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Ecological Dynamics of Oral Microbial Communities

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The microbiota of the oral cavity have been a topic of research for quite some years. Yet, this research was mostly focused on isolated pathogenic species, with limited attention for the ecosystem and what a healthy ecosystem encompasses.
The general aim of this thesis was to investigate the processes that underlie the oral ecosystem with a special interest in maintaining or obtaining a healthy state. This research includes clinical as well as *in vitro* research and addresses the effects of fluoride, arginine and nitrate on oral microbiota.

Summary

In **Chapter 2**, we investigated the effect of a fluoride mouthwash and fixed orthodontic appliances on the oral microbiome of adolescents. In this controlled randomized clinical trial, 10-16.8 year old individuals (n = 91), who were scheduled to receive orthodontic treatment with fixed appliances, participated. Half of the participants received a mouthwash containing 100 ppm amine-fluoride and 150 ppm sodium-fluoride (Elmex caries protection, Colgate-Palmolive Europe, Therwil, Switzerland), while the other half received a placebo mouthwash without fluoride. The mouthwash was used from the time of bonding until debonding twice daily. Supragingival plaque samples were taken at six time-points during this study; once before the placement of the fixed appliances, twice during the treatment, at the time of debonding and twice after the fixed appliances were removed. In addition, a bleeding by probing score was recorded for each subject at these time-points. The bacterial composition of the supragingival plaque was determined by 16S rDNA sequencing using the 454 FLX Titanium platform (Roche, Basel, Switzerland).
We did not observe differences in bacterial composition between the subjects who received the fluoride mouthwash and the subjects who received the placebo mouthwash. There was a slight difference in the bacterial profile as well as in specific genera in relation to gingival health status. The genera *Selenomonas*, *Porphyromonas* and *Johnsonella* were associated with gingivitis, while *Derxia*, *Haemophilus* and *Rothia* were associated with healthy gingiva. In addition, we observed changes in bacterial abundance in time during the study period. Although time did not seem to affect the bacterial profile, a range of genera significantly increased or decreased with time. Most notably were the increase of *Streptococcus*, *Rothia* and *Actinomyces* and the decrease of *Leptotrichia* and *Campylobacter* in time.
This study demonstrates the resilience of the oral ecosystem in regard to the perturbations it was presented with, namely a fluoride mouthwash and fixed orthodontic appliances. Time appeared to be of the greatest influence on the bacterial composition. These differences in time can partially be explained by the effect of the orthodontic treatment. The alignment of the teeth and the subsequent removal of the orthodontic appliances reduces the number of retention sites, disenabling the colonization of certain bacteria. In addition, during adolescence, the body experiences many changes, some of which might influence the oral ecosystem[1, 2].

In **Chapter 3**, we observed the effect of toothpaste containing 8% arginine on the oral microbial composition, lactate and ammonium production. In this pilot study, the subjects (n = 9) used a toothpaste containing 8% arginine (1.45 mg g^{-1} fluoride, Colgate-Palmolive, New York, USA) for eight weeks. In the two weeks prior to the arginine toothpaste use, and in the two weeks after the arginine toothpaste use was stopped, the participants used a control toothpaste (1.45 mg g^{-1} fluoride, Prodent, Sara Lee Household & Bodycare, Exton, PA, USA). To test the bacterial arginolytic capacity and sucrose metabolic activity, saliva was collected prior to the start of the toothpaste usage, four weeks and eight weeks after the start and two weeks after 8% arginine toothpaste usage was stopped. DNA was isolated from plaque and saliva samples taken prior to the start of the experiment and eight weeks after the start. These samples of all the subjects were used for 16S rDNA sequencing, while the saliva samples of four of the subjects were also used for metagenome shotgun sequencing. Sequencing was performed using the 454 FLX Titanium platform (Roche, Basel, Switzerland).
We observed that during the use of the 8% arginine toothpaste, the arginolytic capacity of the microbiota increased, while the sucrose metabolic activity decreased. These effects reversed after the toothpaste use was discontinued. No clear change in the composition of the plaque microbiome was observed, in contrast to the bacterial composition of the saliva, which significantly changed during the course of the study. Most notably was the increase of the proportion of the genus *Veillonella* in saliva.
The addition of arginine clearly affects the metabolic activity of salivary microorganisms, in addition to its effect on the bacterial composition in saliva.

The effect of arginine on oral bacteria is also described in **Chapter 4**, although this chapter concerns the influence of arginine on oral microcosms. The multi-plaque artificial mouth (MAM) biofilm model[3-5] was inoculated with saliva from a healthy donor. During this four week experiment, all eight microcosms received a continuous supply of defined mucin medium (DMM)[6]. Four of the microcosms (Arginine group) received an additional continuous supply of 1.6% (w/v) arginine, which was added to the DMM. The other four microcosms functioned as a control (Control group). All microcosms received eight 6-minute pulses of 10% (w/v) sucrose daily, with two-hour intervals to mimic cariogenic conditions. To observe the ammonium and short chain fatty acid concentrations, samples were taken before and directly after a 6-minute sucrose pulse. Samples to be used for DNA isolation were only taken prior to a sucrose pulse. The obtained DNA was used to determine the bacterial composition by 16S rDNA sequencing using the 454 FLX Titanium platform (Roche, Basel, Switzerland), and the amount of *Candida* and arginine deiminase system (ADS) genes *sagP* and *arcA* (*Streptococcus sanguinis* and *S. gordonii*, respectively) by qPCR. Additionally, *in situ* pH profiles of two of the microcosms during the fermenting phase (sucrose supplementation) were established.
The composition of the arginine treated microcosms remained stable in time, while the composition of the Control group diverged significantly in time. The genera *Streptococcus*, *Veillonella* and *Actinomyces* were highly abundant in both groups. Additionally, *Megasphaera* and *Johnsonella* were highly abundant in the Control group, while *Peptostreptococcus* and *Neisseria* were highly abundant in the Arginine group. The concentration of *Candida* had increased a 100-fold in the Control group compared to the Arginine group, while the abundances of the *sagP* and *arcA* genes were higher in the Arginine group compared to the Control group. The pH in the Arginine microcosms was higher in the resting phase and recovered faster after the addition of sucrose compared to the Control microcosms. Accordingly, the concentration of ammonium was significantly higher in the Arginine group compared to the Control group. The formation of lactate after the addition of sucrose was similar in both groups, while the concentration of butyrate was significantly higher in the Control group compared to the Arginine group.
The higher concentration of butyrate in the Control group most likely coincides with the higher abundance of *Megasphaera*, which is a butyrate producer[7, 8]. The supplementation of arginine, which is a substrate for the ADS that amongst others produces ammonia, explains the elevated levels of ammonium and pH in the Arginine group. This pH raise might also explain the lower abundance of *Candida* in the Arginine group[9, 10]. Arginine supplementation clearly influences the composition as well as the function of oral microcosms and creates a less cariogenic environment.

The MAM[3-5] was also used for the experiment described in **Chapter 5**. Here, we investigated the effect of nitrate on oral microcosms during a four week experiment. The eight MAM stations were inoculated with the saliva of two healthy donors. All of the stations received a continuous supply of defined mucin medium (DMM)[6] with trace element solution DSMZ SL-4. Four of the microcosms (two per donor) received a continuous supply of nitrate (1 mM) that was added to the DMM (Nitrate group). The other four microcosms functioned as control (Control group). All microcosms received eight 6-minute 10% (w/v) sucrose pulses daily, at two-hour intervals. Additionally, the microcosms received a weekly (manual) pulse of nitrate and sucrose, both on separate days. The concentrations of different short chain fatty acids were measured before the sucrose pulse and 6 minutes and 60 minutes after the pulse. The same measurements were performed before and after the nitrate pulse in addition to the concentrations of nitrate and nitrite. A sucrose metabolism assay was performed measuring the concentration of short chain fatty acids and phosphate as well as the pH at the start and end of the 10-minute assay. A nitrate reduction assay was performed and the concentrations of nitrate, nitrite and ammonium were measured at the start and end of the 1 hour assay, both aerobically and anaerobically. Samples for DNA isolation were taken twice a week, when the microcosms were in the resting phase. The 16S rDNA was sequenced using the MiSeq platform (Illumina, San Diego, CA, USA).
The development of the microcosms was different for both donors. For one of the donors the microbial composition of the microcosms remained similar in time, notwithstanding treatment, while for the other donor, the composition of the microcosms was significantly different between the treatments at all time-points. The genus *Neisseria* was highly abundant in the nitrate microcosms of both donors, while *Veillonella* was high in abundance in the nitrate microcosms of only one of the donors. The formation of lactate after the sucrose pulse was similar in all microcosms. On the other hand, the concentration of butyrate decreased in the Nitrate microcosms after the nitrate pulse compared to the Control microcosms. In the sucrose metabolism assay, the concentration of phosphate had increased significantly only in the Control microcosms after 10 minutes.
Although nitrate did have an influence on the composition and function of the oral microcosms, we found that the origin of the saliva had the largest influence on the observed differences.

General conclusions

The results of these experiments emphasize the complexity of the oral microbiome. On one hand, the results show that the microbiome can be steered, while on the other hand, resilience of the microbiome is demonstrated.
Especially the influence of arginine on the oral microbiome was very clear, *in vitro* as well as in a clinical situation. Although arginine has been recognized as one of the pH-elevating agents in the oral cavity for quite some time, its effect on the oral ecosystem is not fully understood. In our *in vitro* experiment, we showed that the composition of microcosms, which received a constant supply of arginine, remained stable in time and were dominated by *Veillonella*, *Streptococcus* and *Neisseria*. Moreover, arginine halted the outgrowth of potentially pathogenic *Candida*. In addition, the concentration of the short chain fatty acid butyrate was lower in the microcosms that received arginine. The pH-raising effect of arginine was confirmed by *in situ* pH-measurements and the higher production of ammonium in the microcosms that received arginine, compared to those that did not receive arginine. The higher production of ammonium was measured as well in the oral cavity of individuals using arginine-containing toothpaste. During the use of this toothpaste, the concentration of lactate decreased. Corresponding to the *in vitro* study, the genus *Veillonella* increased in abundance during the use of arginine-supplemented toothpaste. Based on these two studies, we can conclude that arginine can be regarded as a probiotic that steers the oral microbiome toward a less cariogenic environment.
Conversely, the oral microbiome was not greatly affected by the use of an amine-fluoride and sodium-fluoride containing mouthwash during orthodontic treatment. Indeed, the effects of the orthodontic treatment itself, and time, were more pronounced. Yet, these factors only affected a few taxa and not the complete microbial composition. During the course of the treatment, the abundance of periodontal pathogens such as *Porphyromonas* and *Selenomonas* decreased, while the abundance of the health associated *Streptococcus*, *Rothia* and *Haemophilus* increased. This study demonstrates the remarkable resilience of the oral microbiome of adolescents toward diverse perturbations, yet it also demonstrates that orthodontic treatment does not have a negative influence on the oral ecosystem.
The supplementation of nitrate did influence the composition as well as the metabolism of oral microcosms. The health benefits of nitrate only recently have been recognized and the influence of this compound on the oral ecosystem is not fully understood. In this *in vitro* experiment, we demonstrated that the concentration of butyrate had decreased in microcosms that received a constant supply of nitrate. Additionally, the presence of nitrate appeared to have an influence on the phosphate concentration in the sucrose metabolism assay; the concentration of phosphate was higher in the Control microcosms compared to the Nitrate microcosms. Yet, the presence of nitrate did not influence the production of lactate. The presence of nitrate was associated with a higher abundance of *Neisseria*. Although nitrate clearly affects the microbial composition and metabolism of oral microcosms, the result is strongly dependent on the inoculum.

Future research

The general aim of this thesis was to investigate the influence of certain compounds on the microbial composition and metabolism of the oral ecosystem. Although some questions have been answered in this thesis, many still need to be solved.
For instance, we observed that the influence of fixed orthodontic appliances on the composition of the oral microbiome was minimal. Moreover, the individuals receiving orthodontic treatment were adolescents and during this stage in life, the human body experiences many hormonal as well as behavioral changes, making the resilience of the oral microbiome even more remarkable. The resilience of a bacterial community can be of great interest for the maintenance of human health. Knowing and understanding the mechanisms behind the resilience of the oral microbiome of individuals undergoing quite drastic perturbations will be of great value for future health care.
Another observation that should be addressed in future research is the effect of the amino acid arginine on *Candida*. *In vitro*, the presence of arginine showed to prevent the outgrowth of *Candida*. Whether this was a consequence of the pH change, indirectly caused by arginine, creating a 'stressful' environment for *Candida*, while favoring the growth of bacteria that function as a *Candida* antagonist, or of the arginine itself is not clear. Moreover, the effect of arginine on *Candida* was not assessed in the clinical study. Assessment of the presence of *Candida* should be included in future clinical studies to determine if arginine can function as a possible anti-*Candida* agent, since *Candida* outgrowth can be quite problematic, especially in the oral cavity of immunocompromised patients. Additionally, in neither the *in vitro* nor the clinical study did we investigate the long-term effect of arginine on the oral ecosystem. This would be of

interest for future research in relation to possible adverse effects of arginine on the healthy state of the periodontium, for an elevated pH is related to a less cariogenic environment, yet also to a higher risk of periodontal disease[11].

We observed that nitrate supplementation influenced the composition and metabolism of oral microcosms. We did not observe the presence of nitrate leading to less cariogenic microcosms in relation to lactate formation and pH, even though nitrate has been suggested to have an anti-caries affect[12-14]. In future experiments it might be useful to assess carbohydrate metabolism while sucrose and nitrate are supplied simultaneously. In addition, the development of the microcosms was strongly dependent on the origin of the saliva. It might be suitable to perform similar experiments using the saliva of a higher number of donors to see if there is a common response to nitrate supplementation. The role of nitrate and its derivative nitric oxide as a possible health benefactor, in the oral cavity as well as in the whole human body, has only recently been recognized. Since nitrate metabolism in the human body is complex because bacterial as well as human pathways are involved and it is influenced by factors such as pH and the presence of oxygen, it will be a topic of research for many years to come.

Remarkably, both nitrate supplementation and arginine supplementation in the MAM influenced the concentration of butyrate in the microcosms. Although the production of butyrate by gut bacteria is associated with a healthy state of the human colon[15-17], butyrate produced by certain oral bacteria is thought to play a role in the development of periodontal disease[18, 19]. This shows that a compound may positively influence one ecosystem of the human body, while adversely affecting another; hence, the effects of supplements to benefit oral health on other parts of the human body require further investigation. This will go hand in hand with the rapidly changing DNA sequencing techniques.

Obviously, there is still a lot of research that needs to be done to fully comprehend the processes that underlie the oral ecosystem, because only when we truly understand what health is, we can prevent disease.

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