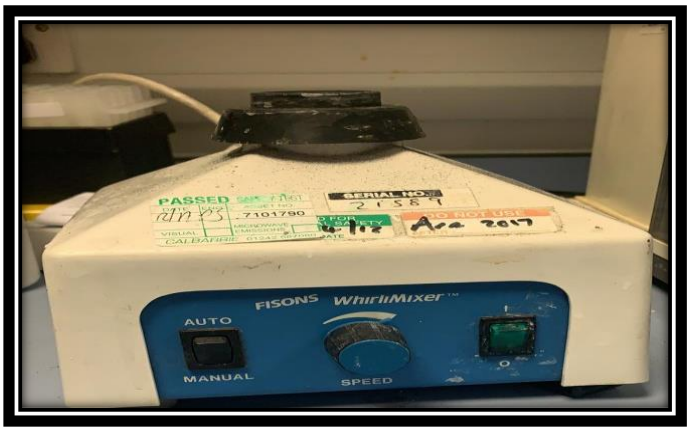


Figure 11: Work Flow charts for phase A- The pH cycling regime with erosive challenge

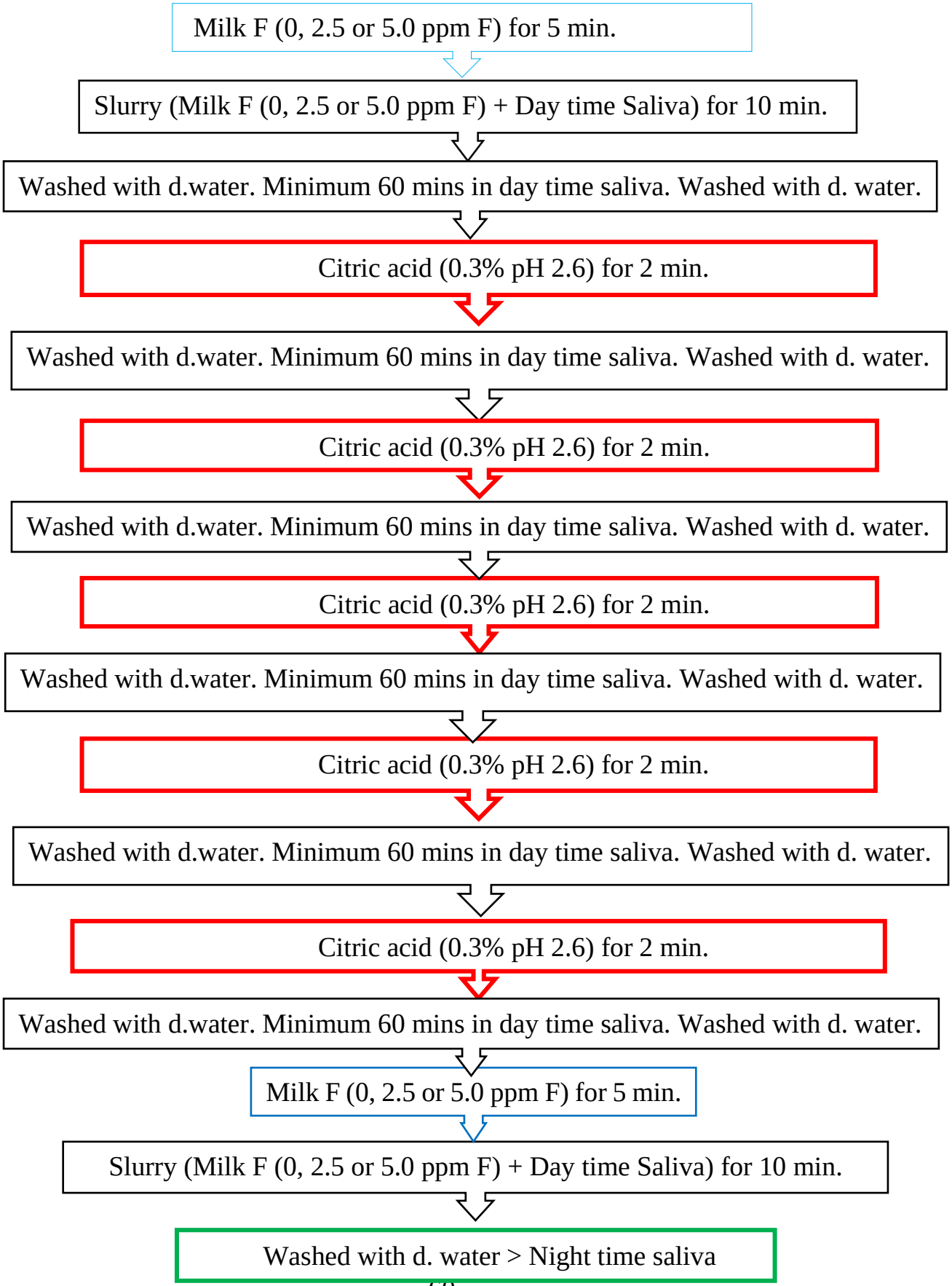


Figure 12: Work Flow charts for Phase B- The pH cycling regime with erosive and abrasive challenges (brushing)

3.11 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 acetone and the enamel surface was cleaned with ethanol to ensure that all residues were removed.

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 150mm 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 (Figure 13). Then 3 point height was selected in the primary plan view (Figure 14) and the result was recorded which can be seen in figure 15 showing the different surfaces of the scan for example after 7 days.

Figure 13: Grid view of a scan of a sample after 7 days with A, B and C the three points of levelling

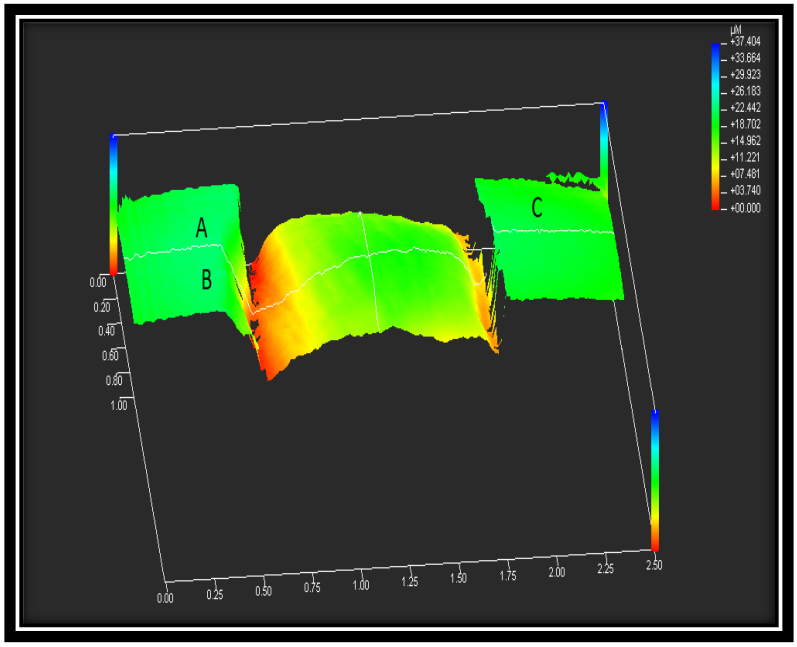


Figure 14: 3 points height sample measurements with the result of the difference in height recorded at 13.603 µm

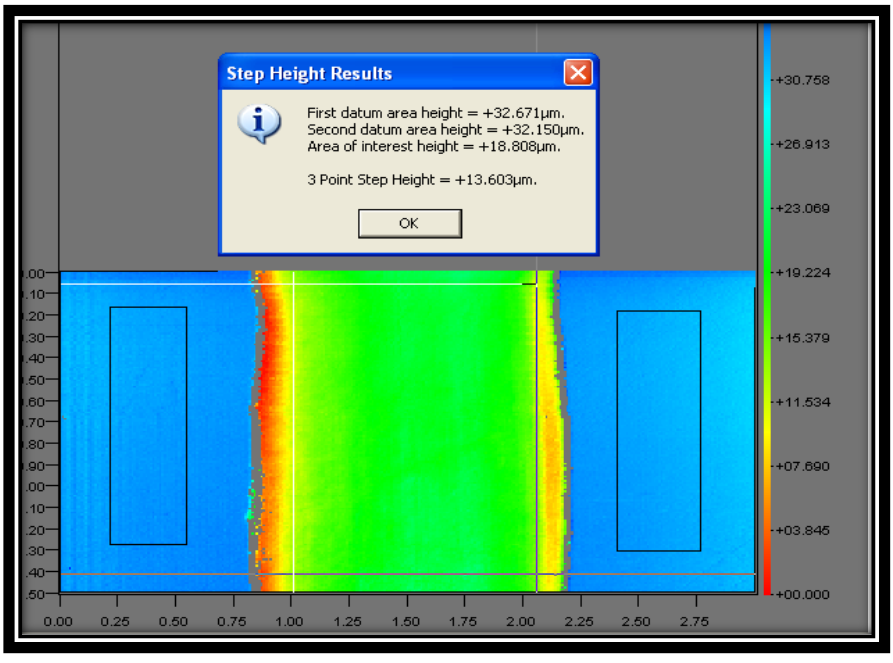
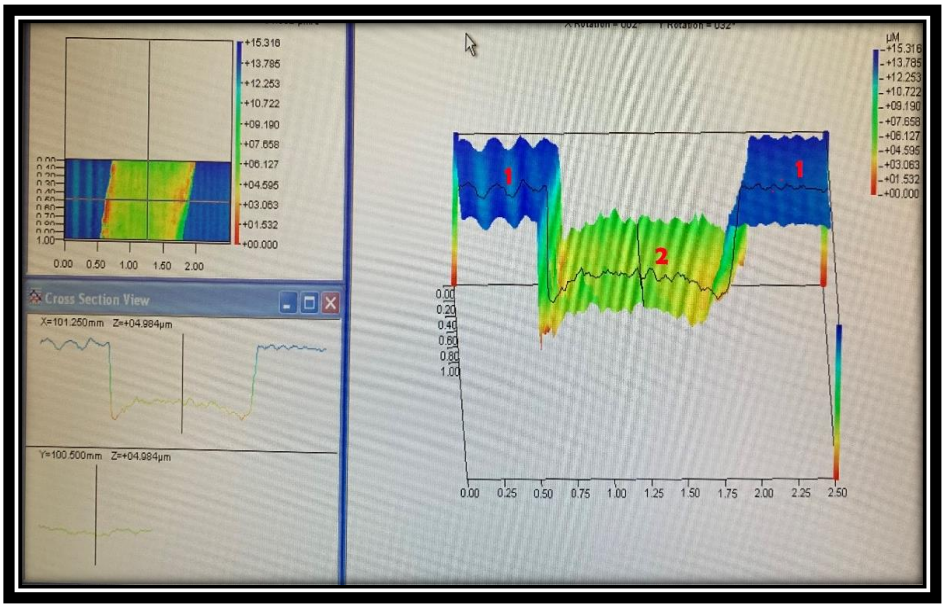


Figure 15: Grid view of a scan of a sample after 7 days with 1 enamel intact surface, 2 step of erosive surface after 7 days.



3.12 Statistical analysis:

For the analysis, the data were uploaded in a SPSS Version 26. Descriptive statistics were used to calculate the mean, median, range, and standard deviation for continuous data. The normality of the data was tested in order to proceed with the appropriate analysis. Data taken from all groups were normally distributed (Table 5). One way ANOVA was used to compare between (0, 2.5 and 5.0 ppm F milk) groups under erosive challenge after 28 days. The same test was used to compare between (0, 2.5 and 5.0 ppm F milk) groups under erosive and abrasive challenges after 28 days. Furthermore, Bonferroni correction was used to assess if there was significant difference between each of the groups.

Independent t-test was performed to compare the amount of surface loss between groups with the same concentrations with and without brushing after 28 days of erosive pH cycling.

95% confidence intervals are presented. The significance level was set at 0.05.

4. Results

4.1 Tooth surface loss

All the prepared slabs were analysed after 28 days. Data were taken from (0, 2.5, and 5.0 ppm F milk) groups on both phases (erosive/ erosive and abrasive challenges) were analysed. Shapiro-Wilk test was used to test the normality distribution of the data. All groups were normally distributed (Table 5). So, one way ANOVA was used for the analysis between groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive challenge and, separately, between the groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive and abrasive challenges. Also, Independent t-test was used for the comparisons between the groups with the same concentration of fluoride in milk on the surface loss after 28 days erosive challenge with and without abrasive challenge (brushing).

Table 5: Results of normality test for enamel slabs.

After 28 days	groups	Shapiro-Wilk		
		Statistic	df	Sig.
Erosive challenge	1 (0 ppm F milk)	.882	15	.051
	2 (2.5 ppm F milk)	.884	15	.055
	3 (5.0 ppm F milk)	.964	15	.761
Erosive and abrasive challenges	4 (0 ppm F milk)	.963	15	.749
	5 (2.5 ppm F milk)	.972	15	.888
	6 (5.0 ppm F milk)	.911	15	.139

4.2 Descriptive statistics and mean comparisons of slabs surface loss for groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive challenge.

Tables 6 shows the descriptive statistics for groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive challenge. It is evident that the mean of enamel surface loss for all concentration levels seemed to decrease as the fluoride concentration level in milk increased. The lowest enamel surface loss was seen in the 5.0 ppm F milk group with mean (8.2 ± 1.02), followed by the 2.5 ppm F milk with the mean (12.180 ± 1.45). The highest enamel surface loss was noticed in the control group (0 ppm F milk) group with a mean (15.436 ± 1.55).

Table 6: Descriptive statistics of slabs surface loss for groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive challenge.

Challenge	Fluoride Conc. (ppm F milk)	Mean (µm)	Std. Deviation	Min. (µm)	Max. (µm)	Confidence interval. (µm)	
						Lower	Upper
Erosive challenge.	0	15. 436	1.55	13.19	17.42	14.58	16.29
Erosive challenge.	2.5	12.180	1.45	9.325	15.64	11.38	12.98
Erosive challenge.	5.0	8.200	1.02	6.17	9.87	7.63	8.77

Statistical analysis of the results:

As the distribution of data was normal the one way ANOVA test and the Bonferroni correction were used in the statistical analysis.

The One Way ANOVA test was performed to assess if the difference in enamel surface loss was statistically significant between the three groups (0, 2.5, and 5.0 ppm F milk). It showed that there was a statistically significant difference ($p < 0.05$) in the mean changes in surface loss between treatment groups (0, 2.5, and 5.0 ppm F milk) after 28 days erosive challenge (Table 7).

Table 7: One way ANOVA results between groups (0, 2.5 and 5.0 ppm F milk) for the difference in enamel surface loss after 28 days erosive challenge.

Erosion after 28 days

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	394.086	2	197.043	106.643	.000
Within Groups	77.603	42	1.848		
Total	471.688	44			

This indicated that the different F milk concentrations used in the study had a different effect on slabs surface loss during the pH cycling period. However, it does not provide any further information for the differences between each of the groups. Therefore, post-hoc test with Bonferroni correction was used for further statistical analysis to see where the difference lay (Table 8).

It can be seen that the mean difference in enamel surface loss of the 0 ppm F milk (control group) after 28 days was significantly higher compared with the 2.5 ppm F milk and 5.0 ppm F milk. Also, the mean difference in enamel surface loss of the 2.5 ppm F milk after 28 days was significantly higher compared with the 5.0 ppm F milk after 28 days, (all adjusted P-values < 0.05).

Table 8: Multiple comparisons between different concentrations of fluoride in milk (0, 2.5 and 5.0 ppm F milk) on the surface enamel loss after 28 days erosive challenge.

(I) flu	(J) flu	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0 ppm	2.5 ppm	3.26*	.000	2.02	4.49
	5 ppm	7.24*	.000	5.99	8.47
2.5 ppm	0 ppm	-3.26*	.000	-4.49	-2.01
	5 ppm	3.98*	.000	2.74	5.22
5 ppm	0 ppm	-7.24*	.000	-8.47	-5.99
	2.5 ppm	-3.98*	.000	-5.22	-2.74

*. The mean difference is significant at the 0.05 level.

4.3 Descriptive statistics and mean comparisons of slabs surface loss for groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive and abrasive challenges.

Tables 9 shows the descriptive statistics for groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive and abrasive challenges. It is evident that the mean of enamel surface loss for all concentration levels seemed to decrease as the fluoride concentration level in milk increased. The lowest enamel surface loss was seen in the 5.0 ppm F milk group with mean (14.513 ± 2.70), followed by the 2.5 ppm F milk with the mean (17.246 ± 1.67). The highest enamel surface loss was noticed in the control group (0 ppm F milk) with a mean (20.921 ± 2.61).

Table 9: Descriptive statistics of slabs surface loss for groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive and abrasive challenges.

Challenge	Fluoride Conc. (ppm F milk)	Mean (µm)	Std. Deviation	Min. (µm)	Max. (µm)	Confidence interval. (µm)	
						Lower	Upper
Erosive and abrasive.	0	20.921	2.61	15.23	25.40	19.47	22.37
Erosive and abrasive.	2.5	17.246	1.67	13.98	19.79	16.32	18.17
Erosive and abrasive.	5.0	14.513	2.70	11.00	18.53	13.02	16.01

Statistical analysis of the results:

As the distribution of data was normal the one way ANOVA test and the Bonferroni correction were used in the statistical analysis.

The One Way ANOVA test was performed to assess if the difference in enamel surface loss was statistically significant between the three groups (0, 2.5, and 5.0 ppm F milk). It showed that there was a statistically significant difference ($p < 0.05$) in the mean changes in surface loss between treatment groups (0, 2.5, and 5.0 ppm F milk) after 28 days erosive and abrasive challenges (Table 10).

Table 10: One way ANOVA results between groups (0, 2.5 and 5.0 ppm F milk) for the difference in enamel surface loss after 28 days erosive and abrasive challenges.

Erosion and abrasion after 28 days

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	310.094	2	155.047	27.518	.000
Within Groups	236.642	42	5.634		
Total	546.736	44			

This indicated that the different F milk concentrations used in the study had a different effect on slabs surface loss during the pH cycling period. However, it does not provide any further information for the differences between each of the groups. Therefore, post-hoc test with Bonferroni correction was used for further statistical analysis to see where the difference lay (Table 11).

It can be seen that the mean difference in enamel surface loss of the 0 ppm F milk (control group) was significantly higher compared with the 2.5 ppm F milk and 5.0 ppm F milk. Also, the mean difference in enamel surface loss of the 2.5 ppm F milk was significantly higher compared with the 5.0 ppm F milk (all after 28 days, all adjusted P-values < 0.05).

Table 11: Multible comparisons between different concentrations of fluoride in milk (0, 2.5 and 5.0 ppm F milk) on the surface enamel loss after 28 days erosive and abrasive challenges.

(I) flu	(J) flu	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0 ppm	2.5 ppm	3.67*	.000	1.51	5.84
	5 ppm	6.41*	.000	4.24	8.57
2.5 ppm	0 ppm	-3.67*	.000	-5.84	-1.51
	5 ppm	2.73*	.009	.571	4.90
5 ppm	0 ppm	-6.417*	.000	-8.57	-4.2
	2.5 ppm	-2.73*	.009	-4.90	-.57

*. The mean difference is significant at the 0.05 level.

4.4 Comparisons between the groups with the same concentration of fluoride in milk on the surface loss after 28 days erosive challenge with and without abrasive challenge (brushing).

4.4.1 Slab surface loss for groups containing 0 ppm F milk

The mean of slabs surface loss of group 0 ppm F milk after 28 days erosive and abrasive challenges was higher compared with the mean of slabs surface loss of group 0 ppm F milk after 28 days erosive challenge only (20.921 ± 2.61; 15.436 ± 1.55), respectively. And, to assess whether this enamel surface loss was significantly different. Independent t-test was carried out and the results (Table 12) showed that there was a statistically significant difference between these two groups.

Table 12: Independent t-test results for comparing means of surface loss in groups 0 ppm F milk after 28 days erosive challenge with and without brushing.

		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
					Lower	Upper
after 28 days	Equal variances assumed	.000	-5.484	.783	-7.09	-3.88
	Equal variances not assumed	.000	-5.484	.783	-7.11	-3.86

4.4.2 Slab surface loss for groups containing 2.5 ppm F milk

The mean of slabs surface loss of group 2.5 ppm F milk after 28 days erosive and abrasive challenges was higher compared with the mean of slabs surface loss of group 2.5 ppm F milk after 28 days erosive challenge only (17.246 ± 1.67; 12.180 ±1.45), respectively. And, to assess whether this enamel surface loss was significantly different. Independent t-test was carried out and the results (Table 13) showed that there was a statistically significant difference between these two groups.

Table 13: Independent t-test results for comparing means of surface loss in groups 2.5 ppm F milk after 28 days erosive challenge with and without brushing.

		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
					Lower	Upper
after 28 days	Equal variances assumed	.000	-5.067	.571	-6.24	-3.90
	Equal variances not assumed	.000	-5.067	.571	-6.24	-3.90

4.4.3. Slab surface loss for groups containing 5 ppm F milk

The mean of slabs surface loss of group 5.0 ppm F milk after 28 days erosive and abrasive challenges was higher compared with the mean of slabs surface loss of group 5.0 ppm F milk after 28 days erosive challenge only (14.513 ± 2.70; 8.2 ± 1.02), respectively. And, to assess whether this enamel surface loss was significantly different. Independent t-test was carried out and the results (Table 14) showed that there was a statistically significant difference between these two groups.

Table 14: Independent t-test results for comparing means of surface loss in groups 5.0 ppm F milk after 28 days erosive challenge with and without brushing.

		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
					Lower	Upper
after 28 days	Equal variances assumed	.000	-6.314	.746	-7.84	-4.79
	Equal variances not assumed	.000	-6.314	.746	-7.88	-4.75

4.5 Intra-examiner correlation

From all the enamel slabs measurements (n=90 slabs) 18 (20%) enamel slabs were randomly selected and re-analysed. The intra-class Correlation Coefficient (Table 15) was found to be (0.99, 95% CI (0.976, 0.997) which represent excellent reproducibility.

Table 15: Intra-class Correlation Coefficient

	Intra-class Correlation	95% Confidence Interval	
		Lower Bound	Upper Bound
Single Measures	.991	.976	.997
Average Measures	.996	.988	.998

4.6 Summary of the results

A) Comparison between different concentrations of fluoride in milk 0, 2.5 and 5.0 ppm F on the surface enamel loss after 28 days of pH cycling.

1- There was a dose dependent trend as the enamel surface loss decreased as the fluoride concentration level in milk increased in both phases.
2- The means of enamel surface loss of groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive and abrasive challenges were higher than those with erosive challenge alone.
3- The results showed that there was statistically significant difference in the enamel surface loss between the groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive challenge. The lowest enamel surface loss was seen in 5.0 ppm F milk group followed by 2.5 ppm F milk group. The highest enamel surface loss was seen in 0 ppm F milk group.
4- The results showed that there was statistically significant difference in the enamel surface loss between the groups (0, 2.5 and 5.0 ppm F milk) after 28 days erosive and abrasive challenges. The lowest enamel surface loss was seen in 5.0 ppm F milk group followed by 2.5 ppm F milk group. The highest enamel surface loss was seen in 0 ppm F milk group.

B) Comparisons between the groups with the same concentration of fluoride on the surface loss after 28 days erosive challenge with and without abrasive challenge (brushing).

0.0 ppm F milk:

There was statistically significant reduction in the slabs surface loss of group (0 ppm F milk) after 28 days erosive challenge compared with group (0 ppm F milk) after 28 days erosive and abrasive challenges.

2.5 ppm F milk:

There was statistically significant reduction in the slabs surface loss of group (2.5 ppm F milk) after 28 days erosive challenge compared with group (2.5 ppm F milk) after 28 days erosive and abrasive challenges.

5.0 ppm F milk:

There was statistically significant reduction in the slabs surface loss of group (5.0 ppm F milk) after 28 days erosive challenge compared with group (5.0 ppm F milk) after 28 days erosive and abrasive challenges.

5.0 Discussion

5.1 Justification of study aims

The potential of remineralisation of eroded enamel has been investigated by El-Aidi *et al*. (2011) and Nahas *et al*. (2011), who both noticed a reduction in the prevalence of tooth surface loss with milk consumption. It was evident from the literature reviewed that the protective ability of saliva might be increased by rinsing with milk as it contains a higher level of calcium and phosphate than saliva (Gedalia *et al*., 1991).

Following the development of community-based programmes in the 1980s, it was clear from the available literature that milk can provide an alternative, cost-effective vehicle for the delivery of fluoride owing to the remineralisation properties of the protein, fat and mineral contents of the milk. However, it is also important to consider the applicability of this kind of programme, where salt and water fluoridation are not possible (Bancozy *et al*., 2009; Bancozy *et al*., 2013). Fluoridated milk is reported to be able to increase tooth remineralisation and reduce tooth demineralisation *in vitro* (Giacman et al., 2012; Malinowski *et al*., 2012), *in situ* (Malinowski *et al*., 2012), and *in vivo* (Skold-Larsson *et al.*, 2013).

Most of the available studies were related to the clinical effectiveness on the caries' prevention of fluoridated milk, comparing the effect of different fluoride delivery methods on the remineralisation / demineralisation process or assessing the effect of fluoridated milk on erosion alone. However, there appears to be a

lack of research investigating the effect of milk containing different concentrations of fluoride on tooth surface wear (erosion and abrasion).

Therefore, the current *in vitro* study has aimed to investigate the effect of fluoridated milk 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive condition and to investigate the effect of fluoridated milk with 2.5 ppm F and 5.0 ppm F on surface loss of dental hard tissue under erosive and abrasive conditions (all after 28 days).

5.2 *In vitro* model

The present study used an *in vitro* pH cycling model to investigate the effectiveness of different concentrations of fluoridated milk on the surface loss of dental enamel under erosive/ erosive and abrasive challenges. The minerals that are gained (remineralisation) or lost (demineralisation) are proof of the efficacy of such experiments, having been widely accepted and used in many studies (Magalhaes *et al*., 2014; Cassiano *et al*., 2016).

One of the key advantages of the *in vitro* model is the ability to do a single variable experiment under well-controlled conditions which can be easily modified. *In vitro* models were used to facilitate understanding of the demineralisation/ remineralisation process by explaining how fluoride applies its properties. Furthermore, this model enables the use of a wide range of analytical methods for substrate analysis that may not be possible with the *in vivo* model. Other reported advantages of this model are its simple and quick approach, in

addition to it being inexpensive with fewer resources needed (White, 1995; Buzalaf *et al*., 2010).

Nevertheless, there are potential limitations with the *in vitro* models. For example, the difficulty of mimicking the complex biological process involved with tooth surface loss, as the oral environment differs between individuals and changes over time. Additionally, it shows a lack of many significant protective biochemical processes present in the oral environment such as the saliva composition, the salivary flow rate, formation of the salivary pellicle on the enamel surface, which can enhance the remineralisation and reduce the demineralisation process (White, 1992; West *et al*., 1998).

5.3 Study design

This was a randomised, single-blinded (examiner) study *in vitro.* This study comprised two phases: phase A- erosive pH cycling challenge and phase B- erosive pH cycling and abrasive challenge.

Phase A- Erosive challenge: groups 1)- 0 ppm F milk (control group), 2)- 2.5 ppm F milk and 3)- 5.0 ppm F milk.

Phase B- Erosive and abrasive challenges: groups 4)- 0 ppm F milk (control group); 5)- 2.5 ppm F milk, and 6)- 5.0 ppm F milk. In this phase, the enamel slabs were brushed twice per day after treatment using fluoride free toothpaste slurry. Two artificial saliva solutions were used in this study. The first solution

was used in daytime during the pH cycling, between the acid exposures. The second solution was used to store the slabs at night. The study was run for 28 days. Efforts were made to eliminate the risk of bias while the study was being performed. The present study was a single blind randomised study. The enamel slabs were coded during analysis with surface profilometry and the codes were released to the investigator at the end of the study. Given this, the chance of bias arising could be considered low. Furthermore, the ninety enamel slabs in this study were randomly allocated to 6 groups (15 slabs in each group) using an online randomisation engine.

5.4 Bovine teeth

Over past years, the use of bovine teeth has been very popular and has dramatically increased in dental research (Yassen *et al*., 2011). Many remineralisation / demineralisation research studies have used bovine enamel as a substitute for human dental enamel (Magalhaes *et al*., 2014; Cassiano *et al*., 2016).

Human teeth are the most appropriate source of dental substrate that can be used in pH cycling regimes in regard to clinical relevance. However, human teeth composition is variable because of environmental factors, age and genetic impact. Buzalaf *et al*. (2010) reported that these differences can cause large differences in their response under erosive condition. Moreover, obtaining human teeth in large numbers, along with the challenges in regard to supplying them and the

associated cost means the sources of human teeth are very limited for dental research. After reviewing the literature, it was decided that bovine teeth (enamel surface) could be used a best-alternative to human teeth.

They are available and easier to obtain from local abattoirs in large quantities and they have a more uniform composition compared to human teeth. Bovine teeth are mostly free of carious lesions or other enamel defects. Their large flat size makes them easy to handle and process in laboratory experiments as well as their large flat surface area allowing researchers to have several slabs from each single tooth (Laurance-Young *et al*., 2011). Furthermore, there is no difference in the fluoride uptake between etched bovine and human teeth (Yassen *et al*., 2011).

Moreover, it has been documented in research literature that both bovine and human teeth behave similarly under acidic and remineralisation conditions (Attin *et al*., 2007; Costa *et al*., 2015).

However, there is a slight variance between human and bovine teeth in terms of mineral content and enamel porosity. It has been reported that bovine enamel is softer than human enamel (Edmunds *et al*., 1988; Amaechi *et al*., 1999). Nevertheless, some evidence in the literature has reported that these differences are not sufficiently significant to exclude using bovine teeth in dental research as both substrates behaved in the same manner to acidic and remineralisation conditions (Mellberg *et al*., 1992; Laurance-Young *et al*., 2011). Additionally, several *in vitro* studies (Magalhaes *et al*., 2014; Cassiano *et al*., 2016) have

successfully used bovine teeth to evaluate the effect of fluoridated milk against dental erosion.

5.5 Enamel slabs preparation and storage

The enamel slabs in the present study were prepared from the buccal section of bovine incisor teeth as the buccal surface offers flatter surfaces and a more uniform thickness of enamel. Based on the available literature, several agents were used to maintain the structural integrity of extracted teeth and to preserve their baseline conditions, such as formalin, sodium hypochlorite, glutaraldehyde, alcohol and thymol (Dominici *et al*., 2001; Kumar *et al*., 2005; Shellis *et al*., 2011). In the present study, distilled water and 0.1% thymol (Sigma Aldrich) were utilised as a storage medium for the extracted teeth and the enamel slabs later. Thymol has largely been utilised in *in situ* and *in vitro* studies due its ability to prevent bacterial and fungal growth as well as inhibiting enamel slabs' dehydration. Thymol's antimicrobial characteristics have been shown in the literature through its ability to penetrate cell membranes and destroy the pathogens that may grow on tooth surfaces (Shapiro and Guggenheim, 1995).

A few studies have argued that thymol may affect dentine permeability (Preston *et al*., 2007). However, Humel *et al*. (2007) reported that thymol has no effect on dentine permeability, microleakage or bond strength. No detrimental effect on enamel has been reported.

Although the bovine enamel slabs utilised in this study came from different teeth, these slabs were within a standard hardness and porosity to reduce any errors that may have occurred due to natural biological variations. All the chosen enamel slabs had a 60-70 μm indentation length and all were polished so they were flat and within 1.0 μm. Also, all enamel slabs were randomly assigned between the groups.

5.6 pH cycling model with erosive/ erosive and abrasive challenges *in vitro*

The pH cycling model was introduced by Ten Cate and Duijster (1982), aiming to mimic the continuous process of remineralisation and demineralisation in the oral environment. In this present model, the process of tooth surface loss can be simulated by involving alternating demineralisation and remineralisation using protective and erosive agents.

Many *in vitro* studies from the literature used pH cycling regimes to investigate the effect of fluoride on dental erosion (Ren *et al*., 2008; Cassiano *et al*., 2016). Therefore, the role of pH cycling regimes has facilitated a generation of sufficient quantitative data, thereby giving researchers the confidence to appropriately design clinical trials.

The duration of pH cycling models that have been used in published *in vitro* studies was for a limited period and mostly over 14 days (Magalhaes *et al*., 2014).

However, in the present study, a 28-day period of pH cycling was implemented to allow sufficient time to produce changes in the demineralised enamel slabs.

The enamel slabs (Phase A and Phase B) in the current study were dipped in one of the concentrations of F milk (0 (control), 2.5 and 5.0 ppm F milk) for 5 minutes twice daily. Then the enamel slabs were dipped for 10 minutes in a slurry of 1 part of milk and 3 parts of artificial saliva twice daily as an attempt to mimic the dilution of milk by saliva in the oral environment (Magalhaes *et al*., 2014).

The slabs were immersed in an erosive solution of 0.3% citric acid (pH 2.6) for two minutes five times daily for a period of 28 days. On each occasion, before immersion in citric acid, the slabs were taken out of the artificial saliva and rinsed with de-ionised water. The slabs were also rinsed in de-ionised water after treatment before being returned to the artificial saliva, which was changed twice daily.

Several methods to develop erosive lesions on tooth enamel or dentin using different protocols have been discussed in the literature. Several *in vitro* studies have tried to create an erosive lesion by simply immersing teeth slabs into various types of erosive challenges such as citric acid or soft drinks (i.e. orange juice), by using different durations (mostly for prolonged periods). This might provide some useful information on the erosive potential of such kind of products. However, it may exaggerate the potential erosive effects as a result of absence of modifying influence of factors present in the oral environment such as the

salivary pellicle, the buffering capacity of saliva, and saliva remineralisation factor (Hunter *et al*., 2000; Eisenberger and Addy, 2001).

Amaechi *et al*. (1999) used a modified model to develop erosion lesions using an *in vitro* technique. Erosive lesions were created by immersing the teeth continuously in stirred orange juice (20 mL/specimen) for 5 minutes at regular intervals, 6 times a day for a period of 24 days, giving 30 minutes' daily exposure to orange juice at room temperature. The 5 minutes' exposure was determined based on observation in one study that the pH value of saliva and its saturation of calcium and phosphate reverted to the baseline level after a five minutes rinse with citric acid (Bashir and Lagerlof, 1996). They concluded that this technique can be utilised to mimic the condition *in vivo* as well as it being suitable to assess the effects of different parameters on dental erosion.

The protocol used for the present study was similar to the one used by Abdullah (2009). This protocol was developed at the University of Leeds and is a slightly modified version from the method used by Amaechi *et al*. (1999). In the present study, the enamel slabs were immersed in 0.3% citric acid (pH 2.6) for 2 minutes, five times per day for 28 days. The six times dipping for 5 minutes immersion technique used by Amaechi *et al*. (1999) was thought to be an overestimation of the actual situation. Therefore, five times dipping for 2 minutes immersion was used instead.

One limitation of *in vitro* models is the absence of plaque or the overlying pellicle, which acts as a reservoir for mineral deposits and fluoride. These can be later released with a lower plaque pH level as a result of an acidic challenge. However, Koulourides *et al*. (1965) reported that artificial saliva made up to match the ions' concentration present *in vivo* showed a greater potential enamel remineralisation compared to natural saliva taken from several individuals. The supersaturated saliva with respect to tooth mineral provides calcium, phosphate and fluoride ions, which is important for remineralisation. Furthermore, it dilutes and neutralise the acids in addition to providing protein that leads to the formation of acquired pellicle (Buzalaf *et al*., 2012). Peterson *et al*. (2002) highlighted the effect of saliva after ingestion of fluoridated milk, reporting a significant increase in the fluoride level of saliva after ingesting fluoridated milk. Similar results were found by Gedalia *et al*. (1991) who concluded that the impact of saliva can be maximised by the presence of food containing high amounts of phosphate and calcium such as milk. Two artificial saliva solutions were used in this study. The first was used for daytime, between the acid exposures, for a 60-minute minimum period. The second solution was used to store the slabs at night. The daytime artificial saliva was supersaturated with calcium and phosphate, which allowed remineralisation of the enamel slabs. The night saliva was a saturated solution that maintained the enamel condition and did not provide any minerals' exchange.

5.7 Surface Microhardness

In the last three decades, the use of surface microhardness machines has increased due to their ability to evaluate the remineralisation and demineralisation of dental hard tissue. Surface microhardness testing can measure the resistance of enamel surfaces to indenter penetration and is a function of the degree of porosity of the superficial enamel layer. Evidence from the literature has shown microhardness is reliable, non-destructive, quick, simple and easy to use as well as being a sensitive method of monitoring hard tissue dissolution especially at early stages and in describing mineral density changes (Featherstone and Zero, 1992).

Research trials (Jaeggi and Lussi, 1999; Joiner *et al*., 2004) have been conducted to use microhardness techniques to measure the amount of tooth surface loss caused by erosive/abrasive challenges. They have measured the depth of indentation before and after abrasion. However, they were not able to measure the amount of tooth surface loss by the erosive attack as acids caused surface loss in the body of indentation, not only from its surroundings.

In vitro studies published in the literature have applied various loads of the Knoop diamond ranging from 50g up to 500g. The recommended load to be applied is one between 50-200g (Featherstone, 1992). Graig and Peyton (1958) observed that a 50g load can give a well-defined indentation with minimal fractures around the edges. However, Davidson *et al*. (1974) reported that to facilitate optical perceptibility, a 100g load was necessary. For this present study a 100g load was

chosen. The initial surface microhardness of the enamel slabs was measured in order to exclude from the study any slabs with very soft enamel, or slabs where areas with exposed dentine were present.

5.8 Surface Profilometry

In the current study, the measurement of enamel surface loss was represented by differences in height, using unexposed areas as a reference point. Using the profilometry software, the three-point height difference was performed.

Surface profilometry technique has been widely used for *in vitro* studies to assess the erosive potential of different acid challenges (Hughes *et al*., 2000; Cassiano *et al*., 2016). Additionally, it has been used in clinical trial studies. It is a simple, and quick technique that can measure the tooth surface loss of a large area with high precision (Hooper *et al*., 2003).

All tested slabs should be flat prior to using surface profilometry. This is to allow reliable detection of minimal loss even below 1μm (Barbour and Rees, 2004). However, it may result in enamel surface becoming more susceptible to acid dissolution than it would be have under clinical conditions (Meurman and Frank, 1991). In the present study, the main issue was to achieve flat and reproducible enamel surfaces. This step of the study proved time consuming and it was necessary to repeat the surface grinding for some slabs 2-3 times as care was taken not to fully abrade the enamel surface. The enamel slab's surfaces were

covered with nail varnish except for a small window approximately 2x3 mm size in the middle of each slab that was left exposed. After 7, 14, 21, 28 days, the nail varnish was removed. This allow to check the average depth surface loss of the exposed compared to unexposed reference surfaces (Ali, 2012; Abdullah, 2009).

5.9 The effect of fluoridated milk on tooth surface loss

A semi-skimmed milk pasteurised cow's milk (Tesco, UK) was used for this experiment. In the literature, milk has been proven to increase the dental remineralisation process and reduce demineralisation as it contains protein (casein), calcium, phosphate, fat and other trace elements. Comparing bovine milk to human milk, Cow's milk had higher casein, calcium and phosphorus compared to human milk (Hambraues, 1994). The Food Standards Agency (2002) reported that semi-skimmed milk contains higher amounts of calcium compared to whole milk.

Bancozy *et al*. (2013) stated that the bioavailability of added fluoride to milk is shown to be satisfactory in all types of milk consumed (whole, low fat, fresh, pasteurised, sterilised, liquid or powder).

The non-fluoridated milk groups (0 ppm F) were used as the negative controls. The other fluoride concentrations added to milk were (2.5 and 5.0 ppm F). Based on milk fluoridation schemes worldwide, the fluoride concentration usually added to milk ranges between 0.5 and 1.0 mg per day (Bancozy *et al*., 2013). Since a glass of 200 ml milk is offered daily by the community-based milk fluoridation programmes to children, the fluoride concentration in milk typically ranges between 2.5 and 5.0 ppm (Magalhaes *et al*., 2014).

Tooth enamel is largely composed of calcium hydroxyapatite. Once fluoride enters the enamel lattice, it replaces the hydroxyl groups to form fluoro-

hydroxyapatite and fluorapatite, which is reported to have a lower solubility rate at a lower pH than calcium hydroxyapatite. It has been reported that fluorapatite does not dissolve until the pH drops below 4.4 (Tenuta *et al*., 2008).

From the analysis of the bovine enamel slabs used in the current study n=90 (15 slabs/6 groups), there was no loss of slabs with a 100% completion rate for the duration of the study (28 days). It was obvious from the results of the present study that fluoridated milk in all concentrations (0, 2.5 and 5.0 ppm F) showed a dose-dependency effect on tooth surface loss under erosion/erosion and abrasion challenges.

Phase (A) of the present study investigated the effect of fluoridated milk (2.5 and 5.0 ppm F) on tooth surface loss under erosive challenges *in vitro* after 28 days. Non-fluoridated milk was used as a control.

In comparison of the amount (microns) of enamel surface loss between groups, results exhibited that 2.5 ppm F milk and 5.0 ppm F milk groups had a significant ($p < 0.05$) reduction on tooth surface loss compared with the control group (0 ppm F milk). Also, there was a significant reduction in enamel surface loss between 2.5 ppm F milk and 5.0 ppm F milk. The lowest enamel surface loss was achieved by the 5.0 ppm F milk group followed by the 2.5 ppm F milk.

Phase (B) of the current study investigated the effect of fluoridated milk (2.5 and 5.0 ppm F) on tooth surface loss under erosive and abrasive challenges *in vitro*

after 28 days. Non-fluoridated milk was used as a control. Similar findings to phase A was noted. The results exhibited that 2.5 ppm F milk and 5.0 ppm F milk groups had a significant ($p < 0.05$) reduction in enamel surface loss compared with the control group (0 ppm F milk). Also, there was a significant reduction in enamel surface loss between 2.5 ppm F milk and 5.0 ppm F milk. The lowest tooth surface loss was achieved by the 5.0 ppm F milk group followed by the 2.5 ppm F milk.

The results of this study are consistent with a study that was published by Magalhaes *et al*. (2014). They undertook an *in vitro* study following pH cycling for 14 days. They examined the effectiveness of milk containing varying concentrations of fluoride (0, 2.5, 5.0 and 10.0 ppm F) on enamel and dentine surface loss. They found that the addition of fluoride to milk reduced enamel and dentine erosion compared with whole fluoridated free milk and showed a trend of reducing the enamel surface loss as the fluoride concentration increased in milk. However, rinsing with fluoridated-free milk after an erosive challenge did not significantly reduce the enamel erosion. Additionally, there was a negative and significant correlation between fluoride concentration in milk and tooth surface loss.

The findings of the current study are in contrast with an *in vitro* study published by Cassiano *et al*. (2016). Their work examined the protective ability of whole and fat-free fluoridated milk with different concentrations (0, 2.5, 5.0 and 10 ppm

F) applied before or after an acid challenge (citric acid pH 2.5). They reported that for enamel, whole milk containing 10 ppm F led to the lowest tooth enamel surface loss. Additionally, they reported that no dose-response was noted in their study. There are several possible explanations that may have caused these contradictory results. It could be due to the larger number of slabs and 19 treatment groups in their study. Another possible explanation is the length of the study, as their demineralisation/ remineralisation model was only for 5 days compared to 28 days in this present study, which might have reduced the probability of finding a significant difference among them. Additionally, another possible reason could be the time of application of the fluoridated milk, since they only applied it either before or after the erosive challenge, while in our study all fluoridated milk groups were applied before and after the erosive challenge.

5.10 The effect of abrasion on dental erosion

Phase B of the present study used the same protocol that was used in phase A. However, in order to assess the effectiveness of abrasion on dental erosion, all phase B groups (0, 2.5, 5.0 ppm F milk) were subjected to two applications of toothbrushing for 2 minutes each time, after each dipping in fluoridated milk slurry.

The mean surface loss of all groups (0, 2.5 and 5.0 ppm F milk) under erosive and abrasive challenges were significantly higher than similar groups under erosive challenge only.

The findings of the current study are consistent with an *in vitro* study that was published by Eisenburger *et al*. (2003). They reported that the combination of erosion and abrasion challenges resulted in significant differences in enamel surface wear compared with erosive challenge alone. They concluded that eroded enamel is highly unstable and potentially removed by any short and relatively gentle physical action. The finding was in agreement with other previous studies (Davis and Winter, 1980; Eisenburger *et al*., 2000).

It was interesting to investigate the period of remineralisation required to make the enamel more resistant against brushing abrasion, following demineralisation caused by an acidic challenge.

The two times applications of abrasive challenge in the present study was at least 60 minutes before first erosive challenge and last erosive challenge. Similar findings were reported by Attin *et al*. (2006). They carried out an *in vitro* study. Enamel slabs were immersed in an erosive soft drink for 1 minute followed by dipping in artificial saliva for different periods of time (0, 10, 60, and 240 minutes). The slabs were then brushed after one hour using an automated brushing machine. It was reported that even after a one-hour period of remineralisation, the tooth surface loss caused by abrasion of the previously eroded enamel slabs was increased.

5.11 Suggestions for future research

- Due to the limitation of the *in vitro* studies, future *in situ* and *in vivo* studies assessing the remineralising / demineralising potential of different concentration of fluoridated milk are needed in order for the results to be of more clinical value.
- In the present study, artificial saliva was used as an alternative to natural human saliva. As such, future intraoral clinical research involving the use of human saliva to assess the effectiveness of fluoridated milk on human teeth would be of value.
- Even though the results of adding fluoride to milk were significant on reducing enamel surface loss, the pH cycling model used in the current study had a duration of 28 days, which might have not been sufficient

duration to detect further changes caused by remineralisation and demineralisation. Given this, it might be interesting to extend this period in future studies to more than 28 days, such as 3 or 6 months and then compare the findings with the current study's outcomes.

5.12 Problems encountered

This study's sample size contained 90 bovine enamel slabs. To fulfil the strict standardisation criteria of this study many slabs were rejected, mostly as they did not have the required indentation length between 60-70 μm. The process of polishing enamel slabs to ensure that they are flat was very time consuming. Many slabs were polished to the extent that dentin was exposed, therefore they had to be excluded from the current study.

5.13 Null hypothesis

The null hypotheses:

A)- There is no difference in the effect of fluoridated milk (2.5 and 5.0 ppm F) on surface loss of dental hard tissue under erosive condition after 28 days *in vitro.* This null hypothesis can be rejected as significant differences were found in the tooth surface loss reduction between test groups and the control group (0 ppm F milk).

B)- There is no difference in the effect of fluoridated milk (2.5 and 5.0 ppm F) on surface loss of dental hard tissue under erosive and abrasive conditions after 28 days *in vitro.* Again, this null hypothesis can be rejected as significant differences were found in the tooth surface loss reduction after 28 days between test groups and the control group (0 ppm F milk).

6. Conclusion:

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

1- There was evidence of enamel surface loss in all groups (0, 2.5 and 5.0 ppm F milk) under erosive/ erosive and abrasive challenges.

2- A dose-response effect was evident with decreased enamel surface loss as the fluoride dose in milk became higher. The lowest tooth surface loss was achieved by the 5.0 ppm F milk group followed by the 2.5 ppm F milk in both phases of the study.

3- There was evidence of significant reduction in tooth surface loss when comparing the groups (0, 2.5 and 5.0 ppm F milk) under erosive condition after 28 days.

4- There was evidence of significant reduction in tooth surface loss when comparing the groups (0, 2.5 and 5.0 ppm F milk) under erosive and abrasive conditions after 28 days.

5- There was evidence of significant reduction in the enamel surface loss of group 0 ppm F milk after 28 days of erosive challenge compared with group 0 ppm F milk after 28 days of erosive and abrasive challenges.

6- There was evidence of significant reduction in the enamel surface loss of group 2.5 ppm F milk after 28 days of erosive challenge compared with group 2.5 ppm F milk after 28 days of erosive and abrasive challenges.

7- There was evidence of significant reduction in the enamel surface loss of group 5.0 ppm F milk after 28 days of erosive challenge compared with group 5.0 ppm F milk after 28 days of erosive and abrasive challenges.

7. References

- Abdullah, A.Z. 2009. *In vitro and in situ studies to investigate the erosion of human dental tissue*. PhD Thesis, Leeds Dental Institute. Leeds, University of Leeds.

- Abdul-Manaf, Z., Tee, L.M., Hazirah, M.A.N., Selvamary, S., Phor, J.Y., Hasnani, I.N., Ying, Y.B.H., Seng, Y.W. and Asyikin, Y.N. 2012. Relationship between food habits and tooth erosion occurrence in Malaysian university students. *The Malaysian journal of medical sciences: MJMS*, **19**(2), pp.56-63.

- Addy, M. and Shellis, R.P. 2006. Interaction between attrition, abrasion and erosion in tooth wear. In *Dental Erosion.* **20**, pp. 17-31.

- Addy, M., and Hunter M.L. 2003. Can tooth brushing damage your health? Effects on oral and dental tissues. *International Dental Journal*. **53**(86), pp.85-93.

- Agency, F. S. 2002. *McCance and Widdowson's The composition of foods*. Sixth summary edition. Cambridge: Royle Society of Chemistry.

- Agency, F. S. 2008. *Masnual of nutrition*. Eleventh edition. Norvich: TSO.

- Al-Kandari, J, J. 2017. *Evaluation of the remineralisation of enamel by different formulations and concentrations of fluoride toothpastes in vitro*. Doctoral Thesis, Leeds Dental Institute. Leeds, University of Leeds.

- Al-Khateeb, S., Forsberg, C. M., de Josselin de Jong, E. & Angmar-Mansson, B. 1998. A longitudinal laser fluorescence study of white spot lesions in orthodontic patients. *American Journal of Orthodontics & Dentofacial Orthopedics.* **113**(6), pp. 595-602.

- Amaechi, B.T., and Higham, S.M., 2005. Dental erosion: possible approaches to prevention and control. *Journal of dentistry*, **33**(3), pp.243-252.

- Amaechi, B.T., Higham, S.M., and Edgar, W.M. 2003. Influence of abrasion in clinical manifestation of human dental erosion. *J Oral Rehab*. **30**, pp. 407-413.
- Amaechi, B.T., Higham, S.M., Edgar, W.M. 1999A. Techniques for the production of dental eroded lesions in vitro. *Journal of Oral Rehabilitation*. **26**, pp. 97-102.
- Amaechi, B.T., Higham, S.M., Edgar, W.M., and Milosevic, A. 1999b. Thickness of Acquired Salivary Pellicle as a Determinant of the Sites of Dental Erosion. *J Den Res*.**78**, pp. 1821- 1828.

- Arends, J., and ten Bosch, J.J. 1992. Demineralisation and remineralisation evaluation techniques. *Journal of Dental Research*,.**71,** pp.924-928.

- Arnold, W.H., Cerman, M., Neuhaus, K. and Gaengler, P. 2003. Volumetric assessment and quantitative element analysis of the effect of fluoridated milk on enamel demineralisation. *Archives of Oral Biology*. **48**(6). pp, 467–473.

- Arnold, W.H., Heidt, B.A., Kuntz, S. and Naumova, E.A. 2014. Effects of fluoridated milk on root dentin remineralization. *PloS one*. **9**(8), pp, 104-112.

- Attin, T., Buchalla. W., Gollner, M., and Hellwig, E. 2000. Use of variable remineralisation periods to improve the abrasion resistance of previously eroded enamel. *Caries Res*. **34**, pp, 48-52.
- Attin, T., Wegehaupt, F., Gries, D. and Wiegand, A. 2007. The potential of deciduous and permanent bovine enamel as substitute for deciduous and permanent human enamel: Erosion-abrasion experiments. *Journal of Dentistry*. **35**(10), pp. 773-777.

- Austin, A. 2011. *The role of fluoride on erosion, attrition and abrasion of human enamel and dentin in vitro*. Ph.D Thesis, King's college London.
- Bánóczy, J., Rugg-Gunn, A. and Woodward, M. 2013. Milk fluoridation for the prevention of dental caries. *Acta Medica Academica*. **42**(2), pp. 156-167.
- Barbour, M.E. and Rees, J.S. 2004. The laboratory assessment of enamel erosion: a review. *Journal of Dentistry*. **32**, pp. 591-602.
- Bartlett, D. 2005. The implication of laboratory research on tooth wear and erosion. *Oral diseases*. **11**(1), pp.3-6.
- Bartlett, D. and Smith, B.G. 2000. Definition, classification and clinical assessment of attrition, erosion and abrasion of enamel and dentine. *Tooth Wear and Sensitivity. London, Martin Dunitz*. **12**, pp.87-92.
- Bartlett, D.W. 1995. *The relationship between gastro-oesophageal reflux and palatal dental erosion*. Doctoral dissertation, University of London.
- Bartlett, D.W. and Coward, P.Y. 2001. Comparison of erosive potential of gastric juice and a carbonated drink in vitro. *Journal of Oral Rehabilitation.* **28**, pp. 1045-1047.
- Bartlett, D.W. and Shah, P., 2006. A critical review of non-carious cervical (wear) lesions and the role of abfraction, erosion, and abrasion. *Journal of dental research*, **85**(4), pp.306-312.
- Bashir, E. and Lagerlof, F. 1996. Effects of citric acid clearance on the saturation with respect to hydroxyapatite in saliva. *Caries Research*. **30**, pp. 213-217.
- Bataineh, M. 2014. *Comparison of the newer preventive therapies on remineralisation of enamel in vitro*. D.Clin.Dent thesis. University of Leeds.

- Beerens, M., Van Der Veen, M., Van Beek, H. & Ten Cate, J. 2010. Effects of casein phosphopeptide amorphous calcium fluoride phosphate paste on white spot lesions and dental plaque after orthodontic treatment: a 3-month follow-up. *European journal of oral sciences.* **118**, pp. 610-617.

- Bian, J.Y., Wang, W.H., Wang, W.J., Rong, W.S. And Lo, E. 2003. Effect of fluoridated milk on caries in primary teeth: 21-month results. *Community Dentistry and Oral Epidemiology*. **31**(4), pp. 241-245.

- Birkhed, D., Imfeld, T. & Edwardsson, S. 1993. pH changes in human dental plaque from lactose and milk before and after adaptation. *Caries research.* **27**, pp. 43-50.

- Bizhang, M., Riemer, K., Arnold, W.H., Domin, J. and Zimmer, S. 2016. Influence of bristle stiffness of manual toothbrushes on eroded and sound human dentin–an in vitro study. *PloS one*. **11**(4), pp. 145-151.

- Borsboom, P. C., Vd Mei, H. C., and Arends, J. 1985. Enamel lesion formation with and without 0.12 ppm F in solution. *Caries Research.* **19**, pp. 396-402.

- Buzalaf, M.A., Hannas, A.R., Magalhaes, A.C., Rios, D., Honorio, H.M. and Delbem, A.C. 2010. pH cycling models for in vitro evaluation of the efficacy of fluoridated dentifrices for caries control: strengths and limitations. *J Appl Oral Sci.* **18**, pp. 316-34.

- Buzalaf, M.A.R. 2017. Current guidance for fluoride intake is it appropriate? [Online].[13/12/2019]. http:/www.Borrowfoundation.com

- Buzalaf, M.A.R. and Levy, S.M.. 2011. Fluoride intake of children: considerations for dental caries and dental fluorosis. *Fluoride and the oral environment.* **22**, pp. 1-19.

- Buzalaf, M.A.R., Cardoso, C.D.A.B., Magalhães, A.C. and Amaechi, B.T. 2015. Prevention and control of dental erosion: patient self-care. *Dental Erosion and Its Clinical Management.* **14**, (pp. 133-150).

- Buzalaf, M.A.R., Hannas, A.R. and Kato, M.T. 2012. Saliva and dental erosion. *Journal of Applied Oral Science*. **20**(5), pp.493-502.

- Campus, G., Congiu, G., Cocco, F., Sale, S., Cagetti, M.G., Sanna, G., Lingstroem, P. and Garcia-Godoy, F. 2014. Fluoride content in breast milk after the use of fluoridated food supplement. A randomized clinical trial. *American journal of dentistry*. **27**(4), pp.199-202.

- Carvalho, T.S., Baumann, T. and Lussi, A. 2016. In vitro salivary pellicles from adults and children have different protective effects against erosion. *Clinical oral investigations*. **20**(8), pp.1973-1979.

- Cassiano, L.P., Charone, S., Souza, J.G., Leizico, L.C., Pessan, J.P. Magalhães, A.C. and Buzalaf, M.A.R. 2016. Protective Effect of Whole andFat-Free Fluoridated Milk, Applied before or after Acid Challenge, against Dental Erosion. *Caries research*. **50**(2), pp.111-116.

- Centerwall, B.S., Armstrong, C.W., Funkhouser, L.S. and lzay R.P. 1986. Erosion of dental enamel among competitive swimmers at a gas-chlorinated swimming pool. *American Journal of Epidemiology.* **123**(4), pp. 641-647.

- Chana, H. Kelleher, M. Briggs, P. and Hooper, R. 2000. Clinical evaluation of resin-bonded gold alloy veneers. *The Journal of Prosthetic Dentistry.* **83**(3), pp. 294-300.

- Costa, B. M., Iwamoto, A. S., Puppin-Rontani, R. M. & Pascon, F. M. 2015. Comparative Analysis of Root Dentin Morphology and Structure of Human Versus Bovine Primary Teeth. *Microsc Microanal J*. **21**, pp. 689-94.

- Davidson, C.L., Hoekstra, I.S., and Arends, J. 1974. Microhardness of sound, decalcified and etched tooth enamel related to the calcium content. *Caries Researc*h. **8,** pp. 135-144.

- Davies, R. M., Ellwood, R. P. and Davies, G. M. 2003. The rational use of fluoride toothpaste. *Int J Dent Hyg*. **1**(1), pp. 3-8.

- Davis, WB., and Winter, P.J. 1980. The effect of abrasion on enamel and dentine after exposure to dietary acid. *Br Dent J*. **148**, pp. 253-256.
- Dominici, J.T., Eleazer, P.D., Clark, S.J., Staat, R.H., Scheetz, J.P. 2001. Disinfection/sterilisation of extracted teeth for dental student use. *J Dent Educ*. **65**(11), pp. 1278-1280.

- Dreisbach, R. H. 1980. *Handbook of poisoning:prevention,diagnosis and treatment.*, Los Altos, CA: Lange Medical Publishers.

- Edmunds, D. H., Whittaker, D. K. & Green, R. M. 1988. Suitability of human, bovine, equine, and ovine tooth enamel for studies of artificial bacterial carious lesions. *Caries Res*. **22**, pp. 327-36.

- Eisenburger M, Hughes J, West NX, Jandt KD, Addy M. 2000. Ultrasonication as a method to study enamel demineralisation during acid erosion. *Caries Res.* **34**, pp.289–294.

- Eisenburger, M., and Addy, M. 2001. Evaluation of pH and erosion time on demineralisation. *Clin Oral Invest*. **5**, pp. 108-111.
- Eisenburger, M., and Shellis, RP., and Addy, M. 2003. Comparative study of wear of enamel induced by alternating and simultaneous combinations of abrasion and erosion in vitro. *Caries Res*. **37**, pp. 450-455.

- Espelid, I. 2009. Caries preventive effect of fluoride in milk, salt and tablets: a literature review. *European Archives of Paediatric Dentistry: Official Journal of the European Academy of Paediatric Dentistry*. **10**, pp. 149-56.

- Featherstone, I.D. 2000. The science and practice of caries prevention. *The Journal of the American Dental Association*. **131**, pp. 887-899.

- Featherstone, J. 1996. Modelling the caries-inhibitory effects of dental materials. *Dental Materials*. **12**, pp. 194-197.

- Featherstone, J.D. 1992. Consensus conference on intra-oral models: evaluation techniques. *Journal of Dental Research*. **71**, pp. 955-956.
- Featherstone, J.D., and Zero, D.T. 1992. An in situ model for simultaneous assessment of inhibition of demineralisation and enhancement of remineralisation. *Journal of Dental Research*. **71**, pp. 804-810.
- Featherstone, J.D.B. and Lussi, A. 2006. Understanding the chemistry of dental erosion. In *Dental erosion*. **20**, pp. 66-76.
- Featherstone, J.D.B. and Rodgers, B.E. 1981. Effect of acetic, lactic and other organic acids on the formation of artificial carious lesions. *Caries research*, **15**(5), pp.377-385.

- Fejerskov, O., Ekstrand, J. and Burt, B. A. 1996. *Fluoride in dentistry*, Copenhagen: Munksgaard.

- Ganss, C. 2006. Definition of erosion and links to tooth wear. In: Dental Erosion from Diagnosis to Therapy. A Lussi editor. Basel: Karger.

- Gedalia, I., Dakuar, A., Shapira, L., Lewinstein, I., Goultschin, J. and Rahamim, E. 1991. Enamel softening with Coca-Cola and rehardening with milk or saliva. *American journal of dentistry*. **4**(3), pp.120-122.

- Giacaman, R.A., Munoz, M.J., Ccahuana-Vasquez, R.A., Munoz-Sandoval, C. and Cury, J.A. 2012. Effect of fluoridated milk on enamel and root dentin demineralisation evaluated by a biofilm caries model. *Caries research*. **46**(5), pp.460-466.

- Gilmour, A.G., and Beckett, H.A. 1993. The voluntary reflux phenomenon. *British Dental Journal*. **175**(10), pp. 368-372.

- Giunta, J.L. 1983. Dental erosion resulting from chewable vitamin C tablets. *Journal of American Dental Association*. **107**, pp. 253-256.

- Graig, R.G. and Peyton, F.A. 1958. The microhardness of enamel and dentin. *Journal of Dental Research*. **37**, pp. 661-668.

- Hambraeus, L. 1994. Milk composition in animals and humans: Nutritional aspects. Dairy products in human health and nutrition. Proceedings of the 1st World Congress, Searrano Rios et al.eds. Rotterdam, A.A. Balkema. *In: pp.* 13-23

- Hanein, A. 2012. *The effects of smoothies on enamel erosion: an in situ study*. Doctoral Thesis, Leeds Dental Institute. Leeds, University of Leeds.

- Hannig, C., Hannig, M., and Attin, T. 2005. Enzymes in the acquired enamel pellicle. *European Journal of Oral Science*. **113**, pp. 2-13.

- Hannig, M. and Joiner, A. 2006. The structure, function and properties of the acquired pellicle. *In The teeth and their environment.* **19**, pp. 29-64.

- Hara, A.T. and Zero, D.T. 2014. The potential of saliva in protecting against dental erosion. *In Erosive Tooth Wear*. **25**, pp. 197-205.

- Hara, A.T., Ando, M., Gonzalez-Cabezas, C, Cury, JA., Serra, M.C., and Zero, D.T. 2006. Protective effect of the dental pellicle against erosive challenges in situ. *Journal of Dental Research*. **85**(7), pp. 612-616.

- Heifetz, S. B. and Horowitz, H. S. 1986. Amounts of fluoride in self-administered dental products: safety considerations for children. *Pediatrics.* **77**, pp. 876-82.

- Hellwig, E. and Lussi, A. 2014. Oral hygiene products, medications and drugs-hidden aetiological factors for dental erosion. *In Erosive Tooth Wear*. **25**, pp. 155-162.

- Hooper, S., Seong, J., Macdonald, E., Claydon, N., Hellin, N. and Barker, M.L. 2014. A randomised in situ trial, measuring the anti-erosive properties of a stannous-containing sodium fluoride dentifrice compared with a sodium fluoride/potassium nitrate dentifrice. *Int Dent J.* **64** (1), pp. 35-42.

- Hooper, S., West, N.X., Pickles, M.J., Joiner, A., Newcombe, R.G., Eisenburger, M., and Addy M. 2003. Investigation of erosion and abrasion on enamel and dentine:a model in situ using toothpastes of different abrasivity. *Journal of Clinical Periodontology*. **30**, pp. 802-808.
- Hughes, J.A., West, N.X., Parker, D.M., van den Braak, M.H., and Addy, M. 2000. Effects of pH and concentration of citric, malic and lactic acids on enamel, in vitro. *Journal of Dentistry*. **28**, pp. 147-155.
- Humel, M.M.C., Oliveira, M.T., Cavalli, V. and Giannini, M., 2007. Effect of storage and disinfection methods of extracted bovine teeth on bond strength to dentin. *Brazilian Journal of Oral Sciences*. **6**, pp. 1402–1406.

- Hunter, M.L., Addy, M., Pickles, M.J. and Joiner, A. 2002. The role of toothpastes and toothbrushes in the aetiology of tooth wear. *International dental journal*. **52**(S5), pp.399-405.

- Hunter, ML, West, NX, Hughes, J.A., Newcombe, R.G., and Addy M. 2000. Erosion of deciduous and permanent dental hard tissue in the oral environment. *J Dent*. **28**, pp. 257-263.

- Imfeld, T. 1996. Prevention of progression of dental erosion by professional and individual prophylactic measures. *European Journal of Oral Sciences*. **104**(2), pp.215-220.

- Jaeggi, T. and Lussi, A. 1999. Toothbrush abrasion of erosively altered enamel after intraoral exposure to saliva: An in situ study. *Caries Res*. **33**, pp. 455-461.

- Joiner, A., Pockles, M.J., Tanner, C., Weader, E., and Doyle, P. 2004. An in situ model to study the toothpaste abrasion of enamel. *Journal of Clinical Periodontology*. **31**, pp. 434- 438.

- Jones, R., and Lydeard, S. 1989. Prevalence of symptoms of dyspepsia in the community. BMJ. **298**, pp. 30-32.

- Koulourides, T. 1971. The challenge of prevention in dentistry. *Alabama Journal Medical Sciences*. **8**(3), pp. 369-371

- Koulourides, T., Feagin, F. & Pigman, W. 1965. Remineralization of dental enamel by saliva in vitro. *Annals of the New York Academy of Sciences.* **131**, pp. 75.

- Kumar, M., Sequeira, P.S., Peter, S., and Bhat, G.K. 2005. Sterilisation of extracted human teeth for educational use. *Indian J. Med. Microbiol*. **23**(4), pp. 25-68.

- Laurance-Young, P., Bozec, L., Gracia, L., Rees, G., Lippert, F., Lynch, R. J. M. and Knowles, J. C. 2011. A review of the structure of human and bovine dental hard tissues and their physicochemical behaviour in relation to erosive challenge and remineralisation. *Journal of Dentistry*. **39** (4), pp. 266-272.

- Lindquist, B., Lingström, P., Fändriks, L. and Birkhed, D. 2011. Influence of five neutralizing products on intra-oral pH after rinsing with simulated gastric acid. *European journal of oral sciences*. **119**(4), pp.301-304.

- Lippert, F., Arrageg, M.A., Eckert, G.J. and Hara, A.T., 2017. Interaction between toothpaste abrasivity and toothbrush filament stiffness on the development of erosive/abrasive lesions in vitro. *International dental journal*, **67**(6), pp.344-350.

- Lussi, A., and Hellwig, E. 2001. Erosive potential of oral care products. *Caries Research*. **35**, pp. 52-56.

- Lussi, A., and Jaeggi, T. 2006. *Chemical factors. In: Dental Erosion from Diagnosis to Therapy*. A Lussi editor. Basel: Karger.

- Lussi, A., Buzalaf, M.A.R., Duangthip, D., Anttonen, V., Ganss, C., João-Souza, S.H., Baumann, T. and Carvalho, T.S. 2019. The use of fluoride for the prevention of dental erosion and erosive tooth wear in children and adolescents. *European archives of paediatric dentistry*. **20**(6), pp.517-527.

- Magalhães, A. C., Levy, F. M., Souza, B. M., Cardoso, C. A. B., Cassiano, L. P., Pessan, J. P., and Buzalaf, M. A. R. 2014. Inhibition of tooth erosion by milk containing different fluoride concentrations: An in vitro study. *Journal of dentistry*. **42**(4), pp. 498-502.

- Malinowski, M. 2010. *In situ studies with fluoridated milk for the remineralisation of dental enamel.* Ph.D thesis. University of Leeds.

- Malinowski, M., Duggal, M.S., Strafford, S.M. and Toumba, K.J. 2012. The effect on dental enamel of varying concentrations of fluoridated milk with a cariogenic challenge in situ. *Journal of dentistry*. **40**(11), pp.929-933.
- Malinowski, M., Duggal, M.S., Strafford, S.M. and Toumba, K.J. 2012. The effect of varying concentrations of fluoridated milk on enamel remineralisation in vitro. *Caries research*. **46**(6), pp.555-560.
- Marinho, V. C. C., Higgins, J. P. T., Logan, S. and Sheiham, A. 2003. Fluoride mouthrinses for preventing dental caries in children and adolescents. *Cochrane Database of Systematic Reviews*, CD002284.
- Marinho, V. C. C., Higgins, J. P. T., Sheiham, A. and Logan, S. 2004. Combinations of topical fluoride (toothpastes, mouthrinses, gels, varnishes) versus single topical fluoride for preventing dental caries in children and adolescents. *Cochrane Database of Systematic Reviews*, CD002781.
- Marino, R., Villa, A., Weitz, A. and Guerrero, S. 2004. Prevalence of fluorosis in children aged 6-9 years-old who participated in a milk fluoridation programme in Codegua, Chile. *Community Dental Health.* **21**, pp. 143-148.
- Martínez-Mier, E.A. 2012. Fluoride: its metabolism, toxicity, and role in dental health. *Journal of Evidence-Based Complementary & Alternative Medicine*. **17**(1), pp.28-32.
- Mcdonagh, M. S., Whiting, P. F., Wilson, P. M., Sutton, A. J., Chestnutt, I., Cooper, J., Misso, K., Bradley, M., Treasure, E. and Kleijnen, J. 2000. Systematic review of water fluoridation. *BMJ*. **321**, pp.855-9.
- Mellberg, J.R. 1992. Hard-tissue substrates for evaluation of cariogenic and antiicariogenic activity in situ. *J Dent Res*. **71**, pp. 913-919.

- Meurman, J. and Frank, R. 1991. Scanning electron microscopic study of the effect of salivary pellicle on enamel erosion. *Caries Research*. **25**, pp. 1-6.

- Meurman, J.H. and Frank, R.M. 1991. Progression and surface ultrastructure of in vitro caused erosive lesions in human and bovine enamel. *Caries research*. **25**(2), pp.81-87.

- Milosevic, A, Agrawal, N., Redfearn, P., Mair, L. 1999. The occurrence of toothwear in users of Ecstasy (3,4-methylenedioxymethamphetamine). *Community Dentistry & Oral Epidemiology*. **27**(4), pp. 283-287.

- Milosevic, A., and Slade, P.D. 1989. The orodental status of anorexics and bulimics. *British Dental Journal*. **167**, pp. 66-70.

- Milosevic, A., Bardsley, P.F., and Taylor, S. 2004. Epidemiological studies of tooth wear and dental erosion in 14-year old children in North West England. Part 2: The association of diet and habits. *British Dental Journal*. **197**(8), pp. 479-483.

- Milosevic, A., Brodie, D.A., and Slade, P.D. 1997. Dental erosion, oral hygiene, and nutrition in eating disorders. *International Journal of Eating Disorders*. **21**(2), pp. 195-199.

- MOYNIHAN, P. 2000. Foods and factors that protect against dental caries. *Nutrition Bulletin*. **25**, pp. 281-286.

- Nahas Pires Correa, J.P., Murakami, C. and Mendes, F.M. 2011. Prevalence and associated factors of dental erosion in children and adolescents of a private dental practice. *International Journal of Paediatric Dentistry*. **21**(6), pp.451-458.

- Nunn, J.H. 2000. *Prevalence and distribution of tooth wear*. *Tooth Wear and Sensitivity*. London, Martin Dunitz.Office of National Statistics. 1994. Children's Dental Health in the United Kindgom 1993. London, Office of National Statistics.
- Office of National Statistics. 2015. Children's Dental Health in the United Kindgom 2013. London, Office of National Statistics.
- O'Sullivan, E.A., and Milosevic, A. 2007. *Diagnosis, prevention and management of dental erosion*: Faculty of Dental Surgery of the Royal College of Surgeons of England. London.
- Pandey, P., Reddy, N.V., Rao, V.A.P., Saxena, A. and Chaudhary, C.P. 2015. Estimation of salivary flow rate, pH, buffer capacity, calcium, total protein content and total antioxidant capacity in relation to dental caries severity, age and gender. *Contemporary clinical dentistry*. **6**(1), pp. 65.
- Petersen, P.E., and Gormsen, C. 1991. Oral conditions among german battery factory workers. *Community Dental Oral Epidemiology.* **19**, pp. 104-106.
- Peterson, L.G., Arvidsson, I., Lynch, E., Engstrom, K. and Twetman, S. 2002. Fluoride Concentrations in Saliva and Dental Plaque in Young Children after Intake of Fluoridated Milk. *Caries Research*. **36**(1), pp. 40-43.
- Poulsen, S. 2009. Fluoride-containing gels, mouth rinses and varnishes: an update of evidence of efficacy. *European Archives of Paediatric Dentistry: Official Journal of the European Academy of Paediatric Dentistry.* **10**, pp. 157-61.

- Preston, K., Higham, S. And Smith, P. 2007. The efficacy of techniques for the disinfection of artificial sub-surface dentinal caries lesions and their effect on demineralisation and remineralisation in vitro. *Journal of dentistry*. **35**, pp. 490-495.

- Ramalingam, L., Messer, L. & Reynolds, E. 2005. Adding casein phosphopeptide-amorphous calcium phosphate to sports drinks to eliminate in vitro erosion. *Pediatric dentistry.* **27**, pp. 61-67.

- Ranjitkar, S., Kaidonis, J. A., Richards, L. C. & Townsend, G. C. 2009. The effect of CPP–ACP on enamel wear under severe erosive conditions. *Archives of oral biology.* **54**, pp. 527-532.

- Ren, Y.F., Liu, X, Fadel, N., Malmstrom, H., Barnes, V., and Xu, T. 2011. Preventive effects of dentifrice containing 5000 ppm fluoride against dental erosion in situ. *J Dent.* **39**, pp. 672-678.

- Ren, Y.F., Zhao, Q., Malmstrom, H., Barnes, V. and Xu, T. 2009. Assessing fluoride treatment and resistance of dental enamel to soft drink erosion in vitro: applications of focus variation 3D scanning microscopy and stylus profilometry. *journal of dentistry*. **37**(3), pp.167-176.

- Reynolds, E. 2009. Casein phosphopeptide-amorphous calcium phosphate: the scientific evidence. *Advances in Dental Research.* **21**, pp. 25-29.

- Riley, J. C., Klause, B. K., Manning, C. J., Davies, G. M., Graham, J. and Worthington, H. V. 2005. Milk fluoridation: a comparison of dental health in two school communities in England. *Community Dental Health,* **22**, pp.141-5.

- Robb, N.D., and Smith, B.G.N. 1990. Prevalence of pathological tooth wear in patients with chronic alcoholism. *British Dental Journal*. **169**, pp. 367-369.

- Rusoff, L. L., Konikoff, B. S., Frye, J. B., Jr., Johnston, J. E. and Frye, W. W. 1962. Fluoride addition to milk and its effect on dental caries in schoolchildren. *American Journal of Clinical Nutrition.* **11**, pp. 94-101.

- Salas, M.M.S., Nascimento, G.G., Vargas-Ferreira, F., Tarquinio, S.B.C., Huysmans, M.C.D.N.J.M. and Demarco, F.F. 2015. Diet influenced tooth erosion prevalence in children and adolescents: Results of a meta-analysis and meta-regression. *Journal of dentistry*. **43**(8), pp.865-875.

- Şener, Y., Tosun, G., Kahvecioğlu, F., Gökalp, A. and Koc, H. 2007. Fluoride levels of human plasma and breast milk. *European journal of dentistry*. **1**(01), pp. 021-024.

- Shapiro, S. and Guggenheim, B. 1995. The action of thymol on oral bacteria. *Oral microbiology and immunology*. **10**, pp. 241-246.
- Shellis, R.P., Ganss, C., Ren, Y., Zero, D.T., and Lussi, A. 2011. Methodology and models in erosion research: discussion and conclusions. *Caries Res*.**45**(1), pp. 69-77.
- Sköld-Larsson, K., Sollenius, O., Karlsson, L., Petersson, L.G. and Twetman, S. 2013. Effect of fluoridated milk on enamel demineralisation adjacent to fixed orthodontic appliances. *Acta Odontologica Scandinavica*. **71**(3-4), pp.464-468.

- Smith, B.G.N., and Knight, J.K. 1984. A comparison of patterns of tooth wear with aetiological factors. *British Dental Journal.* **157**, pp. 16-19.

- Soi, S., Vinayak, V., Singhal, A. and Roy, S., 2013. Fluorides and their role in demineralization and remineralization. J Dent Sci Oral Rehabil. **4**, pp.7-10.

- Somani, R., Jaidka, S., Singh, D.J. and Arora, V. 2014. Remineralizing potential of various agents on dental erosion. *Journal of oral biology and craniofacial research*. **4**(2), pp.104-108.

- Spak, C. J., Ekstrand, J. and Zylberstein, D. 1982. Bioavailability of fluoride added by baby formula and milk. *Caries Research.* **16**(3), pp. 249-256.

- Spak, C. J., Ekstrand, J., Erricsson, S. and Leksell, L. J. 1986. Distribution of fluoride to the central nervous system. *Caries Research.* **20** (2), pp. 157-162.

- Srinivasan, N., Kavitha, M. and Loganathan, S.C. 2010. Comparison of the remineralization potential of CPP–ACP and CPP–ACP with 900 ppm fluoride on eroded human enamel: an in situ study. *Archives of oral biology*, **55**(7), pp.541-544.

- Stecksén-Blicks, C., Sjöström, I. and Twetman, S. 2009. Effect of long-term consumption of milk supplemented with probiotic lactobacilli and fluoride on dental caries and general health in preschool children: a cluster-randomized study. *Caries research*. **43**(5), pp.374-381.

- Stephen, K. W., Boyle, I. T., Campbell, D., McNee, S. and Boyle, P. 1984. Five-year double-blind fluoridated milk study in Scotland. *Community Dentistry & Oral Epidemiology*. **12**, pp. 223-229.

- Stephen, K.W., Banoczy, J., Pakhomov, G.N. and World Health Organization. 1996. *Milk fluoridation for the prevention of dental caries* (No. WHO/ORH/MF/DOC96. 1). Geneva: World Health Organization.

- Sundaram, G., Bartlett, D.W., and Watson, T. 2004. Bonding to and protecting worn palatal surfaces of teeth with dentine bonding agents. *Journal of Oral Rehabilitation.* **31**(5), pp. 505-509.

- Takagi, S., Liao, H. and Chow, L. C. 2000. Effect of tooth-bound fluoride on enamel demineralization/ remineralization in vitro. *Caries Research*, **34**, pp. 281-8.

- Ten Bosch, J. and Ngmar-Mansson, B. 1991. Invited Review: A Review of Quantitative Methods for Studies of Mineral Content of Intra-oral Incipient Caries Lesions. *Journal of Dental Research.* **70**, pp. 2-14.

- Ten Cate, J. M., and Duisters, P. P. E. 1982. Alternating demineralisation and remineralisation of artifial lesions. *Caries Research*. **16**, pp. 201-210.

- Ten Cate, J., Exterkate, R. And Buijs, M. 2006. The relative efficacy of fluoride toothpastes assessed with pH cycling. *Caries research*. **40**, pp.136-141

- Ten Cate, J.M., Larsen, M.J., Pearce, E., and Fejerskov, O. 2008. *Chemical interactions between the tooth and oral fluids. Dental caries. The Disease and Its clinical Management*. Copenhagen, Blackwell Munksgaard.

- Torres, E., Oliva, M., Lecannelier, B., TORRES, E., OLIVA, M. and Lecannelier, B. 2016. Efficacy of Milk Fluoride Prevention of Dental Caries in Children Under 12 Years Old: A Review. *Int. j. odontostomatol.* **12**, pp.197-206.

- Twetman, S. 2009. Caries prevention with fluoride toothpaste in children: an update. European Archives of Paediatric Dentistry: *Official Journal of the European Academy of Paediatric Dentistry*. **10**, 162-167.

- Viswanathan, G., Jaswanth, A. and Gopalakrishnan, S. 2009. Mapping of fluoride endemic areas and assessment of fluoride exposure. *Science of the total environment*. **407**(5), pp.1579-1587.

- Voronets, J. and Lussi, A. 2010. Thickness of softened human enamel removed by toothbrush abrasion: an in vitro study. *Clinical oral investigations*. **14**(3), pp.251-256.

- Warren, J.J., Levy, S.M., Broffitt, B., Cavanaugh, J.E., Kanellis, M.J. and Weber-Gasparoni, K. 2009. Considerations on optimal fluoride intake using dental fluorosis and dental caries outcomes–a longitudinal study. *Journal of public health dentistry*. **69**(2), pp.111-115.

- Weatherell, J. A., Deutsch, D., Robinson, C. & Hallsworth, A. S. 1977. Assimilation of fluoride by enamel throughout the life of the tooth. *Caries Research.* **11**(1), pp. 85-115.

- West, N., Maxwell, A., Hughes, J., Parker, D., Newcombe, R. And Addy, M. 1998. A method to measure clinical erosion: the effect of orange juice consumption on erosion of enamel. *Journal of Dentistry*. **26**, pp. 329-335.

- West, N.X., Sanz, M., Lussi, A., Bartlett, D., Bouchard, P. and Bourgeois, D. 2013. Prevalence of dentine hypersensitivity and study of associated factors: a European population-based cross-sectional study. *Journal of dentistry*. **41**(10), pp.841-851.

- Whelton, H.P., Ketley, C.E., McSweeney, F. and O'Mullane, D.M. 2004. A review of fluorosis in the European Union: prevalence, risk factors and aesthetic issues. *Community dentistry and oral epidemiology*. ***32***, pp.9-18.

- White D. 1995. The application of *in vitro* models to research on demineralisation and remineralisation of the teeth. *Adv Dent Res.* **9**, pp.175-93.

- White, D., Nelson, D. and Faller, R. 1994. Mode of action of fluoride: application of new techniques and test methods to the examination of the mechanism of action of topical fluoride. *Advances in Dental Research*. **8,** pp.166-174

- White, D.J. 1992. The comparative sensitivity of intra-oral, in vitro, and animal models in the "profile" evaluation of topical fluorides. *J. Dent. Res*. **71**, pp. 884-894.

- Whitford, G. M. 1987. Fluoride in dental products: safety considerations. *Journal of Dental Research*. **66**, pp. 1056-60.

- Whitford, G. M. 1990. The physiological and toxicological characteristics of fluoride. *J. Dent. Res.* **69**, pp. 539-549.

- Whitford, G.M. 2011. Acute toxicity of ingested fluoride. *Monogr. Oral. Sci*. **22**, pp. 66-80.

- Whittaker, D.K. 2000. Historical and forensic aspects of tooth wear. Tooth wear and Sensitivity - clinical advances in restorative dentistry. M Addy, G Embery, WM Edgar and R Orchardson editors. London: Martin Dunitz.

- Wiegand, A. and Attin, T. 2014. Randomised in situ trial on the effect of milk and CPP-ACP on dental erosion. *Journal of dentistry*. **42**(9), pp.1210-1215.

- Wiegand, A., Kuhn, M., Sener, B., Roos, M. and Attin, T. 2009. Abrasion of eroded dentin caused by toothpaste slurries of different abrasivity and toothbrushes of different filament diameter. *Journal of dentistry*. **37**(6), pp.480-484.

- Wiegand, A., Schwerzmann, M., Sener, B., Carolina Magalhães, A., Roos, M., Ziebolz, D., Imfeld, T. and Attin, T. 2008. Impact of toothpaste slurry abrasivity and toothbrush filament stiffness on abrasion of eroded enamel–an in vitro study. *Acta Odontologica Scandinavica*. **66**(4), pp.231-235.

- World Health Organisation. 1992. Application of the international classification of diseases to dentistry and stomatology, ICD-DA Geneva.

- Yassen , G. H., Platt, J. A. and Harra, A. T. 2011. Bovine teeth as substitute for human teeth in dental research: a review of literature. *Journal of oral science*. **53**, pp. 273-282.

- Zero, D. T. 1995. In situ caries models. *Advances in Dental Research.* **9**, pp. 214-230.

- Zero, D.T., and Lussi, A. 2005. Erosion. Chemical and biological factors of importance to the dental practitioner. *International Dental Journal*. **55**, pp. 285-290.

- Ziegler, E. 1953. Caries prevention through fluoridation of milk. *Schweizerische Medizinische Wochenschrif.* **83**, pp.723-724.

- Ziegler, E. 1959. Basic data from the large -scale milk-fluoridationexperiment in winterthur. *Mitteilungen der Naturewissenschaftlichen Gesellschaft Winterthur*. **29**, pp. 139-156.

8. Appendices

Appendix I: Means of enamel surface loss (µm) for group 0 ppm F milk after 7, 14, 21, and 28 days erosive challenge.

Group 1 (0 ppm F)	MEAN after 7 days	MEAN after 14 days	MEAN after 21 days	MEAN after 28 days
slab 1	4.530	8.773	10.293	16.409
slab 2	4.837	8.985	12.346	17.263
slab 3	6.630	9.110	14.075	16.185
slab 4	6.481	10.119	11.988	15.530
slab 5	4.213	5.240	9.721	13.195
slab 6	4.911	8.651	12.067	16.900
slab 7	4.568	8.058	11.690	13.248
slab 8	4.160	8.271	9.020	13.392
slab 9	4.930	9.380	11.700	16.802
slab 10	5.402	8.270	11.529	17.420
slab 11	3.996	7.185	11.134	13.237
slab 12	3.405	8.285	9.409	16.057
slab 13	4.480	7.022	11.886	14.602
slab 14	4.166	8.104	9.753	15.075
slab 15	6.008	8.398	12.647	16.230
Mean of group	4.848	8.257	11.284	15.433
SD	0.92	1.14	1.39	1.55

Appendix II: Means of enamel surface loss (µm) for group 2.5 ppm F milk after 7, 14, 21, and 28 days erosive challenge.

Group 2. (2.5 ppm F)	MEAN after 7 days	MEAN after 14 days	MEAN after 21 days	MEAN after 28 days
slab 16	4.320	7.617	8.541	12.635
slab 17	3.106	6.263	9.152	12.080
slab 18	3.656	6.315	9.994	11.758
slab 19	3.282	5.672	7.780	11.679
slab 20	3.627	4.783	9.461	13.859
slab 21	5.820	9.612	12.514	15.640
slab 22	3.508	6.981	8.103	11.985
slab 23	3.047	5.724	8.654	11.635
slab 24	3.493	6.948	9.746	11.957
slab 25	3.931	6.933	9.285	11.544
slab 26	3.638	7.555	9.242	11.975
slab 27	5.412	8.038	9.677	11.776
slab 28	5.377	8.314	10.674	13.920
slab 29	3.595	6.412	8.951	9.235
slab 30	5.237	8.035	11.004	11.022
mean of group	4.070	7.014	9.518	12.180
SD	0.93	1.23	1.20	1.45

Appendix III: Means of enamel surface loss (µm) for group 5.0 ppm F milk after 7, 14, 21, and 28 days erosive challenge.

group 3. (5.0 ppm F)	MEAN after 7 days	MEAN after 14 days	MEAN after 21 days	MEAN after 28 days
slab 31	4.516	6.444	7.165	9.027
slab 32	3.978	6.824	8.231	9.873
slab 33	3.205	6.361	8.305	9.461
slab 34	2.699	3.935	6.131	6.172
slab 35	1.528	5.274	6.227	7.931
slab 36	3.943	4.830	6.780	7.854
slab 37	3.571	4.468	7.400	7.826
slab 38	3.817	5.539	7.991	8.617
slab 39	4.246	5.773	6.968	8.726
slab 40	4.164	5.154	9.535	8.769
slab 41	3.244	3.829	6.328	6.542
slab 42	2.803	4.593	7.836	8.933
slab 43	3.373	4.378	7.613	8.105
slab 44	3.902	4.942	7.226	7.971
slab 45	2.334	2.581	5.623	7.187
Mean of group	3.422	4.995	7.291	8.200
SD	0.81	1.12	1.01	1.02

Appendix IV: Means of enamel surface loss (µm) for group 0 ppm F milk after 7, 14, 21, and 28 days erosive and abrasive challenges.

0 ppm F milk (with brushing)	MEAN after 7 days	MEAN after 14 days	MEAN after 21 days	MEAN after 28 days
slab 46	3.879	8.562	17.689	20.429
slab 47	5.000	11.219	18.636	22.388
slab 48	4.926	10.204	15.803	22.955
slab 49	6.349	15.168	20.099	22.246
slab 50	4.076	9.743	15.052	18.257
slab 51	6.280	11.521	14.464	20.520
slab 52	5.833	7.950	13.480	17.472
slab 53	6.631	10.862	17.466	21.403
slab 54	4.539	10.260	17.518	18.625
slab 55	6.603	12.240	20.311	25.399
slab 56	3.633	10.008	18.504	22.511
slab 57	6.549	8.208	10.308	15.233
slab 58	7.883	9.237	18.064	23.532
slab 59	4.913	9.402	20.125	21.758
slab 60	3.775	11.552	15.118	21.079
Mean of group	5.391	10.409	16.842	20.921
SD	1.30	1.83	2.79	2.61

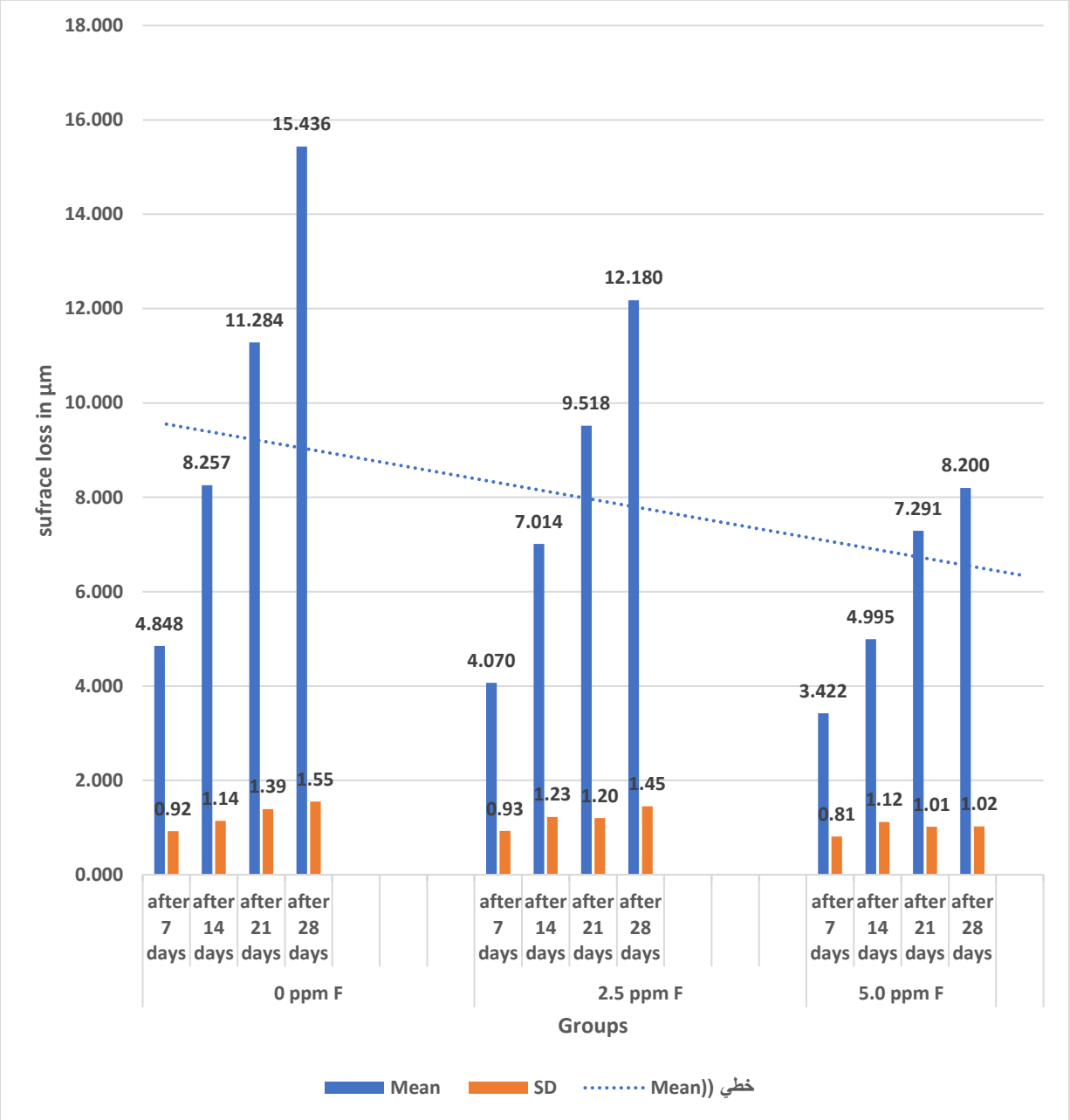
Appendix V: Means of enamel surface loss (in microns) for group 2.5 ppm F milk after 7, 14, 21, and 28 days erosive and abrasive challenges.

2.5 ppm F milk. (with brushing	MEAN after 7 days	MEAN after 14 days	MEAN after 21 days	MEAN after 28 days
slab 61	5.691	10.286	15.390	18.594
slab 62	5.229	9.194	13.243	15.012
slab 63	4.557	9.262	14.156	17.253
slab 64	6.029	10.330	17.104	18.026
slab 65	4.493	9.571	13.741	15.506
slab 66	4.152	8.482	10.619	19.236
slab 67	5.721	9.944	11.351	17.713
slab 68	5.735	9.100	12.576	19.266
slab 69	4.134	8.290	11.601	17.483
slab 70	6.715	10.630	16.033	19.785
slab 71	3.873	8.034	11.795	15.739
slab72	5.384	9.073	14.221	17.650
slab 73	4.266	9.434	13.825	16.496
slab 74	4.122	8.573	11.511	13.982
slab 75	3.625	6.233	10.863	16.953
Mean of group	4.915	9.096	13.202	17.246
SD	0.93	1.10	1.96	1.67

Appendix VI: Means of enamel surface loss (µm) for group 5.0 ppm F milk after 7, 14, 21, and 28 days erosive and abrasive challenges.

5.0 ppm F milk (with brushing)	MEAN after 7 days	MEAN after 14 days	MEAN after 21 days	MEAN after 28 days
slab 76	4.627	9.812	12.235	17.227
slab 77	3.168	6.479	10.808	11.001
slab 78	4.819	6.028	10.224	15.260
slab 79	4.388	6.305	10.829	12.650
slab 80	4.921	15.516	16.447	18.530
slab 81	3.084	6.055	8.409	11.250
slab 82	5.326	7.380	9.696	12.150
slab 83	3.493	7.099	11.464	11.150
slab 84	5.055	6.422	12.134	15.136
slab 85	5.352	8.86	10.133	17.146
slab 86	7.817	10.673	14.023	18.245
slab 87	4.503	8.807	12.484	17.630
slab 88	4.496	5.217	9.465	12.890
slab 89	4.970	6.927	9.024	13.815
slab 90	4.663	8.392	9.682	13.622
Mean of group	4.712	7.998	11.137	14.513
SD	1.11	2.59	2.10	2.70

Appendix VII: The effect of different concentrations of fluoride milk (0, 2.5 and 5.0 ppm F) on the enamel surface loss after 7, 14, 21 and 28 days erosive challenge.



Appendix VIII: The effect of different concentrations of fluoride milk (0, 2.5 and 5.0 ppm F) on the enamel surface loss after 7, 14, 21 and 28 days erosive and abrasive challenges.

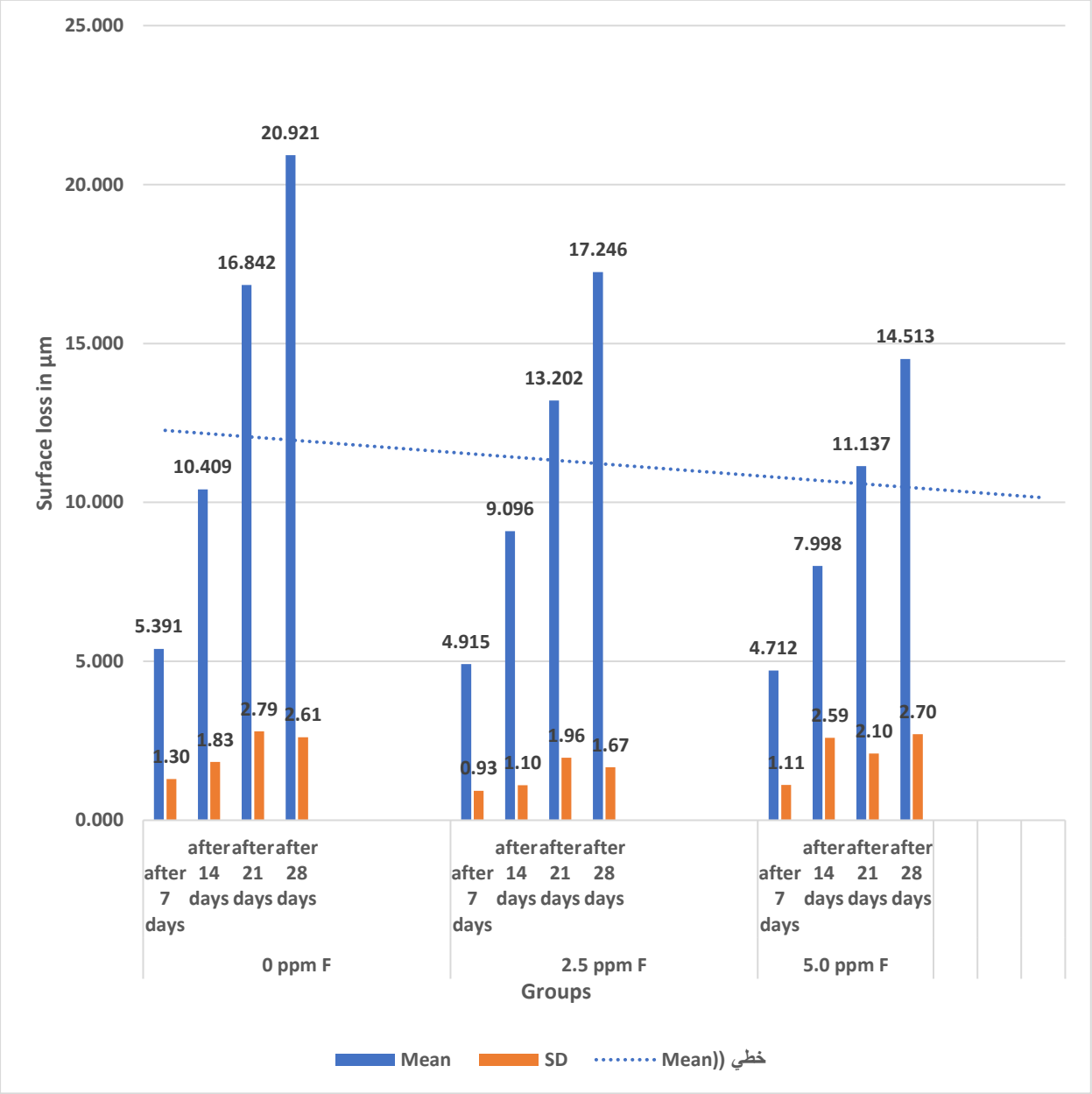


Figure 26-27 show the means of slabs surface loss and standards deviation of all groups (0, 2.5 and 5.0 ppm F milk) in both phases after 7, 14, 21 and 28 days. There was progression on enamel surface loss on all groups (0, 2.5 and 5.0 ppm F milk) in both phases with time increased.

Justifications for not performing statistical analysis for after 7, 14, 21 days of cycling:

1- The aims and objectives of this study were to investigate the effect of fluoridated milk 2.5 ppm and 5.0 ppm F on surface loss of dental hard tissue under erosive/ erosive and abrasive conditions *in vitro* after 28 days.
2- On advice from statistician, statistical analysis was not performed for 7, 14, and 21 days as it would have created the problem of multiple comparisons, leading to increased type 1 error which would have led in detecting false positives.

Appendix IX: Descriptive statistics of slabs surface loss for group (0 ppm F milk) after 28 days erosive challenge.

Group 1)- 0 ppm F milk/phase A (erosive challenge).	
Mean	15.436 µm
Median	16.06 µm
Standard Deviation	1.55
Range	4.23
Minimum	13.19 µm
Maximum	17.42 µm
Count	15 slabs
95% Confidence interval for mean	
Lower bound	14.58 µm
upper bound	16.29 µm

Appendix X: Descriptive statistics of slabs surface loss for group (2.5 ppm F milk) after 28 days erosive challenge.

Group 2)- (2.5 ppm F milk)/phase A (erosive challenge).	
Mean	12.180 µm
Median	11.96 µm
Standard Deviation	1.45
Range	6.41
Minimum	9.235 µm
Maximum	15.64 µm
Count	15 slabs
95% Confidence Level for mean	
Lower bound	11.38 µm
Upper bound	12.98 µm

Appendix XI: Descriptive statistics of slabs surface loss for group (5.0 ppm F milk) after 28 days erosive challenge.

Group 3)- (5.0 ppm F milk)/phase A (erosive challenge).	
Mean	8.200 µm
Median	8.10 µm
Standard Deviation	1.02
Range	3.70
Minimum	6.17 µm
Maximum	9.87 µm
Count	15 slabs
95% Confidence Level for mean	
Lower bound	7.63 µm
Upper bound	8.77 µm

Appendix XII: Descriptive statistics of slabs surface loss for group (0 ppm F milk) after 28 days erosive and abrasive challenges.

Group 4)- (0 ppm F milk)/phase B (erosive and abrasive challenges).	
Mean	20.921 µm
Median	21.40 µm
Standard Deviation	2.61
Range	10.17
Minimum	15.23 µm
Maximum	25.40 µm
Count	15 slabs
95% Confidence Level for mean	
Lower bound	19.47 µm
Upper bound	22.37 µm

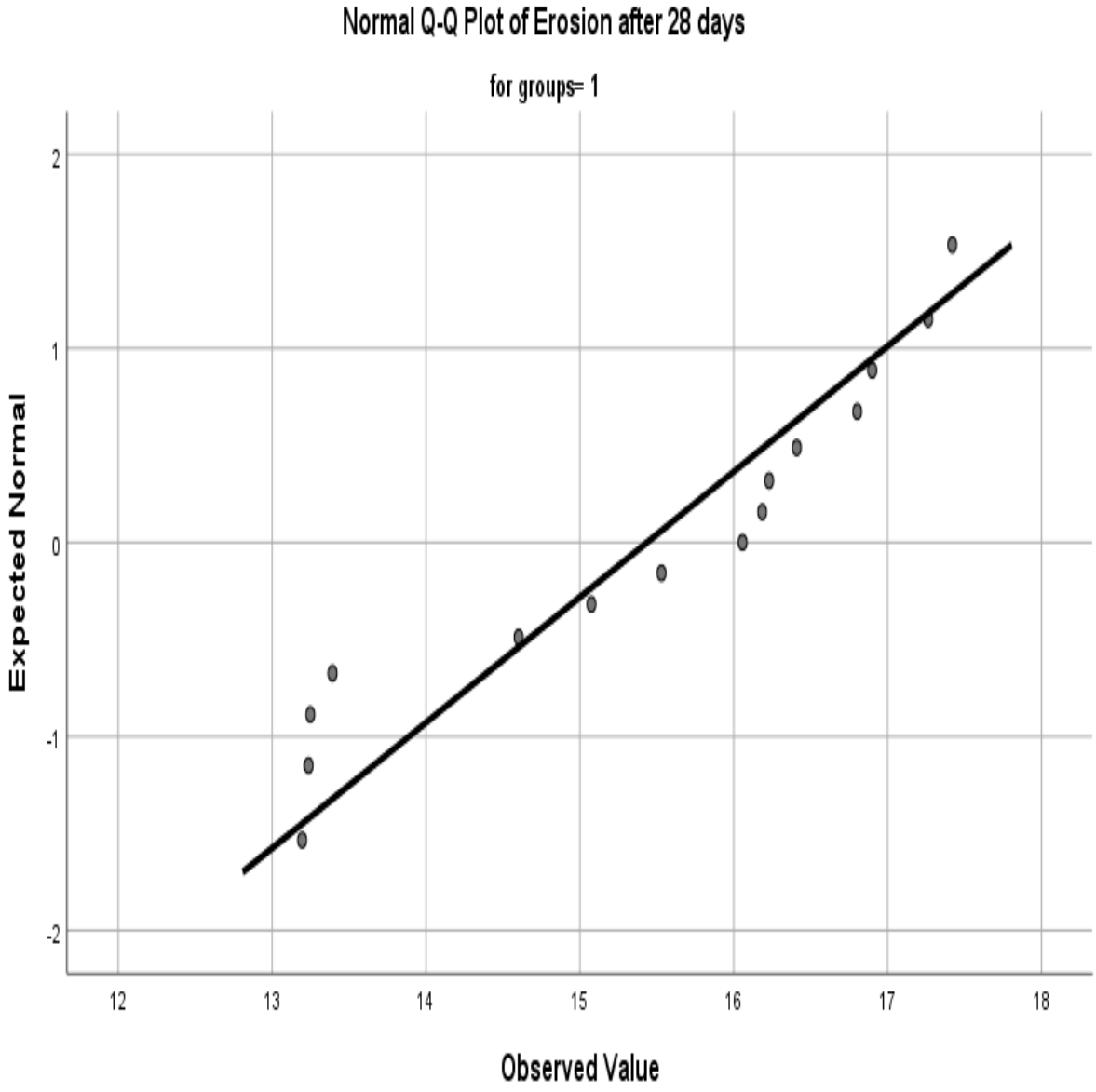
Appendix XIII: Descriptive statistics of slabs surface loss for group (2.5 ppm F milk) after 28 days erosive and abrasive challenges.

Group 5)- (2.5 ppm F milk)/phase B (erosive and abrasive challenges).	
Mean	17.246 µm
Median	17.48 µm
Standard Deviation	1.67
Range	5.80
Minimum	13.98 µm
Maximum	19.79 µm
Count	15 slabs
95% Confidence Level for mean	
Lower bound	16.32 µm
Upper bound	18.17 µm

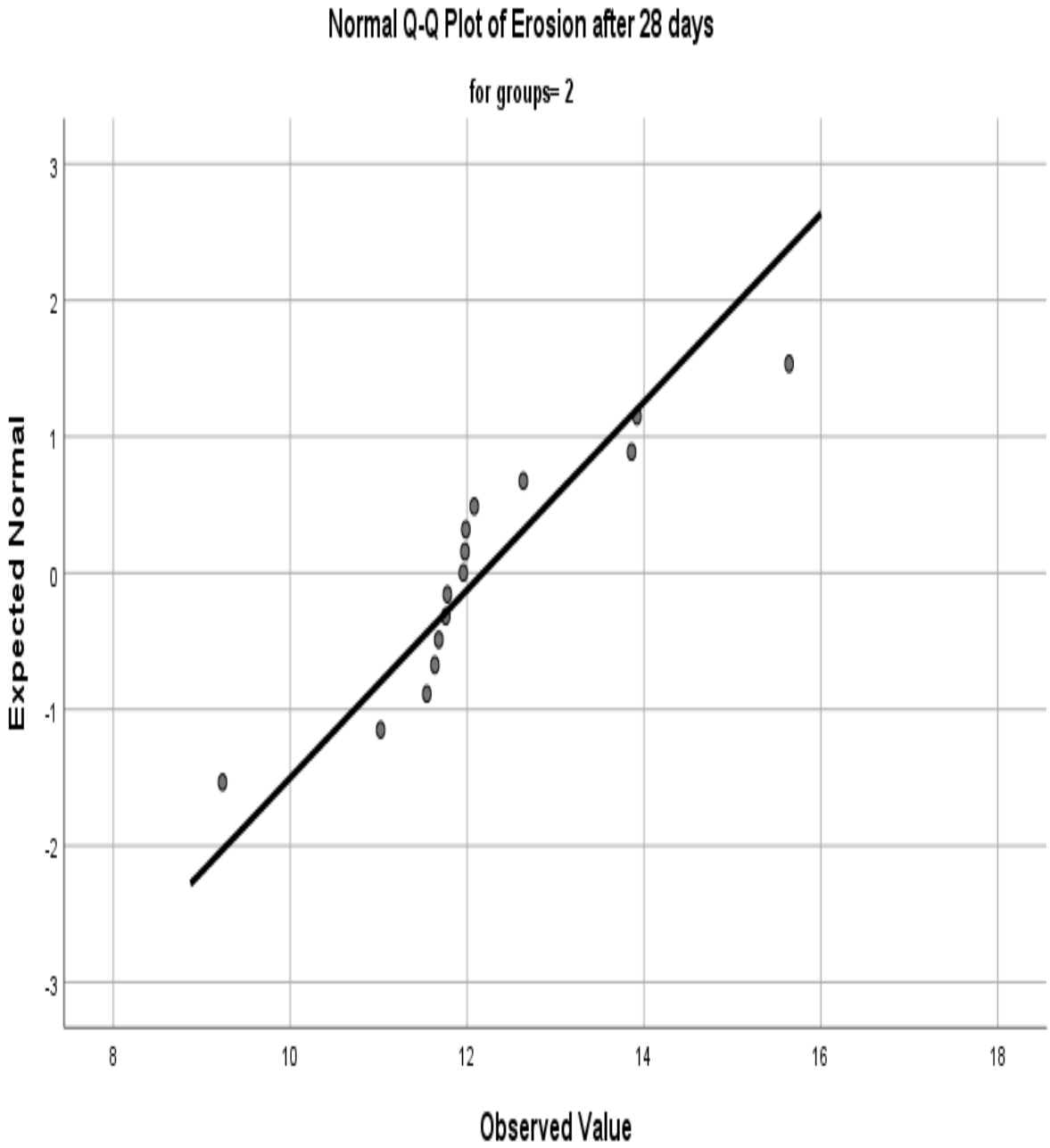
Appendix XIV: Descriptive statistics of slabs surface loss for group (5.0 ppm F milk) after 28 days erosive and abrasive challenges.

Group 6)- (5.0 ppm F milk)/phase B (erosive and abrasive challenges).	
Mean	14.513 µm
Median	13.82 µm
Standard Deviation	2.70
Range	7.53
Minimum	11.00 µm
Maximum	18.53 µm
Count	15 slabs
95% Confidence Level for mean	
Lower bound	13.02 µm
Upper bound	16.01 µm

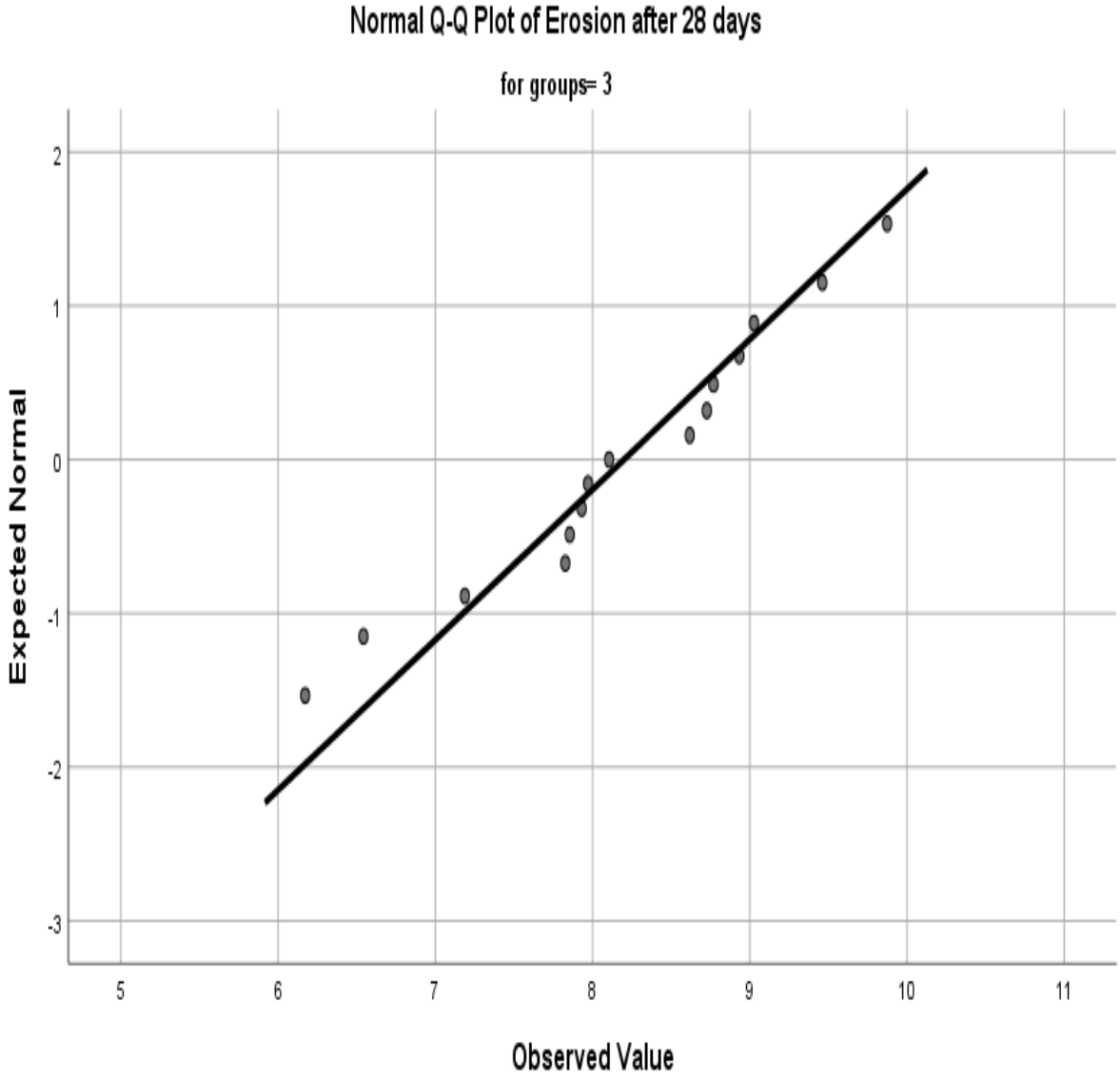
Appendix XV: Q-Q plot test of normality group for group (0 ppm F milk) after 28 days erosive challenge.



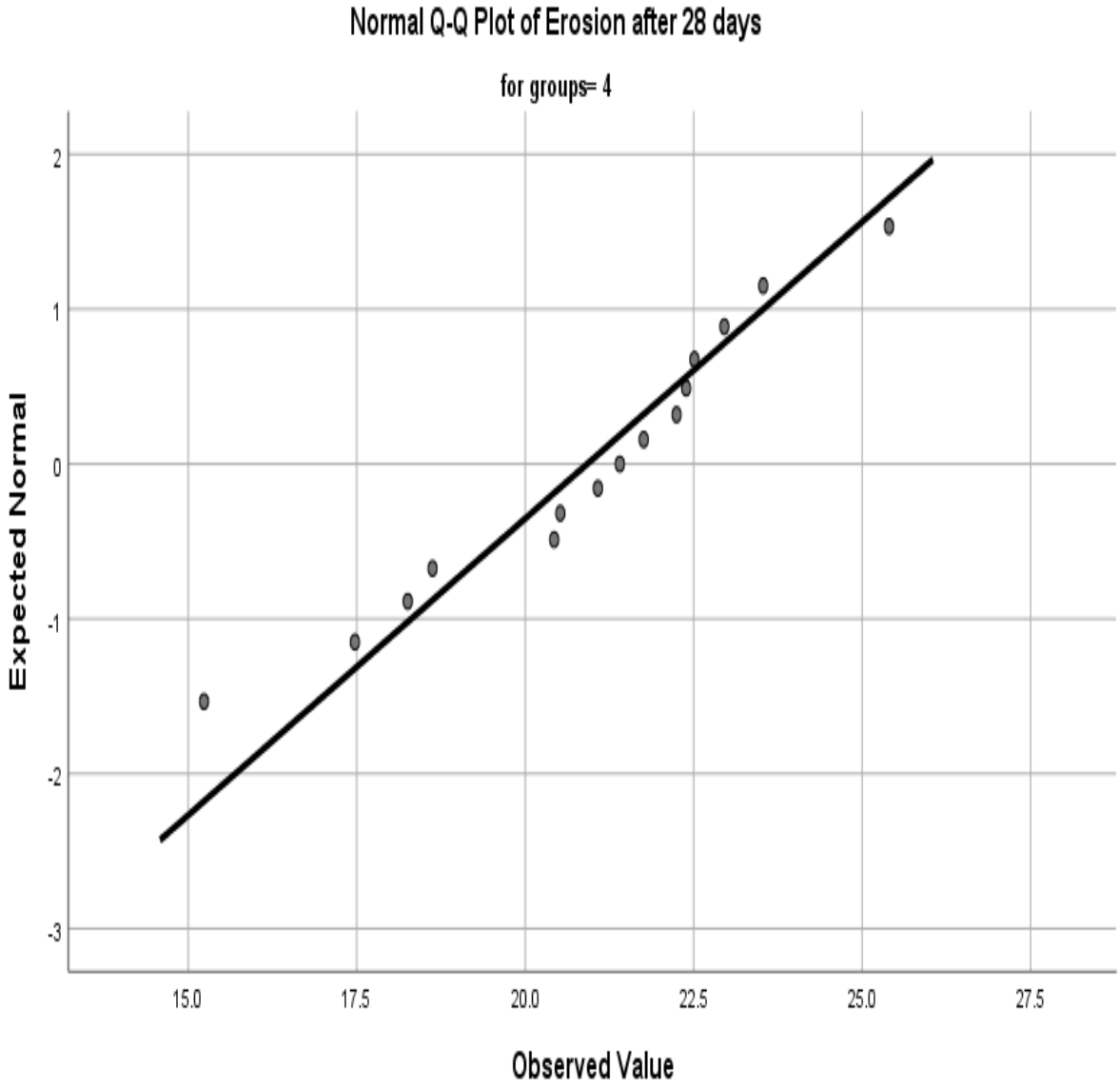
Appendix XVI: Q-Q plot test of normality group for group (2.5 ppm F milk) after 28 days erosive challenge.



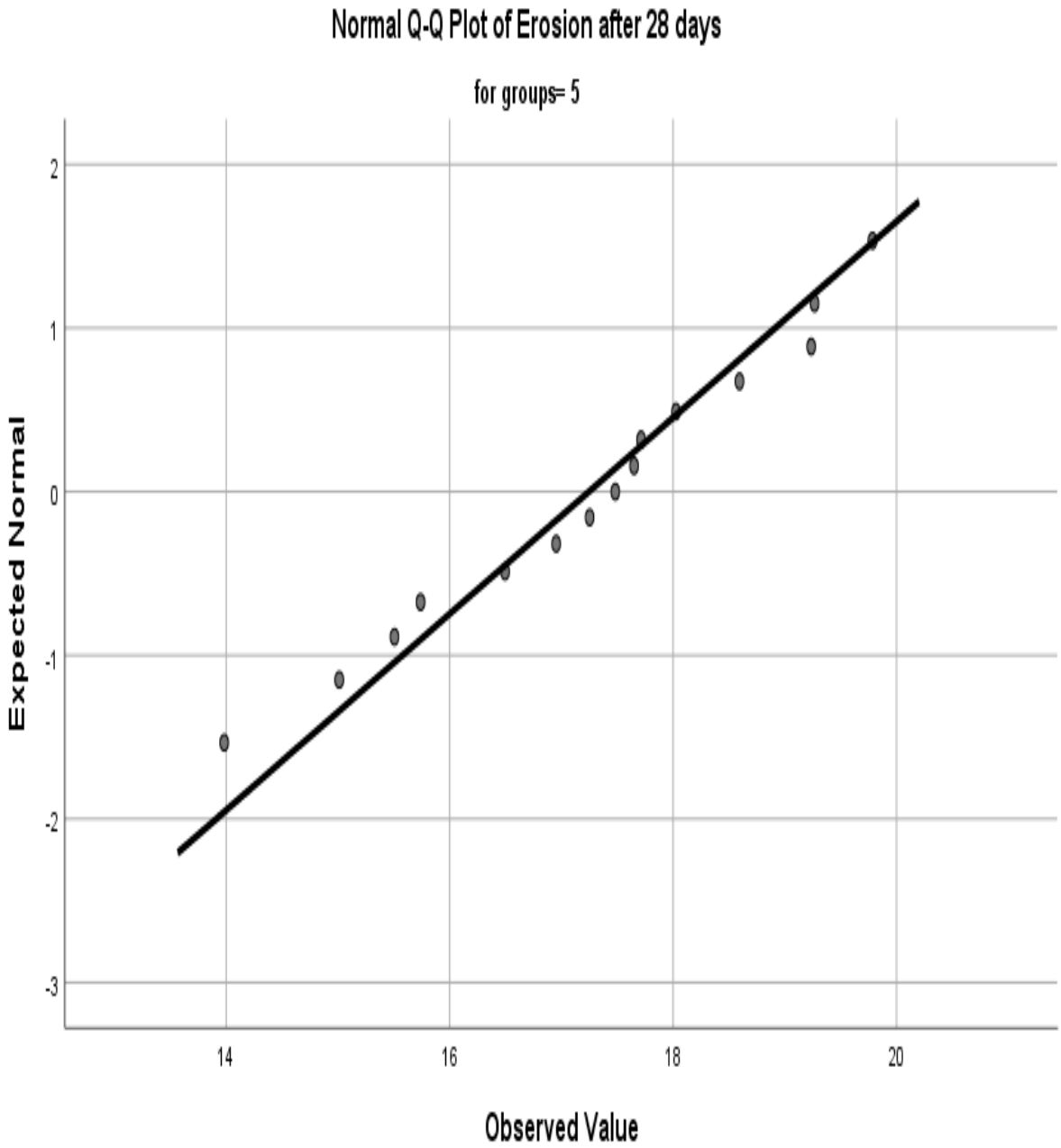
Appendix XVII: Q-Q plot test of normality group for group (5.0 ppm F milk) after 28 days erosive challenge.



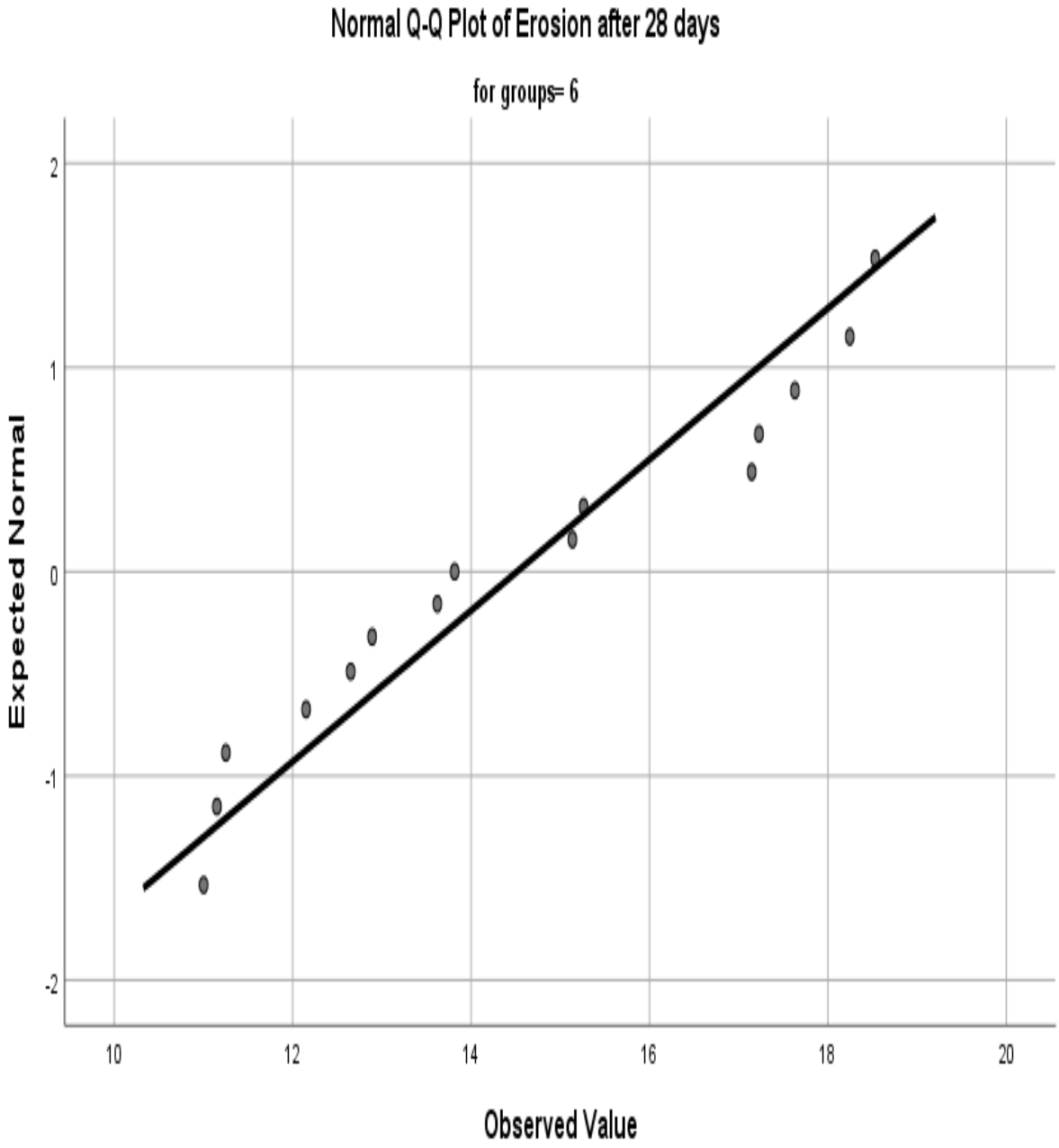
Appendix XVIII: Q-Q plot test of normality group for group (0 ppm F milk) after 28 days erosive and abrasive challenges.



Appendix XIX: Q-Q plot test of normality group for group (2.5 ppm F milk) after 28 days erosive and abrasive challenges.



Appendix XX: Q-Q plot test of normality group for group (5.0 ppm F milk) after 28 days erosive and abrasive challenges.



Appendix XXI: Statistical analysis of results to compare between groups at 0 ppm F milk with and without brushing after 28 days erosive challenge.

t-test

Group Statistics

	brushing	N	Mean	Std. Deviation	Std. Error Mean
Erosion after 28 days	no	15	15.43633333333	1.54607607771	.39919512673
	yes	15	20.92053333333	2.61062975981	.67406170552

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Erosion after 28 days	Equal variances assumed	2.273	.143	-7.00	28	.000	-5.484	.783	-7.091	-3.879
	Equal variances not assumed			-7.00	22.745	.000	-5.484	.783	-7.110	-3.863

Appendix XXII: Statistical analysis of results to compare between groups at 2.5 ppm F milk with and without brushing after 28 days erosive challenge.

t-test

Group Statistics

	brushing	N	Mean	Std. Deviation	Std. Error Mean
Erosion after 28 days	no	15	12.180	1.450932114194183	.374629060983439
	yes	15	17.246	1.667784053370593	.430619990918307

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Erosion after 28 days	Equal variances assumed	.789	.382	-8.876	28	.000	-5.066	.5708	-6.23	-3.90
	Equal variances not assumed			-8.876	27.474	.000	-5.066	.5708	-6.23	-3.90

Appendix XXIII: Statistical analysis of results to compare between groups at 5.0 ppm F milk with and without brushing after 28 days erosive challenge.

t-test

Group Statistics

	brushing	N	Mean	Std. Deviation	Std. Error Mean
Erosion after 28 days	no	15	8.1996	1.023	.265
	yes	15	14.513	2.703	.698

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Erosion after 28 days	Equal variances assumed	20.067	.000	-8.461	28	.000	-6.314	.7462	-7.842	-4.786
	Equal variances not assumed			-8.461	17.934	.000	-6.314	.746	-7.882	-4.746

Appendix XXIV: Intra-class Correlation Coefficient.

Case Processing Summary

		N	%
Cases	Valid	18	100.0
	Excluded[a]	0	.0
	Total	18	100.0

Reliability Statistics

Cronbach's Alpha	N of Items
.996	2

Intra-class Correlation Coefficient							
		95% Confidence Interval		F Test with True Value 0			
	Intra-class Correlation[b]	Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.991	.976	.997	223.981	17	17	.000
Average Measures	.996	.988	.998	223.981	17	17	.000