UCL Eastman Dental Institute

The natural history of the dental pellicle and its relationship to dental erosion.

Thesis submitted by

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I, Hesham Matabdin, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed…..

Date……

Abstract

Oral health is integral to general health, wellbeing and quality of life. Based upon the findings that dental erosion is highly prevalent in elite athletes, the aim of this PhD was to investigate the potential of the salivary dental pellicle to prevent dental erosion in the athlete population.

Four studies were conducted: 1) development of an in vitro dental erosion model which would be able to detect early erosive lesions, 2) a systematic review and meta-analysis on the impact of intense physical training on saliva parameters, 3) a systematic review on the impact of dental pellicle modification on the magnitude of dental erosion, and 4) the effects of micellar casein on the structural and functional properties of the dental pellicle against dental erosion.

Study 1 concluded that optical coherence tomography was not a promising tool to detect early erosive lesions. The systematic review and meta-analysis from study 2 found the evidence neither clearly supported nor refuted a relationship between intense physical activity and alterations in the qualitative and quantitative parameters of saliva which were hypothesised to cause dental erosion in athletes. Methodological issues including the high heterogeneity and low certainty of evidence contributed to the inconclusive overall findings. The systematic review from study 3 found that dental pellicle modification could offer a therapeutic approach to prevent dental erosion. However, the absence of a meta-analysis could not confirm the magnitude of dental erosion protection resulting from the interventions into the dental pellicle. The results from study 4 provided some indication that micellar casein might be protective for DE by reducing the decrease in surface microhardness of bovine enamel surfaces during erosion, although the effect did not quite achieve statistically significant difference. Similarly, there were no statistically significant differences in calcium ion concentration in citric acid and % calcium ion content following dental erosion. Overall, the results were inconclusive as the dental pellicle was not observed to be adsorbed on to polished enamel surfaces raising questions about the possibility of pellicle modification occurring in previous studies. There were no statistically significant effects of micellar casein on the ultrastructural properties of the dental pellicle, suggesting that pellicle modification may not occur with micellar casein indicating that micellar casein may not

be an effective intervention. Therefore, with the limitations of the experiments mentioned, further research is required to confirm the findings and come to a conclusive result.

The results from these studies suggest that further evaluation of the role of saliva in maintaining the oral health of elite athletes is needed. In addition, investigation of the dental pellicle in laboratory and clinical studies and its potential for modification would appear to have value for development of interventions to reduce dental erosion.

Impact statement

The overall aim of this PhD was to modify the salivary dental pellicle to prevent against dental erosion in order to promote general health, wellbeing and performance.

The findings obtained from the studies have created questions for further research into the field of dental erosion in athletes and alternative preventive measures to be adopted. The draft for the systematic review reporting the impact of intense physical training on saliva is being prepared for publication. The findings from the in vitro dental erosion model and the systematic review have also been presented via posters and oral presentations in various conferences such as the International Association of Dental Research (IADR), British Society of Dental Research (BSODR) and International Olympic Committee (IOC). The purpose of participation at the IOC was to raise awareness about the impacts of oral health and dental erosion on the performance of athletes and provide insights into the preventive measures been taken to reduce the impacts of poor oral health on performance.

The findings from the meta-analysis performed on the impact of intense physical training on saliva will hopefully alert researchers to explore other possible risk factors behind dental erosion. The hypothesis was that saliva parameters would change after periods of intense physical activity which may be a factor for dental erosion in athletes. However, the findings from the meta-analysis neither supported nor refuted the hypothesis.

A potentially important finding during the intervention study was the failure of the dental pellicle to be adsorbed onto the polished enamel surface. All previous intervention studies were performed on polished enamel surfaces, however none of the studies confirmed the formation of dental pellicle onto the enamel surface. On using imaging outcome measures during the intervention studies in the PhD, it was observed that pellicles were being adsorbed onto non-polished surfaces as opposed to polished surfaces. The findings obtained raise questions about the possibility of pellicle modification occurring in the previous published studies and will be submitted for publication soon to generate debate about using non-polished surfaces for intervention studies into the dental pellicle.

Scientific output

Publications

Conference abstract: Matabdin, Hesham; Ashley, Paul; Wilde, Pete; Needleman, Ian (2020**) How can we protect athletes from dental erosion?** British Journal of Sports Medicine; London Vol. 54, Suppl 1. DOI:10.1136/bjsports-2020-IOC Abstracts.352

Poster presentation

Oral/Poster presentation "**How can we protect athletes from dental erosion"** – International Olympic Committee (IOC) Monaco November 2021

Poster presentation "**Impact of physical training on saliva"** British Society of Oral and Dental Research (BSODR) conference, University of Birmingham 2021

Virtual oral/poster presentation "**Development of in vitro model to detect early erosive lesions"** International Association of Dental Research (IADR) USA 2021.

Oral presentation

Oral presentation "**Development of in vitro model to detect early erosive lesions with optical coherence tomography**" British Society of Oral and Dental Research (BSODR) conference, University of Leeds 2019

3-minute thesis (3MT) presentation UCL Deans research prize March 2020

3-minute thesis (3MT) presentation UCL Eastman research away day June 2019 (Winner of runner's up prize)

3-minute thesis (3MT) presentation UCL Eastman research away day July 2018

Poster presentation UCL Doctor School Research Poster competition 2019 (Winner of runner's up prize)

3-minute thesis (3MT) presentation GSK Science symposium 2018

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List of abbreviations

AJ	Apple juice
BCA	Bicinchoninic acid assay
BEWE	Basic erosive wear examination
BPE	Basic periodontal examination
BPM	Beats per minute
CC	Case control
CI	Confidence interval
CLSM	Confocal laser scanning microscope
COWR	Cabrera Open Water Race
CPP-ACP	Casein phosphopeptide amorphous calcium phosphate
CT	Controlled trial
DC	Dental caries
DE	Dental erosion
DEFRA	Department of Environment, Food and Rural Affairs
DEJ	Dentine enamel junction
DIW	Deionised water
DLS	Dynamic light scattering
DP	Dental pellicle
EDX	Energy dispersive x-ray spectroscopy
EGCG	Epigallocatechin gallate
ELISA	Enzyme linked assay
ETW	Erosive tooth wear
EV.I.D. E	Evaluating index of dental erosion
FPLC	Fast protein liquid chromatography
GC	Gas chromatography
GORD	Gastro-oesophageal reflux disorder
GRADE	Grading of Recommendations Assessment, Development and Evaluation
HCL	Hydrochloric acid
HR	Heart rate
IC	Ion chromatography
IOC	International Olympic Committee
IR	Integrated reflectivity

LC-MS	Liquid chromatography mass spectroscopy
AFM	Atomic force microscopy
MC	Micellar casein
MD4	Match Day minus 4
MeSH	Medical subject headings
MP	My Protein
N/A	Not Applicable
OCT	Optical coherence tomography
OJ	Orange juice
PC	Prospective cohorts
PRISMA	Preferred reporting
PRPs	Proline-rich proteins
PW	Protein works
QCM-D	Quartz crystal microbalance with dissipation
RCT	Randomised controlled trial
REML	Random effects model
RM	Repeated measure
RN	Reflex nutrition
ROBINS-I	Risk of bias in non-randomised studies - of interventions
RPE	Rating of perceived exertion
rSMH	Relative surface microhardness
RW	Red wine
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopes
SIgA	Salivary immunoglobulin A
SMH	Surface microhardness
SRI	Surface reflection intensity
S-T.W.I.	Simplified tooth wear index
STP	Saliva total protein
STP	Sodium tri polyphosphate
TA	Titratable acidity
TEM	Transmission electron micrograph
TG	Test group

TOF-MS	Time-of-flight mass spectrometry
UCL	University College London
UTST	Ultra-trail Serra de Tramuntana
VO2	Max volume of oxygen
XMT	X-ray microtomography

1. Introduction.

This PhD thesis presents the results from four research studies into the potential of the salivary dental pellicle to prevent against dental erosion (DE). Dental erosion describes non-carious tooth surface loss, usually caused by exposure of acid from dietary sources or gastro-oesophageal reflux (Lussi et al., 2009). Progressive loss of tooth surface initially causes increased sensitivity but can ultimately lead to pulpal involvement if unchecked (Donovan et al., 2021). Data from a systematic review found that up to 85% of athletes had erosive tooth wear of their teeth with the lesions progressing into the dentine (Ashley et al., 2015). Athletes are thought to be at particular risk due to consumption of sports drinks which have been linked with an increased risk of dental erosion (Freese et al. 2015, Sovik et al. 2015). In view of the high prevalence of DE in athletes, there is a need both to better understand the risks for DE in athletes and to develop approaches to reduce or mitigate these risks.

An approach was proposed whereby the erosive potential of a sports drink could be reduced by strengthening the dental pellicle (DP). The DP is a protein rich film of saliva that forms on all surfaces within the mouth and provides a barrier to dissolution of enamel by dietary acids (Hannig and Joiner, 2006). As the pellicle is the primary interface between the oral environment and the hard and soft tissue of the mouth it plays an important role in oral physiological and pathological processes (Hannig and Hannig, 2014). Understanding the mechanisms by which the dental pellicle protects the tooth against erosion might prove a promising intervention strategy to help reduce erosion, not only in elite athletes, but also in other vulnerable groups.

Therefore, the aims were achieved by investigating the following objectives. The first was to develop an in vitro dental erosion model which would detect early dental erosive lesions with optical coherence tomography (OCT). The second was to assess the impact of intense physical training on the saliva parameters via a systematic review and meta-analysis. The third was to assess the impact of dental pellicle modifications on the magnitude of dental erosion, again via a systematic review. The fourth and final objective was to assess the effects of micellar casein on the structural and functional properties of the dental pellicle against dental erosion.

A review of the literature will provide background information regarding dental erosion and the salivary pellicle. This will be followed by a statement of the study hypotheses and related research questions. The aims, methods and results of each study will then be presented and discussed in subsequent chapters. The final chapter will provide an overall summary of the results and discuss the implications for modification of the dental pellicle to prevent dental erosion and the implications for future research.

2. Literature review

2.0. Background

To perform at the highest level, athletes need to be well-prepared, fit and healthy. Oral health contributes towards optimum general health and wellbeing. An imbalance of oral health in the form of dental erosion, dental caries or gum disease may result in a negative impact on their performance. This chapter will provide a background to understanding the determinants and consequences of the imbalance of oral health, particularly focusing on dental erosion, its causes and effects. The protective capabilities of the protein layer of saliva, the dental pellicle will also be discussed. The various in vitro and in vivo methods to assess and measure dental erosion will also be reviewed. Finally, a summary of the various interventions and modification of the dental pellicle based on the literature to prevent dental erosion will be given.

2.1. Importance of oral health in elite sport

There is a substantial amount of evidence suggesting that oral diseases and conditions are common in elite athletes (Ashley et al., 2015, Gallagher et al., 2018, Tripodi et al., 2021, Gallagher et al., 2019). Elite athletes follow programmes of intense physical training to challenge and increase their performance levels which are usually combined with a specific diet pattern (Bryant et al., 2011). Diet and physical training are key elements necessary to promote effective optimal competition and performance (Burke, 2015). To maintain immune function, athletes should eat a well-balanced diet sufficient to meet their energy requirements. Consuming carbohydrates during exercise attenuates rises in stress hormones such as cortisol and appears to limit the degree of exercise-induced immunosuppression, at least for non-fatiguing bouts of exercise (Gleeson and Williams, 2013). Due to the unique and extensive training schedule and nutritional habits, there is a high possibility for the athletes to be at a higher risk of poor oral health as compared to the general population (Julia-Sanchez et al., 2015). Athletes including distance runners have been reported to engage in disordered eating habits (Needleman et al., 2018) which may be a contributing factor to poor oral health.

A decreased saliva flow can result from intense physical activity (Frese et al., 2015a). During exercise, an athlete switches from nose to mouth breathing, which causes increased cooling and drying of the respiratory and oral mucosa (Brukner and Khan, 2014). One study that investigated the impact of endurance training on oral health in athletes and controls found a decrease in saliva flow ($p = 0.001$ stimulated; $p = 0.01$

unstimulated) with a significant increase in saliva pH (p = 0.003) (Frese et al., 2015b). In addition to saliva flow and pH, other components of saliva such as salivary immunoglobulin A (Francis et al., 2005), alpha-amylase (Trochimiak et al., 2015), viscosity (Ligtenberg et al., 2016) and the total protein content (Kiani et al., 2015) have been suggested to have been altered as a result of intense physical activity and sports training. The change in the physical and chemical characteristics of saliva may be a possible factor for the poor oral health in athletes.

Poor oral health has been reported to create a negative impact on the performance of the athletes (Needleman et al., 2013), suggesting that oral health is a determinant of sports performance. A cross-sectional study performed on elite and professional UK athletes reported 49% of the study group suffering from dental caries (DC) and 41% from dental erosion (DE) (Gallagher et al., 2018).

2.1.1. Epidemiology

The prevalence of poor oral health within the athlete population can be suggested with the help of a systematic review on the association of oral health of athletes with performance which concluded that poor oral health was prevalent in the athlete population (Ashley et al., 2015). However, the quality of studies included in the systematic review was considered low as the eligibility assessment was carried out by only one researcher, which might have introduced bias in the selection of studies. The results of the systematic review can be compared to other various studies (Table 2.1) which also suggested the prevalence of poor oral health in athletes.

Table 2.1: Studies assessing the oral health of athletes (DE: Dental Erosion, DC: Dental Caries).

Author/Country	**Type of study**	**Population group/*n***	**Results**
(Nijakowski et al., 2020), Poland.	Cross-sectional.	Secondary school athletes, n= 155.	DE: 59.8%
(Reis et al., 2019), Portugal.	Observational.	Athletes from three sport clubs, n= 30.	Decreased saliva flow Increased saliva pH.
(Gallagher et al., 2018).	Cross-sectional.	Elite and UK professional athletes.	DC= 49% DE= 41% Periodontal disease (PD)= 77%

(D'Ercole et al., 2016), Italy.	Cross-sectional.	Competitive swimmers, n= 54.	Altered saliva biomarkers, IgA.
(Frese et al., 2015a), Germany.	Clinical Trial.	Triathlon athletes, n=35.	Altered saliva markers Increased risk of DE and DC.
(Ashley et al., 2015), United Kingdom.	Systematic review.	UK professional athletes.	Dental trauma 14% - 47% DC 15–75%, DE 36–85%, PD 15%
(Needleman et al., 2013), United Kingdom.	Cross-sectional.	London 2012 olympic athletes, n= 302.	DC= 55% DE= 45% PD= 76%
(Mulic et al., 2012), Norway.	Cross-sectional.	Young athletes from fitness center, n= 220.	DE= 64% Decreased saliva flow rate.

The data captured from all the studies suggest poor oral health to be prevalent among the athlete population with dental erosion being one of the common oral health conditions.

2.2. Dental erosion in the athlete population

The data from table 1 suggests dental erosion (DE) to be one of the most prevalent dental problems in the athlete population. Dental erosion is the loss of hard tissues of the tooth (enamel and dentin), due to the action of intrinsic and extrinsic acids without the involvement of bacteria (Hannig and Hannig, 2014) One of the possible reasons for the high incidence of DE in athletes would be the frequent consumption of acidic sports drinks to maintain their energy and electrolyte levels (Needleman et al., 2015). Sports drinks have a pH below the critical pH of 5.5 and therefore have the tendency to demineralize the teeth by causing the minerals to diffuse out of the tooth hence reducing the tooth volume (Kaye, 2017). However, various studies could not establish any link between the association of isotonic drinks and dental erosion (de Queiroz Gonçalves et al., 2020, Sirimaharaj et al., 2002, Antunes et al., 2017). On the other hand, a recent study concluded that regular physical activity was associated with an increased risk of dental erosion, especially under the influence of frequent consumption of sports drinks (Nijakowski et al., 2022). In vitro studies have also confirmed the association of sports and energy drinks to dental erosion (Coombes, 2005). Therefore, based on the results from the above studies it is yet too early to

propose the high incidence of dental erosion to be caused by consumption of sports beverages.

DE is multifactorial with acid as the main cause, but the progression can be halted if the aetiological factors can be controlled. Ultimately, dental erosion can be classified as a pathological condition when it is associated with loss of function, subjective reduction in aesthetics, or pain (Schlueter et al., 2012).

2.2.1. Determinants of dental erosion

Dental erosion is caused by acidic substances from a variety of sources, however, saliva flow rates, buffering capacity and differing clearance rates from various parts of the mouth may modify the severity and distribution of erosion. Current knowledge suggests that the variation in predisposition to dental erosion is due to differences in susceptibility of dental hard tissues to dissolution, and to differences in saliva (Buzalaf et al., 2018).

Regular physical activity has been reported to be a risk factor towards dental erosion (Nijakowski et al., 2020). One of the reasons behind the disease maybe the reduced salivary flow rate during exercise (Frese et al., 2015). However, flow rate can also be reduced by systemic disease (Sjögren's syndrome) and certain medications (Carvalho et al., 2018).

Reflux/vomiting can also increase the risk of DE. However, to be significant, frequent reflux over an extended period of time is necessary. A raised prevalence of DE has been identified in groups of people with underlying medical conditions such as gastro-oesophageal reflux disorder (GORD) (18-23%), as well as rumination and eating disorders with frequent vomiting such as bulimia nervosa (45-98%) (Schlueter and Luka, 2018). Although the evidence to associate DE with dietary factors is weak, evidence linking dental erosion with soft drink consumption is emerging (Lussi et al., 2004; Carvalho et al., 2018). The intake depends on the drinking habits (sipping or swishing) the drink around the oral cavity. Carbonated beverages, fruit juices, smoothies, and fruit flavoured mineral waters, are tangy or refreshing because of the acidity. Artificially sweetened diet drinks can be as acidic as normal varieties (Omid et al., 2016). Fresh fruits, and in particular citrus fruit, have erosive potential as do foods pickled in vinegar (Bartlett et al., 2011).

DE is a chemical process which occurs onto the tooth enamel structure hence softening the enamel and dissolving it. Therefore, to understand the chemical process of DE onto the enamel, it is crucial to have an understanding of the composition and structure of the human enamel. The next section will provide a brief overview of the composition of human enamel and the chemistry of dental erosion during an acid attack on the enamel structure.

2.2.2. Composition of human enamel

Human enamel is composed of an impure form of calcium hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. The imperfections in pure calcium hydroxyapatite arise from the incorporation of tissue fluids ions into the crystals during hard tissue formation. These ions are substituted by the carbonate (CO_3^{2-}) ions in place of the phosphate (PO_4^{3-}) or hydroxyl (OH^-) ions; and sodium (Na^+) ions and magnesium (Mg^{2+}) ions for calcium (Ca^{2+}) ions. The substituted ions differ from the native hydroxyapatite ions in size and charge and as a result they have the effect of introducing strain in the crystal structure of the hydroxyapatite and this in turn increases the solubility of the enamel structure (Shellis et al., 2014).

2.2.3. Comparison of human and bovine enamel

Enamel specimens obtained from human teeth are preferred for in vitro and in situ dental research because they allow for testing of the study hypothesis in a more clinically relevant substrate. However, some disadvantages and limitations with the use of human teeth exist. They are often difficult to obtain in sufficient quantity and with adequate quality, since many are extracted due to extensive caries lesions and other defects. It can also be challenging to control the source and age of the collected human teeth, which may lead to larger variations in the outcome measures of the study. Furthermore, the relatively small and curved surface area of human teeth may also be a limitation for specific tests requiring flat surfaces of uniform thickness. Finally, awareness of the infection hazard and ethical issues have increased. Therefore, alternative substrates have been proposed and used in dental research (Yassen et al., 2011).

Bovine teeth have become a valuable substitute for dental research studies due to a number of reasons. They are easy to obtain in large quantities, in good condition and with a more uniform composition than that of human teeth. Furthermore, bovine teeth

have a relatively large flat surface, and do not have caries lesions and other defects that might affect outcomes (Soares et al., 2016).

In comparison to human teeth, elemental analysis showed that human substrates have greater similarity to bovine than to porcine and ovine substrates. Based on their chemical composition, bovine teeth should be the first choice as substitutes for human teeth (Teruel Jde et al., 2015). Studies have indicated the possibility of human tooth replacement in enamel evaluations based on morphology, organic, inorganic aspects and optical reflection (Martinez et al., 2022). For the study of morphological aspects of dental erosion, the use of bovine teeth are considered capable of replacing. The surface ultrastructure of erosive lesions in prismatic human enamel and bovine specimens do not differ. However, structural factors in enamel greatly modify the surface ultrastructure and the progression of erosion suggesting studies assessing abrasion and erosion on bovine teeth should be performed with caution (Martinez et al., 2022).

2.2.4. Chemistry of dental erosion

The loss of the enamel surface during dental erosion, has been suggested to be a two-step process. First, the direct acid attack leads to the demineralization of hydroxyapatite of enamel, which is then followed by the abrasion and attrition forces acting onto the tooth surface which eventually leads to tooth surface loss (Shellis and Addy, 2014).

The chemistry of dental erosion is also a two-step process: first, the dental hard tissue minerals are dissolved by attack from the dissociated hydrogen ions (H^+) from the acid, and the resultant mineral dissolving being complexed by anions, especially those with strong chelating action such as citric acid. Chelation is a process where the anions bind with metal ions such as calcium and causes the destabilization of hydroxyapatite surface at a low pH, weakening the phosphate coordination bonds (Zulkapli et al., 2020). The H^+ are derived from the acid dissociating in water and then combine with the carbonate and/or phosphate ions to release the ions from the crystal surface leading to direct surface demineralization. The anions of the acid interact with the calcium ions depending on the strength of the interaction which is represented by the stability constant (K). The stronger the bond, the more likely the anion is to pull the

calcium ions out of the apatite mineral surface and into the solution, hence demineralizing the surface. The strength of the acid is represented by its pKa value which is the negative log of the acid dissociation constant Ka. The lower the pKA value, the higher the acidity and greater the erosion. For example, acetic acid (pKa 4.76) is a weak acid with weak calcium/acetate bonds not rendering very erosive. On the other hand, lactic acid (pKa 3.86) binds stronger to calcium because of the added OH side group on the molecule. Lactic acid is also stronger than acetic acid and gives off H^+ ions more readily.

2.2.5. Mechanics of dental erosion

As the enamel is exposed to an acidic medium, dissolution initiates at the tooth surface. This is followed by the demineralization within the surface enamel, through diffusion of the acidic solution into the enamel and is accompanied by partial dissolution of enamel crystals. The process creates a 'softened layer' at the tooth surface which is not more than a few micrometres thick. As the lesions advances, the demineralization occurs at the prism junctions and the rate of mineral loss reaches a steady state. Theoretically, advancement of demineralization into the tissue is balanced by total loss of mineral from the surface. The acid solution diffuses into the narrow enamel pores and starts to dissolve the mineral (Shellis et al., 2014). The dissolution of the mineral eventually leads to the signs and symptoms in an individual which can range from increased sensitivity to severe pulpal pain.

2.2.6. Mediators of dental erosion - Titratable acidity and pH

The pH and titratable acidity are two indicators of the erosive potential of acids. The pH value corresponds to the measure of the hydrogen ion concentration in the acid medium, however it gives no indication of the overall acidic content of the beverage or food. On the other hand titratable acidity (TA) gives a measure of all free hydrogen ions and concentration of the acid available to cause erosion (Benjakul and Chuenarrom, 2011). The critical pH below which enamel begins to erode is 4.5 which is a nature for most acidic beverages (Antunes et al., 2017). However, one study reported that TA was a more important indicator than pH value for determining the erosive potential of beverages (Edwards et al., 1999).

There have been attempts to raise the pH and reduce the erosive potential of sports and energy drinks by adding phosphates (Jensdottir et al., 2005), nanohydroxyapatite

particles (Min et al., 2011) and fluoride (Dundar et al., 2018). The studies presented with favorable results, however, it was observed that the resulting formulations had a metallic taste and were unpalatable by the athletes. Therefore, other options have to be considered to reduce the incidence of dental erosion.

2.2.7. Clinical signs and symptoms of dental erosion

2.2.7.1. Early Lesions

It is not easy to assess and identify early dental erosion due to the faint appearance of the lesion on the tooth surface. However, the absence of perikymata on the facial and lingual sides of the teeth, with the enamel acquiring a rounded and shiny surface is a key feature. The groove-fossa aspect of the posterior teeth becomes less defined resulting in flattening of the occlusal surfaces (Carvalho et al., 2018).

2.2.7.2. Late Lesions

Further developed lesions often present as a loss of the enamel on the palatal surfaces of maxillary anterior teeth resulting in the incisal edges becoming more transparent and irregular. This may at times lead to a formation of a bluish shadow identified upon slightly opening the mouth. The occlusal surface of premolars and molars become more flattened with the presence of cupping and invaginations (Carvalho et al., 2018).

2.2.7.3. Advanced Lesions

Advanced dental erosion presents as a reduced tooth height due to loss of the incisal edges and is most often combined with attrition. In general, the incisal edges are irregular, and yellowish areas of exposed dentine may be present on facial surfaces. The groove-fossa-system on the occlusal surfaces is no longer identifiable with localised exposure of the dentine. The individual may complain of symptoms such as pain and sensitivity (Carvalho et al., 2018).

2.2.8. Diagnosis

Dental erosion is diagnosed on the basis of the clinical signs and symptoms presented by the individual which can range from aesthetic concerns to pain and sensitivity. A complete medical and dental history of the individual must be recorded in order to identify the intrinsic and extrinsic causes (Ganssa and Lussi, 2006). The history is an

important step as it aids in identifying the preventive and management strategies to be adopted in order to avoid further progression of the lesions.

2.2.9. Management of erosive lesions

The management of dental erosion depends on the extent of the erosive lesions. Management of early erosive lesions involves preventive strategies which include the use of saliva substitutes based on formulations with calcium phosphate and fluoride, changes in dietary habits and removal of the risk factors. Advanced lesions can be managed by restorative treatments such as the use of resin-based materials, fissure sealants or composite resins in more severe erosion lesions (Carvalho et al., 2018).

2.2.10. In vivo assessment of dental erosion (Erosive tooth wear indices)

The severity of dental erosion has been measured using a system of indices (Ganss and Lussi, 2014). The earliest indices relied on descriptive terms such as slight, mild, moderate, severe and extensive. A scoring chart was designed to classify dental erosion according to the extent of lesion (Eccles, 1979) (Table 2.2) which was further refined based on the basis of the descriptive criteria (Bardsley, 2008).

Table 2.2: Eccles index for dental erosion.

Class	**Surface**	**Criteria**
Class I		Early stages of erosion, absence of developmental ridges, smooth, glazed surface occurring mainly on labial surfaces of maxillary incisors and canines.
Class II	Facial.	Dentine involved for less than one third surface
Class IIIa	Facial.	More extensive destruction of dentine, affecting anterior teeth particularly.
Class IIIb	Lingual.	Dentine eroded for more than one third of the surface area. Gingival and proximal enamel margins have white, etched appearance. Incisal edges translucent due to loss of dentine.
Class IIIc	Incisal.	Surfaces involved into dentine, appearing flattened or with cupping. Incisal edges appear translucent due to undermined enamel.
Class IIId	All.	Severely affected teeth, where both labial and lingual surfaces are extensively involved.

The Basic Erosive Wear Examination (B.E.W.E.) (Bartlett et al., 2008), the Simplified Tooth Wear Index (S-T.W.I.) (Bardsley, 2008), and the Evaluating Index of Dental Erosion (EV.I.D.E) (Lopez-Frias et al., 2012) were designed to measure the degree of erosion. One of the popular tooth wear indices used is the one proposed by Smith and

Knight (Smith and Knight, 1984). In this index, four sections of each tooth (i.e., buccal/labial, palatal/lingual, cervical and incisal/occlusal) are examined visually and recorded separately, scoring each surface, ranging from 0 (no loss of enamel surface characteristics) to 4 (complete loss of enamel and pulp exposure). These values are then compared to a set of maximum acceptable tooth wear scores that have been proposed for each decade of life allowing the extent and distribution of the tooth wear in patients to be measured. An important aspect of this index is that it measures tooth wear irrespective of etiology and hence is not exclusively designed for diagnosis of erosion (Taji and Seow, 2010).

Detecting early signs of change can be difficult and require expertise to apply them for clinical diagnosis (Margaritis et al., 2011). Recently, the Basic Erosive Wear Examination (B.E.W.E.) was introduced to quantify the severity of dental erosion. In this method all teeth, except third molars, are examined in each case from the vestibular, occlusal, and palatal aspects for acid damage. The most severely affected surface in a sextant is recorded with a score. The sum of the scores defines the severity of erosive wear and guides the further management of the condition (Bartlett et al., 2008).

Recent reviews have shown that the Tooth Wear Index (TWI) and the Basic Erosive Wear Examination (BEWE) are two commonly used indices in epidemiological surveys (Jaeggi and Lussi, 2014, Bartlett and O'Toole, 2020, Schlueter and Luka, 2018). Both indices measure wear irrespective of the cause, although the BEWE, perhaps confusingly, is named erosive wear examination to highlight to the clinician that significant wear rarely occurs without an erosive component. The TWI classifies severity based upon the level of dentine and secondary dentine exposure. BEWE classifies severity based upon the percentage of surface area of the tooth affected and scores the worst affected surface in each sextant (Bartlett et al., 2008).
A commonly recognised fault of the BEWE is that it is difficult to quantify different severity of wear (O'Hara and Millar, 2020). A BEWE score of 3 could represent loss of all enamel on a surface or complete wear of tooth structure to the level of the gingiva. A recent study indicated that the use of the BEWE method on 3D models could offer a substantial advantage compared with a clinical examination alone when assessing and monitoring erosive tooth wear. However, it was difficult to differentiate between

intact enamel and initial loss of surface texture on 3D models (Alaraudanjoki et al., 2017).

There has not been much reported evidence regarding the in vivo measurement of erosive lesions using imaging techniques. One study reported of using optical coherence tomography (OCT) to quantify dental erosive lesions in patients suffering from GORD. However, it was time consuming and required preparation which is time consuming for the observer as well as the participant (Wilder-Smith et al., 2009). Therefore, no accurate method has been introduced as yet which can help diagnose and quantify erosive lesions in vivo. One of the reasons for the inaccuracy has been reported to be the absence of fixed intra-oral reference points (Attin, 2006). Therefore, the diagnosis is made based on clinical examinations.

2.2.11. In vitro assessment of dental erosion

Majority of the studies assessing the effects of dental erosion on the dental hard tissues have been performed either in vitro or in situ with the outcome measures ranging from microscopic imaging to physical and chemical analysis (Table 2.3) (Attin and Wegehaupt, 2014).

Table 2.3: Various methods of in vitro assessment of dental erosion (Attin, 2006).

Outcome measure	**Principle**	**Advantages**	**Disadvantages**
Scanning electron microscope (SEM).	Qualitatively estimation of surface alterations.	Applicable for wet samples.	Only qualitative assessment.
Optical coherence tomography.	Measuring the intensity of the echo of the reflected or backscattered infra-red light.	Non-invasive. No sample preparation. Excellent image resolution.	Cost. Lack of commercial availability. Lack of reference point.
Surface microhardness.	Determining changes in surface hardness.	Relatively low costs. Not time consuming.	Errors due to non-demineralized deeper layers. Flat surfaces needed.

Surface profilometer.	Determine the irreversible loss of dental hard. Tissue and surface roughness by a laser beam or a contact stylus.	Measurement in natural dentition.	Time-consuming. Stylus could damage surface.
Iodide permeability test.	Measures the amount of iodide recovered using Millipore pre-filter paper discs which provides information about the pore volume of the enamel.	Low costs.	Provides only. information about increased pore volumes.
Microradiography.	Quantification of mineral loss using X-rays.	Determination of both mineral loss and demineralization possible.	Limited resolution. Demanding sample preparation.
Confocal laser scanning microscopy (CLSM).	3-D reconstructions.	High resolution.	Only qualitative assessment.
Quantitative light-induced fluorescence.	The longitudinal assessment of early caries/erosive lesions using fluorescent line.	Surface scan is not time-consuming.	Limited resolution. Not much evidence in literature for erosion studies.
Atomic force microscopy (AFM)	High resolution imaging of measuring surface topography.	High resolution, non-destructive.	Time consuming. Only limited measurements.
Nano indentation.	Determines changes of surface hardness in nanometres.	Very sensitive; also provides information of material properties.	Time consuming demanding sample preparation.

The assessment methods listed above provide both qualitative and quantitative outcomes of dental erosion on the dental hard tissues. The majority of the assessment

tools are invasive or require sample preparation which can be time consuming. However, an optical tool that has recently gained interest in the field of dentistry is optical coherence tomography which has the benefit of being non-invasive and does not require extensive sample preparation (Machoy et al., 2017).

2.2.12. Optical Coherence Tomography

Optical coherence tomography (OCT) is a non-invasive diagnostic technique that provides cross-sectional images of biologic structures based on the scattering intensity profiles of infra-red radiations from a coherent light source (Katkar et al., 2018). OCT has been widely used in ophthalmic departments and it was applied for the first time in dentistry in 1998 in an in vitro study where the hard and soft tissues of the oral cavity were recorded. Since then, OCT has developed popularity and has been used for diagnosis of primary and recurrent/residual caries, chair-side composite restorations, and identification of pulp canals and extent of alveolar bone loss (Podoleanu et al., 2018).

2.2.12.1. Principle of OCT

The phenomenon of optical coherence tomography is based on the interference of two partially coherent light beams coming from a single source. 1) The reference beam and 2) the probe beam. One of the characteristics of biological objects, such as tissues and organs, is that they reflect light with different refractive indices (Hsieh et al., 2013). OCT images show light-scattering intensity from different layers of tissue. Because enamel and dentine have different scattering properties and the optical properties of healthy sound enamel differ from the demineralized tissue, the OCT images can provide information about the enamel thickness, morphology and porosity which may be a possible method to quantify demineralisation of enamel and dentine.

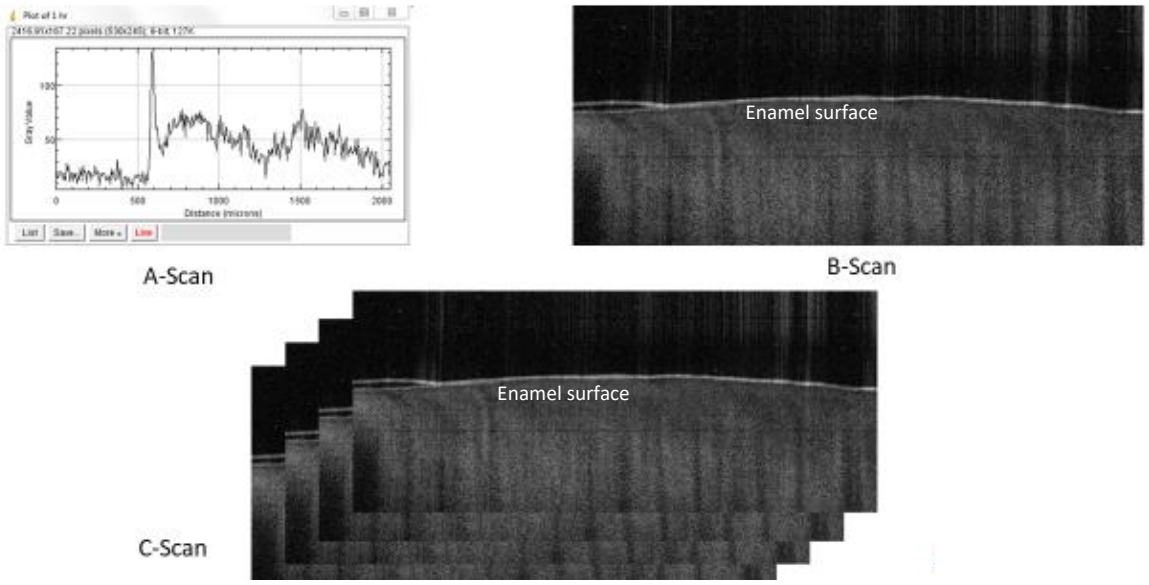
A scan: The graph of reflected wave power density as a function of the position of the reflective point, which is the source of the wave, is called an A-scan.

B scans give sagittal scans of the object, and

C scans is the lateral scanning images at a constant depth.

The combination of results lying in one plane creates a two-dimensional image of the section of the test (Machoy et al., 2017) (Figure 2.1).

Figure 2.1: The data/images obtained from OCT scanning in the form of A-scan, B-Scan and C-scan.



2.2.12.2. OCT and dental erosion

Detection of early erosive tooth wear is a problem of increasing clinical concern. Despite recent improvements in the understanding of the risk factors, currently there are no clinically accepted techniques to measure early erosion in vivo. Optical coherence tomography (OCT) has significant potential for detection of the earliest signs of enamel erosion (Huysmans et al., 2011), such as changes in surface texture (Bartlett et al., 2008; Austin et al., 2015) or enamel mineral loss (Amaechi et al., 2003; Popescu et al., 2008; Chew et al., 2014). There have been significant in vitro developments for the detection of early erosion using OCT (Chew et al., 2014); however, early erosive wear remains a very challenging clinical condition to detect in vivo. Therefore, further clinical research into early erosion detection is required, to inform on preventive measures.

Detection of enamel erosion using OCT signal changes is thought to rely on surface texture changes and increased sub-surface enamel porosity due to acid exposure, and thereby quantitatively changing the backscattered OCT signal in the immediate sub-surface layers (Huysmans et al., 2011). The histology of the early erosion lesion is thought to involve partial demineralization of the enamel surface and immediate sub-

surface enamel (ten Cate et al., 2008). The sub-surface demineralization significantly changes the refractive index of the demineralized tissue which can then be accentuated by dehydration effects to enhance OCT detection (Nazari et al., 2013; Popescu et al., 2008; Meng et al., 2009; Hariri et al., 2013). In vitro OCT erosion research has recently demonstrated that unpolished natural enamel surfaces demonstrated optical changes after 10 min of orange juice erosion; however, it was not clear how these changes should be optimally detected using the OCT A-scan profiles (Chew et al., 2014). Previous research into optimization of dental OCT for enamel demineralization assessment has proposed that using near-infrared wavelengths (1,310 nm) is ideal to improve the axial imaging depth in enamel, However, there is no consensus on the optimal technical specifications for in vivo dental OCT. In addition, the impact of clinical variables influencing in vivo enamel OCT imaging is not clearly understood. Thus, quantitative detection of early enamel erosion using OCT in vivo is complicated by multiple interacting surface phenomena which are poorly defined, including the composition and function of the acquired enamel pellicle (Hannig et al., 2003; Carpenter et al., 2014; Moazzez et al., 2014), the degree of hydration of the dental hard tissue during imaging (Nazari et al., 2013; Chan et al., 2014), and the micro-textural changes of the curved enamel surface in vivo during erosion (Austin et al., 2015). Therefore, carefully controlled clinical studies simulating early erosion are required to elucidate the optimal analytical techniques for imaging early erosive changes.

The data achieved from OCT scans has been analysed in various studies by detecting the change in refractive index (Aden et al., 2017a), change in back scattering intensity (Austin et al., 2017) or change in enamel thickness (Alghilan et al., 2019a) after periods of exposure to acidic mediums (Table 2.4).

Table 2.4: Dental erosion studies using OCT as an assessment tool.

Author/Country	**Type of study**	**Type of OCT data**	**Results**
(Wilder-Smith et al., 2009), Switzerland	In vivo, human enamel.	Change in enamel thickness.	Loss of enamel thickness.
(Kang et al., 2010), USA.	In vitro, bovine enamel.	Change in integrated reflectivity (IR).	Significant difference in (IR).
(Chan et al., 2013), USA	In vitro, bovine enamel.	Change in enamel thickness.	Loss of enamel thickness.

(Chew et al., 2014), United Kingdom.	In vitro, bovine enamel.	Change in intensity of backscattered light.	Significant change in intensity.
(Algarni et al., 2016), USA.	In vitro, human enamel.	Change in enamel thickness.	Significant change in enamel thickness.
(Aden et al., 2017a), United Kingdom.	In vitro, bovine enamel.	Change in reflection intensity.	Linear relationship with micro-hardness.
(Aden et al., 2017b), United Kingdom.	In vitro, bovine enamel.	Lesion optical properties.	Linear relationship with lesion depth.
(Austin et al., 2017), United Kingdom.	In vivo, Randomised Control Trial.	Change in backscattering intensity.	Increased back scattering intensity.
(Mylonas et al., 2018), United Kingdom.	In vitro, human enamel.	Change in surface reflectivity.	Decrease in surface reflectivity.
(Alghilan et al., 2019b), USA.	In vitro, human enamel.	Thickness of enamel.	No significant decrease in thickness.

The studies summarised in table 4 suggest the different methods adopted to analyse the data obtained after OCT investigations. The change in surface reflection intensity (SRI) and back scattering was adopted to detect initial dental erosion based on the principle of increased surface roughness of the enamel as a result of acid exposure and demineralization (Chew et al., 2014). Demineralization presents as a white margin which is obtained due to the reflection of the laser beam, and together with the quantitative analysis of the data, OCT can provide both qualitative and quantitative results (Katkar et al., 2018). The white margin representing demineralisation of enamel has also been observed while detecting early carious lesions in proximal areas of the teeth confirming the scattering of the laser beam due to demineralisation (Ei et al., 2019). However, the quantification of erosive lesions by measuring the surface reflection intensity (SRI) of the backscattering light requires expertise by a trained professional and may not be practical during clinical use.

The other method adopted to measure erosive lesions was by measuring the thickness of enamel via B-scans obtained from the OCT imaging data (Alghilan et al., 2019). However, the erosive lesions were measured after 12-24 hours of acid exposure suggesting OCT images to be beneficial in detecting and measuring advanced surface

lesions with enamel surface loss. Disadvantages of the OCT include difficulty in accessing all positions within the oral cavity, relatively high costs and limited penetration depth (less than 3 mm) (Attin and Wegehaupt, 2014).

2.2.13. X-Ray Microtomography (XMT)

XMT has been shown to be an excellent tool to evaluate morphological features, such as changes in shape and the presence of voids in ex vivo studies of root canal instrumentation and obturation (Peter et al., 2001, Jung et al., 2005) . With appropriate beam-hardening correction and calibration, the technique can also be used to study dental hard tissue loss following acidic challenge, by quantification of mineral concentration (Wilmott et al., 2007). The apparatus involves the use of x-rays to create cross-sections of a physical object which recreates a three dimensional model without destroying the original object. XMT has been widely used in dental research; enamel thickness and tooth measurement, analysis of root canal morphology, craniofacial skeletal development and tissue engineering (Swain and Xue, 2009). It has also been used for measuring the extent of enamel demineralization and has proved to be one of the efficient measures to assess demineralisation of the the the dental hard tissues (Davis et al., 2018) due to its accuracy and ability to montior the progression of demineralisation with time. On the other hand, the due to the specimen preparation and alignment, and the geometry of the X-ray, the imaging precision at the sample edge is limited and the outermost 5–10 μm cannot be exactly reproduced. Additonaly, the technique demands sample preparation which is time consuming (Attin and Wegehaupt, 2014).

2.2.14. Surface microhardness

Surface microhardness has been used in many studies, in fact has been the main outcome measure of choice in many dental erosion studies (Niemeyer et al., 2020, Carvalho et al., 2020b, Jordao et al., 2017, Cheaib and Lussi, 2011, Min et al., 2011). As erosion involves softening of the enamel, early stages of enamel and dentine dissolution without surface loss, can be determined. Surface microhardness involves the indentation of a diamond tip of known geometrical dimensions for a given load and duration (Voort and Lucas, 1998). For microhardness assessments of eroded tooth surfaces, mostly Knoop or Vickers diamonds are used on polished surfaces. Polished surfaces are recommended to produce well-defined indentations. The lengths of the

indentations on the surface are measured under a microscope requiring indentation lengths of about 30–40 µm for precise measurements.

Microhardness measurements allow discrimination of different erosive potentials of various substances on dental hard tissue, even after short exposures (3 min) to acidic agents (Lussi et al., 2000). In other studies, immersion periods of at least 20 min were chosen to investigate the impact on surface hardness (Lussi et al., 1993, Lussi et al., 2004). However it should be noticed that the differences between different aggressive solutions might be less pronounced, since long expositions to different acidic substrates lead to similar depth of the surface softened layer.The main advantages of microhardness determinations are the relatively low costs and the fact that it is a well-established method and could be combined with measurements of abrasive surface loss. On the other hand, on erosively altered surfaces, the outlines of the indentations are sometimes fuzzy, rendering precise measurements difficult.

2.2.15. Calcium ion release

Determination of dental enamel dissolution by assessing the amount of calcium released from the apatite crystals of dental hard tissues is also be regarded as a possible tool for assessing early dental erosive lesions. Studies have performed calcium determination in erosive, acidic solutions after prolonged contact of the solutions with dental hard tissues using calcium sensitive electrodes, atomic absorption spectrophotometer or the highly sensitive method of inductively coupled plasma mass spectrometry (Sieber et al., 2019, Baumann et al., 2016, Carvalho et al., 2020a). The method of measuring calcium ion release is highly sensitive, allowing detection of very small amounts of mineral loss, however is susceptible to errors due to contamination. Additionally, the method has only been used for in vitro experiments.

2.2.16. Calcium ion content – Energy dispersive X-ray spectroscopy

Scanning electron microscopy (SEM) can be coupled with energy-dispersive X-ray spectroscopy (EDX) for the microanalysis of the elemental composition of the top few micrometers of a sample surface. An electron beam hitting the surface leads to excitement of atoms resulting in the emission of X-rays, which provides quantitative information about the distribution of various elements such as calcium, phosphate, stannous or fluoride. However, suitability of the method for evaluating erosive

processes has not been clearly demonstrated as yet. The major drawback of this method is the formation of precipiates or organic elements onto the enamel surface which may hinder the electron beam from striking the enamel surface and produce errors in the results (Hannig et al., 2005).

The oral cavity offers a physiological protective mechanism against DE with the help of the saliva and its proteinaceous layer, the salivary dental pellicle. The next section will provide an account of the properties of the saliva and dental pellicle and the mechanisms by which they have the potential of reducing dental erosion.

2.3. Saliva

The oral cavity is kept moist by saliva which is secreted by the salivary glands. It is composed of both organic and inorganic components. The inorganic component comprises the mineral contents in the form of Ca^{2+}, PO_4^{2-} and other ions, whereas the organic component is made up of various proteins and glycoproteins (Carpenter et al., 2014). Saliva plays a major role in protecting the oral cavity from both external and internal insults. The protective property is achieved by its vast compositional elements each having a specific function (Table 2.5).

Table 2.1: Functions of saliva (Almeida et al., 2008).

Function	**Component**
Buffering.	Carbonic anhydrase, histatin.
Digestion.	Amylase, mucins.
Mineralisation.	Cystatin, statherins, proline-rich proteins.
Lubrication.	Statherins, mucins.
Anti-bacterial.	Peroxidases, histatins.
Anti-fungal.	Histatins.
Anti-viral.	Cystatins, mucins.

Saliva acts as a buffer to maintain the pH of the oral cavity during a low pH environment formed due to acidic intakes, or reflux due to gastro oesophageal reflux disease (GORD) and eventually helps to maintain the integrity of the enamel preventing demineralization of the tooth. The buffering action is achieved by the combination of the carbonic anhydrases enzyme (Hegde and Sajnani, 2017), which neutralises the acid, and the calcium and phosphate ions present in the saliva which provide enamel

homeostasis. Despite the preventive properties of saliva, dental erosion is still prevalent in the athlete population. The possible reasons maybe dry mouth encountered due to the reduced salivary flow during intense physical activity (Horswill et al., 2006).

Despite the presence of reduced saliva flow during training, the proteinaceous layer of saliva known as the salivary pellicle, remains adsorbed to the tooth surface which may have the potential to prevent dental erosion (Lindh et al., 2014).

2.4. Dental Pellicle

The dental pellicle, a thin acellular film predominantly consisting of salivary proteins, covers surfaces that are exposed in the oral cavity (Lindh et al., 2014). The formation of the dental pellicle starts soon after brushing or scaling with an initial layer of 20nm and reaches its maximum consistency and thickness of 1000nm within 24 hours (Zimmermann et al., 2019). This protein rich layer acts as a diffusion barrier preventing the erosive attack by the dietary acid on the tooth surface (Vukosavljevic et al., 2014).

2.4.1. Formation of dental pellicle

The first step of dental pellicle formation involves the adsorption of the salivary proteins onto the tooth surface. The first group of proteins to adsorb onto the enamel surface are the pellicle precursor proteins (PRPs) due to their high affinity to bind to hydroxyapatite. These can be classified into acidic proline rich proteins, statherins and histatins (Hannig and Joiner, 2006). The calcium ions present in the enamel crystallites have a stronger tendency than the phosphate ions to dissolve in saliva. This results in an overall negative charge on the enamel surface due to the remaining phosphate ions. This surface is then coated with a positively charged layer of calcium counter-ions. Hence, dental pellicle is primarily adsorbed at the enamel surface as a result of the electrostatic interactions between the ionic double layer (calcium and phosphate ions) on one side, and correspondingly charged groups of the pellicle proteins on the other giving the dental pellicle its mechanical stability.

Dental pellicle formation occurs as a result of the combination of electrostatic interactions, entropic changes of adsorbed molecules, hydrophobic aggregations and Van Der Waal's forces between the enamel surface and the proteins of the saliva (Hannig and Joiner, 2006).

2.4.2. Structure

Transmission Electron Micrograph (TEM) studies of saliva films on bovine enamel have shown that the structure of the dental pellicle consists of two layers; a dense inner layer tightly adhered to the substrate, and a loosely arranged outer layer (Lindh et al., 2014). The inner layer is the initial layer of the dental pellicle to form on the enamel surface and consists of mostly statherins, histatins and proline-rich proteins (PRPs).

The young two hour pellicle has been found to be 100-500 nm thick (Hannig, 1999). This can increase to 1000nm within 24 hours on the buccal and lingual sides. This rapid increase in thickness is due to the adsorption of protein aggregates on to the initial surface. These protein aggregates appear in the form of globular structures having a hydrophobic interior and a negatively charged surface attaining a micelle like structure. Recently, these micelle like structures have been identified to be the major components of the dental pellicle (Hannig and Joiner, 2006). It has been suggested that only mature, several day-old pellicles can prevent enamel demineralisation. However, when 24-h and 7-day-old pellicles were compared, there was no significant difference in the protective ability of the pellicle (Hannig et al., 2004).

2.4.3. Composition of the dental pellicle

The dental pellicle is a dense protein layer composed of a combination of proteins, carbohydrates and lipids. (Hannig and Joiner, 2006). The major proteins consist of phosphoproteins, acidic proline-rich proteins, histatins, statherin, cystatin SA-I, albumin, a-amylase, lysozyme, lactoferrin, carbonic anhydrase, immunoglobulins and mucins (Hannig and Homburg/Saar, 2002). There are phosphoproteins such as statherin, histatin and proline-rich proteins (PRPs) and are capable of exchange reactions with the phosphate ions of the enamel.

2.4.4. Proteins

Several techniques have been applied to identify and quantify the number of proteins present in the dental pellicle using SDS-polyacrylamide gel electrophoresis (PAGE) and fast protein liquid chromatography (FPLC) and have given variable results (Ash et al., 2014). All of the studies have reported that the dental pellicle contains a fraction of

the proteins present in the whole mouth saliva (Table 2.6). One study showed that individuals with dental erosion have half the number of proteins within the acquired pellicle as compared to patients without dental erosion (Carpenter et al., 2014).

Table 2.2: Assessment of dental pellicle proteins.

Author	**Country**	**In vivo /In vitro**	**Method used**	**Proteins identified**
(Taira et al., 2018).	Brazil.	In vivo.	Shotgun label-free quantitative proteomic analysis using mass spectrometer.	180
(Cassiano et al., 2018).	Brazil.	In vivo.	Shotgun label-free quantitative proteomic analysis using mass spectrometer.	260
(Delecrode et al., 2015).	Brazil.	In vivo.	Liquid chromatography coupled to mass spectrometry.	72
(Cukkemane et al., 2014).	Netherlands	In vitro.	Phage display.	12 peptide sequences.
(Carpenter et al., 2014).	United Kingdom.	In vivo.	SDS-PAGE.	30% less proteins in erosion patients.
(Ash et al., 2014).	United Kingdom.	In vitro.	Sodium dodecyl sulphate (SDS) and sodium tri polyphosphate (STP).	84
(Zauber et al., 2012).	Germany.	In vitro.	LC-MS/MS Gas chromatography (GC) coupled to time-of-flight mass spectrometry (TOF-MS).	Total 1405 proteins out of which 203 were quantified.
(Siqueira and Oppenheim, 2009).	U.S.A.	In vitro.	Tandem mass spectrometry.	78
(Vitorino et al., 2008)	Portugal.	In vivo.	Matrix-assisted laser desorption/ionization-Mass spectrometer.	90
(Vitorino et al., 2007).	Portugal.	In vivo.	Liquid chromatography and mass spectrometry analysis. Tandem mass spectrometry.	6 phosphorylated peptides 30 fragment peptides
(Siqueira et al., 2007).	U.S.A	In vivo.	Liquid chromatography and mass spectrometry analysis.	130.
(Proctor et al., 2005).	United Kingdom.	In vitro.	Electrophoresis and immunodetection	Statherin having a molecular mass of 5380 Da

The studies which detected acid resistant proteins identified serum albumin, statherin (Taira et al., 2018) and cystatin-B (Delecrode et al., 2015) to be in abundance after acid exposure in vitro. The proteins identified are part of the pellicle precursor proteins (PRPs) which are the initial proteins to adsorb during pellicle formation. An abundance in the identified PRPS after acid exposure suggests their potential to protect against DE.

2.4.5. Carbohydrates

The dental pellicle contains several sugars with glucose being the most abundant. The glucose originates from the enzyme glucosyltransferases which is adsorbed in the pellicle layer. This enzyme will synthesise glucans that will be the main source for the pellicle's glucose content. Glycolipids, which are the major pellicle lipids, have also been proposed as a source of glucose, as the carbohydrate portion of the glycolipids consists of glucose only (Hannig and Joiner, 2006).

2.4.6. Lipids

Lipids account to about 22% of the weight of the dental pellicle. These are mostly neutral lipids, phospholipids and glycolipids. However, further studies are still required to identify the characteristics and functions of lipids in the dental pellicle (Hannig and Joiner, 2006)

2.4.7. Functions of the dental pellicle

The pellicle is of great importance when it comes to physiological and pathophysiological functions in the oral cavity.

2.4.7.1. Mineral Homeostasis

Saliva is supersaturated with calcium and phosphate ions and provides the potential for remineralisation of the demineralised enamel surface. Acidic proline rich proteins (PRPs) reveal a high affinity for hydroxyapatite and are important components of the in vivo-formed pellicle layer. These pellicle proteins can act as inhibitors for surface induced precipitation of calcium salts onto the enamel thus providing mineral homeostasis at the enamel–saliva interface (Hannig and Joiner, 2006).

2.4.7.2. Lubrication

The pellicle tends to lubricate the surface of the oral cavity with its protein component, the mucin glycoproteins I and II as well as statherins. The heavy molecular mucin glycoproteins MG1 prevent friction from abrasive and attrition forces hence inhibiting the process of tooth wear (Hannig and Joiner, 2006).

2.4.7.3. Semi Permeable Barrier

The pellicle acts as a semi permeable barrier by providing a medium through which fluoride, calcium and phosphate are delivered which is essential for enamel homeostasis. This suggestion was in accordance with high-resolution SEM and AFM images of the dental pellicle layer which indicated a porous mesh-like surface pattern (Hannig and Joiner, 2006).

2.4.8. Mechanical Characteristics of Dental Pellicle

It has already been mentioned that dental pellicle formation occurs as a result of the combination of electrostatic interactions, entropic changes of adsorbed molecules, hydrophobic aggregations and Van Der Waal's forces between the enamel surface and the proteins of the saliva hence providing the adhesive strength to it. However, mechanical stability is not the only factor contributing to the pellicle's preventive effect; the thickness of the dental pellicle also contributes to prevention from acid attack (Zhang et al., 2016). The thickness of the dental pellicle has been studied using null ellipsometry, atomic force microscope (AFM) (Boyd et al., 2021), transmission electron microscope (TEM) (Hannig et al., 2009) and optical coherence tomography (OCT) (Baek et al., 2009). In a recent study, the thickness of the inner layer of the dental pellicle was measured using nano-indentation techniques and was calculated to be 18 nm thick (Zhang et al., 2018). OCT studies calculated the thickness of the dental pellicle to range from 0.29-1.86 mm (Algarni et al., 2016). The thickness can vary according to different sites in the oral cavity. For instance, the thickest pellicle was found at the lower posterior lingual surface (1.06µm) and the thinnest at the upper anterior palatal surface (0.3µm) (Amaechi et al., 1999). On the other hand, in one study no significant difference in thickness was found between incisors and molars (Zhang et al., 2016). Generally, the pellicle layer towards the vestibule has been found to be much thicker than the pellicle found towards the oral side.

The dental pellicle is a heterogeneous layer with an increasing gradient in density and the density increases as the thickness of the pellicle increases with time. According to Coulomb's law, the magnitude of the electrostatic force has a direct influence on the thickness and structural pattern of the adsorbed salivary pellicle layer. As a result, the electrostatic interactions gradually increase giving the dental pellicle its adhesive strength (Zhang et al., 2018).

Adsorption time is also a key factor in increasing the adhesive power of the dental pellicle with the enamel surface. A study proved that 30 minute pellicles had a higher adhesive and shear strength as compared to 5 minutes pellicles, and a greater potential of protection (Zhang et al., 2015). The adsorption and thickness of the pellicle has also been studied using quartz crystal microbalance with dissipation.

2.4.8.1. Quartz crystal microbalance with dissipation

QCM-D determines the rate of mass deposition in thin film deposition systems under vacuum or the adsorption of molecules or deposition of nanoparticles on to a crystal sensor surface in liquid by monitoring the frequency change and dissipation responses of an oscillating quartz crystal sensor (Chen et al., 2016). QCM-D has been used in studies to assess the effects of several interventions on the dental pellicle. One study has reported a more elastic and an increase in hydrated mass of the dental pellicle after addition of calcium ions (Ash et al., 2013). Another study reported a less dense but with a higher elastic pellicle after addition of sodium dodecyl sulphate (SDS) and sodium tripolyphosphate (STP), resulting in a more diffused pellicle. (Ash et al., 2014). A third study reported changes in structure and physical properties of the dental pellicle due to protein dehydration and protein-polyphenol complexation upon introduction of polyphenolic molecules, increasing the astringency of the dental pellicle (Lei et al., 2021).

2.4.9. Role of Pellicle in Erosion

The dental pellicle is an insoluble network of adsorbed salivary proteins. Demineralization of the tooth surface occurs after diffusion of the acid through its mesh-like structure and semi permeable layer, or after removal of the acquired pellicle by the interacting acid. Hence, the pellicle cannot prevent direct contact between the

acid and the tooth surface; however, it can reduce and retard the immediate interaction between acids and the tooth surface. In addition to the structural features of the pellicle, the proteins present in the dental pellicle play a role in the prevention of erosion. The acidic proline-rich proteins, histatins or statherins contain maintain high concentrations of calcium within the pellicle layer and close to the apatite surface, hence stabilizing the enamel surface and reducing the erosive demineralization (Hannig and Hannig, 2014).

The thickness and viscoelasticity of the pellicle plays a major role in prevention of dental erosion. The thickness of the pellicle is mainly contributed by the adsorption of proteins onto the enamel surface. As the number of proteins increase in number, the pellicle becomes thicker. A thicker pellicle would prevent the direct acid attack onto the tooth surface preventing demineralisation. Similarly, a more viscoelastic the pellicle would allow the diffusion of calcium ions into the pellicle aiding in remineralisation of the dental hard tissue aiding in enamel homeostasis (Ash et al., 2013).

Despite the structural properties of the dental pellicle, dental erosion is still quite abundant in high-risk populations. Calcium ions have been reported to have been released due to dissolution as a result of constant exposure to the acid (Houghton et al., 2020). Transmission electron microscope studies have also suggested the removal of the globular layers of the pellicle after a 120 minutes of exposure to various soft drinks ranging from pH 2.8 to 3.8 hence decreasing the protective effect of the pellicle (Hannig et al., 2009). Proteomic analysis of the pellicle has also shown a decrease in the total protein content after exposure to 3% citric acids for 120 minutes (Taira et al., 2018). Another study reported a protective effect from pellicle in healthy patients compared to those with dental erosion (Moazzez et al., 2014) suggesting that pellicle has the potential to protect against dental erosion, however not for prolonged periods of time.

Preventive measures have been performed by modifying the dental pellicle with interventions and the details of the studies attempting to modify the pellicle to prevent DE are explained in a later chapter.

2.5. Summary

In summary, the evidence suggests that oral disease is prevalent in athletes which is negatively affecting the training and performance of athletes. The reason behind the high incidence of DE is unknown, however maybe multifactorial ranging from reduced salivary flow to frequent consumption of sports/energy drinks. One method for reducing the erosive potential of sports drinks is to raise the pH through the addition of buffering agents. However, the resulting formulations are often unpalatable. Therefore, a different approach whereby the erosive potential of a sports drink is reduced by strengthening the dental pellicle (DP) is proposed and is the main aim of the PhD. Understanding the mechanisms by which the dental pellicle protects the tooth against erosion will allow to develop effective intervention/prevention strategies to help reduce dental erosion, not only in elite athletes, but also other vulnerable groups.

3. Study hypothesis and research question.

Chapter 2 presented a review of the literature and identified gaps in the knowledge base to inform on the potential of the salivary dental pellicle to prevent dental erosion. The resulting study hypotheses and related research questions are presented in this chapter.

Study hypothesis 1: Optical coherence tomography detects early dental erosive lesions.

Research question 1: Is optical coherence tomography capable of detecting early dental erosive lesions? – An in vitro model

Study hypothesis 2: Intense physical training has an impact on saliva parameters

Research question: Does intense physical training have an impact on saliva parameters which would possible be a risk factor contributing to dental erosion.

Study hypothesis 3: Salivary dental pellicle modification impacts dental erosion.

Research question 2: Does salivary dental pellicle modification have an impact on dental erosion? – A systematic review

Study hypothesis 4: Pellicle modification with micellar casein prevents dental erosion.

Research question 3: Will incorporating micellar casein into the dental pellicle reduce the magnitude of dental erosion?

Table 3.1 outlines the research questions, corresponding study designs and chapter numbers as a brief summary of the research presented in the remainder of this thesis.

Table 3.1: Thesis structure.

Research question	**Study design**	**Chapter**
Is optical coherence tomography capable of detecting early dental erosive lesions? – An in vitro model.	In vitro observational study.	Chapter 4
Does intense physical training have an impact on saliva parameters which would possible be a risk factor contributing to dental erosion?	Systematic review and meta-analysis.	Chapter 5
Does salivary dental pellicle modification have an impact on dental erosion?	Systematic review.	Chapter 6
Will incorporating micellar casein into the dental pellicle reduce the magnitude of dental erosion?	In vitro randomised controlled trial.	Chapter 7

Therefore, the starting point for this PhD was to develop an in vitro dental erosion model capable of detecting early dental erosive lesions with optical coherence tomography which could potentially be used for the intervention studies. The following chapter presents a detail of the aims, methods and results of the model.

4. Development of an in vitro model to detect early dental erosion lesions

4.0. Background

The main introduction chapter identified the need for development of an in vitro model to investigate early dental erosion. Therefore, this chapter will discuss the development of an in-vitro erosion model which can be used to detect and quantify early dental erosion lesions.

4.1. Introduction

Previous studies have adopted various methods to create dental erosion lesions in vitro using both human (Assuncao et al., 2019) and bovine (Zanatta et al., 2019) teeth as substrates of enamel. Citric acid has been used as the erosive medium in most studies as it is one of the most common dietary acids found in citrus fruits and many common sports and energy drinks (Zheng et al., 2009). Secondly, citric acid has been shown to produce softened enamel after 60 seconds of exposure (O'Toole et al., 2017), hence suggesting it to be used as an erosive medium capable to produce dental erosion lesions in in vitro studies.

4.1.1. Measuring an in vitro erosive lesion

Detecting early signs of erosion can be difficult and require expertise to apply them for clinical diagnosis (Margaritis et al., 2011). Early dental erosion may be defined as demineralisation of enamel without enamel surface loss (Huysmans et al., 2011). Optical coherence tomography (OCT) is a non-invasive diagnostic tool which captures cross-sectional images of biological tissues (Katkar et al., 2018). It has recently gained interest in both in vitro (Alghilan et al., 2019b) and in vivo (Wilder-Smith et al., 2009) erosion studies but the studies did not detect the initiation of the erosive process or early erosive lesions, but advance erosive lesions. Erosive lesions resulting in enamel surface loss may be termed as advanced lesions as opposed to the early erosive lesions (Huysmans et al., 2011). On the other hand, another study managed to quantify early dental erosion lesions with OCT by measuring the mean thickness of enamel from the surface to the dentine-enamel junction before and after erosion (Alghilan et al., 2019b), but that could have been more accurate if the thickness of enamel would have been the similar for all teeth.

X-ray microtomography (XMT) is another possible method to detect early dental erosion by detecting and quantifying morphological changes such as changes in shapes and presence of voids in the dental hard tissues. However, this imaging technique can only be used in in vitro studies (Davis et al., 2018). It is the currently accepted gold standard for the measurement of mineral loss by measuring the change in X-ray beam intensity as demineralization progresses (Darling et al., 2009) producing three-dimensional images of a material's internal structure (Landis and Keane, 2010). However, so far not many studies have been performed to assess dental erosion using XMT and we shall use it to validate the results from OCT.

4.2. Aim and objectives

The aim of this study was to develop an in vitro dental erosion and dental pellicle model which would be capable of detecting early erosive lesions with and without the presence of dental pellicle. The purpose of the model was subsequently to apply it for interventions into the dental pellicle in order to reduce dental erosion.

- To create an in vitro erosive lesion on enamel surfaces that can be detected and quantified using OCT and X-ray microtomography.
- To create an in vitro erosive lesion on enamel surfaces in the presence of a layer of dental pellicle that can be detected and quantified using OCT and X-ray microtomography.
- To compare the development of erosive lesions in the in vitro model in the presence and absence of dental pellicle.

4.3. Materials and methods

4.3.1. In vitro erosion model experiment

4.3.1.1. Collection of study teeth

Bovine incisors were used in this study as a substitute for human teeth due to them being readily available from abattoirs. Secondly their increased size relative to human teeth made them easier to handle and process, while maximising the available surface area to form erosive lesions.

Fresh bovine incisors were extracted and collected from slaughtered bovine jaws at an abattoir (Newman's Abattoir T/As P.C.Turner & Ockwells Meat Co. Farnborough)

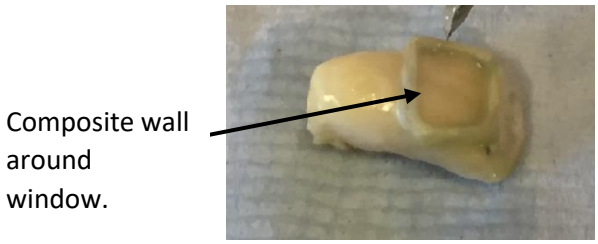
after obtaining approval from the Department of Environment, Food and Rural Affairs (DEFRA). The cows had been slaughtered for normal meat consumption and not for research purposes. A circular saw was used to separate the crowns from the roots as the enamel surfaces were only required for the study. The teeth were transported back to the Eastman Dental Institute (EDI) where they were disinfected by placing them in 70% ethanol for five days. Each tooth was then cleaned and scraped with a rough sponge in order to remove any dead soft tissue and blood stains after which they were stored in 0.1% thymol solution (pH 8.30). The thymol solution was prepared by dissolving 1gm of thymol powder in 1000ml of distilled water and stirred at 60ºC using a temperature-controlled stirrer.

4.3.1.2. Preparation of study teeth

A sample size calculation was not possible for the study as no similar study had been performed before from which the sample size could be estimated and calculated from the standard deviations of previous studies. Twenty bovine incisors were divided into two groups (n=10) each to create in vitro erosive lesions as the first objective.. A sample size of ten teeth per group was chosen due to time restraints. The teeth were removed from thymol and air dried for thirty minutes before embedding them in acrylic moulds in a way that the labial surfaces to be eroded were exposed and lay perpendicular to the optical coherence tomography (OCT) probe.

140 grams of acrylic resin was prepared in the ratio of 1:2.5 where 40 grams of resin was mixed and stirred with 100 grams of the activator. The mixture was poured into twenty individual moulds, each containing one tooth and left to set for 24 hours. After 24 hours the exposed labial surfaces were polished with 500 grit and finished with 2000 grit to remove the surface amorphous enamel and obtain a flat surface. They were sonicated for 3 minutes in deionized water to remove the residual smear layer resulting from polishing. Light cure composite (Constic) was used to construct a 1 mm high wall around a 5 x 5 mm window on the exposed polished labial surface, to act as a reservoir for the erosive medium and pellicle to adsorb on the tooth surface (Figure 4.2). It also acted as a reference point to differentiate the enamel layer before and after exposure to the erosive medium.

Figure 4.1: Light cure composite wall constructed around a 5 x 5 mm window on the labial surface of bovine incisor.

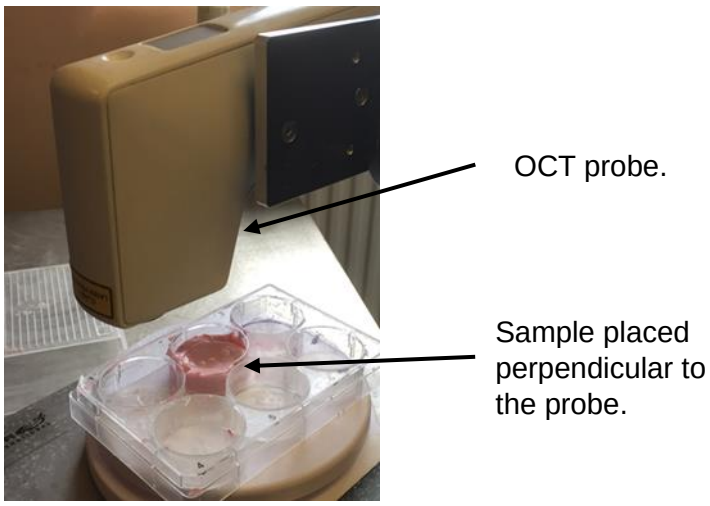


The teeth were divided into two groups; Group 1 (n =10): Teeth exposed to erosion experiment; and Group 2 (n=10): control group exposed to artificial saliva. Artificial saliva (AS; 1.5mM $Ca(NO_3)_2$, 0.90mM KH_2PO_4, 130mM KCl and 60mM Tris buffer; pH 7.4) (Baumann et al., 2016) was preferred over human saliva due to the incidence of protein denaturation and alterations in the composition of human saliva due to environmental exposure.

4.3.1.3. Measurement of enamel thickness with OCT

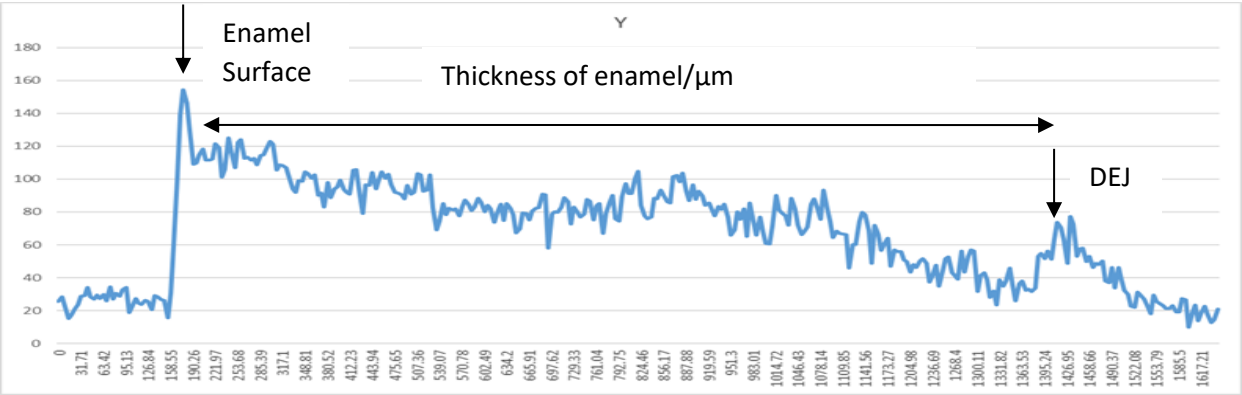
The teeth were then scanned with OCT VivoSight (Michelson Diagnostics) and the thickness of enamel before erosion was recorded by measuring the distance between the enamel surface and the dentine--enamel junction. The OCT probe was calibrated to scan 500 slices from an area of 4x4 mm of the erosion window. The tooth sample was placed perpendicular to the laser beam which was set to oscillate to capture half of the erosion window and half composite wall to mark a reference point (Figure 4.1).

Figure 4.2: Bovine sample placed perpendicular to the OCT probe to scan the labial surface.



A mark was placed with the help of a permanent black marker on the labial surface at the point where the oscillation started in order to scan the same area after exposure to the erosive medium.. Forty A-scans were extracted from one B-Scan of each tooth at different time points and the distance between the enamel surface and the dentin-enamel junction (DEJ) was measured bythe method described by a study measuring enamel thickness with OCT (Algarni et al., 2016). The captured images were transferred to the software ImageJ [1.50i(r2), National Institute of Health)] which extracted the A-scan graphs out of a selected area of the B-scans after erosion (Figure 4.3). Enamel surface-DEJ distance was measured by drawing a line perpendicular to the specimen's surface extending through the DEJ. After plotting the data, each of the graph lines was compared against their referred lines drawn on the specimen micrographs. The first peak of the graph line, near the enamel surface, indicated the change in reflectivity between the background and the specimen, and therefore was selected to represent the enamel surface. The second peak was selected as being the peak that indicated the most evident change in reflectivity between enamel and dentin, near the DEJ area. The peaks were visually identified by the examiner. The difference between the magnitude of the first surface peak and the second (DEJ) peak was calculated using the coordinates of the peaks to determine the enamel thickness... The mean thickness of enamel before and after erosion was calculated for both groups from which the mean loss of enamel after erosion was calculated

Figure 4.3: A-scan used to calculate thickness of enamel before and after erosion.



4.3.1.4. Measurement of enamel thickness using X-ray Microtomography (XMT)

All samples from the in vitro erosion study as well as the in vitro pellicle model were scanned under x-ray microtomography at the Queen Mary, University of London (Perkin Elmer PE 4343) to calibrate the results obtained from OCT. The samples

were transferred to the QMUL in individual containers per group and scanned over night to obtain the images. The images were analysed using the software Tomview to measure the thickness of enamel.

4.3.1.5. Preparation of erosive medium

The erosive medium used was in the form of 0.1M citric acid with pH 3.2 based on literature which has reported citric acid being used as a medium for erosion experiments (Aden et al., 2017, Aykut-Yetkiner et al., 2014, Siber et al. 2019). For this purpose, 19.2 grams of citric acid powder was dissolved in 1000 ml of distilled water in a beaker and stirred until it was dissolved. The initial pH was measured using a pH scale which measured the pH (2.2). The pH was brought to 3.2 by adding sodium hydroxide (NaOH) pellets into the acid and maintained at room temperature of 22^{o}C.

4.3.1.6. Creation of the erosive lesion

Group 1 was immersed in 10 ml of citric acid solution in culture dishes which were agitated on a rocking table at an RPM of 60 at room temperature. The teeth were removed at each time point of, 1 hour, 2 hours, 3 hours and 4 hours, sonicated for three minutes in deionized water (Zimmermann et al., 2019) to remove any precipitates settled on the tooth surface as a result of demineralization from the acid, which may make it difficult to locate the enamel surface during scanning with OCT. The samples were then scanned under OCT. The same protocol was applied for the control group and the images for both groups recorded and analysed.

4.3.2. In vitro pellicle model experiment

4.3.2.1. Preparation of study teeth

Thirty bovine incisors that were stored in thymol were used to develop the in vitro pellicle model. The teeth were prepared as per protocol similar to the in vitro erosion model. However, the teeth were divided into three groups; Group 1 (n =10): Teeth exposed to erosive medium; Group 2 (n=10): Teeth exposed to erosive medium in the presence of a layer of dental pellicle, and Group 3 (n=10): control group exposed to artificial saliva.

4.3.2.2. Whole mouth saliva collection

Whole mouth saliva was obtained from one male participant aged 32 years following informed consent. The experiment was carried out in accordance with the approved guidelines and regulations of the UCL research and ethics committee. The participant was fit and healthy, non-smoking and not under any regular medication for any systemic illness. He was asked to avoid any food consumption one hour before saliva collection to avoid any contamination. He was then asked to passively drool 5 ml of his saliva into a labelled falcon tube. Immediately after collection, the whole saliva was centrifuged for 20 min at 4°C (4,000 g). The supernatants were collected in another falcon tube and stored at –80°C (Bhattarai et al., 2018).

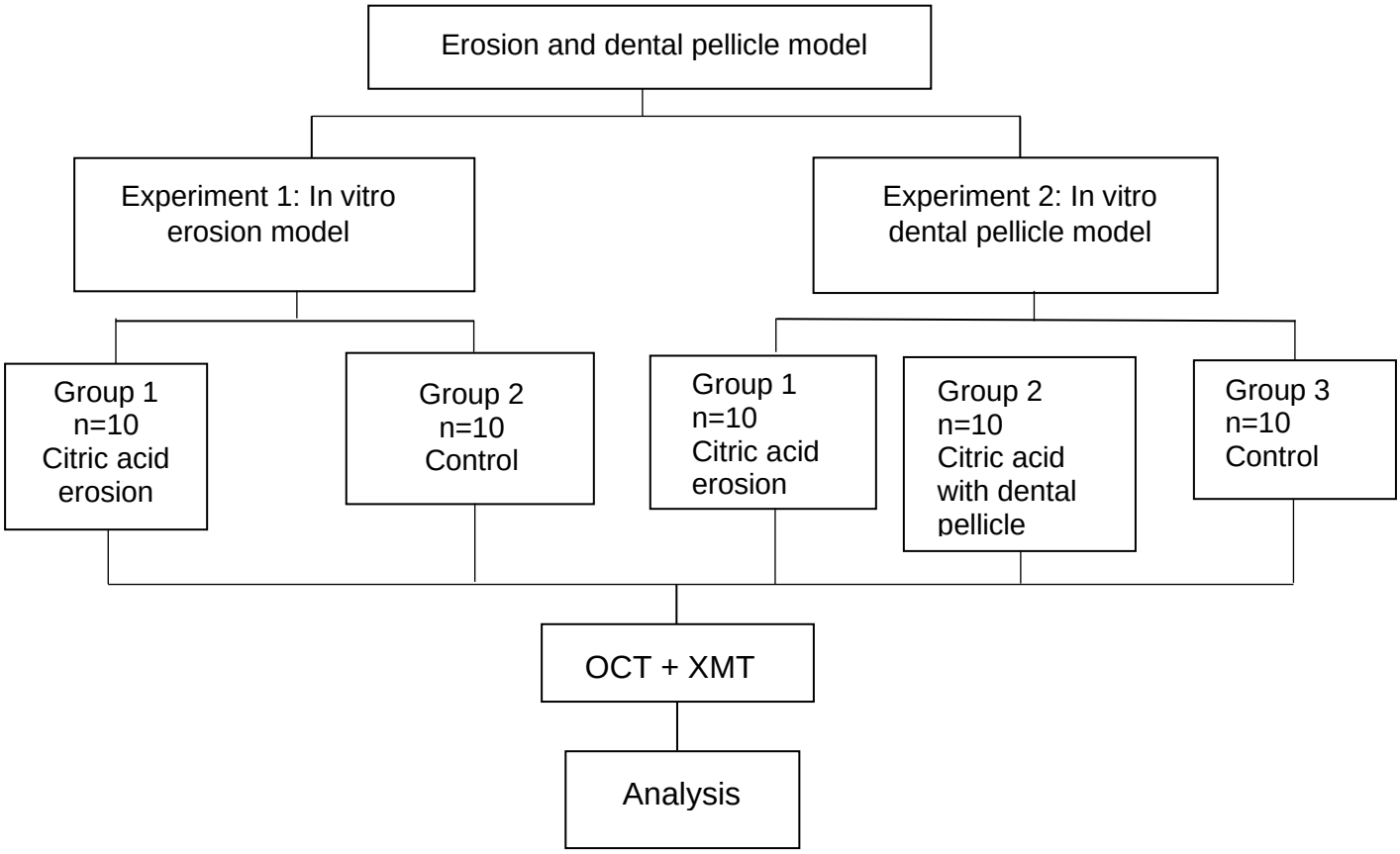
4.3.2.3. In vitro pellicle formation

The time based incubation method of pellicle formation (Baek et al., 2009) was used where a drop of the supernatant from the centrifuged saliva was placed on the windows of group 2 samples for thirty minutes to allow pellicle to be adsorbed on to the tooth surface and allow pellicle formation. The teeth were gently rinsed with deionized water to remove the excessive saliva.

4.3.3. Erosion experiment

Group 1 and group 2 samples were immersed in 10 ml of 1M citric acid (pH 3.2), while group 3 was immersed in 10 ml of artificial saliva under constant agitation on a rocking table at room temperature. The teeth were removed at each time point of, 1 hour, 2 hours, 3 hours and 4 hours, sonicated for three minutes in deionized water and scanned under OCT and the images recorded for all groups (Figure 4.4).

Figure 4.4: Flowchart for protocol followed for in vitro erosion model.



4.3.4. Statistical Analysis

The mean percentage surface loss of enamel was calculated by for the in vitro erosion and in vitro dental pellicle model. The mean percentage loss was obtained by calculating the differences in enamel thickness before and after exposure to the erosive medium. Shapiro-Wilk was used to test the normality of the data. As the data was not normally distributed, a non-parametric test (Mann-Whitney U test) was used to compare the mean percentage loss obtained after each time point and also between groups. The significance level was set at 0.05 where the p value less than 0.05 was considered significant.

4.4. Results

4.4.1. Optical coherence tomography

Figure 4.5 displays the B-scans obtained before and after erosions with OCT without a layer of dental pellicle on the enamel surface as well as the control group.

Figure 4.5: OCT B-Scans obtained at different time points: baseline to 4 hours.

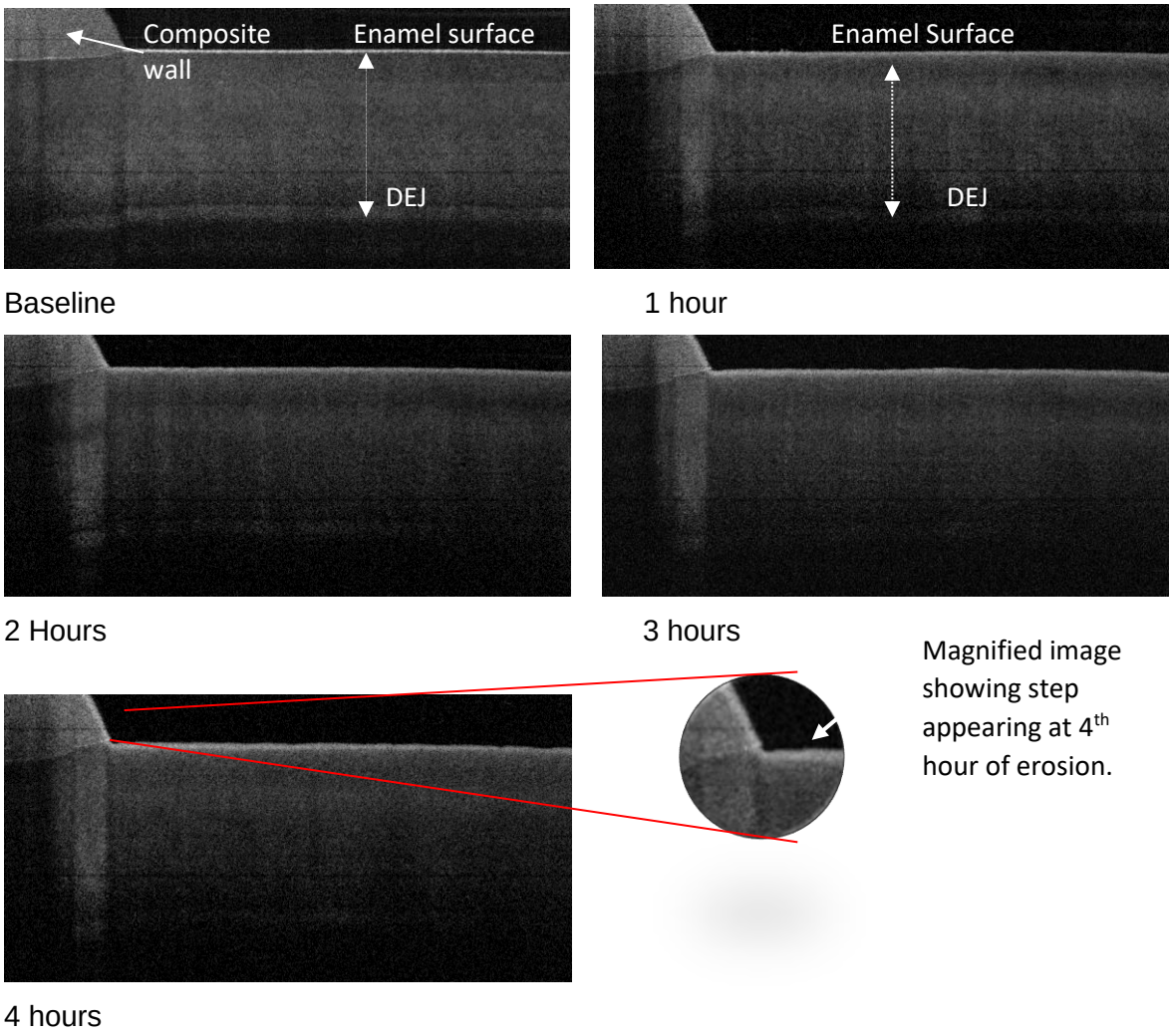
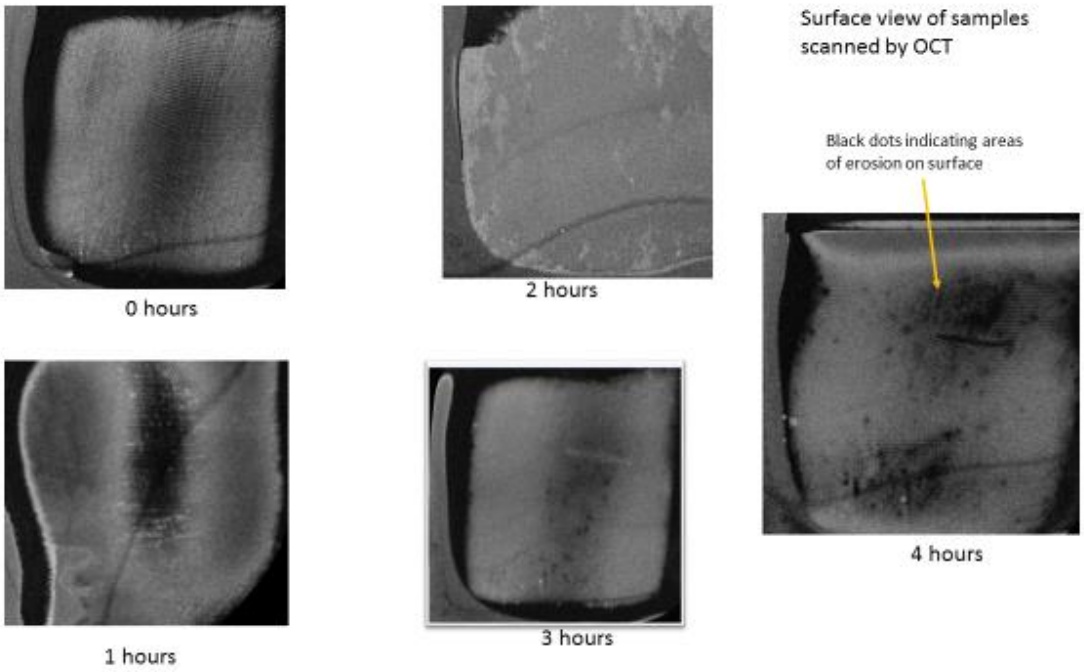
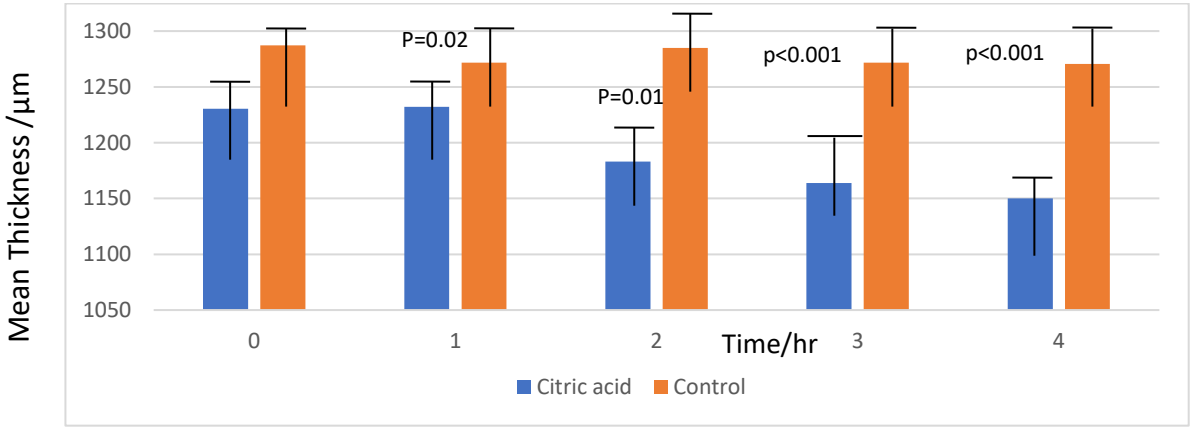


Figure 4.6: Enamel surface topography of samples obtained by OCT at 0, 1, 2 3 and 4 hours.



There was no loss of surface observed on the B-scans in the first two hours of immersion in citric acid. However, a small gap began to appear in the junction between the reference point and the enamel surface at the third hour indicating the initiation of loss of the enamel surface. The DEJ started to disappear as the process of erosion progressed with time. This can be due to the increase in scattering intensity of the infra-red laser beam due to increase in surface roughness. Surface topography indicated black dots appearing at the fourth hour of immersion possibly indicating loss of enamel surface or appearance of enamel prisms (Figure 4.6).

Figure 4.7: Mean thickness of enamel/µm after immersion in citric acid (blue) (n=10) and artificial saliva (orange) (n=10).



The distance between the enamel surface and the dentin-enamel junction was measured using imageJ (Figure 4.3) and the mean thickness of enamel was calculated and compared between both groups (Figure 4.7). The bars of the test group (blue bars) display significant decrease in heights as immersion in the acidic medium progresses with time as compared to the control group (red bars) ($p<0.001$) where there are no significant differences in heights as immersion progresses with time ($p=0.40$). In the test group, the mean thickness of enamel before immersion into the erosive medium was 1230.3±220.0 µm. There was no erosion observed in the first hour of immersion, however loss of surface commenced at the second hour where a loss of 3.84% ($p=0.003$) enamel was calculated (Table 4.1). A total loss of 6.5% ($p<0.001$) enamel was calculated after four hours of immersion. In the control group, no significant change in thickness of enamel was observed after four hours of immersion in artificial saliva, hence indicating no loss of enamel surface ($p=0.40$). There were statistical differences ($p<0.001$) between group 1 (erosion group) and group 2 (control group) after immersion in their respective mediums for four hours (Table 4.2).

Table 4.1: Mean thickness ± SD with % loss of enamel in citric acid (test group) and artificial saliva (control) measured by OCT.

	Time/ hours	**0**	**1**	**2**	**3**	**4**
Erosion group	*Mean±SD enamel thickness (% loss of enamel) µm*	1230.3±220.0	1232.0±175.9 (-0.13%) gain (*p*=0.06) from baseline	1183.0±171.2 (3.84%) loss (*p*=0.003) from baseline	1163.9±183.3 (5.39%) loss (*p*=0.005) from baseline	1150.2±182.0 (6.51%) loss (*p*<0.001) from baseline
Control group	*Mean±SD enamel thickness/µm*	1287.1±67.8	1271.6±72.1 (p=0.05) from baseline	1284.8±62.3 (p=0.77) from baseline	1271.6±76.3 (p=0.88) from baseline	1270.5±46.1 (p=0.40) from baseline

Table 4.2: Mean % enamel loss and significance between erosion group and control group with OCT.

Time/ Hour	**Mean % enamel loss erosion group**	**Mean % enamel loss control group**	**Significance**
1	-0.13	1.24	p=0.02
2	3.84	0.23	p=0.01
3	5.39	1.24	p<0.001
4	6.51	1.32	p<0.001

4.4.2. In vitro pellicle model

Figure 4.8: OCT B-Scans obtained at A: 0 hours, B: 1 hour, C: 2 hours, D: 3 hours and E: 4 hours with dental pellicle in citric acid.

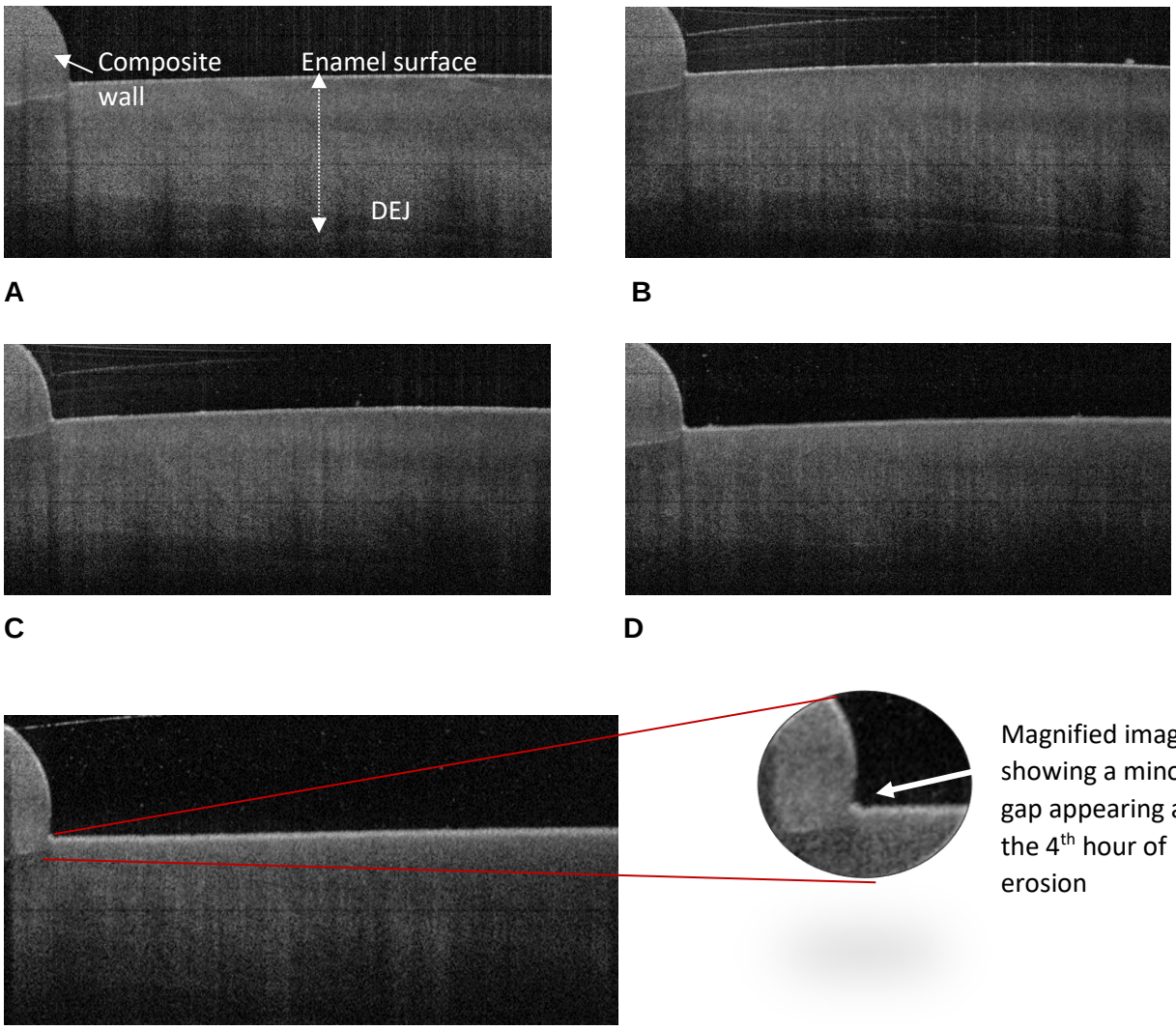
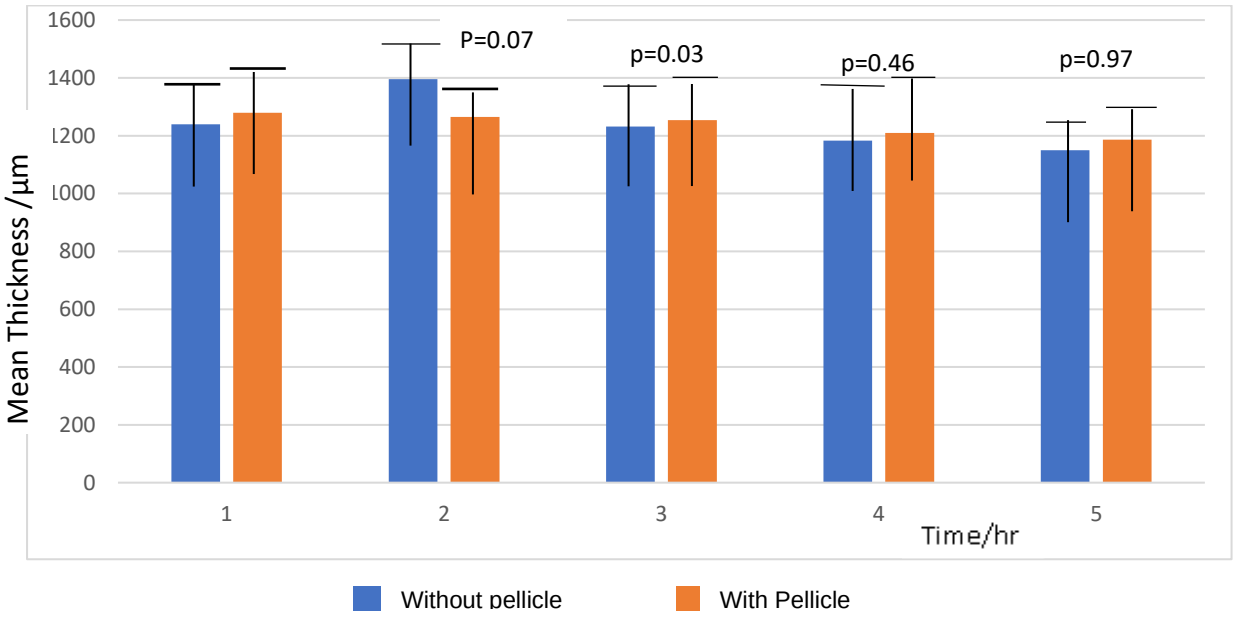


Figure 4.8 shows the OCT B-scan images obtained after every time point from 0 to 4 hours. There were no significant signs of demineralization or loss of enamel surface in the pellicle coated samples as observed in the B-scans. A minute gap appeared between the composite wall and enamel surface at the fourth hour of immersion signifying surface loss.

Figure 4.9: Mean thickness of enamel/µm with dental pellicle (orange) (n=10) and without pellicle (blue) (n=10) after erosion as measured with OCT.



The mean thickness of enamel was calculated and compared between both groups (Fig 4.9). The bars display non-significant differences in heights between group 1 (without pellicle) and group 2 (with pellicle) (p=0.975), however there were significant differences in heights within each group as immersion progressed with time (p<0.001) (Table 4.3). There were no significant differences in the percentage loss of enamel between group 1 and 2 (Table 4.4).

Table 4.3: Mean thickness ± SD with % loss of enamel in citric acid with and without dental pellicle as measured with OCT.

	Time/hours	**0**	**1**	**2**	**3**	**4**
Without pellicle	Mean±SD enamel thickness (% loss of enamel)	1240.06±186.5	1396.2±111.5 (-12.5 %) (p=0.002) from baseline	1232.3±175.9 (0.64%) (p=0.027) from baseline	1183.05±134.9 (4.60%) (p=0.032) from baseline	1150.21±182.0 (7.24%) (p<0.001) from baseline
With pellicle	Mean±SD enamel thickness (% loss of enamel)	1279.4±132.3	1265.6±175.6 (1.08%) (p=0.07) from baseline	1254.5±151.3 (1.92%) (p=0.08) from baseline	1210.4±143.7 (5.39%) (p=0.023) from baseline	1186.2±135.3 (7.26%) (p<0.001) from baseline

Table 4.4: Mean % enamel loss and significance between pellicle and without pellicle as measured with OCT.

Time/ Hour	Mean % enamel loss without pellicle	Mean % enamel loss with pellicle	Significance
1	0.64	1.085	p=0.07
2	4.60	1.92	p=0.03
3	6.13	5.39	p=0.46
4	7.24	7.26	p=0.97

4.4.3. X-Ray Microtomography

Figure 4.10: XMT images of samples immersed in citric acid for 4 hours.

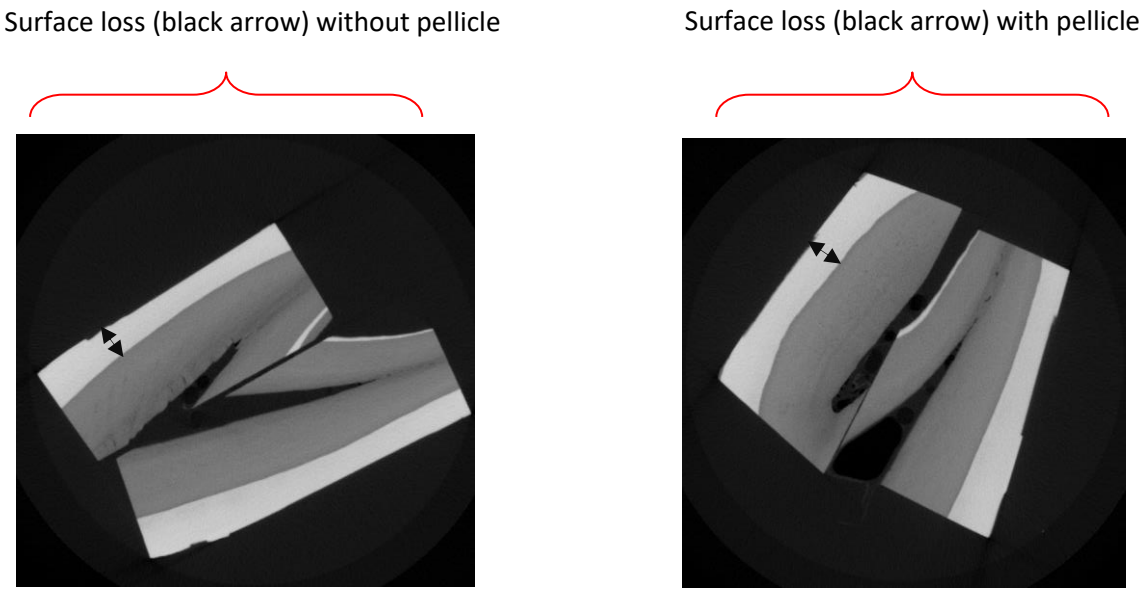


Figure 4.10 shows the images obtained by XMT after 4 hours of exposure to citric acid. Enamel surface loss was observed as a thin grey area on the superior margin of the enamel surface. Tomview (version 2.1, Tomlab Optimization Inc.) was used to measure the thickness of the white (enamel) area and the mean loss of enamel was measured after erosion in both groups. The mean % surface loss of enamel (SD) without pellicle was 16.5% (5.4) while with pellicle was 13.9% (4.6). There was no statistically significant difference between the two groups (p=0.314).

4.5. Discussion

4.5.1. OCT

The study focused on developing an in vitro erosion model where the ability of optical coherence tomography (OCT) to detect early erosive lesions in enamel was assessed. The thickness of enamel layer was measured before erosion using the A-scans from the surface to the dentine enamel junction (DEJ) where the DEJ acted as a reference point. However, the DEJ started to disappear as the process of erosion progressed with time. This can be due to the increase in scattering intensity of the infra-red laser beam due to increase in surface roughness. However, the increase in scattering should have presented as a white margin as observed in previous studies representing demineralisation of enamel due to caries (Ei et al., 2019) which was not the case in the current study. Probably, the acid would not have been strong enough to cause demineralisation, or OCT itself may not have the ability to detect early demineralisation. To compensate for the loss of DEJ, it was advisable to collect maximum number of A-scans to compensate the ones where the DEJ was not identified in order to obtain a normal distribution. This limitation of OCT was also reported by Alghilan et al., which might suggest that OCT is not a useful tool to measure tooth wear if using the DEJ as a reference point (Alghilan et al., 2019b).

4.5.2. XMT

XMT provides three dimensional images of biological structures and also measures the mineral densities of hard tissues. Although microradiography has been predominantly used in the investigation of caries, it has also been used to study erosion in vitro. The current study used XMT to calibrate the findings observed with OCT. Erosive lesions were observed by XMT, however, the enamel samples were scanned only after four hours of the erosive challenge as it was challenging to transport the samples after every one hour to the location for XMT scanningSecondly, each XMT scan is a 24-hour process making it impossible to run scans after every hour.The limitations, hence, made it impossible to detect and identify the point at which erosive lesions initiated which were originally detected by OCT in the second hour. In order to avoid this limitation and obtain quality images, it is suggested to immerse the tooth in a circulating acid or remineralization solution throughout the scanning period, which may be several days, or even weeks (Davis et al., 2018).. At 4 hours, there was no statistically significant mean percentage loss of enamel.

The aim of the study was to detect erosive lesions on the polished surfaces. However, the images obtained by XMT were from non-polished surfaces. Despite numerous attempts to capture images of the polished labials surfaces, the images could not be obtained, hence the images of non-polished surfaces were captured which may have caused discrepancies in the validation of the results.

Another major problem with XMT systems using conventional impact X-ray sources is beam hardening. Beam hardening occurs when an x-ray beam comprised of polychromatic energies passes through an object, resulting in selective attenuation of lower energy photons. As attenuation is increased, the proportion of higher energy photons in the transmitted beam increases leading to reconstructed images of specimens appearing less dense in the centre as rays passing through the centre are generally more attenuated (Davis et al., 2018).With the limitations of XMT, the use of XMT may not be a promising tool to calibrate the results obtained from OCT and suggests further work is required to the investigate the reliability of XMT to detect early erosive lesions.

4.5.3. Polishing

The mean thickness of enamel was measured before and after erosion, but due to variable forces applied during polishing, there was a high probability for the enamel specimens to have variable thickness measurements. The protocol followed for polishing by a study was to apply a force of 10N to all samples for a fixed period of time in order to achieve equal quantities of removal of amorphous enamel (Mylonas et al., 2018). The current study involved pressure applied manually by hand leading to different pressures applied for each tooth resulting in variable quantity of removal of surface enamel. Therefore, the percentage loss of enamel is a more meaningful statistic to summarise change than enamel thickness.

4.5.4. Enamel changes

The first hour showed a minute gain in the enamel thickness, but this is likely to represent an experimental error, as there might be a change in the positioning of the sample for OCT after erosion. But despite the change in positioning, loss of enamel after erosion was expected, but that was not the case as observed in the calculated results as well as the B-scans obtained from OCT. This might be possibly due to the fact that erosion is a chemical process which involves softening of the enamel. The

erosion process should be coupled with attrition and abrasion forces which is observed in vivo that results in enamel surface loss. However, since this was an in vitro study which did not involve attrition and abrasion forces, surface loss was not observed initially by OCT. However, as exposure time increased, a 3.8% loss of enamel was observed, however, despite the loss, B-scans could not identify erosive lesion formation after two hours. Even though, if erosive lesions were to be detected after 2 hours, it should be noted that 2-hour erosive lesions are not identified as early erosion. The other point to be taken into account is that, in in vivo environments, a human tooth is unlikely to be exposed to citric acid constantly for two hours. Surface loss was observed at the 3rd hour of exposure to the acid which was observed both in the B-scans as well as the surface topographic views. On the other hand, studies have observed changes in surface micro-hardness and profiles after sixty seconds (Sieber et al., 2019) which detected erosive lesions at early stages. This indicates that OCT would not begin to detect erosive lesions at earlier stages but only in advanced developed lesions if applied in in vivo experiments as observed in patients suffering from GERD (Wilder-Smith et al., 2009)

4.5.5. Comparison with other studies

The results obtained in the current study can be calibrated with other studies which aimed to observe and detect initial dental erosion lesions with OCT. One study measured change in reflection intensity (Chew et al., 2014) and the other measured change in enamel thickness (Alghilan et al., 2019a) in human specimens. There was no significant change in reflection intensity nor enamel thickness in the first eight hours of exposure in both studies. The current study used bovine specimens but despite that, there were no difference in enamel thickness in the first three hours. On the other hand Aden et al observed changes in the reflection intensity in bovine teeth in the first few minutes of exposure to an erosive challenge (Aden et al., 2017). Based on the results of the previous studies, a conflict might arise with regard to the results of the current study suggesting investigating possible reasons for not detecting early erosion in the study. In order to calibrate the results, surface micro-hardness might be a useful tool to detect early changes in the enamel surface after erosive challenge. Early erosive lesions are lesions without surface loss. Erosion involves the softening of the enamel layer following acid exposure. Surface microhardness measures the

increase/decrease in hardness of a tissue and would be a suitable alternate to measure early erosion.

4.5.6. In vitro pellicle model

The in vitro pellicle model consisted of a layer of pellicle being adsorbed to the tooth surface for thirty minutes and then assessing its potential to prevent or reduce the incidence of dental erosion. We were unable to observe the pellicle layer being formed on the B-scans. This creates an unsure possibility about the presence of a pellicle layer on the surface. However, Baek et al., managed to view pellicle islands using 2-D OCT after 24 hours (Baek et al., 2009) when the pellicle achieves a maximum thickness of 1000 nm (Hannig and Hannig, 2014). TEM studies and fluorescence studies have confirmed the development of a pellicle layer on the surface after 30 minutes of incubation (Hannig, 1997). Considering there has been only one study regarding the optical approach to the dental pellicle, OCT may be able to detect a pellicle layer but after long periods of incubation and might also consider using alternative OCT kits and transmission electron microscopy which would be able to detect pellicle islands on surfaces.

In the presence of a pellicle layer, (assuming the deposition after incubation in saliva), loss of enamel surface was observed at the 4^{th} hour of exposure to the acid. This compares to the specimens without a layer of pellicle where loss of surface occurred at the 3^{rd} hour. The apparent delay in detection of erosion suggests the preventive effect of pellicle. However, the loss of enamel surface in both groups was equivalent to each other after 4 hours of erosion suggesting the disruption of the pellicle layer. In addition, the release of calcium ions due to dissolution as a result of constant exposure to the acid as has also been evidenced in previous studies (Houghton et al., 2020). TEM studies have also suggested the removal of the globular layers of the pellicle after a 120 minutes of exposure to various soft drinks ranging from pH 2.8 to 3.8 hence decreasing the protective effect of the pellicle (Hannig et al., 2009). Proteomic analysis of the pellicle has also shown a decrease in the total protein content after exposure to 3% citric acids for 120 minutes (Taira et al., 2018). This suggests that pellicle has the potential to protect against dental erosion, however not for prolonged periods of time. Considering this an in vitro study where the teeth were exposed to the acid constantly, as opposed to in vivo conditions, the potential is still questionable. This is due to the

fact that the teeth in the oral cavity are exposed to various acids of varying concentrations in the diet. On the other hand, the pellicle is refreshed frequently soon after mechanical removal by cleaning, brushing and tongue movements. Therefore, in order to replicate the conditions of the oral cavity, an in vitro model needs to be developed which would involve a combination of all three forces which can possible be an aim for future projects.

4.5.7. Strengths of the study

The principle of OCT being non-invasive did not require sample preparation and it was easy to achieve a perpendicular surface during scanning which would have been uneasy to achieve in vivo. OCT scans are not time consuming, and samples were scanned in short periods of time. The visible DEJ layer provided a reasonable reference point to measure the thickness of enamel before and after erosion.

4.5.8. Limitations

The continuous four hours of acid exposure in this study was planned to test the capability of the device to evaluate enamel surface loss which is not the case in vivo. The pH of the oral cavity has shown to fall to 5.6 immediately after the intake of an acidic beverage but risen to normal within 15 minutes (Hans et al., 2016). The pH in the current study was maintained at 3.2 for four hours of exposure without any buffering agent which would not replicate the conditions of the oral cavity.

As demineralization progressed, the dentine enamel junction faded due to the increase in scattering intensity of the laser beam, hence some of the B-scan slices had to be excluded. Tooth surfaces, particularly actively eroding surfaces may be rough and not perfectly flat and coupled with the fact that the DEJ may not present a sharp demarcation makes it very challenging to measure the remaining enamel thickness. This is further complicated by the strong attenuation that may occur from scattering from the roughened surface and any subsurface erosion that occurs. (Chan et al., 2013). To overcome the limitation, maintaining hydration is critical, however a layer of water or saliva on the surface will also influence the optical path length and the measurement of the remaining enamel thickness. In addition, different variations of OCT have been invented for the quantification of early tooth erosion. Polarization-sensitive OCT, and particularly cross-polarization OCT, allows high-contrast images between sound and demineralized enamel areas (Attin and Wegehaupt, 2014),

providing more sophisticated erosion quantification which may be a potential for use in future studies. Within the above-mentioned limitations, this work provides sufficient information regarding the capabilities and drawbacks of OCT for enamel erosive surface loss measurement estimated from the remaining enamel thickness.

Although bovine teeth are preferred in in vitro studies because of their easy accessibility, greater surface are and availability from abattoirs they do not completely mimic the properties of human teeth (Soares et al., 2016). Human teeth possess a layer of fluorapatite on the enamel, is known to inhibit demineralization (Zanatta et al., 2019). The failure to completely mimic the substrate used in the experiments is a limitation where probably results may differ between bovine and human teeth.

It is yet too early to use the approach of this study for clinical applications to detect early stages of enamel erosive surface loss, owing to the unreliable surface loss measurements. On the other hand, the measurement approach showed to be practical and repeatable. Based on the effects on OCT measurement, future studies will be aimed to identify the ability of OCT to identify advanced erosive tooth wear levels and to clarify the effects of depth of demineralized enamel on the enamel thickness measurement by OCT. In addition to OCT, other outcome measures shall be considered to detect early dental erosion which may include surface micro-hardness and profilometry.

4.6. Conclusion

Optical coherence tomography aided in detecting and quantifying advanced erosive lesions with surface loss, however, was not suggestive to be a promising tool to detect early erosive lesions. Quantifying lesions was not possible as the DEJ disappeared as duration of exposure increased. The dental pellicle offered protection against erosion in the initial stages but failed to do so as the duration of exposure increased. Other strategies and outcome measures such as surface micro-hardness and profilometer need to be introduced not only to measure and quantify early erosive lesions, but also assess the effects of the interventions made into the dental pellicle to prevent dental erosion.

5. The qualitative and quantitative effects of intensive physical training on saliva – A systematic review and meta-analysis.

5.0. Background

The literature review identified that dental erosion is a multifactorial condition with yet no definite cause identified in the athlete population. However, there may be a possibility of a change in the nature of the saliva and dental pellicles as a result of intense physical activity which may prevent saliva from offering its protective function against dental erosion. Therefore, a systematic review was conducted to identify the qualitative and quantitative effects of intensive physical training on saliva and dental pellicle. This chapter will discuss the main elements of the systematic review and the meta-analysis.

5.1. Introduction

Elite athletes follow programmes of intense physical training to challenge and increase their performance levels. Intense physical training involves activities leading to markedly increased heart and respiratory rates. Scientifically, the definition can further be elaborated to activities ranging from 70–90% HR Max, 6–9 metabolic equivalents (MET) or 60-85% VO_2 Max (Norton et al., 2010). Metabolic equivalents [METs], are multiples of resting metabolic levels and are often used to quantify physical activities and set limitations (Powell et al., 2011).

In addition to the intensive physical training, the athletes have to follow a specific diet pattern which might also include acidic intakes in the form of sports and energy drinks. The specific diet pattern in the form of sugars and sports drinks increases the prevalence of acid in the oral cavity which is a factor for both dental caries and dental erosion (Bryant et al., 2011). The other factor to consider is the change in the properties of the saliva during periods on intense physical training which has been identified through various studies (Hopkins et al., 2020, Ligtenberg et al., 2016).

5.2. Dental erosion

The loss of dental hard tissues due to dental erosion can lead to symptoms ranging from increased sensitivity to pulpal pain which may be factors affecting the performance of athletes (Gallagher et al., 2018). One of the possible reasons for the high incidence of DE in athletes might be the frequent consumption of acidic sports

drinks to maintain their energy and electrolyte levels (Needleman et al., 2015). Sports drinks have a pH below the critical pH of 5.5 and therefore have the tendency to demineralise the teeth by causing the minerals to diffuse out of the tooth hence reducing the tooth volume (Kaye, 2017). However, studies have not established a statistically significant association between isotonic drinks and dental erosion (de Queiroz Gonçalves et al., 2020, Sirimaharaj et al., 2002, Antunes et al., 2017). So, it is yet too early to identify the high incidence of dental erosion in athletes to be caused by consumption of sports beverages.

5.2.1. Saliva and dental erosion

Saliva has been considered as the most important biological factor in the prevention of dental erosion due to its ability to neutralise and buffer the acids, forming a protective membrane acquired pellicle over the tooth surface, reduce the demineralisation rate and enhance remineralisation by providing calcium, phosphate and fluoride to the eroded tooth surface. Overall, the best indicator of the protective properties of saliva would be the flow rate, since all the above-mentioned salivary parameters depend on it. Saliva starts its protective effect against erosion by the increase of the flow rate with the help of the parasympathetic nervous system. The higher salivary flow rate increases the organic (proteins) and inorganic constituents of saliva (bicarbonate, calcium and fluoride ions) which protect the tooth from demineralisation.

The changes in the properties of saliva have been reported through various studies that contrasted the saliva properties and characteristics between populations with and without clinical signs of erosion. One study observed no difference in the flow rate, pH level, buffering capacity, bicarbonate, buffer base, calcium, phosphorus and urea concentrations of whole saliva between a dental erosion population and its age-matched and sex-matched controls (Wang et al., 2011). Another study assessed the different properties of saliva between erosion and non-erosion patients, in various age groups, and observed greater unstimulated salivary buffering capacity when no erosion was present in younger participants. In the elder group, stimulated salivary total protein was significantly higher in the group with enamel erosion (Piangprach et al., 2009). Another study tested the saliva collected from adolescents with erosion and aged-matched controls and assessed the flow rate, pH, buffer capacity, total protein content, calcium and phosphate. Interestingly, only unstimulated flow rate was found to be significantly different between the studied populations (Zwier et al., 2013).

5.3. Change in properties of saliva in athletes

Dental diseases are further exacerbated by a change in the nature of saliva during intensive physical activity which may eventually reduce the protective capabilities of saliva from the various insults (Horswill et al., 2006).

5.3.1. Saliva flow

Intense physical activity and training stimulates the sympathetic nervous system of the human body which is thought to result in a decreased production of saliva. A reduced volume of saliva in the oral cavity could lead to a decrease in the protective capabilities to prevent from caries and dental erosion (Frese et al., 2015). In individuals with reduced salivary flow rate, acid clearance is reduced, and less dilution of acid will be present upon attack of the tooth surface, hence contributing to dental erosion (Buzalaf et al. 2012). Reduced salivary flow from other causes such as radiotherapy, diabetes mellitus and Sjögren's syndrome is recognised as a risk factor for dental and periodontal diseases (Boutsi et al., 1999). A salivary flow rate of 0.3 ml/minute is considered to be normal (Humphrey and Williamson, 2001) and any level below is considered to be a reduction in saliva flow.

5.3.2. Compositional changes

Compositional changes that have been reported after periods of intense physical activities include decreased salivary immunoglobulin A (SIgA), anti-microbial protein levels (Papacosta and Nassis, 2011) and alpha amylase levels (Walsh et al., 1999). An observational study performed on endurance training cyclists also reported changes in salivary protein levels after periods of cycling where some salivary proteins were significantly increased while others were unchanged (Zauber et al., 2012). Consistent findings from studies have reported significant systematic metabolic changes in the steroids and hormone levels (Horswill et al., 2006), including increased androgen and cortisol after periods of intensive physical activity (Tremblay et al., 2004). Similarly, changes in the nature of saliva has also been reported after periods of intense physical activity through various studies (Frese et al., 2015) possibly leading to a decrease in the protective capabilities of the saliva from caries and dental erosion (Mulic et al., 2012). There are reports of changes in the composition of salivary immunoglobulins and proteins, mucosal alterations and increased incidence of dental

caries and dental erosion in individuals undergoing radiotherapy (Kaplan et al., 2008, Atkinson and Fox, 1993) which supports the theory that reduced salivary flow maybe a contributing factor to dental erosion.

However, there has not yet been a systematic review and analysis of these data. Therefore, the consistency and strength of the evidence remain unclear. The importance of establishing the relationship between intense physical activity and saliva quantity and composition is that changes could lead to altered homeostasis in the oral cavity eventually leading to conditions such as dental erosion and caries.

Therefore, the hypothesis was that intense physical training might produce a significant effect on the physical, chemical and structural properties of saliva could affect their protective capabilities against dental erosion

5.4. Aims

To assess effects of intensive physical training on physical and chemical properties of saliva to relate associations with dental erosion.

5.5. Objectives

- To assess the effects of intense physical training on saliva
- To assess the association of possible saliva and changes following intensive physical training with dental erosion.

5.6. Materials and methods

5.6.1. Protocol registration

The systematic review of the qualitative and quantitative effects of intensive physical training on saliva and dental pellicle was based on the guidelines of PRISMA and prospectively registered on the PROSPERO database (https://www.crd.york.ac.uk/PROSPERO/), CRD 42020193694.

5.6.2. Research question

The systematic review was conducted to answer the following two questions: (1) “what are the qualitative and quantitative effects of intense physical training on the composition and structural properties of saliva and dental pellicle?” and (2) “is there

an association between altered properties of saliva as a result of intense physical activity and increased prevalence of dental erosion in the athlete population?"

5.6.3. Literature search strategy

A sensitive search strategy was developed including electronic searching and hand searching in collaboration with the University College of London (UCL) library. There were no language restrictions imposed and non-English language records were planned to be translated though the network of language speakers at University College London and using google translate.

For the selection of studies included in this systematic review, the electronic databases searched were Medline (Ovid), Web of science, SPORTdiscus and Google Scholar (2000 – latest). Studies ranging from the year 1997 - 2020 were searched as it was anticipated that the effects of intensive training on saliva and oral health have been considered and studied within that timeframe.

The search strategy used a combination of controlled vocabulary (MeSH terms and free text terms). The keywords and subject headings were merged and exploded in accordance with the thesaurus of each database. The search was based on the following MeSH and free text terms and the Boolean operators "AND" and "OR" were applied to combine the keywords and to refine the results (Table 5.1).

Table 5.1: Search strategy.

	Medline (Ovid)	**Web of science**	**SPORTdiscus**
P	1. exp Athletes/ 2. (runners adj3 cyclists adj3 swimmers).mp. 3. (athletes adj1 olympic athletes).mp. 4. (athletes adj1 endurance athletes).mp. 5. running/ or jogging/ 6. (military adj1 navy).mp. 7. 1 or 3 8. 1 or 4 9. 1 or 5 10. 1 or 6 11. Military Personnel/ 12. military personnel/ or police/	# 1 (athletes) # 2 (elite athletes # 3 (olympic athletes) # 4 (endurance athletes) # 5 (recreational athletes) # 6 (runners, cyclists, swimmers) #7 (#2 **OR** #1) #8 (#5 **OR** #4) #9 (#7 AND #8)	S1 athletes S2 elite athletes S3 recreational athletes S4 endurance athletes S5 Olympic athletes S6 runners, cyclists and swimmers S7 military or veterans or soldiers or armed forces S8 athletes S9 athletes **OR** elite athletes S10 athletes **OR** elite athletes OR endurance

	13. 1 OR 2 OR 3 OR 4 OR 5		athletes OR runners, cyclist swimmers S11 (S9 **AND** S10)
E	1. Athletes/ or exp Exercise/ or exp Physical Exertion/ 2. exp Exercise/ 3. exp Physical Endurance/ or exp Endurance Training/ 4. Sports/ 5. Gymnastics/ 6. 1 **OR** 2 7. 1 **OR** 4 8. Exercise Test/ 9. 1 **OR** 8 10. 1 **OR** 4 **OR** 5 **OR**8	# 1 (intensive physical training) # 2 (physical exercise) #3 (endurance training) # 4 (exercise) # 5 (sports) # 6 (gymnastics) # 7 (#4 **OR** #3 **OR** #2) # 8 (#5 **OR** #1) #9. (#7 **AND** #8)	S1 intensive physical training S2 physical activity or exercise S3 sports S4 endurance training S5 endurance performance S6 endurance exercise S7 endurance sports S8 CPET S9 (S1 OR S2 OR S3 OR S4 OR S8)
O	1. Saliva/ 2. (saliva adj1 saliva flow).mp. 3. exp "Salivary Proteins and Peptides"/ 4. Immunoglobulin A/ 5. Saliva/ **OR** Immunoglobulin A/ **OR** Immunoglobulin A, Secretory/ 6. Dental Pellicle/ 7. 1 **OR** 3 **OR** 4 **OR** 6 8. 1 **AND** 7	#1 saliva #2 saliva flow rate #3 saliva pH #4 salivary immunoglobulins #5 dental pellicle #6 (#1 **OR** #2 **OR** #3) #7 (#1 **AND** #5)	S1 saliva S2 salivary flow S3 saliva pH S4 salivary IgA S5 saliva proteins S6 dental pellicle S7 acquired pellicle S8 S1 **OR** S2 **OR** S3 **OR** S4 **OR** S5 S9 S1 **AND** S2 **AND** S3 **AND** S4 **AND** S5 S10 S8 **AND** S9
Combined	13(P) **AND** 11(E) **AND** 3(C) **AND** 8(O).	#9(P) **AND** #9 (E) **AND** #1(C) **AND** #7 (O)	S11 (P) **AND** S9 (E) **AND** S1(C) **AND** S10 (O)

Key: / = MeSH terms; mp = multi-purpose or free text terms.

5.6.4. Eligibility criteria

All cohort studies, observational studies, cross-sectional studies, randomised control trials that include the reports of quantitative and/or qualitative analyses of saliva with intense physical training were included in the search strategy. The studies were screened and included according to Population (P), Exposure (E), Comparability (C) and Outcome (O).

Populations (P)

Anybody taking part in intense physical exercise, for example:

- Members of the elite (professional or Olympic) training squads

- Recreational athletes, runners, cyclists, swimmers
- Members of armed forces (military/navy)
- Participants taking part in school/university sports competitions.

Types of exposure (E)

- Intensive physical training and exercise including cardiopulmonary exercise testing (CPET)
- Sports – all physically active sports including football, rugby, swimming, cycling and gymnastics

Comparison (C)

The comparisons groups included non-athletes – participants not involved in physical training or exercise, different levels of training intensity and before and after intensive physical training in the same participants.

Outcome measures (O)

The outcome measures included quantitative as well as qualitative analysis of salivary flow rate, proteins, pH and immunoglobulins (Table 5.2).

Table 5.2: Outcome measures included.

Saliva component	**Outcome**	**Type of measurement (Study)**
Saliva flow.	Reduced saliva flow, dry mouth	Quantitative, but not self-reported.
Saliva proteins.	Statherins, cystatins, mucins, remineralisation.	Quantitative.
Immunoglobulins.	IgA.	Quantitative.
Enzymes.	Carbonic anhydrases.	Quantitative.
pH.	Altered pH if any.	Quantitative.
Dental pellicle.	Pellicle structure, pellicle proteins.	Quantitative (proteins), Qualitative (Thickness, adsorption).

In addition, all studies involving participants undergoing intense physical activity, assessment of saliva or dental pellicle, prospective or retrospective studies and involving adult populations >18 years were also included in the search strategy. On

the other hand, editorial letters, literature reviews, book chapters, guidelines, animal studies, studies involving adolescents and young athletes <18 were excluded.

5.6.5. Study screening

Titles and abstracts were screened for inclusion by one reviewer (HM) against the key eligibility criteria:

1. Investigation of intensive physical training
2. Investigation of saliva or dental pellicle
3. Human, not animal
4. Research study

All eligible studies were retained and the full text obtained for potential inclusion in the review against the full eligibility criteria. A random 10% of titles and abstracts and 10% of full-text articles were independently screened by one of two experienced systematic reviewers (IN & PA) to examine levels of agreement.

5.6.6. Data Extraction Strategy

Data was extracted by one reviewer (HM) using a specially designed data extraction spread sheet. The data extraction form was piloted on ten papers and modified as necessary prior to commencing the study data extraction. A second experienced reviewer (IN, PA) independently extracted data on 10% of studies to check consistency of process.

Two main categories of data were extracted as listed below:

Study Characteristics Data

- Study authors
- Year of publication
- Country where study performed
- Study design
- Type of physical activity/sport

- Duration of physical activity
- Study population group.
- Age > 18 years (Adult population)
- Number of subjects recruited
- Overall sample size

Outcome and/or Confounders Data

- Saliva flow rate
- Salivary total protein content
- Salivary @-amylase
- Salivary immunoglobulin A (IgA)

5.6.7. Risk of Bias

The Newcastle Ottawa scale was used to assess the quality of observational and non-randomised studies, whereas the Cochrane Collaboration RoB 2.0 tool was used for the interventional, randomised controlled trials. Due to the lack of established standard criteria, authors subjectively considered studies achieving 7 to 9 stars in the scale to be of high quality, studies with 5 to 6 stars of medium quality whilst studies with less than 5 stars were deemed of low methodological quality. Disagreements between reviewing authors over the risk of bias were resolved by discussion.

5.6.8. Certainty of evidence

The quality of the certainty of the main outcomes (i.e. saliva flow rate, salivary IgA, STP, salivary amylase and salivary mucin) was graded (high, moderate, low, or very low certainty) using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach (Goldet and Howick, 2013)Four different GRADE factors were used in the meta-analysis: risk of bias (i.e., the methodological quality of the studies), inconsistency (i.e., unexplained inconsistency of results across studies), indirectness (i.e., evidence from different populations than the population of interest in the review) and imprecision (i.e., total sample size of the available studies. The quality of evidence was subsequently downgraded by one or two levels per factor to very

serious, serious, or not serious when there was a risk of bias, inconsistency, imprecision or indirect results according to the following categories

i. Risk of bias:

Low risk of bias – not serious

Moderate risk of bias - serious

High risk of bias – very serious

ii. Inconsistency:

0% to 40% heterogeneity: not serious

50% to 90% heterogeneity: serious

90% to 100% heterogeneity: very serious

iii. Imprecision:

0 – 50 participants: very serious

50-100 participants: serious

100 – above: not serious

5.6.9. Study synthesis

The data extraction sheets were used to construct evidence tables, grouping studies by similar design, populations and outcomes. The evidence tables were examined to determine whether there were multiple studies investigating similar research questions with outcomes that could be combined quantitatively in meta-analysis.

5.6.10. Quantitative analysis

Due to the variation in the outcome values, a meta-analysis was planned with a random effect model for observational studies and both fixed and random effects models for randomised controlled trials. The data was presented in a forest plot and measures of heterogeneity including I^2 and chi-square testing were estimated using the software Stata 16.

5.6.11. Heterogeneity

The causes of heterogeneity from the included studies were explored based on the expected following factors (Subgroup analysis was carried out to investigate the possible causes of heterogeneity on the basis of the sports/physical activity, saliva collection method and saliva collection time after cessation of activity (Table 5.3).. Sensitivity analysis was performed for each outcome when subgroup analysis could not investigate the cause of heterogeneity. Results for both individual studies and meta-analyses were reported with a point estimate together with an associated confidence interval (95%) a p-value (0.05).

Table 5.3: Possible factors causing heterogeneity in the results of meta-analysis.

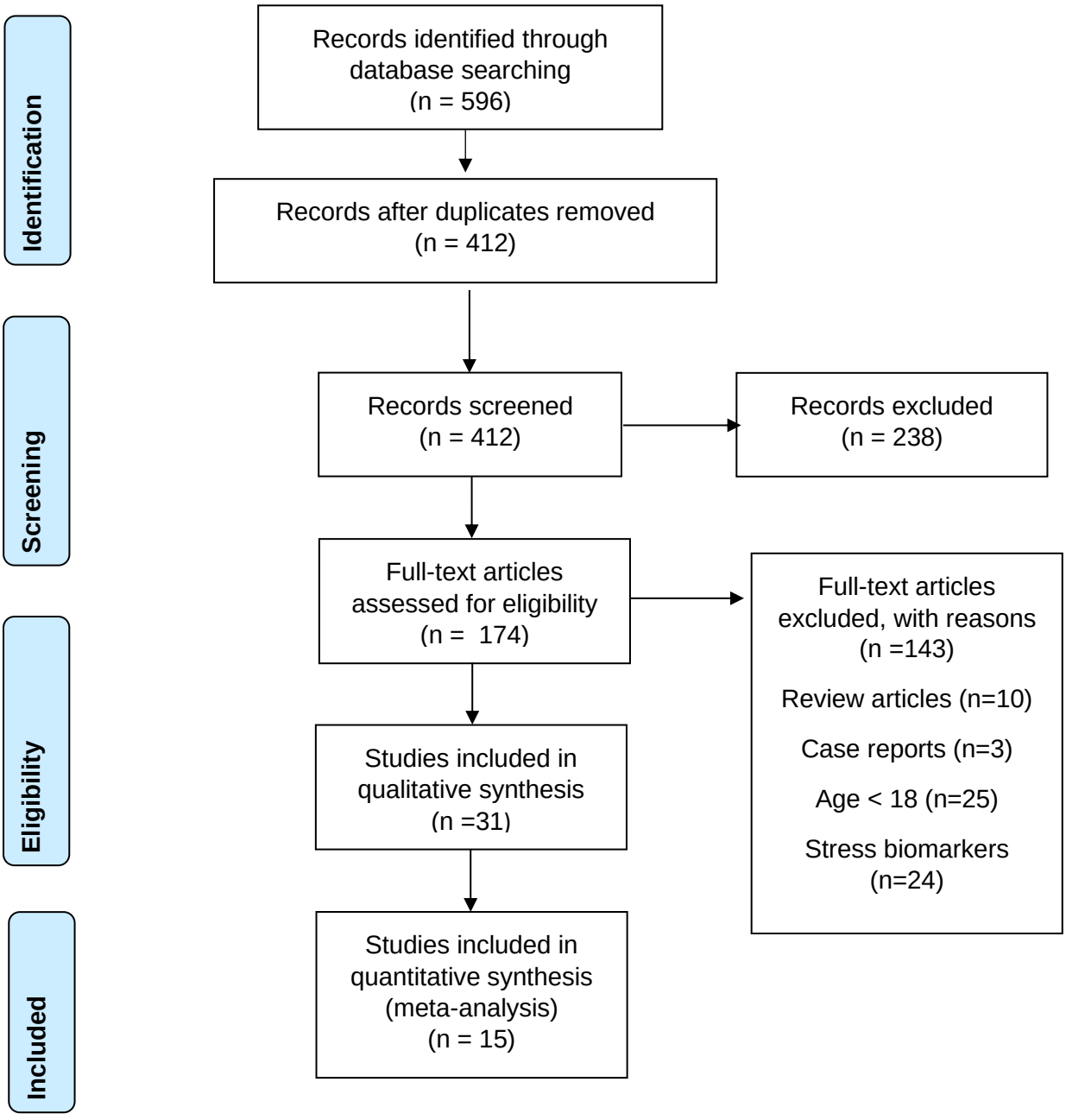
P (Population	**E (Exposure)**	**O (Outcome)**
Types of participants included (Olympic, endurance and recreational athletes). Age of participants.	Intensity of physical training. Length of training. Nature of sport.	Saliva collection method. Saliva collection times (soon after/ after a gap). Saliva analytical methods.

5.7. Results

The inter examiner agreement at full-text screening was excellent (kappa score = 0.72). A total of 596 studies were obtained from the search performed (Figure 5.1) and following removal of 184 duplicates 412 potentially suitable titles and abstracts were screened. After excluding 238 non-relevant citations, 174 publications were selected and retrieved for full-text review. Among these full text articles and abstracts, 143 were excluded; [review articles *(n=10),* case reports *(n=3),* age < 18 *(n=25),* stress biomarkers *(n=24),* hormones *(n=26)* salivary cortisol *(n= 35),* inflammatory biomarkers (*n= 20)*]. This resulted in a total of 31 publications eligible to be included in the systematic review after the screening procedure. Of these, 15 papers were included in the meta-analyses. 10 publications did not contain the full information necessary for the meta-analyses, however it was possible to obtain the required data from the authors of 5 publications. The remaining 5 studies did not contain the numerical data but mentioned significant and non-significant outcomes in their results. The total number of participants included in the review was 737. The number of

participants included in the meta-analysis for salivary flow were 206; salivary IgA were 435; STP were125; salivary α-amylase were 104 and salivary mucin were 71.

Figure 5.1: PRISMA flowchart representing the results of the workflow identify eligible studies.



5.7.1. Descriptive results

The full body of data obtained from the included studies was tabulated according to the chronological date of publication and sorted according to the outcome measure. The characteristics of the studies were summarized in individual tables, each for salivary flow rate, salivary IgA, saliva total protein content and salivary α-amylase.

5.7.1.1. Salivary flow

Eight observational studies measured salivary flow rate out of which seven were prospective cohorts (PC) and one study was a case control (CC) study. Four studies had professional athletes as participants, two had endurance athletes and two had university students as participants involved in intensive physical activity (Table 5.4). Two studies involved participants participating in running competitions; two studies involved participants playing football matches; one study assessed saliva flow before and after a kick boxing match; one study involved swimming and two studies involved resistance training for a specific period of time. Saliva samples in all studies were collected before and after the respective physical activity; three studies collected unstimulated saliva via expectoration and four studies collected saliva via passive drooling. The saliva flow rate was calculated by dividing the volume of saliva collected by the duration of collection. On the other hand, 3 studies did not report any environmental conditions or temperature at which the saliva was collected, or which may have affected the saliva flow rate (Li et al., 2015, Milanez et al., 2014, Moreira 2011). Moreover, whole mouth saliva (WMS) was reported to be collected from all participants included in the review. Overall, 3 studies showed a statistically significant decrease in saliva flow (Li et al., 2015, Tauler et al., 2014, Moreira et al., 2011), one study showed a statistically significant increase in saliva flow (Tauler et al., 2014) and 4 studies showed no statistically significant difference in saliva flow before and after physical activity (Milanez et al., 2014, Ligtenberg et al., 2016, Moreira et al., 2010, Moreira et al., 2009)

Table 5.4: Characteristics of studies measuring salivary flow rate

Author/Year/ Country	Study design	Population group/Sample size/Gender Age	Physical activity/ Intensity (RPE/VO_2 max/HR Max, BPM)	Environmental conditions Temperature/ humidity	Nature/Method of saliva collection	Collection time before activity	Collection time after activity	Assessment method	Saliva flow rate ml/min
Ligtenberg 2016, Netherland	PC	University students; 20; Mixed; 25±3	Cycle ergometer; 130-140 bpm	21.6°C	Unstimulated/ Expectoration	Immediately before	Immediately, and after 30 minutes	Dividing saliva volume by time	Before: 0.56±0.21 After: 0.58±0.2 (p>0.05)
Li 2015, Japan	PC	Endurance athletes - males VS females; 18; Mixed; 9.3 ± 0.7	5000 m race; M= 59.9 ± 3.8 VO2 Max, F= 47.5 ± 3.9 VO_2 Max	N/R	Unstimulated/ Passive drooling	10 minutes	10 minutes	Dividing saliva volume by time	Before: 0.51±0.22 After: 0.3±0.17 (p<0.05)
Frese 2015, Germany	CT	Endurance athletes;15; Mixed; 36.8 ± 7.2	Incremental running field test (IRFT); 1200 m distance stage	N/R	Unstimulated	5 minutes	5 minutes	Weighing, with saliva density	Before: 1.8±0.7 After: 1.1±0.4 (p=0.01)
Tauler 2014, Spain	PC	Endurance athletes; 107; Mixed; UTST:38.5±8.7 COWR: 39.8±10.0	106 km running and 25 km swimming; Training load: UTST: 10.9±8.5 COWR: 8.58±2.61 h/week	UTST Water intake: 4296±3238mL COWR Water intake: 4486±1709mL	Unstimulated/ Passive drooling.	2 hours before	Within 15 minutes	Weighing, with saliva density	UTST Before: 0.78±0.44 After: 0.67±0.35 (p<0.05) COWR: Before: 0.63±0.32 After: 1.06±0.62 (p<0.05)

Milanez 2014, Spain	PC	Professional futsal players; 13; Females; 22.1 ± 4.2	Resistance training; HR max 190 bpm VO_2 Max 53.1±7.0	N/R	Unstimulated/ expectoration	Immediately before	N/R	Dividing saliva volume by time	Before: 0.84±0.26 After: 0.83±0.46 (p>0.05)
Moreira 2011, Brazil	PC	Professional futsal players; 10; Males. 24 ± 6.3	Professional futsal games; RPE= 6.5, 172 bpm.	N/R	Unstimulated/ passive drooling	5 minutes	10 minutes	Dividing saliva volume by time	Before: 0.12±0.02 After: 0.07±0.01 (p<0.05)
Moreira 2010, Brazil	PC	Professional kickboxers: 20; Males; 23 ± 4	Kickboxing match; Training five times per week for about 120 min	Water intake during rest intervals	Unstimulated/ Passive drooling.	10 minutes	10 minutes	Dividing saliva volume by time	Before: 0.4±0.2 After: 0.44±0.5 (p>0.05)
Moreira 2009, Brazil	CC	Professional soccer players; 28; Males. 23 ± 6.4	Soccer match; RPE:16.4	Water intake during rest intervals	Unstimulated/ passive drooling	10 minutes	5 minutes	Dividing saliva volume by time	Before: 0.5±0.34 After: 0.55±0.36 (p>0.05)

Key: PC: Prospective Cohort; CC: Case Control; RM: Repeated Measure; RCT: Randomized Control Trial; UTST: Ultra-trail Serra de Tramuntana; COWR: Cabrera Open Water Race; RPE: Rating of Perceived Exertion; HR: Heart Rate; BPM: Beats per Minute; VO_2 Max: Volume of oxygen; N/R: Not reported; p>0.05 (exact P-values not reported)

5.7.1.2. Salivary IgA

23 observational studies measured salivary IgA where participants varied from university students to professional athletes involved in various intense activities (Table 5.5). Sixteen studies involved elite athletes, two studies involved endurance athletes, two studies involved /university/college teams, two studies included military personal, one study involved recreational athletes and one study involved weightlifters. Ten studies had participants involved in increased resistance and physical training, four studies had participants involved in professional football matches, two studies had participants involved in swimming and running, one study had participants involved in judo competitions; the military personnel (two studies) were involved in peace keeping missions and various training loads. The saliva samples were collected before and after the activities. Five studies collected saliva via expectoration, nine studies collected saliva via the passive drooling method, seven studies collected saliva using the oral fluid collector device and two studies did not report the method adopted to collect saliva. Various methods were adopted in the studies to measure the concentration of Immunoglobulin A; twenty studies used Enzyme Linked Immunoassay (ELISA), two studies used the real-time lateral flow device, and one study used a spectrophotometer. Overall, five studies showed a statistically significant increase in salivary IgA concentration (Monje et al., 2020, Milanez et al., 2014, Kvietkauskaite et al., 2014, Reid et al., 2001, L. et al., 2000), ten studies showed a statistically significant decrease in salivary IgA concentration (Maya et al., 2016, Tauler et al., 2014, Morgans et al., 2014, Morgans et al., 2015, Fredericks et al., 2012, OWEN et al., 2014a, Min-Lung et al., 2012, Moreira et al., 2011, Francis et al., 2005, Paul et al., 2015) and eight studies showed no difference in salivary IgA concentration before and after physical activity (Papacosta et al., 2016, Fisher et al., 2015, Guilhem et al., 2015, Leicht et al., 2012, Moreira et al., 2010, Moreira et al., 2009, Vardiman et al., 2011, Koch et al., 2007b, Nunes et al., 2011)

Table 5.5: Characteristics of studies measuring salivary IgA

Author/Year/ Country	Study design	Population group/ Sample size/ Gender/ Age	Physical activity /Intensity (RPE/VO2 max/HR Max, BPM)	Environmental conditions Temperature/ humidity	Nature/ method of saliva collection	Collection time before activity	Collection time after activity	Assessment method	Salivary IgA (µg/ml)	
Monje 2020, Chile	PC	Endurance athletes; 20. M: 21.9±0.8 F: 25.8±6.2	High intensity interval training; 90% of VO2 peak	N/R	Unstimulated/ expectorated	5 minutes	20 minutes	ELISA	Before: 138±14 After: 184±16 (p=0.004)	
Papacosta 2016, United Kingdom	PC	Elite athletes (judo competitors); 23; M; 22±4	Olympic sport (Judo competitions)	Water intake during rest intervals	Unstimulated/ Passive drooling.	Immediately before	15 minutes	ELISA	Winners Before: 78.46±56.2 After: 95.60±53.1 (p=0.07)	Losers: Before: 85.02±75.46 After: 89.3± 58.45 (p>0.05)
Maya 2016, Chile	PC	Elite football players; 16; F; 22.5±2.1	Two football games; RPE M1: 5.63±2.13 M2: 5.81±1.42	N/R	N/R	30 minutes	5 minutes	ELISA	Before: 56.21±27.86 After: 20.73±9.63 (p=0.04)	
Fisher 2015, Australia	RM	Elite surf lifesaving (SLS) athletes; 18; Mixed; 24.4±7.5	Variable intense sport (soft sand beach running, surf swimming, surfboard paddling surf ski paddling)	N/R	Unstimulated/ Oral Fluid Collector	15 minutes	15 minutes	IPRO Lateral Flow Device (LFD),	Males: 54h prior: 33.55±8.8 30h prior: 38.60±8.7 morning: 37.41±8.6 Post event: 44.3±8.9 17 Hours:	Females: 54h prior: 31.00±6.2 30h prior: 39.76±7.4 morning: 34.0±7.2 Post event: 38.9±8.4 17 hours:

									39.7±9.3 37 hours: 39.8±8.6 (p>0.05)	32.4±7.9 37 hours: 39.8±6.7 (p>0.05)
Guilhem 2015, France	PC	Elite world class level athletes (sprinters, runners); 24; Mixed; 25±4	High intensity interval training	N/R	Unstimulated /Passive drool	N/R	N/R	Spectrophotometer	Males: Before: 16.84±6.3 After: 23.49±10.53 (p=0.006)	Females: Before: 17.17±15.8 After: 21.7±8.91 (p=0.23)
Morgans 2015, United Kingdom	RM	Elite athletes (soccer players); 13; M; 25±3	Physical training	N/R	Unstimulated/ Oral Fluid Collector	Immediately before	N/A	Real-time lateral-flow device	MD4:365±127 MD3: 348±154 MD1: 256±90 (p<0.05)	
Paul 2015, India	RM	Military; 12; 27.1±3.6	Carrying variable training loads	25-28 ^{o}C 35-40%	Stimulated/ Expectoration	Immediately before	Immediately after	Sandwich ELISA	Data not reported Statistically significant decrease	
Tauler 2014, Spain	PC	Endurance athletes; 107; Mixed; UTST:38.5±8.7 COWR: 39.8±10.0	106 km Running and 25 km swimming; Training load: UTST: 10.9±8.5 COWR:8.58±2.61 h/week	UTST Water intake: 4296±3238mL COWR Water intake: 4486±1709mL	Unstimulated/ Passive drooling.	2 hours	15 minutes	ELISA	UTST Before: 162.4±82 After: 127.7±83.4 (p<0.05) COWR: Before: 152.6±47 After: 64.9±41 (p<0.05)	
Morgans 2014, United Kingdom	PC	Elite athletes (premier leagues soccer players); 21; 26±4	Professional Sport - soccer games	N/R	Unstimulated/ Oral Fluid Collector	N/R	2 days	Real-time lateral-flow device	Before: 139±25 After: 41±10 (p<0.05)	

Milanez 2014, Spain	PC	Elite athletes (futsal players); 13; Females; 22.1±4.2	Resistance training; HR max 190 bpm VO2 Max 53.1±7.0	N/R	Unstimulated/ Expectoration	Immediately before	N/R	ELISA	Before: 52.2±32.1 After: 59.8±31.4 ($p<0.05$)
Owen 2014, United Kingdom	PC	Elite athletes (soccer players); 10; Males; 26.8±4.1	Intense training sessions	N/R	Unstimulated/ Oral Fluid Collector	30 minutes	Immediately after	ELISA	Before:125.11±64.66 After: 58.11±44.98 ($p<0.05$)
Kvietkauskaite 2014, Lithuania	PC	Military; 30; Males; 33±4	Missions	N/R	Saliva collection system Oracol	N/R	N/R	Sandwich ELISA	Data not reported Statistically significant increase
Fredericks 2012, Brunei Darussalam	PC	Elite athletes (footballers); 33; 26±4	Training and football matches	N/R	Omni-sal absorbent pad	N/A	20 minutes, and next morning	Immunoturbido metric assay	Before: 133.4±75.3 After: 82.5±36 ($p<0.05$)
Leicht 2012, United Kingdom	RM	Elite tertaplegic athletes; 14 ;mixed 33±5	Training for world cup matches; 14±3 h/wk	N/R	Unstimulated/ Passive drooling.	Immediately before	N/R	ELISA	Data not reported No statistically significant difference
Min-Lung 2012, Taiwan	RM	Weightlifters; 11; Males; 26.7±2.8	Intense resistance training with variable training loads	N/R	Unstimulated/ passive drooling.	Immediately before	N/R	ELISA	Before: 231.4±85.8 After: 165.8±44.5 ($p<0.001$)
Vardiman 2011, U.S.A	CC	College athletes; 20; Females; 20±0.37	Soccer training; 86±34.55 mins	N/R	N/R	2 hours	15 minutes	ELISA	Data not reported No statistically significant difference

Nunes 2011, Brazil	RM	Elite athletes (basketball players); 14; Females; 26.2±3.9	Three endurance training schemes; 4 sets of 12 reps	N/R	N/R	2 hours, and immediately before	15 minutes, and 2 hours after	Salivary Secretory IgA, salimetrics	Data not reported No statistically significant difference
Moreira 2011, Brazil	PC	Elite athletes (futsal players); 10; Males; 24±6.3	Professional football;RPE = 6.5, 172 BPM.	Water intake during rest intervals	Unstimulated/ Passive drooling.	5 minutes	10 minutes	ELISA	Before: 175±43 After: 99±17 ($p<0.05$)
Moreira 2010, Brazil	PC	Elite athletes (kickboxers): 20; Males; 23±4	Kickboxing match; Training five times per week for about 120 min	Water intake during rest intervals	Unstimulated/ Passive drooling.	10 minutes	10 minutes	ELISA	Before: 54±25 After: 58.5±55 ($p=0.43$)
Moreira 2009, Brazil	CC	Elite athletes (soccer players); 28; Males; 23±6.4	Soccer match; RPE:16.4	Water intake during rest intervals	Unstimulated/ Passive drooling.	10 minutes	5 minutes	ELISA	Before: 380±22 After: 384±22 ($p>0.05$)
Koch 2007, U.S.A	PC	College players; 16; Males; 20.6±1.2	College rugby match; 3 strenuous 2 hours training/week	N/R	Unstimulated/ Passive drooling.	10 minutes	5 minutes	ELISA	Before: 311.4±162.1 After: 355.8±175.5 ($p=0.176$)
Francis 2005, Australia	RM	Elite athletes (swimmers); 53; Mixed; 21.4±2.3	Swimming and exercise	N/R	Unstimulated/ Expectoration	Immediately before	60 minutes	ELISA	Elite: 65.2±17.3 Active: 31.6±14 ($p<0.002$)
Reid 2001, Australia	PC	Recreational athletes; 28; mixed; 34±11	Cycle ergometer; 30% HR max 106± 10 beats/min, 60% HR max	24 ^{o}C	Unstimulated/ Passive drooling.	Immediately before	Immediately after, and 30 minutes after	ELISA	Data not reported Statistically significant increase

			136± 9 beats/min						
Sandra 2000, U.S.A	CC	Elite women rowers; 39; Females; 22.6±0.5	2 hours rowing in training camp; 189±3	15.5 °C; 70%	Unstimulated/ gentle suction with a plastic pipette	N/R	N/R	ELISA	Data not reported Statistically significant increase

Key: ELISA: Enzyme Linked Assay; MD4: Match Day minus 4; N/A: Not Applicable p>0.05 (exact P-values not reported) UTST: Ultra-trail Serra de Tramuntana; COWR: Cabrera Open Water Race

5.7.1.3. Saliva total protein

Thirteen studies measured the salivary total protein (STP) content out of which seven were observational studies and one was a randomised controlled trial (RCT) (Table 5.7). One study involved elite athletes, two studies involved endurance athletes, two studies involved university/college players, one study involved weightlifters and two studies involved subjects not involved in any physical or athletic activity. One study involved participants competing in a rugby match and in one study participants competed in a football match. Two studies had participants being involved in cycling events and four studies had participants being involved in intense training activities. Saliva samples were collected before and after each training/activity period. Four studies collected saliva via expectoration, two studies collected saliva via the passive drooling method, two studies collected saliva using the oral fluid collector device, and one study collected saliva using a low-adsorptive polypropylene-based swab. Four studies did not report the methods of collection of saliva. Seven studies used the bicinchoninic acid assay (BCA) method to measure the STP; four studies used the Bradford method; one study used the Coomassie protein assay and one study used liquid chromatography/mass spectroscopy (LC/MS) to measure STP (Table 5.6). Overall, four studies showed a statistically significant increase in STP (Li et al., 2015, Zauber et al., 2012, Vardiman et al., 2011, Moreira et al., 2009), two studies showed a statistically significant decrease in STP (Trochimiak et al., 2015, Kiani et al., 2015) and four studies showed no difference in STP before and after physical activity (Ligtenberg et al., 2016, Nehlsen-Cannarella et al., 2000, Min-Lung et al., 2012, Koch et al., 2007a). In the RCT, the test group showed a statistically significant increase in STP after training while the control group showed a statistically significant decrease in STP following training (Hopkins et al., 2020). Two studies did not quantify the STP levels as results were displayed on graphs and the respective authors could not be contacted.

Table 5.6: Characteristics of assays used for protein analysis.

Assay	Description
BCA: bicinchoninic acid assay	Depends on the conversion of Cu2+ to Cu+ under alkaline conditions. The Cu+ is then detected by reaction with BCA spectrophotometrically. One step process (Walker, 2009)
Bradford technique	The Bradford assay relies on the binding of the dye Coomassie Blue G250 to protein. Proteins quantified by the amount of dye in the blue ionic form (Kruger, 2009).
Lowry's method	Similar to BCA. Two-step process (Walker, 2009).
Coomassie assay	Similar to Bradford technique

Table 5.7: Characteristics of studies measuring saliva total protein (STP).

Author/Year/ Country	Study design	Population group/Sample size/Gender Age	Physical activity /Intensity (RPE/VO2 max/ HR Max, BPM)	Environmental conditions Temperature/ humidity	Nature/method of saliva collection	Collection time before activity	Collection time after activity	Assessment method	Saliva total Protein (STP) (µg/ml)	
Hopkins, 2020 United Kingdom	RCT	Non-athletes; 22; F; 24.4±1.8	Moderate intensity cycling; 75% HR Max	Thermo-neutral environment 22 °C	Unstimulated/ Passive drooling.	Immediately before	Immediately after, and 60 minutes after	BCA assay	TG Before: 686.5±59.8 After: 949.9±132.2 (p<0.05)	Control: Before: 612.6±90.2 After: 532.2±62.2 (p<0.05)
Ligtenberg 2016, Netherland	PC	University students; 20; Mixed; 25±3	Cycle ergometer; 130-140 bpm	21.6^{o}C	Unstimulated/ Expectorated	Immediately before	Immediately after, and 30 minutes after	BCA assay	Before: 1180±410 After: 1200±500 (p>0.05)	
Trochimiak 2015, Poland	CC	Collegiate wrestlers; 57; Mixed; 20.7±2.9	Wrestling training	N/R	N/R	Immediately before	After 6 weeks of preparation and before actual event	Lowry's method	Data not reported Statistically significant decrease	
Paul 2015, India	RM	Military; 12; 27.1±3.6	Variable training loads	25-28 ^{o}C 35-40%	Stimulated expectoration	Immediately before	Immediately after	BCA assay	N/R	
Li 2015, Japan	PC	Endurance athletes - males VS females; 18; Mixed; 9.3±0.7	5000 m race; M= 59.9±3.8 VO2 Max, F= 47.5±3.9 VO2 Max	N/R	Unstimulated/ Passive drooling	10 minutes	10 minutes	BCA assay	Males Before: 997±74 After: 1748±293 (p<0.05)	Females Before: 997± 180 After: 1644± 135 (p<0.05)
Kiani 2015, Iran	PC	Non-athletes; 10; Males; 23.2±2.35	Activities of different intensities; 50-75% VO2 max	N/R	N/R	Immediately before	Immediately after,	Bradford method	Before: 50% VO2 Max: 6560±1410 75% VO2 Max: 5450±1450	After: 50% VO2 Max: 1900±1070 75% VO2 Max: 3160±810

Zauber 2012, Germany	PC	Endurance cyclist;2; mixed	Cycling event; 62 hours of cycling	N/R	Low-adsorptive polypropylene-based swab	4 times during cycling 4 days before	2 days after 4 times	LC-MS/MS analysis of peptides	Data not reported Statistically significant Increase
Min-Lung 2012, Taiwan	RM	Weightlifters; 11; Males; 26.7±2.8	Intense resistance training	N/R	Unstimulated/ Passive drooling.	Immediately before	N/R	BCA assay	Before: 1768±760 After: 1945±715 (p>0.05)
Fredericks 2012, Brunei Darussalam	PC	Elite athletes (footballers);33; 26±4	Football training and matches	N/R	Omni-Sal collection device	N/R	20 min after games Following morning	Bradford method	N/R
Vardiman 2011, U.S.A	CC	College athletes; 20; Females; 20±0.37	Soccer games	N/R	N/R	2 hours	5 minutes	BCA assay	Data not reported Statistically significant increase
Moreira 2009, Brazil	CC	Elite athletes (soccer players);28; Males; 23±6.4	Soccer match; RPE:16.4	Water intake during rest	Unstimulated/ Passive drooling.	10 minutes	5 minutes	BCA assay	Before:1460±400 After: 2000±700 (p<0.05)
Koch 2007, U.S.A	PC	College players; 16; Males; 20.6±1.2	College rugby match; 3 strenuous 2 hours training/week	N/R	Unstimulated/ Passive drooling.	10 minutes	5 minutes	Dye binding colorimeter	Before: 1670± 230 After: 1730 ± 320 (p=0.469)
Sandra 2000,U.S.A	CC	Elite women rowers; 39; Females; 22.6±0.5	Rowing competitions	15.5 °C; 70%	Gentle suction with a plastic pipette	N/R	N/R	Coomassie protein assay	Data not reported No statistically significant difference

Key: BCA: bicinchoninic acid assay; TG: Test group; (p>0.05) exact values not reported.

5.7.1.4. Salivary α-amylase

There were six studies that measured salivary α-amylase levels out of which five were observational studies and one was an RCT (Table 5.8). Two studies included endurance athletes whereas one study involved elite athletes. Two studies included university/college players. The RCT included non-athletes being exposed to an intense physical activity as the test group (TG) while the control group were exposed to a period of resting. Three studies used the salimetrics α-amylase kit to measure the salivary α-amylase levels; two studies used ELISA and one study spectrophotometrically measured the amylase levels in the saliva. Overall, two studies showed a statistically significant increase in salivary alpha-amylase (Li et al., 2015, Monje et al., 2020), two studies showed a statistically significant decrease in salivary alpha-amylase (Hopkins et al., 2020, Trochimiak et al., 2015) and two studies showed no difference in salivary alpha-amylase before and after physical activity (Ligtenberg et al., 2016, Guilhem et al., 2015)

Table 5.8: Characteristics of studies measuring salivary amylase.

Author/Year/ Country	Study design	Population group/Sample size/Gender/ Age	Nature/method of saliva collection	Physical activity /Intensity (RPE/VO2 max/HR Max, BPM)	Environmental conditions Temperature/ humidity	Collection time before activity	Collection time after activity	Assessment method	Salivary α -amylase (U/ml)	
Hopkins, 2020, United Kingdom	RCT	Non-athletes; 22; F; 24.4±1.8	Unstimulated /Passive drooling.	Moderate intensity cycling; 75% HR Max	Thermo-neutral environment 22 °C	Immediately before	Immediately after, and 60 minutes after	The salimetrics α-amylase kit	TG: Before: 40±4.7 After: 59.6±4.9 (p<0.05)	Control: Before: 37.4±6.6 After: 31.6±5.3 (p<0.05)
Ligtenberg 2016, Netherland	PC	University students; 20; Mixed; 25±3	Unstimulated/ Expectoration	Cycle ergometer; 130-140 bpm	21.6^{o}C	Immediately before	Immediately after, and 30 minutes after	EnzCheck Ultra Amylase assay kit	Before: 128±74 After: 140±77 (p>0.05)	
Li 2015, Japan	PC	Endurance athletes - males VS females; 18; Mixed; 9.3±0.7	Unstimulated/ Passive drooling	5000 m race; M= 59.9±3.8 VO2 Max, F= 47.5±3.9 VO2 Max	N/R	10 minutes	10 minutes	The salimetrics α-amylase kit	Males Before: 32.3±5.5 After: 59.2±9.5 (p<0.05)	Females: Before: 66.2±16.3 After: 88.6±18 (p<0.05)
Trochimiak 2015, Poland	CC	Collegiate wrestlers; 57; Mixed; 20.7±2.9	N/R	Wrestling training	N/R	Immediately before	6 weeks of competition After 6 weeks of preparation Before actual event	The salimetrics α-amylase kit	Data not reported Statistically significant decrease	
Guilhem 2015, France	PC	Elite athletes; 24; Mixed; 25 ± 4	Unstimulated/ Passive drooling	High intensity interval training	N/R	N/R	N/R	Spectrophoto -metre	Males: Before: 112.6±50.7 After: 142.2±82.7 (p>0.05)	Females: Before: 106.7±98.3 After: 109.8±55.6 (p>0.05)
Monje 2020, Chile	PC	Endurance athletes; 20; M: 21.9 ± 0.8 F: 25.8 ± 6.2	Unstimulated/ Expectoration	High intensity interval training; 90% of VO2 peak	N/R	5 minutes	20 minutes	ELISA	Before:117±15 After: 284±61 (p=0.007)	

Key: ELISA: Enzyme Linked Assay

5.7.1.5. Salivary Mucins

Three studies measured salivary mucin levels (MUC5B) out of which two were prospective cohort studies and one was a randomised controlled trial (RCT) (Table 5.9). The observational studies involved university/college students whereas the RCT recruited non-athletes being exposed to an intense physical activity as the test group (TG) while the control group were exposed to a period of resting for the study. Two studies used ELISA to determine the concentration of MUC5B whereas one study used the BCA assay. Overall, two studies showed a statistically significant increase in salivary MUC5B (Ligtenberg et al., 2015, Ligtenberg et al., 2016), while one study showed no difference in salivary MUC5B before and after physical activity (Hopkins et al., 2020).

Table 5.9: Characteristics of studies measuring salivary mucin MUC5B.

Author/Year/ Country	Study design	Population group/Sample size/Gender/ Age	Nature/method of saliva collection	Physical activity /Intensity (RPE/VO2 max/HR Max, BPM)	Environmental conditions Temperature/ humidity	Collection time before activity	Collection time after activity	Assessment method	Salivary Mucin MUC5B	
Hopkins, 2020, United Kingdom	RCT	Non-athletes; 22; F; 24.4±1.8	Unstimulated /Passive drooling.	Moderate intensity cycling; 75% HR Max	Thermo-neutral environment 22 °C	Immediately before	Immediately after, and 60 minutes after	The salimetrics α-amylase kit	TG: Before: 7.85±2.37 After: 6.72±1.73 (p>0.05)	Control: Before: 6.86±0.07 After: 7.77±2.34 (p>0.05)
Ligtenberg 2016, Netherland	PC	University students; 20; Mixed; 25±3	Unstimulated/ Expectoration	Cycle ergometer; 130-140 bpm	21.6^{o}C	Immediately before	Immediately after, and 30 minutes after	EnzCheck Ultra Amylase assay kit	Before: 1.28±0.94 After: 1.47±1.15 (p<0.05)	
Ligtenberg 2015, Netherland	PC	University students; 29; Mixed; 21.6±1.5	Unstimulated/ Expectoration	Moderate (130 BPM) and high intensity running (N/R)	7-15 ^{o}C	N/R	Immediately after	ELISA	Before: 2.77± 3.49 After: 4.48±4.99 (p<0.0001)	

Key: ELISA: Enzyme Linked Assay

5.8. Bias assessment

According to the Newcastle-Ottawa scale for the observational studies, eight studies were considered at low risk of bias (Guilhem et al., 2015, Tauler et al., 2014, Milanez et al., 2014, Trochimiak et al., 2015, Kvietkauskaite et al., 2014, Vardiman et al., 2011, Moreira et al., 2009, Nehlsen-Cannarella et al., 2000, Leicht et al., 2012), seventeen studies were considered moderate risk of bias (Monje et al., 2020, Papacosta et al., 2016, Maya et al., 2016, Ligtenberg et al., 2016, Li et al., 2015, Morgans et al., 2014, OWEN et al., 2014a, Fredericks et al., 2012, Moreira et al., 2010, Koch et al., 2007a, Frese et al., 2015, Paul et al., 2015, Morgans et al., 2015, Fisher et al., 2015, Min-Lung et al., 2012, Zauber et al., 2012, Nunes et al., 2011) and two studies were considered as high risk of bias (Mori et al., 2016, Francis et al., 2005) (Table 5.10). There were some concerns in the RCTs which were assessed by the RoB2 scale (Table 5.11).

Table 5.10: Bias assessment for observational studies using Newcastle Ottawa Scale (NOS).

Study	**Selection**	**Comparability**	**Exposure**	**Overall**
Prospective Cohort				
Monje, 2020	***	*	*	*****(5/9) Moderate
Papacosta, 2016	***	*	*	*****(5/9) Moderate
Maya 2016	***	*	*	*****(5/9) Moderate
Mori 2016	*	*	*	***(3/9) High
Ligtenberg 2016	***	*	*	*****(5/9) Moderate
Li, 2015	***	*	*	*****(5/9) Moderate
Guilhem 2015	***	*	***	*******(7/9) Low
Tauler 2014	***	*	***	*******(7/9) Low
Milanez 2014	***		***	******(6/9) low
Owen 2014	***		*	****(4/9) Moderate
Morgans 2014	***	*	*	*****(5/9) Moderate
Kvietkauskaite 2014	***		***	******(6/9) low
Fredericks 2012	***	*	*	*****(5/9) Moderate
Moreira 2010	***		*	****(4/9) Moderate
Koch 2007	***		*	****(4/9) Moderate
Case-control				
Trochimiak 2015	****	*	***	********(8/9) Low
Frese 2015	***	*	*	*****(5/9) Moderate
Vardiman 2011	****	*	***	********(8/9) Low
Moreira 2009	****	*	*	******(6/9) low
Sandra 2000	****	*	*	******(6/9) low
Repeated measure				

Paul 2015	***		*	****(4/9) Moderate
Morgans 2015	***		*	****(4/9) Moderate
Fisher 2015	***	*	*	*****(5/9) Moderate
Min-Lung 2012	***		*	****(4/9) Moderate
Leicht 2012	***		***	******(6/9) low
Zauber 2012	***		*	****(4/9) Moderate
Nunes 2011	***	*	*	*****(5/9) Moderate
Francis 2005	**		*	***(3/9) High

Table 5.11: Bias assessment for RCT using RoB2.

Study	Method of randomisation	Randomisation bias	Deviation bias	Missing outcome data	Measurement bias	Result selection bias	Total
Hopkins 2020	Simple	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns
Kiani 2015	Simple	Some concerns	Some concerns	Some concerns	Some concerns	Some concerns	Some concerns
Reid 2001	Simple	Some concerns	Some concerns	Some concerns	Some concerns	Low	Some concerns

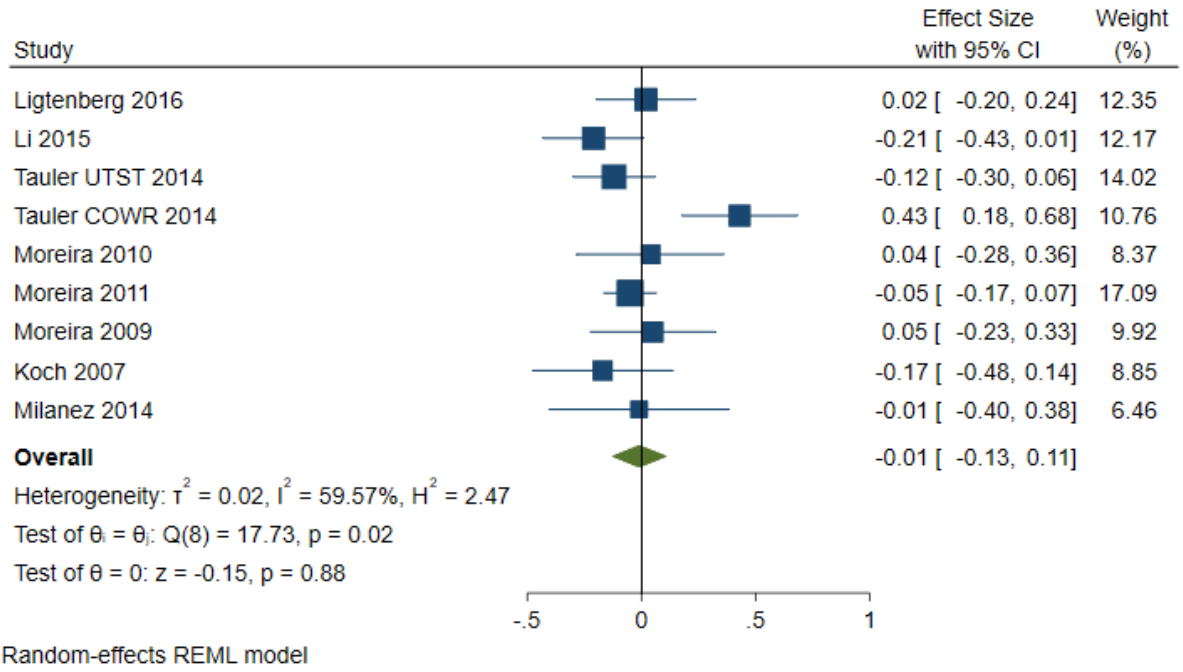
5.9. Quantitative results

15 out of 31 studies were included in the meta-analysis of intensive physical activity on the saliva flow rate, salivary IgA, alpha-amylase and saliva total protein. The reasons for exclusions were graphical representation of outcome data (Trochimiak et al., 2015, Mori et al., 2016, Paul et al., 2015, Morgans et al., 2014, Francis et al., 2005, Owen et al., 2014b, Leicht et al., 2012), failure of reporting of pre-activity values (Morgans et al., 2015, Nehlsen-Cannarella et al., 2000), providing the median rather than the mean values (Fredericks et al., 2012) and failure of contact from the respective authors (Frese et al., 2015, Kvietkauskaite et al., 2014, Zauber et al., 2012, Nunes et al., 2011).

5.9.1. Saliva flow rate

Overall, there was no statistically significant change in saliva flow following physical activity (mean difference of -0.01 ml/min (95% CI -0.13, 0.11) (p=0.88) with high heterogeneity (I^2= 59%) (Figure 5.2). One study appeared to be an outlier contributing to the high heterogeneity (Tauler et al., 2014).

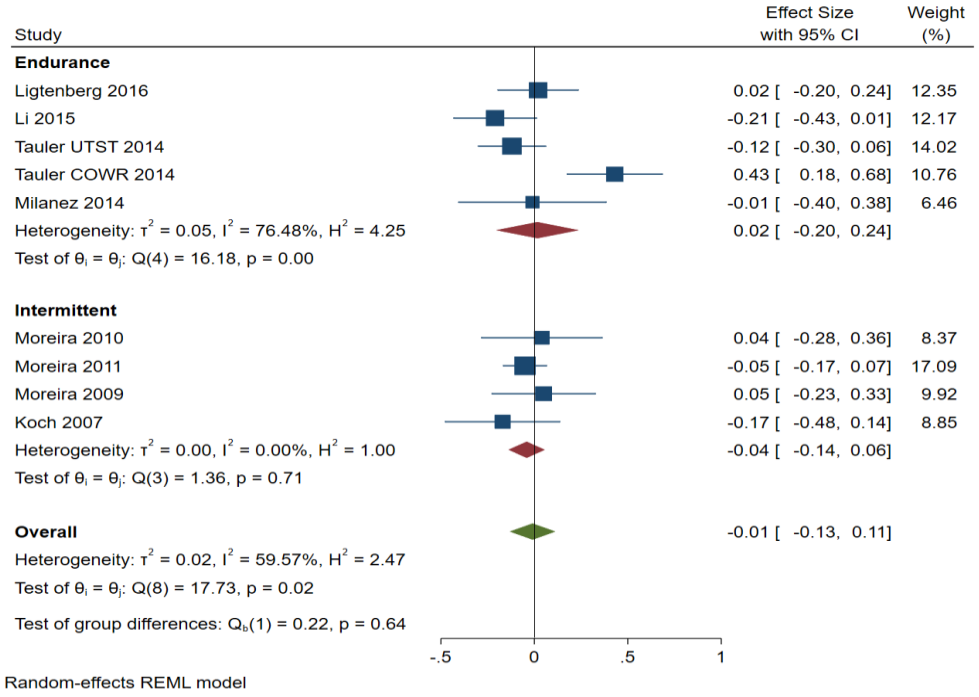
Figure 5.2: Forest plot for mean difference in salivary flow rate (ml/min).



As planned subgroup analysis was carried out to investigate the possible causes of heterogeneity. The subgroup analysis was carried out on the basis of the sports/physical activity, saliva collection method and saliva collection time after cessation of activity.

5.9.1.1. According to sport/physical activity

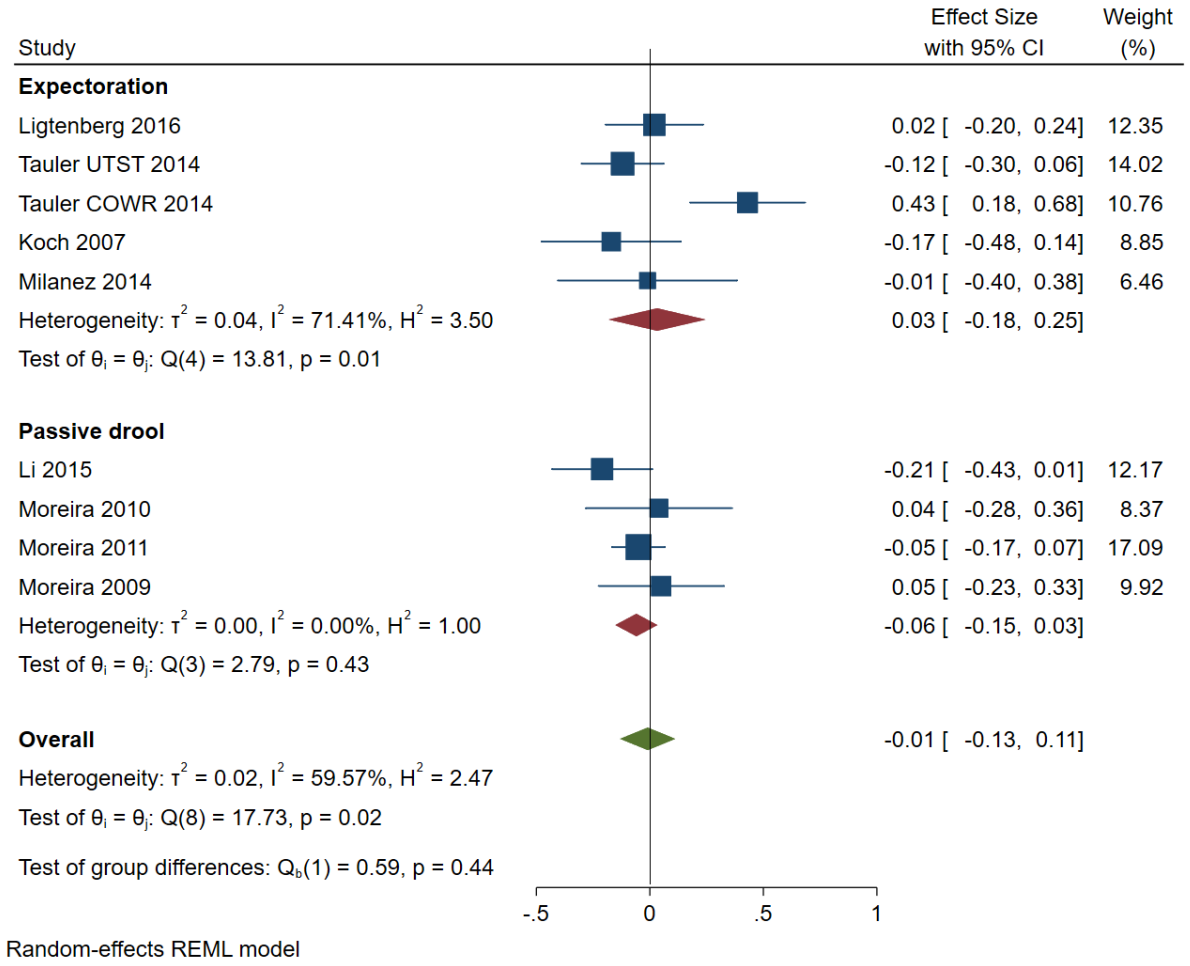
Figure 5.3: Forest plot for mean difference in saliva flow according to sports/physical activity (ml/min).



The sports/physical activities were stratified into intermittent and endurance sports (Figure 5.3). The mean difference in saliva flow in the endurance activity subgroup was 0.02 (95% CI -0.2, 0.24) (p=0.00) with a high heterogeneity (I^2=76%). The mean difference in saliva flow in the intermittent activity subgroup was -0.04 (95% CI -0.14, 0.06) (p=0.71) with no heterogeneity. There were no statistically significant differences in flow rate between both subgroups (p=0.64).

5.9.1.2. According to saliva collection method

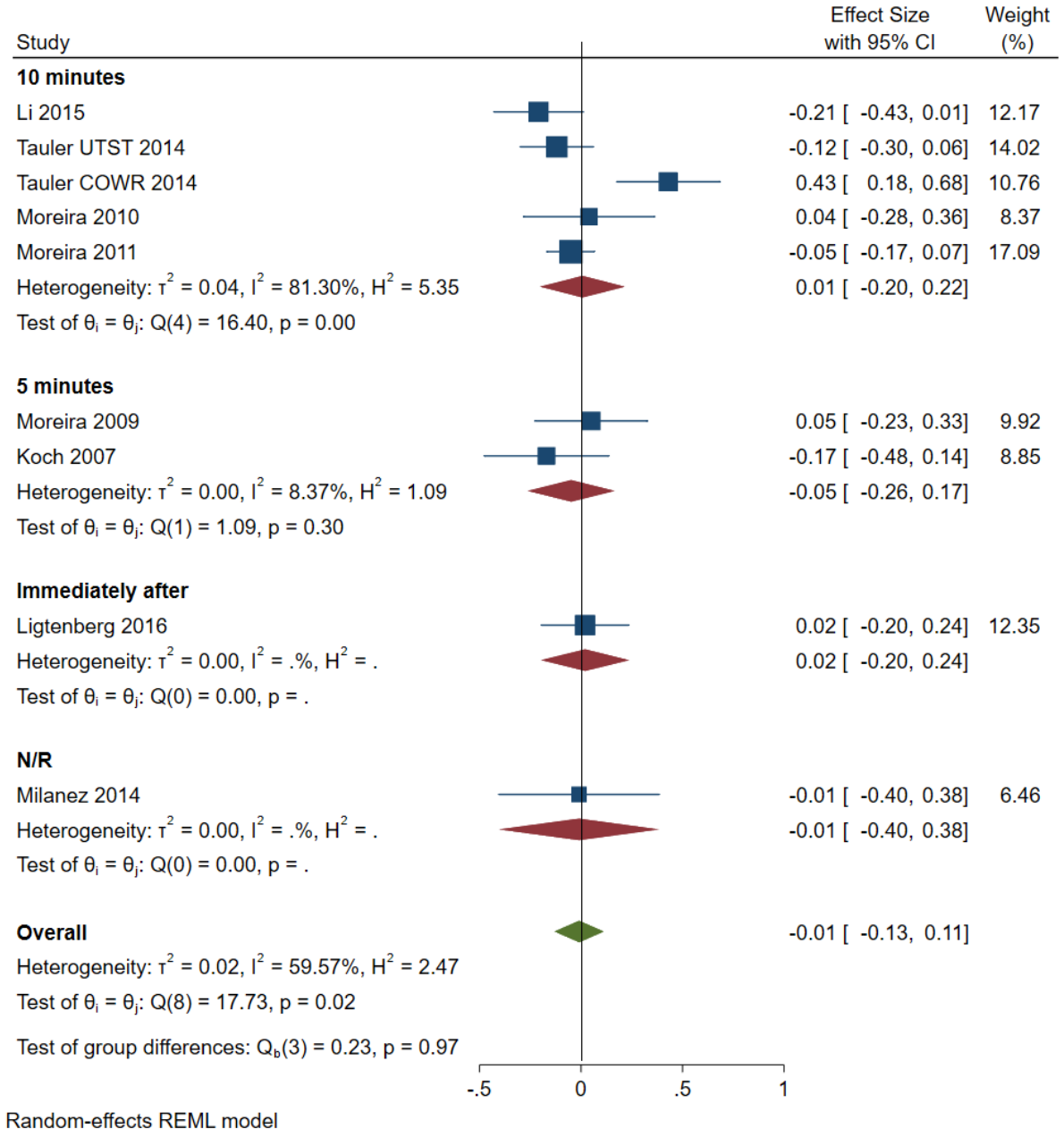
Figure 5.4: Forest plot measuring mean difference in saliva flow according to saliva collection methods (ml/min).



The saliva collection methods were stratified into passive drooling and expectoration (Figure 5.4). The mean difference in saliva flow in the expectoration method subgroup was 0.03 (95% CI -0.18, 0.25) (p=0.01) with a high heterogeneity (I^2=71%). The mean difference in saliva flow in the passive drooling method subgroup was -0.06 (95% CI -0.15, 0.03) (p=0.43) with no heterogeneity. There were no statistically significant differences in flow rate between both subgroups (p=0.64).

5.9.1.3. According to saliva collection time after cessation of activity

Figure 5.5: Forest plot measuring mean difference in saliva flow according to collection times (ml/min).



The saliva from the participants in the study after the cessation of the activity was either immediately collected, or after five and ten minutes (Figure 5.5). Subgroups were made on the basis of the collection times. The mean difference in saliva flow after ten minutes was 0.01 (95% CI -0.20, 0.2) (p=0.00) with high heterogeneity (I^2=81%). The mean difference in saliva flow after five minutes of activity was -0.05 (95% CI -0.26, 0.17) (p=0.30) with low heterogeneity (8%). Only one study collected saliva immediately after activity and one study did not report the collection times. There

were no statistically significant differences in flow rate between both subgroups (p=0.97).

5.9.1.4. Sensitivity analysis

Sensitivity analysis was performed to further investigate the causes of heterogeneity which were not resolved during subgroup analysis.

Table 5.12: Sensitivity analysis for saliva flow rate.

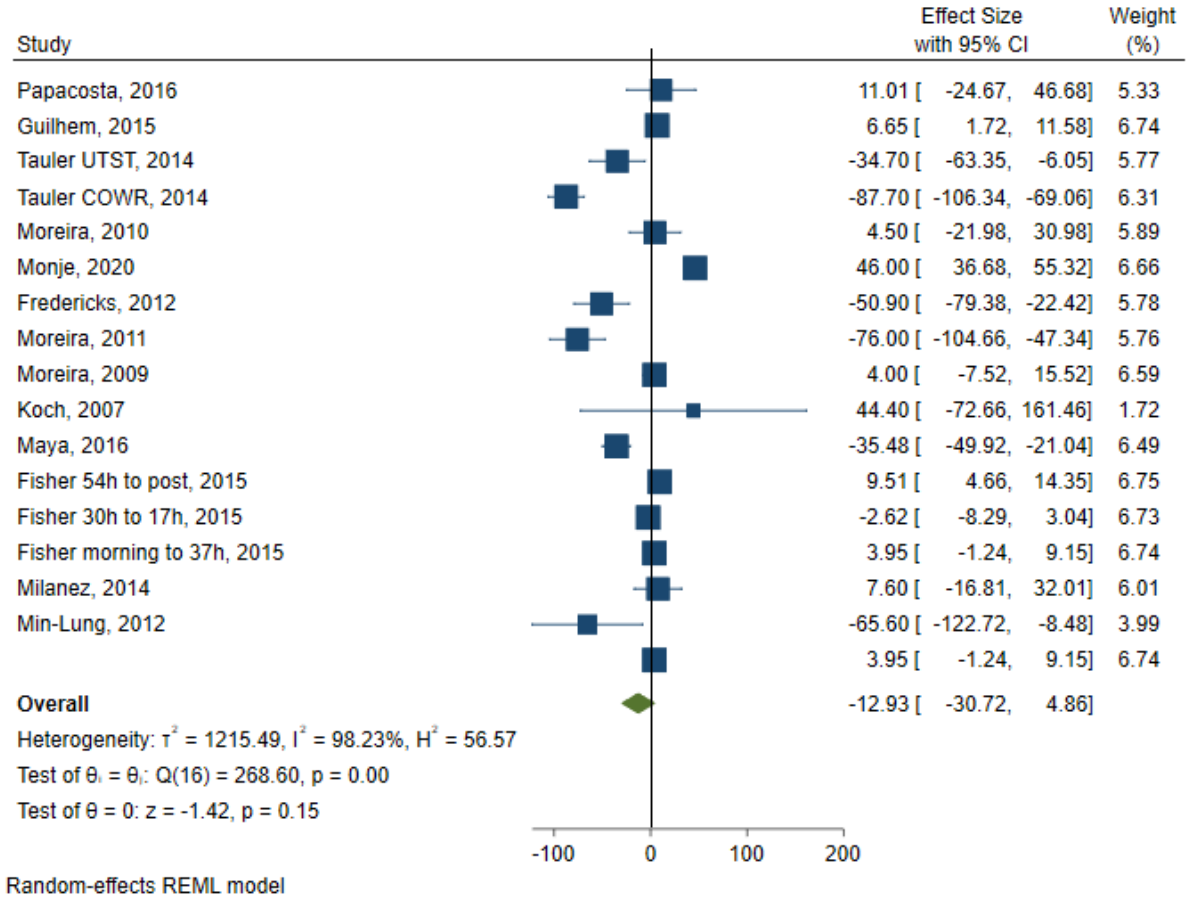
	Effect size	95% CI	I^2	Homogeneity test	Effect test
All data	-0.01	(-0.13,0.11)	59.57%	0.023	0.87
Excluded Tauler 2014	-0.054	(-0.13, 0.02)	0.00%	0.69	0.18

There was 0.0% heterogeneity observed after excluding one segment of Tauler et al., 2014 as there may have been a possibility that the saliva flow rate may have been affected by the sodium chloride present in sea water, hence increasing the saliva flow rate. The effect size decreased to -0.054 and remained not statistically significant (Table 5.12).

5.9.2. Salivary IgA

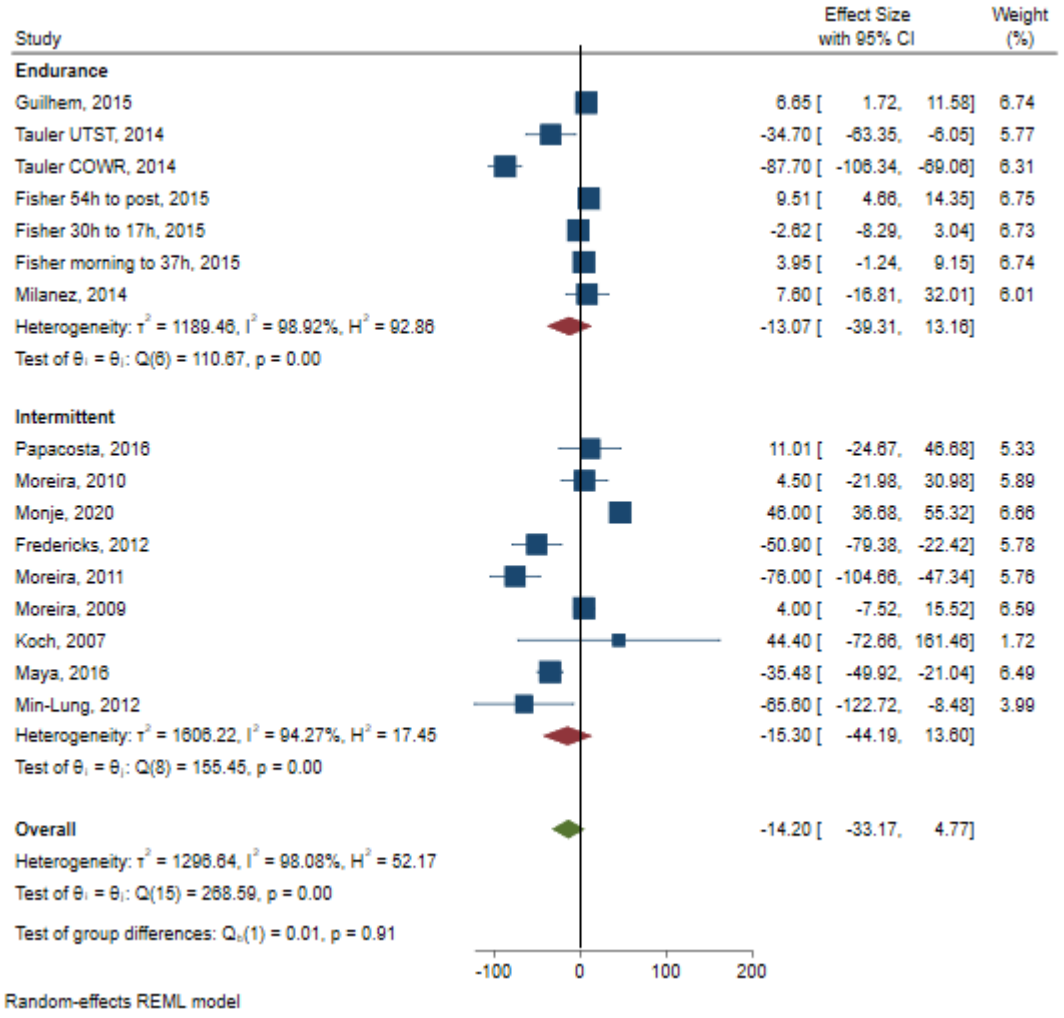
Thirteen studies were included in the meta-analysis for salivary IgA (Figure 5.6). Overall, there was no change in the concentration of salivary IgA following physical activity (mean difference of -19.43 µg/ml (95% CI -45.23, 6.67) with high heterogeneity (I^2= 98%)). There was no statistically significant difference between the saliva IgA concentration before and after the periods of activity (p=0.14). One study appeared to be outlier with a mean difference of -218 µg/ml (Maya et al., 2016). Subgroup analysis was performed to investigate the causes of heterogeneity.

Figure 5.6: Forest plot measuring mean difference in salivary IgA (µg/ml)



5.9.2.1. According to sports/physical activity

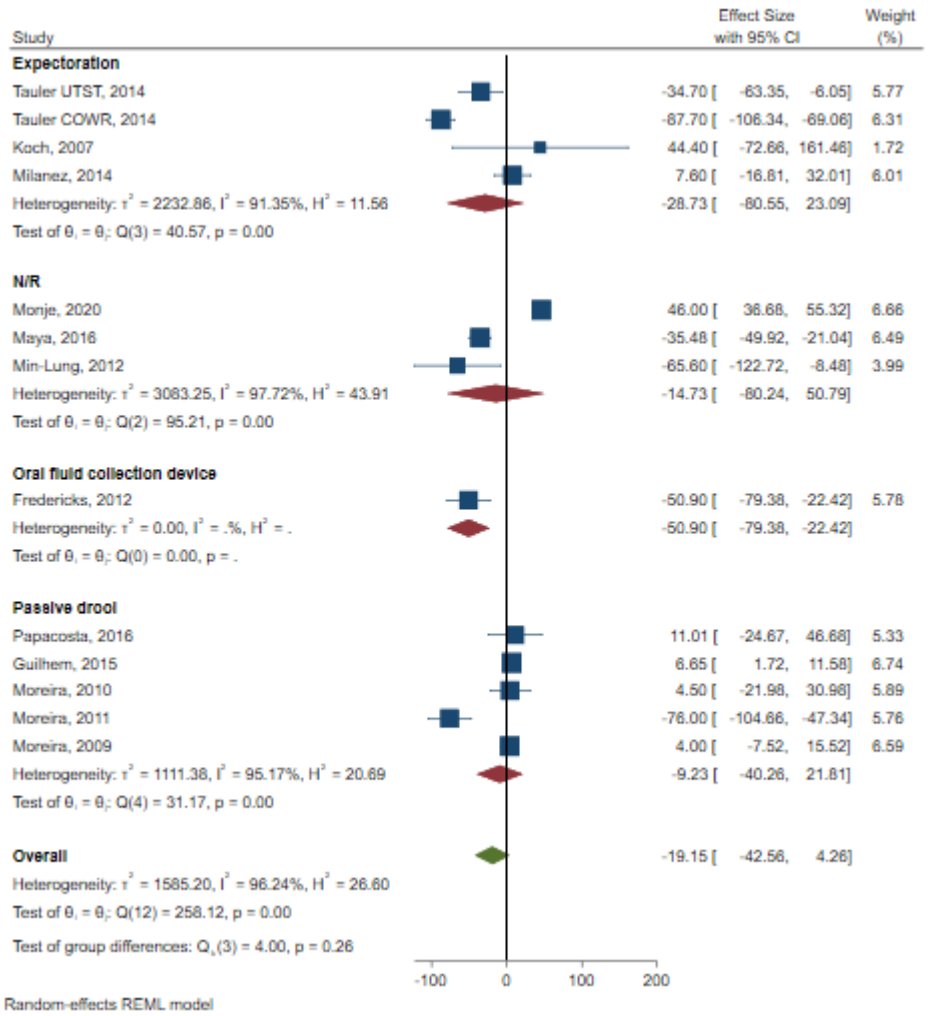
Figure 5.7: Forest plot measuring mean difference in salivary IgA according to sport activity (µg/ml)



The mean difference in salivary IgA in the endurance sport subgroup was 10.72 µg/ml (95% CI -39.62, 18.18) (p=0.00) with high heterogeneity (I^2=98%). The mean difference in salivary IgA intermittent sport subgroup was -30.01 µg/ml (95% CI -76.48, 16.47) (p=0.00) with high heterogeneity (I^2=97%). There were no statistically significant differences in salivary IgA between both subgroups (p=0.49) (Figure 5.7).

5.9.2.2. According to collection method

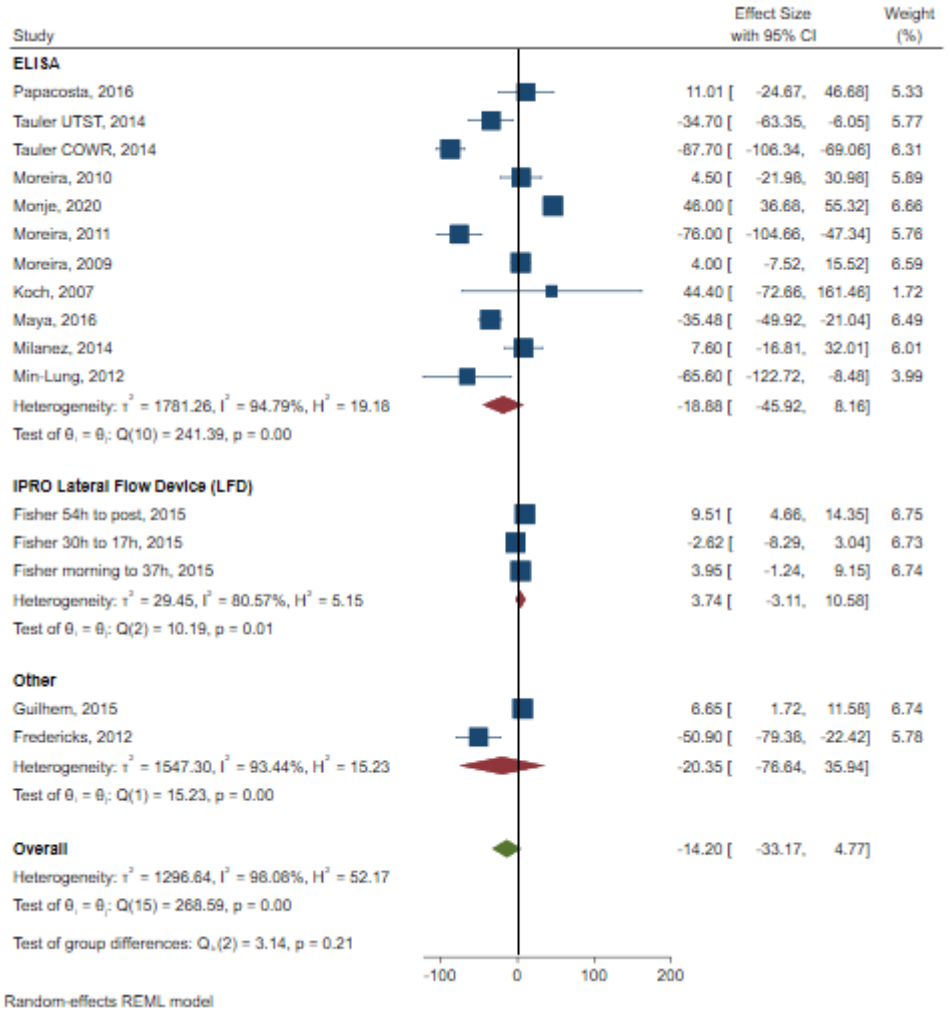
Figure 5.8: Forest plot measuring mean difference in salivary IgA according to collection methods (µg/ml)



The mean difference in salivary IgA in the expectoration method subgroup was -74.16 µg/ml (95% CI -222.98, 74.66) (p=0.00) with a high heterogeneity (I^2=96%). The mean difference in salivary IgA in the passive drooling method subgroup was -5.44 µg/ml (95% CI -41.41, 30.52) (p=0.00) with high heterogeneity (I^2=89%). The overall mean difference was -26.49 µg/ml (95% CI -59.76, 6.77) There were no statistically significant differences in salivary IgA between the three subgroups (p=0.25) (Figure 5.8).

5.9.2.3. According to methods of assessment

Figure 5.9: Forest plot measuring mean difference in salivary IgA according to assessment methods (µg/ml).



Salivary IgA was assessed by ELISA, an IPRO lateral flow device (LFD) and immunoturbidometric assay (Figure 5.9). Three subgroups were made on the basis of the three assessment methods. The mean difference in salivary IgA using ELISA was -30.05 µg/ml (95% CI -68.60, 8.510) (p=0.00) with high heterogeneity (96%). The mean difference in salivary IgA using LFD was 3.74 µg/ml (95% CI -3.11, 10.58) (p=0.01) with high heterogeneity (80%). The mean difference in salivary IgA using immunoturbidometric assay was -10.01 µg/ml (95% CI -92.61, 6.37) (p=0.00) with high heterogeneity (I^2=91%). Overall, there were no statistically significant differences in salivary IgA between the three subgroups (p=0.23).

5.9.2.4. Sensitivity analysis

Sensitivity analysis was performed to investigate the cause of heterogeneity in salivary IgA.

Table 5.13: Sensitivity analysis of salivary IgA.

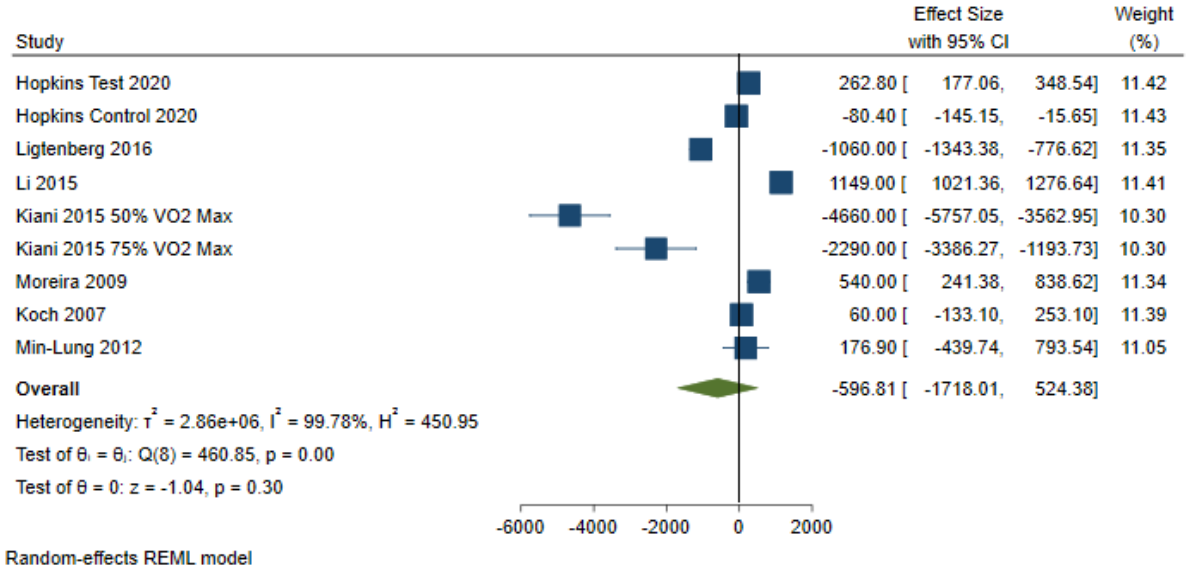
Author	Effect size	95% CI	I^2	Homogeneity test	Effect test
All data	-12.931	(-30.722, 4.86)	98.23%	0	0.1543
Excluding Tauler 2014	-5.663	(-21.716, 10.39)	97.71%	0	0.4893
Excluding Moreira 2011	-8.943	(-25.971, 8.086)	98.04%	0	0.3033
Excluding Min-Lung 2012	-10.737	(-28.719, 7.244)	98.30%	0	0.2419

There was no significant change in heterogeneity observed after exclusion of three studies which were suspected of causing the high heterogeneity as they were observed to be outliers in the forest plot of the meta-analysis indicating a significant decrease in IgA levels as compared to the other studies (Table 5.13).

5.9.3. Saliva Total Protein

Overall, there was no statistically significant change in saliva total protein content following physical activity and had a mean difference of -596.81 µg/ml (95% CI -1718.0, 524.38) with high heterogeneity (I^2= 99%) (Figure 5.10). One study was divided into two according to the intensity and produced outliers which might have been the cause of the high heterogeneity (Kiani, et al., 2015)

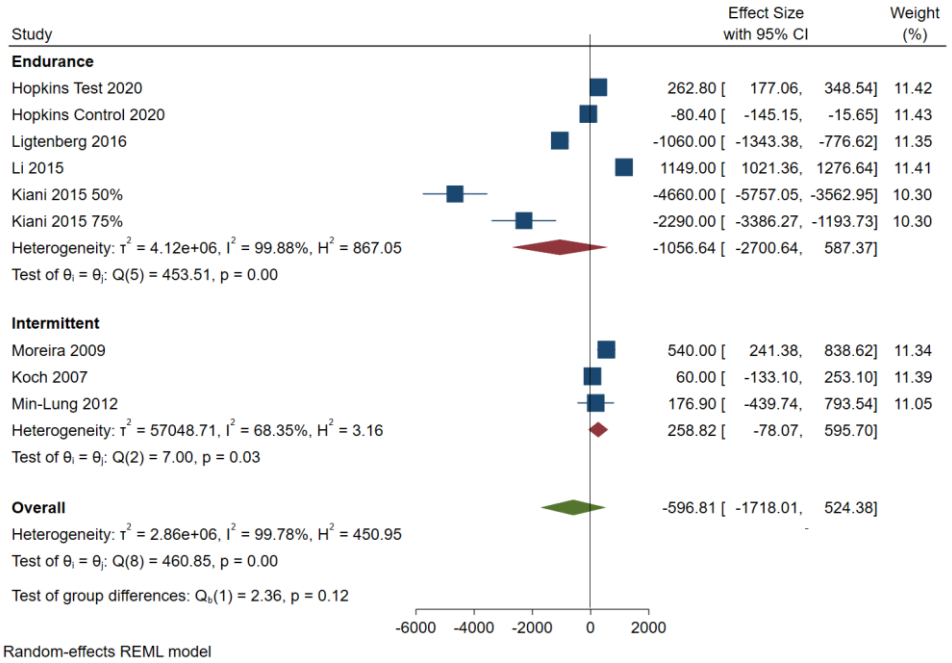
Figure 5.10: Forest plot measuring mean difference in saliva total protein (STP) (µg/ml).



Subgroup analysis was performed to investigate the causes of heterogeneity.

5.9.3.1. According to sports/physical activity

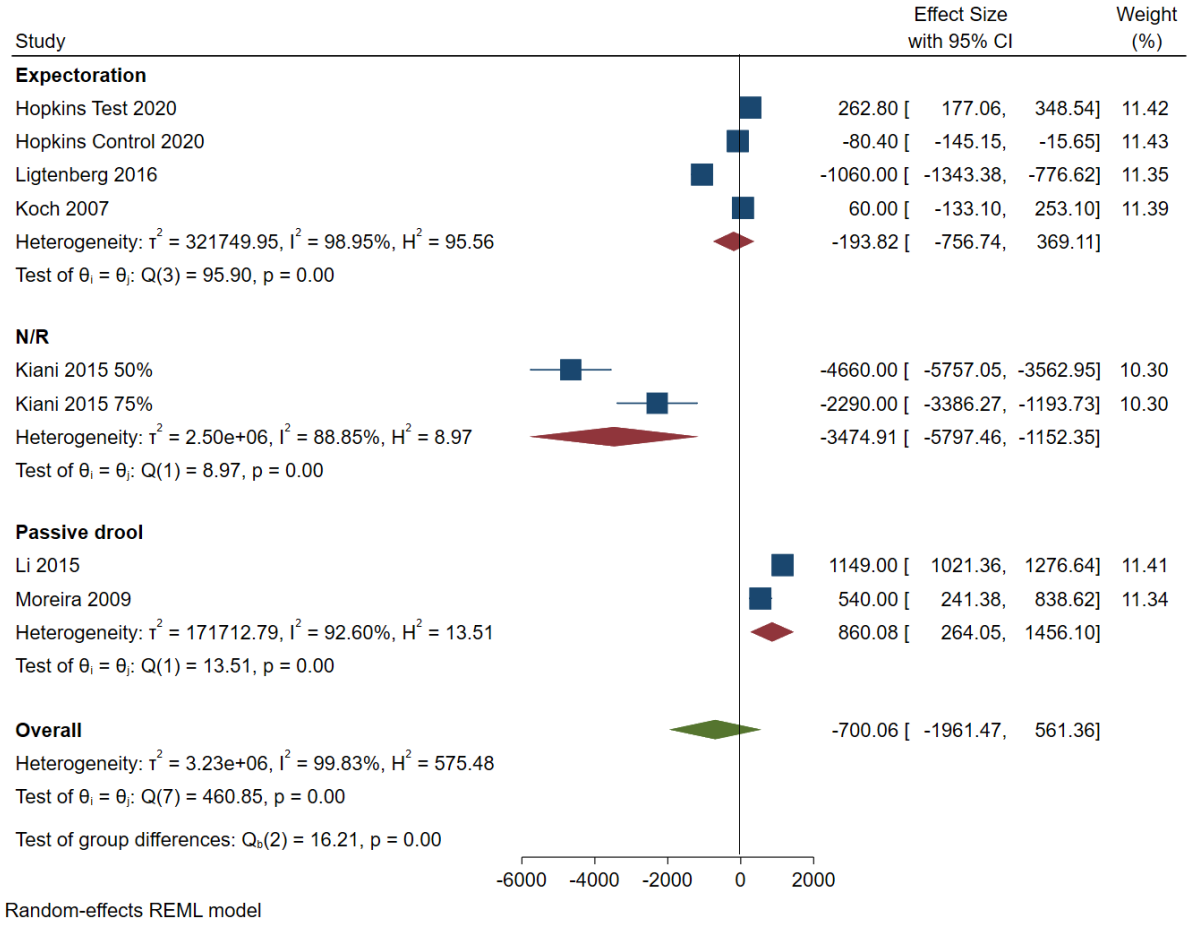
Figure 5.11: Forest plot measuring mean difference in STP according to sports activity (µg/ml)



The mean difference in STP in the endurance sport subgroup was -1056.64 µg/ml (95% CI –2700.6, 587.37) (p=0.00) with a high heterogeneity (I^2=99%). The mean difference STP in the intermittent sport subgroup was 258 µg/ml (95% CI -78.07, 595.70) (p=0.03) with high heterogeneity (I^2=97%). There were no statistically significant differences in salivary IgA between both subgroups (p=0.12) (Figure 5.11).

5.9.3.2. According to collection method

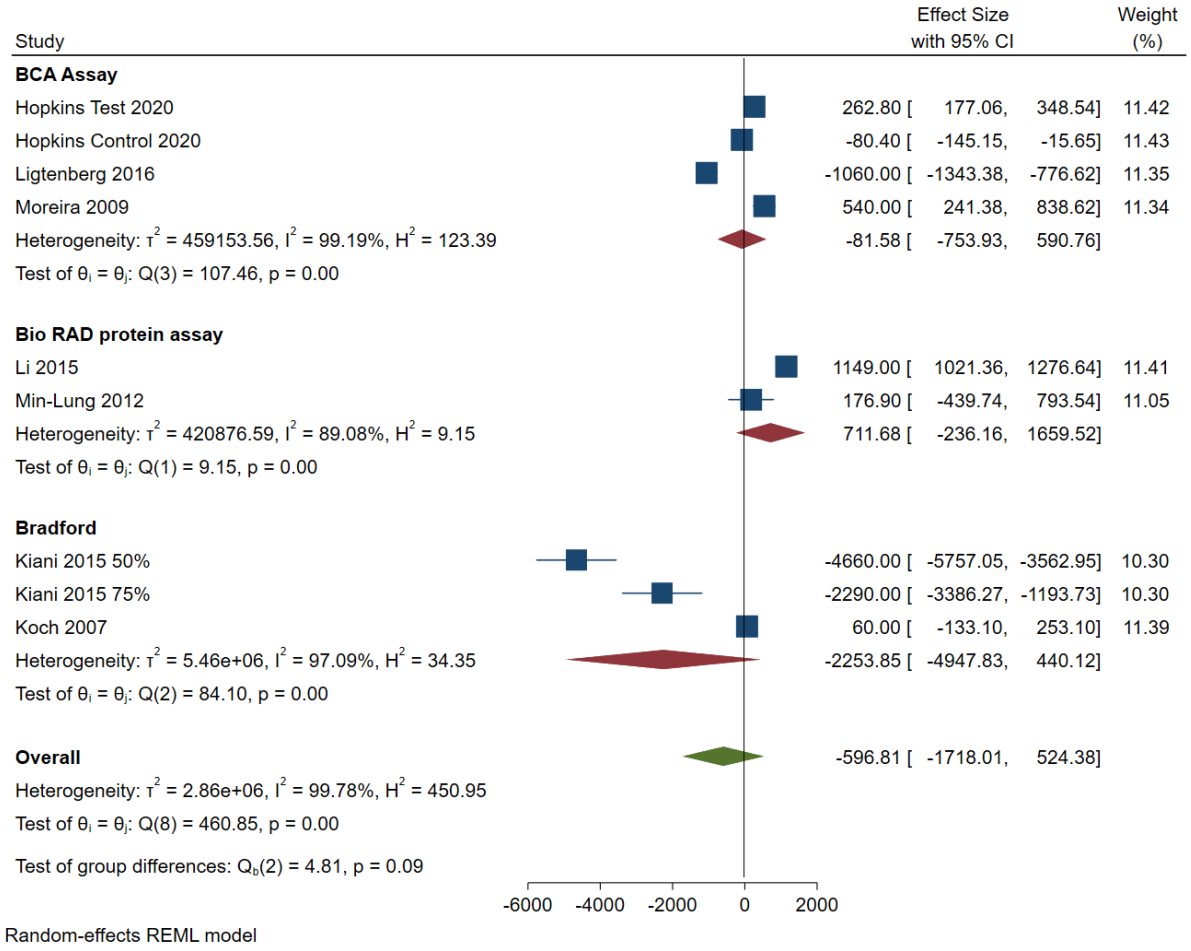
Figure 5.12: Forest plot measuring mean difference in STP according to saliva collection methods (µg/ml)



The mean difference in STP in the expectoration method subgroup was -193.82 µg/ml (95% CI -756.74, 369.11) (p=0.00) with a high heterogeneity (I^2=98%) (Figure 5.12). The mean difference in STP in the passive drooling method subgroup was 860.08 µg/ml (95% CI -264.05, 1456.10) with high heterogeneity (I^2=92.6%). There were no statistically significant differences in salivary IgA between the three subgroups (p=0.00). The mean difference in STP in the subgroup that did not report collection method was -3474.9 µg/ml (95% CI -5797.4, -1152.3) with high heterogeneity (I^2= 88.8%).

5.9.3.3. According to assessment methods

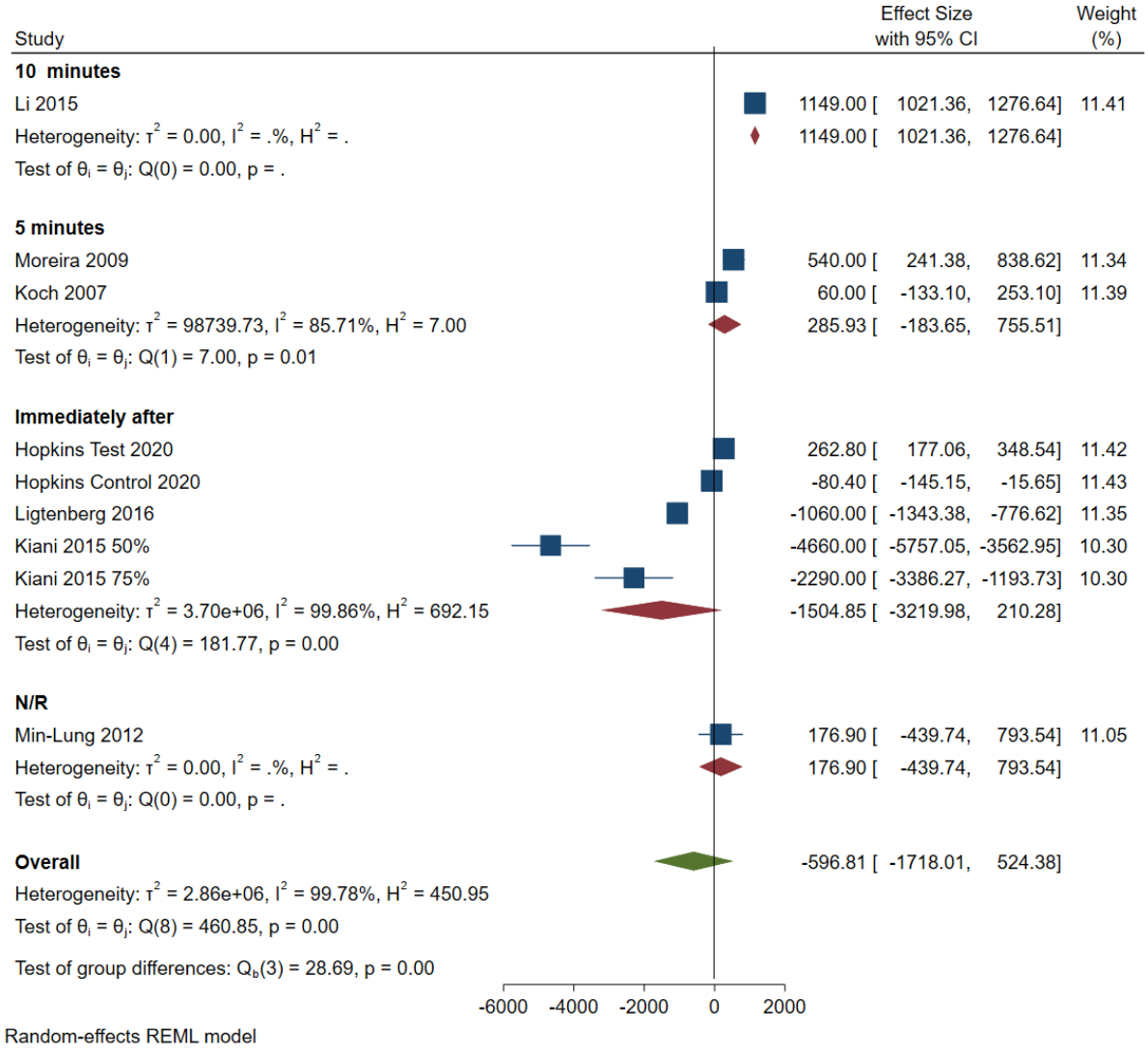
Figure 5.13: Forest plot measuring mean difference in STP according to assessment methods (µg/ml)



STP was measured using the BCA assay, BIO-RAD protein assay and the Bradford technique. The mean difference in STP using the BCA was -81.58 µg/ml (95% CI -753.93, 590.76) (p=0.00) with a high heterogeneity (I^2=99%). The mean difference in STP using the BIO-RAD protein assay was 711.68 µg/ml (95% CI -236.16, 1659.52). The mean difference in STP using the Bradford technique was -2253.85 µg/ml (95% CI --4947.83, 440.12). There were no statistically significant differences in STP between the three subgroups (p=0.09) (Figure 5.13).

5.9.3.4. According to collection time after cessation of activity

Figure 5.14: Forest plot measuring mean difference in STP according to collection times (µg/ml)



One study collected saliva after ten minutes of cessation of activity (Figure 5.14). The mean difference in STP of the subgroup collecting saliva after 5 minutes was 285.96 µg/ml (95% CI -183.65, 755.51) (p=0.01) with a high heterogeneity (I^2=85%). The mean difference in STP of saliva collected immediately after activity was -1504 µg/ml (95% CI -3219.9, 210.2) (p=0.00) with high heterogeneity (I^2=99%). One study did not report collection time after the activity. Overall, there were no statistically significant differences in STP between the three subgroups.

5.9.3.5. Sensitivity analysis

Sensitivity analysis was performed to further identify the causes of heterogeneity (Table 5.14). There was no significant change in heterogeneity observed after exclusion of one study which was suspected of causing the high heterogeneity as the participants included in the study were non-athletes which may be a reason for the significant decrease in STP as observed in the forest plot of the meta-analysis.

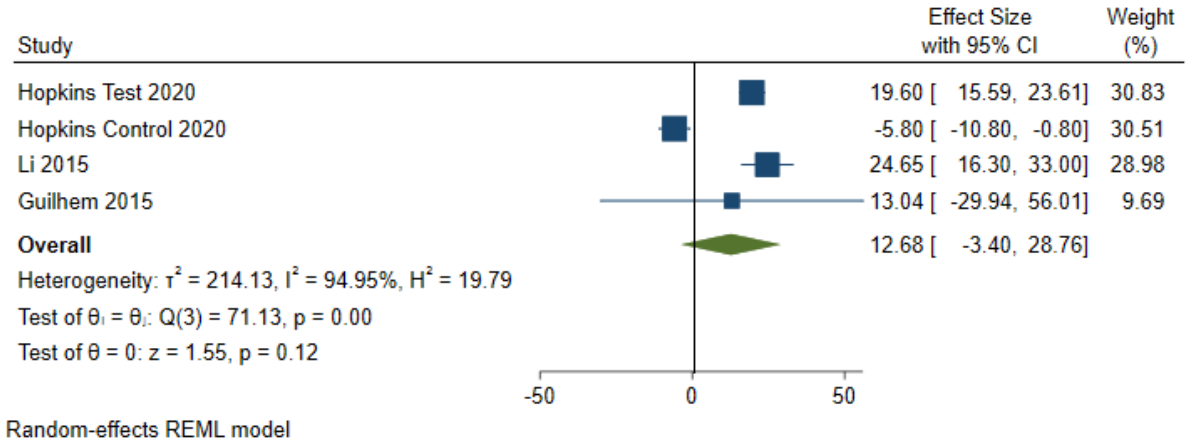
Table 5.14: Sensitivity analysis for STP.

	Effect size	95% CI	I^2	Homogeneity test	Effect test
All data	-596.81	(-1718.0, 524.35)	99.78%	0	0.29
Without Kiani	153.16	(-350.25, 656.58)	98.92%	0	0.55

5.9.4. Salivary α-amylase

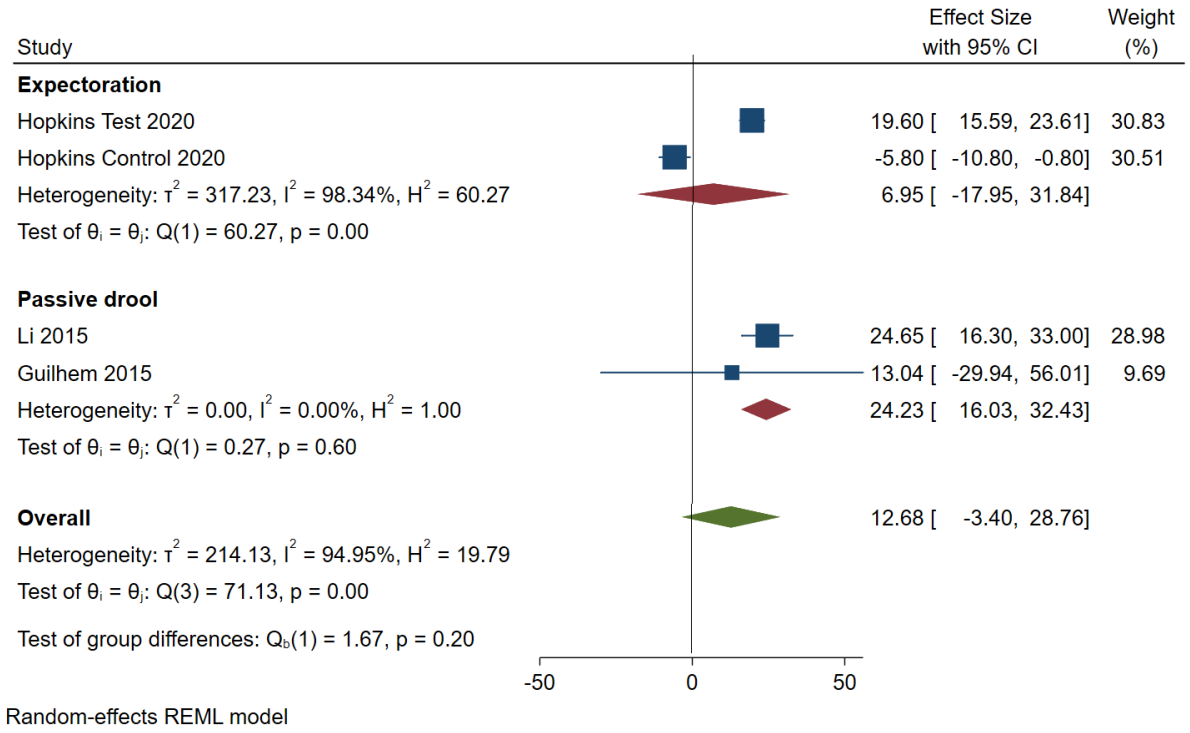
Three studies were included in the meta-analysis for salivary α-amylase (Figure 5.15). Overall, there was no statistically significant change in salivary α-amylase levels following physical activity with a mean difference of -12.68 µg/ml (95% CI -3.40, 28.76) and high heterogeneity (I^2= 94%). There was no statistically significant difference between salivary α-amylase levels before and after the periods of activity (p=0.12). Subgroup analysis was performed to investigate the causes of heterogeneity.

Figure 5.15: Forest plot measuring mean difference in salivary α-amylase (µg/ml).



5.9.4.1. According to collection method

Figure 5.16: Forest plot measuring mean difference in salivary amylase levels according to collection methods (µg/ml).

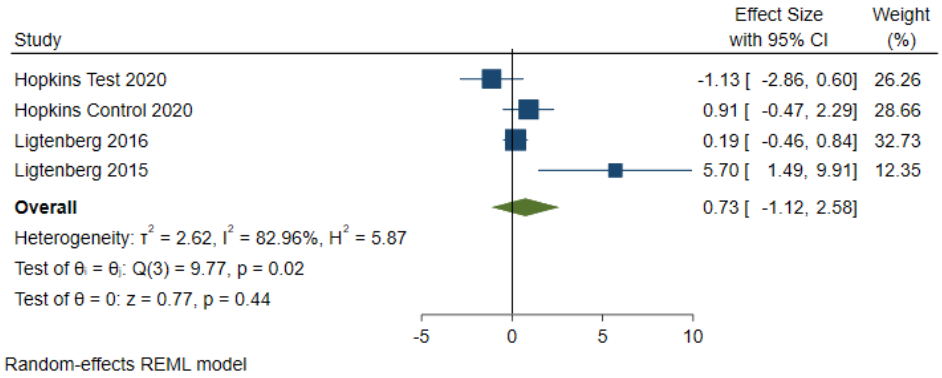


The mean difference in salivary α-amylase using expectoration was 6.95 (95% CI -17.95, 31.84) (p=0.01) with high heterogeneity (I^2=98%). The mean difference in amylase using passive drooling was 24.23 (95% CI -16.03, 32.43) (p=0.60) with no heterogeneity (Figure 5.16) between the two included studies. There was no statistically significant difference between the two subgroups (P=0.20)

5.9.5. Salivary mucins (MUC5B)

Three studies measured salivary mucin levels (Figure 5.17). Although two studies reported a statistically significant increase in salivary mucins and one reported no change, the meta-analysis did not report a statistically significant change in salivary mucins MUC5B levels following physical activity (mean difference of -0.73 U/ml (95% CI -1.12, 2.58) with high heterogeneity (I^2= 82%) (p=0.44).

Figure 5.17: Forest plot showing mean difference of salivary mucins (U/ml).



5.9.6. Certainty of evidence

Overall, GRADE results suggested a low certainty in the evidence for one outcome, and very low certainties in four outcomes (Table 5.15). The ratings were downgraded due to moderate risk of bias, extremely high heterogeneity and small sample sizes.

Risk of bias

The outcomes, saliva flow and STP had moderate risk of bias due to loss of follow up and had no description of the non-exposed cohorts due to which they were downgraded and considered serious.

Inconsistency

According to the criteria, four outcomes had an extremely high heterogeneity (>90%) due to which they were considered very serious. One outcome had moderate heterogeneity (57%) due to which it was considered serious and downgraded the quality of the evidence and hence a very low certainty.

Table 5.15: Certainty of evidence.

	Certainty assessment							No. of patients	Effect		Certainty
	No. of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**	**Physical activity**	**Relative**	**Absolute (95% CI)**	
Salivary amylase	3	observational studies	not serious	very serious [b]	not serious	serious	none	53		MD **0** (3.4 lower to 28.7 higher)	⨁◯◯◯ VERY LOW
Salivary mucin	3	randomised trials	not serious	very serious [b]	not serious	serious	none	60		MD **0** (1.12 lower to 2.58 higher)	⨁⨁◯◯ LOW
Saliva flow	9	observational studies	serious [a]	serious [b]	not serious	not serious	none	228		MD **0** (0.13 lower to 0.11 higher)	⨁◯◯◯ VERY LOW
Salivary IgA	13	observational studies	not serious	very serious [b]	not serious	not serious	none	302		**0** (0 to 0)	⨁◯◯◯ VERY LOW
STP	7	observational studies	serious [a]	very serious [b]	not serious	serious	none	99		MD **0** (1718 lower to 524 higher)	⨁◯◯◯ VERY LOW

a: Moderate risk of bias due to loss of follow up and no details of the non-exposed cohorts
b: High heterogeneity with unexplained reasons.

5.10. Discussion

5.10.1. Key findings

The systematic review was performed to assess the qualitative and quantitative effects of intense physical activity and training on saliva. Our findings indicate that there was no statistically significant effect of intense physical activity on the saliva flow rate, concentrations of salivary IgA or salivary α-amylase, saliva total protein (STP) and salivary mucin levels. However, the certainty of the evidence for all outcomes was very low suggesting that the summary estimate of the meta-analysis might not be totally reliable.

5.10.2. Strength and limitations of included evidence

This is the first systematic review to be performed on the effects of physical activity on saliva flow rate, saliva total protein content, salivary mucins, and salivary IgA, supported by a thorough literature search, a priori defined protocol, and following recommended guidelines for data reporting (Appendix A). The review included participants involved in intense physical training and all studies involved above college level athletes involved in both intermittent and endurance sports and physical activities. Apart from athletes, two studies also involved military personnel who carried various loads (Paul et al., 2015) or were involved in peace keeping missions (Kvietkauskaite et al., 2014). All studies collected saliva from the participants, however the collections were made at different time points after cessation of physical activity. The factor contributing to the strength of the evidence was the identical collection method (unstimulated, passive drooling) in 24 out of 31 studies which indicated that whole mouth saliva (WMS) was collected as whole mouth saliva is collected by either spitting or drooling (Mutahar et al., 2020).The unstimulated passive drooling collection has been the most preferred method because 1). It minimises the dilution of analytes and 2). There is minimal effect of flow rate on the saliva composition (Bellagambi et al., 2020). However, it should also be noted that differences in composition exist between WMS and parotid saliva (PS). PS is a serious fluid which consists predominantly of proteins such as proline-rich proteins (PRPs), histatins and statherin but lacks mucins whereas mucin 7 and mucin 5b are salivary mucins secreted by submandibular and sublingual glands but are absent from parotid glands (Humphrey and Williamson, 2001).

Studies performing mass spectroscopic analysis of WMS and PS have also suggested a greater variety of proteins adhering from WMS compared to parotid saliva with the total protein concentration nearly double to that from parotid saliva. Examples include mucin 5b

and albumin rich within the WMS, but on the other hand statherin/ carbonic anhydrase rich, mucin/ albumin deficient in PS (Mutahar et al., 2020).

On the other hand, the included evidence had a few limitations. The intensity and length of physical activity was different for each included study. The saliva collection times were also different for each study ranging from five minutes to two days post activity There were a large number of studies reporting IgA levels, but there has been no reported evidence of associations of salivary IgA to dental erosion. Still, IgA was included in the review because the search strategy included proteins in saliva, and it could not be omitted. Secondly, salivary IgA is also used as a stress biomarker to measure stress levels and increased stress levels have been suggested to be associated with bruxism. Since dental erosion is a multifactorial process which includes abrasion and attrition, there may be a possibility for salivary IgA to be indirectly associated with dental erosion. The studies measuring STP did not specifically quantify proteins responsible for enamel homeostasis except for mucin MUC5B. MUC5B is a globular protein which plays a role in lubrication of the oral cavity, hence preventing dental erosion. However, only three studies assessed salivary mucin (MUC5B) levels with respect to dental erosion. Furthermore, the dental pellicle was also not assessed in any of the studies included.

5.10.3. Limitations of the review

Only 10% of studies were checked for eligibility and duplicate data abstraction, despite the gold standard being 100% for both leading to a potential increased risk of reviewer bias and error rate. On the other hand, the kappa score was high suggesting the eligibility checking process was well conducted.

The causes of the high heterogeneity could not be identified. There were variations in saliva collection methods, outcomes assessment methods and the activities performed but the subgroup analysis performed according to the variations could not identify the causes of the high heterogeneity. The extremely high heterogeneity had an impact on the certainty of the evidence as observed after assessing the evidence though GRADE. The high heterogeneity downgraded the quality of the evidence leading to a very low certainty, suggesting the results of the review and meta-analysis to be unreliable.

5.10.4. Significance of findings

5.10.4.1. Saliva flow rate

The findings suggested that there was no statistically significant change in saliva flow rate immediately after physical activity. The mean difference in saliva flow rate before and after the physical activity was 0.1ml/min. The average saliva flow rate suggested by one review ranged from 0.21 - 0.84 ml/min with an average flow rate of 0.3 ml/min (Humphrey and Williamson, 2001). Another recent review also reported the average saliva flow rate to be 0.5 ml/min (Bellagambi et al., 2020). Therefore, a mean difference of 0.1 ml/min, as per the results of the current study's analysis, could not be considered a clinically relevant change as the change still fell within the normal range of saliva flow. The findings of the analysis were surprising as physiologically, the saliva flow rate is reported to decrease as a result of increased sympathetic activity during intense physical activity (Frese et al., 2015). Physical activity causes a repression of the cholinergic parasympathetic innervation causing a significant vasoconstriction in salivary glands (Proctor & Carpenter, 2007). Additionally, fluid and electrolyte deficit as a consequence to water and sweat loss might add to the effect (Mulic et al., 2012; Allgrove et al., 2013).

On the other hand, two studies showed an increase in saliva flow as a result of intense physical activity. Keeping the physiology of saliva flow in mind, the increase in flow rate was surprising. The possible reason for the increase saliva flow rate could be that the participants performed a non-competitive 25 km swimming race in fresh sea water. Sodium chloride (being one of the components of sea water) has been suggested to be one of the most important oral stimuli for increased salivary flow rate which probably might be a factor for the increased secretion of saliva. In addition, one study reported significantly low levels of salivary flow rate of 0.12 ml/min before activity as compared to the other included studies in the review (Moreira et al.,2011). Keeping in mind the physiological salivary flow rate of 0.3 ml/min at rest, the flow rate measured in the mentioned study raises questions on the methodology of collection of saliva or calculation of saliva flow rate.

The heterogeneity was moderately high, and the cause could not be identified using subgroup analysis according to the sports/physical activity, collection methods and collection times after cessation of activity. However, heterogeneity disappeared during sensitivity analysis with the exclusion of the only study performed in sea water (Tauler et al., 2014) accompanied by a non-statistically significant reduction in salivary flow p= 0.89.

The results of the meta-analysis conflict with some previous studies that have reported a decrease in salivary flow rate after intense physical activity. However, after assessing the quality of the included evidence, the evidence was downgraded due to the fact that the studies were observational studies, had moderate risk of bias and moderate heterogeneity. Hence, due to the very low certainty of evidence, the summary estimate of the meta-analysis might not be reliable and as GRADE highlighted, is likely to change with further research.

5.10.4.2. Salivary IgA

The findings suggested that there was no statistically significant change in salivary IgA levels immediately after physical activity. Salivary IgA levels have been suggested to be influenced by intense physical activity in several studies (Nakamura et al., 2006). It has been reported that acute moderate exercise has either no effect or decreases salivary IgA levels (Klentrou et al., 2002). One study reported that running at intensities of 50-80% VO_{2max} for 15 – 45 minutes did not affect salivary IgA levels (McDowell et al., 1991). Another study also did not report changes in IgA levels following 30 minutes of treadmill running at 80% VO_{2max} at different temperatures (Housh et al., 1991). In contrast, several studies have also reported decrease in salivary IgA levels immediately and thirty minutes post high intensity exercise (Morgan, & Kolokouri, 2002, Davis, et al., 2002). However, the results of this meta-analysis showed a non-statistically significant change of -19.43 µg/ml after intense physical activities. The results of the current analysis conflict with a recent meta-analysis which reported a statistically significant increase in salivary IgA levels after periods of intense physical activity (Chastin et al., 2021). The differences might be related to the inclusion criteria of studies since our review included elite athletes whereas they were excluded in the latter. Additionally, the meta-analysis of the study comprised of 53 intervention studies in the form of randomised controlled trials whereas the current meta-analysis comprised of only 3 RCTs. However, the recent published study aimed to perform the meta-analysis only on RCTs and observational studies and included studies where participants received multiple interventions which was the reason for the higher number of RCTs included in the meta-analysis. Additionally, the study excluded studies which reported only acute response to exercise which was not the case in the current meta-analysis. Therefore, the minimal amount of RCTs in the current meta-analysis might be a reason for difference in results. As evidenced in literature, RCTs are second in line in the

hierarchy of evidence pyramid (Alper and Haynes, 2016) which might have probably improved precision of the estimates in the published study. Moreover, the heterogeneity was extremely high in the current meta-analysis with a very low certainty of evidence which may have further created disparity between the results. Normal salivary IgA levels are hard to define as they may vary with age (Jafarzadeh et al., 2009). Therefore, it is unclear whether the decrease in IgA levels calculated for the age group in the current meta-analysis is clinically relevant. Only one author has observed that a fall in SIgA to levels less than 40% of the baseline value indicates a 50% chance of contacting infection within 3 weeks (Neville et al., 2008).

The cause of the extremely high heterogeneity in the meta-analysis could not be identified. Subgroup analysis was also performed according to the various factors (saliva collection methods, assessment methods, physical activity). Despite, the heterogeneity remained consistently high. No significant change was observed in the sensitivity analysis after excluding three studies.

The results of the meta-analysis conflict with some previous studies that have reported a decrease in salivary IgA levels after intense physical activity (Mackinnon and Jenkins, 1993). However, after assessing the quality of the included evidence, the evidence was downgraded due to the studies being observational and having extremely high heterogeneity (>90%). Hence, due to the very low certainty of evidence, the summary estimate of the meta-analysis might not be totally reliable.

5.10.4.3. Saliva Total Protein (STP)

The findings suggested that there was no statistically significant change in STP levels immediately after physical activity. Prolonged exercise and heavy mouth breathing during exercise leads to reduced salivary flow and dehydration, hence resulting in a concomitant increase in protein and MUC5B concentration (Ligtenberg et al., 2015). Protein secretion is managed by the sympathetic nervous system and physiologically is meant to increase as a reflux mechanism to protect against any hazards to the oral tissues. However, the meta-analysis did not detect any statistically significant difference in protein concentration after physical activity. This would indicate that the proteins responsible for protecting the oral cavity tissues remained unchanged where an increase was expected based on the physiological mechanism. The results suggest that an unchanged concentration of

proteins may lead to an increased incidence of dental erosion in the athlete population due to the limited protection being offered by the proteins. The findings are opposite to one study which observed a statistically significant increase in protein concentration immediately after intense exercise (Walsh, 1999). On the other hand, another study can be compared to these findings which also observed no differences in protein concentration after intense physical activity (Bortolini et al., 2009).

Extremely high heterogeneity was observed between the studies. One study (Kiani et al., 2015) which was the only one that included non-athletes participating in intense physical training appeared to be an outlier. The participants included in the study performed activity in three separate sessions for seven days as opposed to the other included studies which measured STP after acute physical activity. The study did not report the method of collection of saliva as well which collectively may be reasons for the study to be an outlier. However, despite excluding the study during sensitivity analysis, the heterogeneity remained high. No cause was identified for the heterogeneity during subgroup analysis.

The results of the meta-analysis conflict with some previous studies that have reported both an increase and decrease in STP levels after intense physical activity. However, after assessing the quality of the included evidence, the evidence was downgraded due to the studies being observational, moderate risk of bias and having extremely high heterogeneity (>90%). Hence, due to the very low certainty of evidence, the summary estimate of the meta-analysis might not be reliable.

5.10.4.4. Salivary α-amylase

The findings suggested that there was no statistically significant change in salivary α-amylase levels immediately after physical activity. Salivary α-amylase secretion, like salivary IgA, is a protein which is used also as a stress indicator (Takai et al., 2004). Several studies have reported an increase in amylase levels following intense physical activity (Kivlighan and Granger, 2006, Granger et al., 2007, Chatterton et al., 1996). There were some statistically significant increases in amylase levels within individual studies included in the meta-analysis, although overall, the meta-analysis did not find any statistically significant difference in amylase levels as a result of physical activity. However, due to limited evidence to report the association of salivary α-amylase levels to dental

erosion it would be too early to conclude that changes in salivary α-amylase levels due to intense physical activity would lead to increased risk oral disease.

The results of the meta-analysis conflict with some previous studies that have reported a decrease in salivary amylase levels after intense physical activity. However, after assessing the quality of the included evidence, the evidence was downgraded due to the studies being observational, smaller sample size and having extremely high heterogeneity (>90%). Hence, due to the very low certainty of evidence, the summary estimate of the meta-analysis might not be reliable.

5.10.4.5. Saliva mucin (MUC5B)

MUC5B is a high molecular weight protein responsible for the viscosity of saliva and lubrication of the oral cavity. During exercise, the viscosity of saliva increases (Dawes, 1981) due to enhanced evaporation resulting in elevated concentrations of proteins and MUC5B (Walsh et al., 2004). Three studies were included that measured salivary mucin levels and its association to saliva viscosity before and after intense physical activity. One study was a randomised controlled trial which showed no association of mucin levels to the increase in viscosity of saliva. In the remaining two observational studies that included university students, the mucin levels increased significantly with increase in viscosity. On the other hand, the meta-analysis of the three studies resulted in a non-statistically significant change in mucin levels after physical activity. However, it should be noted that the salivary mucin levels measured in two studies (Hopkins et al., 2020 and Ligtenberg et al., 2015) before physical activity was higher than the normal resting salivary mucin levels of 1.1-1.9 µg/mg (Sanchez et al 2011, Sanchez et al., 2013, Kejriwal et al., 2014). The higher levels of mucin at rest could be a possible reason for the increased heterogeneity in the meta-analysis. However, with the limited number of studies measuring mucin levels in the meta-analysis and literature, it is not possible to confirm or refute an effect of physical activity on salivary mucin concentration and amount and to associate salivary mucins to dental erosion in the athlete population.

The results of the meta-analysis were comparable to one study which also reported no statistically significant change in salivary mucin levels after intense physical activity. However, after assessing the quality of the included evidence, the evidence was downgraded due to the limited number of studies, small sample size and having extremely

high heterogeneity (>90%). Hence, due to the very low certainty of evidence, the summary estimate of the meta-analysis might not be reliable.

5.10.5. Comparison with other reviews

This is the first systematic review to be performed on the effects of physical activity on saliva flow rate, saliva total protein content, salivary mucins, and salivary IgA. Previous narrative reviews have reported statistically significant changes in salivary flow (Mulic et al., 2012), and both statistically significant and non-significant changes in salivary IgA (MacKinnon et al., 1993), STP (Dawes, 1981) and salivary amylase (Walsh, 1999) levels after intense physical activity. A recent comprehensive review has summarised the following findings: physical exercise seems to increase the salivary flow rate and protein (e.g., amylase, lysozyme and MUC5B) secretion; salivary α-amylase increased in acute exercise and the magnitude depended on the intensity of exercise; with regards to salivary IgA, the review reported increase, decrease and no change in salivary IgA after exercise (Ntovas et al., 2022). However, although narrative and comprehensive reviews are written by experts based on their intuition and experience in focused topics, they are not based on the findings of a comprehensive and systematic literature search as compared to a systematic review which is focuses on a predefined selection criterion, protocol and a research question, all minimizing the chances of bias (Pae, 2015).

The findings of the current systematic review and meta-analysis do not coincide with the long since held belief that saliva parameters change significantly after episodes of intense physical training Therefore, the meta-analysis was performed to combine the results of the individual studies to come to an agreement of the exact nature of saliva after physical activity and the analysis resulted in non-statistically significant changes in the measured salivary parameters. The results obtained from the meta-analysis of salivary IgA can be related to a review that also reported that salivary IgA may not be significantly suppressed after bouts of intense physical activity (Walsh and Oliver, 2016). But due to the low certainty of the evidence obtained following GRADE, there is a high possibility for the results of this meta-analysis to be unreliable. Therefore, based on the results of the meta-analysis and the outcomes obtained from GRADE, it is yet too early to make a conclusion about the effects of intense physical activity on the properties of saliva.

5.10.6. Implications for dental erosion in athletes

Intense physical training stimulates the sympathetic nervous system and would therefore be expected to result in a decreased salivary flow and hence alterations in the composition of the saliva (Ntovas et al., 2022). The hypothesis of the review was that intense physical activity alters the physiological and compositional characteristics of saliva, which might be a factor for DE in the athletes. However, the results of the meta-analysis did not support the hypothesis. Therefore, based on the results of the meta-analysis and the outcomes obtained from GRADE, currently a conclusion cannot be made confirming the association of salivary parameters with dental erosion during intense physical training. Therefore, other potential causes of dental erosion in athletes must also be considered. The frequent intake of isotonic sports beverages may be a factor; however, a recent systematic review reported no association of sports drinks with dental erosion in the athlete population (de Queiroz Gonçalves et al., 2020). On the other hand, in vitro studies have confirmed association of sports drinks to dental erosion (Coombes, 2005). Further research is therefore required to identify the causes for the high prevalence of DE in athletes, in order to aid the development of preventive measures. The fact that WMS was collected may also be a factor as WMS has been reported to offer better protection to dental erosion when compared to PS and the reason may be related to the mucin 5b component present in WMS (Mutahar et al., 2020).

5.10.7. Implications for future research

Future studies should focus on interventional randomised controlled trials (RCTs) rather than observational studies where the element of physical activity can be considered to be the intervention following which the outcomes may be measured. RCTs would allow researchers to compare the outcomes of saliva parameters between the intervention group (intense physical activity) and the control group (no physical activity or different intensities of activity).

Apart from study design, further research should focus on collecting saliva (methods and collection times). Unstimulated saliva collected immediately after the cessation of physical activity is recommended as there is evidence supporting that saliva parameters might return to their normal levels after 60 minutes of physical activity (Ljungberg et al., 1997). The fact that only WMS was collected also raises a potential question for future research due to the differences in composition between PS and PS collections must also be

encouraged in order to evaluate the differences in composition after intense physical activities. Focusing on similar intensities of physical activity must also be considered to minimise exposure bias and would further enhance the reliability of the saliva outcomes obtained after physical activity. Further research must also focus on salivary proteins specific for enamel homeostasis (statherins, histatins) in order to probably have a better understanding and reasoning behind increased incidence in dental erosion and hence decide on preventive interventions if required. The limited number of studies assessing mucin levels also provides a platform to perform in depth research on the science of MUC5B during intense physical training for future. Last but not least, a significant element to consider is the properties of the dental pellicle during intense physical activities.

5.11. Conclusion

The systematic review and meta-analysis suggested that intense physical activity did not cause statistically significant alterations in the saliva flow and composition of saliva which were hypothesised to cause dental erosion in athletes. The results of the meta-analysis conflict with some of the previous studies assessing saliva parameters after intense physical activity. However, due to the very low certainty of the evidence, the results may not be reliable. Therefore, it is too early to support or refute an association between intense physical activity and saliva parameters. Further research is required to investigate their interrelationship. The results also suggest identifying other possible factors causing dental erosion in the athlete population.

6. The impact of dental pellicle modification on dental erosion – A systematic review.

6.0. Background

The introduction chapter (chapter 1) summarised the various in vitro and in vivo intervention studies performed to modify the dental pellicle to reduce and prevent the incidence of dental erosion. Therefore, a systematic review was conducted to assess preventive effects of the various interventions into the dental pellicle against dental erosion. This chapter will provide a detailed explanation of the systematic review.

6.1. Introduction

The dental pellicle, a thin acellular film predominantly consisting of salivary proteins, covers surfaces that are exposed in the oral cavity (Lindh et al., 2014). The formation of the dental pellicle starts soon after brushing or scaling with an initial layer of 20nm and reaches its maximum consistency and thickness of 1000nm within 24 hours (Zimmermann et al., 2019). This protein rich layer acts as a diffusion barrier preventing the erosive attack by the dietary acid on the tooth surface (Vukosavljevic et al., 2014).

6.1.1. Role of dental pellicle in dental erosion

The dental pellicle is an insoluble network of adsorbed salivary proteins. Demineralisation of the tooth surface occurs after diffusion of the acid through the mesh-like structure of the dental pellicle and semi permeable layer, or after removal of the acquired pellicle by the interacting acid. Hence, the pellicle cannot prevent direct contact between the acid and the tooth surface; however, it can reduce and retard the immediate interaction between acids and the tooth surface (Taira et al., 2018). In addition to the structural features of the pellicle, the proteins (acidic proline-rich proteins, histatins and statherins) present in the dental pellicle play a role in the prevention of erosion. They aid by maintaining high concentrations of calcium within the pellicle layer by binding to the calcium ions and then releasing the ions when required during demineralisation, hence stabilizing the enamel surface (Hannig and Hannig, 2014a).

6.1.2. Collection of dental pellicle

Two methods for collecting dental pellicle have been reported in literature. The in vitro method involves the “time-based incubation method” where the enamel specimens are

immersed in collected saliva for a period of time to allow pellicle formation (Zhang et al., 2018). On the other hand, two methods have been reported for the in vivo method of pellicle formation. The first method is by introducing a splint comprising of a 5 x 5 mm enamel disc attached on the buccal aspect of the splint which is then inserted into the oral cavity of the individual for a period of time to allow pellicle formation on the attached enamel disc (Kensche et al., 2019). The other in vivo method involves rubbing an electrode filter paper pre dipped in 3% citric acid on the surfaces of teeth for pellicle to be adsorbed on the filter paper. The filter paper is then stored at -80 ^{O}C until use for experiments (Martini et al., 2019).

6.1.3. In vitro modification of dental pellicle to prevent dental erosion

Several approaches have been made in vitro and in vivo to modify the dental pellicle to prevent dental erosion, for example, by the addition of organic elements (casein, mucin) (Sieber et al., 2019) and inorganic elements (stannous and fluoride) (Zanatta et al., 2019). Evidence suggests that casein forms micelle like structures that increase the thickness of the pellicle, hence preventing acidic elements to attack the enamel surface (Smith and Bowen, 2000). Mucins have been reported to increase the lubrication properties of the dental pellicle, hence preventing dental abrasion which occurs after softening of the enamel after the erosive attack (Amerongen et al., 1987). Stannous ions have been reported to increase the adsorption of acid resistant proteins which may prevent acid attack (Hara et al., 2013). Nevertheless, the evidence of the impacts of the various types of interventions on dental erosion remains unclear. For this purpose, a systematic review was required to identify the various interventions that have been used to modify pellicle, potentially important approaches to preventing DE and the impact of the various interventions on dental erosion.

6.1.4. Summary

The modification of dental pellicle by organic and inorganic elements could have an impact on the severity of dental erosion and could therefore contribute to the reduction of dental erosion in the athlete population. A better understanding of the effects of the added elements would also inform on preventive interventions to reduce in the incidence of dental erosion not only in the high-risk group but also in the general population.

6.2. Aims

To assess the impact of dental pellicle modification on the severity of dental erosion.

6.3. Objectives

- To assess the effects of the dental pellicle modification on the magnitude of dental erosion.
- To identify the most promising interventions for further research.

6.4. Materials and methods

6.4.1. Protocol registration

The systematic review of the impact of dental pellicle modification on dental erosion was based on the guidelines of PRISMA (Moher et al., 2009) and prospectively registered on the PROSPERO database (https://www.crd.york.ac.uk/PROSPERO/), CRD 42021229478.

6.4.2. Research question

The systematic review was conducted to answer the following two questions: (1) “what is the impact of dental pellicle modification on the severity of dental erosion?” and (2) “does dental pellicle modification affect the composition and structural properties of the dental pellicle?”

6.4.3. Literature search strategy

A sensitive search strategy was developed including electronic searching, and hand searching in collaboration with UCL online library. Detailed search strategies deemed appropriate for each database were developed as well as for unpublished studies. No language restrictions were imposed, and non-English language records were translated though the network of language speakers at University College London.

For the selection of studies included in this systematic review, the electronic databases searched were Medline (Ovid), Web of science, EMBASE and Google Scholar (2000 – latest). Studies ranging from January 2000 – December 2020 were searched as it was anticipated that in vitro modification of dental pellicle had been performed and studied

within that timeframe. The search strategy used a combination of controlled vocabulary (MeSH terms and free text terms) (Table 6.1). The keywords and subject headings were merged and exploded in accordance with the thesaurus of each database. The search was based on the following MeSH and free text terms:

Table 6.1: Search strategy.

	Medline (Ovid)	**Web of science**	**EMBASE**
P	Dental Pellicle/ 2. Dental Pellicle/ 3. Acquired pellicle/ or Dental Enamel/ 4. Salivary Pellicle/ 5. Modification of pellicle 6. 3 and 5 7. 4 or 5 8. Dental Pellicle bovine teeth 9. Dental pellicle hydroxyapatite 10. limit 8 to yr="2000 - 2020" 11. (Dental pellicle adj3 modification adj3 tooth erosion).mp.	# 1 (dental pellicle) # 2 (salivary pellicle # 3 (acquired pellicle) # 4 (modification of dental pellicle) #5 (#4 **OR** #1) #6 (#5 **OR** #4)	1. Dental pellicle 2. Salivary pellicle 3. Acquired pellicle 4. Modification of pellicle
I	1. Caseins/ 2. Mucins/ 3. Catechin/ 4. Chitosan/ 5/ Stannous Fluoride 6. 1 or 5 7. 1 and 5 8. 2 or 5 9. 2 and 5 10. 3 or 5 11. 3 and 5 12. (casein adj3 dental pellicle).mp.	# 1 (Caseins) # 2 (Mucins) #3 (Chitosan) # 4 (Catechin) # 5 (Stannous fluoride) # 6 (Fluoride) # 7 (#1 **OR** #2 **OR** #3) # 8 (#1 AND #2 AND #3 AND #4) #9. (#7 **AND** #8)	1. Caseins 2. Mucins 3. Catechins 4. Stannous fluoride 5. Chitosan 6. 1 OR 2 OR 3 OR 4 OR 5 7. 1 AND 2 AND 3 AND 4 AND 5
O	Tooth Erosion/ 2. Tooth Erosion/ 3. Pellicle thickness 4. Dental Pellicle/ 5. "Salivary Proteins and Peptides"/ or Dental Pellicle/ 6. 2 AND 3 AND 5	#1 Tooth erosion #2 Dental erosion #3 Pellicle proteins #4 Pellicle thickness #5 Pellicle adsorption #6 (#1 **OR** #2 **OR** #3 OR #4) #7 (#1 **AND** #6)	1. Dental erosion 2. Tooth erosion 3. Pellicle proteins 4. Pellicle thickness 5. Pellicle adsorption 6. (1 OR 2 OR 3 OR 4 OR 5) 7. (1 AND 6)
Combined	10(P) **AND** 12(I) **AND** **AND** 6(O).	#6(P) **AND** #9 (I) **AND** #7 (O)	4 (P) **AND** 7 (I) **AND** **AND** 7 (O)

6.4.4. Hand searching

The bibliographies of papers and review articles were checked for studies not retrieved through other search methods. Online hand searching of issues of dental journals such as British Dental Journal (BDJ), Caries Research, Colloids and Surfaces B: Biointerfaces, Archives of Oral Biology and Journal of Dental Research were also searched for the relevant topic.

6.4.5. Eligibility criteria

Types of studies

All in vitro and in vivo studies including randomised controlled trials (RCTs), non-randomised controlled trials (non-RCTs) and control trials that reported the quantitative analysis of dental erosion and qualitative analyses of dental pellicle as a result of modification of dental pellicle were included. Studies aiming to modify the dental pellicle by altering the thickness, adsorption properties and protein composition of dental pellicle with the aid of interventions were also included. On the other hand, studies targeting hard tissue modification, for example hydroxyapatite of enamel were excluded.

Study populations (P)

- In vivo dental pellicles collected from healthy participants using fabricated splints or electrode filter paper.

- In vitro pellicle formation on different substrates including, bovine, human, hydroxyapatite surfaces following saliva collection from healthy participants.

Types of intervention (I)

Any additive to dental pellicle including:

Organic additives – casein, mucins, epigallocatechin gallate (EGCG), chitosan.

Inorganic additives – fluoride ions, stannous ions, calcium ions, tetrafluorides.

Comparison (C)

The comparisons groups were no interventions, control or any other intervention to prevent dental erosion.

Outcome measures (O) (Table 6.2)

Table 6.2: Outcome measures included

Primary outcome	Outcome anticipated	Type of measurement (Study)
Dental erosion	Reduction in dental erosion	Quantitative
Secondary outcomes		
Pellicle proteins	Increased protein content	Quantitative
Pellicle structure	Increased thickness	Quantitative/ qualitative
Pellicle adsorption	Increased adsorption	Quantitative/ qualitative

6.4.6. Study eligibility assessment

Inclusion/exclusion criteria were set as follows:

Inclusion criteria

- Randomised controlled trials, non-randomised controlled trials and uncontrolled studies.
- In vivo and in vitro studies
- Saliva collected from healthy participants for in vitro pellicle formation.
- In vivo dental pellicles collected from healthy participants using fabricated splints/electrode filter paper
- Any age of participants.
- Pellicles formed on human or bovine teeth
- Modification of dental pellicle by intervention
- Pellicles modified to alter thickness, adsorption and composition
- Dental erosion quantified as outcome measure following pellicle modification
- Pellicle properties measured (composition, structure).

Exclusion criteria

- Animal studies
- Smoking participants or with systemic diseases

- Studies primarily aiming at hard tissues modification: hydroxyapatite, calcium levels of enamel
- Reviews
- Letters to editors and opinion articles

6.4.7. Study screening

Titles and abstracts were screened for inclusion by one reviewer (HM) against a key eligibility criteria:

- Intervention in the dental pellicle
- Assessment of dental pellicle
- Assessment of dental erosion
- Research study

All possibly eligible studies were retained, and the full text obtained for potential inclusion in the review against the full eligibility criteria above. A random 10% of titles and abstracts and 10% of full-text articles were also independently screened by one of two experienced systematic reviews (IN & PA) to examine levels of agreement.

6.4.8. Data extraction strategy

Data was extracted by one reviewer (HM) using a specially designed data extraction spread sheet. The data extraction form was piloted on five papers and modified as necessary prior to commencing the study data extraction. A second experienced reviewer (IN, PA) independently extracted data on 10% of studies to check accuracy and consistency of process.

Two main categories of data were extracted as listed below:

Study characteristics data

- Study authors
- Year of publication
- Country where study performed

- Study design
- Method of saliva/pellicle collection
- Method of pellicle formation
- Substrate for pellicle formation
- Nature of intervention
- Study population group.
- Age
- Overall sample size

Outcome and/or confounders data

- Magnitude of dental erosion
- Thickness of dental pellicle
- Pellicle proteins
- Dental pellicle adsorption

6.4.9. Risk of bias

The Cochrane Collaboration RoB 2.0 tool was used to assess the risk of bias in the interventional, randomised controlled trials (Sterne et al., 2019). For the non-randomised controlled trials, the Risk of Bias In Non-randomised Studies - of Interventions (ROBINS-I) tool was used (Sterne et al., 2016).

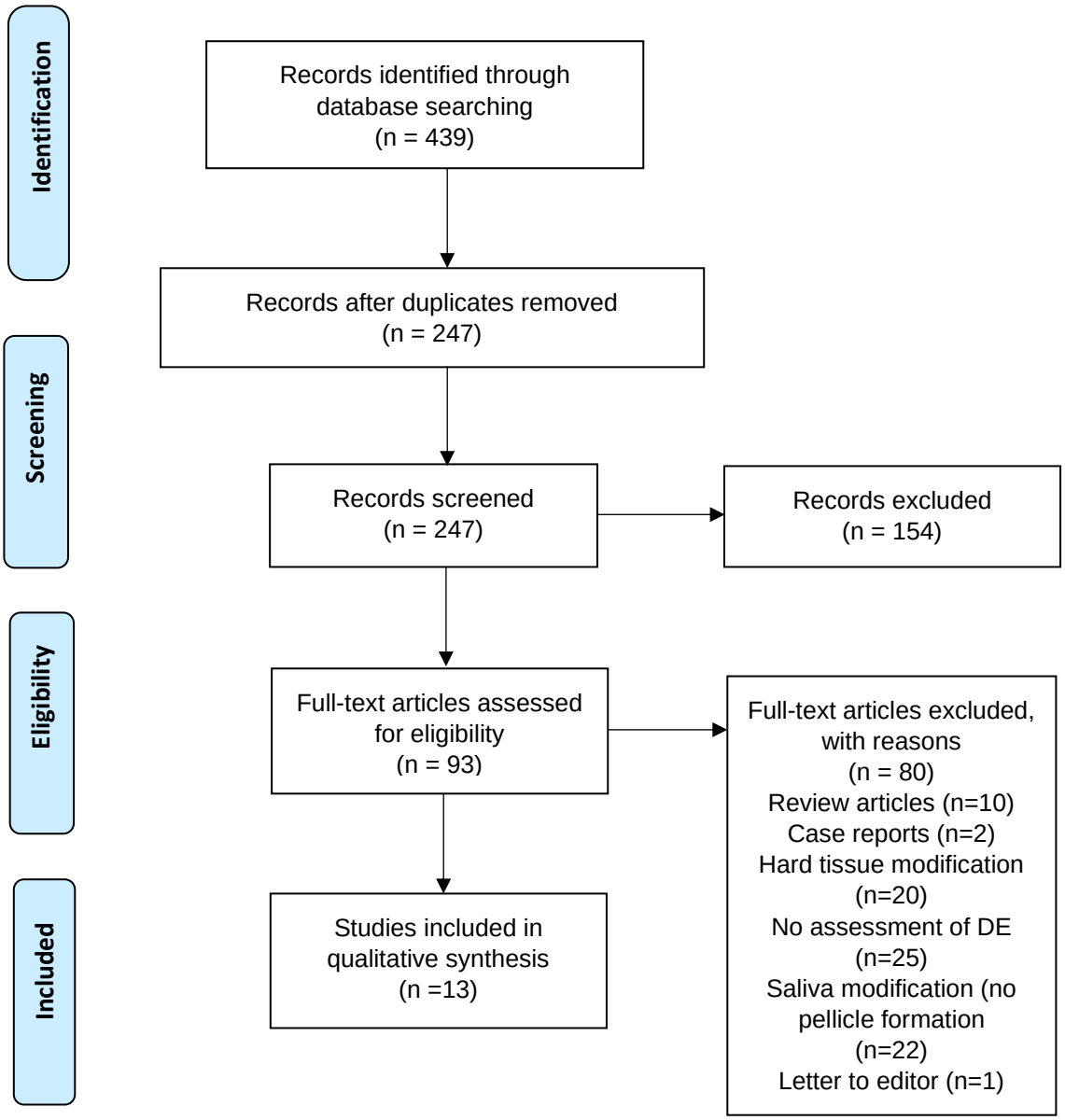
6.4.10. Study synthesis

Data extraction sheets were used to construct evidence tables, grouping studies by similar design, populations and outcomes. The evidence tables were examined to determine whether there were multiple studies investigating similar research questions with outcomes that could be combined quantitatively in meta-analysis. However, a meta-analysis could not be conducted due to insufficient data available. The respective authors were contacted multiple times to acquire the quantitative data, but it was only possible to obtain data from four of the authors which remained insufficient for meta-analysis.

6.5. Results

The inter examiner agreement at full-text screening was excellent (kappa score = 0.87). A total of 439 studies were obtained from the search performed (Figure 6.1) and following the removal of 192 duplicates, 247 potentially suitable titles and abstracts were screened. After excluding 154 non-relevant citations, 93 publications were selected and retrieved for full-text reviewing. Among these full text articles and abstracts, 80 were excluded; [review articles *(n=10),* case reports *(n=2)*, hard tissue modification *(n=20*), no assessment of DE *(n=25),* saliva modification without pellicle formation *(n=22)* and letter to the editor *(n=1)*]. This resulted in a total of 13 publications eligible to be included in the systematic review after the screening procedure. 8 publications did not contain the full information necessary for the meta-analyses, however it was possible to obtain the required data from the authors of 4 publications. The remaining 4 studies did not contain the numerical data but mentioned significant and non-significant outcomes in their results. Overall, a total of 1,365 teeth were used for the in vitro studies (human teeth: 617; bovine teeth: 748). 16 participants were involved in the in vivo studies.

Figure 6.1: PRISMA flowchart representing the results of the workflow to identify eligible studies.



6.5.1. Bias assessment

The review included eight randomised controlled trials and five non-randomised controlled trials. According to the RoB2 tool for RCTs, one study was considered a low risk of bias (Carvalho et al., 2020a) while seven studies had some concerns (Carvalho et al., 2020b, Baumann et al., 2020, Sieber et al., 2019, Weber et al., 2015, Ionta et al., 2017, Cheaib and Lussi, 2011, Algarni et al., 2015) (Table 6.3). On the other hand, according to the ROBINS-I tool for non-RCTs, one study was considered a moderate risk of bias (Hertel et al., 2016), while four were considered a low risk of bias (Niemeyer et al., 2020, Hertel et al., 2017, Hannig et al., 2012, Kensche et al., 2017) (Table 6.4).

Table 6.3: Bias assessment for RCTs using RoB2.

Study	Method of randomisation	Randomisation bias	Deviation bias	Missing outcome data	Measurement bias	Result selection bias	Total
Carvalho 2020b	Block	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns
Carvalho 2020a	Crossover	Some concerns	Low	Low	Low	Low	Low
Baumann 2020	Simple	Some concerns	Some concerns	Low	Some concerns	Some concerns	Some concerns
Sieber 2019	Simple	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns
Ionta 2017	Block	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns
Weber 2015	Crossover	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns
Algarni 2015	Simple	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns
Cheab and Lussi 2011	Block	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns

Table 6.4: Bias assessment for non RCT using ROBINS-I.

Study	Confounding bias	Selection bias	Intervention bias	Deviation bias	Missing outcome data	Measurement bias	Result selection bias	Total
Niemeyer 2020	Low	Low	Low	Low	Low	Moderate	Low	Low
Hertel 2017	Low	Low	Low	Low	Low	Low	Moderate	Low
Kensche 2017	Low	Low	Low	Moderate	Low	Low	Serious	Low
Hertel 2016	Low	Low	Moderate	Moderate	Low	Low	Serious	Moderate
Hannig 2012	Low	Low	Low	Low	Low	Low	Moderate	Low

6.5.2. Methods of assessment of dental erosion

Overall, four methods were used to assess the effects of the interventions on dental erosion. Surface microhardness was used to measure the degree of hardness of enamel achieved following treatment with the respective interventions (Niemeyer et al., 2020, Carvalho et al., 2020b, Baumann et al., 2020, Sieber et al., 2019, Ionta et al., 2017, Cheaib and Lussi, 2011). The second method assessed the surface reflection intensity (SRI) of enamel before and after treatment (Niemeyer et al., 2020, Carvalho et al., 2020b, Sieber et al., 2019). The third most common method used was the chemical analysis method

which measured the amount of calcium release following treatment and comparing the release to the control groups (Niemeyer et al., 2020, Carvalho et al., 2020a, Sieber et al., 2019, Kensche et al., 2017, Hertel et al., 2017, Hertel et al., 2016, Weber et al., 2015, Hannig et al., 2012). The fourth method used was detection of enamel surface loss using profilometer which was used in three studies (Baumann et al., 2020, Cheaib and Lussi, 2011, Algarni et al., 2015).

6.5.3. Descriptive results

The full body of data obtained from the included studies was tabulated according to the chronological date of publication and sorted according to the interventions introduced. The characteristics of the studies were summarized in individual tables, each for polyphenols, casein-mucin, vegetable oils, plant extracts, ions and engineering peptides.

6.5.3.1. Polyphenols

Five studies modified the dental pellicle using polyphenols, out of which one study was a randomised controlled trial (RCT) (Carvalho et al., 2020b), one study was a crossover RCT (Weber et al., 2015) while three were non-randomised controlled trials (CT) (Niemeyer et al., 2020, Hertel et al., 2017, Hertel et al., 2016) (Table 6.5). Two studies were performed in vitro where pellicles were formed using the time-based incubation method in which enamel specimens were immersed in expectorated whole mouth fresh saliva from 30 minutes to two hours to allow pellicle formation (Niemeyer et al., 2020, Carvalho et al., 2020b). The three in vivo studies involved pellicles being formed on enamel specimens attached to prefabricated splints and being worn by the participants for 30 minutes to allow pellicle formation on the attached tooth surfaces (Hertel et al., 2017). The in vitro studies used human teeth as substrates for pellicle formation and erosive experiments, while the in vivo studies involved bovine incisors. The polyphenols introduced in the studies to modify the dental pellicle were used in the form of red wine (Carvalho et al., 2020b), polyphenol-rich teas (Niemeyer et al., 2020) and tannins (tannic acid) (Hertel et al., 2017) plant extracts (Weber et al., 2015) and inula viscosa tea (Hertel et al., 2016). The erosive mediums used were in the form of 1% citric acid (pH 3.6) for one minute (Niemeyer et al., 2020), citric acid (pH 3.48) for four minutes (Carvalho et al., 2020b) and hydrochloric acid (HCL) (pH 2.0, 2.3 and 3.0) for 120 seconds (Hertel et al., 2017, Hertel et al., 2016, Weber et al., 2015). The studies involving the use of red wine and polyphenol-rich teas did not investigate any structural or compositional changes in the dental pellicle after intervention,

however, the study involving tannins reported a higher electron density of the dental pellicle as compared to baseline after intervention as observed under the transmission electron microscope (TEM). The study using plant extracts reported a thickened and more electron-dense pellicle layer after treatment as observed under TEM, while the study using inula viscosa tea reported round electron-dense complexes with embedded vacuoles attached to the basal layer of the dental pellicle. Relative surface microhardness (rSMH) and photometric determination of calcium release was used to assess the preventive effects against dental erosion. rSMH was calculated using the formula rSMH = (SMH final/SMH initial) × 100. Overall, the green and black teas and grapeseed reported a statistically significant higher (rSMH) of the enamel following exposure to the erosive medium, while tannins resulted in a statistically significant decrease in calcium and phosphate loss from the enamel surface. On the other hand, a combination of R. nigrum and oregano resulted in a statistically significant decrease in calcium loss from the enamel surface as compared to the calcium release from the physiological pellicle. On the other hand, no statistically significant change in calcium loss was reported after the intervention of inula viscosa when compared with the control (no rinsing with intervention).

Table 6.5: Characteristics of studies using polyphenols to modify dental pellicle.

Author/ Year/ Country	Study design	Nature/ method of saliva collection/ sample size/age	Pellicle formation method	Substrate used (bovine/ human enamel)	Acidic medium/ concentration/ exposure time	Intervention/ proposed mechanism of action/outcome measure used	Control/ comparison	Main findings (Dental erosion)	Pellicle findings
Niemeyer 2020, Switzerland	CT In vitro.	Stimulated/ expectoration N/R, 20-30 years.	In vitro pellicle formation using time-based incubation (30 minutes).	150 human molars assigned to 10 groups.	1 min, 1% citric acid, pH 3.6.	Experiment 1 Teas Experiment 2 Natural extracts Increased adsorption of acid resistant proteins with increase in thickness/ rSMH, SRI and Ca^{2+} release	Deionized Water.	**Experiment 1** Green and black tea significantly increased rSMH ($p<0.05$) * and significantly decreased SRI. **($p<0.05$)** *. Rosehip tea most erosive. **Experiment 2** Grape and grapefruit seed: highest rSMH and SRI. Propolis extract: no statistically significant difference in rSMH. **($p = 0.555$).** Grapefruit seed and propolis: statistically significant lower Ca^{2+} release as compared to control* Cranberry extract: higher Ca^{2+} to control*.	N/R
Carvalho 2020, Switzerland	RCT In vitro.	Stimulated/ expectoration N/R	In vitro pellicle formation using time-based incubation (2 hours, 37ºC).	100 human molars: presence of acquired enamel (n = 60)	30 ml test solution per specimen for 1 min, at 25°C, and shaking at 70 rpm.	Red wine (RW) (Salicylic and malic acids) Increased adsorption of acid resistant	Absence of dental pellicle	**rSMH%** RW with P 89.3±7.3 RW without P 73.0±3.6 **($p<0.05$)***	N/R

				absence (n = 40); four subgroups.	Orange juice **(OJ)** (pH= 3.48), apple juice **(AJ)** (pH 3.48) citric acid **(CA)** (3.48).	proteins with increase in thickness/ rSMH and SRI		OJ with P 57.7±6.2 OJ without P 68.6±5.1 **(p<0.05) *** AJ with P 45.1± 8.6 AJ without P 32.0± 5.4 **(p<0.05)*** CA with P 31.8± 5.2 CA without P 36.7±3.8 **(p<0.05)*** **SRI** RW and OJ least SRI loss than AJ. CA no stat significant difference b/w RW and OJ. **(p=0.48).**	
Hertel 2017, Germany	CT In vivo.	N/A 6; 24-42 years.	In vivo pellicle formation for 30 minute using fabricated splints.	Bovine incisors attached to splints	HCl at pH 2.0, 2.3 and 3.0 for 120 seconds	Tannins (Tannic acid) Increased adsorption of acid resistant proteins with increase in thickness/ Ca^{2+} release	Fluoridated mouthwash.	Tannic acid significantly reduced calcium and phosphate loss. **(p=0.025).**	Higher electron density as compared to control

Weber 2015, Germany	Crossover RCT In vivo.	N/A 12; 22-26 years;	In vivo pellicle formation for 30 minutes using fabricated splints.	Enamel slabs from 72 bovine incisors attached to splints.	HCL (pH 2, 2.3 or 3) for 120 seconds	R. nigrum, Oregano/ Increased adsorption of acid resistant proteins with increase in thickness/ Ca^{2+} and PO_4^{2-} release and TEM	Elmex 250 ppm amine fluoride (fluoridated mouthwash).	Statistically significant reduction of calcium loss as compared to control: pH 2: 36%, pH 2.3: 43%, pH 3: 50%. **(p<0.05) *** Statistically significant reduction of phosphate loss at pH 2: 28%, pH 2.3: 32%, pH 3: 41%. **(p<0.05)*.**	A thickened and more electron-dense pellicle layer. Application of fluoride increased rigidity of the pellicle.
Hertel 2016, Germany	CT	N/A 3; 22-29 years.	In vivo pellicle formation for 30 minute using fabricated splints.	Enamel slabs from 18 bovine incisors attached to splints.	HCl (pH 2.0, 3.0 and 3.2) for 120 seconds	Inula viscosa tea (flowering plant). Formation and adsorption of vacuoles on the pellicle layer/ Ca^{2+} and PO_4^{2-} release and TEM	Control: no rinsing with intervention.	No statistically significant difference in Ca^{2+} and PO_4^{2-} release when compared to the physiological pellicle. **(p>0.05)***.	Round electron-dense complexes with embedded vacuoles attached to the basal layer.

Key: RCT: *: Actual p-value not reported; AJ: Apple juice; CT: Controlled trial; HCL: Hydrochloric acid; OJ: Orange juice; RCT: Randomised controlled trial; RW: Red wine; rSMH: Relative surface microhardness; SRI: Surface reflection intensity.

6.5.3.2. Casein/Mucin

The three studies which modified the dental pellicle using casein/mucin compounds were RCTs (Baumann et al., 2020, Sieber et al., 2019, Cheaib and Lussi, 2011) (Table 6.6). All studies were performed in vitro using the time-based incubation method where human enamel specimens were immersed in expectorated whole mouth fresh saliva for two hours to allow pellicle formation. The enamel specimens were randomly assigned into different groups to receive the interventions. The casein compounds were obtained from bovine milk while the mucin compounds were obtained from pig gastric mucin and were introduced into the dental pellicle in various concentrations. The studies did not investigate the structural or compositional changes in the dental pellicle after intervention. With regards to dental erosion, relative surface microhardness (rSMH) was used to calculate the change in surface microhardness using the formula rSMH= (SMH final/SMH initial) × 100. One study reported a statistically significant decrease in rSMH ($p=0.004$) of the control group as compared to that of casein-mucin (CM) and pellicle (P) groups, but group pellicle-casein-mucin (PCM) was not different to any of the groups ($p=1.00$) (Baumann et al., 2020). The study also assessed the effects of pellicle modification on abrasion and reported no statistically significant difference in surface loss ($p=0.92$) or %rSMH between the groups after the last abrasion cycle. Another study that used casein/mucin in various concentrations, reported to have achieved the maximum rSMH in the 1.0/0.27 casein/mucin concentration group compared with other concentrations (Sieber et al., 2019). The third study also reported a significant increase in rSMH in the group which used the combination of casein/mucin, as compared to the groups with casein and mucin being used individually ($p<0.001$) (Cheaib and Lussi, 2011). However, the study did not report the statistical data comparing between groups. The %rSMH obtained from casein and mucin had a wide range from 49% (Baumann et al., 2020) to 80% (Sieber et al., 2019). However, Baumann et al., reported 49% rSMH after five cycles of erosive challenge as compared to the other study which reported 80% rSMH after only one cycle of erosive challenge. Overall, the results obtained from the studies suggested that the combination of casein and mucin would enhance the protection against dental erosion as compared to when casein and mucin intervened individually.

Table 6.6: Characteristics of studies using casein/mucin to modify dental pellicle.

Author/ Year/ Country	Study design	Nature/ method of saliva collection/ sample size/age	Pellicle formation method	Substrate used (bovine/ human enamel)	Acidic medium/ concentration/ exposure time	Intervention/ proposed mechanism of action/outcome measure assessed/used	Control/ comparison	Main findings (Dental erosion)	Pellicle findings
Baumann 2020, Switzerland	RCT In vitro.	Stimulated/ expectoration N/R.	In vitro pellicle formation using time-based incubation (200 µL) 37°C for 2 h.	72 human enamel specimens randomly assigned to four (n = 18) experimental groups. Experiment 1: erosion Experiment 2: Abrasion	1% citric acid (10 mL, pH 3.6) at 25 °C for 3 min, at 70 rpm.	Pellicle-casein-mucin **(PCM)** Casein-mucin **(CM)** Pellicle only **(P)** Formation of casein micelles increasing thickness of pellicle /Dental erosion and abrasion/SMH and profilometer	No saliva (no pellicle) and no protein modification.	**Erosion** **%rSMH** PCM: 49.25±7.35 **(p=1.00) to all groups** CM: 52.3±8.97 **(p=0.02) to control** P: 52.26±7.56 **(p=0.004) to control** **Abrasion** **SL µm** PCM: -1.88±0.43 CM: **-**1.77±0.35 P: **-**1.81±0.47 (p=0.92) **%rSMH** PCM: 60.780± 6.544 CM: 60.698±9.895 P: 65.386±6.899 No statistically significant difference b/w groups.	N/R
Sieber 2019, Switzerland	RCT In vitro.	Stimulated/ expectoration 38, mixed, aged 20–30 years.	In vitro pellicle formation using time-based incubation 1.2 mL of saliva, for 2h at 30°C with constant agitation (70 rpm.	195 human enamel. **Experiment 1**: Various casein/mucin concentration/ randomly assigned ten (n = 15) groups	0.65% citric acid (10 mL pH 3.5, 30°C) for 60 s with constant agitation (70 rpm).	Varying mucin and casein concentration/ Formation of casein micelles increasing thickness of pellicle.	No pellicle modification.	**Experiment 1** **Varying casein concentrations** **0.1 / 0.27** %rSMH: 78.93±2.04 Ca^{2+}: 22.63±8.36 **0.25 / 0.27** %rSMH: 78.01±1.81 Ca^{2+}: 23.76±10.1 **0.5 / 0.27** %rSMH: 79.94±4.52	N/R

				Experiment 2: Increasing protein concentrations at the same protein ratio/ randomly assigned to 3 (n = 15) groups.				Ca^{2+}: 30.0±12.6 **1.0 / 0.27** %rSMH: 80.55±2.10 Ca^{2+}: 22.0±8.2 **Varying mucin concentrations** **0.5 / 0.13** %rSMH: 75.27±6.96 Ca^{2+}: 28.6±13.4 **0.5 / 0.54** %rSMH: 80.64±6.02 Ca^{2+}: 23.3±5.6 **0.5 / 1.08** %rSMH: 78.41±5.33 Ca^{2+}: 22.6±5.2 No statistically significant difference in %rSMH between groups **(p>0.05).** %rSMH of 1.0/.027 group statistically significant from control **(p=0.031).** No statistically significant difference in Ca^{2+} release between groups (**p=0.16).** **Experiment 2** Casein/mucin 1.0/0.27 %rSMH: 52.67±2.68 2.0/0.54 rSMH%: 56.43±4.67 3.0/0.81 %rSMH: 58.71±3.41 Significant differences between the rSMH of the 1.0/0.27 and the 3.0/0.81 groups **(p=0.0002).**	

Cheaib 2011, Switzerland	RCT In vitro.	Stimulated/ expectoration N/R (26-53 years);	In vitro pellicle formation using time-based incubation 13 mL of saliva, for 2h at 30°C	100 human enamel specimens assigned to 5 experimental groups (n = 20).	0.65% citric acid solution (pH = 3.5) for 1 min at 30 °C.	Mucin and casein/ Formation of casein micelles increasing thickness of pellicle/rSMH	Deionized water.	(Mucin-casein) group was statistically significant more protective than the pellicle in the single protein and control group Comparative p-values N/R	N/R

Key: *: Actual p-value not reported; b/w: between; CM: Casein-Mucin; N/R: Not reported PCM: Pellicle-Casein-Mucin; P: Pellicle; RCT: Randomised controlled trial; rSMH: Relative surface microhardness; SL: Surface loss

6.5.3.3. Vegetable oils

Two studies modified the dental pellicle using vegetable oils as the intervention, out of which one study was an RCT (Ionta et al., 2017) while the other was a CT (Hannig et al., 2012) (Table 6.7). Both involved in vivo pellicle formation where pellicles were formed on enamel slabs obtained from bovine incisors attached to prefabricated splints being worn by the participants for 30 minutes to allow pellicle formation on the attached tooth surfaces. In the RCT, following pellicle formation, the attached bovine incisors were removed from the splints and randomly assigned to different groups and treated with palm oil, coconut oil, safflower oil, sunflower oil and olive oil in vitro. The rSMH was assessed and compared to the baseline SMH after immersion in 0.5% citric acid, (pH 2.4), for 30 seconds, to identify the most effective intervention (Ionta et al., 2017) The CT used safflower oil to modify the dental pellicle followed by exposure to HCL (pH 3.0) in vitro, after which preventive effects were assessed by photometric determination of calcium release from enamel (Hannig et al., 2012). The study also reported a decrease in the density of the dental pellicle, and appearance of irregularly shaped vesicles like structures under transmission electron microscope (TEM). In the RCT, only pure palm oil reported a statistically significant change of 7.89% in SMH as compared to the other experimental groups. There were no statistically significant differences in rSMH reported between other groups with the rSMH ranging between 21.7% - 28.7% in all groups. On the other hand, in the CT, safflower oil led to a statistically significant increase ($p<0.001$) in calcium ion release as compared to the control (pellicle without safflower oil). Overall, pure palm oil reported to be the most protective against dental erosion as compared to the remaining oils.

Table 6.7: Characteristics of studies using vegetable oils to modify dental pellicle.

Author/ Year/ Country	Study design	Nature/ method of saliva collection/ sample size/age	Pellicle formation method	Substrate used (bovine/ human enamel)	Acidic medium/ concentration/ exposure time	Intervention/ Proposed mechanism of action/outcome measure used	Control/ comparison	Main findings (Dental erosion)	Pellicle findings
Ionta 2017, Brazil	RCT In vivo.	N/A 2, 22 years; In vivo pellicle.	In vivo pellicle formation for 2 hours using fabricated splints.	140 bovine incisors randomly assigned to be attached to splints to be worn for 6 days.	17.6 ml of 0.5% citric acid pH 2.4, under constant agitation, for 30 seconds.	Palm oil. extra virgin coconut oil; safflower oil; sunflower oil; olive oil. Formation of lipid micelles increasing thickness of pellicle/ microhardness	**Negative control**: deionized water **Positive control**: fluoridated commercial mouthwash containing 125 ppm F− F− as NaF, 800 ppm Sn2+ as AmF, 375 ppm as SnCl2; pH 4.5 (Elmex® Erosion Protection Dental Rinse/EP – CP GABA GmbH; Hamburg, Germany	**% change in SMH from baseline** GP5 5% palm oil 23.24±8.43[a] **GP100 pure palm oil** **7.89±7.5[b]** GC-5% coconut oil 24.65±11.5[a] GC100 pure coconut oil 28.47±13.37[a] GSa5 5% safflower oil 28.74±11.53[a] GSa100 pure safflower oil 26.56±9.51[a] GSu5 5% sunflower oil 22.92±12.94[a] GSu100 pure sunflower oil 21.73±14.83[a] GO55% olive oil 25.35±12.76[a] GO100 pure olive oil. 25.91±12.51[a]	N/R

Hannig 2012, Germany	CT In vivo.	N/A 6: 24-42 years.	In vivo pellicle formation for 30 minutes using fabricated splints.	398 enamel slabs from bovine incisors attached to splints. 6 slabs in each splint.	HCL (pH 2, 2.3 or 3) for 120 seconds	Safflower oil **(C)**/ Formation of lipid micelles increasing thickness of pellicle/Ca^{2+} and PO_4^{2-} release/TEM	Enamel without pellicle **(A)** Enamel with 30-minute pellicle **(B)**	**Ca^{2+} release** **pH 2** **(A)**: 249.92±13.41[a] **(B):** 199.85±37.11[b] **(C):** 221.71±14.53[b] **(p<0.001)** **pH 2.3** **(A):** 87.69±20.35[a] **(B):** 65.32±20.39[b] **(C):** 82.01±18.67[a,b] **(p=0.012).** **pH 3.0** (**A):** 12.63±3.09[a] **(B):** 4.55 0.62[b] **(C):** 7.31±2.26[c] **(p<0.001)**	Lower density pellicle; irregularly shaped vesicle-like structures

Key: Data significantly different within one group are followed by distinct superscripts ($p<0.05$); RCT: Randomised controlled trial; TEM: Transmission electron microscope; A: Enamel without pellicle; B: Enamel with 30-minute pellicle; C: Safflower oil

6.5.3.4. Ionic compounds

Two studies modified the dental pellicle using ionic compounds in the form of stannous (Sn^{2+}) and fluoride (F^-) ions as the interventions, out of which one study was an RCT (Algarni et al., 2015) while the other was a CT (Kensche et al., 2017) (Table 6.8). The RCT collected both in vitro and in vivo pellicles as it performed a proteomic experiment, which assessed pellicle proteins, as well as a functional experiment which assessed the protection against dental erosion. In vitro pellicles were formed via the time-based incubation method where human enamel specimens were immersed in expectorated whole mouth fresh saliva for two hours. In vivo pellicles for the proteomic experiment were collected with the help of an electrode filter paper being rubbed onto the tooth surface (Algarni et al., 2015). The CT was performed in vivo, where pellicles were formed on enamel slabs obtained from bovine incisors. The bovine incisors were then attached to prefabricated splints which were worn by the participants for 30 minutes to allow pellicle formation on the attached tooth surfaces (Kensche et al., 2017). Both studies used bovine incisors as substrates. In the functional experiment of the RCT study, the bovine incisors were randomly assigned to different groups and the pellicle covered enamel specimens were rinsed in the stannous and fluoride solutions and then exposed to the erosive medium after which the enamel specimens were assessed for surface loss using profilometry. The proteomic counterpart involved participants rinsing with the stannous and fluoride solutions after pellicle formation after which pellicles were collected and proteins were quantified using Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS). The study reported a higher abundance of proteins when stannous and fluoride ions were used in combination as compared to when used individually. The proteins identified to be in abundance were mucins (MUC7 and MUC5B) ($p<0.001$) albumin ($p=0.012$), histatins 1 and 3 ($p=0.002$), acidic proline rich proteins (PRP) 1 and 2 ($p<0.001$), small PRP ($p<0.001$), and basic PRP ($p<0.001$) and cystatins ($p=0.002$).

The CT involved participants rinsing four stannous and fluoride mouth rinses: Elmex Kariesschutz **(A)** (150 ppm NaF, 100 ppm Amine fluoride), Dontodent Med Care Extra-Schutz **(B)** (500 ppm NaF), Meridol **(C)** (125 ppm Amine fluoride 125 ppm SnF), and Elmex Zahnschmelzschutz Professional **(D)** (375 ppm NaF, 125 ppm Olaflur Amine Fluoride and 800 ppm SnCl), following the insertion of the splints, and then the enamel specimens were exposed to the erosive medium in vitro and assessed for calcium release. However, the study did not report any effects on pellicle's thickness or density. With regards to dental

erosion, the RCT reported a statistically significant decrease in surface loss with the combination of SnF_2 as compared to the groups with Sn^{2+} and F^- ions intervened individually. The other study reported a statistically significant decrease in calcium loss after rinsing with fluoride and stannous mouthwashes. The pellicle modifying effect of the investigated preparations depended on the acid's pH-value. At pH 3 and pH 2.3, there was a statistically significant reduction of percentage calcium release by the application of mouth rinse (A) (pH 3: 52%, pH 2.3: 22%; ($p<0.01$) and mouth rinse (D) (pH 3: 51%, pH 2.3: 27.73%) ($p<0.01$). However, at pH 2, there was a statistically significant reduction of calcium release from mouth rinse (D) (−22%, $p<0.01$). Mouth rinse (B) and (C) had no statistically significant impact on the protective properties of the physiological pellicle at any of the investigated pH-values ($p>0.01$). Pairwise comparison between the mouth rinses revealed significant differences among the mouth rinses only at pH 3 ($p<0.01$). Both mouth rinse (A) and (D) promoted the protective properties of the pellicle more effectively than B.

In the case of phosphate release, the effect of pellicle modification was generally less pronounced. At pH 3, mouth rinse A as well as D reported a statistically significant decrease in phosphate release compared to the physiological 30-minute pellicle. At lower pH level, a statistically significant decrease of phosphate release compared could only be achieved by the application of mouth rinse D (pH 2.3: 15%; pH 2: 15%; $p<0.01$). According to the pairwise comparison, the mouth rinse A was superior to the purely 500 ppm B at pH 3 and D at pH 3 and pH 2.

Table 6.8: Characteristics of studies using ionic compounds to modify dental pellicle.

Author/ Year/ Country	Study design	Nature/ method of saliva collection/ sample size/age	Pellicle formation method	Substrate used (bovine/ human enamel)	Acidic medium/ concentration/ exposure time	Intervention/ Proposed mechanism of action/outcome measure used	Control/ comparison	Main findings (Dental erosion)	Pellicle findings
Algarni 2015, U.S.A	RCT In vitro.	Stimulated/ expectoration 6; N/A.	In vitro pellicle formation using time-based incubation 2 mL of saliva, for 2h. In vivo pellicles collected using electrode filter paper.	Functional experiment (erosion) 40 bovine enamel slabs. Proteomics experiment: 32 bovine enamel slabs.	15.6 mM citric acid (pH 2.6), 4 ml.	Fluoride Stannous ions/ Increased adsorption of acid resistant proteins.	Deionized water.	Mean surface loss (µm) **Sn+F** 2.05±0.72[a] 89% reduction to control. **Sn** 6.14±1.5[b] 67% reduction to control. **F** 10.85±1.51[c] 42% reduction to control. **DIW** 18.60±3.32[d] **(p<0.05)***	**Sn^{2+}+F^-** 166 proteins **F^-** 158 proteins **Sn^{2+}** 146 proteins **Control** 157 proteins **(p-values N/R**).
Kensche 2017, Germany	CT In vitro, In situ.	N/A; 8, 25-43 years	In vivo pellicle formation for one minute using fabricated splints.	Enamel slabs from 48 bovine incisors attached to splints.	HCl (pH 2.0, 3.0 and 3.2) for 120 seconds	**Elmex Kariesschutz(A)** 150 ppm NaF, 100 ppm (Amine fluoride) **Dontodent Med Care Extra-Schutz (B)** 500 ppm NaF **Meridol (C)** 125 ppm Amine fluoride	Native enamel without rinsing.	Decrease in Ca^{2+} release: **Enamel with pellicle** pH 3.0: 37%, pH 2.3: 26% pH 2.0: 18% **(A)** pH 3: 52% pH 2.3: 22% **(p<0.01)** **(B)** pH 3: 51% pH 2.3: 27.73%	No effects on pellicle thickness or density.

						125 ppm SnF **Elmex Zahnschmelzschutz Professional (D)** 375 ppm NaF 125 ppm Olaflur Amine Fluoride 800 ppm SnCl/ Increase density of pellicle/ Ca^{2+} $PO_4^{2-/}$ release and SEM		pH 2: 22% **(p<0.01)** **(C)** No statistically significant impact **(p>0.01)** **(D)** No statistically significant impact **(p>0.01)** A and D more protective than B **(p<0.01).** Decrease in PO_4^{2-} release Enamel with pellicle pH 3: 33%, pH 2.3: 17% pH 2: 20% **(D)** pH 2.3:15% pH 2: 15% **(p<0.01)** A protective than B at pH 3.	

Key: *: actual p-value not reported; [abcd]: statistically significant difference ($p<0.05$); DIW: Deionised water; RCT: Randomised controlled trial; Sn: Stannous; F: Fluoride; A: Elmex Kariesschutz(A); B: Dontodent Med Care Extra-Schutz; C: Meridol; D: Elmex Zahnschmelzschutz Professional

6.5.3.5. Engineering peptides

The study which modified the dental pellicle using engineering peptides was a crossover triple blind RCT (Carvalho et al., 2020a) consisting of five parallel phases in which ten participants randomly participated in all study groups. (Table 6.9). The study used sugarcane cystatin (CaneCPI-5), haemoglobin (Hb) and a statherin derived peptide (StN15) as interventions into the dental pellicle. In each phase, the participants rinsed with 10 mL with each of the interventions for one minute after which pellicles were allowed to form for 120 minutes and then collected in vivo with the help of an electrode filter paper being rubbed onto tooth surface for proteomic analysis. For the erosion experiment, 10 µL of 1% citric acid (pH 2.5) was applied on to the tooth surface after pellicle formation. The drop was collected after 10 s with a pipette for calcium analysis, using the arsenazo III method. Proteomic analysis was performed by label-free proteomics which reported a statistically significant increase in acid resistant proteins (PRPs up to 32-folds with StN15) following treatment as compared to the control group. Treatment with the peptides also resulted in a statistically significant decrease in calcium loss as compared to the control group (deionized water).

Table 6.9: Characteristics of studies using engineering peptides to modify the dental pellicle.

Author/ Year/ Country	**Study design**	**Nature/ method of saliva collection/ sample size/age**	**Pellicle formation method**	**Substrate used (bovine/ human enamel)**	**Acidic medium/ concentration/ exposure time**	**Intervention/ Proposed mechanism of action/outcome measure used**	**Control/ comparison**	**Main findings (Dental erosion)**	**Pellicle findings**
Carvalho 2020, Brazil	In vivo crossover triple blind RCT.	N/A 6 female, 4 males; age 18–35 years.	In vivo pellicles collected using electrode filter paper.	Human upper and lower teeth.	10 µL of 1% citric acid (pH 2.5) for 10 seconds.	Sugarcane cystatin (CaneCPI-5), Haemoglobin (HB), Statherin derived peptide (StN15)/ formation of a reinforced basal layer altering the proteome. Ca^{2+} release/ (nLC-ESI-MS/MS	Deionized water.	**Ca^{2+} release (mol/mm)** CaneCPI-5[bc] 1.23±0.28 Hb 1.09±24[bc] StN15 1.05±0.28[b] Combined 1.60±0.54[ac] Deionised water[a] 2.36±0.77	**Proteins Mean ± SD (µg)** CaneCPI-5 21.7 ± 5.2 Hb 17.5 ± 3.9 StN15 16.2 ± 3.7 Combined 19.6 ± 4.3 Deionised water 8.2 ± 2.5 **(p<value N/R)**

Key: *Actual p-value not reported; CaneCPI-5: Sugarcane cystatin; Hb: Haemoglobin; RCT: Randomised controlled trial; StN15: Statherin derived peptide; nLC-ESI-MS/MS: nano reverse phase liquid chromatography coupled to mass spectrometry; [bc,b]: significantly lower Ca release compared to the negative control ($p<0.0001$); [ac]: combination did not significantly reduce the Ca release when compared to control; [b:] StN15 significantly reduced Ca release compared to the combination

6.6. Discussion

6.6.1. Key findings

The systematic review was performed to assess the impact of modifying the dental pellicle on the prevention against dental erosion. The findings identified six categories of interventions which were used to modify the pellicle and assess their preventive effects. The six categories were polyphenols, casein/mucin compounds, vegetable oils, plant extracts, ionic compounds and engineering peptides. The compounds reporting statistically significant effects on DE were green and black tea, red wine, while the compounds reporting statistically non-significant effects on DE were edible oils and casein-mucin compounds. Moreover, seven out of the thirteen included studies reported changes in pellicle structure and composition following intervention.

6.6.2. Strengths and limitations of the included evidence

The systematic review reported the impact of dental pellicle modification on the prevention against dental erosion, supported by a thorough literature search, a priori defined protocol, and followed recommended guidelines of the PRISMA checklist of 2020 (Page et al., 2021) for data reporting (Appendix B). All studies aimed to modify the dental pellicle and assess the effects on dental erosion both in vivo and in vitro. All studies collected dental pellicle either by in vitro or in vivo methods. The time-based incubation method for in vitro pellicle formation has been the preferred method in previous studies as pellicle age plays a role in defining the in vivo dentition's response to acid attack (Baek et al., 2009). However, since there is a high chance of some components of saliva to denature following in vitro sampling, in vivo collection of pellicles is preferred specifically for proteomic analysis (Delecrode et al., 2015). There is a very low possibility of proteins being denatured once the pellicle is frozen at -80^{o}C soon after in vivo collection by either the electrode filter paper or by using prefabricated splints (Papacosta and Nassis, 2011). The studies in the current systematic review adopted the in vivo method not only for proteomic analysis but also structural analysis following intervention.

Eight out of the thirteen included studies used citric acid at pH 3.0 as the erosive medium. Citric acid being used in the study was an advantage as it is a common ingredient in beverages with a potential to erode the dental hard tissues (Zheng et al., 2009). Similarly, the remaining five studies used hydrochloric acid at pH 2.0, 3.0 and

3.2, as the erosive medium which also contributed to the strength of the evidence. Gastric hydrochloric acid (HCL) comes into direct contact with teeth as a consequence of chronic vomiting or reflux and is an etiological factor of prime importance for dental erosion (Turssi et al., 2012). Both acids are common acids that are exposed to the dental hard tissues and using them for erosive experiments mimic the oral conditions and has an added advantage of comparability across studies of using the same acidic challenge.

Relative surface microhardness (rSMH) and analysis of calcium release were the two main outcome methods adopted to measure dental erosion following intervention. Both methods share the advantage of detecting the initial stages of dental erosion (Attin and Wegehaupt, 2014). Dental erosion begins as softening of the dental hard tissues which makes surface microhardness a valuable tool to measure the degree of hardness soon after an erosive challenge. Similarly, measuring the release of calcium ion soon after the dissolution of enamel after erosion is an efficient method to measure early erosion lesions (Joshi et al., 2016). The exposure time to the erosive mediums ranged from 30 seconds to three minutes similar to the normal conditions of the oral cavity and the methods adopted to detect early dental erosion may have been appropriate.

On the other hand, the evidence had a number of limitations. There were some concerns of the evidence to be biased as the personnel performing the intervention were aware of the intervention and there was no concealment of the allocation of the groups. Another major limitation encountered was the missing quantitative data preventing meta-analysis. Out of the thirteen included studies, quantitative data from only six studies could be obtained from the authors. Even then, the data were insufficient for meta-analysis as they were incomparable because of the interventions or different erosive mediums. Additionally, the actual statistical data (p-values) were not reported, hence creating difficulties in interpreting the findings. The other important element not taken into consideration in the studies was validating the presence of a pellicle layer adsorbed onto the surface of the tooth after incubation in saliva and rinsing in deionised water. No tests or visual aids were reported to confirm the presence of the pellicle layer and therefore, the presence of the dental pellicle layer and the occurrence of dental pellicle modification remains questionable.

6.6.3. Strength and limitations of the review

All the included studies were interventional with eight out of thirteen being randomised controlled trials which contributed to the strength of the review. In addition, one RCT was a triple blind study (Carvalho et al., 2020a) which minimised the possibility of information or ascertainment bias (Schulz and Grimes, 2002). However, blinding was only possible in the in vivo crossover RCTs where the multiple interventions to modify the pellicle were administered in the oral cavity. The studies where blinding was not possible were the single intervention in vitro modifications of dental pellicle as the investigator already had knowledge of the intervention being introduced. Secondly, as all studies were measuring short intervals of intervention effects (30 seconds to 3 minutes), there was no possibility of loss of follow up which further enhanced the quality of the outcome.

On the other hand, the insufficient quantitative data did not allow the possibility of a meta-analysis therefore, the combined effects of the outcomes could not be assessed which was the main aim of the review. Only 10% of studies were checked for eligibility and duplicate data abstraction, despite the gold standard being 100% for both, leading to a potential increased risk of reviewer bias and error rate. On the other hand, the kappa score was high suggesting the eligibility checking process was well conducted.

In vitro studies are not capable of accurately replicating the biological characteristics of the oral cavity, such as the presence of human saliva and the formation of AEP. In vitro saliva collection can lead to the salivary proteins to be degraded during collection and storage (Esser et al., 2008). In one study, during erosive cycling, storage of enamel and dentine specimens in saliva in vitro showed a reduced protective effect compared to storage in an in-situ environment (Hall et al., 1999). Therefore, in vivo saliva collection might be a preferred method for intervention experiments.

6.6.4. Significance of key findings

6.6.4.1. Polyphenols

Polyphenols have been reported to increase the adsorption of proteins hence increasing the thickness of the dental pellicle as observed by ellipsometry (Joiner et al., 2004). The polyphenols incorporated in the three studies resulted in a statistically significant increase in surface microhardness and a statistically significant decrease

in calcium release, however the exact p-values were not reported. The polyphenols present in green tea are in the form of epigallocatechin gallate (EGCG) which reported a statistically significant increase in SMH and reduction in erosion (Niemeyer et al., 2020). The increase in thickness of the dental pellicle after the addition of EGCG has also been reported via scanning electron microscope (SEM) and transmission electron microscope (Rehage et al., 2017). Another study reported an increase in proteins of the dental pellicle, specifically statherins and albumins after addition of polyphenols in the form of EGCG (de Souza et al., 2017). Statherins, together with PRPs, helps to maintain a state of supersaturation with respect to calcium and phosphate in the oral fluids, since they inhibit their precipitation under neutral pH and release these ions after an acidic attack (Proctor et al., 2005). Ovalbumin has been reported to reduce the dissolution of hydroxyapatite by acids solutions in vitro (Hemingway et al., 2008).

It must also be noted that red wine, being rich in polyphenols, also has a low pH (3.6-3.8) which may have also caused demineralisation of the tooth during the experiment. Therefore, there may be a possibility of variations in the results achieved from rSMH. Despite of the possible variations, red wine resulted in a statistically significant increase in rSMH as compared to the other acidic mediums of the same pH. The increase in rSMH by red wine suggests that the polyphenol present in red wine was effective in preventing dental erosion. Possibly, buffering the red wine before modifying the pellicle, may be an aspect to consider for future studies.

On the other hand, a factor to be considered regarding the use of polyphenols is their astringency which may cause drying of the oral cavity and sensations of increased roughness of the oral tissues. Polyphenols appear to reduce saliva lubricity by precipitating and aggregating salivary proteins which may include mucins (MUC5B). Mucins play an important role in the lubrication of the oral cavity, and a loss of lubrication can play a factor in contributing to the loss of enamel surface after an episode of dental erosion (Wang et al., 2020).

The mechanism of action suggested by the above-mentioned studies along with their effects may suggest polyphenols to be a promising intervention to be approached to modify the dental pellicle to prevent dental erosion. However, the studies only assessed dental erosion without the presence of abrasion or attrition, hence the effects

of astringency caused by polyphenols could not be investigated which may provide an opportunity for future research to further investigate the effects of pellicle modification using an erosion-attrition or erosion-abrasion model.

6.6.4.2. Casein/Mucin compounds.

Casein has been a great interest for researchers in oral health for at least two decades due to its ability to thicken the dental pellicle by forming micelle like structures by adsorbing to the proteins present in the dental pellicle. The structural interaction of casein with dental pellicle was been observed by SEM (Smith and Bowen, 2000) on hydroxyapatite discs which suggested that the characteristic structural feature may prevent the acids from penetrating the pellicle and demineralising the tooth; additionally, the feature may also inhibit the dissolution of calcium ions from the enamel (Barbour et al., 2008). Mucins are mucus forming proteins which increase the protein–protein interactions between each other, which could increase their ability to bind to the pellicle. They are also responsible for increasing the lubrication of saliva hence preventing from abrasive forces following softening of the dental hard tissues (Sieber et al., 2019).

One of the included studies in the review (Baumann et al., 2020) compared the combined effects of casein-mucin with casein and mucin intervened individually and reported no statistically significant difference in rSMH between any groups. The reason for lack of effect might have been the lengthy erosion time of three minutes as a strong erosion challenge also decreases the possibility of protection from the modified pellicle, as the dental pellicle itself and the casein/mucin layer are liable to dissolution by the acid and only offer protection in the short term (Hannig et al., 2004). Another possible reason for the rSMH findings would be the high pH of casein used during interventions. Increasing the pH affects the internal structure of casein micelles giving rise to more negatively charged micelles. The higher negative charges strengthen the repulsive forces of the caseins chain, which in turn, produces loose and expanded structures in the micelles weakening the cohesive interaction in the micelles. These conditions lead to the breakage of the hydrophobic bonds amongst caseins which eventually dissociate the casein micelles (Sinaga et al., 2016). Therefore, the results of the included studies remain questionable. None of the studies

were performed in vivo, and in vivo interventions may be a consideration for future research.

6.6.4.3. Vegetable oils

Several edible oils have been discussed for application in preventive dentistry and preventive medicine (Buchalla et al., 2003, Wiegand et al., 2007). The possible impact of the lipids present in oil on the protective properties of the dental pellicle has been discussed previously (Hannig and Joiner, 2006) reporting the formation of lipid micelles increasing the thickness of pellicle creating a barrier for acid attack. The TEM images obtained after rinsing with safflower oil indicated that the application of safflower oil did not retard pellicle disintegration under acidic conditions despite accumulation of lipid vesicles at the pellicle surface. In addition, the structure of the 120-min pellicle after initial rinsing with safflower oil was even of lower tenacity and loosened density. Moreover, an enhanced mineral loss was recorded after rinsing with safflower oil. In the other included study, a statistically significant change in surface microhardness was reported from only pure palm oil when compared to the other forms of edible oils used in the study. With only two studies reporting the modification of pellicle with edible oils with no effects on magnitude of DE, the evidence is limited and maybe a potential for future research to assess whether oils can be an approach to modify pellicle to prevent dental erosion.

6.6.4.4. Ionic compounds

Fluoride precipitates (CaF_2) have been found to be more stable and protective in the presence of the dental pellicle (Ganss et al., 2007) however the mechanism of the fluoride interaction with the dental pellicle still remains unclear. On the other hand, increased protection from dental erosion has been reported for dental surfaces treated with stannous ions which have reported to increase the adsorption of acid resistant proteins, when associated with the dental pellicle (Hove et al., 2007). The preventive effect was also observed in another study which reported a statistically significant decrease in surface loss after stannous ions interacting with the hard tissues in the presence of the dental pellicle (Hara et al., 2013). However, the aim of this study was not to modify the dental pellicle to prevent dental erosion but to observe how stannous ions interacted in the presence of pellicle. The included studies involved both stannous and fluoride ions to modify the pellicle, out of which one study reported a statistically

significant decrease in surface loss and statistically significant increase in enamel specific proteins when used together as compared to when F^- and Sn ions were used individually (Algarni et al., 2015).

Surprisingly, low F^- concentration mouth rinses have been reported to offer better protection against DE as compared to the mouth rinses with higher F^- concentration (Kensche et al., 2017). In addition, despite mouth rinse C comprising a combination of both stannous and fluoride ions, there was no statistically significant protection offered against DE. One of the low fluoride mouth rinses offered the best protection against severe erosive challenge, which can be expected as the mouth rinse comprised a combination of amine fluoride, sodium fluoride and stannous chloride and there is evidence to suggest that a combination of stannous and fluoride ions offer better protection when compared to when intervened individually. However, the combined stannous and fluoride mouth rinse did not offer protection against DE. The reason might be the low concentration of both components when compared to the other low concentration mouth rinse suggesting that a concentration ranging from 200ppm fluoride would be sufficient to protect against DE. Possibly, modifying the pellicle with various concentrations of F^- ions or combinations of calcium-fluoride (Ash et al., 2013) or stannous-fluoride ions could be a potential for future research.

Apart from fluoride and stannous ions, the additions of calcium ions into the pellicle have resulted in a dramatic impact on the hydrated mass, polymer mass, thickness and polymer concentration of the pellicle with the pellicle becoming predominantly elastic (Ash et al., 2013). The results from the study suggest that calcium can easily diffuse in and out of the pellicle, under physiological conditions, which may potentially facilitate the mineralisation of enamel. Another study investigated the impact of sodium triphosphate (STP) on the salivary pellicle. STP have shown to remove pellicle proteins and also prevent plaque development. After the pellicle was exposed to STP the pellicle became less dense but with a higher elastic component implying a structural transformation from a soft but dense structured pellicle, to a more diffuse pellicle (Ash et al., 2014). The evidence to modify the dental pellicle with ionic compounds to prevent erosion is limited, however the mechanism of action of fluoride and stannous ions on the dental hard tissues against dental erosion has been studied

for a long time and can be a possible approach to modify the pellicle to protect against dental erosion.

Five studies involved using fluoride compounds either as interventions in the form of mouth rinses (Kensche et al., 2017, Algarni et al., 2015), or as positive controls (Ionta et al., 2017, Hertel et al., 2017, Weber et al., 2015). Fluoride protects against mineral loss by its affinity to substitute hydroxyapatite with fluorapatite making it less soluble and preventing demineralisation (Cate and Featherstone, 1991). However, the mechanism by which fluoride effects the dental pellicle has still not been reported and requires further research. One theory suggests that fluoride application promotes the adsorption of specific proteins such as mucins, histatin 3 and PRP at the enamel surface, influencing the pellicle's density as well as molecular interaction (Algarni et al., 2015). The findings of the review found that vegetable oils, oregano and R. nigrum did not show any statistically significant difference in rSMH when compared to fluoride (control), while tannic acid offered better protection against DE than fluoride (control). On the other hand, fluoride ions intervened alone were not protective as compared to Sn^{2+}+F^{-} ions used as a combination. The mouth rinse containing 150 ppm and 375 ppm sodium fluoride were most protective against DE suggesting higher concentrations of F^{-} ion would offer better protection. Modifying the pellicle with various concentrations of F^{-} ions could be a potential for future research. Nevertheless, due to the variation in the finding, it is too early to support that fluoride would be either similar or better in protection against DE when compared to the other interventions.

6.6.4.5. Engineering peptides

A number of proteins have been reported to remain adsorbed to the pellicle even after a severe erosive challenge (Hannig et al., 2009b) out of which cystatins, hemoglobin and statherins have been identified due to their high affinity to hydroxyapatite (Delecrode et al., 2015). The concentrations of the added peptides corresponded to the mean range of concentrations originally found in natural human saliva and hence, resulted in a statistically significant increase in acid resistant proteins as well as a statistically significant decrease in calcium ion release. Statherin is a salivary protein with most of its charge located at its N terminus and due to the high density of the negative charge, to have a high affinity to interact with hydroxyapatite surfaces (Shah et al., 2011). Despite the results obtained after modification of dental pellicle with the

engineering peptides, the evidence is limited as the results are based on one study. Therefore, further studies are required to strengthen the findings and confirm the potential of engineering peptides to prevent DE.

6.6.5. Comparison with other reviews

This is the first systematic review to be performed on the impact of dental pellicle modification on dental erosion. So far, there are a limited number of narrative reviews published on the association of dental pellicle and dental erosion with a minor detail on pellicle modification. The narrative reviews that have summarised the various interventions and their effects on dental erosion (Hannig and Hannig, 2014b, Buzalaf et al., 2012) have reported casein and polyphenolic compounds to be used as possible interventions for pellicle modification, however, the scientific data is limited. Additionally, narrative reviews do not provide a comprehensive and systematic insight on the methods, mechanism of action and outcomes of the interventions (Pae, 2015). A systematic review on the published studies on the modification of dental pellicle to prevent against dental erosion may help in understanding and planning interventions for future clinical research.

6.6.6. Summary of effects of interventions

The systematic review was performed to assess the impact of dental pellicle modification on dental pellicle. Additionally, the aim was also to identify the most effective intervention against dental erosion. After the thorough review, it was observed that polyphenols, casein-mucin compounds and ionic compounds (F^- and Sn^{2+}) individually reported statistically significant effects on rSMH, calcium release and the composition and structure of the dental pellicle. However, due to the absence of a meta-analysis, the combined statistically significant effect of pellicle modification could not be confirmed.

Thickness, density and continuity of the pellicle layer are three factors that affect the protection of the underlying enamel (Hannig and Hannig, 2014), and six out of the thirteen included studies reported the effects of the interventions on the ultrastructural properties of the dental pellicle. The effects ranged from the increase in density of the pellicle to increase in proteins leading to an increase in thickness of the pellicle. However, it should also be noted that the thickness of the pellicle tends to decrease

with increase in acid exposure due to removal of the surface globular proteins. However, the basal layer remains intact (Hannig et al., 2009a). As dental pellicle contains mainly proteins, an increase in protein content leading to an increase in thickness of pellicle, might help to prevent acid influx towards the tooth surface, hence preventing DE.

The majority studies were performed on polyphenols and then casein. Polyphenols are a large family of compounds, so it is difficult to pinpoint exactly which polyphenolic compound is more effective. But considering the products with best results, it can be speculated that the compounds with best interaction with the salivary pellicle were EGCG (from green tea), theaflavins (from black tea), OPC (from grape seed extract) and flavanones and naringenin (from grapefruit seed extract). However, the fact that many other polyphenols are present in these products in minute quantities cannot be excluded, and hence the interaction of different polyphenols might influence their mechanism of action. Polyphenol molecules can easily react with salivary proteins, forming protein-polyphenol complexes interconnected with the proteins of the acquired enamel pellicle. Erosion was prevented best with casein and mucin combined together as mucins are large molecular proteins which have the ability to replace the smaller weight molecular proteins in the pellicle and form a thicker layer. The in vitro investigations of the proteomic profile of the pellicle suggested that fluoride application promotes the adsorption of specific proteins such as mucins, histatin 3 and PRP at the enamel surface, influencing the pellicle's density as well as molecular interaction. However, there was only one study which assessed the proteomic profile of the dental pellicle after application of fluoride ions, therefore future studies would be recommended to confirm the findings. Similarly, there was only one study which attempted to modify the pellicle with engineering peptides, and also has the potential for future studies. Palm oil reported to be the most effective in preventing DE. Palm oil is the second largest produced and consumed vegetable oil in the world, due to its high productivity, low production cost, and rich nutritional content. Oils are comprised of lipids and studies have confirmed the interaction of lipids with the dental pellicle using TEM (Kensche et al., 2013). However, the mechanism by which lipids interact with the dental pellicle requires further research. Although structural changes were reported after the addition of edible oils and plant extracts, no statistically significant effects on dental erosion were reported. Based on the findings of further research, the

practicality and palatability of edible oils can be considered during interventions. Introducing the oils to the diet on a daily basis could possibly be effective not only in preventing DE but could be a cost-effective measure for therapeutic purposes.

Overall, all interventions in the included studies reported to achieve some protection against DE, however possibility of pellicle modification occurring is questionable due to lack of assessment and investigation of the formation and adsorption of pellicle onto the enamel surfaces. Additionally, due to the limited statistical data and absence of a meta-analysis, a conclusion could not be made about the effectiveness of all interventions on dental erosion. However, after assessing the mechanism of action of casein on the dental pellicle (increased number of acid resistant proteins and thickness of pellicle by forming casein micelles) through the systematic review, casein suggests being an effective intervention to be incorporated into the dental pellicle in order to reduce/prevent dental erosion. In addition, casein is used widespread in commercially available sport protein supplements and therefore likely acceptability to athletes if used in this application.

6.6.7. Implications for future research

The limited number of studies in each category of interventions provide a platform for further insights into the impact of dental pellicle modification on dental erosion. An important element to consider is investigating the mode of action of pellicle modification against DE which could be achieved by first investigating the formation and adsorption of dental pellicle on enamel surfaces. The adsorption of pellicle to the enamel surfaces can be investigated by confocal laser scanning microscopy or scanning electron microscopy. The effects of pellicle modification can be visualized by transmission electron microscopy to assess changes in pellicle thickness. Additionally, quartz crystal microbalance can also be an option to assess the effects of interventions on the ultrastructural properties of the dental pellicle.

Buffering the red wine before modifying the pellicle, provided that the buffering agent does not change the structural and compositional characteristics of polyphenols, may be an aspect to consider for future studies. The various classes of polyphenols such as flavonoids and gallic acid must also be considered as intervention measures as they are known to have high affinity for calcium ions (Niemeyer et al., 2021). But on

the other hand, the astringency occurring after the use of polyphenols must be considered using an erosion-abrasion or erosion-attrition model. A tribology model could be an important element to assess the astringency while investigating erosion protection by polyphenols. Modifying the pellicle with casein-mucin compounds in vivo must be considered to assess the impacts of modification in the oral environment as all studies were performed in vitro. Moreover, micellar casein which is a slow calcium releasing protein could be an effective intervention measure to remineralise the enamel after acid exposure. Oils have resulted in structural changes to the pellicle increasing lubrication, however, their remineralisation potential should be assessed further. Engineering peptides and fluoride administered in various concentrations can be a potential for future studies to modify the pellicle to protect against dental erosion. Furthermore, the mechanism of action of fluoride on dental pellicle against DE is an important element of future research as the limited number of studies do not report the mechanism or changes to the pellicle following pellicle modification with fluoride ions.

6.7. Conclusions

The systematic review concluded that dental pellicle modification might be possible to reduce dental erosion. The effects should be further investigated by studies designed and reported to allow combination in meta-analysis. Additionally, further research is required to investigate the mechanism of effects and in particular the role of the dental pellicle and its ultrastructural and compositional properties.

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7.0. Salivary pellicle modification with micellar casein to improve protection against dental erosion – Intervention

7.1. Background

The previous chapter (chapter 6) gave a detailed account of the systematic review investigating interventions to modify the dental pellicle to reduce and prevent the incidence of dental erosion (DE). The systematic review reported casein to be one of the interventions frequently used to modify the dental pellicle to prevent dental erosion. Micellar casein has not been previously studied to modify the dental pellicle against dental erosion. This chapter will provide a detailed explanation of studies performed to investigate the effects of modifying the dental pellicle with micellar casein to prevent dental erosion which will also include a study performed to differentiate between casein and micellar casein and studies performed to confirm the formation of in vitro dental pellicles on enamel surfaces.

7.2. Introduction

7.2.1. Casein

Casein has been an area of interest for researchers in oral health for many years, comprising about 80% of the proteins in cow's milk with a family of four related phosphoproteins (αS1, αS2, β and κ). A suggested mechanism of action of casein is directly binding to enamel due to its high affinity to calcium hydroxyapatite, forming a dissolution barrier at the surface (Cheaib and Lussi, 2011). Studies have also reported the anti-cariogenic effect of casein after interventions in the form of casein phosphopeptide amorphous calcium phosphate (CPP-ACP) which is reported to inhibit the adherence of Streptococcus mutans, one of the key bacteria responsible for dental caries. (Goncalves et al., 2021). Research on casein has also reported that the application of casein in custom-made mouth guards is able to directly counteract the adhesion of plaque on the surface of the teeth and to increase pH values, salivary flow, amount of stimulated saliva and buffer capacity, improving the oral health of the athletes (Tripodi et al., 2021). Additionally, due to its ability to bind to calcium and proteins, casein has been suggested to have high anti-erosive properties after being incorporated into the dental pellicle (Sieber et al., 2019). On the other hand, another form of the casein protein, which may have a potential to prevent dental erosion, is micellar casein.

7.2.2. Micellar Casein

Micellar Casein is considered a high-quality protein mainly consumed by weightlifters to increase lean muscle mass (muscle hypertrophy) while maintaining a lean physique containing approximately 90% un-denatured protein and is the purest form of casein. Naturally in milk, caseins form structured aggregates around 200nm in diameter (so called “micelles”) which allows them to stabilise colloidal calcium phosphate in the milk as an important nutritional source of calcium. The micelles form a gel under the acid pH of the stomach and take a long time to digest and therefore micellar casein is digested slower than other milk proteins.

7.2.3. Structure of micellar casein

Various models of casein micelle structure have been proposed but the most enduring is the submicelle model. In this submicelle model, the caseins first aggregate via hydrophobic interaction into subunits of 15–20 molecules each. The pattern of interaction is such that it brings about a variation in the κ-casein content of these submicelles. Those rich in κ-casein congregate on the micelle surface. Whereas those submicelles poor or totally deficient in κ-casein are located in the interior of the micelle (Horne, 2006) and the interior submicelles are linked by colloidal calcium phosphate. The slow digestion of micellar casein can be beneficial in terms of providing calcium and phosphate ions being slowly released for longer periods of time when needed by the body as well as the dental hard tissues during DE promoting enamel homeostasis.

While casein has been investigated in various oral health related studies and has reported significant results in caries and erosion prevention, micellar casein might also have the potential to prevent dental erosion but has not been investigated for this effect.

In addition to prevention against DE, MC has not been assessed for particle size distribution (PSD) and would be an interesting element to investigate the difference in particle size of casein and micellar casein which may be performed by dynamic light scattering (DLS). The hypothesis is that the larger molecular size of micellar casein would have a higher affinity to adsorb to the pellicle and form a thicker pellicle which would eventually reduce dental erosion. Additionally, the larger particle size would also

have a higher affinity to bind to calcium ions and release them during acidic conditions to remineralise the enamel surface.

Moreover, little approach has been made to confirm the formation of dental pellicle on enamel surfaces prior to pellicle modification. None of the pellicle modification studies included in the systematic review confirmed the formation of pellicle on the enamel surface, which may raise the question of the validity of the results. Therefore, for the pellicle modification to be effective, it is important to investigate the formation of dental pellicle which may be performed by confocal laser scanning microscope (CLSM) or scanning electron microscope (SEM).

7.2.4. Summary

Micellar casein has never been investigated for prevention against DE. In addition, no previous pellicle modification studies have confirmed the formation of dental pellicle onto the enamel surface. Therefore, the aim of the study was to investigate the preventive potential of micellar casein modified dental pellicle against DE. In addition, the difference in particle size between casein and micellar casein using DLS, and the formation of dental pellicle onto the tooth surface using CLSM was also investigated.

7.3. Aims

To investigate the effects of modifying the dental pellicle with micellar casein to prevent against dental erosion.

7.4. Objectives

- To assess the difference in particle size distribution between casein and micellar casein using dynamic light scattering (DLS).
- To investigate the formation of pellicle layer on the tooth surface using confocal laser fluorescence microscope.
- To identify the most effective concentration of micellar casein against dental erosion.

- To determine the effect of micellar casein on the viscoelasticity and thickness of dental pellicle using quartz crystal microbalance with dissipation.

7.5. Study design

The study was an in vitro study and was performed in four steps. Experiment 1 involved the measurement of the particle size of casein and micellar casein. Experiment 2 comprised of in vitro pellicles formed on bovine teeth and assessing for pellicle formation. Experiment 3 involved the intervention where dental pellicles were modified with MC on bovine teeth in vitro. The bovine teeth were randomly assigned to different groups and the effect of micellar casein against dental erosion was investigated. Finally, experiment 4 comprised of forming in vitro pellicles on quartz crystal surfaces and assessing the change in thickness and hydrated mass after intervening with three concentrations of micellar casein solutions.

7.6. Materials and methods

7.6.1. Experiment 1 – DLS (Figure 7.5A)

The first experiment aimed to differentiate between the particle sizes of casein and micellar casein.

7.6.1.1. Preparation of casein and micellar casein solutions

The concentration of 1% casein and micellar casein was chosen based on an earlier study which identified the concentration as best for erosion protection (Sieber et al., 2019). The solutions were prepared as follows: 10 g of casein from bovine milk (Sigma-Aldrich, C7078) and 10 g each of micellar casein (DMV Micelate^{TM} Prestige (DMV), Friesland Campina, Netherland; Protein Works (PW) United Kingdom; My Protein (MP), Manchester, UK; and Reflex Nutrition (RN), (UK) (Table 7.1) was dissolved in deionised water at 40°C. The four brands of micellar casein were commercially available and were tested to calibrate the results and not to rely on only one brand of micellar casein.

Casein is insoluble in water as it contains a high number of proline amino acids which hinder the formation of common secondary structural motifs of proteins without disulfide bridges making it is relatively hydrophobic and poorly soluble in water.

However, since casein is an acidic protein, the technique applied to dissolve it is to add the powder in water and add sodium hydroxide (NaOH) at high temperature (approximately 40^{o}C) (Dalgeish, 1998). Therefore, after addition of casein and micellar casein powders, NaOH pellets (125mg) (Fisher Scientific) were added in increments and stirred continuously with a magnetic stirrer at room temperature overnight till the pH was 7.0 and powder was dissolved. The solutions were then filtered using the 0.2µm syringe filter to remove the larger particles as the resultant diameter was hypothesised to be around 200 nm. The solutions were assessed for PSD under DLS.

Table 7.1: Four brands of micellar casein investigated for particle size diameter.

1.	DMV Micelate^{TM} Prestige (DMV), Friesland Campina, Netherland
2.	Protein Works (PW) United Kingdom
3.	My Protein (MP), Manchester, UK
4.	Reflex Nutrition (RN), (UK)

7.6.1.2. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a tool for studying the diffusion behaviour of macromolecules in solution. The diffusion coefficient, and hence the hydrodynamic radii calculated from it, depends on the size and shape of macromolecules and the viscosity of the fluid (Stetefeld et al., 2016). As a particle diffuses through the known volume, it scatters photons, causing fluctuations in the light intensity. The process involves a laser light being emitted from a source which is scattered as a result of the Brownian motion of the particles in the solution. The time-dependent fluctuations in the intensity of scattered light that occur are analysed using an autocorrelator which determines the autocorrelation function of the signal. The correlation of the signal decays at an exponential rate which is dependent upon the diffusion coefficient of the particles being measured (Kaszuba et al., 2007).

Casein has been investigated under different conditions (temperature, pH, pressure) using DLS over the years (Tran Le et al., 2008, Gebhardt et al., 2006, Beliciu and Moraru, 2009). The studies assessed the effects of the different conditions on the particle size distribution of casein micelles and their impacts on the properties of milk.

For the current study, the measurements were made at 25.0±0.1°C and at a scattering angle of 90° to the incident beam, using a laser light-scattering instrument (Brookhaven, model: Nanobrook Omni) equipped with a 22mWHe–Ne laser at 632 nm in combination with a digital correlator (Figure 7.1). Experiment duration was in the range of 5–10 min, and each experiment was repeated three times. 3 ml of the prepared casein and micellar solutions was placed in 4 ml plastic cuvettes designed for DLS experiments. Baseline measurements were taken at first, after which depending on the outcomes of the scattering profiles, the solutions were diluted and filtered.

Figure 7.1: Dynamic Light Scattering (DLS) apparatus: Courtesy of UCL School of Chemical Engineering.

7.6.1.3. Statistical evaluation

Shapiro-Wilk was used to test the normality of the data. As the data was not normally distributed, a non-parametric test (Mann-Whitney U test) was used to compare the mean PSD of all groups from each other. Tukey's test was used for the pairwise comparison of the means. The significance level was set at 0.05 where the p value less than 0.05 was considered to be statistically significant.

7.6.2. Experiment 2 – Investigation of pellicle formation (Figure 7.5B)

The next study aimed to investigate the formation of dental pellicle using confocal laser scanning fluorescence microscopy (CLSM).

7.6.2.1. Collection and preparation of study teeth

Bovine incisors were used for this study using the same protocols as described in chapter 4 section 4.3.1.2. The labial surfaces of the bovine incisors were polished by wet grinding with abrasive paper (400–4000 grit) (Struers, Accutom-50, Denmark). After grinding and polishing, the samples were washed in distilled water for 5 min using ultra sonification, and finally disinfected in ethanol (70%) for another 10 min under ultrasonification. Following this, the samples were stored in distilled water at 4ºC until use. Twenty bovine incisors were randomly assigned (block randomisation) (Suresh, 2011) to two groups where one group (n=10) was the test group (pellicle formation) and the other group (n=10) was the negative control group (deionised water).

7.6.2.2. Saliva collection and pellicle formation

Unstimulated fresh human saliva was obtained from one healthy donor (male), aged 34 years. The donor was asked to avoid eating or drinking (except water) for 2 h before the saliva collection. The donor expectorated his saliva into falcon tubes and the saliva was centrifuged for 20 min at 4◦C (4000 g). The supernatant was collected and divided in small aliquots and used for pellicle formation immediately. For pellicle formation, the enamel specimens from first group (group 1, n=10) were immersed in the 1.5 ml of centrifuged saliva via time-based incubation method for 24 h at 37°C; no agitation to achieve maximum thickness of pellicle. The other group was immersed in 1.5 ml deionised water.

7.6.2.3. Investigation of pellicle formation by confocal laser scanning fluorescence microscopy (CLSM)

CLSM is a tool for obtaining high-resolution images, 3D reconstructions and optical sections through 3D specimens. The principle involves illumination by a gas laser (e.g. Ar/Kr or He/Ar), is focused by an objective lens into a small focal volume within an specimen labelled with fluorescent dyes. The laser beam is focused on the focal plane. A mixture of emitted fluorescent light as well as reflected laser light from the illuminated spot is then recollected by the objective lenses and a photon multiplier detector. The laser spot is scanned across a 2-dimensional (X, Y) focal plane to assemble a 2-D image. Information about the specimen can be collected from different focal planes by raising or lowering the microscopes stage. The computer can then generate a 3D

picture of a sample by assembling a stack of these 2D images from successive focal planes (Attin and Wegehaupt, 2014).

The formation and thickness of dental pellicle on the enamel surface has been detected in studies by transmission electron microscopy (TEM) (Hannig et al., 2005), however the preparation of the samples for TEM is a lengthy process. Confocal microscopy benefits by differentiating between organic and inorganic elements with the help of the fluorescent dyes (Nwaneshiudu et al., 2012) and may aid in identifying the organic pellicle layer being deposited on the enamel surface. Until now, no studies have been performed to detect the formation of pellicle layer using CLSM.

Rhodamine B (aqueous, 0.1 mM, pH 8) was used to stain proteins adsorbed to the enamel samples. The samples were incubated with the dye for 24 hours within a fridge at 4^{o}C in total darkness to prevent photo bleaching. After staining, the samples were rinsed with deionised water for 10 seconds to remove surplus dye. The samples were then then mounted onto a glass slide (No. 1.5) with an immersion oil (Olympus UPlanApo20x/070, Japan). Images were captured using an inverted confocal laser-scanning microscope (Olympus BX51) and processed using the software Aurox Visionary.

Scanning electron microscopy (SEM) was used to validate the findings obtained from CLSM images. However, since the samples used for CLSM had been stained with Rhodamine dye, fresh bovine teeth were subjected to the similar method of pellicle formation as for the CLSM images. A stepwise standard protocol was followed to prepare the samples for SEM following pellicle formation: The samples were fixed in a solution containing 2.5% glutaraldehyde for fixation of the pellicle proteins, and 0.1 M cacodylate buffer for 24 h at 4°C. After dehydration with an ascending series of ethanol (70%, 90% and 100%), specimens were chemically dried using hexamethyldisilazane (HMDS) and sputtered with carbon (sputter coater SCD 030, Balzers, Liechtenstein). The pellicle's surface micromorphology was then imaged using a scanning electron microscope (Zeiss Sigma 300 VP (ZEISS House, Cambourne, Cambridge, UK) at a magnification up to 100-fold. The images were calibrated with non-pellicle coated samples as control groups.

7.6.3. Experiment 3 – Intervention with MC (Figure 7.5C)

The third experiment aimed at modifying the pellicle with MC and assessing the anti-erosive potential using surface microhardness and calcium ion concentration.

7.6.3.1. Intervention for pellicle modification

The study consisted of seventy bovine incisors which were labelled and randomly divided (block randomisation) into seven groups (Table 7.2):

Table 7.2: Experimental groups for intervention study

Groups	**Description**
Group 1	1% casein pH 7.0 (*n=10).*
Group 2:	0.5% micellar casein pH 7.0 (*n=10).*
Group 3:	1% micellar casein solution pH 7.0 (*n=10).*
Group 4	2% micellar casein solution pH 7.0 (*n=10).*
Group 5:	Dental pellicle with no modification (*n=10).*
Group 6:	1% citric acid pH 3.0 (positive control) (*n=10).*
Group 7	Deionised water (negative control) (*n=10).*

7.6.3.2. Micellar casein solution preparation

Three solutions from the brand, The Protein Works were prepared for the pellicle modification. The Protein Works was selected as it reported the largest particle size diameter as compared to the other three brands. The hypothesis was that a larger particle size would offer better preventive potential. The solutions were prepared as follows:

For 1% micellar casein concentration, 1 g micellar casein (The Protein Works) was dissolved in 100 ml deionised water at 40°C pH 7.0 and the solution will be allowed to cool down to room temperature.

For 0.5% micellar casein concentration 500mg, micellar casein (The Protein Works) was dissolved in 100 ml deionised water at 40°C pH 7.0.

For 2% micellar casein concentration, 2 g micellar casein of (The Protein Works) was dissolved in 1000 ml deionised water at 40°C pH 7.0.

Previous studies have prepared casein solutions at the pH of 10.0 (Baumann et al., 2020, Sieber et al., 2019), however, there is evidence that casein particles dissociate at high pH which may possibly affect the protective effect (Sinaga et al., 2016). Therefore, the pH in this study was maintained at 7.0.

7.6.3.3. Preparation of erosive medium

The erosive medium used in the study comprised of 1% citric acid pH 3.2. Citric acid is a commonly used to model dental erosion (chapter 4, section 4.3.1.5.). The solution was prepared by dissolving 19.2 g of citric acid powder in 100 ml of deionised water. The pH was maintained at 3.2 by the addition of NaOH pellets (125mg) (Fisher Scientific) in increments and continuously stirring at room temperature.

7.6.3.4. Pellicle modification and experimental design

Seventy enamel specimens were randomly assigned (blocked randomisation) (Suresh, 2011) to one of the seven (n = 10) experimental groups mentioned above. The procedure involved groups 1, 2, 3 ,4 and 5 being incubated in saliva in a humid chamber for 24 hours to allow pellicle formation. The specimens were gently rinsed with deionised water to remove excess saliva and dried with air. This was followed by 2 hours of incubation in the prepared protein solutions for pellicle modification.

Group 1, 2, 3, 4, 5 and 6 were submitted to an erosive challenge in 1% citric acid (10 mL, pH 3.0) at 25°C for 3 min, at 70 rpm. Group 7 was immersed in deionised water for the same periods of time. All samples from all groups were removed from the erosive mediums, rinsed with deionised water and assessed for erosion using microhardness. Additionally, calcium release was measured from the citric acid using ion chromatography and calcium levels present in the enamel following erosion were quantified using EDX.

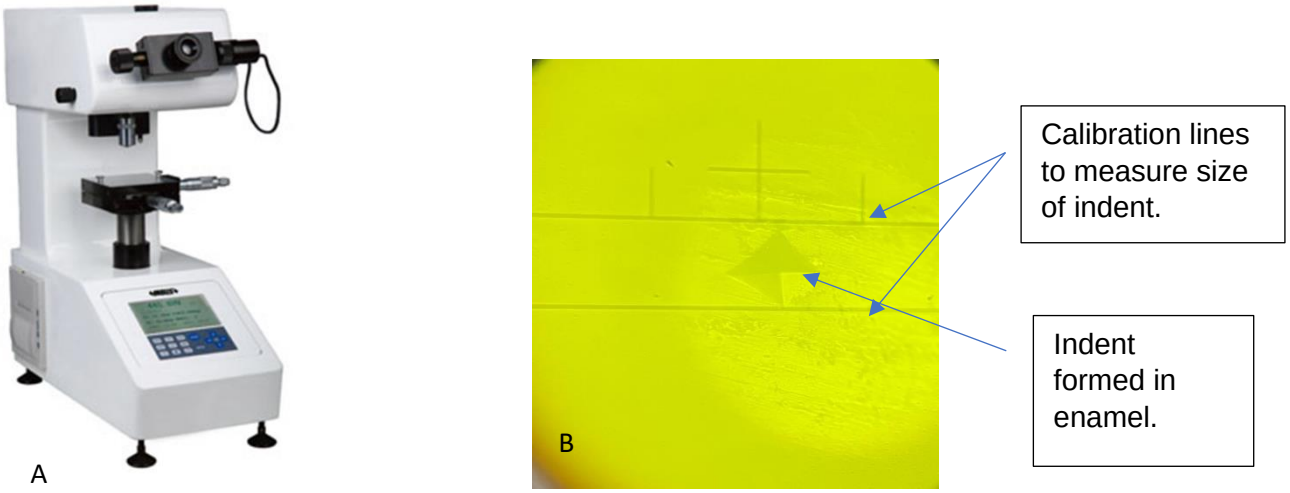
7.6.3.5. Assessment of DE

7.6.3.5.1. Surface microhardness (SMH)

Surface microhardness of the seven groups (*n=10 each group*) was measured with a Vickers diamond, with 10 g load and dwell time of 15 s (Insize, ISHV-D120 Automatic digital micro-Vickers hardness tester, United Kingdom) (Figure 7.2A) for both baseline and post erosion measurements. For each SMH measurement, six indentations were

performed with 25 µm distance from each other (Figure 7.2B). The size of the indent was measured by placing the calibration lines on the corners of the shape of the indent. The smaller the size of the indent measured indicates a harder surface and vice versa. The mean value from the six indentations were computed as the SMH of each specimen. The SMH was analysed at baseline (SMH initial) and after the final cycle (SMH final). The relative SMH (rSMH) was calculated using the following formula: rSMH = (SMH final/SMH initial) × 100 and expressed as percentage (%) decrease in microhardness of each group.

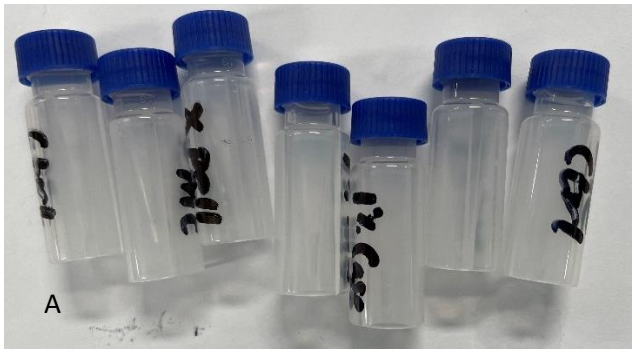
Figure 7.2 A: Insize Vickers hardness tester; B: Image of indent formed which will be measured.



7.6.3.5.2. Ion chromatography (IC)

The concentration of calcium ions in the citric acid after the erosive challenge, was determined using ion chromatography (Dionex Cation system, ICS1000, USA) for all seven groups (n=10, each group). For this purpose, 5 ml of the citric acid used for erosion was collected in falcon tubes. The samples were filtered with ammonia (Dionex OnGuard^{TM} IIA) to remove the phosphate ions as ammonia has a high affinity to bind to phosphate ions. Finally, 1 ml of the filtrate from each specimen was dispensed in 1 ml ion chromatography capsules (Figure 7.3A) and subjected for IC (Figure 7.3B). The samples were calibrated against standard solutions of 5, 10, 20, 50 and 100 ppm of calcium chloride ($CaCl_2$).

Figure 7.3: A. 1ml Ion chromatography capsules; B: IC apparatus.



7.6.3.5.3. Energy dispersive X-ray spectroscopy

Energy dispersive X-ray spectroscopy (EDX) is a chemical microanalysis technique used for elemental analysis and chemical characterisation of a sample. It is often used in conjunction with a scanning electron microscope (SEM). Its characterisation capabilities are mainly due to the fundamental principle that each element has a unique atomic structure allowing a unique set of characteristic X-ray signals to be generated when bombarded with electrons. It has the advantage of studying the elemental compositions of a sample from a very small area. The SEM-EDX technique represents a useful tool for quantitatively evaluating the elemental distributions in biological materials including calcified tissues like teeth (Wang et al., 2021)

Traces of calcium ions in the enamel samples from all seven groups (n=5, each group) after erosion were detected using EDX (Zeiss Smart EDX (ZEISS House, Cambourne, Cambridge, UK). The samples were rinsed with deionised water to remove the residual acid and then ultrasonicated in 10 ml acetone for five minutes to remove the adsorbed pellicle layer. The removal of the pellicle layer was an important step for EDX analysis to allow the electron beam to pass through and directly strike the enamel surface for elemental analysis. Finally, the samples were sputtered with carbon (sputter coater SCD 030, Balzers, Liechtenstein) and loaded on the stubs to be analysed under EDX. The detected calcium ions were measured in percentages of ions present.

7.6.3.6. Statistical evaluation

Data for relative surface microhardness rSMH, calcium ion concentration in citric acid, and % calcium in the enamel were analysed separately for each experiment. Shapiro-Wilk test was used to test the normality of the data. As the data was not normally distributed, a non-parametric test Mann–Whitney U test was used to compare the mean rSMH, calcium release and % calcium ions. Tukey's test was used for the pairwise comparison of the means of rSMH. The significance level was set at 0.05 where the p value less than 0.05 was considered to be statistically significant.

7.6.4. Experiment 4 - The effect of micellar casein on the viscoelasticity and thickness of dental pellicle using quartz crystal microbalance with dissipation (Figure 7.5D)

The effects of MC on the dental pellicle with regards to viscoelasticity, adsorption and quantification of pellicle thickness was investigated using quartz crystal microbalance with dissipation (QCM-D).

7.6.4.1. Micellar casein solution preparation

Three solutions of 0.5%, 1% and 2% micellar casein were prepared by dissolving 5 grams, 10 grams and 20 grams of micellar casein of (Protein Works, UK) in 1000 ml deionised water at 40°C pH 7.0 and allowing to cool down to room temperature

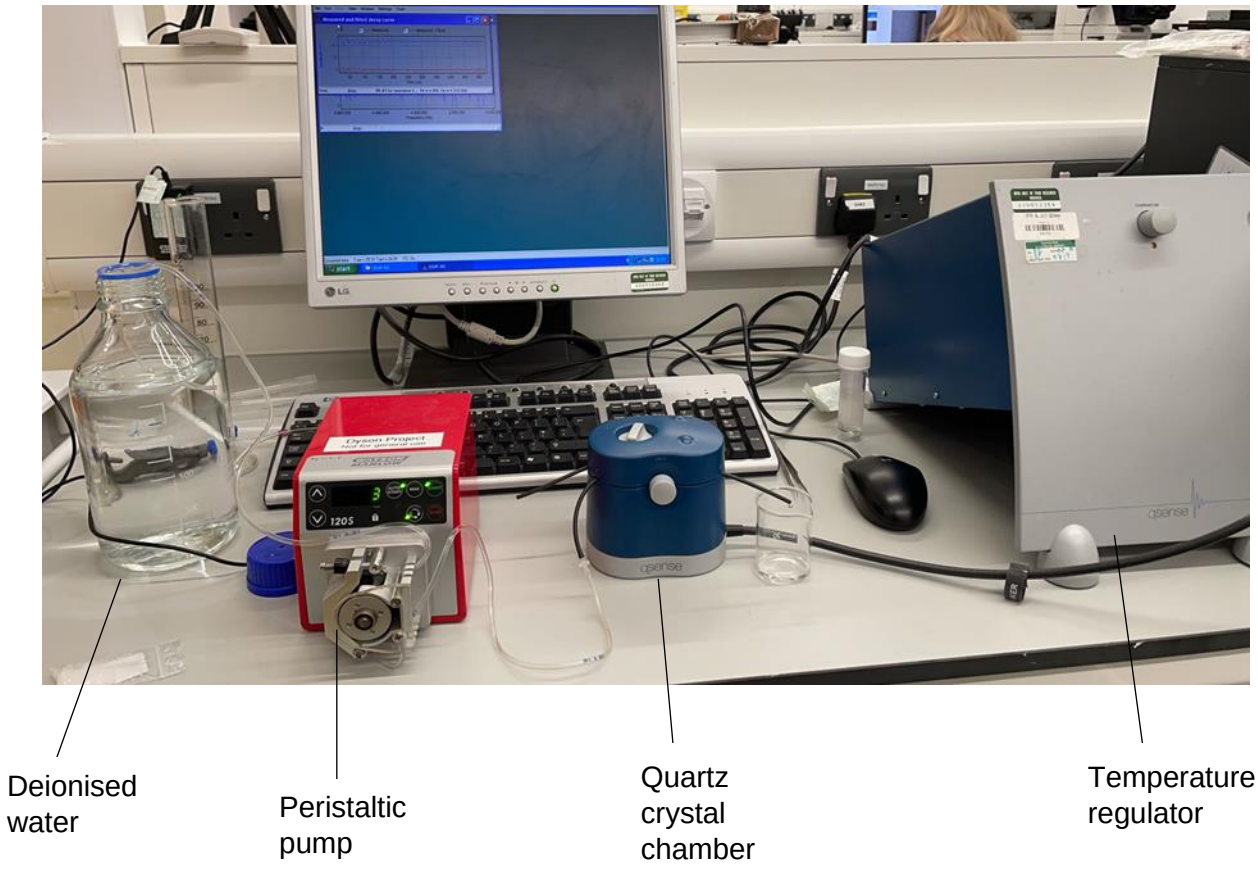
7.6.4.2. Saliva adsorption and treatment with MC

Saliva was collected as per protocols described in section 7.6.2.2. The QCMD apparatus was set up as shown in figure 7.6 The measurements were conducted using a D300 QCMD (QSense AB, Vastra Frolunda, Sweden) with a QAFC 302 axial flow measurement chamber maintained at 25^{o}C. Silicon dioxide coated AT-cut piezoelectric quartz crystals sandwiched between gold electrodes (QSX-303, Q-Sense AB, Vastra Frolunda, Sweden) were used as the substrate. The flowrate was adjusted to 80µL/min using a peristaltic pump (Watson-Marlow 102R, UK). The baseline was recorded by injecting deionized water till a baseline equilibrium was maintained. Each measurement included four stages. The measurement started with the injection of saliva into the measuring chamber (stage I). The flow was stopped after 15 min and was resumed during rinsing with deionised water (stage II). After rinsing for 5 min, the prepared MC solution was injected into the measuring chamber

(stage III) for a period of 10 min. Finally, deionised water was injected into the measuring chamber to rinse the chip (stage IV). The ending time of the four stages were referred to as TI TII, TIII and TIV.

The sensor was excited to oscillate by applying an alternating current across the sensor electrodes at its fundamental resonant frequency (i.e. 5 MHz). The frequency change (Δf) and the dissipation change (ΔD) were measured and calculated using the software (Qtools, version 3.0, United Kingdom).

Figure 7.4: QCMD apparatus setup to determine pellicle properties.

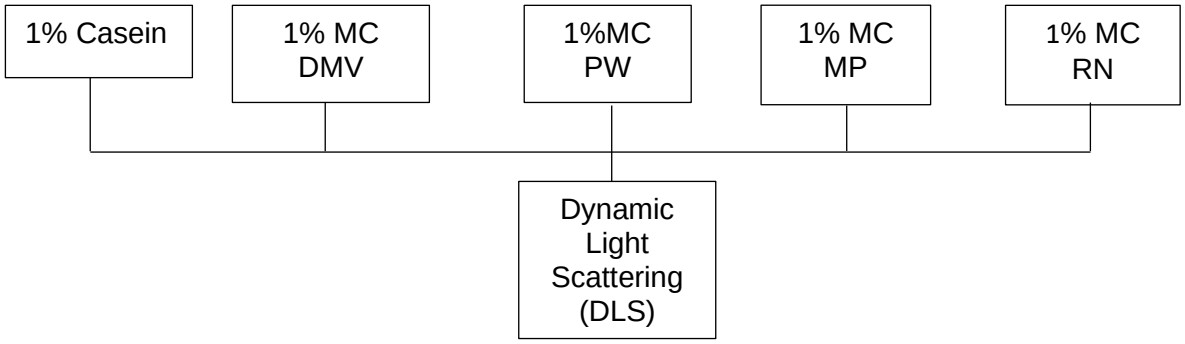


7.6.4.3. Statistical evaluation

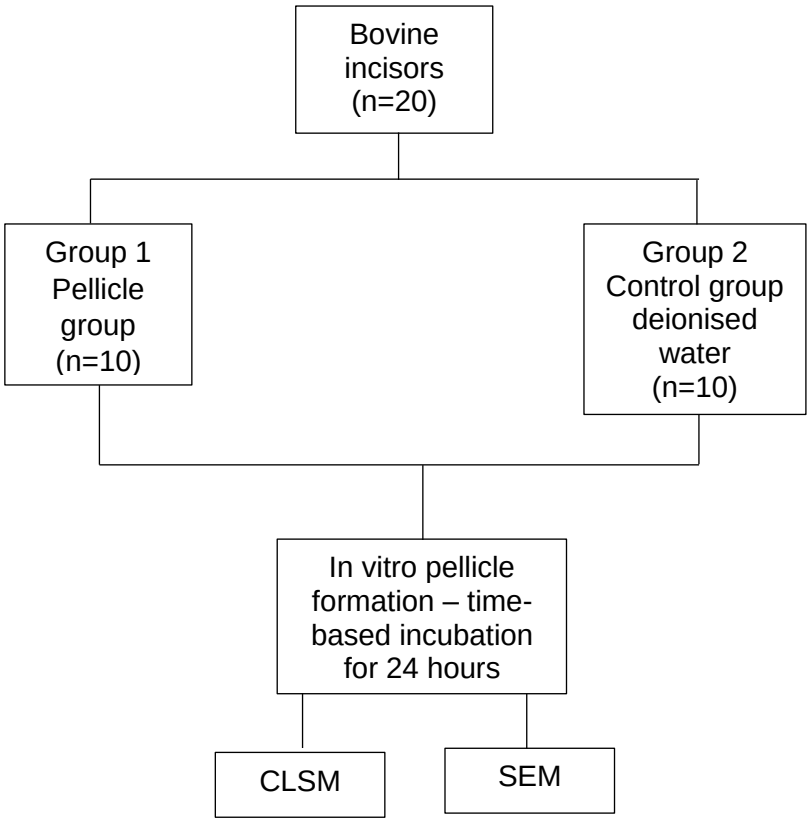
Significant differences in pellicle mass, thickness and density in relation to micellar casein concentration was determined by one-way ANOVA and Tukey post hoc analysis at three points of the experiment (i.e. peak, post MC rinse and post water rinse values) using OriginPro 11, USA). The significance level was set at 0.05 where the p value less than 0.05 was considered to be statistically significant.

Figure 7.5 A-D: Flowcharts summarising methods for intervention studies A: Dynamic Light Scattering (DLS) for particle size; B: Confirmation of pellicle formation; C: Modification of pellicle with outcome of DE; D: QCM-D experiment

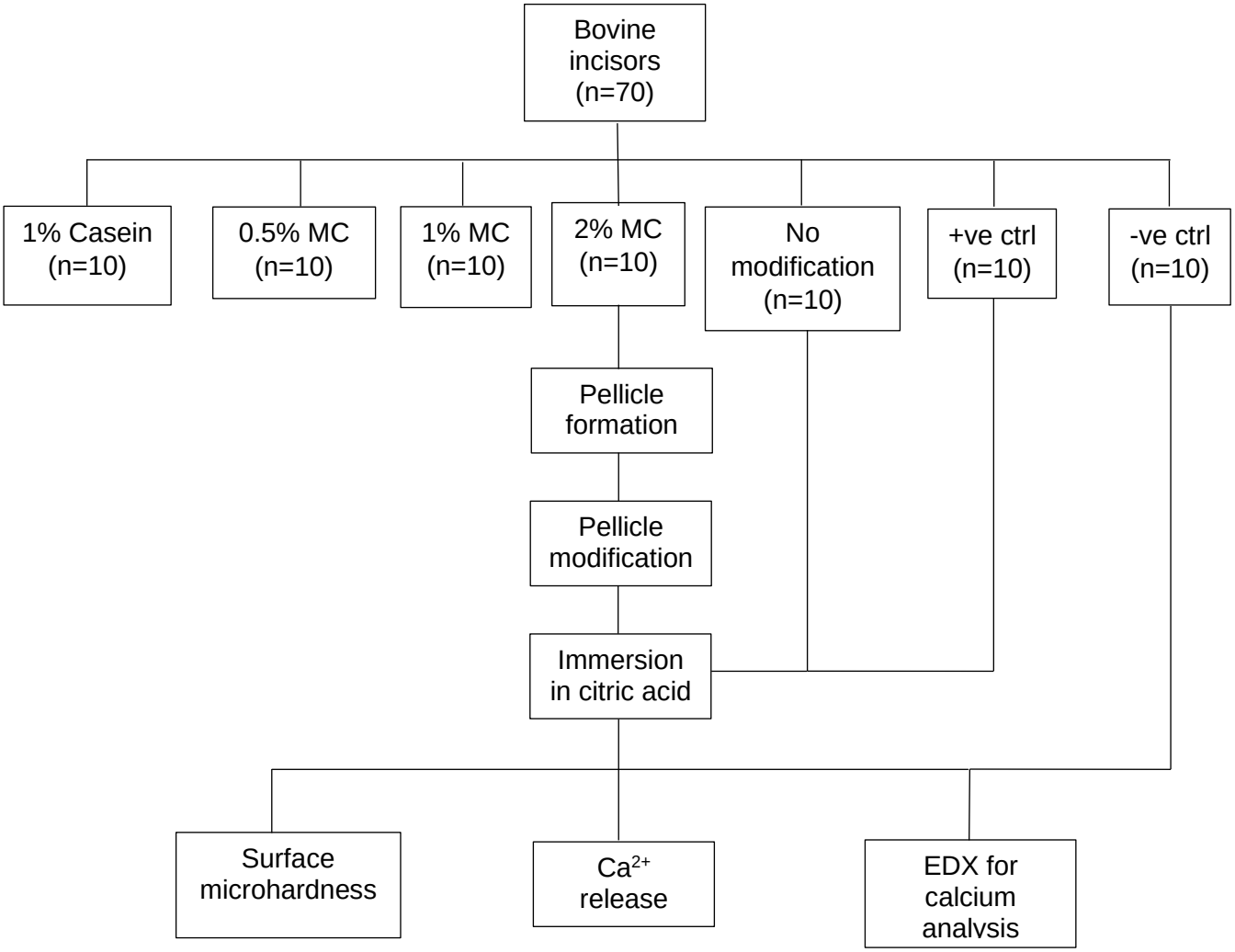
A: Experiment 1: DLS



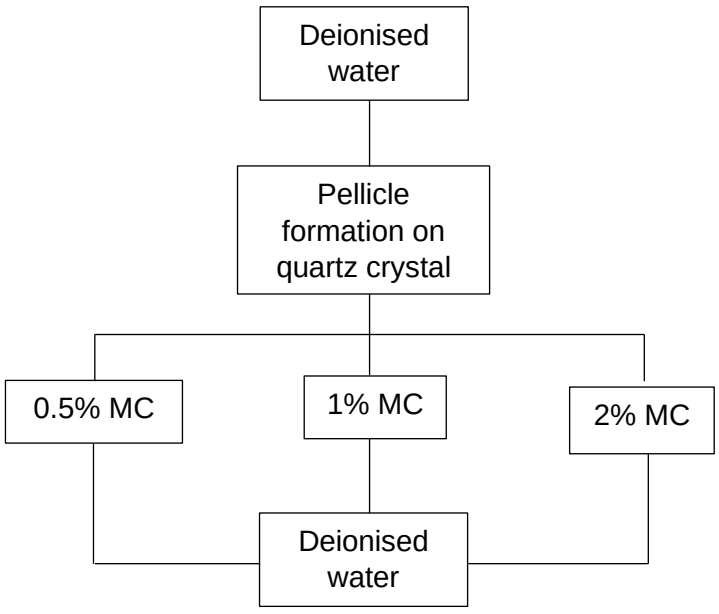
B*: Experiment 2: Investigation of pellicle formation*



C: *Experiment 3- Intervention study*



D: *Experiment 4: Determination of pellicle properties using QCMD*.

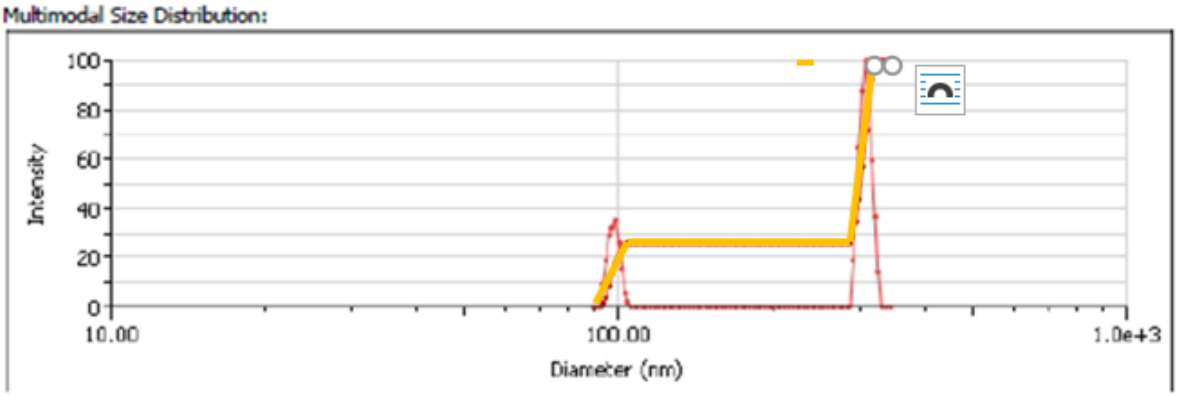


7.7. Results

7.7.1. DLS

Before the results of the DLS are presented, a brief discussion of the representation of DLS results is necessary. The results of particle size measurements can be displayed either as a lognormal distribution or as a multimodal size distribution (Figure 7.6). The lognormal distribution offers a simplified representation of the cumulative particle size, making it easy to compare between different samples and to calculate the single effective diameter for the analysed sample. The multimodal size distribution, on the other hand, offers more information regarding the presence of populations of particles or molecules of different sizes.

Figure 7.6: Multimodal size distribution graph with intensity (yellow) and number of particles (red).



The yellow curve indicates the proportion (intensity) of particles below the specific diameter within the specific intensity, whereas the red curve signifies the number of particles within a specific size band giving a particle size diameter (PSD) obtained as a result of the relative scattering intensity.

7.7.1.1. Particle size diameter

The multimodal size distribution graphs obtained from the DLS measurements of the four brands of micellar casein are shown in figure 7.7 A-D.

Figure 7.7 A: Multimodal size distribution of DMV.

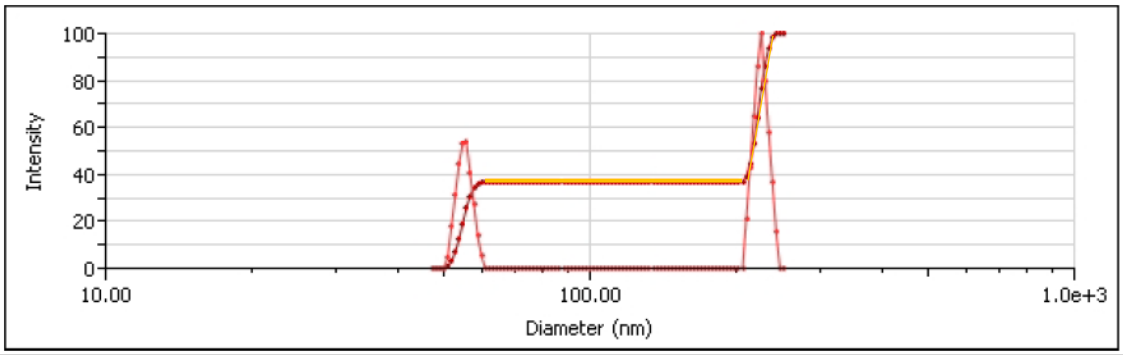


Figure 7.7 B: Multimodal size distribution of My Protein (MP).

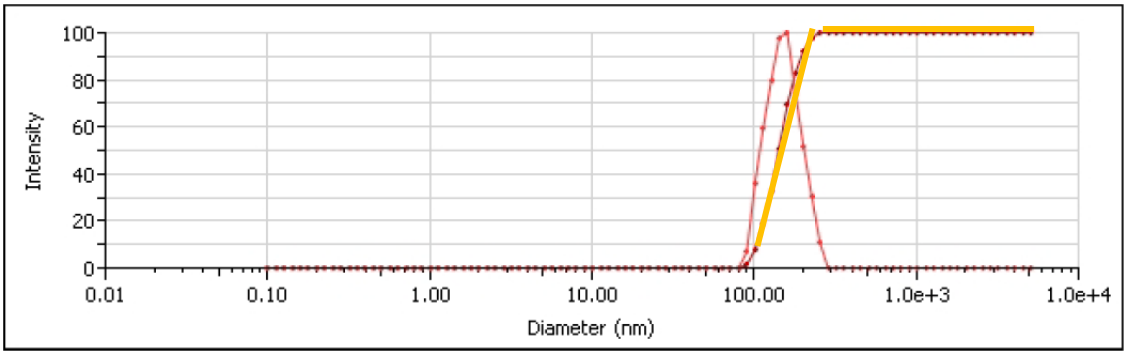


Figure 7.7 C: Multimodal size distribution of Protein works (PW).

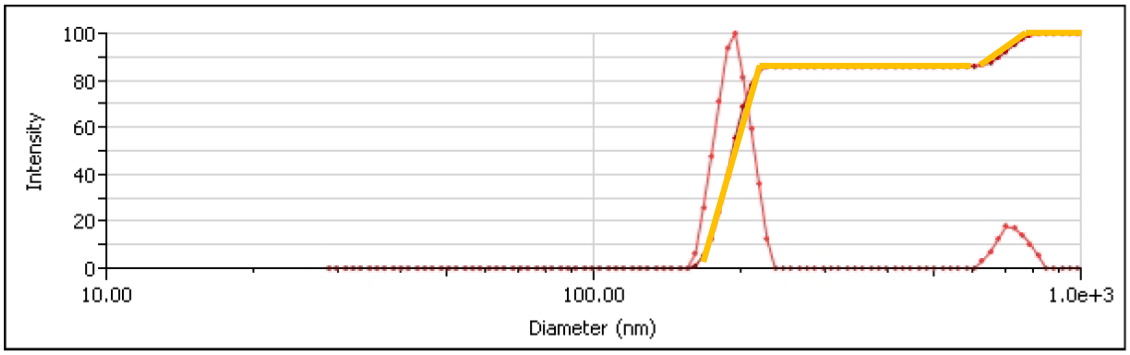
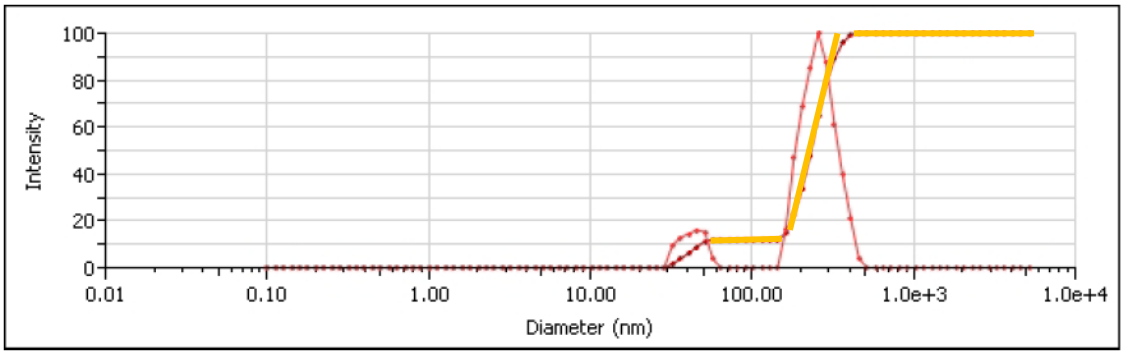
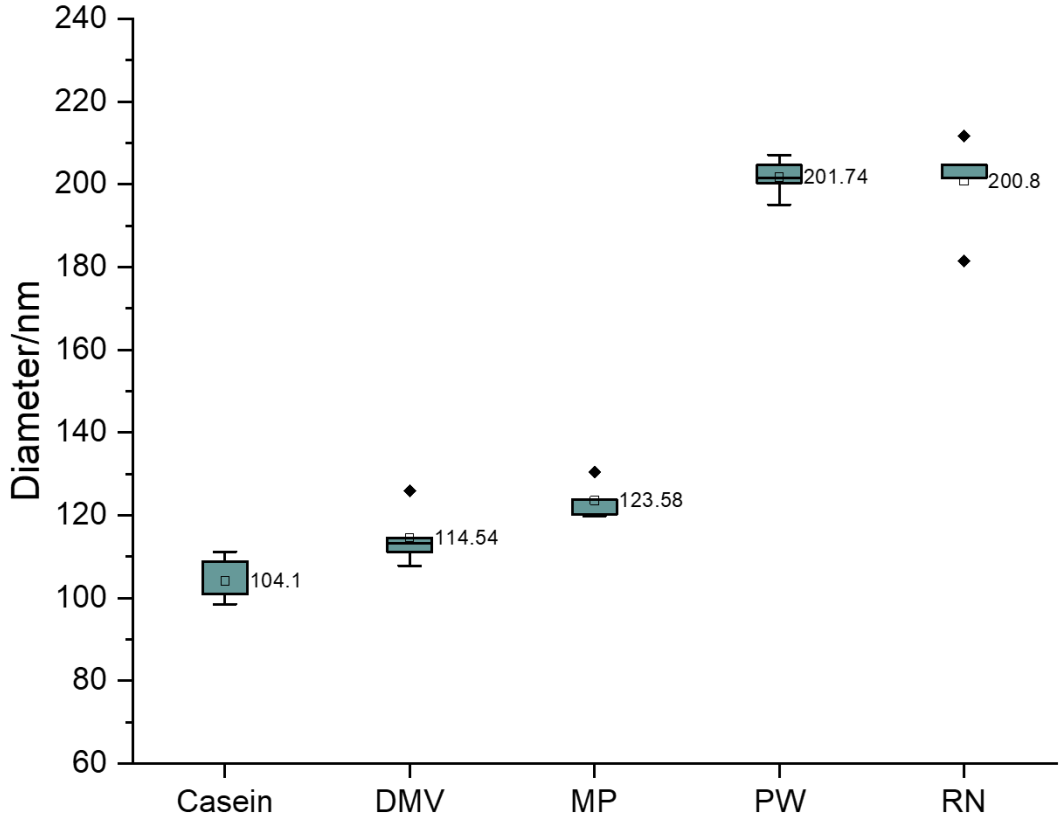


Figure 7.7 D: Multimodal size distribution of Reflex Nutrition (RN).



After assessing the values of the overall mean diameters, there appeared to be two groups of sizes, Protein Works (PW) (201.7±4.5 nm) and RN (200.8±11.4 nm), followed by a second group of MP (123.5±4.3 nm), DMV (114.5±6.8) and casein (104.1±5.9 nm) (Figure 7.8).

Figure 7.8: Box plots of mean PSD of all brands.



Pairwise comparison indicated statistically significant differences in diameters of micellar of PW, RN, and MP from casein (Table 7.3) indicating micellar casein particles to have larger particle sizes as compared to casein particles. However, there were no statistically significant differences between DMV and casein.

Table 7.3: Pairwise comparison of statistically significant differences in PSD among all brands. p<0.05 to be statistically significant.

	Casein/nm	DMV/nm	My Protein (MP)/nm	Protein Works (PW)/nm	Reflex Nutrition (RN)/nm
Casein/nm		p=0.055 (NS)	p=0.007 (Stat significant)	p=0.007 (Stat significant	p=0.007 (Stat significant
DMV/nm			p=0.09 (NS)	p=0.007 (Stat significant)	p=0.007 (Stat significant)
My Protein (MP)/nm				p=0.007 (Stat significant)	p=0.22 (NS)
Protein Works (PW)/nm					p=0.80 (NS)

7.7.2. Investigation of pellicle formation - Confocal microscopy (CLSM).

Dental pellicle appeared as inconsistent red patches stained by rhodamine dye denoting organic material settled onto the non-polished enamel surface (green) after 24 hours of pellicle formation (Figure 7.9 A-D). On the other hand, the red patches were not observed to be settled onto the polished surfaces (Figure 7.10).

Figure 7.9 A-D: Confocal images of different enamel surfaces confirming pellicle formation (red) on non-polished enamel surface (green).

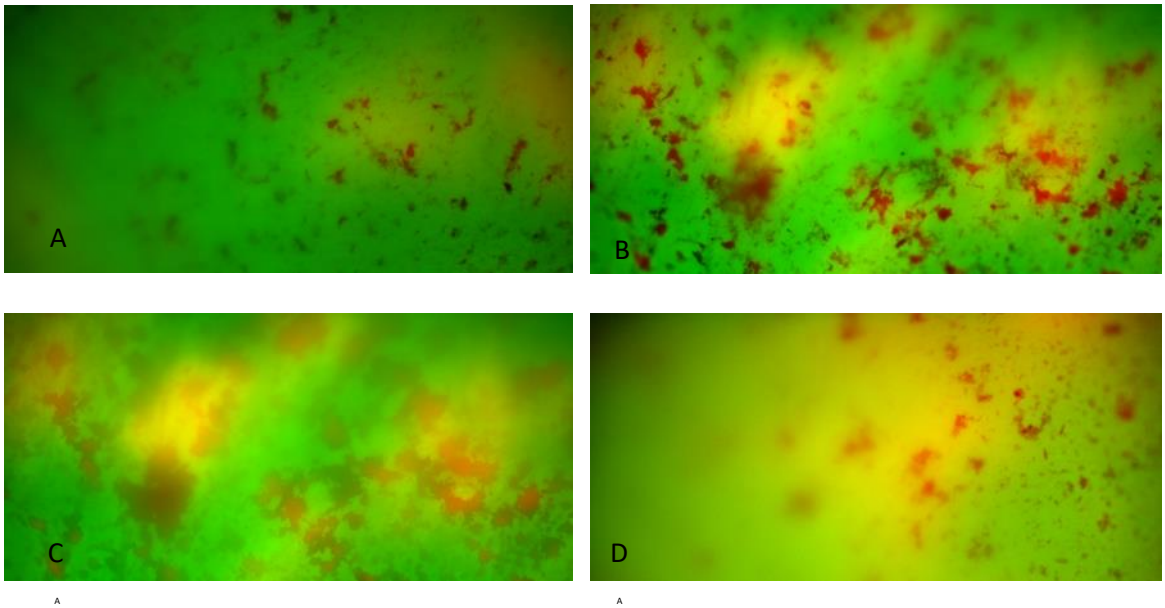
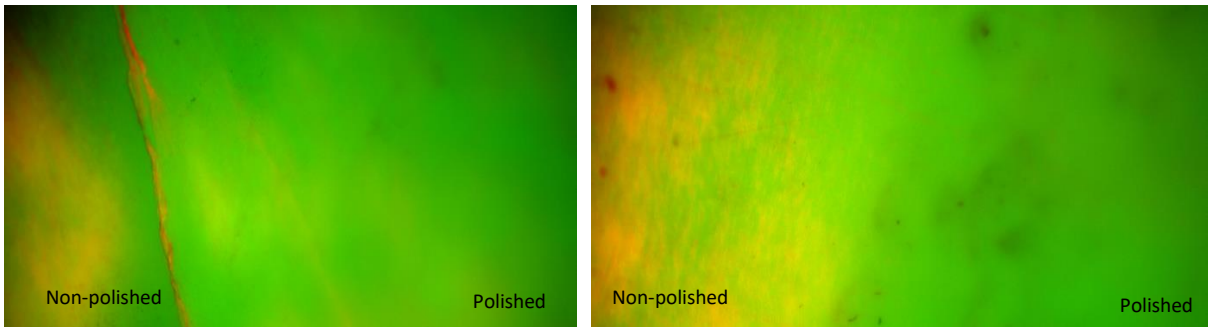


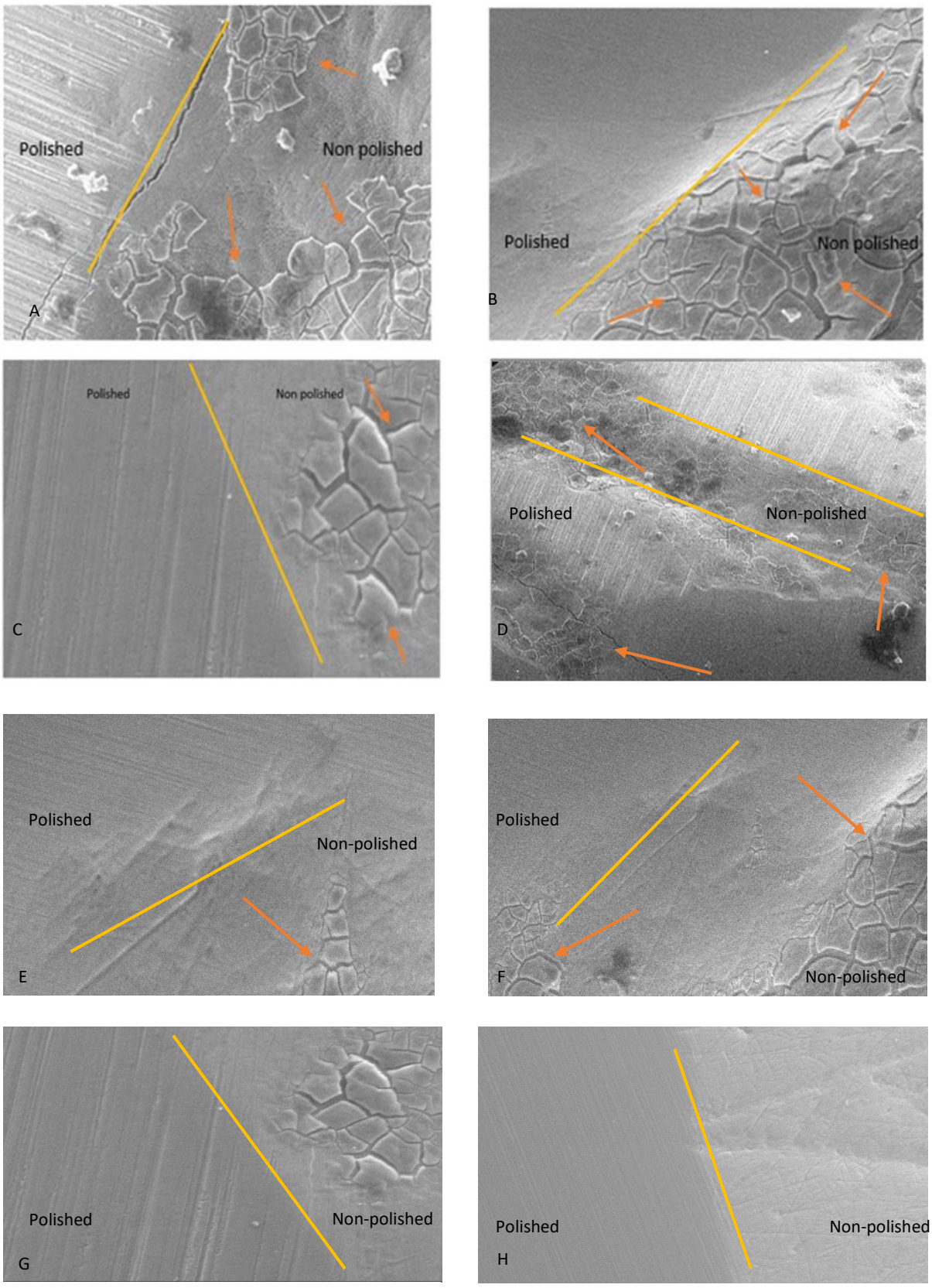
Figure 7.10: Confocal images of two enamel surfaces comparing pellicle formation between polished and non-polished surfaces.



7.7.2.1. Confirmation of pellicle formation – scanning electron microscope (SEM)

Due to the inconsistent layers of pellicle deposition observed via CLSM, SEM was performed to investigate the deposition of pellicle and also compare to non-pellicle coated controls. Two sections from each specimen of pellicle coated samples were captured at the junction between the polished and non-polished enamel labial surface (Figure 7.11 A-H-yellow line). were captured. The images were also revealed inconsistent patches of dental pellicle deposited on the enamel surface at 200x magnification (Figures 7.11 A-H orange arrows). However, the pellicle did not seem to deposit on to the polished surface as observed in figures 11 A-H. No deposition was observed in the negative control group (at 200x magnification (Figures 7.11 H.)

Figure 7.11: Scanning electron microscope (SEM) images of different enamel surfaces confirming pellicle formation on non-polished enamel surfaces as opposed to polished surfaces (A-G). Image H acting as control. Yellow line differentiating between polished and non-polished surfaces. Orange arrows representing pellicle islands.



7.7.3. Surface microhardness

The group treated with 1% micellar casein showed the least decrease in microhardness of 2.0% (Table 7.4) when compared to the groups treated with 0.5% (4.8%) and 2% (6.3%) micellar casein solutions. The group treated with 1% casein, and the group with no modification of pellicle, both showed a non-statistically significant decrease in microhardness (12.8%, p=0.2) demonstrating them to be similar in preventing dental erosion. The positive control group displayed a statistically significant decrease in microhardness (15.9%) (p=0.007). On the other hand, the negative control group showed a non-statistically significant increase in surface microhardness after erosion cycling. Pairwise comparison between groups displayed statistically significant differences in surface microhardness reduction between the positive and negative control group, and the positive control and 'pellicle without treatment' group (Table 7.5). There were no statistically significant differences in surface microhardness between the remaining groups although comparison of the reduction in SMH between the positive control and the 1% MC group was only just outside statistical significance at P<0.05 (P=0.051). However, there was significant variation of surface microhardness in certain groups (baseline 0.5% MC, post erosion 2%MC).

Table 7.4: Mean SD surface microhardness of baseline and post erosion for all groups.

Group	Mean ± SD Baseline microhardness/ HV	Mean ± SD Post erosion microhardness/ HV	rSMH=SMHf-SMHi x 100 SMH initial
Positive control	333.2 ± 13.0	280.29 ± 24.2	- 15.9% (p=0.007)
Negative control	340.4 ± 20.8	363.8 ± 45.4	+6.8% inc. (p=0.53)
Pellicle no treatment	351.7 ± 35.8	306.4 ± 26.8	-12.8% (p=0.2)
0.5% Micellar casein	267.9 ± 35.8	254.5 ± 36.7	-4.8% (p=0.31)
1% Micellar casein	286.5 ± 14.4	280.0 ± 29.8	-2.0% (p=0.56)
2% Micellar casein	251.9 ± 59.3	235.5 ± 22.2	-6.3% (p=0.11)
1% Casein	333.35 ± 33.1	290.6 ± 29.3	-12.8% (p=0.2)

Table 7.5: Pairwise comparison of surface microhardness between the seven treatment groups.

	Positive control	**Negative control**	**Pellicle no treatment**	**0.5% MC**	**1% MC**	**2% MC**
Negative control	p=0.007					
Pellicle no treatment	p=0.03	p=0.73				
0.5% MC	p=0.2	p=0.43	p=0.64			
1% MC	p=0.051	p=0.42	p=0.91	p=0.73		
2% MC	p=0.12	p=0.12	p=0.47	p=0.83	p=0.39	
1% Casein	p=0.41	p=0.01	p=0.057	p=0.31	p=0.11	p=0.47

7.7.4. Calcium release - Ion Chromatography

There were no statistically significant differences in the mean calcium ion concentration between all groups (n=10 per group) (p=0.2-0.9) (Table 7.6). Although not statistically significant, the lowest calcium ion concentration was recorded from the no pellicle treatment group while the highest being from the 1% MC group (21.98 ± 8.6 mmol).

Table 7.6: Mean ± SD of calcium release from all groups after erosion.

	+ve Ctrl	**No treatment**	**0.5% MC**	**1% MC**	**2% MC**	**1% Casein**
Ca^{2+} release /mmol	19.18±4.06	19.11±4.29	19.4±9.5	21.9±8.6	20.1±6.4	20.7±7.8

7.7.5. Calcium analysis – EDX

There were no statistically significant differences between the mean percentage calcium ions present in the enamel of all groups (p=0.3-1.0) (Table 7.7). Although not statistically significant, the highest calcium ion content was recorded from the positive control group.

Table 7.7: Mean±SD of % calcium ions detected in enamel with EDX in all groups

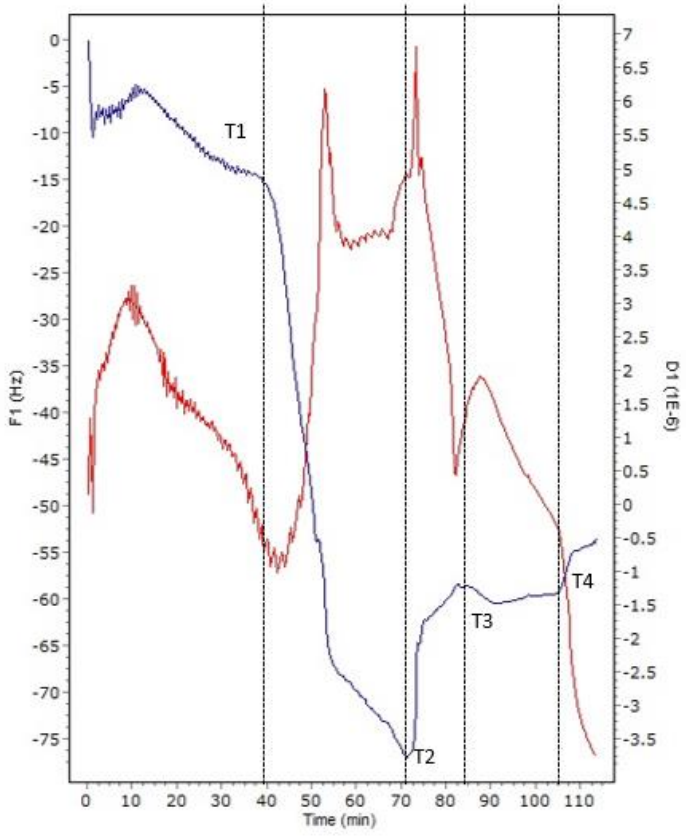
	+ve ctrl	**No pellicle treatment**	**0.5% MC**	**1% MC**	**2% MC**	**1% Casein**	**-ve ctrl**
Ca^{2+} %	35.6±1.30	36.4±6.5	41.8±2.8	38.0±6.0	39.86±1.49	37.3±5.27	42.6±1.13

7.7.6. QCM-D

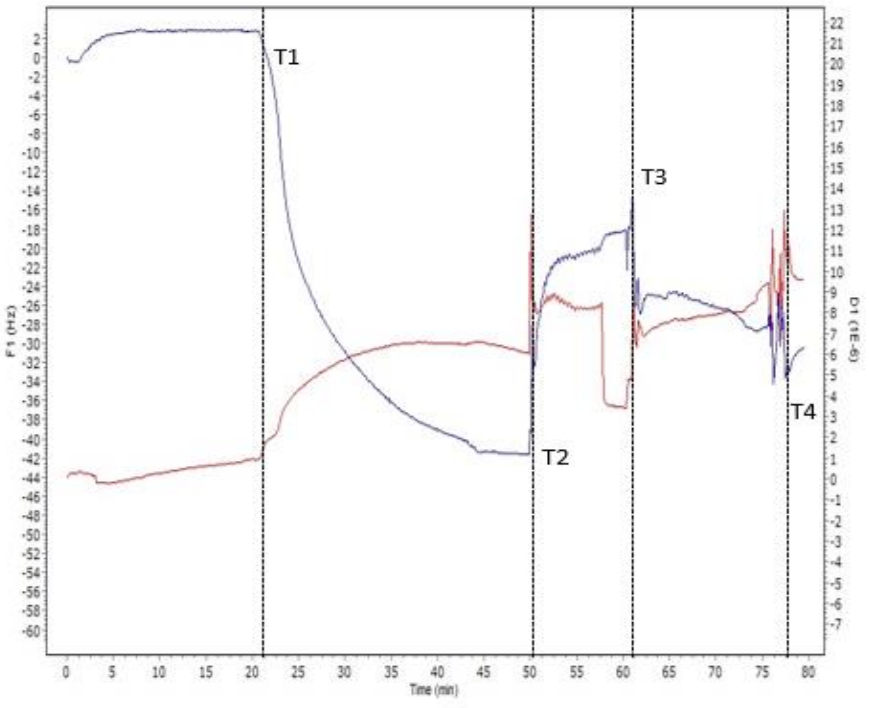
Changes in the frequency of the oscillating sensor were related to the changes in the hydrated mass adsorbing on to the quartz crystal sensor via the Sauerbrey model. In addition, pellicle thickness was calculated from the hydrated mass by assuming a value for the density of 1000 kg/m^3. The viscous properties of softer films dampen the sensor's frequency of oscillation, and in such cases the Sauerbrey model underestimates the mass, whereupon another method of analysis is needed to fully characterise the film. Therefore, in addition to recording frequency changes, the QCMD measures a second parameter known as dissipation.

The interaction between the salivary pellicle and the micellar casein solutions was monitored by the changes in frequency (blue line) and dissipation (red line), of the gold sensor (Figure 7.12 A, B and C). During T1, the injection of saliva into the QCM-D chamber led to a rapid decrease in frequency and a rapid increase in dissipation which means that a highly viscoelastic salivary pellicle was formed on the chip. An increase in frequency and a decrease in dissipation were detected during stage II of water rinsing, indicating the removal of some weakly adsorbed proteins from the outer layer of the pellicle. After sequentially injecting micellar casein (stage III), the frequency decreased instantly and then increased to a stable value, with the decrease using the 2% MC solution being more intense, which demonstrated a strong association of the salivary pellicle with micellar casein at 2% MC concentration. Meanwhile, the dissipation revealed an inverse relation with the frequency, where the dissipation increased with decrease in frequency. After flushing with water to remove the MC molecules (stage IV), an apparent increase in frequency along with a decrease in dissipation occurred all treated samples.

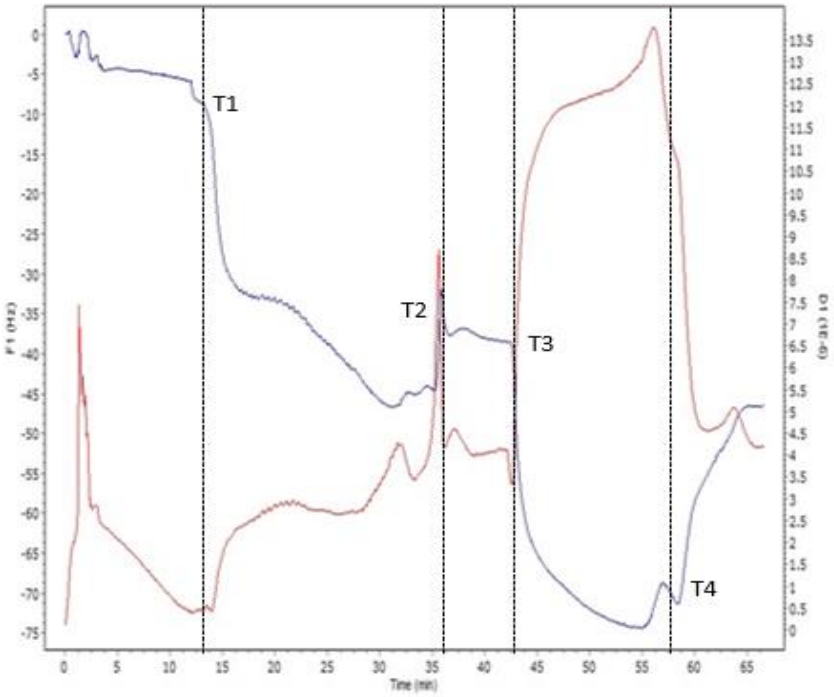
Figure 7.12: Frequency (F) (blue) and dissipation (D) (red) curves of pellicles interacting with gold sensors at 0.5% MC (A), 1% MC (B) and 2% MC (C). T1: Baseline pellicle; T2: Post rinsing; T3: MC injection and T4: post rinsing.



A.



B.



C.

The hydrated mass measured via QCMD takes into consideration the hydration, or water, contained within salivary pellicle. There were no statistically significant changes in the pellicle thickness (Table 7.8) and hydrated mass (Table 7.9) of the pellicle after injection of all concentrations of micellar casein. However, there was substantial variation observed in the outcome data.

Table 7.8: Mean±SD pellicle thickness at baseline and post MC injection

	0.5% MC	**1% MC**	**2% MC**
Baseline thickness/nm	34.25±34.86	13.59±8.62	4.51±2.99
Thickness post MC/nm	13.8±8.05 (p=0.7) from baseline	19.0±12.8 (p=0.4) from baseline	1.8±8.8 (p=1.0) from baseline

Table 7.9: Mean±SD hydrated mass of pellicle at baseline and post MC injection

	0.5% MC	1% MC	2% MC
Baseline mass/ng	1454.3±603.6	1722.5±1983.1	246.8±297.0
Mass post MC/ng	1615.1±986.7 (p=1.0) from baseline	471.8±1714.9 (p=1.0) from baseline	625.9±262.9 (p=0.66) from baseline

7.8. Discussion

7.8.5. Main findings

Dynamic light scattering of micellar casein reported a larger particle size diameter than casein. Under confocal microscopy and scanning electron microscope, the pellicle was observed to be inconsistent with patchy deposits on the non-polished enamel surfaces. Despite the large particle size, there were no statistically significant differences in surface microhardness between baseline and post erosion. There were statistically non-significant differences in calcium ion release between all groups as measured by ion chromatography. There was a statistically significant lower number of calcium ions in enamel in the pellicle modified with 2% MC as detected by energy dispersive x-ray spectroscopy. There were no statistically significant differences in thickness and the hydrated mass of dental pellicles after modification with micellar casein. However, one aspect that was common to most experiments was the high variability in results which shall be discussed in the sections below.

7.8.6. Dynamic light scattering (DLS).

The results indicated that overall micellar casein had a larger diameter than casein. However, there was substantial variation in the detected diameter in all the different brands investigated. One possibility is that some of the products are not micellar in composition as differences with casein were minimal. The other factor contributing to the variability in diameters could be a difference in the ultra-purification procedures of milk to prepare MC. There are two purification methods used in the preparation of MC: one is the microfiltration technique while the other is re-micellisation (acid drying) from acid casein (Mounsey et al., 2005). Microfiltration involves the removal of whey

proteins from milk filtering (0.1 - 0.2 mm), a casein micelle enriched product. The powders used in the current study did not signify the method used to prepare the micellar casein powders. A difference in the technique used to prepare and purify milk could be a possible reason for the variation in diameter of MC observed with DLS.

Another problem associated with dairy protein powders containing increased amounts of micellar casein, is their poor reconstitution properties which can lead to poor dissolving properties (Schokker et al., 2011) which could be another factor behind the variability in diameter. Improvement in solubility can be achieved by allowing sufficient rehydration time (overnight) which was performed during the current study. Despite, there was a variability in diameter. A study has suggested that the growth of MC is due to a balance of the crosslinking action of the multi-functional calcium-sensitive caseins and a loop formation. The loop formation involves two dangling phosphoseryl functionalities at the end of extended chains coming to reside on the same nanocluster core, effectively closing the loop and terminating growth. However, since the loop formation is a random event, there is a possibility for the diameters to be random and occur in a range of sizes (Horne, 2006).

In addition to the ultra-purification, there was a variation in the ingredients of the samples with additives such as flavouring agents and artificial sugars added which may have contributed to the varying particle size observed.

Another possibility is that the methods employed in the study were unable to reliably determine particle size. For instance, the prepared solutions were diluted and filtered before DLS scanning which may have allowed and blocked certain larger and smaller particles which may have resulted in variable diameters which is the reason why the DLS scanning was repeated three times. An option to improve the accuracy of the results would be to use filters of different sizes (0.1 and 0.3 µm) and performing DLS after each filtering cycle.

7.8.7. CLSM and SEM

There was inconsistency in the deposition of the dental pellicle observed on the enamel surface with CLSM and SEM imaging. Under CLSM, inconsistent red patches of dental pellicle were observed on the enamel surface. Similarly, inconsistent patches

of dental pellicle were also observed in the SEM images at 200x magnification. The inconsistent patches observed on the enamel surface could be possibly due to the handling of the samples during preparation. Preparing the samples for imaging under both CLSM and SEM is a lengthy process involving numerous steps of immersion in glutaraldehyde, ethanol and HMDS due to which there may be a chance of the dental pellicle to be partially dislodged during immersion and extraction of the samples from the liquid mediums. A similar observation has also been reported in another study which also reported patchy appearance of the pellicles due to preparational damage induced by the experimental and dehydration processes (Meurman and Frank., 1991). An alternative protocol for preparing samples not involving numerous steps of fixation and dehydration may aid in avoiding the chances of the inconsistency in pellicle formation. The resolution of the CLSM lenses may not be capable to capture images of the dental pellicle. So far, there has been no attempts to capture images of the dental pellicle using CLSM, therefore the methods or results cannot be compared to any previous study which would inform on the resolution or staining time to be used to capture images. On the other hand, a consistent pellicle layer has been observed in a study after 30 minutes of pellicle formation at 10000x (Rehage et al., 2017b). Capturing SEM images at higher magnification provide information by focussing on a selected area and do not provide information regarding the whole sample. The images obtained in the current study were at low magnification providing information about the inconsistency of pellicle formation on a larger scale.

Despite the variability, an interesting observation in the SEM and CLSM images was that the dental pellicle was not deposited on the polished surfaces as opposed to the non-polished surfaces (Figures 7.11 A-H). The deposited pellicle extended from the polished enamel surface till the junction between the polished and non-polished surface. The reason for the remarkable finding is a potential area for future research and needs to be identified to inform on protocols for future intervention studies. So far, SEM has been used to investigate the erosion inhibiting properties of the dental pellicle (Brevik et al., 2013, Hannig and Balz, 1999), however the images were captured after removal of the pellicle surface by rinsing with sodium hypochlorite. On the other hand, studies have identified the formation of dental pellicle on polished surfaces (Rehage et al., 2017a, Deimling et al., 2004), however the pellicles were collected in vivo by introducing splints with bovine teeth into the oral cavities of human subjects. There is

continuous replenishment with saliva in vivo that might have resulted in a larger number of proteins being adsorbed to the enamel specimens resulting in a thicker pellicle that could be observed onto the polished enamel surface. The current study used the in vitro time-based incubation method in which the enamel specimens were immersed in centrifuged saliva and were not replenished with fresh saliva over time which possibly would have been a reason for pellicle not being deposited and observed on the polished surface. The reason may be justified as several differences have been found between in vitro and in vivo pellicles, including amino acid composition and electrophoretic/ chromatographic patterns (Siqueira et al., 2012). However, the pellicle being observed to be formed on non-polished surfaces as opposed to polished surfaces raises the question about the possibility of pellicle modifications taking effect not only in the current study but also previous studies attempting to modify the dental pellicle to prevent DE and requires further investigation. These are important areas for research for studies employing in vitro models.

7.8.8. Surface microhardness

The 1% micellar casein group seemed to be the most protective against dental erosion as it showed the least reduction in surface microhardness as compared to the 0.5% and 2% MC groups. However, the pairwise comparison did not display any statistically significant differences in surface microhardness between all the treatment groups. There were statistically significant differences in surface microhardness between the positive control (citric acid) and negative control (deionised water) and 'pellicle without treatment' group. The pairwise comparison results signified that none of the concentrations of MC used in the study were effective in reducing dental erosion. Hypothetically, the most protective group should have been the 2% MC group due to higher concentration encouraging a thicker pellicle to be formed on the enamel surface. The prevention of erosion by increasing the concentration of casein was observed in a study but the casein had been coupled with another protein mucin which increases the lubricative properties of dental pellicle (Sieber et al., 2019). The sample size was calculated post-hoc using data from previous experiment (Seiber et al., 2019) considering $\alpha = 0.05$ and $1-\beta = 0.8$. The effect size (% microhardness) was considered as 5% which was appropriate as the % microhardness measured in the current study

ranged between 2 – 12%. The estimated number of samples was 10 per group with 7 study groups included in the study based on previous studies.

The surface microhardness measurement of the 1% casein group and the no pellicle modification group was similar (-12.5%) demonstrating that there was no preventive effect of casein on the dental pellicle. Similar findings were also reported by the three studies that have attempted to modify the dental pellicle with casein (Cheaib and Lussi, 2011, Seiber et al., 2019, Baumann et al., 2020). The first study (Cheaib and Lussi, 2011) reported no statistically significant difference in microhardness with pellicle being modified with casein alone but reported statistically significant differences when casein was combined with mucin. Similar findings were reported from the remaining two studies, but the studies combined casein with mucin. Therefore, the results from the previous studies demonstrate that casein may only be effective when combined with other proteins such as mucin.

The substantial variation observed in the microhardness values would have been due to the polishing technique applied during the specimen preparation. The specimens were polished by manually holding the specimens resulting in variable pressures being applied during polishing which probably may resulted in varying hard areas of the enamel. The application of the variable pressure during polishing may have resulted in the baseline levels below the normal surface hardness levels of 311-377 Vh (Habelitz et al., 2001) of the 0.5% (267 Vh), 1% (286 Vh) and 2% (251 Vh) MC groups. The baseline hardness levels below the normal threshold levels indicate that the enamel was already softer before erosion and polishing may have exposed the dentine which is known to be softer than enamel. Possibly, the erosion on the 3 groups was performed on dentine rather than the enamel. The other reason would be that during microhardness testing, a different section was measured pre and post immersion which may have coincided with the different hard areas of the enamel.

However, it should be noted that the microhardness was measured on the polished surfaces of enamel where there remains an uncertainty about the pellicle being deposited on the polished enamel surface as observed during CLSM and SEM imaging.

7.8.9. Calcium ion release – ion chromatography

There were no statistically significant differences in calcium concentration in the citric acid between all groups. However, the pellicle group without being treated with MC displayed the minimum amount of calcium release whereas, the positive control group, being unprotected, was expected to exhibit the greatest calcium release. Calcium ion concentration in citric acid is a technique that has been used to measure the amount of calcium from dental hard tissues that dissolves into the citric acid during an erosive challenge (Attin and Wegehaupt, 2014). Hypothetically, the calcium release of the 1% MC was expected to be consistent with the surface microhardness results obtained from the 1% MC group which was also the least out of all concentrations of MC introduced into the dental pellicle. However, the results obtained from calcium release cannot be calibrated with the results obtained from the surface microhardness because the surface microhardness was measured from polished surfaces which may not have pellicles formed as observed by SEM and CLSM. Additionally, calcium concentration was measured from the whole of the enamel samples immersed in citric acid which comprised of both polished and non-polished surfaces which would result in calcium ions being released from a larger surface area.

Similar findings in calcium ion release have been reported in another study which assessed the protective properties of dental pellicle modified with different concentrations of caseins (Sieber et al., 2019). Possibly, pellicles may not have been adsorbed onto the polished enamel surfaces in the study, as was observed in the current study which maybe a reason for similar findings. Another possibility for the findings of calcium release could be that the calcium release values measured originate not only from the dental hard tissue, but also from the pellicle itself or from calcium deposits formed during the incubation time. It should be noted that calcium does not only arise from enamel, but also from the pellicle which has also been reported by another study (Jager et al. 2011) that showed that calcium in the pellicle was released during erosive challenge. The combined release of calcium from the pellicle as well as the enamel surface can falsify the results of calcium release measurements and hence, the results should be interpreted with caution.

7.8.10. Calcium analysis – EDX

Overall, there were no statistically significant differences in the calcium ion content present in the enamel between all groups. No previous study has reported the detection of calcium ions after intervention with MC or casein therefore, the results cannot be compared. However, the studies which aimed to modify the dental pellicle to prevent DE with other compounds such as fluoride and tin, only did not report measurement of calcium ions as outcome measures, but also did not report any changes in tin (Sn^{2+}) ions after acid exposure (Kensche et al., 2019, Rehage et al., 2017).

The highest amount of calcium ions present in the enamel was detected in the positive control group whereas, physiologically, the lowest calcium ion content was expected to be from the positive control group which was unprotected from pellicle or MC against citric acid as it would have released more calcium ions in solution. The negative control group which involved the non-pellicle coated enamel samples being immersed in deionised water reported lower calcium ion levels than the positive control. The inconsistency in the detection of the calcium ions in the enamel of the positive and negative control group raises the question about the methods adopted during the experiment or whether EDX is an accurate tool to detect and measure calcium ions in enamel. The latter possibility can be verified by the fact that studies have reported that the method does not allow specific quantitative evaluation of ions (Kensche et al., 2019). Therefore, based on the results of previous studies and the current studies, EDX may not be a promising tool to be used as a method to assess DE in this model.

7.8.11. QCM-D

This is the first time that the effect of casein and micellar caseins on the structural properties of the dental pellicle has been assessed using QCM-D. There were no statistically significant changes in the thickness and mass of the pellicle detected by the QCM-D. The graphs in figures 7.17 a, b and c displayed a decreasing trend in frequency as the concentration of micellar casein increased which might suggest the oscillations of the crystal to reduce as a result of the increase in mass of the pellicle. However, the results of the current study conflict with the results of other studies where the effects of casein on the ultrastructural properties of the pellicle were observed under transmission electron microscopy (TEM) (Kensche et al., 2020, Kensche et al.,

2019) where a thicker pellicle with casein micelles adsorbed to the pellicle surface was observed. If casein micelles were formed on the pellicle, a thicker pellicle would have been formed onto the crystal. Especially, with the decrease in frequency from -25 to -75 Hz, a significant change in thickness was expected. But studies have managed to quantify the thickness and hydrated mass of pellicle and have observed significant changes with calcium ions (Ash et al., 2013) and polyphenols (Lei et al, 2021). However, the latter study measured and calculated the thickness of the pellicle using scanning probe microscopy (SPM) where the modified pellicle coated crystals were kept under the microscope to measure the thickness, a method which can be adopted in future studies to validate the results obtained from QCM-D.

One of the reasons for the non-statistically significant results in the current study could be that the pellicle formed in the study was from the saliva collected from only a single participant. The studies mentioned above collected saliva from several participants and the results did not depend on one pellicle and could not be compared to the pellicles formed from saliva collected from a number of participants which had an impact on the reliability of the results. The current study was unable to collect saliva from various participants due to ethical issues raised due to the ongoing pandemic (COVID-19). Probably, pellicles formed from saliva collected from several participants would produce more reliable results.

Another reason might be that upon rinsing with deionised water, the density of the pellicles returned to the same level as observed for saliva with no added MC, suggesting that the protein adsorbed was easily removed, leaving a denser and more robust basal layer. The rapid loss of protein from the pellicle on rinsing with deionised water highlights the importance of maintaining the pH and ionic balance of saliva in order to preserve the stability of the pellicle. It is possible that upon introduction of deionised water to the pellicle, ions and proteins, leached out into the water, primarily due to the hypotonicity of the deionised water. This rapid decrease in the ionic concentration of the pellicle would have increased the electrostatic repulsion between anionic moieties within proteins and reduced intermolecular interactions destabilising the pellicle facilitating its displacement, which also maybe a reason for non-significant change and substantial variation in pellicle thickness and mass with concomitant increase in density (Macakova et al., 2010). There was a steady increase in adsorbed

mass as MC concentration increased (T3 period) suggesting that the MC was adsorbing to the surface or pellicle, but the rinsing step with distilled water, removed the protein / pellicle.

Data from the dissipation (red line) might have been helpful to determine the viscoelasticity of the dental pellicle. Dissipation is inversely proportional to the decay time and resonant frequency of the oscillating sensor. The QCMD measures the decay time by stopping the current to the sensor and allowing the sensor to freely oscillate to a standstill. The decrease in the amplitude of the oscillation with time is dependent on the viscoelasticity of the adsorbed layer. The viscous properties of softer films dampen the sensor's frequency of oscillation, and in such cases the Sauerbrey model underestimates the mass, whereupon another method of analysis is needed to fully characterise the film.

A more viscoelastic pellicle has reported to increase the movement of calcium ions within the pellicle aiding in remineralisation of the enamel (Ash et al., 2013). The increase in dissipation after the injection of saliva onto the crystal indicated a more elastic pellicle to be formed (Lei et al., 2021). Dissipation is calculated using the Voigt model; however, the Voight model was not available in the current software used, therefore the viscoelasticity could not be calculated and can be a potential for future work.

7.8.12. Implications of micellar casein against DE

Table 7.10: Summary of the four studies performed with the main findings.

	Aims	Main findings
Experiment 1	PSD by DLS (difference in PSD between casein and MC)	Statistically significant difference in PSD between casein and MC with MC having a larger PSD (p=0.007). PW had the largest PSD of all specimens assessed (p=0.007)
Experiment 2	Investigation of pellicle formation by CLSM and SEM	Inconsistent patches of pellicles observed to be formed on non-polished surfaces as opposed to polished surfaces.
Experiment 3	Erosion experiment	**rSMH:** 1%MC group displayed the least decrease in SMH. No statistically significant difference in rSMH between groups (p=0.12 – 0.91). **Ca^{2+} analysis**: No statistically significant difference in calcium ion concentration in solution (ion chromatography) and % Ca^{2+} (EDX) across all study groups. (p=0.1 – 0.7)
Experiment 4	Structural properties	No statistically significant difference in thickness (p=0.7) as well as hydrated mass (p=1.0). of pellicle after introduction of MC onto the pellicle adsorbed onto the quartz crystal

Key: PSD: Particle size diameter; DSL: Dynamic light scattering; MC: Micellar casein; CLSM: Confocal laser scanning microscope; SEM: Scanning electron microscope; rSMH: relative surface microhardness.

The aim of the study was to investigate and determine the potential effect of micellar casein modified pellicle against DE. Several outcome measures were used to assess the effects and after interpretation of the results obtained from surface microhardness, the pellicles treated with the three concentrations MC managed to reduce DE, however there were no statistically significant differences in rSMH across all groups. The lower decrease in rSMH as compared to the other groups suggests the larger particle size of MC may have been effective in forming a preventive barrier on the tooth surface hence preventing the erosive medium to attack the enamel surface. However, the results obtained from the thickness and mass observed with QCMD suggested no effects of MC on the ultrastructural properties of the pellicle. Also, the fact that dental pellicle was not formed on the polished enamel surface suggests that MC may not have interacted with the dental pellicle and raises the question about the occurrence of pellicle modification with MC. On the other hand, calcium release and EDX did not

report any statistically significant changes after erosion, therefore along with the limitations of the study, the results are inconclusive as yet and needs to be further investigated. Therefore, a conclusion cannot be made as yet on the preventive effects of micellar casein and further research required to gain further insights into the capability of MC to prevent against DE.

7.8.13. Strengths and limitations

Strength of the study included bovine teeth being used as a substitute for human enamel. It has been reported that the chemical composition and physical feature of bovine enamel are similar to those of human teeth (Falla-Sotelo et al., 2005) and therefore might be considered as an acceptable alternative for this phase of research. Also, bovine enamel has been used in demineralization and remineralization experiments because it is easier to obtain and to prepare samples (Attin et al., 2007). However, it should also be noted that using bovine teeth for in vitro studies does not mimic the conditions of human teeth as human teeth possess a layer of fluorapatite which aids in preventing demineralisation. Therefore, bovine teeth are considered to be a limitation for in vitro studies.

Fresh human centrifuged saliva was used to form pellicle. Unpublished data (Smith, 1994) has stated that freezing saliva effected the rheological and adsorption properties of saliva, therefore the pellicles were formed immediately after centrifugation as freezing and storing of saliva was to be avoided.

The variable concentrations of MC were used to investigate the most effective concentration of MC against DE as depending on only one concentration would have been unreliable. The prepared solutions were also used immediately to avoid the MC particles to disperse or suspend in the solution due to storage.

On the other hand, there were a few limitations to the study. Due to the new health and safety regulations regarding the recent pandemic, saliva collection was not possible from participants, which is the reason why saliva was collected from only one participant. Therefore, the study depended on only one saliva and saliva from more participants would have produced more reliable results as saliva obtained from more participants would help in assessing any differences in effects.

Ion chromatography was used to determine the amount of calcium release in the citric acid after erosion. Studies which determined calcium release after erosion used atomic absorption spectrometers coupled with microscopes (Sieber et al., 2019, Niemeyer et al., 2020) or photometric analysis (Hertel et al., 2017, Kensche et al., 2017). Due to the unavailability of the device, the current study could not adopt the technique, therefore the results could not be compared.

The exact method for the preparation of the MC solutions has not been reported. The method adopted in previous studies have reported heating the sample in deionised water at 40°C, pH 11.5 (Baumann et al., 2020, Sieber et al., 2019). However, a study has reported that increasing the pH above 7.0 by the addition of alkali solution affects the internal structure of casein micelles. Further increases in milk pH from the isoelectric pH of protein molecules, gives rise to more negatively charged micelles. The higher negative charges strengthen the repulsive forces of the caseins chain, which in turn, produces loose and expanded structures in the micelles (Sinaga et al., 2016). Therefore, the pH of the solutions for the current study was maintained at 7.0, however, no previous study has prepared casein solutions at 7.0 for pellicle modification, so the methodology for solution preparation is still questionable.

The specimens from 3 groups were polished to result in hardness levels below the normal levels of enamel hardness possible exposing the dentine leading to erosion being performed on dentine rather than enamel of the 3 groups.

The reasons for the inconsistency in the formation of the pellicle layers still remains questionable, although one study also reported the formation of the patchy layers of pellicle due to the procedures adopted for drying and fixation. The inconsistency in pellicle formation may result in areas of exposed enamel hence resulting in the modified pellicle not to be fully functional against DE.

The viscoelasticity of the pellicle modified with MC could not be calculated using the Voigt model due to the software Qtools not possessing the function. A later version of the software may be useful in calculating the viscoelasticity using dissipation curves.

7.8.14. Implications for future research

For future studies, certain modifications to the existing protocols can be adopted and new outcome measures may also be introduced to further investigate the effects of MC on dental pellicle and DE.

Collecting in vivo pellicles may be a better option due to the replenishment by the continuous flow of saliva which may have a larger number of proteins resulting in a thicker pellicle being formed which might also avoid the inconsistency observed in the in vitro pellicle in the current study. The pellicles being formed on non-polished surfaces as opposed to polished surfaces as observed with SEM, suggests that further pellicle modification studies must be performed on non-polished surfaces to calibrate the results with the current study.

To further investigate the effects of MC on dental pellicle, transmission electron microscopy can be suggested to assess, if any, the effects of MC on the thickness of dental pellicle. Moreover, quartz crystal microbalance with dissipation may be used to investigate the effects of MC on the adsorption properties of dental pellicle. Alternative outcome measures such as nanoindentation (Attin, 2014) and time of flight secondary ion mass spectroscopy (TOF-SIMS) (Vickerman, 2001) can be considered for the assessment of early dental erosion.

The interaction of MC with dental pellicle at the molecular level using different protein assays and imaging techniques such as fluorescent resonant imaging is another area of interest which needs to be investigated.

7.9. Conclusions

Micellar casein reduced DE by decreasing the surface microhardness, however, there were no statistically significant differences in SMH between all study groups. There were no statistically significant differences in calcium ion concentration (ion chromatography) and % calcium ion (EDX) between all study groups. However, it should also be kept in mind that pellicles were not formed on some enamel surfaces impairing the ability to draw conclusions about pellicle modification. But, regardless of the adsorption of dental pellicles onto the enamel surfaces, the results obtained from QCM-D indicate that there was no statistically significant effect of micellar casein on

the ultrastructural properties of the dental pellicle, suggesting that pellicle modification may not occur with micellar casein. However, with the substantial variation in the results and the limitations of the experiments mentioned, the effect of micellar casein on DE are inconclusive and further research may be required to confirm micellar casein to be an effective intervention against DE.

8. General Discussion

The preceding chapters in this thesis have presented the results of four pieces of research that were collectively designed to investigate the impact of modifying the dental pellicle to prevent dental erosion in elite athletes.

Chapters 1, 2 and 3 of this thesis have presented the study hypotheses and research questions formulated to address the knowledge gaps identified in the review of the literature. The hypothesis proposed in this thesis was that “modifying the dental pellicle will reduce the incidence of dental erosion not only in athletes but also other high-risk population groups”.

Chapters 4, 5, 6 and 7 presented the research conducted to answer the research question; the findings have contributed to the body of evidence on the association between intense physical training and saliva properties, as well as impact of dental pellicle modification on dental erosion. This chapter will present an overall summary of the thesis then discuss the implications of this research for reducing the incidence of dental erosion and potential for future research.

8.1. Key findings

The following section presents a summary of the key findings from Chapters 4, 5, 6 and 7 in relation to the research questions and study hypotheses.

8.1.1. An in vitro model to detect early dental erosion lesions with OCT

The first research question was “can early dental erosion lesions be detected using optical coherence tomography (OCT)?”.

The aim of this study was to develop an in vitro dental erosion model which would be capable of detecting early erosive lesions using OCT with and without the presence of dental pellicle. The purpose of the model was subsequently to apply it for interventions into the dental pellicle in order to reduce dental erosion. The model used bovine incisors onto which erosive lesions were formed using citric acid as the erosive medium and the lesions were assessed using B-scans obtained from the OCT. The lesions were quantified as enamel surface loss by measuring the thickness of enamel

from the enamel surface to the dentin enamel junction to calculate enamel surface loss.

The study concluded that OCT was not capable of detecting and quantifying early erosive lesions. However, it was capable of detecting advanced erosive lesions with surface loss after four hours of constant acid exposure. Nevertheless, keeping in mind that the oral cavity is not exposed to acid for four hours constantly, OCT does not appear to be a promising tool to detect early erosive lesions and was not used for the remaining part of the project.

8.1.2. The effects of intensive physical training on saliva.

The second research question was “is there an impact of intense physical exercise on the saliva parameters?”. The original plan for the project was to collect saliva samples from participants involved in intense physical training via cardiopulmonary exercise testing (CPET) and assessing the effects of the training on saliva parameters (saliva flow, salivary proteins). However, due to the occurrence of the pandemic (COVID-19), the original plan of the project had to be replaced by a systematic review and meta-analysis.

The aim of the systematic review and meta-analysis was to assess the impacts of intense physical training on the saliva parameters and to assess any association of the changes in saliva to dental erosion. This was the first systematic review that reported association of saliva parameters to intense physical activity, supported by a thorough literature search, a priori defined protocol, and following recommended guidelines for data reporting. Studies included a variety of participants ranging from above college level athletes involved in both intermittent and endurance sports and physical activities, as well as military personal.

The results obtained from the meta-analysis of 15 included studies confirmed no statistically significant association of intense physical activity to changes in saliva flow (mean difference of -0.01 ml/min (95% CI -0.13, 0.11) (p=0.88) with high heterogeneity (I2= 59%); salivary IgA (mean difference of -19.43 µg/ml (95% CI -45.23, 6.67) with high heterogeneity (I2= 98%)); saliva total protein (mean difference of -596.81 µg/ml

(95% CI -1718.0, 524.38) with high heterogeneity (I^2= 99%)); salivary α-amylase (mean difference of -12.68 µg/ml (95% CI -3.40, 28.76) and high heterogeneity (I2= 94%)); and salivary mucins (mean difference of -0.73 U/ml (95% CI -1.12, 2.58) with high heterogeneity (I2= 82%) (p=0.44).

Overall, the heterogeneity was extremely high in all studies despite performing subgroup and sensitivity analysis, for which GRADE was performed to assess the certainty of the evidence which suggested a very low certainty in the evidence in all outcomes. The ratings were downgraded due to moderate risk of bias, extremely high heterogeneity, lack of follow-ups and small sample sizes. Due to the low certainty of the evidence obtained following GRADE, it is likely that further research will markedly change the estimated effect. Therefore, the most appropriate conclusion would appear to be that the evidence neither supports nor refutes the long held belief of the association between intense physical activity and changes in saliva. This means other potential causes of dental erosion in athletes must also be considered. The frequent intake of isotonic sports beverages may be a factor; however, recent systematic reviews have reported no association of sports drinks with dental erosion in the athlete population (de Queiroz Gonçalves et al., 2020, Sirimaharaj et al., 2002, Antunes et al., 2017). But in vitro studies have confirmed association of sports drinks to dental erosion non-athlete populations (Moazzez et al., 2000, Sovik et., 2015, Ehlen et., 2008). Crucially, these in vitro studies do not mimic the conditions of the oral cavity (saliva clearance, buffering capacity, pellicle formation, pattern of consumption), therefore, further research is required to identify the causes for the high prevalence of DE in athletes, and then decide on preventive measures.

8.1.3. The impact of dental pellicle modification on dental erosion

The third research question was “is there an impact of modifying the dental pellicle on the magnitude of dental erosion?”. The systematic review was performed due to the inaccessibility of the laboratories because of the prolonging situation of the pandemic. The aim of the systematic review was to assess the effects of the dental pellicle modification on the magnitude of dental erosion and to identify the most promising interventions for further research. This was the first systematic review that reported the impacts of modifying the dental pellicle on dental erosion, supported by a thorough

literature search, a priori defined protocol, and following recommended guidelines for data reporting.

The findings of the systematic review identified six compounds used to modify the dental pellicle: polyphenols, casein/mucin compounds, vegetable oils, plant extracts, ionic compounds and engineering peptides. There was a variation in the effects of the intervention compounds on DE with some studies reporting statistically significant (green and black tea, red wine) and some reporting non-significant effects (edible oils and casein-mucin compounds) on dental erosion. Moreover, seven out of the thirteen included studies reported changes in pellicle structure and composition following intervention. However, the major limitation of the systematic review was the absence of a meta-analysis due to lack of missing quantitative data. Additionally, none of the included studies confirmed the formation and adsorption of the dental pellicle using imaging techniques before intervention raising questions about the possibility of pellicle modification occurring.

The majority of the studies were performed on polyphenols followed by casein which supported the potential of these two compounds to act against DE. The polyphenol with best interaction with the salivary pellicle was EGCG (from green tea). Erosion was reduced best with casein and mucin combined together as mucins are large molecular proteins which have the ability to replace the smaller weight molecular proteins in the pellicle and form a thicker layer. Moreover, the in vitro investigations of the proteomic profile of the dental pellicle suggested that fluoride application promoted the adsorption of specific proteins hence increasing the thickness of the dental pellicle. Lastly, palm oil reported to be the most effective in reducing DE as compared to all other edible oils included in the studies.

Overall, all interventions in the included studies were reported to offer a degree of protection against DE indicating that modification of dental pellicle is possible; however due to the limited statistical data, absence of a meta-analysis and lack of confirmation of pellicle formation, a conclusion cannot be currently made about the effectiveness of all interventions on dental erosion. But with the frequency of studies performed on polyphenols and casein/mucin compounds, the two elements may be of interest to further investigate their effectiveness against DE.

8.1.4. Intervention of micellar casein into the dental pellicle to prevent DE

The fourth and the last research question was “will modifying the dental pellicle with micellar casein prevent or reduce the incidence of dental erosion?”. The study was the most crucial and important study of the PhD and involved several experiments commencing from assessing the difference in particle size distribution between casein and micellar casein using dynamic light scattering (DLS); detecting the formation of pellicle layer on the tooth surface using confocal laser fluorescence microscope; identifying the ability of micellar casein to protect against dental erosion and lastly; determining the effect of micellar casein on the viscoelasticity and thickness of dental pellicle using quartz crystal microbalance with dissipation (QCM-D).

The first experiment was to assess the difference in particle size distribution between casein and micellar casein using dynamic light scattering (DLS) where of micellar casein reported a larger particle size diameter of 201.7±4.5 nm as compared to casein (104.1±5.9 nm). The results of the experiment supported the hypothesis which stated micellar casein to have a larger particle size diameter than casein and provided a level of quality assurance that some products did indeed contain the micellar form of casein.

The second experiment was to detect and confirm the formation of the dental pellicle on the enamel surface which was performed primarily by confocal laser fluorescence microscope and the images calibrated by scanning electron microscope. The experiment was performed as all studies included in the systematic review (presented in chapter 6) did not involve any imaging techniques to confirm the formation of dental pellicle on the enamel surfaces before interventions. It was remarkable to observe dental pellicles not being adsorbed to the polished surfaces as opposed to non-polished surfaces. Possibly, polishing the enamel surfaces could have reduced the retentive properties of the enamel surface due to which the pellicle may not have adsorbed onto the surface. However, no study has been performed to confirm the possibility and this creates a window for further research into the subject of pellicle adsorption on polished enamel surfaces. Therefore, the findings from the current study raise a question on the possibility of pellicle modifications being performed in the previous studies as the intervention studies on pellicle modifications have been performed on polished enamel surfaces. Therefore, it is suggested to adopt imaging

techniques to confirm the adsorption of dental pellicles on both polished and non-polished enamel surfaces before introducing the interventions and then compare the effects of pellicle modifications from both surfaces.

The third experiment was to identify the ability of micellar casein to protect against dental erosion using three concentrations of micellar casein solutions. The dental pellicles were formed on the enamel surfaces and then the pellicle adsorbed enamel samples were incubated in each of the solutions and then exposed to the erosive medium. The outcome measures used to assess dental erosion were surface microhardness, calcium ion release from the enamel surface into the erosive medium, and calcium ion content present in the enamel using EDX. The 1% MC solution reported to be the most effective in preventing DE due to the least decrease in microhardness (2.0% decrease), however there were no statistically significant differences in microhardness across all groups except for the positive (citric acid only) and negative control (deionised water) group. Additionally, there were no statistically significant differences in calcium ion concentration in citric acid from all samples, and % calcium ion content in all enamel samples before and after acid exposure. The results indicated that MC might not be an effective intervention against DE.

The fourth and last experiment was determining the effect of micellar casein on the viscoelasticity and thickness of dental pellicle using quartz crystal microbalance with dissipation (QCM-D). No statistically significant change in the pellicle thickness (p=0.4-1.0) or hydrated mass (p=0.66-1.0) was seen after introduction of all three concentrations of micellar casein on the dental pellicle adsorbed crystals.

Overall, micellar casein might have reduced DE by decreasing the surface microhardness, however, the prevention was reported from only one outcome measure. The results obtained from calcium ion release and calcium ion content make the results inconclusive. However, it should also be kept in mind that pellicles were not formed on the polished enamel surfaces and therefore the effect might be independent of possible pellicle modification. The fact that DE was reduced by MC in the absence of pellicle creates an opportunity to investigate the interactions, if any, of MC directly on hydroxyapatite surfaces. But, regardless of the adsorption of dental pellicles onto the enamel surfaces, the results obtained from QCM-D indicate that there was no

statistically significant effect of micellar casein on the ultrastructural properties of the dental pellicle, suggesting that pellicle modification may not occur with micellar casein. The results from the above experiments currently indicate that micellar casein may not be an effective intervention, however with the limitations of the experiments and the substantial variation observed in the results, further research may be required to confirm the findings and come to a conclusive result.

8.2. Strengths of the PhD

The key strength of the PhD was the planned outline designed for the respective studies to be performed throughout the PhD which aided in designing protocols for each study. The easy access to the supervisors and statisticians further added to the efficiency of the methodology and results of the experiments. The previous studies performed on pellicle modification mentioned in the literature also acted as guidance to adopt similar protocols for the studies to be performed for the PhD.

The studies adopted a variety of outcome measures from assessing particle size diameter to dental erosion which included (DLS, OCT, XMT, rSMH, ICP, CLSM, SEM, EDX and QCM-D) which was beneficial in validation and calibration of the results to confirm the findings. The fact that the study adopted more outcome measures than any of the previous studies added to the strength of the PhD.

The systematic review (chapter 5) was the first systematic review performed on the impact of intense physical training. The findings challenge some views on the effect of intense physical activity on saliva and its potential relevance to DE in athletes. All included studies involved saliva being assessed from participants undergoing intense physical training (sports, military training, endurance training).

Similarly, the second systematic review (chapter 6) was also the first systematic review performed on the impact of dental pellicle modification on dental erosion. Therefore, the PhD comprised of two systematic reviews contributing to the strength of the evidence which is recognised as important in terms of the evidence hierarchy pyramid (Murad et al., 2016).

The micellar casein compounds chosen were readily available from manufacturers based in the United Kingdom and therefore potentially relevant to future use. They were purchased and delivered directly to the institute within two days. The micellar casein compounds were usual ingredients consumed by athletes and physical trainers on a daily basis so there was no concern of health and safety during in vitro laboratory work. The assessment of the four different brands was beneficial as relying on only one brand would have produced unreliable results and would have required further calibration.

The detection and the confirmation of the formation of dental pellicle on the enamel surface was a surprising finding and of significance for researchers attempting to modify the dental pellicle on enamel surfaces. The fact that the dental pellicle was observed to be formed on non-polished surfaces as opposed to polished surfaces may doubt the possibility of pellicle modifications occurring in the previous studies in the literature and opens the window for further research with changes in protocols.

Fresh human centrifuged saliva was used to form pellicle on the enamel surfaces. Storage of saliva by freezing at -80 ^{o}C was avoided in order to mimic almost similar conditions of the oral cavity. Freezing the saliva has reported to hinder the adsorption properties of the pellicle (Francis et al., 2000), therefore in all studies, fresh saliva was preferred and used for all the experiments. Pellicle formation was carried out by time-based incubation for 24 hours in fresh saliva at 37^{o}C to ensure maximum thickness of pellicles is achieved as reported in previous studies (Hannig and Joiner, 2006).

Although the results obtained from the meta-analysis (chapter 5) and the intervention study (chapter 7) were statistically non-significant, they are potentially important findings to guide future research into the areas of salivary pellicle and dental erosion.

8.3. Limitations of the PhD

The major limitation experienced during the PhD was the influx of the pandemic (COVID-19) due to which saliva collection from participants was not possible both for assessment and intervention purposes. Therefore, all experiments were performed on saliva from one participant and the results could not be further calibrated and compared with saliva obtained from various participants. Bovine teeth used for the in

vitro studies do not mimic the conditions of human teeth as human teeth possess a layer of fluorapatite which aids in preventing demineralisation. Therefore, using bovine teeth for the in vitro studies added to the limitation of the PhD. The original plan of the PhD was to collect saliva samples from participants involved in IPT and then assessing the saliva for any impacts on their parameters. However, due to the pandemic the plan/project and design of the PhD had to be changed. The other major limitation experienced was the shifting of the Eastman campus to another site which caused further delays to the initiation of the experiments. Another limitation experienced was a health issue encountered by the PhD candidate where he was admitted to hospital and then was on a four-week bed rest. Collectively, the circumstances led to a delay in commencement of the intervention study and hence, led to a limited timescale.

8.4. Summary of PhD

The aim of the PhD was to modify the dental pellicle to reduce the incidence of dental erosion in the elite athlete population. Evidence has reported high prevalence of dental erosion in athletes (Gallagher et al., 2018) with the intake of sports drinks being one of the reasons behind the disease (Nijakowski et al., 2022). However, association of sports drinks with dental erosion is far from clear (de Queiroz Gonçalves et al., 2020, Sirimaharaj et al., 2002, Antunes et al., 2017) although the link has been reported in non-athlete populations (Moazzez et al., 2000, Sovik et., 2015, Ehlen et., 2008, Chan et al., 2020). On the other hand, in vitro studies performed to assess the impact of sports drinks on dental erosion have reported demineralisation of the enamel after periods of exposure to the sports drinks (Cairns et al., 2002). Therefore, other reasons were investigated which included salivary parameters for which the systematic review was conducted to investigate the impact of IPT on saliva. The meta-analysis reported no association between IPT and saliva which changed the long-held wisdom of changes in saliva parameters occurring as a result of IPT. But due to the extremely high heterogeneity and low certainty of evidence, a conclusion could not be refuted on the impact of IPT on saliva. Therefore, the other approach considered to prevent DE was the modification of the dental pellicle.

But before the pellicle was attempted to be modified, a study was conducted via a systematic review to investigate the effects, if any, of pellicle modification on DE. All included intervention studies reported to reduce dental erosion, but first of all, the

absence of a meta-analysis due to lack of statistical and quantitative data could not confirm the results. Secondly, the other limitation observed in all studies was the absence of techniques to confirm the formation of the dental pellicle on the polished enamel surfaces which raised questions on the occurrence of pellicle modification occurring in the intervention studies. The confirmation of dental pellicle on the polished surfaces of enamel for intervention is an important step to consider for intervention studies and was one of the objectives of the intervention study (chapter 7) which was performed using confocal microscopy and SEM. It was potentially an interesting finding to observe pellicles being adsorbed to non-polished enamel surfaces as opposed to polished surfaces, as all pellicle modification studies performed previously aimed to form pellicles on polished surfaces without using any visual aids to confirm the adsorption on the enamel surface. Therefore, the observation not only raised the question on the reliability of the results obtained from the intervention studies included in the systematic review, but also created opportunities for further insights and modifying protocols for future intervention studies.

Based on the results from the systematic review on pellicle modification, the intervention study was performed where the dental pellicle was attempted to be modified using micellar casein. The protocol applied for the intervention study was slightly different than the protocols applied in the included studies of the systematic review. The modified protocol included investigation of pellicle formation and assessment of ultrastructural properties of the dental pellicle. Micellar casein reported to have a larger particle size than pure casein via DLS and was hypothesised to have better preventive potential than pure casein by forming a thicker pellicle layer, hence preventing the dissolution of calcium ions from the enamel. Initially, OCT was considered to be used as the outcome measure to assess early dental erosion, but the in vitro erosion model did not prove to be successful in detecting early erosive lesions and was not used for the intervention study. Alternate outcome measures were used which included surface microhardness, ion chromatography and EDX. The 1% MC group initially reported maximum protection against DE via surface microhardness, however, was not true when later compared across other groups. Similarly, the results obtained from the calcium ions reported statistically non-significant changes when compared to the positive control. The findings raised the question on the mechanics of MC and the ultrastructural properties of the dental

pellicle. To answer the questions, QCM-D was used which also reported non-statistically significant effects on the pellicle hydrated mass as well as the thickness. However, since the pellicle modification and the studies to assess the ultrastructural properties were performed from pellicles obtained from the saliva of only one individual, further work may be needed which includes saliva being obtained from more than one participant as saliva obtained from more participants would help in assessing any differences in effects. Therefore, within the limitations of the study, the results of modifying the dental pellicle with MC to prevent DE were inconclusive and requires further research.

8.5. Implications for future research

The results obtained from the experiments performed during the PhD has created opportunities for future research. The following section will discuss the limitations and suggest opportunities for future research projects.

8.5.1. Detection of early erosive lesions

Early erosive lesions were not detected by the B-scan images obtained by OCT. On the other hand, previous studies have managed to detect early erosive lesions with OCT, but the studies used the magnitude of surface reflection intensities (SRI) obtained during OCT scanning to detect and measure the erosive lesions. However, for clinical aspects, SRI may not be practically possible as it would require quantification formulas and techniques to quantify the erosive lesions which requires expertise. Therefore, in addition to OCT, other methods should be investigated to detect and quantify early dental erosion which may include nanoindentation and profilometry to calibrate the results.

8.5.2. Impact of IPT on saliva

Alternate causes of dental erosion other than saliva must be considered. It was hypothesised that based on the salivary physiology, there would be a significant impact of ITP on saliva parameters, however the results of the meta-analysis, although statistically non-significant, were inconclusive due to the high heterogeneity and low certainty of evidence. An approach that might help explore the existing data is to conduct individual participant data meta-analysis as this might aid in exploring the impact of individual differences versus having to use the group mean data. To further

explore the effects of ITP on saliva, studies must focus specifically on saliva collection methods and collection times. Similar intensities of physical activity must also be considered to minimise exposure bias and would further enhance the reliability of the saliva outcomes obtained after physical activity. Further research must also focus on salivary proteins specific for enamel homeostasis (statherins, histatins) in order to probably have a better understanding and reasoning behind increased incidence in dental erosion and hence decide on preventive interventions if required. The limited number of studies assessing mucin levels also provides a platform to perform in depth research on the science of MUC5B during intense physical training for future. Last but not least, a significant element to consider is the properties of the dental pellicle during intense physical activities.

8.5.3. Impact of dental pellicle modifications on dental erosion

The absence of a meta-analysis in the systematic review was the major drawback of the review due to which a conclusion could not be deduced on the impacts of modifying the dental pellicle on dental erosion. Although numerous attempts to contact the authors were made, the outcome and statistical data quantifying the magnitude of erosion (rSMH, Ca^{2+} ion) after intervention could not be extracted for a meta-analysis to be performed. Therefore, extraction of quantitative data would aid in a meta-analysis to be performed in the future. Authors should emphasise on publishing the quantification data in future studies in order for a meta-analysis to be performed.

8.5.4. Intervention

For future intervention studies, collecting in vivo pellicles may be a better option due to the replenishment by the continuous flow of saliva which may have a larger number of proteins resulting in a thicker pellicle being formed which might also avoid the inconsistency observed in the in vitro pellicle in the current study. The pellicles being formed on non-polished surfaces as opposed to polished surfaces as observed with SEM, suggests that further pellicle modification studies must be performed on non-polished surfaces to calibrate the results with the current study.

To further investigate the effects of MC on dental pellicle, transmission electron microscope can be suggested to assess, if any, the effects of MC on the thickness of dental pellicle. Moreover, quartz crystal microbalance with dissipation may be used to

investigate the effects of MC on the adsorption properties of dental pellicle. Alternative outcome measures such as nanoindentation (Attin, 2014) and time of flight secondary ion mass spectroscopy (TOF-SIMS) (Vickerman, 2001) can be considered for the assessment of early dental erosion. The interaction of MC with dental pellicle at the molecular level using different protein assays and imaging techniques such as fluorescent resonant imaging is another area of interest which needs to be investigated.

In addition, the surprisingly finding of the pellicle not being adsorbed onto the polished enamel surface creates an important question to investigate in order to understand the reliability of the modification studies performed in the past. A confirmation of the findings of pellicles not being adsorbed onto polished surfaces may aid in diverting the attention of researchers to form pellicles on natural enamel surfaces to carry out intervention studies.

8.6. Conclusions

Erosive tooth wear is common in elite athletes. However, the cause of the high incidence of dental erosion in the population group is still unclear and further research is required to investigate the causes.

Imaging with optical coherence tomography does not appear to be a promising tool for detecting and quantifying early erosive lesions. Additional imaging techniques should be investigated to supplement or supplant current methods assessing early dental erosive lesions which can be used for both in vitro as well and in vivo studies aimed to modify the dental pellicle.

The results of the meta-analysis on the intense physical activity on the qualitative and quantitative parameters of saliva were inconclusive due to the very low certainty of the evidence. Therefore, it is too early to support and refute an association between intense physical activity and saliva parameters. Further research should focus on investigating their interrelationship.

Micellar casein reduced DE however the results are still inconclusive. Dental pellicles were not formed on the enamel surfaces due to which pellicle modification may not

have occurred. But, regardless of the adsorption of dental pellicles onto the enamel surfaces, the results obtained from QCM-D indicate that there was no statistically significant effect of micellar casein on the ultrastructural properties of the dental pellicle, suggesting that pellicle modification may not occur with micellar casein. The results from the above experiments currently indicate that micellar casein may not be an effective intervention, however with the limitations of the experiments mentioned, further research may be required to confirm the findings and come to a conclusive result.

Positive findings produced by addressing and removing the limitations would aid in preventing DE not only in the athlete population but also other high-risk population groups.

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Appendix A – PRISMA checklist chapter 5

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 68
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Page 68-70
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 71
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Page 74
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Page 73
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Page 72
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Page 75
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Page 76
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Page 75
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 77
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Page 77
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Page 77
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Page 77
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Page 77

Section and Topic	Item #	Checklist item	Location where item is reported
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Page 77
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Page 77
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Page 77
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Page 77
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Page 77
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Page 79
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Page 79
Study characteristics	17	Cite each included study and present its characteristics.	Page 79-96
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Page 97
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	99-113
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	103,108,113
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Page 116
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Page 117
	23b	Discuss any limitations of the evidence included in the review.	Page 118
	23c	Discuss any limitations of the review processes used.	Page 118
	23d	Discuss implications of the results for practice, policy, and future research.	Page 124

Section and Topic	Item #	Checklist item	Location where item is reported
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Page 71
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Page 71
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Page 71
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	
Competing interests	26	Declare any competing interests of review authors.	
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

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Appendix B – PRISMA checklist chapter 6

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 126
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	N/A
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	126-127
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 128
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Page 130
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Page 128
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Page 128
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Page 132
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Page 132
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Page 133
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 133
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Page 133
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Page 133
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Page 133
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Page 133

Section and Topic	Item #	Checklist item	Location where item is reported
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	N/A
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	N/A
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Page 133
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Page 134
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Page 79
Study characteristics	17	Cite each included study and present its characteristics.	136-153
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Page 135
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Page 154
	23b	Discuss any limitations of the evidence included in the review.	Page 155
	23c	Discuss any limitations of the review processes used.	Page 156
	23d	Discuss implications of the results for practice, policy, and future research.	Page 164

Section and Topic	Item #	Checklist item	Location where item is reported
OTHER INFORMATION			
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Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	

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