Cariogenic potential of dental biofilm bacteria
- support for caries as a polymicrobial disease

Carolina Robertsson
Soha Abdul Rahim

Supervisors: Gunnel Svensäter, Madeleine Blomqvist and Bertil Kinnby

Thesis in Odontology (30 ECTS)
Dental program
June, 2017

Malmö University
Faculty of Odontology
205 06 Malmö
Cariogenic potential of dental biofilm bacteria

- support for caries as a polymicrobial disease

Carolina Robertsson
Soha Abdul Rahim

Handledare: Gunnel Svensäter, Madeleine Blomqvist and Bertil Kinnby

Examensarbete (30 hp) Malmö högskola
Tandläkarprogrammet Odontologiska fakulteten
Juni, 2017 205 06 Malmö
Table of Contents

Abstract ................................................................................................................................. 1

Sammanfattning .................................................................................................................. 1

Introduction .......................................................................................................................... 2

  Background ......................................................................................................................... 2

  Aim ........................................................................................................................................ 5

Research questions ............................................................................................................... 5

Hypotheses ............................................................................................................................ 5

Materials and methods ......................................................................................................... 6

  Bacterial strains ................................................................................................................... 6

  Growth media ....................................................................................................................... 6

  Bacterial growth in different environments ...................................................................... 7

  Growth measurement ........................................................................................................... 7

  pH measurement ................................................................................................................ 7

    Acid production .................................................................................................................. 7

    Alkali production in artificial consortium ..................................................................... 7

    Alkali production in oral consortium ............................................................................. 8

Statistical analysis ................................................................................................................. 8

Results .................................................................................................................................. 9

  Comparison of growth at neutral and acidic pH .............................................................. 9

  Acid production at low pH ................................................................................................. 11

  Growth in complex media with and without glucose ......................................................... 12

  Acid production in different growth media with and without glucose ............................. 13

  Control media ..................................................................................................................... 14

  Alkali production ............................................................................................................... 15

Discussion .............................................................................................................................. 17

  Project meaning .................................................................................................................. 21

  Conclusion ......................................................................................................................... 21

References ............................................................................................................................. 23
Abstract

Aim: To investigate dental biofilm bacteria for growth and acid production in different environments. The occurrence of inherent buffering capacity by alkali production in an artificial consortium and oral consortium was also examined.

Materials and method: Fresh isolates of Actinomyces, Lactobacillus paracasei, Streptococcus mutans, Streptococcus intermedius and Streptococcus oralis were incubated in growth media with neutral or acidic initial pH and with and without glucose and/ or urea. Growth was monitored by measuring optical density at 600nm. Acid and alkali production was monitored using a pH meter.

Results: L. paracasei and S. intermedius showed growth independent of pH and a more acidic final pH at acidic than neutral initial pH. S. oralis and Actinomyces showed a greater growth at neutral pH and had an acid production insensitive to an acidic environment. All species reached a final pH below the critical pH for enamel (5.5) independent of initial pH. Growth was unaffected by glucose. In oral consortium, the final pH was less acidic in the medium with glucose and urea compared to in the medium with only glucose.

Conclusion: All species showed acidic and aciduric properties and may contribute to tooth demineralization. Further studies with a larger number of measurements are needed to assess the cariogenicity of these species with a higher reliability. Alkali production in saccharolytic species may affect the plaque pH. Further studies are needed to assess the occurrence of alkali production in saccharolytic species related to caries and its effects on the pH in the biofilm.

Sammanfattning

Syfte: Tillväxt och syraproduktion undersöktes i olika suspensioner hos bakterier som är vanligt förekommande i den dentala biofilmen. Förekomst av bufferingskapacitet till följd av proteolytisk aktivitet i artificiellt konsortium och oralt konsortium undersöktes också.


Introduction

Background

The intraoral milieu is thriving with microlife, with a wide diversity of bacterial species that co-exist and interact with one another in different ways (1,2). The microbial plaque that occupies all surfaces inside the mouth harbours a variety of microenvironments. These microenvironments influence and are influenced by the microbes that inhabit that particular local sphere. Different bacteria prosper in different ecological niches. The colonization of a surface takes place through an array of events and is ever changing. (1,3,4)

The features of each surface that influence the conditions for initial colonization vary according to the type of tissue, local oral clearance, type and availability of nutritional supply and pH as well as capacity and accessibility for the host immune mechanisms. (2,5-7)

Oral clearance interferes with static adhesion mechanically. Saliva also has qualities that affect microbial capacity for colonizing and metabolizing, such as pH-buffering HCO₃⁻ ions and a variety of proteins. The proteins in saliva have a broad spectrum of functional mechanisms. They can both inhibit bacterial colonization and growth, like lysozyme and lactoferrin, but also favor bacterial metabolism like mucins. (2,6)

Nutritional supply greatly affects what species will be able to reside at a specific location more permanently (1,5,8). Different bacteria tend to be drawn to local niches and if they get the opportunity, settle in the preferred location and contribute to a growing biofilm that organizes over time. Bacterial metabolism will in turn start affecting the habitat, and lead to alterations in the characteristics of the microenvironment, such as changes in pH and oxygen supply. (3,8) This specialization of the local milieu will then allow for succession, previously less common microorganisms may become more competitive and colonize the surface under the newly established environmental circumstances (1,3,8). This dynamic process is what is described in the ecological plaque hypothesis (3,8).

The ecological plaque hypothesis suggests that caries is caused by opportunistic pathogens that become competitive and it is a sequence of events that leads to disease (3-5,8). Different bacteria may fill similar functions in a multibacterial biofilm and may be able to contribute to disease development (3,5,8). There does not seem to exist any single pathogen and therefore there is no point in searching for a treatment to kill a specific pathogen. A broader approach seems to be necessary. (1,3,8)

In caries initiation frequent carbohydrate intake, especially in combination with other risk factors such as reduced flow of saliva, affects the composition of biofilms accumulated on tooth surfaces. As the acid production and oxygen consumption are raised in plaque as a result of the shift in nutritional supply, the proportion of saccharolytic acidogenic, aciduric species are increased and a gradual change in the local milieu follows. (1,2,9) Species such as streptococci, lactobacilli and actinomyces are given an advantage to establish themselves in
increasing numbers since they have the ability to adjust to a more acidic environment (1,2). The pH of the biofilm and plaque fluid declines gradually due to the accumulation of acidic waste products. The mean pH in saliva is 6.75-7.25 under normal conditions. When the local pH in the biofilm decreases below the “critical pH” of 5.5 for enamel, demineralization of the tooth surface takes place and a lesion is established. (2) Oral streptococci and lactobacilli have been shown to continue to grow and metabolize at pH values well below the critical pH (1). Bacteria obtain energy in the form of ATP from glycolysis. Metabolite residues are acids such as formic acid, acetic acid and ethanol. At higher sugar concentrations lactic acid is produced, which gives the most deleterious effects on the tooth surfaces because of its very low pH. Veillonella species utilizes lactic acid and other metabolites from saccharolytic fermentation for its own metabolism. Earlier colonizers enable attachment and their metabolism creates more suitable environments for later colonizers. (2)

The specific characteristics of the bacteria associated with caries are:

1. Sugar transport; a more effective sugar uptake in comparison to other microorganisms present in the plaque.
2. Acidogenicity; rapid conversion of carbohydrates through the glycolytic pathway.
3. Aciduricity; adaptation to the altered environment with lowered pH.
4. Production of extracellular polysaccharides (EPS); increasing the plaque volume and thereby promoting bacterial colonization by enabling attachment, and altering the diffusion properties of the dental plaque. (2,9)
5. Production of intracellular polysaccharides (IPS); storing nutrition (2)

It is known that some oral bacteria, mainly described for S. mutans, have an ability to induce an acid tolerance response (ATR) to keep a functional metabolism when subjected to acidic environments (1). The response is due to changes in the cellular physiology (10). ATR is attributed to a change in gene expression, but the full mechanism of action is not yet known (1,11). Lactobacilli are speculated to have an inherent ATR while the ATR of S. mutans is suggested to be adaptive (10). This means that different bacteria have different prerequisites to survive in an acidic environment. A previous study (12) showed that S. mutans in established biofilms have a higher ATR than planktonic bacterial cells.

Some of the most commonly found resident microbes in oral plaque are the four main groups of Streptococci, namely the Mutans-, Salivarius-, Anginosus- and Mitis-groups. Other commonly found species are Actinomyces and Lactobacillus. Neisseria- and Veillonella-groups can normally also be found. S. mutans has been considered the main pathogen for caries since the 1960s. This study will focus on three species of Streptococci (S. intermedius, S. oralis, S. mutans), one strain of Actinomyces and one strain of Lactobacillus (L. paracasei). These strains constitute a large proportion of common oral bacteria found in supragingival plaque. (2)

A study made in vitro showed that mutans streptococci have the ability to produce acid at the highest rate and that they produce the highest amount of acid, in comparison to other oral streptococci (13). S. mutans has been seen to produce a final pH in the range between pH
3.95-4.10 (2). The acid that is converted from carbohydrates is mainly lactic acid (2,14). *S. mutans* also has the cariogenic ability to produce IPS and EPS (2,15).

Another streptococcus that frequently occurs in the resident microflora is *S. intermedius*, which belongs to the Anginosus-group. This strain is acidogenic and aciduric, but does not produce EPS. *S. oralis* is a strain of the bigger Mitis-group and is one of the most common aciduric species in the mouth. (2,16) Some strains produce extracellular glucan (2,9). *S. oralis* is commonly recovered from the lips, cheek, dorsum of the tongue and supragingival plaque in health, but also from carious sites. It has been shown that *S. oralis* can produce a final pH in the range between pH 4.05-4.5. (2) An extensive variation in biochemical and physiological features in different strains of *S. oralis* has been found, which may indicate a great adaptability to different environmental stresses of the species (13).

Lactobacilli have recently been associated with advanced caries, because of their high acid tolerance and acidogenicity. A high prevalence of Lactobacilli in root caries has been seen. The *L. paracasei* is a hetero-fermentative species which produces lactic acid and acetate. (2) *Lactobacillus* has been associated with caries progression to a higher extent rather than the initiation of caries lesions (2,9).

*Actinomyces* species are heterotrophic rods, they are mainly short rods but can also be filamentous. They are common in oral plaque, especially on approximal sites and in the gingival crevice. *Actinomyces* are saccharolytic and metabolic end products are succinate, acetate and lactic acid. Urease production has been seen in some species. (2)

During cariogenic progression, bacteria from the resident microflora with proteolytic abilities that produce alkali products are partly outrivaled when the plaque becomes dominated by acid producing, aciduric species (17,18). However, cariogenic species such as *S. mutans* and *Lactobacillus* are also known to be able to excrete proteases. The breakdown of peptides from the saliva and collagen leads to accumulation of alkaline waste products that may help buffer the continuously decreasing pH from the saccharolytic metabolism. (2,18,19) Bacterial urease hydrolyzes urea to ammonium and CO₂ (18). It has been shown that some oral Streptococci such as *S. mutans* can induce urease synthesis at low pH, which may contribute to the buffering system and protect them against harmful acidification (1).

A previous study (13) investigated the acid production per time unit (\(V_{ap}\)) at different pH of nine different species of Streptococci. Among these were *S. mutans*, *S. oralis* and *S. intermedius*. In this study, some strains of *S. oralis*, and also *S. mitis*, were found to have a higher \(V_{ap}\) than some strains of *S. mutans*, especially at pH values around 6-7. This suggests that these strains may play important roles in initiating cariogenic lesions. When the pH value decreased, some strains of *S. mutans* and *S. mitis* maintained a relatively high \(V_{ap}\). This suggests that these strains may also play important roles in maintaining caries. To our knowledge, this study was the only one that included non-mutans Streptococci. No studies were found that investigated other species than Streptococci, or where consortia of multiple species were used.
Previous studies (17,20) showed that the urease-activity was higher in oral plaque from caries free subjects compared to caries active subjects. The aim of our study was not to conduct a comparison, but to examine the alkali production of certain bacteria associated with caries. A pilot study (21) showed no statistically significant effect when measuring pH before and after an adaptation period of rinsing with urea. However, a slightly larger increase in pH was noted in caries active subjects 5-10 minutes after rinsing, compared to caries free subjects. A suggested explanation for this was that the cariogenic plaque contained microorganisms that were already adapted to a more acidic environment, and therefore had a greater capacity for urease-activity as a protecting mechanism.

Aim

The aim was to investigate dental biofilm bacteria for growth and acid production in different environments. The occurrence of inherent buffering capacity by alkali production in an artificial consortium and oral consortium was also examined.

Research questions

How does the growth and acid production of *Lactobacillus paracasei*, *Streptococcus intermedius*, *Streptococcus oralis*, *Streptococcus mutans* and *Actinomyces* vary in different growth media with and without glucose and with an initial pH of 7.2 vs 5.5?

Can production of NH$_3$ from breakdown of urea counteract pH drops in plaque or consortium?

Hypotheses

1. All tested strains will be able to adapt to an initial pH of 5.5 due to aciduric properties, but since an acidic environment constitutes a stress factor, their growth will not be as great as at neutral initial pH. Their acidurity will also enable a continued acid production at acidic initial pH. However, because of differences in metabolic products between species the final pH may vary.

2. Because of their saccharolytic nature, all tested strains will have an increased growth in suspensions with glucose compared to suspensions with only 25% or 10% Todd Hewitt medium with an initial pH of 7.2. Due to accumulation of acidic waste products from their saccharolytic metabolism, all strains will also reach a final pH below the critical pH of enamel (5.5) in all media with glucose.

3. In the experiments with the consortium with added urea, an increase in pH will be seen after some time due to bacterial expression of urease and thereby breakdown of urea which will reverse the fall in pH.
Materials and methods

Bacterial strains

The bacterial strains used in this study were fresh isolates collected from patients. *Lactobacillus paracasei*, *Streptococcus intermedius*, *Streptococcus oralis*, *Streptococcus mutans* and *Actinomyces* were used. Species were identified and classified by sequencing the 16S rRNA gene at the department for Clinical microbiology in Lund and with enzyme- and fermentation test at the department for Oral Biology, Faculty of Odontology, Malmö. The strain of *Actinomyces* used in this study is currently being identified by genetic sequencing by the Faculty of Odontology, in Malmö. The fresh isolates were stored in cryotubes with skimmed milk at -80°C. The bacteria were thawed for 10 minutes at room temperature, and then spread on blood agar with an inoculation loop. The bacterial isolates were then grown overnight in 37°C in an atmosphere of 5% CO₂.

Growth media

For bacterial growth monitoring and pH measurement for acid production, six different growth media based on Todd Hewitt broth (Becton Dickinson, Sparks, MD 21152, USA) were used. Todd Hewitt medium is a broth containing Beef heart infusion 3.1g/L, Neopeptone 20g/L, Dextrose 2g/L, Sodium Chloride 2g/L, Disodium Phosphate 0.4g/L, Sodium Carbonate 2.5g/L. Todd Hewitt with different concentrations was used to model conditions in the oral cavity.

The growth media were (a) 100% Todd Hewitt medium pH 7.2, (b) 100% Todd Hewitt medium pH 5.5, (c) 25% Todd Hewitt medium pH 7.2, (d) 25% Todd Hewitt medium pH 7.2 + 10mM glucose, (e) 10% Todd Hewitt medium pH 7.2 and (f) 10% Todd Hewitt medium pH 7.2 + 10mM glucose. Distilled water was used for dilution.

For pH measurements investigating alkali production in an artificial consortium, the growth media were (1) 25% Todd Hewitt medium pH 7.2, (2) 25% Todd Hewitt medium pH 7.2 + 10mM glucose (3) 25% Todd Hewitt medium pH 7.2 + 10mM glucose + 500mM urea. Distilled water was used for dilution.

For pH measurements investigating alkali production in oral consortium, the growth media were (α) 10mM PBS pH 7.2, (β) 10 mM pH 7.2 + 10mM glucose (γ) 10 mM PBS pH 7.2 + 10mM glucose + 500mM urea. Distilled water was used for dilution.

pH 7.2 was chosen as an initial pH because it is close to salivary pH and favors growth of most of the oral bacteria (2). Streptococci and Lactobacilli are known to be aciduric and therefore a pH of 5.5 was also tested to see how well they managed to adapt to this lower pH. pH in plaque has been measured to be even lower than this during cariogenic activity. 5.5 is known to be the critical pH at which demineralization of enamel starts. (22)
Bacterial growth in different environments

Growth of single species was investigated in different environments as follows. 2 µl of each bacteria was inoculated from blood agar with inoculation loops separately into 2.5 ml growth media a-f in sterile tubes. Each suspension was vortexed for 30 seconds. The OD\textsubscript{600} was measured at 0 hours at 600 nm with a spectrophotometer, using 800 µl of bacterial suspension. 1ml of each suspension was added to polystyrene twelve-well plates for measuring OD\textsubscript{600} after 24 hours. The twelve-well plate was placed in a humid chamber to reduce the evaporation and was incubated between measurements in 37°C with an atmosphere of 5% CO\textsubscript{2}. The OD\textsubscript{600} was measured in 800 µl of each suspension after 24 hrs of incubation.

Growth measurement

In the current study, a spectrophotometer was used for estimating the number of bacteria present in a liquid medium by measuring the optical density (OD\textsubscript{600}). This method is commonly used for monitoring bacterial growth over time. Light is transmitted through a sample of the bacterial suspension to a photometric cell that registers the amount of light that passes through the medium. More cells give a higher turbidity which leads to scattering of the light and less light is able to pass through the medium. The amount of light that passes through is measured in percent transmission, %T. Thus fewer cells will give a higher value of %T. The OD\textsubscript{600} is the opposite of %T as it describes the amount of light scattered. This enables a more straightforward visualization since fewer cells will instead give a lower value of OD\textsubscript{600}. To get a reading on the spectrophotometer, a minimum of 10-100 million cells per ml suspension is needed. (23) Fold change was calculated by dividing the mean growth in the neutral medium with the mean growth in the acidic medium.

pH measurement

All pH measurements were carried out using a calibrated pH meter. The pH meter was rinsed with distilled water between measurements.

Acid production

Investigation of acid production was carried out using single species. 2 µl of each bacteria were inoculated from blood agar with inoculation loops to 2.5 ml of growth media a-f separately. Each suspension was vortexed for 30 seconds. 100 µl of each suspension was added in duplicates to two 96-well-plates. As a control, duplicates of 100 µl of growth media was also added to the 96-well plates. The initial pH was measured at 0 hours in one of the 96-well plates. The other 96-well plate was placed in a humid chamber to reduce evaporation and incubated for 24 hrs in 37°C with an atmosphere of 5% CO\textsubscript{2}. After 24 hours the pH was measured in the incubated 96-well plate.

Alkali production in artificial consortium

These experiments were carried out with the five bacterial species in a consortium. 2µl of each bacterial strain was inoculated from blood agar with inoculation loops to 5 ml of growth media 1-3 separately. Each suspension was vortexed for 30 seconds. 100µl of each bacterial suspension was added to 96-well-plates in duplicates for each point of measurement, in total
14 wells. As a control, duplicates of 100 µl of growth media was also added to the 96-well plates. The two 96-well plates were placed in a humid chamber to reduce evaporation and incubated between measurements in 37°C with an atmosphere of 5% CO₂. After 0, 30, 60 and 120 minutes and 24, 48, 72 hours the pH was measured in the incubated 96-well.

Alkali production in oral consortium
These experiments were carried out with dental plaque. Plaque was collected with sterile Quick-sticks® from approximal and gingival surfaces from one subject before breakfast. The plaque was suspended in 1.3 ml of growth media α-γ separately. Each suspension was vortexted for 30 seconds. 100µl of each bacterial suspension was added to 96-well-plates in duplicates for each point of measurement, in total 10 wells. As a control, duplicates of 100 µl of growth media was also added to the 96-well plates. The 96-well plates were placed in a moist container to reduce evaporation and incubated between measurements in 37°C with an atmosphere of 5% CO₂. After 0, 30, 60 and 120 minutes and 72 hours the pH was measured in the incubated 96-well.

Statistical analysis
Growth from 0h to 24h within species was tested for significance by paired t-test. Comparison in growth between species was tested for significance by a one-way ANOVA (analysis of variance); Kruskal-Wallis test with Dunn’s multiple comparison as post test. Differences in pH measurements for acid production were tested for significance by paired t-test. p<0.05 was selected as significance level for all tests.
Results
Comparison of growth at neutral and acidic pH

The purpose of this experiment was to compare growth of different species of oral bacteria in Todd Hewitt broth at neutral and acidic initial pH. This was done by measuring OD$_{600}$ at 0 and 24 hours. A mean value (0.174) was calculated for the initial OD$_{600}$ of all species. The main results are presented in fig. 1-3.

In the medium with a neutral initial pH, *S. mutans* reached an OD$_{600}$ of 1.2–1.3 in all three experiments, while the other species reached a mean OD$_{600}$ of 0.4-1.0 and showed a larger diversity between experiments, see fig. 1. However, the differences were not statistically significant (ANOVA p=0.09). In the medium with an acidic initial pH, all species reached a mean OD$_{600}$ of between 0.3-0.8, see fig. 2. The differences were not statistically significant (ANOVA p=0.09).

![Figure 1](image1.png) **Figure 1. Growth at neutral initial pH.** Depicting bacterial growth in 100% Todd Hewitt broth with an initial pH of 7.2, measured with spectrophotometer at 0 and 24 hours.

![Figure 2](image2.png) **Figure 2. Growth at acidic initial pH.** Depicting bacterial growth in 100% Todd Hewitt broth with an initial pH of 5.5, measured with spectrophotometer at 0 and 24 hours.

Fold change was calculated by dividing the mean growth in the neutral medium with the mean growth in the acidic medium *L. paracasei, S. mutans, S. oralis* and *Actinomyces* showed a more extensive growth in the medium with a neutral initial pH compared to an acidic initial pH (fold change 1.2, 1.5, 2.2 and 2.0 respectively), see fig. 3. On the contrary, growth for *S. intermedius* was higher in the medium with an acidic initial pH (fold change 0.8). None of these differences were significant at the 5% level. All species showed growth at both neutral and acidic initial pH (p= 0.0046 and 0.0054 respectively).
Figure 3. Comparison of growth at neutral and acidic pH.
Depicting bacterial growth in 100% Todd Hewitt broth with an initial pH of 7.2 and 5.5 respectively, measured with spectrophotometer at 0 and 24 hours. Dashed lines connect experiments inoculated at the same time to ensure congruity.
Acid production at low pH

The purpose of this experiment was to compare acid production in different species of oral bacteria in Todd Hewitt broth at neutral and acidic initial pH. This was done by measuring pH at 0 and 24 hours. The main results are presented in Fig. 4.

**Figure 4. Final pH in neutral and acidic media.** Depicting pH change in 100% Todd Hewitt broth with an initial pH of 7.2 and 5.5 respectively, measured with a pH meter at 0 and 24 hours.

All species reached a final pH below pH 5.5 in both media, see fig. 4.

All species showed a decrease in pH in the medium with a neutral initial pH (p=0.0001). *L. paracasei, S. intermedius, S. mutans* and *Actinomyces* showed a decrease in pH in the medium with acidic initial pH (p=0.0001). *S. oralis* also showed a decrease in pH in the medium with an initial acidic pH (p=0.0017).

Of all species, *S. mutans* showed the lowest mean pH in the medium with a neutral initial pH after 24 hours, pH 4.89. *L. paracasei* and *S. mutans* both showed the lowest mean pH in the medium with an acidic initial pH after 24 hours, pH 4.21.
Growth in complex media with and without glucose

The purpose of this experiment was to compare growth in different species of oral bacteria in Todd Hewitt broth with and without 10 mM glucose at pH 7.2. This was done by measuring OD$_{600}$ at 0 and 24 hours. A mean value was calculated for the initial OD$_{600}$ of all species (0.197 in 25% TH and 0.212 in 10% TH). The main results are presented in Table 1.

**Table 1. Effects of glucose on growth.** Depicting fold change in growth from 0 to 24 hours in 25% Todd Hewitt with glucose compared to 25% Todd Hewitt without glucose, and 10% Todd Hewitt with glucose compared to 10% Todd Hewitt without glucose.

<table>
<thead>
<tr>
<th>Species</th>
<th>25% TH + glucose (Fold change)</th>
<th>p-value (Wilcoxon t-test)</th>
<th>10% TH + glucose (Fold change)</th>
<th>p-value (Wilcoxon t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. paracasei</em></td>
<td>0.6</td>
<td>0.5</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>2.0</td>
<td>0.25</td>
<td>1.6</td>
<td>0.25</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>1.1</td>
<td>0.5</td>
<td>0.8</td>
<td>0.25</td>
</tr>
<tr>
<td><em>S. oralis</em></td>
<td>1.2</td>
<td>0.25</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td><em>A. spp.</em></td>
<td>0.3</td>
<td>0.25</td>
<td>1.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Fold change <1 = Growth was more extensive in the medium without glucose. 
Fold change = 1 = The growth did not differ between the media. 
Fold change >1 = Growth was more extensive in the medium with glucose.

*L. paracasei* and *Actinomyces* showed a pattern of more extensive growth in the growth media with 25% TH without glucose compared to in a growth media with glucose. On the contrary, the pattern for *S. intermedius*, *S. mutans* and *S. oralis* showed a more extensive growth in the media with glucose. All species showed growth in 25% TH both with and without glucose (p= 0.007 and 0.03 respectively).

*L. paracasei*, *S. intermedius*, *S. oralis* and *Actinomyces* showed a more extensive growth in the growth media with 10% TH + glucose compared to in a growth media without glucose. On the contrary, growth for *S. mutans* was more extensive in the media without glucose. All species in the media with 10% TH with and without glucose showed growth (p= 0.111 and 0.172 respectively).

In the media with 25% and 10% TH with glucose, *S. intermedius* showed a fold change of 2.0 and 1.6 respectively, see table 1. In the medium with 25% TH + glucose, the fold change was
statistically significantly higher for *S. intermedius* compared to *Actinomyces* (ANOVA p=0.03).

Acid production in different growth media with and without glucose

The purpose of this experiment was to compare acid production in different species of oral bacteria in growth media with and without 10 mM glucose at pH 7.2. This was done by measuring pH at 0 and 24 hours. The main results are presented in table 2 and 3.

**Table 2.** Final pH after 24 hrs of incubation in 25 % Todd Hewitt (TH), with and without 10 mM glucose.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th><em>L. paracasei</em></th>
<th><em>S. intermedius</em></th>
<th><em>S. mutans</em></th>
<th><em>S. oralis</em></th>
<th><em>Actinomyces spp.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours 25% TH without glucose</td>
<td>4.9</td>
<td>5.3</td>
<td>4.9</td>
<td>5.4</td>
<td>5.9</td>
</tr>
<tr>
<td>24 hours 25% TH with glucose</td>
<td>4.1</td>
<td>4.4</td>
<td>4.1</td>
<td>5.2</td>
<td>4.5</td>
</tr>
</tbody>
</table>

**Table 3.** Final pH after 24 hrs of incubation in 10 % Todd Hewitt (TH), with and without 10 mM glucose.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th><em>L. paracasei</em></th>
<th><em>S. intermedius</em></th>
<th><em>S. mutans</em></th>
<th><em>S. oralis</em></th>
<th><em>Actinomyces spp.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours 10% TH without glucose</td>
<td>5.1</td>
<td>5.7</td>
<td>5.0</td>
<td>5.5</td>
<td>6.7</td>
</tr>
<tr>
<td>24 hours 10% TH with glucose</td>
<td>3.9</td>
<td>4.5</td>
<td>4.1</td>
<td>5.3</td>
<td>4.6</td>
</tr>
</tbody>
</table>

*L. paracasei, S. intermedius, S. mutans* and *Actinomyces* showed a decrease in pH in both media with 25% TH (p=0.0001). *S. oralis* also showed a decrease in pH in the medium without glucose (p=0.0026) and with glucose (p=0.003). The general pattern of acid production in 10% TH was the same as in 25% TH for these experiments, see table 2 and 3. All species showed a decrease in pH in the media with 10% TH with and without glucose (p=0.0001).

*L. paracasei, S. intermedius, S. mutans* and *S. oralis* reached a final pH below pH 5.5, in both media with 25% TH. *Actinomyces* reached a final pH of 5.9 in the medium without glucose, but the final pH in the medium with glucose was below 5.5. In the experiments with 10% TH, *S. intermedius* and *Actinomyces* were the only species that did not reach a final pH below 5.5 in the medium without glucose. All species reached a final pH below 5.5 in the medium with 10% TH with glucose.

Of all species, *S. mutans* showed the lowest mean pH in the medium with 25% TH without glucose after 24 hours, pH 4.9. *S. mutans* also showed the lowest mean pH in the medium
with 25% TH with glucose after 24 hours, pH 4.08. In the experiments with 10% TH, *S. mutans* showed the lowest mean pH in the medium without glucose after 24 hours, pH 5.03. Contrary to in the medium with 25% TH with glucose, *L. paracasei* showed the lowest mean pH in the medium with 10% TH with glucose after 24 hours, pH 3.91.

Control media

The mean pH in all the neutral control media at 0 hours was 7.36 with a standard deviation of 0.09. The mean pH in all the acidic control media at 0 hours was 5.79 with a standard deviation of 0.05. After 24 hours the mean pH in all the neutral control media was 7.06 with a standard deviation of 0.19. After 24 hours the mean pH in all the acidic control media was 5.75 with a standard deviation of 0.075.
Alkali production

The purpose of this experiment was to investigate if breakdown of urea can counteract pH drops in artificial or oral consortium in growth media. This was done by measuring pH. The main results are presented in Fig. 6 and 7.

**Figure 6. Alkali production in an artificial consortium.** Depicting pH change in 25% Todd Hewitt broth with and without glucose and urea with an initial pH of 7.2 measured with a pH meter at 0, 30, 60, 120 minutes and 24, 48 and 72 hours for an artificial consortium.

In the artificial consortium the pH dropped to 4.41 after 24 hours in the medium with glucose, see fig. 6. In the medium with glucose and urea the pH dropped to 4.47 after 24 hours. At 72
hours the pH had risen and the final pH was 4.85 in both media. In the oral consortium the pH dropped to 6.78 after 30 minutes in the medium with glucose, see fig. 7. In the medium with glucose and urea the pH dropped to 6.96 after 30 minutes. At 180 minutes the final pH was 6.42 in the medium with glucose. In the medium with glucose and urea the pH had risen and the final pH was 7.44 at 180 minutes.
Discussion

To better understand the etiology of dental caries, it is important to assess the acidogenic and aciduric properties of bacteria present in the supragingival biofilm. *S. mutans* and *L. paracasei* have traditionally been considered the main pathogens of caries. (24) In recent time, a more polymicrobial etiology for caries has been suggested (25) and it is therefore of interest to investigate the aciduric profile of other saccharolytic bacteria that might contribute to the development of caries. The aim of this study was to examine the acid production and growth in an acidic environment in bacterial species commonly found in supragingival biofilms. The species selected were *S. intermedius*, *S. mutans*, *S. oralis*, *L. paracasei* and *Actinomyces*. All these microbes are considered saccharolytic and can be found in supragingival dental biofilms on intact as well as carious tooth surfaces. *S. oralis* and *Actinomyces* are early colonizers and generally constitute a large proportion, up to 40%, of supragingival biofilms. Both species have also been associated with the early stages of enamel demineralization. *S. intermedius*, *S. mutans* and *L. paracasei* are normally present in supragingival plaque only in small numbers, up to 2%. However, *S. mutans* is clearly associated with early as well as more advanced caries lesions while *L. paracasei* is more related to advanced caries. Non-mutans streptococci and *Actinomyces* have been found to be more prevalent in initial caries lesions compared to *S. mutans*. Of interest is that no single bacterial species can be uniquely related to caries in the sense that demineralization would occur only in the presence of a specific species that would never be found at sites without evidence of demineralization. Overall, this brings up the polymicrobial etiology of caries in which the contribution by different microbes is not yet fully understood. (2,25,26)

As a first step, the ability to grow at pH 5.5 was examined in order to determine the aciduric properties of the strains selected for this investigation. One clinical study (27) showed that the proportion of dental plaque that exhibited acid tolerance was constituted of a large variety of cell morphologies. This suggests that a number of bacterial species may contribute to the acid tolerance of the microbiota in dental plaque. A larger proportion of acid tolerant flora in the dental plaque may lead to an increased risk of developing caries. A previous study (28) reported that *Lactobacillus* and *S. mutans* exhibited slightly greater growth at acidic initial pH compared to non-mutans streptococci (*S. oralis* and *S. intermedius*) and *Actinomyces*. In accordance with our Hypothesis 1, growth was seen for all test strains at pH 5.5 in the current study. Contrary to the hypothesis however, *L. paracasei* and *S. intermedius* showed a pattern of approximately the same growth independent of neutral or acidic initial pH. This leads to the assumption that the growth of these species may be less affected by an acidic environment than the other species. Contrary to previous findings, no statistically significant differences in growth between neutral and acidic initial pH was shown for any species. This may be due to a wide dispersion of values, and no conclusion can be drawn in comparing the species. It is also worth noting that in the current study, only fresh isolates of clinical strains were used since they would more properly reflect the real life situation than type collection strains. The latter have the disadvantage of being in storage for a long period of time and exposed to multiple
growth conditions in the laboratory that may lead to alterations in their properties, such as acid tolerance.

Many previous studies have investigated the cariogenicity of mutans streptococci and lactobacilli, but few studies have focused on other oral bacteria, that are likely to have similar acidogenic and aciduric properties. In the current study, the tested strains of *S. intermedius*, *S. mutans*, *S. oralis*, *L. paracasei* and *Actinomyces* showed growth and acid production in TH broth at pH 7.2, giving rise to final pH values below 5.5, the critical pH below which demineralization of enamel occurs. Also in highly diluted TH broth, 25% and 10% respectively, *L. paracasei*, *S. mutans* and *S. oralis* exhibited a significant acid production resulting in final pH-values below 5.5. It is important to note that the pH drops occurred even though no additional glucose support was given to the growth medium. TH is traditionally used when cultivating oral bacteria and has a high nutritional content consisting of different proteins, peptides, carbohydrates and minerals, which makes it difficult to discern what molecules constitute the source of nutrition and take part in the metabolic processes that results in acid production and lower pH-values. *Actinomyces* and *S. intermedius* did not reach a final pH below 5.5 in diluted TH indicating that these strains are more dependent on the surrounding environment and nutritional supply compared to the other species tested and may therefore be considered less acidogenic when nutrient supply is limited. Contrary to our Hypothesis 2, no effect of glucose on the growth was seen in this study. All species showed growth in both 25% and 10% TH with and without glucose. Furthermore, no relation was seen between the growth and acid production. Further studies are required to investigate the correlation between these mechanisms more thoroughly.

The expression ‘feast and famine’ have been used to describe the general nutrient supply available to bacteria in supragingival biofilms. ‘Famine’ is the presumed prevailing condition during much of the day while the ‘feast’ occurs during intake of rapidly degraded carbohydrates. (11) The pH in dental biofilms can change up to four units, depending on the age of the biofilm and concentrations of the sugar (29). In the current study, the addition of glucose to TH resulted in pronounced pH drops to a final pH ranging from 3.9 to 5.3, a finding that was in accordance with our Hypothesis 2. One previous study (30) compared the extent of the lesions formed on root and enamel surfaces on 192 molars *in vitro* after being exposed to different carbohydrate solutions with *S. mutans* and *Actinomyces viscosus* respectively over three weeks. In some cases, presence of *S. mutans* led to statistically significantly deeper lesions in root as well as enamel surfaces compared to *A. viscosus*. In other cases however, presence of *A. viscosus* resulted in similar or more extensive lesions on root and enamel surfaces. This finding suggests that *Actinomyces* actually possesses a cariogenic capacity, even if it is not clear how this compares to that of the *S. mutans* or other potentially cariogenic species. Collectively, our results combined with those of others suggest that if exposed to a frequent carbohydrate intake *in vivo* an array of bacterial species can contribute to the acid production and create an acidic environment that give rise to demineralization of enamel.
The rationale behind investigating acid production at pH 5.5 was that individuals with a frequent intake of carbohydrates, or a low buffering capacity, are expected to experience a lower pH in dental biofilms both between meals and over longer periods of time in relation to carbohydrate intake. Moderately low pH, such as 5.5, can elicit an acid stress response in bacteria (ATR) that allows survival and carbohydrate metabolism in acidic environments. In the present study, all bacterial strains tested were able to decrease pH from 5.5 to levels between 4.2 and 4.8 demonstrating their aciduric properties. This coincides with our Hypothesis 1. Even if the differences in final pH were very small, *S. oralis* and *Actinomyces* showed a tendency of not reaching as low values as the other species. This might be attributed to a weak ATR in these strains. In a previous study, *A. naeslundii* showed no ATR when exposed to an acidic environment, *S. oralis* a weak or no ATR while *L. casei* and *S. mutans* showed a strong ATR (10). However, it is important to keep in mind that aciduric properties vary between strains of the same species. For example, *S. mutans* isolated from biofilms on intact teeth showed a less extensive ATR as compared to isolates recovered from biofilms on carious tooth surfaces (31). It is therefore difficult to draw general conclusions when using only one strain for each species. However, all strains tested here sustained an acidic environment with continued metabolism, further lowering the pH below the critical pH for enamel and may therefore collectively contribute to the development of caries.

Since 1944, it is a well-known fact that pH drops after exposure to glucose are more extensive and last longer in dental plaque collected from caries-active than from caries-free individuals (32,33). It is therefore evident that caries-active individuals possess highly aciduric biofilms comprised of microorganisms that keep a functional metabolism with continued acid production also in acidic environments. A continued saccharolytic metabolism under acidic conditions contributes to a larger and longer pH drop, which in turn contributes to the development of caries as a consequence of the breakdown of enamel. In the current study, *S. mutans* and *L. paracasei* reached the most acidic final pH in all media. This coincides with a previous study (13) where mutans streptococci were shown to produce acid at the highest rate and in the largest quantity in comparison to some non-mutans streptococci. The extensive acid producing ability of *L. paracasei* and *S. mutans* in the media with a relatively low content of nutrients supports the perception that these species are key pathogens, but does not rule out the significance of other species in the caries process. The caries lesion arises through a dynamic process as a consequence of the tooth surface being exposed to a large variety of microbial acids. All acidic products are added together in a pool and give a collective effect on the continued progression of the lesion, where the system should be viewed as a whole and not as an assemblage of parts. Therefore, all tested species may have the ability to effectively establish themselves in a biofilm promoting development of caries.

Many environmental factors influence the pH of multi-species supragingival biofilms. For example, *Veillonella* utilizes lactic acid for its metabolism and produces acetic acid as its end product, and thereby contributes somewhat to neutralizing the plaque pH. Alkali production by acidogenic species may also have an effect in counteracting the pH drop in dental biofilms. Some bacteria produce ureases that can break down the urea in saliva to ammonium, which also may have a neutralizing effect in the plaque. This ability has been seen in species such as
S. mutans (1) and A. naeslundii (2). In this study we investigated ammonium production in an artificial consortium comprised of dental biofilm bacteria. When the artificial consortium was challenged with glucose alone or glucose with urea, similar pH profiles were observed (Fig. 6) indicating that no significant alkali production took place in the presence of urea. Another plausible explanation is that the presence of TH masked the actual pH changes due to nutritional components, both carbohydrates and sources of protein, which may have influenced the final pH in unknown directions. Therefore, another experiment was performed using an oral consortium suspended in PBS. This consortium had a higher microbial diversity and showed much less saccharolytic activity than the artificial consortium. In fact, the pH drop was minimal compared to the pH drop seen in the current single species acid production experiments, see fig.5, and did not reach below the critical pH of enamel. However, the pH profile differed for glucose alone and glucose with urea (see fig. 7). This indicates an alkali production from urea in this oral consortium, which supports our Hypothesis 3.

Based on previous experiments (13,21) a greater pH drop was expected for the oral consortium. In one previous in vivo study (21) where the subjects rinsed with 10% sucrose, pH measurements after 30 minutes showed a final pH in oral consortium in vivo of below 5.5. Because of the environmental differences, an oral consortium may behave differently in vitro compared to in vivo. This may be one reason for the minimal pH drop in the current study. The oral consortium was also collected from only one subject, with low caries activity, which may have affected the results since this sample probably contained a minor proportion of acidogenic bacteria. In addition, the amount of urea used does not correspond to the urea level in the saliva, which may vary between individuals and occasions. Future studies on buffer capacity of dental biofilms are needed since it may reveal some new aspects on how different microorganisms interact to counteract environmental change and maintain homeostasis in the biofilm that in turn may contribute to a more thorough understanding of caries etiology.

To our knowledge, few studies have investigated alkali production in single or multi-species biofilms. Previous studies (17,20) have shown that the alkali production was higher in oral plaque from caries-free compared to caries active individuals. This is not surprising since a healthy plaque is composed of a more mixed flora containing a higher proportion of bacteria with proteolytic metabolism and abilities, compared to the more specialized cariogenic acidic plaque dominated by saccharolytic species. However, even if it is lower in cariogenic plaque, an alkali production has been seen in acidogenic species which may contribute to the plaque homeostasis. Further studies on single species, saccharolytic consortia and cariogenic plaque are needed to assess the occurrence of alkali production in saccharolytic species related to caries and it is effects on the pH in cariogenic plaque. In conclusion, breakdown of urea in oral plaque may affect the pH in the biofilm.

Limitations

This study was made in vitro. It is important to keep in mind that the growth environments in vitro differ from the environment in vivo. In the current experiments, the bacteria grew in suspensions rather than on oral surfaces. In the consortia, predetermined amounts of bacteria
were added at one time, whereas in the mouth there are early and late colonizers that affect the local niche and attachment and enables the establishment of subsequent species.

In the cases were no statistical significance was found, the values were widely dispersed between experiments. Diverging values affect the results greatly, due to the fact that there were few values. In the experiment with growth at neutral initial pH, one value for *S. intermedius* is clearly divergent. In repeating these experiments, a higher quantity of measurements would be of importance.

The term “acid production” is used loosely in this study. The final pH is of the most clinical relevance since it is related to tooth demineralization. Different bacteria produce different kinds of acids. For example, *Actinomyces* produces succinate (2) which is a weaker acid than the lactic acid produced by *L. paracasei* and the streptococci.

One limitation with the current study was that it did not include a non-saccharolytic species in the experiments as a control, which would have been of interest.

In order to avoid artefactual effects, no buffer was used when preparing the suspensions and controls. Because of this, the initial pH values vary somewhat. The mean value of controls was calculated and considered acceptable. At pH measurements, the temperature also differed in some cases, which may affect the values. In future attempts, the temperature should be consistent at all measurements.

Project meaning

To predict caries it is interesting to look at shared properties of cariogenic bacteria, which is enlightened in this study. Since the 1960’s, the prevailing opinion has been that *S. mutans* is the primary etiologic agent for caries due to its acidic and aciduric properties. Estimation of salivary counts of mutans streptococci and Lactobacilli is a clinical tool to identify individuals with increased caries risk. In this study, non-mutans streptococci and *Actinomyces* are also being investigated for their aciduric and acidic properties. A comparison is interesting to examine whether the traditionally considered cariogenic pathogens showed statistically significantly more extensive acid production and acidurity compared to other saccharolytic species present in oral plaque. This is to give additional information for and to provide evidence for new microbiological risk assessment of caries. To better understand the etiology of dental caries, it is important to assess the roles of the microorganisms present in the plaque and the course of caries progression as thoroughly as possible. The results can be used to support the suggestion that caries is a multibacterial disease, and thus that the present multifactorial treatment is suitable.

Conclusion

All species showed acidic and aciduric properties and may contribute to tooth demineralization. Further studies with a larger number of measurements are needed to assess the cariogenicity of these species with a higher reliability. Alkali production in saccharolytic
species may affect the plaque pH. Further studies are needed to assess the occurrence of alkali production in saccharolytic species related to caries and its effects on the pH in the biofilm.
References


