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Effects of an Arginine-containing Dentifrice on Arginolytic Bacterial Population Levels

by

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

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in the

GRADUATE DIVISION

of the

ABSTRACT

Effects of an Arginine-containing Dentifrice on Arginolytic Bacterial Population Levels. Chen TY*, Hoover CI, Rechmann P, Gansky SA, Featherstone JDB, (University of California San Francisco, San Francisco, CA)

Purpose: To determine if use of an arginine-containing dentifrice increases the population of arginolytic bacteria in orthodontic patients with white spot lesions.

Methods: Forty-seven orthodontic patients, aged 12-21 years with white spot lesions were recruited from the UCSF orthodontic clinic and randomized to 1 of 4 groups: (1) arginine-containing dentifrice (AD)+fluoride rinse (FR) group, (2) AD+FR+antiseptic rinse (AR) group, (3) 1100 ppm fluoride dentifrice (FD)+AR group, or (4) FD+placebo rinse (PR) group. Saliva and plaque samples were collected at baseline, 2 weeks, 2, 4 and 6 months. Mutans streptococci (MS), lactobacilli (LB), and arginolytic bacterial colonies were cultured on MSSB, Rogosa, and BHI agar, respectively. MS and LB were enumerated by observing colony morphologies with a dissecting microscope. Arginolytic bacteria were enumerated using an arginine overlay technique and arginolytic detection reagent.

Results: There were no baseline differences in the age, sex, ethnicity and bacterial levels among the study groups. MS levels ranged from non-detectable to 2.1×10^7 colony-forming units/ml saliva (cfu/ml), LB levels ranged from non-detectable to 1.6×10^7 cfu/ml, and arginolytic bacterial levels ranged from 0.1% to 76.9% of the total cfu. The ratio of arginolytic bacteria to cariogenic bacteria (MS+LB) ranged from <0.1 to ~1,400,000. No consistent changes in arginolytic bacterial populations were observed within individual participants or among the groups throughout the 6-month study period.

Conclusion: Population levels of MS, LB, and arginolytic bacteria in orthodontic participants with white spot lesions were not consistently affected by the use of either fluoride-containing dentifrice or arginine-containing dentifrice.

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1. STUDY AIM/PURPOSE

The purpose of this study was to determine the effect of using an arginine-containing dentifrice on population levels of arginolytic bacteria and cariogenic bacteria in orthodontic patients with white spot lesions. The hypothesis to be tested was that participants using an arginine-containing dentifrice would exhibit increased populations of arginolytic bacteria and decreased populations of cariogenic bacteria.

2. LITERATURE REVIEW

Dental caries remains a significant public health problem in the United States. The National Health and Nutrition Examination Survey (NHANES, 1999-2004) found that 42% of 2-11 year-old children had dental caries experience in their primary teeth, whereas 59% of 12-19 year-old children and adolescents and approximately 92% of adults 20-64 had dental caries experience in their permanent dentition (Dye *et al*., 2007.)

Orthodontic patients, in particular, with fixed appliances are at high risk for caries and white-spot lesion formation due to challenges in maintaining good oral hygiene which leads to plaque accumulation (Gorelick *et al*., 1982). Demineralized white spot lesions occur frequently during orthodontic treatment with approximately a third of orthodontic patients developing at least one white spot lesion until treatment was completed. Some teeth are more prone to demineralization, typically the maxillary lateral incisors and mandibular canine teeth. The distogingival area of the labial enamel surface is the most commonly affected. In the first few weeks after removal of appliances there is typically an exponential reduction of white spot lesion size due to remineralization, with about half of the original lesion being remineralized after 6 months with no specific

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treatment. There are reports in the literature of interventions to prevent and manage white-spot lesions during fixed orthodontic treatment, with weak evidence that untilregular use of fluoride mouthrinses or cementing brackets with fluoride-releasing glass ionomer can reduce white-spot severity (Benson *et al.*, 2004; Wilmot, 2004; Gorton & Featherstone 2003).

Solutions containing arginine can be metabolized by the oral flora to produce ammonia which can neutralize the acid and arrest the demineralization process. Therefore, using arginine during orthodontic treatment may prevent or reduce the formation of white spot lesions. Conventional caries clinical trials measure frank cavitation as the primary outcome and are cost-intensive. However, recognition that caries is a dynamic process (Featherstone, 2004) facilitates the development of abbreviated caries trials utilizing the measurement of caries at the pre-cavitation (white-spot lesion) level (Pretty, 2006).

At least three categories of risk factors have been associated with caries development: microorganisms, substrate/oral environment, and host/teeth. Several studies strongly suggest that the first step in the development of caries is colonization by mutans streptococci (MS; *Streptococcus mutans* and *Streptococcus sobrinus*). *Streptococcus mutans* has been found to be acidogenic (produce large amounts of acid) and aciduric (survive at low pH). Acid is the central virulence agent in dental caries development and is associated with the availability of fermentable carbohydrate in the diet as the substrate for acid byproducts to dissolve tooth structure by the process of demineralization (Fitzgerald and Keyes, 1960; Kleinberg, 1977; Loesche, 1986).

Several anticaries therapeutic methods have been utilized, including fluoride supplementation either in gel or toothpaste form to remineralize tooth enamel, chlorohexidine mouthrinse to reduce MS levels, and xylitol gum to reduce transmission of MS from mother to child. Researchers have shown that xylitol significantly reduces the mother-child transmission of *S. mutans* as compared to other preventative measures used by the mothers, such as chlorhexidine and fluoride (Soderling *et al.* 2001; Thorild *et al.* 2006). However, the majority of xylitol products on the market in the US do not contain enough xylitol for anti-cariogenic effects and require such frequent use as to make it impractical for widespread use. Therefore, there are not enough effective methods available for caries prevention and caries remains a worldwide health problem.

Several hypotheses have been formulated to explain the etiology of dental diseases. The non-specific plaque hypothesis (Theilade, 1986) proposes that micro-organisms have several virulence factors which promote colonization, destroy host defense mechanisms, and promote disease. Different combinations of indigenous bacteria, rather than just a single species, possess the pathogenic potential necessary to cause disease progression. Therefore, oral disease is primarily dependent on plaque mass and metabolic activity and in general not dependent on the presence of specific pathogenic species. In contrast, the specific plaque hypothesis (Loesche, 1976) proposes that out of the numerous bacterial species present in dental plaque(s) only a few are actively involved in the disease process. The specific plaque hypothesis suggests that the way to deal with the microbial aspect of dental caries is targeted elimination of specific pathogens.

In regards to the specific plaque hypothesis, the bacteria most closely associated with caries are the MS (*S. mutans* & *S. sobrinus*). These bacteria are able to rapidly metabolize dietary sugars to acid, creating a low pH environment which demineralizes enamel and in which they grow and metabolize optimally. Glucosyltransferases produced by mutans streptococci synthesize glucan polymers from sucrose and starch hydrolysates. The ability of mutans streptococci to produce large amounts of extracellular, sticky glucans from sucrose is considered to be an important component of their ability to facilitate plaque formation and cause caries.   In the study performed by Vacca *et al.* (2007), there is a strong correlation between MS populations in saliva and caries activity. The data show that glucosyltransferase levels in saliva correlated with caries activity and with number of carious lesions in young children.

High oral colonization levels of lactobacilli (LB) are also associated with dental caries incidence. Studies have shown that increases in fermentable carbohydrate in the diet leads to increases in *Lactobacillus* counts, whereas lowering of such carbohydrate resulted in reductions (Becks *et al.,* 1944, Becks, 1950; Stecksen-Blicks, 1985). LB does not produce glucan to adhere to tooth surface, however, LB can coaggregate with MS and both bacterial species are acidogenic and aciduric. They thrive in pH below 4.4, while other bacteria, such as *Streptococcus sanguinis* and *Actinomyces,* begin to die. Acidification at pH 4.0 for 1 hour impaired the ability of *Streptococcus sanguinis* cells to grow and to temporarily go through a reversible inhibition of glycolytic enzymes. Impaired cells started to grow again at pH 7.0 with a longer lag phase (Kleinberg 1961, 1970). Persistent acidification supports the growth of MS and LB, inhibits the growth of other health-associated microbes, and results in demineralization of enamel.

Specific dentition sites and dental appliances increase retention of fermentable dietary carbohydrate and have also been reported to increase LB counts and caries incidence (Sakamaki and Bahn, 1968). However, caries lesions can develop in the absence of LB, so the relationship between LB and dental caries in humans is also not cause-and-effect but associative (Sims, 1985).

Both the non-specific and specific plaque hypotheses support that caries formation is initiated by the presence of significant numbers of acidogenic and aciduric bacteria in plaque. The level of acid production can be observed in the Stephan curve (Stephan, 1940) which demonstrated that dental plaque has the ability to produce acid rapidly and substantially decrease pH immediately following exposure to a variety of carbohydrates, including glucose and sucrose. After reaching a minimum, the pH showed a subsequent slow rise to baseline, which usually took about one hour. Delay in the return of the pH to baseline was observed and explained by continued acid production when the availability of fermentable substrate was prolonged. The dynamics of the plaque pH changes have been attributed to the concentration and types of bacteria present and the carbohydrate substrate available (Kleinberg, 1961). Besides MS and LB, that are sufficiently acidogenic to be cariogenic, other bacteria such as *S. sanguinis*, *S. mitis*, *S. milleri*, *Actinomyces*, and bifidobacteria are also acidogenic and may participate in caries formation (Marsh and Bradshaw, 1999). Marsh proposed the 'ecological plaque hypothesis' that environmental acidification causes a microbial shift towards a more acidogenic and aciduric population, resulting in a cariogenic microflora.

The Stephan curve suggests that the amount of acid production, following dietary carbohydrate intake, and the time required for recovery to neutral pH are both important

in caries formation. With more frequent carbohydrate intake, the acid demineralization phase becomes longer and consequently the alkalinization phase takes longer to restore neutral pH in the mouth. The frequency and length of these phases are also dependent on frequency, form, and chemical composition of carbohydrate intake, acid production, diffusion rates in plaque, rate of salivary flow, salivary buffering capacity, and metabolic activity of alkalinogenic bacteria.

The pH neutralizing aspects of saliva and the alkalinogenic potential of oral biofilms are important caries protective factors which can mitigate the demineralizing potential of acidogenic bacteria. The buffering capacity of saliva is a property that has been shown to have an inverse correlation with caries prevalence and is largely due to salivary bicarbonate (Lilienthal, 1955). Other pH-rising factors in saliva come from two major substrates for alkali generation by oral biofilms colonizing the teeth: urea and arginine.

Urea is in all salivary gland secretions at concentrations approximately the same as in serum, 3-10 mM (Al-Nowaiser et al., 2003). Urease, a nickel-containing oligomeric enzyme (Mobley et al., 1995), is produced by a discrete subset of oral bacteria, including *S. salivarius*, *Actinomyces naeslundii* and oral haemophili. These urease-producing bacteria rapidly metabolize urea to two molecules of ammonia and one of carbon dioxide. Ammonia production by ureolytic bacteria may participate in reducing cariogenic potential.   In this regard, despite consuming a high carbohydrate diet, chronic renal failure patients who have salivary urea levels 5-25 fold higher than healthy controls have a significantly lower caries incidence (Peterson *et al.,* 1985.) In contrast , Shiboski *et al.* (2009) found that renal transplant patients have higher caries prevalence, but the

study population also had low use of preventive dental care. Therefore, the modulation of the alkalinogenic potential of oral biofilms and manipulation of the availability of alkali-yielding substrates may be effective strategies to modify plaque pH and improve oral health.

Arginine in free form is also present in saliva at concentrations averaging about 50uM, and is abundant as a constituent of salivary peptides and proteins (van Wuyckhuyse *et al.,* 1995). Arginine is primarily catabolized to ornithine, ammonia and CO_2 by abundant dental plaque streptococci, such as *S. gordonii*, *S. parasanguis*, and *S. sanguinis*, some lactobacilli and a few other oral bacteria, including spirochetes (Burne and Marquis, 2000). The arginine dihydrolase system (ADS) is a three enzyme pathway that initially converts arginine to cirtulline and ammonia via arginine deiminase. The citrulline generated is acted on by a catabolic ornithine transcarbamylase in the presence of inorganic phosphate to produce ornithine and carbamyl phosphate. The third enzyme in the pathway, carbamate kinase, cleaves carbamyl phosphate to ammonia and CO_2, concomitantly donating the phosphate to ADP to produce ATP. Unlike ureolysis, the net reaction yields ATP for growth in addition to ammonia and CO_2 .

 Many ADS-positive bacteria can grow moderately well with arginine as the sole catabolite, but protection against acid damage is still considered to be the major function of the system. However, the ADS is sensitive to low pH range. This depends, to some degree, on the specific organism but generally the ADS is significantly inhibited below pH 4. In low pH environments, arginolytic bacteria attempt to maintain a pH gradient across the cell membrane by proton pump ATPases (Casiano-Colon and Marquis, 1988).

This enhances the ability for ADS-positive organisms to reverse potentially lethal acidification through arginolysis.

Arginine and urea metabolism have been identified as mechanisms by which oral bacteria are protected against acid killing, derive bioenergetic advantages by ATP production, and assist in restoring and maintaining a relatively neutral environmental pH that may suppress the emergence of a cariogenic microflora. Dental plaque from caries-resistant individuals has been shown to have higher pH values compared to the plaque from caries-susceptible individuals, and in part the increased pH has been correlated with elevated ammonia levels (Margolis *et al.*, 1988). In addition, van Wuyckhuyse *et al.* (1995) reported a strong correlation between elevated levels of free arginine in saliva and caries resistance and Chen (1996) found that oral bacteria rapidly metabolize arginine to create an increase in environmental pH. More recently, urease activity in dental plaque of caries-free subjects has been reported to be about three-fold higher than in caries active subjects (Shu *et al.*, 2007).

A few studies have investigated the effects of modifying the alkalinogenic properties of oral biofilms. Clancy and Burne (1997) infected rats with a recombinant, urease-producing strain of *Streptococcus mutans* and observed a strong inhibitory effect on the development of caries. Lastly, use of an arginine bicarbonate containing dentifrice has been reported to have a profound anti-caries effect, presumably partially due to the ability of arginine to serve as a substrate for ammonia generation by plaque bacteria (Acevedo *et al.*, 2008). A clinical study done by Acevedo *et al.* (2008) demonstrated that the use of a sugarless mint containing CaviStat (an arginine

bicarbonate calcium carbonate complex) in children with mixed dentition was capable of inhibiting both caries onset and caries progression.

Collectively, a substantial body of evidence indicates that the metabolic activities of oral bacteria with alkali-producing potential may be a promising strategy for caries control.   Despite efforts to modulate alkali production in human oral biofilms, a detailed understanding of the role of alkali production in microbial ecology, oral health and disease in humans, is only beginning to develop.

2.1 Hypothesis

Use of an arginine containing dentifrice by orthodontic patients with white spot lesions will increase their arginolytic bacterial populations and lower their cariogenic bacterial populations.

2.2 Significance

The presence of white spot lesions in orthodontic patients indicates the initiation of demineralization in enamel and caries formation. If use of an arginine containing dentifrice is found to increase arginolytic bacterial populations and decrease cariogenic bacterial populations this should affect the dynamics of the Stephan Curve and potentially reduce the risk of developing future caries in patients with high caries risk, such as orthodontic patients. Thus, arginine-containing dentifrice would be a useful adjunctive therapeutic supplement for caries prevention.

3. **MATERIALS AND METHODS:**

3.1 GENERAL STUDY DESIGN

Prior to initiation, study design and subject recruitment were approved by the UCSF Committee on Human Research. Forty-eight subjects were recruited, but one subject withdrew during the study. The final study population consisted of 47 orthodontic patients, ages 12-21 years with white spot lesions and who were recruited from the UCSF orthodontic clinic. The subjects were randomly assigned to four different treatment groups. Group 1 had twelve subjects who received arginine-containing dentifrice and fluoride rinse (AD+FR); group 2 had twelve subjects who received arginine-containing dentifrice, antiseptic rinse and fluoride rinse (AD+AR+FR); group 3 had eleven subjects who received fluoride dentifrice, antiseptic rinse and fluoride rinse (AR+FR); lastly, group 4 had twelve subjects who received fluoride dentifrice and placebo rinse (FD+PR). All the subjects were instructed to use the assigned dentifrice twice a day and the mouth rinse(s) once a day immediately after the dentifrice use. At the baseline visit, each subject received an oral soft tissue exam (including modified sulcus bleeding index), a dental prophylaxis, an International Caries Detection and Assessment System (ICDAS) caries examination, and a quantitative light fluorescence (QLF) caries examination. Saliva and dental plaque samples were collected for microbiological characterization (mutans streptococci (MS), lactobacilli (LB), arginolytic (Arg) bacteria, and total colony formation). The subjects were followed up 2 weeks after the baseline appointment for saliva and dental plaque sample collection for microbiological characterization. Subsequently, at 2 months, 4 months, and 6 months after baseline, subjects returned for oral soft tissue exams (including modified sulcus bleeding index), dental prophylaxis, ICDAS caries examination, QLF caries examination, and saliva and dental plaque collection.

3.2 SALIVA AND DENTAL PLAQUE COLLECTION

Samples of saliva and dental plaque were collected for microbiological characterization. Saliva samples were collected by requesting subjects to chew on a small piece of paraffin and expectorate into a sterile polycarbonate tube until they had collected ~2 milliliters of saliva. Standardized (1 mg) dental plaque samples were collected over clinically-observed white spot lesions using a disposable plastic 1 ul bacteriological loop. Following collection, the loop (containing dental plaque) was aseptically cut off and deposited into a tube containing 1 mL of TE buffer. Samples were immediately refrigerated and subsequently transferred to the microbiology lab for culture within 24-48 hours.

3.3 LABORARY PROCEDURES

3.3.1 Bacteriological culture and enumeration

The test tubes containing the saliva and plaque samples from the subjects were sonicated for 20 seconds, and 0.1 mL portions were removed for microbiology assays, as described below. Ten-fold serial dilutions (10^{-1}-10^{-7}) of each saliva and dental plaque sample were prepared in phosphate buffered saline (PBS, pH 7.2). Portions (0.1 mL) of each sample and each dilution were spin-plated on three different culture media. Mitis Salivarius Sucrose Bacitracin (MSSB) agar (Gold *et al*., 1973) was used for selective growth and enumeration of mutans streptococci. Rogosa tomato juice (RTJ) agar (Rogosa *et al*., 1951) was used for selective growth and enumeration of lactobacilli. Brain heart infusion (BHI) agar was used for non-selective growth and enumeration of total colony-forming units (CFU), as well as for arginolytic bacteria as described

subsequently. All plates were incubated at 37°C for 72-96 hours in an anaerobic atmosphere (85% nitrogen, 10% hydrogen and 5% carbon dioxide). The resulting bacterial colonies were observed and enumerated using a dissection microscope. Data were recorded as CFU/mL of saliva or CFU/mg plaque.

3.3.2 Enumeration of arginolytic bacteria

Arginolytic bacteria were enumerated using a technique developed by Kaufman *et al.* (2004). After incubation and growth, colonies were transferred from BHI plates to filter paper (5.5 cm diameter, coarse porosity, Fisher Scientific) by blotting. Briefly, filter papers were gently pressed into contact with the agar surface and colonies, after 1-2 minutes the filter papers were removed and placed colony-side up in a petri dish lid. Then arginolytic detection reagent (~0.5 to 1 ml) was added until the filter paper had a uniform yellow color. Excess reagent was poured off and the petri dish lid was sealed with Parafilm^{TM} and incubated at 37°C for 15-20 minutes. Colonies which produced ammonia from arginine developed a blue color (Figure 1) or were surrounded by a blue halo (pH change), while other colonies remained colorless or yellow. Arginolytic bacterial colonies were enumerated with the aid of a dissecting microscope and expressed as CFU/mL of saliva or CFU/mg of plaque.

Figure 1: Arginine overlay – blue zone indicates ammonia production

The arginolytic detection reagent consisted of 50 ml of deionized water, with 1.5g of arginine, 0.625 ml of 1.6% bromcresol purple, and 2.5 ml of 0.2% cresol red. The pH was carefully adjusted to 4.0, then the reagent was filter sterilized, and stored at 4°C when not in use.

3.3.3 Most probable number (MPN) assay

Since the overlay technique described above only has the ability to detect arginolytic bacteria when they represent > 0.1-1% ($\geq 10^5$) of the total bacteria present, we attempted to develop a most probable number (MPN) technique to estimate lower colonization levels. The ten-fold serial dilution series described above (10^{-1}-10^{-7}) were used to inoculate sets of tubes in triplicate (i.e. 0.1 ml of each dilution inoculated into a set of 3 tubes each containing 0.9 mL of BHI broth with 2% arginine). The tubes were incubated at 37°C for 72-96 hours in an anaerobic atmosphere (85% nitrogen, 10% hydrogen and 5% carbon dioxide). Ammonia production from arginine was detected by use of Nesslers reagent (0.09 mol/L solution of potassium tetraiodomercurate in 2.5

mol/L potassium hydroxide). The sensitivity as a spot test is about 0.3 µg NH$_3$ in 2 µL.

Briefly, 10 µL from each tube was placed on a glass slide and 10 µL of Nessler's reagent

was added. If the solution turns yellow (and/or forms a brown precipitate), then ammonia

is present (Figure 2), which signifies the presence of arginolytic bacteria. If no ammonia

is present, the spots remain colorless.

**Figure 2: MPN spot test with Nessler's reagent- yellow color indicates ammonia
production**

The MPN of arginolytic bacteria was determined by comparing results from the

set of tubes with the highest dilution exhibiting at least one negative result and the next

two lower dilution sets (set of 9 tubes, 3 from each dilution) to an MPN table (Table 1,

modified from Standard Methods for the Examination of Water and Wastewater, 12th

edition). To estimate the concentration of the undiluted sample, the middle dilution is

used in the calculation. The MPN procedure provides an estimate of cell concentration

and 95% confidence limits of each estimate.

Table 1. Three Tube Most Probable Number (MPN) Determination Chart

Number of 3 tubes with a positive reaction			MPN Index per 100 ml	95% Confidence Limits	
0.1 ml of $10^{(x-1)}$	0.1 ml of $10^{(x)}$	0.1 ml of $10^{(x+1)}$		Lower	Upper
0	0	1	3	0.5	9
0	1	0	3	0.5	13
1	0	0	4	0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800

4. RESULTS:

4.1 Participants

The first participant was screened for eligibility in July, 2007, recruited and examined in August, 2007, and the final assessment was completed in October, 2009. Forty-seven randomized participants completed the study. The mean participant age was 15 years (range 12-21), with 32 females and 15 males. Baseline demographic data for the study participants by treatment group is presented in Table 2. There were no significant differences in age, sex and race/ethnicity between the groups.

Table 2. Baseline demographic characteristics by treatment group (n=47)

Demographic Characteristics	Treatment Group				Statistical Test
	4 FD + PR n=11	3 FR + AR n=12	1 AD + FR n=12	2 AD + FR + AR n=12	
Age, mean (SD)	15.7 (2.9)	14.3 (1.6)	14.5 (1.9)	15.4 (3.1)	Kruskal-Wallis (P-value=0.717)
Gender, n (%)					Fisher's exact test (P-value=0.789)
Male	4 (36.4)	5 (41.7)	3 (25.0)	3 (25.0)	
Female	7 (63.6)	7 (58.3)	9 (75.0)	9 (75.0)	
Race/ethnicity, n (%)					Fisher's exact test (P-value=0.565)
White	2 (18.2)	3 (25.0)	1 (8.3)	5 (41.7)	
African American	2 (18.2)	0 (0.0)	3 (25.0)	3 (25.0)	
Hispanic	4 (36.4)	5 (41.7)	4 (33.3)	3 (25.0)	
Asian	2 (18.2)	3 (25.0)	3 (25.0)	0 (0.0)	
Other/Multiracial	1 (9.1)	1 (8.3)	1 (8.3)	1 (8.3)	

4.2 Comparison of MPN and Overlay Technique

Initially, we used pure cultures and 4 trial samples from lab members that worked well with the arginine overlay technique and found a good correlation between the MPN

and the overlay technique. Figure 3 illustrates the results obtained with the four samples from lab members.
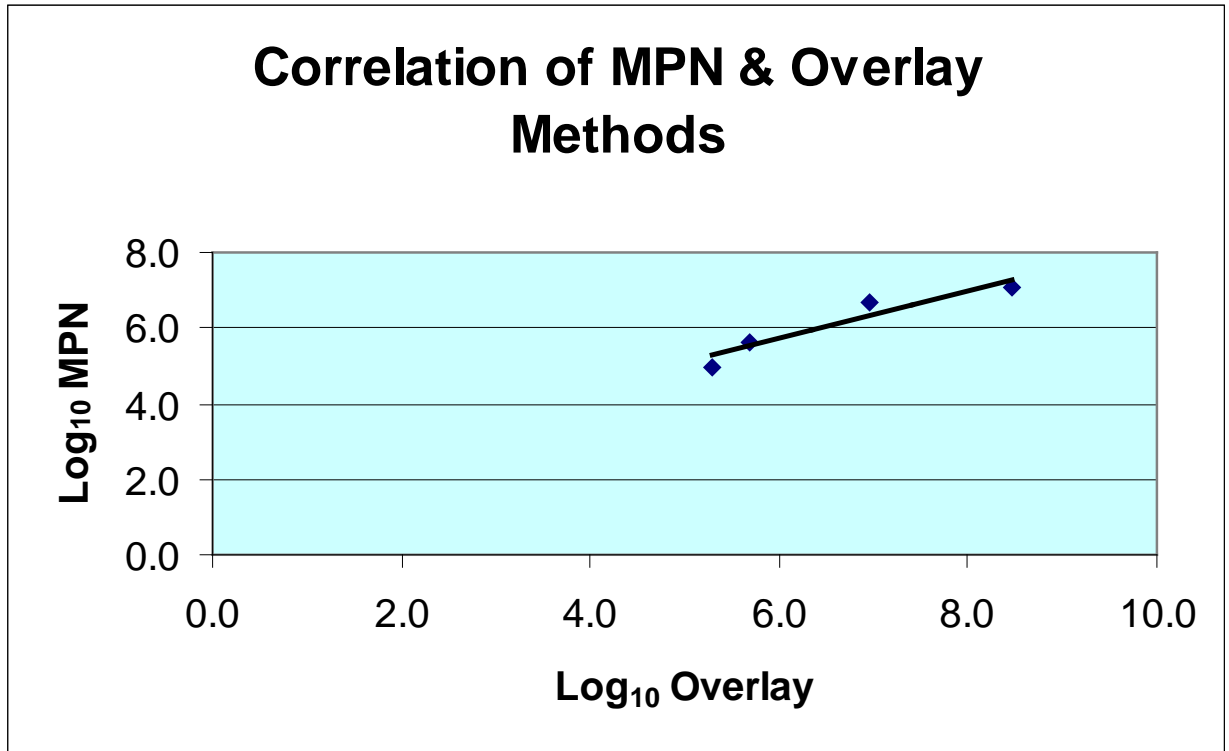
Figure 3: MPN and Overlay Technique for Four Trial Samples

However, subsequent determinations with additional saliva and dental plaque samples were problematic, exhibiting internally inconsistent results and poor correlation with the overlay technique. We attempted to improve the utility of the MPN technique by adding reducing agents DTT (dithiothreitol) and azide. DTT was used to facilitate the growth of anaerobic organisms and azides were used to inhibit the growth and competition from non-streptococcal bacteria. However, the MPN results were still internally inconsistent and did not correlate with the overlay results so we abandoned the use of the MPN technique and only used the arginine overlay technique. Table 3 shows data for 20 subject samples using the MPN technique and arginine overlay technique.

Figure 4 illustrates the data from Table 3 and indicates the poor correlation (Pearson

r=0.16) of MPN and arginine overlay results.

Table 3:MPN and Arginine Overlay Results for Eight Participant Samples.

Subject and Sample	MPN Arginine	Overlay Arginine
1S	4.6×10^5	2×10^7
1P	2.4×10^4	4×10^5
2S	3×10^4	1.8×10^6
2P	6.4×10^4	1×10^5
3S	1.5×10^7	1.1×10^6
3P	4.6×10^5	4.3×10^2
4S	1.1×10^7	1.1×10^7
4P	1.1×10^5	4.6×10^4
5S	1.1×10^6	23
5P	2.1×10^5	0
6S	2.4×10^6	1.5×10^3
6P	4.5×10^5	7
7S	9.3×10^3	2×10^6
7P	1.2×10^2	2×10^5
8S	1×10^5	0
8P	9	0
1S-2	4.6×10^5	6×10^5

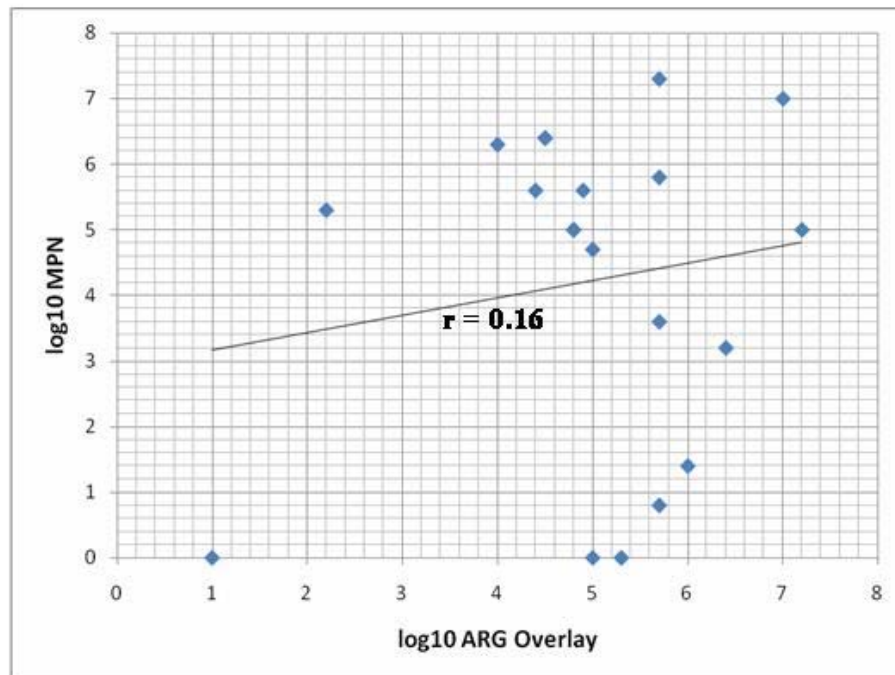|		
1P-2	7.5×10^4	4×10^5
2S-2	3×10^4	1.8×10^6
2P-2	6.4×10^4	1×10^5

Figure 4. Low correlation of MPN and Overlay Techniques for Eight Participant Samples. Pearson correlation co-efficient, r =0.16

4.3 Efficacy Analysis

The data presented in Table 4 demonstrate that the spread (minimum to maximum) of the log10 bacterial counts were narrow within the groups at the beginning of the study (baseline).

Table 4: Baseline Microbiology Group Ranges

Bacterial Levels	Range
$\log_{10}$ MS plaque	3.0-3.2
$\log_{10}$ MS saliva	3.9-4.7
$\log_{10}$ LB plaque	1.9-2.4
$\log_{10}$ LB saliva	4.0-4.8
$\log_{10}$ Arg plaque	6.2-6.7
$\log_{10}$ Arg saliva	6.2-7.1
Ratio C plaque	2.2-2.7
Ratio C saliva	1.4-1.5

After baseline, plaque and saliva samples were collected at 2 weeks, 2 months, 4 months and 6 months. Table 5 shows the 7 pairwise comparisons that resulted in statistically significant changes between treatment groups out of the 192 possible pairwise comparisons (Bonferroni-Holm's pairwise comparisons using overall P-value$\leq$ 0.10).

Table 5. Mean changes from baseline for microbiology data with significant pairwise treatment comparisons

Change from baseline in plaque bacteria	1 AD + FR n=12	2 AD + FR + AR n=12	3 FR + AR n=12	4 FD + PR n=12	Significant pairwise comparisons *
Ratio C in dental plaque at 2-week visit – baseline	0.45	-0.20	-0.07	0.75	3 vs 4, 2 vs 4
$\log_{10}$MS in dental plaque at 2-week visit – baseline	-0.55	0.12	0.18	-0.94	3 vs 4, 2 vs 4
$\log_{10}$LB in dental plaque at 2-week visit – baseline	-0.24	0.35	-0.20	-0.64	2 vs 4
$\log_{10}$MS in dental plaque at 6-month visit – baseline	-0.61	0.42	0.16	-1.24	3 vs 4, 2 vs 4

*Bonferroni-Holm *P*-value≤ 0.10 adjusted for pairwise comparisons between groups. Negative mean value indicates a decrease from baseline.

Overall, there were no consistent significant changes from baseline in bacteria levels among the four treatment groups. The only statistically significant changes from baseline are shown in Table 4. Group 2 (AD+FR+AR) had a decrease (-0.20) in arginolytic bacteria level when compared to group 4 (FD+PR) at two weeks. Group 4 had an increase (0.75) in arginolytic bacteria level at two weeks. When comparing change in MS levels from baseline between these two groups at 2 weeks and 6 months, Group 2's MS levels went up (0.12 and 0.35), whereas Group 4's MS levels went down (-0.94 and -1.24). LB level was also higher at the 2 week visit in Group 2 (0.35) and lower in Group 4 (-0.64).

5 DISCUSSION:

This study compared the effect of a 6-month home application of an arginine-containing dentifrice against that of an anti-bacterial mouth rinse, fluoride mouth rinse

and fluoride-containing dentifrice in orthodontic patients with white-spot lesion. White-spot lesions had been identified in all participants, and it was therefore an ethical imperative to ensure that all participants received standard care. Therefore, all participants received fluoride-containing toothpaste, oral hygiene monitoring and advice, prophylaxes, and background environmental fluoride.

In this study, there were few significant differences between the arginine dentifrice group and fluoride dentifrice group. It appeared that fluoride dentifrice alone was the best regimen, with an increase in number of arginolytic bacteria and decrease in number of mutans streptococci and lactobacilli. The changes in the other groups had an opposite effect of what was expected. In the arginine dentifrice group, we expected that by providing arginine to the arginolytic bacteria, we would be able to increase the number of arginolytic bacteria. This would in return compete with the cariogenic bacteria in the oral flora and result in decreased number of mutans streptococcus and lactobacilli. However, there was a decrease in the number of arginolytic bacteria in the arginine dentifrice group and an increase in MS and LB.

In a previous study, Acevedo *et al.* (2008) conducted a12-month long clinical trial to determine if a sugarless mint containing CaviStat (an arginine bicarbonate calcium carbonate complex) is capable of preventing the development of dental caries in the primary molars and first permanent molars of 10½ to 11-year-old Venezuelan children. The results showed statistically significant reductions in both DMFS and dmfs and the investigators therefore concluded that the mint confections containing CaviStat are able to inhibit both caries onset and caries progression in these children.

The timing and concurrent fluoride use and antiseptic mouthrinse use in our study may have contributed to the lack of effect we observed with arginine dentifrice use. The use of either product may contain ingredients that have eliminated the arginolytic bacteria population, therefore leading to a more challenging environment for growth and utilization of arginine to produce ammonia.

Fluoride and tricolsan, which are commonly used in oral health care products, have been found to inhibit ammonia production by arginolytic bacteria. Presumably, these compounds reduce ammonia production by inhibiting the arginine uptake system. Initial uptake of arginine by arginolytic bacteria over the first few minutes after exposure was insensitive to the inhibitors, but subsequent uptake was highly sensitive. Fluoride and triclosan are weak organic acids that are able to effect changes in pH across the cell membrane and inhibit arginine uptake in a pH dependent way. This suggests that membrane-associated pH changes are the basis for the inhibitory effects demonstrated by fluoride and triclosan on ammonia production by oral bacteria (Marquis *et al*., 2003).

Fluoride has also been found to be a highly effective inhibitor of ammonia production from urea by oral bacteria. Fluoride inhibits ammonia production from urea by two mechanisms: (1) it is a direct inhibitor of urease and (2) it functions as a transmembrane proton conductor which acidifies the cell cytoplasm and diminishes urease activity (Barboza-Silva E *et al*., 2005).

The fact that dental caries is still a major clinical problem encourages more research to be done on exploring new strategies for caries prevention and treatment therapy, as well as for assessment of caries risk. Although our study did not find that arginine-containing dentifrice increased populations of arginolytic and decreased

populations of cariogenic bacteria, a study using an arginine-containing mint has reported

caries reduction (Acevedo *et al*., 2008). The CaviStat mint confection may have

provided persistence of oral arginine concentrations not obtainable from the dentifrice.

Therefore, more studies should be conducted using other forms of arginine-containing

products, such as gums or varnish, to assess alkali-generating capacity and reducing

caries risk. Additional studies to identify novel bacterial strains contributing to total

arginolysis and ureolysis in the oral cavity and to establish the nature and function of

factors controlling their alkali-generating activity are also warranted.

6   CONCLUSIONS:

- Techniques that increase proportions of alkali-producing bacteria in dental plaque may reduce the risk of caries formation.

- Overall, the presence of fluoride may make the metabolism of arginine to ammonia less effective. This could be a reason why we did not detect any differences between the groups.

- Estimation of cariogenic bacterial populations and alkali-producing bacterial populations may provide more accurate caries risk assessments.

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