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Synergistic effects of arginine and fluoride on human dental biofilm control

Nanako Kuriki ^{a,b,*}, Yoko Asahi ^a, Motoki Okamoto ^b, Yuichiro Noiri ^c, Shigeyuki Ebisu ^a, Hiroyuki Machi ^d, Maiko Suzuki ^b, Mikako Hayashi ^a

^a Department of Restorative Dentistry and Endodontology, Osaka University Graduate School of Dentistry, Osaka, Japan

^b Department of Oral Science and Translational Research, College of Dental Medicine, Nova Southeastern University, Fort Lauderdale, FL, USA

^c Division of Cariology, Operative Dentistry and Endodontics, Department of Oral Health Science, Niigata University Graduate School of Medical and Dental Sciences,

Niigata, Japan

^d Osaka University Dental Technology Institute, Osaka, Japan

ARTICLE INFO	A B S T R A C T
Keywords: Biofilms Next-generation sequencing Microbiome In situ Arginine Fluoride	Objectives: The aim of this study was to quantitatively and comprehensively investigate the combined effects of arginine and fluoride on the suppression of pathogenicity using an <i>in situ</i> biofilm model and next-generation sequencing (NGS).Methods: Using the <i>in situ</i> model, dental biofilms were formed and the viable bacterial counts and arginine ac- tivity in the arginine- and fluoride-containing dentifrice and control groups were measured. We also compared their effects on the bacterial microbiota and predictive functional factors in the control, arginine (arg), and arginine + fluoride (argF) groups using NGS analysis. Results: Compared to the control treatment, the use of 8 % arginine and 1450 ppm fluoride toothpaste resulted in significantly high oral NH4 concentrations without affecting the number of viable bacteria ($P < 0.05$). NGS analysis revealed that the oral microbiota of the control, arg, and argF groups were significantly different. Heat map analysis of the predicted functional factors revealed that the arg group had different properties from the other groups and activated specific substrate metabolic pathways; contrastingly, argF treatment inhibited the activity of these pathways and prevented an increase in the abundance of bacterial genera that utilize substrates such as sucrose, suggesting the synergistic effect of arginine and fluoride. Conclusions: This study indicates that the combination of arginine and fluoride has a synergistic effect on the bacterial microbiota and pathogenicity of dental biofilms compared with arginine alone. Clinical significance: Our findings suggest that the combination of arginine and fluoride could be used as an effective prebiotic and may inhibit the growth of bacteria associated with dental diseases.

1. Introduction

The oral cavity is home to over 700 bacterial species that are able to form biofilms [1-3]. Oral biofilms are associated with dental caries and periodontal diseases, and their effects on the entire body have been widely reported [4,5]. Therefore, the elucidation of methods to control and inhibit these diseases is extremely important for maintaining oral and systemic health.

Unlike biofilms formed *in vitro*, human dental biofilms comprise >700 of bacterial species that interact with a wide variety of oral environments and host factors. However, it is difficult to evaluate these parameters quantitatively and over time using conventional oral sample

collection methods, such as swabs. Therefore, we developed an intraoral device that can quantitatively form and evaluate dental biofilms in the human oral cavity [6]. We used next-generation sequencing (NGS) to identify the bacterial components of dental biofilms created over time using this model. We found that facultative anaerobic microbiota predominantly increased in proportion as the biofilm matured at 96 h after formation.

Although antimicrobial therapy is considered as the first choice for the treatment of bacterial infections, it has been found that many biofilms are resistant to antibiotics, and that the balance of the oral microbiota is destroyed by the emergence of resistant bacteria as a result of their use [7]. To address this problem, prebiotics, a concept for

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^{*} Corresponding author at: Department of Restorative Dentistry and Endodontology, Osaka University Graduate School of Dentistry, 1-8, Yamadaoka, Suita, Osaka, Japan.

E-mail address: kuriki.nanako.dent@osaka-u.ac.jp (N. Kuriki).

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Fig. 1. Experimental protocol.

Sampling of the hydroxyapatite (HA) disks and saliva was performed according to the experimental schedule displayed above. These samples were used to conduct DNA sequencing and NH_4 ⁺ concentration measurements.

The black arrow indicates when the appliance was installed. The triangle indicates when sampling was conducted.

intestinal bacteria, have attracted attention. Prebiotics are non-digestible substances (hydrocarbons) that have a favorable effect on the host and improve its health by stimulating the growth and activation of specific groups of bacteria established in the colon [8]. Arginine, which is also a component of saliva, has attracted attention due to its possible applications in the oral cavity [9]. There have been several reports of arginine-induced changes in oral microbiota, with an arginine deiminase system (ADS) being found to increase the pH of the oral environment, thereby inhibiting the growth of acid-tolerant bacteria and resulting in a less caries-prone oral environment [10,11]. Using an *in situ* biofilm model, we demonstrated that arginine increases the pH of the oral environment via ammonium ions and induces changes in the bacterial microbiota, increasing the prevalence of *Neisseria* and *Streptococcus* and decreasing that of *Porphyromonas* [12].

Numerous reports have been published on the caries-preventive effects of fluoride [13,14]. However, caries remains one of the most prevalent chronic infectious diseases worldwide, and the high incidence of root surface caries in Japan, which is a hyper-aged society, has become an issue that necessitates other methods of caries control. In a 2-year clinical study on arginine, 1.5 % arginine toothpaste without fluoride was reported to be more effective in reducing the incidence of caries than the fluoride-only toothpaste [15]. Therefore, we hypothesized that the control and inhibition of dental biofilms might be enhanced when arginine and fluoride, which have been reported to inhibit dental caries, are combined. To verify this synergistic effect, we considered it necessary to evaluate the bacterial microbiota of dental biofilms and their impact on pathogenicity.

The purpose of this study was to quantitatively and comprehensively investigate the combined effects of arginine and fluoride on the suppression of pathogenicity using an *in situ* biofilm model, NGS, and other methods. The null hypotheses tested were as follows: 1) the use of 8 % arginine + 1450 ppm fluoride toothpaste has no effect on the number of viable oral bacteria, 2) the use of 8 % arginine + 1450 ppm fluoride toothpaste has no effect on the number of arginine and fluoride has no effect on oral bacterial flora, and 4) the combination of arginine and fluoride has no effect on oral bacterial function.

2. Materials and methods

2.1. Study participants

Volunteers were recruited from among the students of the School of Dentistry, Osaka University, and the medical staff of the Osaka University Dental Hospital, who agreed to participate in this study after being informed of its content. Ten healthy individuals were recruited based on previously described methods [6,12,16]. Participants were selected based on the fact that they had no systemic or oral abnormalities and had not received any antibiotics or other drugs within the past 6 months. The samples were collected using an intraoral device developed to prepare dental biofilms. The study design was approved by the Ethics Committee of the Osaka University Graduate School of Dentistry (R3-E17).

2.2. In situ model of biofilms

For biofilm formation, we used a previously developed *in situ* dental biofilm model [16]. Briefly, a maxillary splint, used as an *in situ* oral device, was vacuum-formed from a 1.5 mm-thick thermoplastic resin sheet (Erkodule, Erkodent, Pfalzgrafenweiler, Germany). A hydroxyapatite (HA) disk (diameter 6 mm and height 1.5 mm; Olympus Terumo Biomaterials, Tokyo, Japan) simulating the tooth enamel surface was inserted into the buccal side of the device. Four HA disks were placed on each side, yielding a total of eight disks in the device. A dental biofilm was created on the HA disk fixed to the oral device, and samples were collected.

2.3. Experimental protocol

The experimental schedule is shown in Fig. 1. All participants brushed their teeth without a toothpaste or mouthwash before inserting the oral device. They wore the oral appliance from 24:00 h to 8:00 h the next morning, and the disks were collected at 8:00 h immediately after waking (control group). For 28 days, the participants were instructed to brush with a toothpaste containing 8 % arginine and fluoride (1450 ppm fluoride, Colgate-Palmolive, USA). After 28 days of brushing, the



Fig. 2. Graph displaying the viable bacterial cell count per unit area under aerobic (A) or anaerobic (B) conditions. No change was observed in the viable cell count under both conditions. (Wilcoxon-rank sum test).

participants wore the oral appliance once again from 24:00 h to 8:00 h the next morning, and the disks were collected at 8:00 h immediately after waking (arginine + fluoride group). Two HA disks, randomly selected from eight HA disks per person, were used—one for assessing viable counts and the other for DNA extraction for sequencing. For NGS analysis, the arginine group used DNA extraction samples pooled from a previous study [12].

2.4. Determination of viable count and arginine activity

Samples were immersed in sterile distilled water, sonicated for 5 min, stirred for 30 s, and serially diluted (10-fold). The diluted solution was inoculated onto Columbia sheep hemolysis agar medium (Becton, Dickinson and Company, Fukushima, Japan) and incubated at 37 °C for 48 h under aerobic and anaerobic conditions using Anaeropac®/Kenki, and the viable bacterial count was subsequently determined. To evaluate arginine activity, ammonium ion (NH⁺₄) concentrations were measured in saliva samples that had been collected at the same time points using an ammoniometer AT-2000 (Central Scientific Co., Ltd, Japan).

2.5. DNA sequencing

DNA was extracted from the collected samples using the DNeasy[®] PowerSoil[®] DNA Isolation Kit (QIAGEN). The V1-V2 region was then amplified by MiSeq[®] ((Illumina Inc. California, USA) using a universal primer targeting 16S rRNA (27F mod: 5'-ACACTCTTTCCCTA-CACGACGCTCTTCCCGATCTNNNNN-3').

(338R: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNN -TGCTGCCTCCCGTAGGAGT-3') and sequenced according to the manufacturer's instructions. Using universal primers targeting 16S rRNA, sequence analysis was performed by clustering with Operational Treatment Units (OTUs) based on a 97 % homology cutoff. Sequencing results were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline and systematically estimated using the EzBioCloud 16S database. The functional predictors were also analyzed. Using the frequency table output from Qiime2 and the representative sequences as inputs, picrust2 (ver. 2.3.0 b) was used to create a functional composition table based on the EC number (EC), Kyoto Encyclopedia of Genes and Genomes (KEGG), ortholog (KO), Cluster of Orthologous groups (COG), and METACYC (pathway). Principal Coordinate Analysis (PCA) was performed using the diversity and emperor plugins of Qiime2.



Fig. 3. Graph demonstrating that NH₄ ⁺concentration was significantly higher in the 8 % arginine solution-treated group than in the control group. (Wilcoxonrank sum test, *P < 0.05).

2.6. Statistical analysis

The statistical significance of viable counts and ammonium ion concentrations was tested using IBM SPSS[®] Statistics (version 10.02 2012 SAS Institute Inc., Cary, NC, USA). Statistically significant differences in the NGS analysis were also tested using IBM SPSS Statistics, and the data are displayed as box plots. The Wilcoxon rank-sum test was performed at a risk rate of 5 %. In the NGS group, comparison analysis with Linear discriminant analysis effect size (LEfSe, 1.0.8) was used to determine whether the relative abundances of the strains differed between groups. Specifically, a difference was considered as significant when the difference between the three groups had a P-value < 0.05, and a Linear discriminant analysis (LDA) score $(\log 10) > 3$, that is, one digit greater than the default value in the LEfSe method [17]. In addition, Gene Cluster 3.0 was used for heatmap analysis, and clustering analysis was performed for items that were determined to be significant in the aforementioned statistical processing using the numerical value changed to the Z-score.

3. Results

3.1. Determination of viable count and arginine activity

Under both aerobic and anaerobic conditions, we observed no change in the number of viable bacteria in the group that used



Fig. 4. Relative abundance of bacterial phyla in all the participants (n = 10). The graph represents the average of data collected from all the participants.



Fig. 5. Proportional abundance of genera in all the participants (n = 10) The most frequently detected taxa (> 1 % relative abundance) in each level are shown.

toothpaste containing 8 % arginine and fluoride for one month (argF) or in the control group (Fig. 2). However, the NH_4^+ concentration in the saliva was significantly higher in the argF group than that in the control group (Fig. 3).

3.2. Effects on dental biofilm microbiota

Differences were observed after evaluating the effects of the different treatments on oral microbiota. At the phylum level, the arg and argF groups demonstrated an increase in the number of *Firmicutes* and a decrease in the number of *Bacteroidetes* compared to the control group (Fig. 4). At the genus level, the arg and argF groups demonstrated an increase in *Streptococcus* and *Gemella*, and a decrease in *Porphylomonas* (Fig. 5). Furthermore, LEfSe analysis was performed to compare the estimated phylotypes of the microbial communities in the control, arg,

and argF groups. Histograms of the LDA scores were constructed to demonstrate the features that differed in abundance among the three groups. The most significantly abundant bacterial taxon in the control group was *Porphylomonadaceae* (LDA score [log10] > 3), while *Lactobacillales* and *Abiotrophia* were found to be significant microbiomes in the arg group. In addition, the argF group was characterized by a predominance of *Actinomyces, Campylobacter*, and *Episilonproteobacteria* (LDA score [log10] > 3) (Fig. 6A), suggesting that the significant strains in each group had consistent biological differences (Fig. 6B).

3.3. Effects on functional factors

Clustering analysis was performed on the functional predictors that were determined to be significant in METACYC (pathway). Values were changed to Z-scores and represented in a heat map (Fig. 7). The heat A)



Fig. 6. Characterization of microbiomes in the control, arginine, and arginine + fluoride groups using linear discriminant analysis effect size (LEfSe) analysis and linear discriminant analysis (LDA). A) Histogram of LDA scores (log10) used to indicate features that differed in abundance among the three groups (control, arg, and argF). B) Taxonomic representation of statistically and biologically consistent differences in the control, arg, and argF groups.

map displayed the clustering of the top 60 factors contributing to the differences in each group, with each column representing the individual target samples and each row representing the functional factor. Our findings revealed that the argF and control samples were in similar positions, whereas most of the arg samples were located on the right side of the column. Hierarchical clustering indicated that the arg group was significantly separated from the argF samples and control group. This suggested that, unlike the arg group, there was a similar relationship between the functional factors expressed in the control and argF group. Additionally, this visualization highlighted the significantly different factors among the treatment groups, displaying these differences using a boxplot. The top factor that were significantly upregulated in the argF group compared with those in the arg group are shown in Fig. 8.

4. Discussion

In 2019, metagenomic analysis, which is used to analyze bacterial microbiota and their functions, and metabolomic analysis, which is used to identify metabolites, were used to investigate the characteristics of the intestinal environment that predisposes patients to colorectal cancer [18]. In recent years, extensive research has been conducted on intestinal bacteria, and it is widely known that the balance of the human microbiota is closely related to health. Prebiotics, which have favorable effects and improve the health of the host, have attracted attention as potential controls for intestinal bacteria. In the present study, we focused on arginine, which is a component of saliva and a natural dietary supplement, and performed NGS analysis using an *in situ* biofilm



Fig. 7. Heat map of clustering analysis.

The heat map shows the clustering of the top 60 genes contributing to the differences in each group. Each column represents the material, and each row represents the genes. The color key represents the Z-score, which is shown as a relative value for all of the tiles within the total sample. Green, black, and red indicate the lowest, intermediate, and highest expression levels, respectively.

model that can reproduce dental biofilms. We found that arginine increases the ammonium ion concentration in the oral cavity, which in turn alters the bacterial microbiota. Although we expected that the 8 % arginine group would exhibit an increase in the number of oral commensal bacteria without acid resistance or the arginine-related functional factors, our previous study did not demonstrate a significant increase in the number of these bacteria or functional factors [12]. Therefore, we considered it necessary to develop an effective arginine formulation by adding a compound that would enhance its effects. Hence, we combined fluoride with arginine. Many studies have been conducted on fluoride and its caries-preventive effects have been recognized [13,14]. In a clinical study consisting of 12,500 participants, it was reported that toothpaste containing arginine reduced the incidence of dental caries at the crowns and roots of teeth when compared with that containing only fluoride [19]. Although many effects of fluoride on the oral microflora have been reported [20,21], regarding the effects of arginine and fluoride on supragingival plaque, a synergistic effect has been suggested but not clearly demonstrated [22]. Furthermore, no study has comprehensively compared the number of bacteria and bacterial microbiota in human dental biofilms treated with a combination of arginine and fluoride or with arginine alone. In this study, we used an in situ biofilm model and NGS to quantitatively and comprehensively investigate the effects of arginine and fluoride on the suppression of dental diseases.

We used a dentifrice with 8 % arginine in this study because in our previous in vitro study, the effective arginine concentration was significantly higher in the 8 % arginine solution. In addition, a previous clinical study reported that the addition of 1.5 % arginine to a 1450 ppm fluoride toothpaste reduced the incidence of caries when compared with the dentifrice containing fluoride alone [23]. Therefore, a toothpaste containing 8 % arginine and 1450 ppm fluoride was used to test the combined effects of arginine and fluoride.

Using our model, we investigated the effects of arginine and fluoride on the viable cell counts and arginine activity of oral dental biofilms. Our results indicated that the viable bacterial counts under aerobic and anaerobic conditions did not differ between the control and argF groups (Fig. 2). However, the NH_4^+ concentration in the saliva of the argF group was significantly higher than that of the control group (Fig. 3). In other words, significant arginine activity was observed in the oral cavity treated with arginine + fluoride; however, the number of bacteria in the oral cavity was not affected. This result is similar to that obtained when using arginine alone [12]. This is because arginine has an effect on the oral microflora but does not kill the bacteria. [10]. Therefore, we accepted the first null hypothesis and rejected the second one.



Fig. 8. Relative proportion of functional factors (glucose_and_glucose_1_phosphate_degradation)with significant differences between the argF group compared with those in the arg group. Boxes extend from the 25th to 75th percentiles. Circles represent outliers. Asterisks above the whisker indicate a statistically significant difference. (n = 10, *P < 0.05).

After analyzing the bacterial microbiota, we observed individual differences in the data of healthy participants, which is consistent with previous reports [6,12,24]. These trends were demonstrated in Fig. 4-6. First, at the phylum level, the arg group demonstrated an increase in Firmicutes and a decrease in Bacteroidetes abundance compared with those in the control group (Fig. 4). At the genus level, the arg group demonstrated an increase in Streptococcus and Gemella and a decrease in Porphylomonas (Fig. 5). Furthermore, LEfSe analysis was performed to compare the estimated phylotypes of the microbial communities among the control, arg, and argF groups, and histograms of the LDA scores were constructed to demonstrate the features with different abundances among the three groups, which all exhibited statistically significant differences (Fig. 6). The bacterial taxonomic group that was significantly more abundant in the control group was Porphylomonadaceae (LDA score $[\log 10] > 3$), whereas those that were significantly more abundant in the arg group were Lactobacillales and Abiotrophia. In contrast, Actinomyces, Campylobacter, and Episilonproteobacteria were more common in the argF group (LDA score [log10] > 3) (Fig. 6A). Although there was variation in the argF group, significant strains were considered consistent with biological differences (Fig. 6B). As in previous reports, the control group had a significantly higher abundance of Porphyromonas, which is abundant in mature biofilms and periodontal patients [6], while the argF group had a significantly higher abundance of Actinomyces and others, which are abundant in early biofilms (48 h or less). Thus, similar to previous reports, the addition of arginine may prevent the migration of mature biofilms and dysbiosis. Accordingly, the third null hypothesis was rejected. However surprisingly, the arg group had a significantly higher number of *Lactobacillales*, suggesting that they may shift to a bacterial microbiota with a higher risk of dental caries than that in the control and argF groups. In this regard, we suspected that there may be a relationship with complex factors in the oral cavity, and

further analyzed functional predictors.

According to the heat map analysis of functional predictors, it is likely that the control and argF groups had an approximate relationship with the predictive functional factors (Fig. 7). Among these, a feature that was more common in the arg group indicated a significant increase in the number of functional factors. Furthermore, the activity of substrate metabolic pathways, including sucrose, invertase, lactose, and galactose degradation, was significantly increased in the arg group in this study. This finding is consistent with previous clinical reports [25, 26]. The functional factor that was significantly elevated in the arginine group was invertases, also known as saccharases or invertins, which are hydrolytic enzymes that release fructose from oligosaccharides. Sucrose can also be used as a substrate. This enzyme acts on d-fructofuranoside to release beta-d-fructose. When acting on sucrose, beta-d-fructose and glucopyranose are produced [27], which is very common in plants and other organisms. The hexoses produced by invertases are used to meet the energy and substrate requirements of the organism for growth, storage, or energy production. The most common pathway of lactose and galactose degradation also involves the initial hydrolysis of lactose to glucose and galactose by β-galactosidase. Galactose is then converted into galactose-1-phosphate and glucose-1-phosphate [28]. Staphylococcus aureus, Lactococcus lactis, and Streptococcus mutans have been reported to degrade lactose via this pathway [29]. In other words, in the arg group, activating these substrate metabolic pathways may have allowed the bacteria to utilize nutrients, leading to an increase in the Lactobacillales. On the other hand, metabolome analysis, which comprehensively analyzes metabolites in vivo, has reported that fluorine inhibits glycolytic activity [30]. As shown in Fig. 8 of this study, one of the functional factors significantly elevated in the argF group was _glucose_ and _glucose_1_ phosphate_ degradation. One of the reported functions of this factor is that the uptake of exogenous glucose via the phosphatase system (PTS) inhibits the utilization of other exogenous sugars [31]. This suggests that the argF group may inhibit the substrate metabolic pathway activated by the arginine group and inhibit the growth of bacterial genera that utilize substrates such as sucrose. Accordingly, the fourth null hypothesis was rejected.

This study has several limitations. First, we could not determine whether fluorine plays a role in the inhibitory function of the substrate metabolic pathway activated by the arginine group observed in the argF group. Second, previous studies on metagenomic and metatranscriptomic functional profiling with arginine and fluorine combinations have reported a significant increase in the expression of genes involved in the arginolysis pathway (ADS) [23], which was not observed in this study. Future studies with more detailed identification of taxonomic composition and functional factors using whole genome shotgun sequencing and RNA-seq are required.

5. Conclusion

In this study, we investigated the effectiveness of arginine and fluoride preparations for plaque control. The results showed that the use of an 8 % arginine and 1450 ppm fluoride toothpaste resulted in increased $\rm NH_4^+$ concentration in the oral cavity, altering the oral microbiota but having no effect on the number of viable bacteria. These findings suggest that the combination of arginine and fluoride synergistically reduces the risk of dental caries by decreasing the abundance of periodontal disease- and caries-causing bacteria and inhibiting the pathways involved in the bacterial metabolic system, highlighting the potential utility of arginine and fluoride preparations as effective prebiotics. Future studies should also examine their effects on patients at the risk of developing dental caries or periodontal disease.

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CRediT authorship contribution statement

Nanako Kuriki: Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. Yoko Asahi: Writing – original draft, Methodology. Motoki Okamoto: Writing – original draft, Visualization. Yuichiro Noiri: Writing – review & editing. Shigeyuki Ebisu: Writing – review & editing, Supervision. Hiroyuki Machi: Methodology. Maiko Suzuki: Writing – review & editing. Mikako Hayashi: Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jdent.2024.105307.

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