Effect of acid and alkali formation on pH in the dental biofilm with reference to caries

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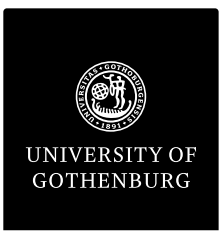

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CONTENT

ABSTRACT

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Dental caries is a common multifactorial disease where a frequently low pH in the dental biofilm (dental plaque) plays an important role for caries occurrence and progress. The plaque-pH is lowered by acid formation from sugars by biofilm bacteria that also help to restore the pH by alkali formation from urea and the amino acid arginine. Despite the importance of pH for caries to occur, *in vivo* studies comparing the dental plaque-pH after acid and alkali formation are scarce. This may be due to the lack of methods that easily can be applied in the clinic.

In this thesis, the dental plaque-pH after acid and alkali formation *in vivo* in relation to the individual caries status were examined. Two chair-side methods were also developed; the 'strip method' to measure *in situ* the interproximal, supragingival plaque-pH (Study I) and a microtiter plate format of RUT (Rapid Urease Test) to grade bacterial urease activity *in vitro* and *ex vivo* (Study III). Hopefully studies on pH and alkali formation in the dental plaque can contribute to finding measures for assessment of the individual caries risk.

Studies on plaque-pH after sugar and urea challenges, pre- and post-adaptation periods to respectively acid and alkali formation from 1-week daily rinses with sucrose and urea, were examined in caries-free and caries-active individuals (Study II). Furthermore, the number of acid tolerant bacteria was examined as well as the ability of isolated acid tolerant bacteria to form acid from sugars and sugar alcohols *in vitro* (Study II, V). The pH response to a sugar challenge after 6-week usage of fluoride toothpaste with arginine was also examined in relation to caries (Study IV).

Similar plaque-pH values and Stephan curves were obtained using the 'strip method' and the well-known 'microtouch method' before and up to 60 min following a sugar challenge (Study I). RUT showed a strong *in vitro* urease activity for the well-known urease active *Helicobacter pylori* and for strains of *Haemophilus parainfluenzae* but not for the more common plaque bacteria *Actinomyces spp.* and *Streptococcus mitis* (Study III). A higher urease activity in plaque at sites in the lower front compared to plaque at other sites was found as well (Study III, IV). Adaptation to acid formation resulted in lower plaque-pH after a sugar challenge and an increased number of acid tolerant bacteria in caries-free (CF) individuals (Study II). Adaptation to alkali formation resulted in somewhat higher pH values after a urea challenge in caries-active (CA) individuals (Study II). In concordance, acid formation was numerically increased in bacteria isolated from the CF group after acid adaptation and decreased in isolates from the CA group after alkali adaptation (Study V). In CA but not CF individuals, the usage of fluoride toothpaste with arginine resulted in increased plaque pH-values as well as increased saliva buffer capacity and pH (Study IV).

It can be concluded that the 'strip method' and 'RUT' are applicable as chair-side methods, for the assessment of plaque acidogenicity and urease activity, respectively. Adaptation to sugar increased the acid formation and decreased the pH in the dental plaque in caries-free individuals. Adaptation to urea and arginine decreased the acid formation and increased pH in the dental biofilm in caries-active individuals

Keywords: acid, alkali, biofilm, caries, pH, supragingival plaque

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SAMMANFATTNING PÅ SVENSKA

Karies är en multifaktoriell sjukdom där lågt pH i den dentala biofilmen (dentalt plack) spelar en stor roll för karies förekomst och utveckling. Plack-pH sänks då bakterier som bygger upp placket bildar syra från socker och höjs av plackbakteriers bildning av ammoniak (alkali) från urea och aminosyran arginin. Trots betydelsen av pH för karies finns det idag få *in vivo* studier som jämför plack-pH efter bildning av syra och alkali vilket kan bero på avsaknaden av enkla metoder för att göra detta.

I denna avhandling har pH i plack på tandytan före och efter bildning av syra respektive alkali undersökts *in vivo* i relation till individens karies-status. Vidare har två chair-side metoder utvecklats; 'stripmetoden', för att mäta pH i supragingivalt plack *in situ* (Studie I) och 'RUT' (Rapid Urease Test) för att utvärdera plackets ureasaktivitet *in vitro* och *ex vivo* (Studie III). Förhoppningen är att studier på pH och alkalibildning i plack kan bidra till att finna metoder för skattning av individens kariesrisk vilket idag saknas.

Plack-pH *in situ* före och efter sköljning med en lösning av socker respektive urea undersöktes på kariesfria och kariesaktiva individer. Det utfördes före och efter adaptation till bildning av syra och alkali genom 1 veckas dagliga sköljningar med lösningar av socker respektive urea (Study II). Förekomsten av syratåliga bakterier undersöktes även liksom deras förmåga att bilda syra från socker och sockeralkoholer *in vitro* (Studie V). Effekten av att använda en fluortandkräm med arginin på plack-pH före och efter sköljning med sockerlösning undersöktes också i relation till individens kariesstatus (Studie IV).

Resultaten visade att mätning av plack-pH med 'stripmetoden' och den etablerade 'microtouch metoden' gav samstämmiga pH-värden och Stephan-kurvor då pH mättes före och upp till 60 min efter sköljning med sockerlösning (Studie I). En stark ureasaktivitet sågs med RUT *in vitro* för välkända, urease-positiva *Helicobacter pylori* liksom för stammar av *Haemophilus parainfluenzae* men inte för de vanliga plackbakterierna *Actinomyces spp*. and *Streptococcus mitis* (Study III). Högre ureasaktivitet i plack på tänder i underkäksfronten jämfört med plack på andra ställen i munnen påvisades (Studie III, IV). Adaptation till syra resulterade i lägre plack-pH före och efter sköljning med sockerlösning och till ökat antal syratåliga bakterier hos kariesfria (CF) individer (Studie II). Adaptation till alkali resulterade i numeriskt förhöjda pH-värden i plack efter sköljning med urealösning hos kariesaktiva (CA) individer (Studie II). Detta är i överensstämmelse med en numeriskt ökad syrabildning hos bakterier isolerade från CF efter adaptation till syra och en minskad syrabildning hos isolat från CA efter adaptation till alkali (Studie V). Användning av tandkräm med arginin resulterade i högre plack-pH liksom högre pH och buffertkapacitet i saliv hos CA men inte CF (Studie IV).

Sammanfattningsvis visar studierna att 'stripmetoden' och 'RUT' fungerar väl som chair-side metoder för bedömning av det dentala plackets syrabildande förmåga och ureasaktivitet. Adaptation till socker resulterade i ökad syrabildning och lägre pH i den dentala biofilmen hos kariesfria individer. Adaptation till alkali från urea och arginin resulterade i mindre syrabildning och högre pH i den dentala biofilmen hos kariesaktiva individer.

PREFACE

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Carlén A, Hassan H, Lingström P.
The 'strip method': a simple method for plaque pH assessment.
Caries Res. 2010;44:341-344.

II. Hassan H, Lingström P, Carlén A.
Plaque pH in caries-free and caries-active young individuals before and after frequent rinses with sucrose and urea solution.
Caries Res. 2015;4:18-25.

III. Dahlén G, Hassan H, Blomqvist S, Carlén A.
Rapid urease test (RUT) for evaluation of urease activity in oral bacteria *in vitro* and in supragingival dental plaque *ex vivo*.
BMC Oral Health. 2018;18:89-95.

IV. Hassan H, Ghali L, Wildeboer D, Sarwar S, Lingström P, Carlén A.
Interproximal in situ plaque pH in relation to caries before and after short-term use of 1.5% arginine toothpaste.
In manuscript.

V. Hassan H, Bjondahl F, Olofsson R, Dahlén G, Carlén A.
Acid formation of supragingival dental biofilm bacteria isolated from caries-free and caries-active individuals - an *in vitro* study.
In manuscript.

LIST OF ABBREVIATIONS AND DEFINITIONS

AA = After using fluoride toothpaste with 1.5% arginine
AB = After using fluoride toothpaste
AAA = After Acid Adaptation
ABA = After Base (alkali) Adaptation
Acidogenicity = To produce/generate acid
Aciduric = To tolerate acidic conditions/environment
ADS = Arginine Dihydrolyse System
$AOC_{7.0}$ = Area Over the Curve, over the neutral pH
ATCC = American Type Culture Collection
ATR = Acid Tolerance Response
AUC = Area Under the Curve, where pH is plotted against time (pH units multiple time)
$AUC_{5.7}$ = Area Under the Curve, below the critical pH of enamel
$AUC_{6.2}$ = Area Under the Curve, below the critical pH of dentin
BA = Before using fluoride toothpaste with 1.5% arginine
BAA = Before Acid Adaptation
Bacterial isolate = Bacteria collected from a specific sample i.e. supragingival plaque
Bacterial species = Collection of similar and related bacteria
Bacterial strain = Individual genetic variant or subtype or clone of bacteria
Baseline pH = Resting supragingival plaque-pH before an acid/alkali challenge
BB = Before using fluoride toothpaste
BBA = Before Base (alkali) Adaptation
CA = Caries-Active
Caries activity = New and recurrent caries lesions over a specific period of time
CCUG = Culture Collection University of Gothenburg
CF = Caries-Free
CFU = Number of Colony-Forming Units
Critical pH = The pH value when the oral fluid is saturated with a particular mineral such as tooth enamel
DMFS = Decayed, Missed and Filled Surfaces
DMFT = Decayed, Missed and Filled Teeth
Dm+iMFS = Decayed, Missed and Filled Surfaces, both manifest and initial caries
DmMFS = Decayed (manifest), Missed and Filled Surfaces
Double-blinded = Blind for the participants and the observer
ex vivo = Outside the living organism with minimal alteration of natural condition
Final pH = The last pH value measured after a sugar/urea challenge
in situ = In the original living location
in vitro = Outside the living organism and within a cultured and controlled system
in vivo = Within the living organism
LB = Lactobacilli
Max pH drop/fall = The difference between baseline-pH and minimum pH after a sugar challenge
Min pH = Minimum pH-value after a sugar challenge
MS = Mutans Streptococci
MT = Microtouch method
NCTC = National Collection of Type Culture

OMGS = Oral Microbiology Gothenburg Sweden
PBS = Phophate-Buffered Saline
PTC = Professional Tooth Cleaning
Quorum-sensing = A bacterial cell–cell communication signal system
RUT = Rapid Urease Test
Single-blinded = Blind for the participants
ST = Strip method

INTRODUCTION

Dental caries - a multifactorial biofilm-induced disease

Presently and in the previous century, different methods, diagnostic tools and strategical procedures have been used for prevention and treatment of dental caries. Despite this, dental caries still represents one of the leading oral diseases (Bowen, 2002; Marthaler, 2004; Taubman and Nash, 2006; Hugoson et al., 2008; SBU, 2008; Marcenes et al., 2013; Lagerweij and van Loveren, 2015; Jin et al., 2016). The wide knowledge of dental caries aetiology and cause, as well as the huge efforts undergone to treat it, has played a significant role to sculpture a clear understanding in order to investigate and to deal with this disease.

The concept of dental caries has been adapted dramatically according to the basis of plaque hypotheses (Loesche, 1975; Loesche, 1976; Theilade, 1989; Marsh, 1994; Hajishengallis et al, 2012; Rosier et al., 2014; Takahashi and Nyvad, 2016). Based on the biofilm-induced concept, dental caries is defined as a multifactorial disease caused by a complex aetiology and is a result of the net outcome of dynamic interactions in the dental biofilm between three elements: microorganisms, the host and the diet (Featherstone, 2004; Fejerskov, 2004; Bowen et al., 2018). This net effect is translated to a negative disturbance of the equilibrium of the dental biofilm, allowing dental caries to occur. According to this definition, there are many risk factors related to dental caries such as diet composition, oral hygiene, behavioural, cultural, psychological, environmental and genetic factors, which may have an effect on the dental biofilm (Reisine and Douglass, 1998; Fejerskov, 2004; Paes Leme et al., 2006).

A low pH milieu of the dental biofilm plays a key role in the development of dental caries. This milieu is a result of a frequent intake of fermentable carbohydrates, irregular and poor oral hygiene, and inadequate usage of oral health substances such as fluoride or alkaline supplements (Lingström, 1994; Krasse, 2001; Fontana, 2016). An acidic environment may result in demineralisation of the tooth structure, which can be terminated by an alkalisation effect resulting in remineralisation of the tooth structure (Takahashi, 2015; Samaranayake and Matsubara, 2017).

Dental plaque in relation to caries

The fundamental of the modern concept of the definition of dental caries dates back to 1994 when Marsh published the ecological plaque hypothesis (Marsh, 1994). However, the historical concept of dental caries has passed many important theories. The first hypothesis, called the non-specific plaque hypothesis, was based on research conducted by Black and Miller in the 19th century (Rosier et al., 2014). This hypothesis was built on the foundation that the quantity of dental plaque has the greatest impact for caries development and the best treatment option is by mechanical elimination of the dental plaque.

In the early 1970's, "the specific plaque hypothesis" was born. According to this hypothesis, dental caries is significantly related to specific bacteria such as *Streptococcus mutans* and lactobacilli. The treatment strategy was aimed specifically against these microorganisms by using e.g. antimicrobial agents (Loesche and Nafe, 1973; Loesche et al., 1973; Loesche et al., 1977). A decade later, the non-specific plaque hypothesis was adjusted as a result of the advancement in laboratory technology and microbiological analysis. This modified hypothesis focused on the plaque as an environment of complex microbiota and the plaque-induced disease is a result of shifting to unhealthy milieu once the virulent microorganisms are dominated in this complex environment (Theilade, 1986). This hypothesis focused on subgingival plaque and periodontal disease; however, it had an impact on cariological research development.

In the final decade of the previous century, all prior efforts had been evaluated and a more scientific and reliable hypothesis was crafted once Marsh published the review article in 1994 on "the ecological plaque hypothesis", explaining that the plaque-induced disease is associated to the disruption of the haemostatic balance of microorganisms in the dental plaque (Marsh, 1994). This imbalance is a product of many factors and therefore the treatment plan will not be aimed at specific pathogenic microorganisms but at multi-factorial strategies to stabilise the haemostatic balance. This hypothesis became more comprehensible once it was suggested to manage dental plaque as a biofilm consisting of a diverse microbial community within a homogenous environment containing different formation stages (Marsh and Bradshaw, 1995). Based on the diverse

types of stress in the host, the final outcome indicates that some species will not be suitable in this community and the disease will occur.

After reviewing the previous hypothesis, the ecological plaque hypothesis is more reliable in relation to the new concept of dental caries as a multifactorial disease. However, this hypothesis does not examine the genetic sensitivity in relation to caries (Rosier et al., 2014). Moreover, a recent review suggested the importance of genetic and environmental risk factors on caries occurrence and development (Opal et al., 2015; Chapple et al., 2017).

Acid and alkali formation in relation to caries

According to the ecological plaque hypothesis, the dental biofilm activity is controlled by environmental and ecological factors such as exposure to different types of nutrients and fermentable carbohydrates (Marsh, 1994). Recurring intake of fermentable carbohydrates leads to a decrease in the pH-values in the dental biofilm community. Frequent decrease in these pH-values results in activation of both acidogenic and aciduric microorganisms within the biofilm environment. The activity of these microorganisms stimulates the acid production and increases the adaptation level to a low pH condition (Belli and Marquis, 1991; Takahashi and Yamada, 1999). Furthermore, a continuously acidic environment within the biofilm resulted in an increased proportion of acid-tolerant microorganisms that could survive and respond to the acidic milieu or supposed acid-tolerance response (ATR) (Svensäter et al., 1997; Marsh, 2003; Welin-Neilands and Svensäter, 2007).

It is well established that bacterial metabolism of fermentable carbohydrates leads to low plaque-pH values, commencing in the 1940´s where Stephan displayed how these low pH-values play a major role in caries development and activity (Stephan, 1940; 1944). The highly acidic conditions of the dental biofilm have negative consequences in the form of demineralisation of the tooth surface, causing dysbiosis within the dental biofilm (Bowen, 2013; Samaranayake and Matsubara, 2017).

The impact of the acidification phase and acidification condition affects the pH-values of the supragingival biofilm which could result in the dissolving of the mineral structure of dentin and enamel if the pH reaches the critical value of demineralisation.

In brief, the critical pH of tooth structure, e.g. dentin or enamel, is the value of the pH when the oral fluid (saliva and plaque) is saturated to specific minerals such as hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and fluorapatite $Ca_{10}(PO_4)_6F_2$. In the case that the fluid-pH is lower than the critical pH, the fluid will be unsaturated with hydroxyapatite respective fluorapatite, which results in the mineral dissolving (Dawes, 2003). The critical pH varies between the dentin and enamel, as it is dependent on the concentration of calcium and phosphate present. Previous studies have revealed that the critical pH for enamel can vary between 5.2 to 5.7 and between 6.2 to 6.7 for dentin (Surmount and Martens, 1989; Delgado et al., 2016; Sung et al., 2016).

In contrast, pH-values are increased by the salivary buffer system as well as by alkalogenic and acid neutralising oral microorganisms metabolising urea and arginine (Kleinberg, 1967; Imfeld et al., 1995; Burne and Marquis, 2000; Kleinberg, 2002; Nascimento et al., 2009; Takahashi, 2015). As a result, the alkali production may resist the acid production and the demineralisation stage, which may lead to the control and inhibition of the caries process (Gordan et al., 2010; Nascimento et al., 2014).

Clinical studies suggest that the high urea level in individuals with renal failure makes them more caries resistant despite their carbohydrate intake (Shannon et al., 1977; Epstein et al., 1980; Peterson et al., 1985). In addition, using arginine oral health product has been reported to have a significant effect on the oral environment by increasing the oral pH and reversing the early stage of the demineralisation process. This may be of special interest for individuals suffering from dry mouth syndrome (Guignon and Nový, 2015).

In relation to caries, clinical studies have presented higher pH-values of supragingival biofilm in caries-free individuals in comparison to caries active-individuals (Lingström et al., 2000; Aranibar et al., 2014).

Oral alkalogenic resources

The main source of the alkalisation phase is ammonia, which is the outcome of a neutralisation effect derived from urea and arginine within the micoroorganisms' metabolism. This outcome has a beneficial role by protecting against the acidification phase and by maintaining a neutral pH in the oral environment, impacting positively in correlation to caries (Nascimento et al., 2009).

Urea is an organic compound falling under a variety of concentrations between 3-10 mM in the salivary gland secretion. The compound is hydrolysed by oral microorganisms resulting in the products ammonia and carbon dioxide (Burne and Marquiz, 2000). The hydrolysis of urea is aided by a nickel-containing oligomeric enzyme (urease) which is activated by acidic conditions and the presence of carbohydrate (Liu et al., 2012) (Figure 1).

Arginine is an amino acid naturally present in both saliva and arginine-containing peptides in the dental biofilm with an average concentration of 50 uM in parotid saliva and is also abundant in salivary peptides and proteins (van Wuyckhuyse et al., 1995). The final products and net reaction of arginine catabolism by oral microorganisms are ammonia, carbon dioxide and ATP. These microorganisms utilise an arginine dihydrolase system (ADS) which consists of three enzymes; arginine deiminase, ornithine carbamoyltransferase, and carbamate kinase (Burne and Marquiz, 2000; Liu et al., 2012) (Figure 1). ADS is activated by stimuli such as a low-pH environment and the presence of arginine (Liu et al., 2012).

Urea:

Arginine:

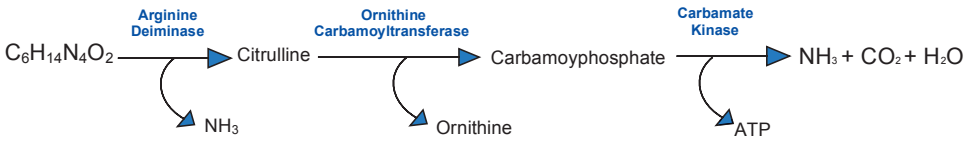

Figure 1. End products of urea hydrolysis and arginine catabolism

Biofilm bacteria associated with acid- and alkali formation

Despite the high variance in the human oral microorganisms (>700 taxa), there is no sole species exclusively related to the occurrence and development of dental caries (Jenkinson, 2011; Marsh and Zaura, 2017). According to the ecological plaque hypothesis, cariogenic bacteria are present in the resident microflora during neutral pH environment. However, the quantity of such microorganisms is minimal and their pathogenic ability is limited which makes their competitive competence restricted (Nyvad et al., 2004).

Once this environment is altered e.g. by frequent sugar intake, the functions of the bacteria will change. The shift of the environment will have an impact on the ability of the bacteria to produce and tolerate acids. This will consequently affect the homeostasis of the biofilm ecology and cause an imbalance resulting in a low pH-milieu and will further lead to the initiation of the demineralisation process. However, this acid formation is accompanied by alkali-formation by some of the oral bacteria, which metabolise arginine and hydrolyse urea to ammonia. Thus, the ammonia production of

this alkalisation process will result in a higher-pH milieu and the commencement of a remineralisation process (Liu et al., 2012) (Figure 2).

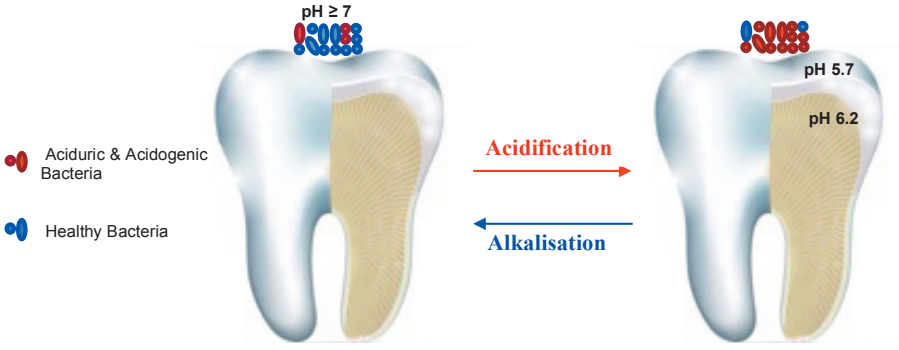

Figure 2. The acid- and alkali-formation role in the dental biofilm homeostasis (modified from Liu et al., 2012)

The traditional microbial aetiology of dental caries is constrained to some bacterial species such as *Streptococcus mutans, S. sobrinus, S. downei, Lactobacillus acidophilus, L. casei, L. fermentum, L. rhamnosus, Actinomyces naeslundii* and *A. odontolyticus*. However, the development in the molecular approach make the bacterial spectrum which relates to dental caries wider, to which it has therefore included species such as *Bifidobacterium dentium, B. longum, B. adolescentis, Scardovia wiggsiae, Prevotella spp.* and *Selenomonas spp.* (Jenkinson, 2011).

The cariogenic bacteria have virulence properties, which specifically relate to three elements: fermentable carbohydrates, acid, and low pH. They have the ability to metabolise fermentable carbohydrates and produce acid (acidogenicity) and to survive at a low pH milieu (aciduric) (Takahashi and Yamada, 1999; Takahashi, 2015). In addition

to these properties, cariogenic *S. mutans* has the capability to produce extra- and intracellular polysaccharides as well as changing the quorum-sensing with other microorganisms (Kuramitsu and Wang, 2006).

In an acidic environment, some non-mutans streptococci such as *Streptococcus sanguinis, S. oralis, S. gordonii* and *S. mitis* increase their capacity to produce acid as well as their acidurance adaptability (Takahashi and Nyvad, 2011). The mechanism behind this adaptation could be linked to the induction of proton-translocating ATPase and increased expression of stress proteins as well as the acceleration of the arginine deaiminase system and alkali production (Takahashi and Yamada, 1999).

The phenotypic and genotypic modification of the microflora, due to acid adaptation and acid-induced selection respectively, results in the imbalance of the de- and remineralisation processes, which will impact the onset caries (Takahashi and Nyvad, 2011). With severely low-pH conditions and prolonged acidification conditions, the composition of the microflora will shift towards a higher number of aidogenic and aciduric bacteria; e.g. mutans streptococci, non-mutans streptococci, lactobacilli and *Bifodobacterium* spp. (van Houte et al., 1996; Jenkinson, 2011). For a reverse shift to homeostasis of the biofilm ecology, alkali production to increase the biofilm-pH by the saliva buffer system and bacterial alkali formation is needed.

Urea is converted to ammonia by bacteria found in the oral cavity such as *S. salivarius, A. naeslundii, Helicobacter pylori, Prevotella tannerae*, *Staphylococcus epidermidis* and oral haemophili (Barboza-Silva et al., 2005; Liu et al., 2012; Piwat et al., 2015). Furthermore, ammonia protects some oral bacteria such as *S. salivarius* and *A. naeslundii* from acid damage and decreases the duration of the glycolytic pH-drop of *S. mutans* (Clancy and Burne, 1997; Chen et al., 2000; Morou-Bermudez and Burne, 2000).

In addition to ammonia, arginine catabolism by the ADS system provides ATP (Huang et al., 2015). The ADS positive oral bacteria or so-called arginolytic bacteria include non-mutans streptococci such as *Streptococcus sanguinis, S. gordonii, S. parasanguis, S. mitis*, *S. oralis, S. rattus, S. faecium, S. cristatus, S. australis,* and some lactobacilli, *Actinomyces* spp. and oral spirochetes (Marquis et al., 1987; Rogers,

1990; Burne and Marquis, 2000; Liu et al., 2012, Huang et al., 2015).

It has been recognised by several *in vitro* and *in vivo* studies that there is a strong inverse association between caries and the ability of oral bacteria to produce alkali (Peterson et al., 1985; Margolis et al., 1988; Clancy et al., 2000; Dawes and Dibdin 2001; Shu et al., 2007; Nascimento et al., 2009; Gordan et al., 2010; Toro et al., 2010). A few of these studies suggested that increased urease activity in supragingival biofilm could be regarded as an indicator for low caries risk (Kleinberg, 1967; Imfeld et al., 1995; Clancy et al., 2000; Morou-Bermudez et al., 2011). Moreover, it was conveyed that oral bacteria in caries-free individuals have higher ADS activity and ammonia-production in comparison to caries-active subjects (Marquis et al., 1987; Nascimento et al., 2009; 2013; 2014; Reyes et al., 2014). This depicts an importance of the biofilm bacteria ADS activity in caries occurrence and progress (Huang et al., 2015).

Methods for pH-measurement of supragingival dental biofilm

Initial attempts to measure the pH of dental plaque was conducted in 1938 by Stephan by mixing the plaque sample with a pH indicator and by registering the outcome using a microscope (Preston and Edgar, 2005). The methods of plaque pH-measurments have been developed by using different techniques with the aim to increase the accuracy and proficiency when performing the measurements. These methods vary in the form of type of biofilm studied (*in vivo, ex vivo)*, the instruments utilised, the accessibility to measure the plaque on the tooth surface, technical difficulties, cost efficiency, and clinical performance. Both advantages and disadvantages of these techniques have been discussed in the literature, which are shown in Table 1 (Harper et al., 1985; Lingström et al., 1993; Preston and Edgar, 2005). Despite the method used, the aim of these methods is to provide an objective tool to measure the net effect of the changes in biological environment of the dental biofilm after exposure to different fermentable substrates as well as the effect of oral health products such as mouthrinses and toothpastes.

The first method is known as the touch method (Stephan, 1940; 1944) and has been further developed throughout the years (Preston and Edgar, 2005). It has been developed, modified and evaluated after 50 years by using multiple types of electrodes to measure different acid and alkali responses to different anti-caries products and sugars

(Preston and Edgar, 2005). In brief, an electrode is placed on the tooth surface or interproximally touching the dental biofilm. The micro-touch electrode is connected to a metre device alongside another reference electrode placed in a salt solution together with the participant´s finger. The latter will create a saltbridge and the pH-value will be presented in the digital screen. This method is easy to use in different dental sites on different tooth surfaces. However, there is a risk for saliva contamination by using this micro-touch electrode, which could therfore increase the risk for cross-infection (Table 1).

The sampling method is an alternative technique to measure the pH of the total dental biofilm *ex vivo* induced by Strålfors, by scraping off supragingival plaque from the tooth surface using a dental instrument such as a carver (Schachtele and Jensen, 1982). The sample will be dispersed in small amounts of distilled water and pH measured using a combination electrode connected to a pH-meter (Fosdick et al. 1941; Frostell, 1970; Lingström et al., 1993). The scraping technique may increase the risk for contamination in a disturbed plaque and therefore the pH-measurement will not reflect the precise pH on the measurement site (Table 1).

The telemetric method has been used to measure the plaque-pH *in vivo* by using an external appliance containing a sensor to record the pH-changes continously. The technique was devised by Graf and Muhlemann (1965) and then further modified and developed by using multiple types of appliances (Preston and Edgar, 2005). The appliance is placed directly adjacent to a tooth surface without disturbance of the biofilm structure. The methods described need that calibration is performed before each measurement (Scheie et al., 1992; Lingström et al., 1993).

Table 1. The advantages and disadvantages of different methods for pH-measurement of supragingival dental biofilm (*in vivo* and *ex vivo*)

Method	Advantages	Disadvantages
Touch Electrodes	+ Clear clinical performance + Accurate, it can discriminate up to two decimal differences of pH unit + Easy accessible in different sites and tooth surfaces + Can be used with a large sample size study + Cost effective	- It cannot be used to measure pH on the metal surfaces - Difficult to sterilise, Risk for cross-infection between individuals - The pH readings are fluctuated and not stable - Risk for fragility and breakage - Technique sensitive - Requires time to calibrate
Plaque Sampling	+ Clear clinical performance + Simple to perform and measure + Cost effective + Enhanced alternative in superior situations such as the posterior region + Relatively accurate + Lower risk for cross-infection	- May be considered uncomfortable by some patients - Technique sensitive - Time consuming - Risk for contamination of plaque sample - Occasionally difficult to collect interproximal plaque samples - Disturbance of biofilm structure
Telemetric Method	+ More accurate with continuous plaque-pH readings + May be used for longstanding testing + Biofilm structure remains maintained + Individual device, therefore no risk for cross-infection	- Not suitable for all ages - Expensive - Difficult to manage - Technique difficulties - Risk for saliva and unnatural plaque retention - Limited access to the sites/surface - Requires time to calibrate - Biofilm formed on an artificial surface

Methods for urease activity evaluation

Scientific research on the dental biofilm has increased towards the concept of a correlation between urease activity and caries status as well as urease activity being one of the fundemental factors of the dental biofilm (Morou-Bermudez et al., 2011). However, there is a lack of knowledge with regards to the standard level and stability of urease activity in the dental biofilm. One key reason behind this issue is the lack of clinically applicable chair-side methods for objective measurements of urease activity in the daily practice.

The most common method used to measure urease activity of supragingival plaque is the usage of Nessler's reagent where the ammonia produced by a sample of dispersed and washed plaque from 50 mM urea is measured (Chen et al., 1996). This method has been used in several clinical studies (Shu et al., 2007; Nascimento et al., 2009; Toro et al., 2010), although there are some disadvantages related to the method. A time and cost consuming laboratory environment is required making the conditions unfit for a chair-side assessment.

HYPOTHESES AND AIMS

Despite several markers and modules to measure and predict the caries risk and progression on a group level, methods to assess the individual caries risk are lacking. This thesis was based on the overall hypothesis that the net effect of acid and/or alkali formation in the dental biofilm as reflected in plaque-pH and/or in the ability of the plaque to form alkali, might be used for assessment of the individual caries risk. To evaluate the hypothesis and to be applicable in the clinic and on larger groups of subjects, non-expensive and easy-to-handle methods were sought.

The hypotheses of the thesis were that:

- A method based on pH-indicator strips could replace the 'microtouch method' for supragingival plaque-pH measurements (Study I) and a modified rapid (R) urease (U) test (T), (RUT) in a microtitre plate format could easily be used for evaluation of the urease activity in bacteria *in vitro* and in dental plaque *ex vivo* in the clinic (Study III).

- Frequent exposure of the dental plaque respectively to sugar, urea and arginine would affect the pH and alkaline activity differently in the dental plaque of non-caries and caries individuals (Study II, IV, V).

The aims were to:

- Evaluate pH values obtained using pH indicator strips with those obtained using the 'microtouch method' for interproximal supragingival plaque-pH measurements *in situ* (Study I).

- Measure the pH by using the 'strip method' in the interproximal supragingival plaque before and after a sugar or a urea challenge, pre and post adaptation to acid and alkali formation by frequent daily rinses with sucrose or urea solutions respectively, in caries-free and caries-active individuals. A second aim was to examine acid tolerant bacteria in saliva and supragingival plaque as well as the saliva secretion rate and buffer capacity (Study II).

- Assess RUT for screening of the urease activity in oral bacteria *in vitro*, and the urease activity of interproximal supragingival plaque at dental sites *ex vivo* (Study III).

- Investigate the plaque-pH, before and after a sugar challenge, pre and post two 6-week periods of using fluoride toothpaste with and without arginine in individuals with different caries status. Salivary secretion rate, pH and buffer capacity, and the plaque alkaline activity *ex vivo* using RUT, were also examined (Study IV).

- To assess acid formation from different sugars and sugar alcohols *in vitro* of plaque bacteria isolated from caries-free and caries-active individuals before and after adaptation to acid and alkali in study II (Study V).

MATERIAL AND METHODS

An overview of the materials, methods and procedures are given below (Table 2). More detailed descriptions are found in respective paper.

Table 2. Study type, number of participants and their age and caries status in the five studies included in this thesis

Study	Study type	Sample size/gender	Age (year, mean ± SD)	Caries Index (mean ± SD)
I	*in vivo*	n=30 (16 female, 14 male)	19-63 (41.6 ± 13.0)	n/a
II	*in vivo*	n=20 (11 female, 9 male)	15-21 (17.0 ± 1.8)	10 CF ($D_{m+i}MFS = 0$) 10 CA($D_mMFS = 3.4±1.8$)
III	*in vitro, ex vivo*	n=18 (11 female, 7 male)	25-69 (37.3 ± 15.4)	DMFT = 9.7±5.5
IV	*in vivo, ex vivo*	n=33 (21 female, 12 male)	19-58 (25.0 ± 10.0)	14 CF (DMFS = 0) 19 CA (DMFS = 3.2±2.7)
V	*in vitro*	n=128 isolates	n/a	73 CF isolates ($D_{m+i}MFS = 0$) 55 CA isolates ($D_mMFS = 3.4±1.8$)

Study I:

The 'strip method' and 'microtouch method' were compared when measuring the interproximal supragingival plaque-pH before and after a sugar challenge. All participants in the study were healthy (Table 2). There were no specific inclusion and exclusion criteria besides having normal salivary secretion rate and no metal prosthetic replacements such as amalgam fillings or metal crowns in the area of measurement.

All participants accumulated supraginigival plaque for three days prior to the test day, by refraining from brushing and from using alternative oral health products such as dental floss or mouthrinse. Furthermore, the participants were, in order to standardise the study procedure, instructed not to eat or drink anything but water two hours prior to the visit. The plaque-pH measurements were performed at two sites: between the upper right first

molar and second premolar (16/15), and between lower left second premolar and first molar (35/36).

Plaque-pH registrations were performed prior to a sugar challenge (baseline), and up to 60 minutes after the challenge by rinsing with 10% sucrose solution for 1 min. The pH was first measured by using the 'strip method' followed by the 'microtouch tmethod'; one examiner performed all the measurements throughout the study. The strip was used prior to the microtouch method since the risk of bias was considered higher if a 2-digit pH-value was determined prior to the assessment of the colour obtained on the strip.

For using the 'strip method', the original indicator strips (Spezialindikator, Merck, Darmstadt, Germany) were cut into four pieces (approximately 2 mm in width), which were easy to insert into the interproximal site (Figure 3). Strips in the pH range of 4.0 to 7.0 were used to measure pH prior to and after a sugar challenge. After 10 seconds insertion, the pH value was assessed by comparing the colour of the strip with the colour index scheme supplied by the manufacturer.

Moreover, whole stimulated saliva was collected, by chewing on a piece of paraffin for the enumeration of mutans streptococci after growth on mitis salivarius-bacitracin agar (MSB).

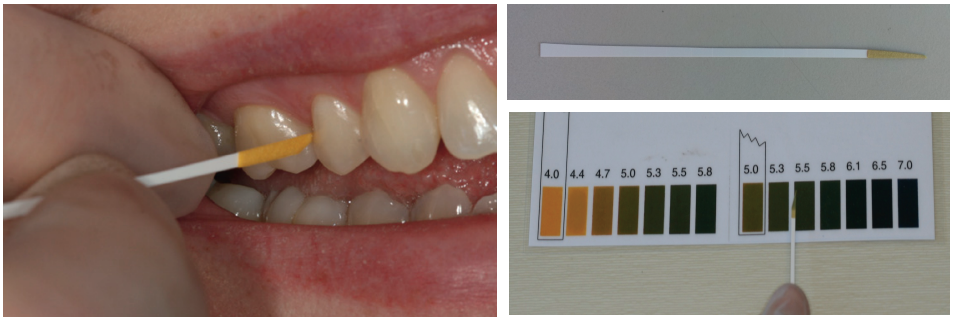

Figure 3. The performance of the 'strip method' for interproximal supragingival plaque-pH measurements

Study II:

The study was a randomised, controlled, two-leg cross over, single-blinded (for the participants) trial including 20 healthy adolescents and young adults randomly selected from four public dental health centres in the Västra Götland region, Gothenburg, Sweden (Table 2). Their caries activity was obtained from their dental records including radiographs within the last three years. A caries active individual was defined as having ≥1 new, primary, manifest caries lesions per year (occlusal and/or approximal) in the last three years. Caries-free was defined as DMFS-index = 0 (decayed, missed and filled surfaces).

This study included 5 visits to a dentist (HH) with a medical and detailed dental history being obtained at the first visit. With a duration of six weeks, each participant should rinse 5 times/day for 1 week with each of two, randomly selected and coded mouthrinses; A=10% sucrose and B= 0.25% urea (Figure 4). To increase the compliance, a message was sent daily as a reminder to the participants during the test periods and before each visit to the lab. Except for the first introductory visit before the first washout period, the participants were asked to accumulate plaque for one day prior to each scheduled visit. At the visits, prior to the sugar and urea challenges, pooled interproximal supragingival dental plaque was collected from two sites between the upper right second and first molar (17/16), and between the upper left first and second molar (26/27) for microbial analysis.

Interproximal plaque-pH was measured by using the 'strip method', between the upper right first molar and second premolar (16/15), and between upper left second premolar and first molar (25/26). The plaque-pH was measured prior to and after the 1-week acid and alkali adaptation period respectively, before (baseline) and up to 30 min after rinsing for 1 minute with 10% sucrose (acid formation) or 0.25% urea (alkali formation). Two types of pH-strips measuring pH 4.0–7.0 after the sugar rinse and pH 6.5–10.0 after the urea rinse were used. At the end of each visit, professional tooth cleaning was performed. The stimulated saliva secretion rate and buffer capacity was determined and a sample was used for microbial analysis. The plaque and saliva samples were cultured on pH 5.2 agar, MSB and Rogosa agar for the enumeration of acid tolerant bacteria and specifically mutans streptococci and lactobacilli, respectively.

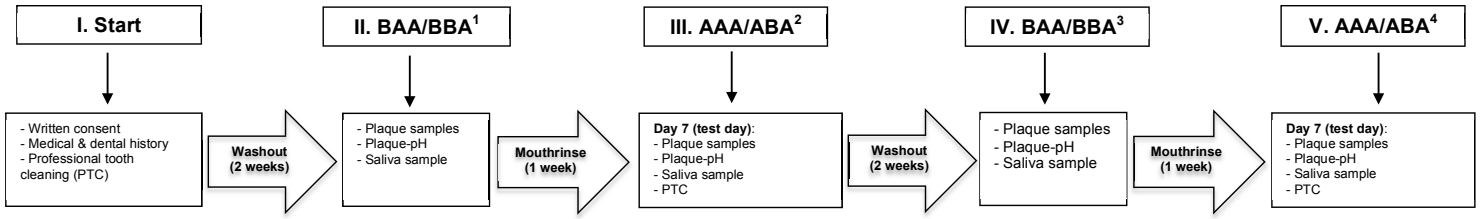

Figure 4. Experimental design of Study II

Samplings and pH measurements performed:

[1)] before start of the first one-weeks acid (**BAA**) or base adaptation (**BBA**) period using a randomly selected 10% sucrose or 0.25% urea rinse

[2)] after the first acid (**AAA**) or base adaptation (**ABA**) period

[3)] before the second one-weeks acid (**BAA**) or base adaptation (**BBA**) period

[4)] after the second acid (**AAA**) or base adaptation (**ABA**) period

Study III:

The rapid urease test (RUT) was modified to assess the urease activity of bacterial strains *in vitro* (Figure 5) and of interproximal, supragingival plaque *ex vivo*.

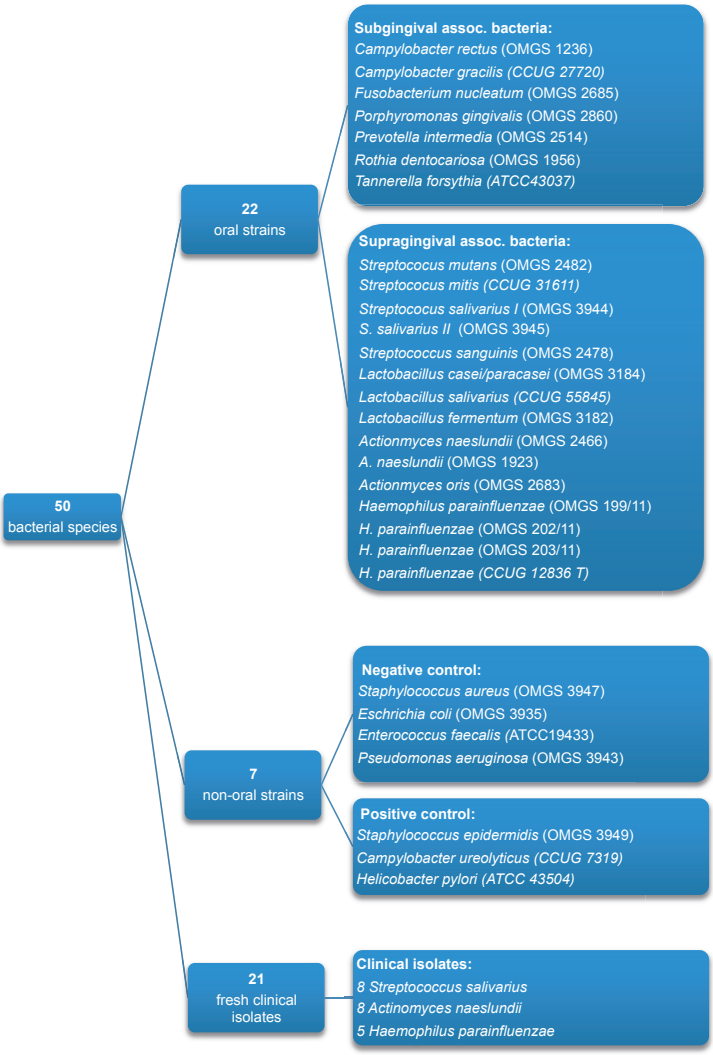

Figure 5. Bacterial species and clinical oral isolates, which were tested for urease activity with the RUT test

After growth on Brucella agar, a sterile inoculation loop was used to transfer approximately 1 µl of bacterial cells into micro-titer plate wells containing urease-broth (Sahlgrenska Hospital, Gothenburg, Sweden) with 2 % urea, pH 6.8, and 0.002% phenol red as an indicator.

The colour outcome was classified into 4 categories (Figure 6):

- (0) : no urease activity displayed as a weak orange colour or yellow as a result of acid production
- (+) : slight urease activity displayed as a visible pink colour
- (++): moderate urease activity displayed as a red colour
- (+++): strong urease activity displayed as a clear purple colour

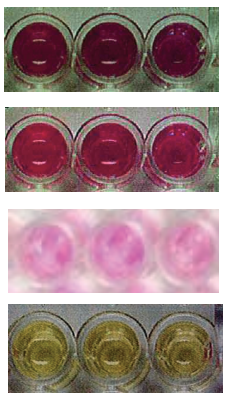

Figure 6. Different colour reactions of interproximal supragingival plaque by using the rapid urease test (RUT)

To evaluate RUT *ex vivo*, 18 healthy volunteers participated in the study (Table 2). As in Study I, there were no specific inclusion and exclusion criteria and the participants should refrain from tooth brushing and from using other oral care products for two days before the test day and from eating or drinking two hours prior to the visit. The interproximal supragingival plaque was collected separately from four sites between the lower central incisors (site 41/31), between the upper central incisors (site 11/21), between the upper left second premolar and first molar (site 25/26), and between lower right first molar and second premolar (site 46/45). Approximately 1 ul plaque sample was transformed to the urease-broth as described above, and the colour was graded 1 hour after incubation at 25°.

Study IV:

This was a controlled, two-leg cross over, double-blinded (for the participants and examiner) study. Thirty-three volunteers among patients, staff and students of the Middlesex University (London, UK) accepted to participate in the study. After obtaining the participants' medical and dental history, the caries status was examined by using a dental probe and clinical loupes and DMFS (manifest decayed, missed and filled tooth surface) was determined. The individuals were then divided into a caries-free group (CF) and a caries group (CA) (Table 2).

The study lasted for approximately 16 weeks and included five visits and 2 washout and 2 test periods (Figure 7). During each test period, the participants brushed their teeth twice a day using one of two, coded and randomly selected toothpastes, A: 1450 ppm fluoride toothpaste with 1.5% arginine (Colgate Maximum Cavity Protection plus Sugar Acid Neutraliser™, Colgate-Palmolive®, New York, USA) and B: 1450 ppm fluoride toothpaste without arginine (Colgate Cavity Protection™, Colgate-Palmolive®, New York, USA). The ingredients of these two toothpastes, which were specifically developed for caries protection according to the manufacturer, are summarised in Table 3. To standardise the tooth brushing, all participants were given one and the same toothbrush and instructed 2 cm toothpaste twice a day; in the morning after breakfast and in the evening before bedtime.

The participants were instructed to avoid using any other toothpaste or other dental care products throughout the study. As in previous studies they should refrain from brushing their teeth for two days prior to the visits, including tests and sample collections (II – V), and not consume any food and drinks two hours before the visit. To improve compliance, a message was, as in Study II, sent daily as a reminder during the test periods and before each visit to the laboratory.

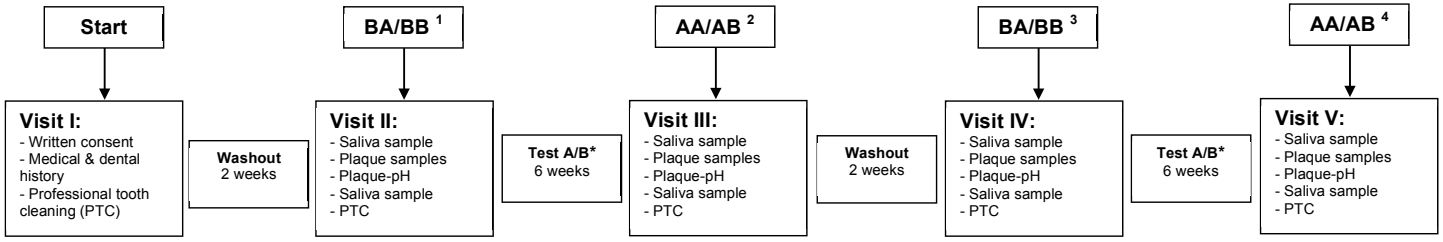

Figure 7. Experimental design of Study (IV).

Samplings and pH measurements performed:

1) before start of the first test period brushing with toothpaste A (**BA**) or toothpaste B (**BB**)

2) after the 6-weeks test period brushing with toothpaste A (**AA**) or toothpaste B (**AB**) and before the 2-weeks washout period using a tooth paste without arginine

3) before the second six-weeks test period brushing with toothpaste A (**BA**) or toothpaste B (**BB**)

4) after the second 6-weeks test period brushing with toothpaste A (**AA**) or toothpaste B (**AB**)

* Randomly selected toothpaste A or B (A= toothpaste with 1.5% arginine and 1450 ppm fluoride B= toothpaste with 1450 ppm fluoride)

Table 3. Composition of the fluoride toothpastes with arginine (A) and without arginine (B)

Type	Ingredients	Function	Tooth-paste A	Tooth-paste B
Active	Arginine 1.5%	Anti-caries effect	✔	
	Sodium monofluorophosphate 1450 ppm	Anti-caries effect	✔	
	Sodium fluoride 450 ppm Sodium monofluorophosphate 1000 ppm	Anti-caries effect		✔
Non-active	Water	Dissolving effect	✔	✔
	Glycerin	Hydration effect	✔	✔
	Sodium Lauryl Sulphate	Debris removal	✔	✔
	Cellulose Gum	Stabilising effect	✔	✔
	Flavour	Taste improvement	✔	✔
	Sodium Saccharin	Sweetener	✔	✔
	Calcium Carbonate	Abrasive effect	✔	
	Sodium Hydroxide	Product pH-regulator	✔	
	Sodium Bicarbonate	Abrasive effect	✔	
	Benzyl Alcohol	Preservation function	✔	
	Dicalcium Phosphate Dihydrate	Abrasive effect		✔
	Tetrasodium Pyrophosphate	Stain removal		✔

At the visits, unstimulated saliva was collected according to Dawes (1987) before plaque sampling and pH measurements were performed as described above. Thereafter, plaque samples were collected between the upper right lateral canine and incisor (site 13/12), between the upper right first molar and second premolar (site 16/15), between the lower left lateral incisor and canine (site 32/33), and between the lower left second premolar and first molar (site 35/36), for urease activity measurement by using RUT method as described in Study III.

Supragingival plaque-pH was measured before and up to 15 min after a 1-min rinse with 10% sucrose at four interproximal sites: between the upper left lateral incisor and canine (site 22/23), between the upper left second premolar and first molar (site 25/26), between the lower canine and right lateral incisor (43/42), and between the lower first

molar and right second premolar (site 46/45), by using the 'strip method'.

Stimulated saliva was finally collected. The salivary secretion rate and pH were determined in samples of unstimulated and stimulated saliva as well as the buffer capacity in the latter by using a chair-side saliva kit (Saliva-Check, GC, Japan) (Maldupa et al., 2011).

Study V:

This *in vitro*, single-blinded study was based on acid tolerant bacteria in interproximal plaque samples, collected in Study II, obtained after culturing on pH 5.2 agar. The plaque samples were collected before/after acid adaptation (BAA/AAA) and before/after alkali adaptation (BBA/ABA). In total 128 isolates were collected; 73 from the caries-free (BAA: n=20, AAA: n=20, BBA: n=19, ABA: n=14) and 55 from the caries-active individuals (BAA: n=20, AAA: n=14, BBA: n=10, ABA: n=11). After growth on pH 5.2 agar, bacteria to be isolated were cultured on blood agar for purity control before being transferred to and preserved on glass beads at -80^{o}C. The bacteria grown on blood agar were characterized by gram-staining and further identified by culturing on mitis-salivarius agar (MS), mitis salivarius-bacitracin agar (MSB), and Rogosa SL agar (RSL) for identification of streptococci, mutans streptococci and lactobacilli, respectively.

Prior to the fermentation test, each isolate was cultured overnight on a blood agar plate. Fresh colonies were cultured in Brain Heart Infusion broth (BHI) and harvested during the mid log-phase. After washing, the bacteria were diluted in phosphate-buffered saline (PBS) to OD_{650} = 1 corresponding to 10^9 cells/ml.

In the fermentation test, sucrose, glucose, fructose, and lactose, and the sugar alcohols sorbitol and xylitol were used. Each isolate was tested in duplicate in a 96 well microtiter plate and using controls with PBS only instead of isolates (Hedberg et al., 2008; Almståhl et al., 2017).

The bacterial solution or PBS was mixed with Basal medium, sugar/sugar alcohols and indicator solution in the microtiter plate wells and after 24 hours incubation at 36^{o}C in a CO_2 atmosphere (10 % CO_2 + 90 % N_2), the colour reaction was registered by using a digital camera (Canon EOD 450D) for later, simultaneous evaluation of the colour

reaction in all tests performed. The pH was classified as <5 (yellow), 5 - 6 (between yellow to purple) and > 6 (purple). The pH was checked in random wells using pH-indicator paper (Merck) and the tests were repeated using 17 of the isolates.

Statistical Analysis

Statistical descriptive analyses were used in all studies (Study I – V). The mean interproximal supragingival plaque-pH (±SD) for all participants in the respective sites was calculated at the different time points (including baseline). Changes in plaque-pH after acid formation were determined as the area of the curve below the critical pH of enamel (pH 5.7; $AUC_{5.7}$) and of dentine (pH 6.2; $AUC_{6.2}$) in Study I, II and IV using a computer-based program (Larsen and Pearce, 1997). Other variables related to acid formation such as maximum pH fall and minimum-pH were also considered in the statistical analysis. For alkali formation, beside the maximum pH increase and maximum pH, the area of the curve above pH 7.0 ($AOC_{7.0}$) was also determined in Study II.

Student's two-sample, paired *t-test* was used to analyse the statistical differences between the 'strip Method' and 'microtouch method' (Study I). This test was also used to analyse differences in plaque-pH variables and salivary parameters (Study II, IV) as well as mean (±SD) logarithmically transferred bacterial numbers (Study I, II), within the same group. Differences in these variables between the groups were analysed using Student's two-sample, unpaired *t-test* (Study II, IV).

Non-parametric Mann-Whitney test and Wilcoxon´s signed rank test was used to analyse differences in the urease activity of interproximal supragingival plaque between sites and groups (Study III, IV) and within groups (Study IV). The distribution of isolates between three pH-intervals after acid formation was calculated and differences between and within the CF and CA groups were analysed using Fischer's exact test (Study V). For all studies (I-V), the figures showing results of statistical analyses were prepared using a computer program (Kaleida Graph 4.1.2, Synerg, Software, Reading, PA, USA). $p < 0.05$ was considered statistically significant in all studies (I-V).

RESULTS

Study I

Comparison of the 'strip method' and 'microtouch method' for plaque-pH measurements

The 'strip method' and the 'microtouch method', demonstrated similar pH-values for the interproximal supragingival plaque before (baseline) and after a sugar challenge. The correlation coefficients for the Stephan curves were high; r = 0.989 for plaque-pH between incisors in the upper front (site 16/15) and r = 0.995 for pH between teeth in the lower premolar-molar region (site 35/36). No statistically significant differences were found between the methods when comparing pH-values and Stephan curves obtained from individuals with respectively $<10^5$ (Figure 8-A) and $> 10^5$ (Figure 8-B) number of mutans streptococci/ml saliva, although the latter group displayed more acidic pH values. Furthermore, other plaque-pH parameters, e.g. $AUC_{5.7}$, $AUC_{6.2}$, maximum-pH fall and minimum pH, as calculated from the pH-values obtained using the respective methods, did not differ between the two methods.

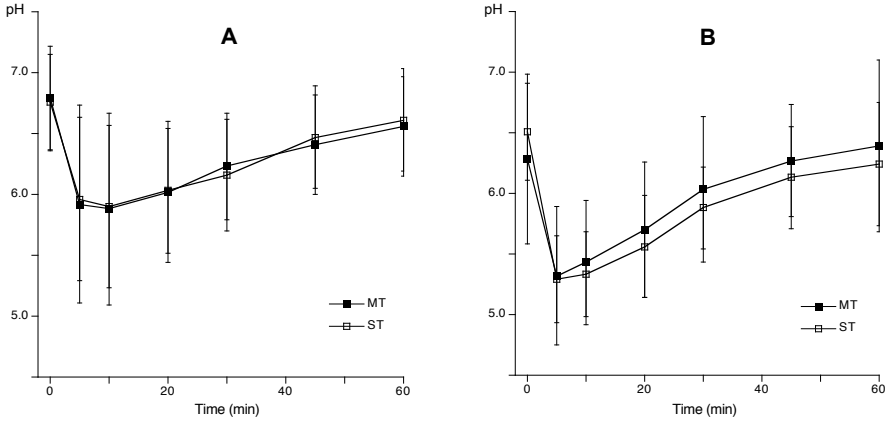

Figure 8. Mean values of interapproximal plaque pH (±SD) before and up to 60 min after sugar challenge from the sites (16/15, 35/36) assessed by the strip (ST) and the microtouch method (MT) in 15 participants with $<10^5$ (A) and 9 participants with $> 10^5$ (B) salivary mutans streptococci

Study II

Effect of acid and alkali adaptation on plaque-pH in caries-free (CF) and caries-active (CA) individuals

The caries-free and caries-active individuals who had consented to participate in the study were healthy and visited the dental practice regularly. Except for the use of mouth-rinse in the CA group, their reported use of dental care products and dietary habits were quite similar (Table 4).

Table 4. The outcome of dental and diet anamneses for caries-free and caries-active individuals

Oral care and dietary habits	Caries-free (n=10)	Caries-active (n=10)
Tooth brushing twice/daily	100%	100%
Fluoride toothpaste (1450 ppm F)	100%	100%
Regular mouthwash (910 ppm F)	0%	40%
Dental floss	30%	40%
Main meals intake (mean ± SD)	2.8 ± 0.8	2.8 ± 0.6
Snack intake (mean ± SD)	1.7 ± 0.8	1.7 ± 0.7

In both the caries-free (CF) and caries-active groups (CA) the shape of the Stephan curves obtained after a challenge by rinsing with sucrose or urea was similar before and after the adaptation period to acid and alkali formation, respectively. Except for the values at 2 and 5 min, adaptation resulted in significantly lower pH in the CF group from baseline up to the final pH 30 min after the sugar challenge (Figure 9). In the CA group, pH was lower at 15 and 30 min after the challenge. Although not statistically significant, lower minimum pH in the CF group and larger $AUC_{6.2}$ in both groups were also noted after the adaptation period.

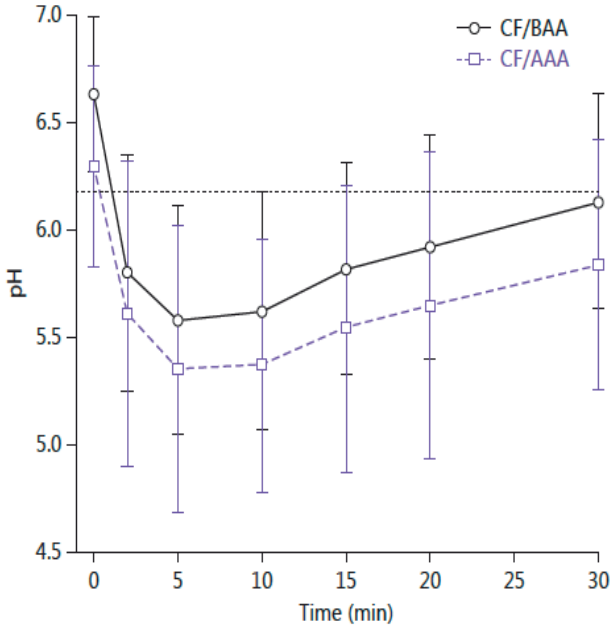

Figure 9. Mean values of interproximal plaque-pH (±SD) before and up to 30 min after a sugar challenge from the site (16/15) in 10 caries-free (CF) participants before (BAA) and after (AAA) acid adaptation

No significant effects on plaque-pH were seen after alkali adaptation in either of the groups. Numerically increased pH-values and the area over the curve ($AOC_{7.0}$) was, however, noted for the CA group (Figure 10). Significant effects of the adaptation periods on bacteria were found for the CF group only. The number of acid-tolerant plaque-bacteria growing at pH 5.2 was increased after both adaptation periods and number of lactobacilli increased in saliva after acid adaptation.

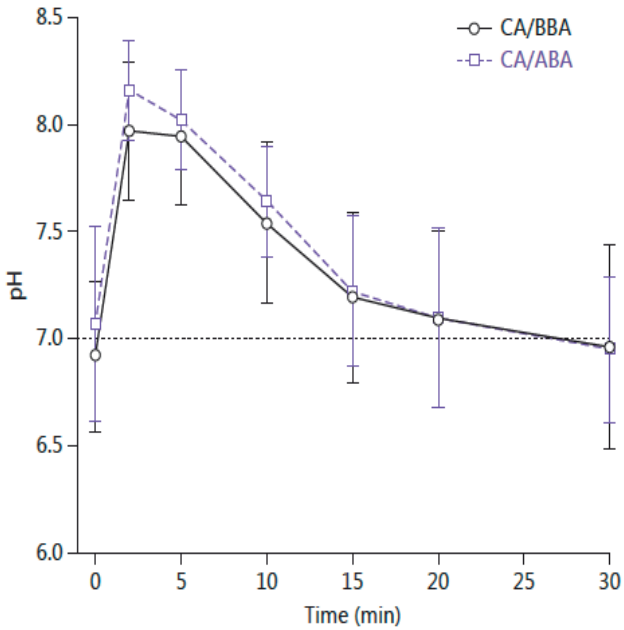

Figure 10. Mean values of interapproximal plaque-pH (±SD) before and up to 30 min after a urea challenge from the site (15/16) in 10 caries-active (CA) participants before (BBA) and after (ABA) alkali (base) adaptation

There were no statistically significant differences between the CA and CF groups after the acid and alkali adaptation periods. However, baseline plaque-pH was higher in CF in comparison to CA before acid adaptation. The saliva secretion rate and buffer capacity did not contrast before and after acid/alkali adaption in either of the groups.

Study III

Evaluation of bacterial urease activity using RUT

From RUT tests using varying incubation times and temperatures, reading after 60 min at room temperature were found to be appropriate for the assessment of high and rapid, and weak and slow urease activities. This procedure could therefore applied in both the *in vitro* test on bacteria and *ex vivo* tests on plaque samples.

In vitro tests

RUT revealed large differences in urease activity both between and within bacteria of different species and even within species such as *S. salivarius* previously reported to be urease active (Table 5).

Out of the reference-strains tested (OMGS, CCUG, ATCC), the non-oral, urease active *S. epidermidis, C. ureolyticus* and *H. pylori* (positive controls) as well as two strains of *H. parainfluenzae* associated with supragingival plaque (OMGS 199/11, OMGS 203/11) demonstrated a rapid, strong urease activity giving a clear purple colour in RUT within the first 15 minutes of incubation at room temperature. However, a moderate, red reaction and no reaction were seen for *H. parainfluenzae* strains OMGS 199/11 and CCUG 12836 T, respectively. Otherwise only none or weak reactions were found for the plaque bacteria tested, with no reaction being typical for the bacteria associated with subgingival plaque.

Similarly, the results for the salivary fresh clinical isolates varied. The majority of the *H. parainfluenzae* isolates displayed a strong reaction whereas no reaction was the most common for the *A. naeslundii* isolates. For the *S. salivarius* isolates the reaction varied between none, weak and moderate.

Table 5. The outcome of RUT in different bacterial strains and isolates tested for urease activity after 1 hour incubation at 36°C

Oral bacterial strains from collection	RUT	Non-oral bacterial reference strains	RUT	Fresh clinical isolates	RUT
Supragingival assoc. bacteria:		**Positive control:**		*S. salivarius (n=4)*	0
S. mutans (OMGS 2482)	0	*S. epidermidis (OMGS 3949)*	+++	*S. salivarius (n=2)*	+
S. mitis (CCUG 31611)	+	*C. ureolyticus (CCUG 7319)*	+++	*S. salivarius (n=2)*	++
S. salivarius I (OMGS 3944)	0	*H. pylori (ATCC 43504)*	+++		
S. salivarius II (OMGS 3945)	+			*A. naeslundii (n=7)*	0
S. sanguinis (OMGS 2478)	0	**Negative control:**		*A. naeslundii (n=1)*	+
L. casei/paracasei (OMGS 3184)	0	*S. aureus (OMGS 3947)*	+		
L. salivarius (CCUG 55845)	0	*E. coli (OMGS 3935)*	0	*H. parainfluenzae (n=1)*	0
L. fermentum (OMGS 3182)	+	*E. faecalis (ATCC19433)*	0	*H. parainfluenzae (n=4)*	+++
A. naeslundii (OMGS 2466)	+	*P. aeruginosa (OMGS 3943)*	0		
A. naeslundii (OMGS 1923)	0				
A. oris (OMGS 2683)	+				
H. parainfluenzae (OMGS 199/11)	++				
H. parainfluenzae (OMGS 202/11)	+++				
H. parainfluenzae (OMGS 203/11)	+++				
H. parainfluenzae (CCUG 12836 T)	0				
Subgingival assoc. bacteris:					
C. rectus (OMGS 1236)	+				
F. nucleatum (OMGS 2685)	0				
P. gingivalis (OMGS 2860)	0				
P. intermedia (OMGS 2514)	0				
R. dentocariosa (OMGS 1956)	0				
C. gracilis (CCUG 27720)	+				
T. forsythia (ATCC43037)	0				

Ex vivo tests

The urease activity varied between plaque samples from different interproximal sites. The highest activity was found for plaque on the central incisors in the lower front (site 41/31). Here, the urease activity was statistically significantly higher than in the upper front (site 11/12) and in comparison to sites in the upper and lower molar regions (sites 25/26 and 46/45). There were numerical but no statistically significant differences between the last three sites.

Study IV:

Effect of using arginine and non-arginine fluoride toothpaste on supragingival plaque-pH and saliva in individuals with and without caries, respectively

The supragingival plaque-pH before and up to 15 min after a sugar challenge as measured before and after periods of brushing with respectivley arginine or non-arginine fluoride toothpaste did not result in any statistically significant differences with plaque-pH variables in the caries-free group.

In the caries group, however, the use of arginine toothpaste resulted in significantly higher pH-values at the four interproximal sites measured (22/23; 25/26; 43/42; 46/45) both before (baseline pH) and after a sugar challenge (Figure 11). Concordantly, numerically and statsitically significant differences were revealed also for other pH-variables indicating a less acidogenic plaque.

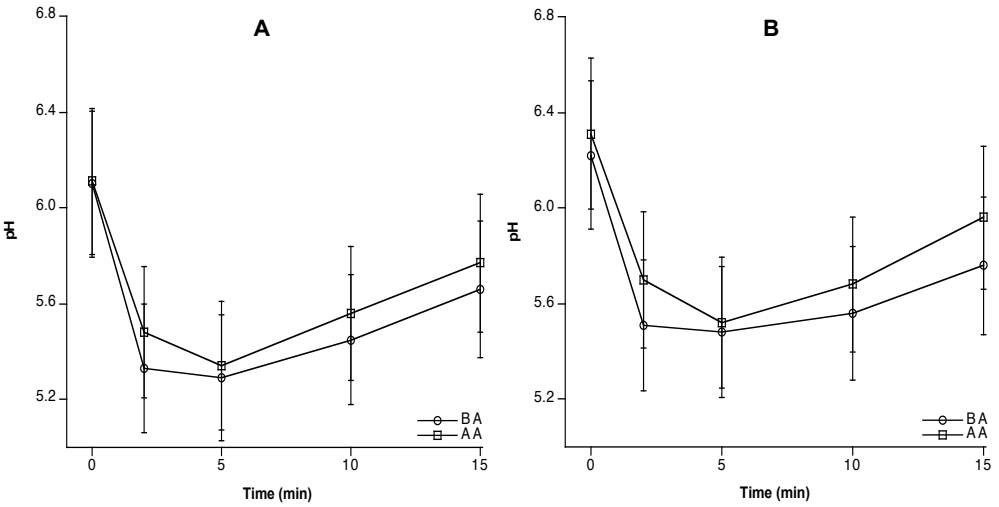

Figure 11. Mean values of interproximal plaque-pH (±SD) before and up to 30 min after a sugar challenge from the sites (22/23, A) and (25/26, B) in the caries group before (BA) and after (AA) using arginine toothpaste

No significant differences were found before and after 6-weeks of using the non-arginine fluoride toothpaste in any of the groups (data not shown).

Ex vivo evaluation of the plaque urease activity with RUT before and after using the arginine toothpaste, suggested increased activity in the upper and lower molar regions in the caries group (sites 16/15 and 35/36; $p<0.05$) (Figure 12).

Site-specific differences in plaque-pH were noticed in both groups regardless of toothpaste usage. The highest pH values were seen in the lower jaw (sites 43/42 and 46/45) resulting in generally smaller $AUC_{5.7}$ and $AUC_{6.2}$ compared with the upper front region. Furthermore, the strongest urease activity was registered in the lower jaw compared with the upper jaw in both the front and molar regions in both groups.

Saliva analyses revealed increased stimulated saliva pH and buffer capacity in the caries group after using arginine toothpaste. There were no differences in the unstimulated saliva in either group.

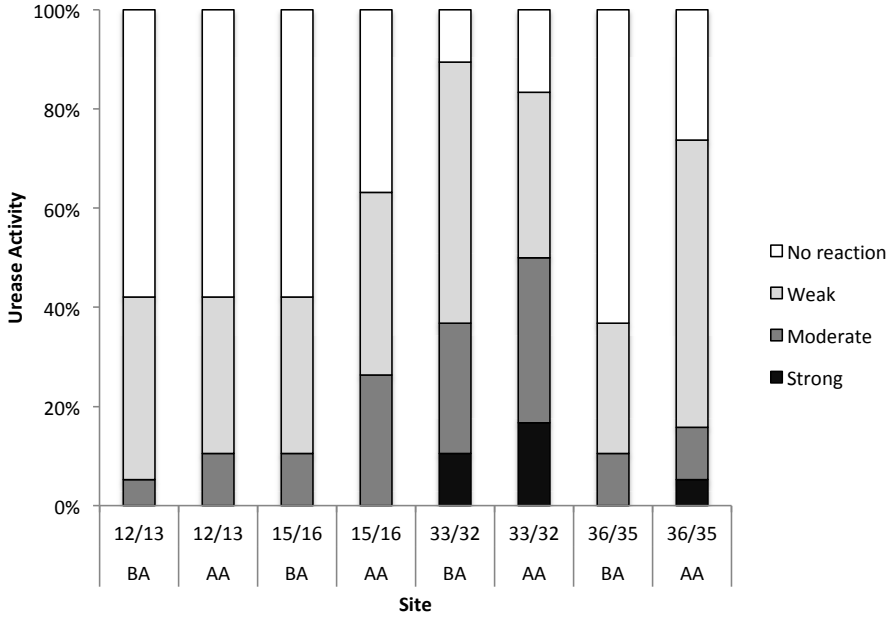

Figure 12. The changes in supragingival plaque urease activity *ex vivo* with RUT at the 4 dental sites before (BA) and after (AA) using arginine toothpaste in the caries group

Study V:

Acid formation of bacteria isolated from caries-free (CF) and caries-active (CA) individuals before and after acid/alkali adaptation

The majority of the bacterial isolates in the CF group were characterised as *Streptococcus mitis* and *Streptococcus oralis*. In the CA group most isolates were characterised as *Streptococcus mitis*, lactobacilli and *Streptococcus mutans*. In addition, isolates of *Streptococcus salivarius* and of *Streptococcus sanguinis* were identified among the CA bacteria.

The distribution of the isolates between the pH-intervals <5, 5 - 6 and >6 did not differ significantly within the CF and CA groups after the acid and alkali adaptation periods as compared to before adaptation. However, there was a numerically higher proportion of isolates in the lower pH-intervals after acid adaptation in the CF group (Figure 13-A) and a numerically lower proportion of isolates in the low pH-intervals in the CA group after adaptation to alkali (Figure 13-B).

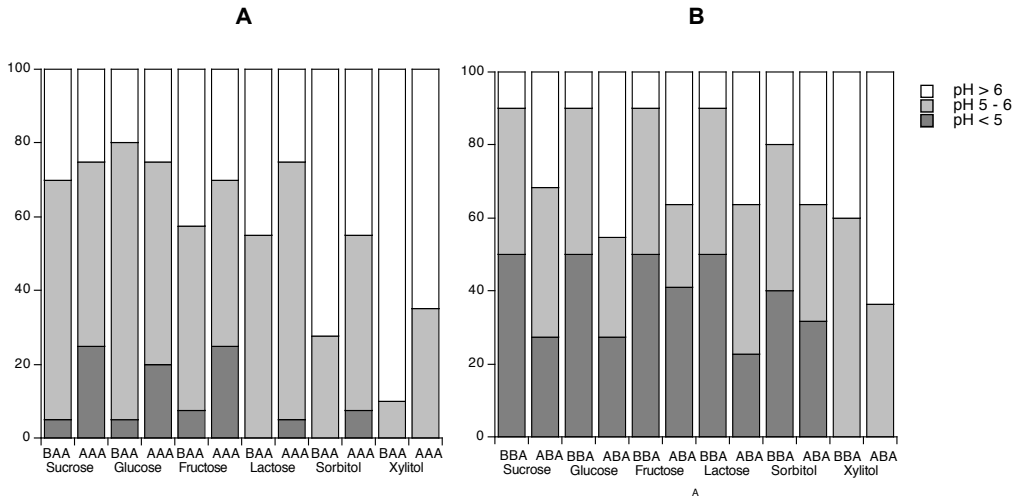

Figure 13. Distribution profile of bacterial isolates (%) between the different pH intervals after fermentation of sugars and sugar alcohols. (A) The bacteria were isolated; before (BAA) and after acid adaptation (AAA) from caries-free individuals and (B) The bacteria were isolated; before (BBA) and after alkali adaptation (ABA) from caries-active individuals

When comparing the CF and CA groups, statistically significant more isolates in the higher pH intervals were seen for the CF group for glucose and lactose, prior the adaptation to acid (BAA). No significant difference between the groups was found neither after acid adaptation nor before or after adaptation to alkali. The main findings of the studies in this thesis are presented in Table 6.

Table 6. Main outcomes in the five studies included in this thesis.

Study	Main outcome
I	There was a high similarity in the values obtained from interproximal supragingival plaque-pH measurements using the 'strip method' and the 'microtouch method'.
II	Acid adaptation resulted in decreased plaque-pH after a sugar challenge, and in increased numbers of acid tolerant bacteria after both acid and alkali adaptation in caries-free individuals. No significant changes were seen for caries active individuals.
III	The RUT test revealed variations in the urease activity between species and between strains of the same species including strains of species reported to have a high activity. *H. parainfluenzae* displayed the highest activity among the oral bacteria tested. The interproximal plaque urease activity determined *ex vivo* using RUT was significantly higher in the lower front than in the molar and upper front regions.
IV	Using fluoride toothpaste with arginine resulted in significantly increased plaque-pH, as well as increased salivary buffer capacity and pH in individuals with caries. No significant effects were noticed for the caries-free individuals.
V	Before adaptation to acid formation, significant less acid formation from sugars and sugar alcohols were seen for isolates from caries-free individuals (CF) compared with isolates from caries-active individuals (CA). The proportion of the most acidogenic isolates was numerically increased after adaptation to acid formation in CF individuals and numerically decreased after adaptation to alkali formation in CA individuals.

DISCUSSION

The net effect of bacterial acid and alkali formation on pH in the dental biofilm (dental plaque), which is affected by several factors including e.g. saliva clearance and buffering, is a key factor for caries to occur. Therefore, measurements of the dental plaque-pH *in situ* could be beneficial in order to investigate the capacity of plaque to form more acidic (acidogenicity) or more alkaline conditions (alkalogenicity). Clinically, if supragingival plaque-pH measurements could reflect the individual´s caries status, negative changes may be discerned by regular plaque-pH measurements. There are several methods and techniques available to examine the supragingival plaque-pH within the research field and many studies show a correlation between caries prevalence and plaque-pH on a group level (Dong et al., 1999; Lingström et al., 2000; Aranibar et al., 2014).

The fundamental aim of this thesis is based on the hypothesis that the individual supragingival plaque-pH may reflect the individual's caries risk. Therefore, a simple, cost-effective and easy to use method is essential to register the supragingival plaque-pH *in situ*, which could be used for comparisons over time and, hopefully, could be used as marker for individual caries risk assessments.

A universal method of measuring supragingival plaque-pH is the 'microtouch method', which has been used in various *in vivo* clinical trials and found to be accurate. The comparison between the 'microtouch method' and the 'strip method', which was developed in our lab, showed a strong coherence between them, which indicate that the 'strip method' may replace the 'microtouch method'.

A high correlation (r = 0.99) between the pH-values obtained using the 'microtouch method' and the 'strip method' with a pH range of 4.0 - 7.0 for measuring plaque-pH after a sugar challenge was seen in Study I. This was true also for tests using the 'strip method' with a pH range of 6.5 - 10.0 before and after a 1 minute rinsing with 10% xylitol. Furthermore, in a recent study that included 136 children (7-9 yrs), following the same study protocol as in Study I, high correlation coefficients were seen for comparison of pH values using the two methods (Cocco et al., 2017). Thus, it could be

concluded that the 'strip method' has as high reliability and validity as the 'microtouch method' and could be used for chair-side evaluation of the individual, interproximal supragingival plaque-pH (Table 7).

Table 7. The advantages and disadvantages of using the 'strip method' for pH-measurement of supragingival biofilm *in situ*

Advantages	**Disadvantages**
+ Easy functionality, in the lab and clinic + Provides easy access to different sites with distinctive prosthetic materials such as amalgam or ceramic + Does not require any sophisticated settings and equipment + Single use, therefore disinfection is not necessary, hence no risk of cross-contamination + Cost-effective	- Cannot discriminate a difference lower than 0.2 pH unit - Colour dependant technique. In some cases, distinguishing between colours is questionable

Additional to the 'strip method', a method based on the rapid urease test (RUT) for urease activity measurements, was developed. The method is a modification of the NCTC micro method (National Collection of Type Cultures, NCTC, Public Health England, UK) into a microtiter plate format, making RUT a simple and rapid test that could be used for screening bacterial urease activity *in vitro* as well as in plaque samples *ex vivo*. The requirements to perform this method are routinely available and there is no need for expensive and complicated lab tools and devices. With a practical time scale (1hour incubation) and temperature required (room temperature at 25^{o} C), 'RUT' has the characteristics for a chair-side method that could be used for assessing the ureolytic activity in supragingival plaque samples of any dental site, in any environment (Appelgren et al., 2014; Piwat et al., 2015).

The strongest and most rapid reaction (within 15 minutes) of using RUT was seen in the non-oral, positive control bacteria (*S. epidermidis, C. ureolyticus, H. pylori*). Strong and rapid reactions were also seen for most of the *H. parainfluenzae* strains, which have been associated with supragingival plaque and reported to be major contributors to a

high urease activity in children (Morou-Bermudez et al., 2015). These findings suggest that *H. parainfluenzae* performs an essential role within the alkalisation phase in the dental plaque. However, the role of *H. parainfluenzae* in relation to the caries process remains to be determined.

The common oral bacteria *Streptococcus salivarius* and *Actinomyces naeslundii* were reported to be ureolytic (Sisson et al., 1988; Salako and Kleinberg, 1989; Kleinberg, 2002). They were considered important for the dental biofilm ecology by operating urease to produce ammonia (Morou-Bermudez and Burne, 1999; Barboza-Silva et al., 2005), which increases the pH of the biofilm and thereby could protect from acid damage and caries (Liu et al., 2012). *Streptococcus salivarius* is also considered as the primary source of urease in the oral cavity (Bowen, 2013). The RUT test showed variations from none up to moderate urease activity among strains of *S. salivarius* with the highest activity seen for the fresh clinical isolates. For *A. naeslundii*, RUT resulted in none or a weak urease activity only in both the reference and in the majority of the fresh isolates. No high urease activity may, however, not be due to an incapability to form urease. It may be due to the environment from which they were obtained since the environment has an impact on the genes encoding for the enzyme activity (Morou-Bermudez and Burne, 1999; Morou-Bermudez and Burne, 2000; Yaling et al., 2006; Liu et al., 2008).

The diversity of urease activity between bacteria associated with caries and periodontitis was obvious with no reactions in RUT for bacteria associated with periodontal disease (*F. nucleatum, P. gingivalis, P. intermedia, T. forsythia*). These bacteria, which are acid intolerant and could produce weak acids only, thrive in the dental pocket, which is normally slightly alkaline (Burne and Chen, 2000; Marsh and Martin; 2009). Thus, they are not in need to produce protective alkali like bacteria in the acidogenic, supragingival plaque environment.

Adaptation to sucrose and acid formation had the largest effect in caries-free (CF) compared with caries-active (CA) individuals. CF had a significantly lower Stephan curve including at baseline, and increased salivary lactobacilli (LB) and acid tolerant plaque bacteria (pH 5.2) compared to the CA. The increased number of salivary LB could be explained by an increased acidic oral environment in this group. The increased

number of acid-tolerant plaque bacteria are in line with previous studies suggesting an increased acidurance adaptability by frequent sugar supply in non-mutans streptococci (*Streptococcus sanguinis, S. oralis, S. gordoni* and *S. mitis*), bifidobacteria and *Actinomyces* spp. (Aas et al., 2008; Mantzourani et al., 2009; Takahashi and Nyvad, 2011). Similar differences in baseline-pH, salivary lactobacilli, and acid tolerant plaque bacteria (pH 5.2) were not found for the CA group.

Before, but not after the acid adaptation period the baseline pH before a sugar challenge was lower in the CA group than in the CF group. This is in concordance with a previous study where it was concluded that a group of individuals with the lowest baseline plaque-pH had the highest number of caries lesions (Aranibar et al., 2014). The previous and present findings of lower baseline pH in individuals with caries suggest that plaque-pH may reflect the individual caries-activity generally used as a marker for caries.

Adaptation to alkali formation from urea had no apparent effect on the plaque-pH and Stephan curve in the CF group. This could be related to a reported higher alkali generating capacity in CF compared with CA individuals (Shu et al., 2007; Nascimento et al., 2009; Gordan et al., 2010; Reyes et al., 2014). The capacity may be at its maximum and, therefore, alkaline supplements will not have any discernible effect with the method used. However, considerably more plaque bacteria growing on pH 5.2 agar were found in this group suggesting a more alkalogenic environment after the adaptation, allowing increased growth of the acid-tolerant bacteria.

In contrast, alkali adaptation resulted in numerically increased plaque-pH before and after a urea challenge giving an elevated Stephan-curve as well as numerically increased area over the curve ($AOC_{7.0}$) in the CA group. The lack of statistically significant effects may be explained by the low number of individuals tested and the duration of the adaptation process, which may be too short to reveal possible significant changes.

Bacteria grown on pH 5.2 agar plates in Study II were isolated, identified and tested for their ability to produce acid from sugars and sugar alcohols in Study V. Non-mutans streptococci such as putative *S. mitis* and *S. oralis* characterized the CF group, both before and after the adaptation to acid formation. This confirms previous findings of

these bacteria in the healthy biofilm of CF individuals with controlled acidic conditions (Aas et al., 2005; Haffajee et al., 2008; Zaura et al., 2009; Wolff et al., 2013). Numerically increased ability of the bacteria to produce acid from sugars and sugar alcohols after adaptation to acid formation confirmed the lower pH-values and Stephan curve seen after acid adaptation (Study II). It could therefore be suggested that a short-term frequent use of sugar supplements may have an impact on the biofilm ecology where the bacteria will adapt to the new environment and become more acidogenic and acid tolerant (Marsh, 1994; Kleinberg, 2002; ten Cate, 2006). No notable effects were witnessed after the alkali adaption period in the CF group.

Within the CA group, *S. mutans, S. salivarius* and lactobacilli were the dominating bacteria isolated. These bacteria reflect an acidogenic and aciduric flora of the biofilm ecology in CA individuals (Hamada and Slade, 1980; Wolff et al., 2013). In this group a notable decrease in acid formation by the isolates was seen after the alkali adaptation period. This effect is analogue to the numerically higher pH-values presented by the Stephan curve and in the area over the curve ($AOC_{7.0}$) (Study II). Thus, a frequent supply of urea may increase the capacity of bacteria to hydrolyse urea to ammonia. This gives a higher intracellular and extracellular bacterial pH-level that will protect bacteria from acid killing (Hamilton and Buckley, 1991; Svensäter et al., 1997; Burne and Marquis, 2000; Senneby et al., 2017).

An increased acid formation by putative *S. mitis* and *S. oralis*, isolated from CF individuals before and after acid adaptation (Study V), representing a less and a more acidic plaque respectively, has not been observed in a recent study on *S. mitis* and *S. oralis* (Banas et al., 2016). Here, bacteria were isolated from occlusal tooth surfaces with and without caries and examined for acid formation from glucose only. Furthermore, the acid formation varied between strains within the same species, which may in part be explained by large difficulties in the correct identification of *Streptococcus* spp. even when using PCR-based methodology (Banas et al., 2016). In Study V, the aim was not to examine acid formation from specific bacterial species or strains but to get an overview of the interproximal plaque-bacteria acidogenicity.

Arginine provides substrate for bacterial ammonia formation and thus may have an impact on the dental biofilm ecology. *In vitro* findings have shown that arginine has a negative effect on the acidogenic oral bacteria by decreasing their numbers (Ledder et al, 2017). Furthermore, arginine could modify the production of the extracellular polysaccharide matrix, resulting in a less criogenic dental biofilm (Koo et al., 2009; Moryl, 2015; He et al., 2016).

The usage of the arginine toothpaste (Study IV) resulted in higher interproximal supragingival plaque-pH in different dental sites in the caries group. This supports previous findings suggesting that arginine could be used as an anti-cariogenic supplement due to its alkalogenic effect (Kraivaphan et al., 2013; Srisilapanan 2013; Yin et al., 2013). Also, this finding supports the effects seen for caries-active individuals after frequent rinsing with 0.25% of alkalogenic urea (Study II).

However, arginine did not result in an increased plaque pH in the caries-free group. This is similar to what was seen for the CF group after frequent urea rinsing (Study II) and could be similarly explained by a high alkalogenic level in the dental biofilm of CF individuals, which may not be increased by any alkalogenic supplements (Nascimento et al., 2009; Reyes et al., 2014).

Additionally, after using an alkaline supplement such as 1.5% arginine toothpaste, the RUT test suggested an increased urease activity as well, in the caries group. However, this finding may result from an increased ammonia levels in the plaque after arginolysis since the method measures the amount of ammonia.

Using arginine toothpaste resulted in a significantly increased stimulated saliva-pH and buffer capacity only for the caries group (Study IV). This may corroborate previous findings in children showing an association between less caries and high pH neutralising ammonia in saliva produced from arginine especially (Moncada et al, 2015).

The pH-values, Stephan curves and urease activity according to RUT were higher in the front and molar regions of the lower jaw in comparison to the upper front region (Study III and IV). These findings correspond with previous studies, which have concluded that a significantly higher urease activity of the mandibular incisiors and a

lower caries prevelance in the lower jaw was related to higher pH and salivary flow rate within these regions (Edgar, 1990; Luan et al., 2000; Demirci et al., 2010; Piwat et al., 2015).

It could be concluded that the cost-effective and simple chair-side 'strip method' and 'RUT' were applicable for assessment of supragingival plaque acidogenicity and urease activity respectively. RUT showed strong urease activity for the well-known urease active *Helicobacter pylori* and strains of *Haemphilus parainfluenzae* associated with supragingival plaque, *in vitro*. A stronger urease activity in supragingival plaque as determined *ex vivo* was found for sites in the lower jaw compared to the upper jaw. In relation to caries, one-week daily rinses with sucrose resulted in decreased pH and increased number of aciduric bacteria as well as a numerically increased proportion of the most acidogenic bacteria in the dental plaque of caries-free individuals. Numerically higher pH-values and lower proportion of acidogenic bacteria was seen for caries-active individuals after one-week daily rinses with urea. In caries-active individuals only, using fluoride toothpaste with 1.5% arginine resulted in a less acidogenic plaque with increased supragingival plaque-pH and alkali formation (RUT) as well as increased salivary pH and buffer capacity.

METHODOLOGICAL CONSIDERATIONS

Strip method

Initial tests previously performed in our laboratory of oral microbiology suggested the rigid and non-bleeding pH indicator strips might be used instead of the 'microtouch method' for *in situ* interproximal plaque-pH measurements. However, as well as for the 'microtouch method' (Table 1, introduction) there are advantages and disadvantages also for the 'strip method' (Table 7). A major disadvantage could be that it is a colour dependant technique and the colour observed may not always be easily assessed. Also, this method could not discriminate differences less than 0.2 pH units. However, there is instability also of pH-measurement readings by using the 'microtouch method' and other pH methods. pH is not a stable value as it reflects a metabolic process which, particularly during the first phase after a challenge is undergoing continuous changes.

Furthermore, in Study I, it was suggested the strip pH 6.5 – 10.0 should be used to measure increased pH in the supragingival plaque in case the level of pH-value is > 7.0. However, using the 6.5 – 10.0 strip in Study II, before a urea challenge and prior to an alkali adaptation period, did not show any significant differences in the baseline pH-value between the CF and CA groups. A significant difference in baseline pH between the groups was found before a sugar challenge prior to an acid adaptation period, by using the pH strip 4.0 – 7.0. This could be explained by the colour readings within the range of pH 6.0 – 7.0 being 6.1, 6.5 and 7.0 of the pH 4.0 – 7.0 strip, whereas there are just two colour readings, 6.5 and 6.8, for the pH 6.5 – 10.0 strips. It could be beneficial to use the pH 4.0 – 7.0 strip for supragingival plaque-pH registration of baseline values before both an acid and an alkali challenge, especially for individuals with caries, since the supragingival plaque-pH is expected to be lower in this group in comparison to a caries-free group.

To minimise the risk that remaining saliva in the plaque could affect the pH measurements, the participants were told to "suck air" between their teeth just before the start of taking measurements.

Rapid Urease Test (RUT)

The outcome of Study III suggested that the microtiter plate format of RUT could be used as a simple, cost effective, and rapid chair-side method in order to assess urease activity in bacteria *in vitro* and in supragingival plaque samples *ex vivo*. To protect from any environmental contamination and evaporation it is recommended the microtiter plate be tightly covered with a plastic foil during the incubation time. Also, the plate must be kept away from direct sunlight exposure. A standardised incubation of 1 hour at room temperature was found to be a reliable protocol.

Supragingival plaque samples

The exact quantity of plaque sample that was collected in Study II for further microbiological analysis was not determined, which could have an impact on the total amount of bacteria in the plaque. The quantity of plaque might also affect the outcome of the *ex vivo* urease activity tests using RUT in Study III and IV. In these studies the same examiner (HH) used standard sterile curettes for similarly performed plaque sampling and a loopful (1 µl Inoculation loop, Sarstedt, Nümbrecht, Germany) of plaque in the RUT tests.

Compliance

In studies II and IV, the rinsing and toothpaste regime was specified in a restricted time scheme, which the participants were encouraged to follow. To increase the compliance, a message was sent daily (Study II) as a reminder to the participants during the test periods and before each visit to the lab (Study II, IV). For further evaluation of compliance, the participants were asked, at each visit, if they had followed the instructions. In Study II, the concentration of the urea mouth rinse (0.25%) was decided after initial tests on a group of volunteers to find a concentration that could be acceptable to use.

Caries registration

In Study IV, the participants were divided into a caries-free and a caries group based on clinical examinations using a sterile probe and clinical loupes with suitable LED light. Undergoing in this manner, initial caries and specifically interproximal caries, may be missed. Thus, a radiographical examination would have been necessary for a more detailed and thorough caries investigation. Lower baseline-pH was found in this study compared with Study II. This may be due to older participants and initial caries or a higher caries activity in the caries group in Study IV.

Statistical considerations

No power analysis was performed in Study II due to the lack of *in vivo* studies on plaque adaptation to acid and alkali in relation to caries activity. The sample size used was based on a previous study (Aranibar et al., 2003) where statistically significant differences between groups of 10-20 individuals with respectively high and low capacity of acid formation in the dental plaque, was revealed. This was also seen in Study IV and no further power analysis was performed for Study IV.

ETHICAL CONSIDERATIONS

All the *in vivo* studies in this thesis (Study I-IV) followed the ethical considerations of the Helsinki declaration. Verbal and written detailed information about each study including possible risks and adverse effects was provided to the participants prior to the start. Each participant had agreed to participate voluntarily in the study, and had given written consent (Study I-IV). Parents or legal guardians had given informed consent for underage participants prior to the study (Study II). All the data remained confidential, and no identifiable personal data has been published.

Any risk for negative consequences and side effects by using 'strip method' orally must be considered minimal or none. All pH-measurements, plaque and saliva collections, were performed in a clinical environment, regulated by health and safety rules. All instruments and materials used were sterilised to avoid any risk for cross-infection.

The Regional Ethical Review Board (Dnr 736-14) in Gothenburg (Sweden) approved Study II (Dnr 282-10) and Study III (Dnr 736-14). Study IV, was ethically approved by the Ethics Committee at the Middlesex University in London, United Kingdom (NSESC 1570). Study V was an *in vitro* study based on bacterial isolates for which no ethical approval was needed.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The main findings of this thesis suggest that measurement of individual supragingival plaque-pH by using chair-side methods such as the 'strip method' and 'RUT', might be useful for individual caries risk assessment. However, for further evaluation of using plaque-pH and alkaline activity for individual caries risk assessment, randomised controlled studies with larger sample sizes, studies on the short- and long-term intraindividual variations and the effect of acid and alkali adaptation in relation to different caries activity (low, moderate and high) as well as prospective studies are of interest.

It is also of interest to confirm the present findings suggesting that dysbiosis, characterized by increased numbers of known cariogenic bacteria, is initiated by the adaptation of bacterial species characterizing the healthy plaque to increased acid formation. For better understanding of the bacteria involved, acid tolerant bacteria isolated after anaerobic growth and identified by molecular-based technique could be used.

The frequent short-term usage of a suggested anti-cariogenic alkali product was found to affect pH and alkali formation in interproximal plaque, as well as saliva pH and buffer capacity in caries-active but not in caries-free individuals. To evaluate possible effects in individuals with none or low caries activity, long-term, prospective studies on larger sample sizes should be performed using the 'strip method' and 'RUT'.

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