Ecological dynamics of oral microbial communities
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Chapter 3

Changes in the oral ecosystem induced by the use of 8% arginine toothpaste

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Bacterial metabolism of arginine in the oral cavity has a pH-raising and thus, potential anti-caries effect. However, the influence of arginine on the oral microbial ecosystem remains largely unresolved.

In this pilot study, nine healthy individuals used toothpaste containing 8% arginine for eight weeks. Saliva was collected to determine arginolytic potential and sucrose metabolic activity at the Baseline, Week 4, Week 8 and after a two weeks Wash-out period. To follow the effects on microbial ecology, 16S rDNA sequencing on saliva and plaque samples at Baseline and Week 8 and metagenome sequencing on selected saliva samples of the same time-points was performed. During the study period, the arginolytic potential of saliva increased, while the sucrose metabolism in saliva decreased. These effects were reversed during the Wash-out period. Although a few operational taxonomic units (OTUs) in plaque changed in abundance during the study period, there was no real shift in the plaque microbiome. In the saliva microbiome there was a significant compositional shift, specifically the genus *Veillonella* had increased significantly in abundance at Week 8.

Indeed, the presence of arginine in toothpaste affects the arginolytic capacity of saliva and reduces its sucrose metabolic activity. Additionally, it leads to a shift in the salivary microbiome composition towards a healthy ecology from a caries point of view. Therefore, arginine can be regarded as a genuine oral prebiotic.

### Introduction

Caries is an old and still common problem among humans. Severe caries can lead to excruciating pain and tooth loss, which in turn can lead to an inability to chew or even eat. Logically, treatment and prevention of caries has been a priority of the dental community for quite some decades. Therefore it is now recognized that the lowered pH, as a result of the acids formed by bacterial fermentation of sugars, leads to demineralization of the enamel. In contrast, the bacterial metabolism of arginine has a pH-raising effect.

The human oral cavity is an ecosystem, and like all ecosystems, the oral ecosystem is a combination of many different components (e.g. bacteria, fungi, metabolic compounds, host cells, salivary constituents) and in one way or another, this system retains a balance. The introduction of a specific compound might shift the balance of an ecosystem towards a different microbiological composition and functional potential. For instance, the frequent intake of sucrose facilitates a favorable environment for fermentative bacteria, leading to a cariogenic environment. In contrast, the bacterial metabolism of arginine elevates the pH in the oral cavity. The pH-raising effect of arginine is, amongst others, facilitated by the bacterial arginine deiminase system (ADS). This ADS metabolizes arginine, forming ammonia in the process and the subsequent protonation of ammonia into ammonium causes the pH to rise.

Compounds that have a positive effect on human health through the selective stimulation of growth or activity of specific microbes are called prebiotics. Some research has been done on...
the effect of prebiotics on the ecosystems of the human gastro-intestinal tract and skin\textsuperscript{14,16}, while knowledge on the use of prebiotics in the oral cavity is very limited.  
Arginine naturally occurs in a variety of foods and has recently been added to oral care products, for its pH-raising effect and anti-caries potential\textsuperscript{12,17-20}. Yet, how arginine affects the total oral ecosystem remains largely unknown. Therefore, the aim was to enhance knowledge on the effect of arginine on the human oral ecosystem and to shed light on the question if this potential prebiotic is active on a functional as well as compositional level.

**Materials & Methods**

**Pilot study design and sampling**

Nine healthy volunteers with no overt caries, who had given written informed consent, participated in this pilot study. The study was approved by the review board of the local Medical Ethical Committee (VU University Medical Center, reference number 2010/147). In the two weeks prior to the first of a total of four sampling moments (Fig. 1), the participants used a control toothpaste (1.45 mg g\textsuperscript{-1} fluoride, Prodent, Sara Lee Household & Bodycare, Exton, PA, USA). Sampling moments consist of two consecutive days. Samples were taken on each day, and the data of these duplicate samples were averaged. After the first samples were taken (Baseline), the subjects were instructed to brush their teeth using toothpaste containing 8\% arginine (1.45 mg g\textsuperscript{-1} fluoride, Colgate-Palmolive, New York, NY, USA). Subsequent samples were taken four weeks (Week 4) and eight weeks (Week 8) after the Baseline. After Week 8, use of the 8\% arginine toothpaste was stopped and the control toothpaste was reintroduced. The last samples, used for the biochemical assays, were taken two weeks after discontinuation of the 8\% arginine toothpaste (Wash-out). On sampling days, the subjects were asked to refrain from oral hygiene in the morning and not to eat and drink 2 h prior to sampling, to obtain overnight plaque.

At each sampling moment both, stimulated saliva samples and supragingival plaque samples were obtained. Stimulated saliva was collected by chewing on gum base (Wrigley, Chicago, Il, USA) for 10 min while expectorating the saliva into a sterile container. During expectoration the tube was kept on ice until further processing. On arrival to the lab, the saliva was vortexed vigorously and distributed into 2 ml portions. Supragingival plaque was collected, by a dentist, from the buccal surface of the upper first molar using a sterile microbrush (Microbrush International, Grafton, WI, USA). The tip of the microbrush was placed in RNAProtect solution (Qiagen, Hilden, Germany). All samples were stored at -80\°C.

**Biochemical assays**

To determine either the arginolytic potential or the sucrose metabolic activity of the salivary pellet, each stimulated saliva sample was washed and concentrated five times in either 10 mM tris-maleate buffer or buffered peptone water, respectively. The concentrate was pipetted, in
duplicate, into the wells of a 96-wells plate and the remaining cell suspension was stored at -20°C for subsequent protein determination. 

**Figure 1**: Sampling scheme of the study. The n represents the number of subjects contributing to the samples from different visits. Duplicate indicates that the samples were taken on two consecutive days. The data from these samples were averaged for subsequent statistical analysis. Single indicates that only one of the samples taken at the two consecutive days was used for further analysis. Saliva samples for the biochemical assays were taken before the participants started using the 8% arginine toothpaste (Baseline), four weeks (Week 4) and eight weeks (Week 8) after the Baseline, and after a two week wash-out period that followed the finish of the 8% arginine toothpaste use (Wash-out). The saliva and plaque samples to be used for DNA analysis were taken at the Baseline visit and Week 8.
To determine the arginolytic potential, an arginine metabolism assay was performed. Arginine was added to the samples to a final concentration of 50 mM. The ammonium produced in the assay was representative for the arginolytic potential of the salivary microbiome. The sucrose metabolic potential was determined by adding sucrose to the samples to a final concentration of 0.5% (w/v). The lactate produced was representative for the sucrose metabolic activity of the salivary microbiome.

For both assays the salivary pellets were incubated for three hours at 37°C. Metabolite samples were taken immediately after the arginine or sucrose addition and after three hours incubation. All samples were heat inactivated, centrifuged and the supernatants were stored -20°C until further analysis. The arginine and sucrose metabolism was analyzed, in duplicate, either by determining the ammonium or lactate produced, respectively.

16S rRNA gene amplicon sequencing
The saliva and plaque samples, taken in duplicate at the Baseline and Week 8, were used for amplicon sequencing. The DNA was isolated as described previously. An amplicon library based on the partial 16S rRNA gene was prepared using degenerate primers annealing to regions V5-V7. Sequencing was performed using 454 GS-FLX Titanium chemistry (Roche, Branford, CT, USA). The sequence data have been submitted to NCBI’s SRA database under accession number PRJNA288541.

Amplicon sequencing data analysis
Quantitative Insights Into Microbial Ecology (QIIME) version 1.5.0 was used to filter the raw amplicon data. Reads with a length < 200 or > 1000 bp, a mean quality score < 30, a homopolymer sequence > 6 nt, N > 0, > 1 barcode error, > 1 error in the forward or > 2 errors in the reverse primer were removed from the dataset. The reads were clustered into operational taxonomic units (OTUs). The samples were randomly subsampled at 2060 reads per sample before further analysis.

Shotgun sequencing of saliva samples
Baseline and Week 8 saliva samples from four subjects who had the highest arginolytic potential after eight weeks of using the arginine containing toothpaste were selected for metagenomics shotgun sequencing. These were the same samples that were used for amplicon sequencing. The DNA to be used for shotgun sequencing was isolated as described previously. The shotgun sequencing was performed using 454 GS-FLX Titanium chemistry. The sequence data have been submitted to the Genbank database under accession number PRJNA288541.

Shotgun sequencing data analysis
Reads were quality filtered using PRINSEQ; sequences with a minimal length of < 70 bp, mean quality score < 20, percentage of ambiguous bases > 10 or an entropy value < 70, were removed from the dataset, as were exact duplicates and (reverse complement) 5’ and 3’end duplicates. Sequences of human origin were removed using BMTagger. HUMAnN v0.99 was used to generate pathway summaries according to the KEGG Orthology.
Statistical analysis
The data from the duplicate samples were averaged and used for further statistical analysis. The Wilcoxon Signed Ranks test was performed, using SPSS v21 (IBM Corp, Armonk, NY, USA) to determine if there was a statistically significant difference in arginolytic potential or sucrose metabolism between any of the four visits (Baseline, Week 4, Week 8 and Wash-out). To visualize similarity of the microbial profiles based on the 16S rDNA of the plaque and saliva samples at the two different visits (Baseline and Week 8), non-metric multidimensional scaling (nMDS) plots, based on the Bray-Curtis similarity index ($\text{stress} < 0.2$), were made in PAST v3.0. The one-way permutational multivariate analysis of variance (PERMANOVA) was used to test the difference between the Baseline and Week 8 visit per sample type in PAST. The Shannon Diversity Index was calculated using PAST. The Wilcoxon Signed Ranks test in SPSS was used to determine if there was a significant difference in Shannon Diversity Index between visits. Linear Discriminant Analysis Effect Size (LDA:Se) was used to determine which taxa and which functions were differentially abundant between the Baseline and Week 8. The default settings were used (factorial Kruskal-Wallis test among classes: $\alpha = 0.05$, threshold on the logarithmic LDA score for discriminative features: 2.0 and the strategy for multi-class analysis was set to be all-against-all).

Results
The effect of 8% arginine toothpaste on the arginolytic potential and sucrose metabolic activity of saliva
The duplicate saliva samples taken at all four time-points (Baseline, Week 4, Week 8 and Wash-out) from all nine participants were used for the biochemical assays.
In the arginolytic assay, the ammonium produced was representative for the arginolytic capacity of the salivary microbiome. The arginolytic capacity increased significantly from the Baseline to the visit at Week 4 and again from Week 4 to Week 8 (Fig. 2). On the other hand, the arginolytic capacity of the salivary pellet decreased significantly from Week 8 to the end of the Wash-out period (Week 10). In the sucrose metabolism assay, the lactate produced indicated the sucrose metabolic potential of the salivary microbiome. The sucrose metabolic potential decreased significantly from the Baseline to Week 4 and on to Week 8, while it increased again significantly at the Wash-out visit (Fig. 3).

The effect of 8% arginine toothpaste on the plaque and salivary microbial composition
Duplicate plaque and saliva samples from all nine subjects, taken at the Baseline and after eight weeks of using the arginine containing toothpaste (Week 8), were used for 16S rDNA amplicon sequencing.
Of the 1010385 raw input sequences, 451514 with an average of 6271 reads per sample (SD 2440, range 2063-13089) remained after filtering and the removal of chimeric sequences. The subsampling threshold was set at 2060 reads. The remaining subset of the plaque samples consisted of 362 OTUs with an average of 101 OTUs per sample (SD 19, range 58-136), while
the saliva samples comprised a total of 415 OTUs with an average of 103 OTUs per sample (SD 19, range 67-148).

**Figure 2.** Arginolytic potential of saliva. The arginolytic potential of saliva was measured as the amount of ammonia produced by the salivary pellet. Statistical significance (p < 0.05) was determined using the Wilcoxon Signed Ranks test. The boxes represent the median and interquartile range (IQR), outliers more than 1.5x IQR are depicted by ○, and more than 3x IQR by *. n = 9.

**Figure 3.** Sucrose metabolism in saliva. Sucrose metabolism was measured as the amount of lactate produced by the salivary pellet. Statistical significance (p < 0.05) was determined using the Wilcoxon Signed Ranks test. The boxes represent the median and interquartile range (IQR), outliers more than 1.5x IQR are depicted by ○, and more than 3x IQR by *. n = 9.

**Figure 4.** Non-metric multidimensional scaling plot based on 16S rDNA derived from plaque, calculated using the three-dimensional Bray-Curtis similarity index. Stress: 0.118. One-way PERMANOVA: p = 0.692, F = 0.68. Baseline: ○, week 8: ●. n = 9.

**Figure 5.** Non-metric multidimensional scaling plot based on 16S rDNA derived from saliva, calculated using the three-dimensional Bray-Curtis similarity index. Stress: 0.121. One-way PERMANOVA: p = 0.007, F = 2.76. Baseline: ○, week 8: ●. n = 9.
The effect of arginine toothpaste on the oral microbiome

The nmMDS plot did not reveal a strong divide in the composition of the plaque microbiome at the Baseline or at Week 8 (Fig. 4), in contrast to the nmMDS plot based on the salivary microbiome (Fig. 5). One-way PERMANOVA indicated a significant difference in the saliva derived microbial profiles between the two visits ($p = 0.007$, $F = 2.76$), contrary to the plaque derived microbial profile ($p = 0.692$, $F = 0.68$). There was no significant change in Shannon diversity observed for both sample types after the use of the arginine containing toothpaste (Figs. S1 and S2).

Descending from the total microbial profile to the separate taxa, *Streptococcus* was the most abundant genus in the plaque microbiome (Fig. 6) as well as in the salivary microbiome (Fig. 7). Their level of abundance remained similar between both visits.

To determine which taxa were significantly different in abundance between the two visits, linear discriminant analysis effect size (LEfSe) was used. In the plaque samples, only *Candidate division TM7* was differentially abundant between the two visits and was associated with the Baseline (LDA score 2.67). The overall abundance of *Candidate division TM7* was < 1% (Baseline: 0.049%, Week 8: 0.003%) (Fig. 6). At the OTU level, seven OTUs were differentially abundant in plaque between the two visits. Both OTU184 (*Candidate division TM7*) and OTU148 (*Dialister*) were associated with the Baseline, while OTU516 (*Treponema*), OTU102 and OTU530 (both *Prevotella*) and OTU150 and OTU 423 (both *Eubacterium*) were associated with Week 8 (Fig. 8).

In the salivary microbiome, twelve genera were differentially abundant between the two visits, with *Veillonella* being the only genus associated with Week 8 (Fig. S3). At the OTU level, 47 OTUs were differentially abundant between the two visits (Fig. 9). The OTU429 (*Porphyromonas*) had the strongest association with the Baseline. Of the seven OTUs that were associated with Week 8, the association of OTU472 (*Veillonella*) was the strongest.

![Figure 6: Relative abundance of the predominant genera in the plaque microbiome. The bars and error bars represent the mean relative abundance and standard deviation, respectively. n = 9.](image-url)
Figure 7: Relative abundance of the predominant genera in the salivary microbiome. The bars and error bars represent the mean relative abundance and standard deviation, respectively. The * indicates that these genera were significantly differentially abundant between the two visits according to LEfSe. n = 9.

Figure 8: Relative abundance of differentially abundant OTUs in plaque. (a) The relative abundance of the OTUs that were found to be significantly differentially abundant by LEfSe between the two visits, displayed per individual per visit. (b) The histogram of the differentially abundant OTUs between the two visits, identified through linear discriminant analysis effect size score. The white bars represent the OTUs that are associated with the Baseline and the black bars represent the OTUs that are associated with Week 8. n = 9.
The effect of arginine toothpaste on the oral microbiome

Figure 9: Relative abundance of differentially abundant OTUs in saliva. (a) The relative abundance of the OTUs that were found to be significantly differentially abundant by LEfSe between the two visits, displayed per individual per visit. (b) The histogram of the differentially abundant OTUs between the two visits, identified through linear discriminant analysis effect size score. The white bars represent the OTUs that are associated with the Baseline and the black bars represent the OTUs that are associated with Week 8. n= 9.
Chapter 3

The effect of 8% arginine toothpaste on the functional potential of the salivary microbiome

Shotgun sequencing was performed on Baseline and Week 8 saliva samples of four of the subjects who had the highest arginolytic potential after using the arginine containing toothpaste for eight weeks.

After filtering the 1408122 shotgun reads using PRINSEQ\textsuperscript{29}, 1181472 reads with an average length of 323 bp remained. The average number of reads per sample was 147684 (SD 46030, range 81199-218604).

The base excision repair (ko03410) and the lysosome (ko04142) KEGG pathways were the most abundant pathways in the Baseline samples compared to the samples taken at Week 8 (Fig. S4). A total of six pathways were more abundant in the samples taken at Week 8 compared to the samples taken at the Baseline visit. Of these six pathways, carbon fixation in photosynthetic organisms (ko00710) and carbon fixation pathway in prokaryotes (ko00720) explained the greatest differences between the two time-points.

Discussion

The findings of this pilot study indicate that using a toothpaste that contains 8% arginine, affects the composition of the oral microbiome. In addition, it enhances the arginolytic potential of the salivary pellet, while the sucrose metabolic activity is repressed.

Indeed, we found the capacity of the salivary pellet to metabolize arginine to have increased during the time the subjects used toothpaste containing 8% arginine. However, this activity was reduced after the subjects stopped using the toothpaste. From our results it is clear that the capacity to metabolize arginine is present in the oral cavity and is adjusted to the amount of substrate present. In contrast to the arginolytic capacity, the capacity to convert sucrose into lactate became lower during the study, yet became higher again after the use of arginine containing toothpaste was discontinued.

These observations seem to relate to the abundance of the genus *Veillonella* in saliva, which increased in the course of this study. Members of this genus have an optimum growth pH of 6.5-8\textsuperscript{36, 37}. Hence, a raised pH of the ecosystem creates a favorable environment for these commensals. *Veillonella* species are lactate fermenters\textsuperscript{36, 37} and might have used the lactate formed in the sucrose metabolism assays, explaining the observed decrease in the lactate concentration. Although we lack the microbiological data of the last time-point, the amount of lactate that was measured at the Wash-out period in the sucrose metabolism assay suggests the decrease in abundance of this lactate fermenter.

Interestingly, *Veillonella* was the only genus that was differentially abundant in saliva at Week 8, in contrast to the Baseline visit where eleven genera were differentially abundant.

Moreover, the genera that showed differential abundance between the two visits were different in the saliva and plaque derived-samples. *Candidate division TM7* as well as OTU184 (*Candidate division TM7*) were both more abundant at the Baseline in plaque. Until now, this still quite elusive member of the oral ecosystem has been related to periodontitis and gingivitis\textsuperscript{38, 39}. In contrast to *Candidate division TM7*, which seemingly did not favor the conditions introduced by
The effect of arginine toothpaste on the oral microbiome

arginine metabolism, were the OTUs identified as members of the genera *Eubacterium*, *Prevotella* and *Treponema*, which became more abundant in plaque at Week 8. Likely, these OTUs represent species that favor the conditions brought about by the arginine metabolism. Some members of these genera are associated with an elevated pH, as well as periodontal disease\(^ {40-45}\). However, Li *et al.* did not find an association between the use of 8% arginine containing toothpaste and an increase in gingivitis\(^ {46}\).

A number of OTUs identified as *Prevotella* and *Treponema* were also present in the salivary microbiome, mostly high in abundance at the Baseline. However, OTU140 and OTU208 (both *Prevotella*) were more abundant at Week 8 in the salivary microbiome, yet these particular OTUs were not significantly abundant in the plaque microbiome. Interestingly, there are members of the genus *Prevotella*, which are associated with caries\(^ {47-49}\). It is possible that the taxa which become lower in abundance in time are those associated with a lower pH, as would be the desired effect of arginine as a prebiotic. Still, the taxonomic resolution is too low to distinguish between species. Nevertheless, these results do emphasize that saliva and plaque are different niches within the same ecosystem\(^ {25, 50, 51}\). It is likely that a compound such as arginine has a different influence on a different habitat. Indeed, it was observed that the use of toothpaste containing 1.5% arginine did induce the activity of the arginine deiminase system in dental plaque and not in saliva\(^ {52}\). In our study we only looked at the arginolytic potential of saliva and not of plaque.

Additionally, in this study we observed the functional potential of saliva through metagenome shotgun sequencing. There was enrichment of the tropane, piperidine and pyridine alkaloid biosynthesis pathway (ko00960) at Week 8. Alkaloids are secondary metabolites, mostly isolated from plants, which have a range of functions within human society, *e.g.* medication or stimulants\(^ {53, 54}\). Interestingly, arginine is a precursor in the tropane alkaloid pathway, where arginine is metabolized into putrescine through arginine decarboxylase (ADC)\(^ {55-57}\). ADC is detected in higher plants and bacteria, generally not in mammals or fungi\(^ {56, 58}\). In contrast, the lysosome pathway (ko04142) was reduced at Week 8. Lysosomes are organelles in animal cells; hence there is a possibility that part of the host DNA was not removed using the stringent filters. However, the origins of the lysosome mechanism are thought to come from primitive protozoans and are strongly conserved in amoeba as well as mammalian cells\(^ {59}\). Therefore, lysosome pathway genes might originate from oral protozoa\(^ {60, 61}\). Although this does not clarify why these genes were reduced at Week 8, it does suggest that oral protozoa might be an interesting topic for future research.

On a different note, we found OTU309 (*Streptococcus*), to be more abundant in saliva after the use of arginine toothpaste. The arginine deiminase system (ADS) is present in a number of *Streptococcus* species\(^ {11, 52, 62}\), yet we did not observe significant enrichment of ADS orthologs in the salivary metagenome.

Why we did not observe enrichment of the ADS after eight weeks of 8% arginine toothpaste use remains largely unexplained, as do the observed changes in functional potential, although it is very likely that the low sample size plays a part.
A placebo-controlled randomized clinical study including a higher number of participants and samples should provide more conclusive answers, including the analysis of the microbiota after cessation of treatment.

In summary, we conclude that arginine added to toothpaste does bring about changes in the oral ecosystem, compositional as well as functional, and serves as a prebiotic.

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**Conflicts of interest**

The study was financially supported by the Colgate-Palmolive Company. The authors declare that otherwise no competing interests exist. The involvement of the Colgate-Palmolive Company in this study by financial support had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References


The effect of arginine toothpaste on the oral microbiome


**Figure S1**: Diversity of the supragingival plaque microbiome based on the 16S rRNA gene. The boxes represent the median and the interquartile range. n = 9.

**Figure S2**: Diversity of the salivary microbiome based on the 16S rRNA gene. The boxes represent the median and interquartile range (IQR), outliers more than 3x IQR are depicted by *. n = 9.

**Figure S3**: Histogram of the differentially abundant genera, between the two visits, derived from saliva and identified through linear discriminant analysis effect size score (LEfSe). The white bars represent the genera associated with the Baseline; the black bar represents the genus associated with Week 8. n = 9.
Figure S4. Histogram of the differentially abundant KEGG pathways between the Baseline and Week 8, identified through linear discriminant analysis effect size score (LEfSe). The white bars represent the KEGG pathways that are associated with the Baseline and the black bars represent the KEGG pathways that are associated with the visit at Week 8. n = 4.