Novel Methods for the prevention of Infectious Diseases involving Microbial Biofilms

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The oral cavity is one of the primary routes of entry for bacterial, viral, fungal and other foreign antigens into the human body. In addition to this extensive exogenous load, abundant indigenous microbial flora colonise the mouth, the dentition, and the mucosal surfaces, thus providing the host with a considerable amount of antigenic stimulation.

Concerning microbial flora colonisation, it is possible to distinguish two different aspects. The first one consists of an isolated form that is observed mainly in saliva. The second one consists of a colonised form that is observed in the dental plaque or on mucosal surfaces. Usually in clinical tests, the microorganisms which are studied, originate from saliva samples, which means that they are in isolated form. Only a few studies have been performed with colonised microorganisms, commonly called biofilm microorganisms. Nevertheless, the percentage of microorganisms living in biofilm forms is considered to be more than 60% compared to isolated microorganisms. Moreover, a microorganism has been observed to be much less resistant in isolated form than in biofilm mass.

A good equilibrium between all these microorganisms is important for a healthy oral condition. The maintenance of this equilibrium is dependent on the presence of number of host defense systems acting in the oral cavity.

The mouth is constantly bathed by saliva, a unique secretion which contains a wide variety of components with antimicrobial activity, essential for the maintenance of oral health (1). These saliva-mediated agents, usually proteins, are mainly synthesized in the major and minor salivary glands (2), but a considerable proportion is also derived from blood via the gingival crevicular fluid or directly from oral polymorphonuclear (PMN) cells (3). The antimicrobial agents in whole saliva are usually divided into non-immunoglobulin, innate factors and immunoglobulins, acquired factors.

There are also other important agents present in the saliva which act indirectly against pathogenic mocro-organisms and in synergy with the antimicrobial agents contained in whole saliva. These agents are small peptides commonly called Growth Factors. Many studies demonstrate the direct relation between the concentration of growth factors in saliva and the presence of growth factor receptors on the surface of mucosal cells which have become inflamed due to the adhesion of biofilm bacteria (4,5).

Table 1 : Major antimicrobial factors and growth factors in human whole saliva

Non-immunoglobulin, innate factors
Lysozyme
Lactoferrin
Salivary peroxidase
SCN^-
H_2O_2
Myeloperoxidase
Cl^-
Agglutinins
Histidine-rich proteins
Proline-rich proteins
Mucins
Phagocytic cells

Immunoglobulins, acquired factors

IgA (sIgA), IgG and IgM

Growth factors

EGF: Epidermal growth factor
PDGF: Platelet growth factors
TGF: Transforming growth factors
FGF: Fibroblast growth factors
IGF I&II: Insulin I&II growth factors

Previous attemps to study the activity of some of these antimicrobial agents such as lactoferrin (6), lysozyme (6), or $OSCN^-$ generated by the lactoperoxidase system (7) have produced controversial results. In fact, it is evident that one antimicrobial agent alone will not prevent periodontal problems. On the contrary, it is the synergistic effect between all these antimicrobial agents, innate, acquired factors and growth factors which produces an active defence against oral disease.

In order to substantiate this hypothesis, a comparative study was conducted using two different toothpaste formulations. The first (A) contained only one antimicrobial agent - $OSCN^-$ generated by the lactoperoxidase system, the second (B) contained all the components. (Table 2)

Table 2 : Active ingredients contained in both formulations

Ingredients	**Toothpaste A**	**Toothpaste B**
Lysozyme		X
Lactoferrin		X
Salivary peroxidase (Lactoperoxidase)	X	X
SCN^-	X	X
H_2O_2 (Glucose/Glucose Oxidase)	X	X
IgA		X
gG		X
IgM		X
EGF: Epidermal growth factor		X
PDGF: Platelet growth factors		X
TGF: Transforming growth factors		X
FGF: Fibroblast growth factors		X
IGF I&II: Insulin I&II growth factors		X

MATERIALS AND METHODS

Subjects and study design
The study was a double-blind crossover design. Each test period was 28 days. Seventeen volunteers presenting a non-healthy oral situation, took part in the investigation. The volunteers, 9 males, 8 females, were aged 30-45 years (mean 37,8 years). The criteria for participating were high levels (10^5-10^6 colony-forming limits CFU/ml) of salivary Streptococcus *mutans* as measured before the trial.

After base-line measurements all participants were given instructions to brush with the experimental toothpastes (toothpaste A or B) twice daily, in the morning and in the evening and during 1 minute each time for 4 weeks. The amount of toothpaste used was +/- 1 gr each time. The subjects were instructed not to use any other oral hygiene or antimicrobial products during the trial period. After the second base-line recordings the other experimental toothpaste was given to the subjects for 4 weeks use. The subjects were instructed not to eat, drink, or smoke for at least 1 hour before sampling and pH measurements.

Test toothpastes
The two experimental products, coded toothpaste A and toothpaste B were produced. The toothpastes were delivered in identical plain white tubes to ensure that neither the examiner nor the subjects were aware of the contents of the tube. Both toothpastes contained sodium monofluorophosphate (0.15% F-), xylitol (1%), and a nonionic surfactant, Arlasolve 200, which is a polyoxyethylene 20 isohexadecyl ether.
The pH of both toothpates was 6.5. The code was opened after all data had been processed. Toothpaste A contained lactoperoxidase (bovine milk: 10.000 ABTS units/100 gr), 0.02% KSCN, and Glucose/Glucose Oxidase (10.000 ABTS units per 100 gr) system for H_2O_2 generation. Toothpaste B contained lactoperoxidase (bovine milk: 10.000 ABTS units/100 gr), 0.02% KSCN, and Glucose/Glucose Oxidase (10.000 ABTS units per 100 gr) system for H_2O_2 generation, Lactoferrin (bovine milk : 0.1%), Lysozyme (egg white : 0.1%), Immunoglobulins (0.05%) and Growth Factors (Epidermal growth factors, Transforming growth factors, Fibroblasts growth factors, Insulin I&II growth factors, nerve growth factors and Derived platelet growth factor).

Collection and treatment of saliva and plaques samples
Paraffin-stimulated whole saliva was collected from each subject for 5 minutes and the secretion rate was determined as millitres per minute. After the saliva collection, 100µl of fresh saliva was used for the hypothiocyanite ($HOSCN/OSCN^-$) assay. For microbiologic analysis a 100µl aliquot was transferred to a tube with 20% glycerol. For the ATP assay, 100µl aliquot was transferred at frozen and maintained at –80° C. The rest of the collected saliva was centrifuged at 18.000 g during 10 minutes at +4°C. A part of the samples were used to analysis the thiocyanate concentration, the lactoferrin, immunoglobulins and lysozyme concentrations.

After saliva sampling, supragingival plaque was collected from all tooth surfaces with a sterile curette for approximatively 5 minutes. The wet weight of the plaque was determined immediately. The sample was then transferred to a tube containing 2 ml phosphate buffer saline solution and sterile glass beads. The tube was vortexed during 30 seconds and a 100µl aliquot was withdrawn and transferred into a tube containing 1 ml of TSB supplemented with 20% glycerol. The TBS tubes were stored frozen at –20°C until microbiologic analysis.
The rest of the plaque suspension was supplemented with 100µl 2M glucose (final concentration of 0,1M) to induce lactic acid production.

Chemical Assays
Concentrations of hypothiocyanite ($HOSCN/OSCN^-$), thiocyanate (SCN^-) and the total peroxidase activity were determined in all saliva samples. $HOSCN/ OSCN^-$concentrations were analyzed by reaction with the colored anionic monomer of 5.5-dithiobis-(2-nitro-benzoic acid) as described by Aune & Thomas (8) and modified by Pruitt et al (9). The thiocyanate was identified by ferric nitrate method described by Betts & Dainton (10). Salivary peroxidase activity was measured at 37°C by following the rate of oxidation of 5-thio-2-nitrobenzoic acid by $OSCN^-$ ions generated during the oxidation of SCN^- by salivary peroxidase or myeloperoxidase. Immunoglobulins were measured by nephelometry method. Lactoferrin concentrations were assessed using an ELISA kit (Bio-X Diagnostics, Belgium). Lysozyme activities were estimated with Micrococcus luteus at pH 6,6 at 20°C.
Lactic acid production in plaque was quantitated using the L-Lactic acid kit (Mannheim Boehringer, Germany)

Microbiological Assays
The specific characteristics of the cultures (morphology, Gram Staining) were determined and the colony forming units (CFU) of all samples were counted. The bacteriological counts were $\log_{10}$ transformed so that the variances would bed normalised. The survival rate (%) was calculated as $\log_{10} CFU_{assay}/\log_{10} CFU_{control} x100$. For this experiment, we used a toothpaste control containing starch in replacement of the active ingredients contained in toothpaste A and in toothpaste B. The measures were normalised based on this toothpaste control (base line in table 4a). Bacterial ATP content was determined according to the method of Schram and Weyes-Van Witzenburg (11) using and LKB Wallac 250 Luminometer (LKB-Pharmacia, Uppsala, Sweden) with an ATP bioluminescence CLS kit (Boehringer Mannheim, Darmstadt, Germany)

RESULTS
A 4-week daily use of toothpaste A and toothpaste B had no effect on salivary flow rate. An increase in the concentration of the

peroxidase system-generated HOSCN/$OSCN^-$ in saliva could be discerned after 4 weeks using toothpaste B compared to toothpaste A. An increase of immunoglobulins and lactoferrin concentrations and lysozyme activities in the saliva samples were observed (Table 1a and 1b).

The accumulation of dental plaque was not affected by toothpaste A, whereas a decrease in the accumulation of dental plaque was observed using toothpaste B (Table 2 and Figure 1).

The acidogenicity of plaque quantitated by glucose stimulated lactic acid production did not change significantly using toothpaste A. This was not the case with toothpaste B (Table 2 and Figure 2). In the presence of glucose (15%), toothpaste B was observed to inhibit the reduction in pH of dental plaque. This was not the case with toothpaste A (Table 3).

Regarding the effects of the two products on oral microorganisms, both have a notable effect in inhibiting the cells present in the saliva. But a significantly better activity was observed for toothpaste B against toothpaste A, comparing the ratio ATP/CFU (Table 4a,4b and the Figure 3). This observation was also confirmed on some individual microorganisms present in saliva samples (Table 4a,4b and Figures 4,5).
Nevertheless a more significant number of colony units was observed in the case of toothpaste B compared to toothpaste A, and the control toothpaste in the saliva samples. (See Figure 6). This significant increase in the microorganisms in the saliva was observed for the subjects using toothpaste B can be explained by the detachment of biofilm bacteria due to the presence and action of growth factors on mucosal cells in conjunction with the ability of immunoglobulins, lactoferrin and lysozyme to inhibit re-adherence of the bacteria to mucosal surfaces. (12,13,14). This observation confirms the results.
However, in the case of subjects using toothpaste B, where more microorganisms were observed in the saliva, (Figure 6), toothpaste B was observed to exert a significantly greater antibacterial effect on these bacteria present in the saliva compared to the antibacterial effect of the toothpaste A (Figure 3).

DISCUSSION

The study indicates that in subjects with normal salivary secretion rate but with bad oral hygiene, the daily use of toothpaste A does not significantly affect the accumulation or acidogenicity of dental plaque. In the case of toothpaste B, an inhibitory effect on the acidogenicity and a decrease of the dental plaque was observed.

In the case of effect on microorganisms, using the ATP method, a decrease in energy of the total amount of bacteria in the saliva was observed. This corresponds to the fact that both toothpastes have a bacteriostatic defence system on the salivary flora.

Similarly, a previous study performed by Dr Courtois at al., (15) demonstrated that HOSCN/$OSCN^-$ generated by the peroxidase system has a bacteriostatic effect instead of a bactericidal effect. Although, this decrease was not observed for dental plaque bacteria it was concluded that the HOSCN/$OSCN^-$generated by peroxidase system and contained in toothpaste A had an effect only on isolated bacteria and not on the biofilm bacteria. The results observed with toothpaste B showed a greater level of bacteriostatic activity against salivary flora. Regarding dental plaque in the form of organized biofilm bacteria, toothpaste B show significant activity.

Based on these results, it was concluded that the improved activity of toothpaste B over toothpaste A against salivary bacteria, is mainly due to the activity of other antibacterial molecules such as lactoferrin, lysozyme and immunoglobulins acting in conjunction with the peroxidase system and to the synergistic effects of their activities as demonstrated in precedent scientific papers (16,17,18,19, 20). Moreover, toothpaste B has an effect on biofilm bacteria. This can be explained by the presence of the growth factors which assist in in the natural healing process of the mucosal cells (21, 22,23) damaged by the lipopolysaccharides-bacteria (24,25), by facilitating the detachment and release of biofilm bacteria into the saliva. This function corresponds to the higher levels of bacteria observed in the saliva of the subjects using the toothpaste B. These experiments demonstrate that the role of HOSCN/$OSCN^-$ is to react against suspended cells and that its dilution in penetrating biofilm bacteria is decreased to a concentration lower than 100µM (minimum concentration for the activity of the molecule) (26), yielding the molecule inactive against biofilm bacteria. This effect was also demonstrated in case of biocide (27) and antibiotic (28,29)

In the case of lactoferrin, lysozyme and immunoglobulins, any inactivity against biofilm bacteria is mainly due to the size of the molecules which prevents their penetration through biofilm. Nevertheless, it was proved that lactoferrin and immunoglobulins will prevent the re-adhesion of the suspended bacteria to the mucosa cells (13,14). This indicates that toothpaste B provides a more effective preventive mechanism than toothpaste A.
Similarly, the increased levels of bacteria found in the saliva of subjects using toothpaste B can be explained by the removal of biofilm bacteria attached to the mucosal cells. The subsequent transformation of these biofilm bacteria into isolated bacteria renders them more accessible to the antibacterial molecules such as $OSCN^-$, lactoferrin and lysozyme. This corroborates the results of the assays performed by Lin et al (30), Chang et al (31), Nordlund et al (32), Whitcomb et al (33) which demonstrated that when mucosa cells are inflamed due to the formation of lipopolysaccharides the presence of growth factorspecific receptors is observed on the surface of these cells, together with an increase in the concentration of the growth factors in saliva (34,35) and in the gingival crevicular fluid (36,37). Evidence shows the presence of these growth factors to be essential for repairing the damage caused to mucosal cells by lipopolysaccharides - also known as bacterial virulence factors. This process of wound healing encourages the detachment of bacteria from the mucosa cell surfaces consequently transforming the bacteria from biofilm form to isolated bacteria.

It is important to note that with the same concentration in vitro of $HOSCN/OSCN^-$ generated by the peroxidase system contained in the two toothpastes, the concentration of $HOSCN/OSCN^-$ in the saliva samples of both groups were not the same after 4 weeks treatments. In fact, the $HOSCN/OSCN^-$ produced in toothpaste A acted only against isolated bacteria and the $HOSCN/OSCN^-$ produced in the toothpaste B acts against isolated bacteria and the biofilm bacteria which have been detached from the mucosa / tooth surfaces and which have become isolated bacteria.
When the subjects brushed their teeth with toothpaste A, the $HOSCN/OSCN^-$ produced inhibited the isolated bacteria only. Over a 4-week period this study demonstrated that the concentration of $HOSCN/OSCN^-$ produced by toothpaste A was still lower than normally would be expected. In the case of the toothpaste B, the concentration of $HOSCN/OSCN^-$ was also found to be lower than the toothpaste would normally produce after 2 weeks. The concentration was even lower than that produced by the toothpaste A, but we recovered to normal levels after 4 weeks.
It was concluded that because the $HOSCN/OSCN^-$ in toothpaste B is used to inhibit the isolated bacteria and the biofilm bacteria released into the saliva, a higher proportion of $HOSCN/OSCN^-$ is used up in order to inhibit all these bacteria.
However, after 4 weeks, the concentration of $HOSCN/OSCN^-$ in toothpaste B was observed to have reached expected levels. The difference between the two toothpastes seems to suggest that toothpaste B has been effective against isolated and biofilm bacteria within the 4-week period. It would have been useful to have completed some tests between 2 to 4 weeks. Nevertheless, in the case of the toothpaste B, it is not only the $HOSCN/OSCN^-$ which inhibits isolated bacteria, but the presence of lactoferrin, lysozyme and immunoglobulins in the formulation facilitate an inhibition of isolated bacteria combined with a prevention of the production biofilm bacteria.

If precedent studies have indicated that oral $HOSCN/OSCN^-$ may inhibit the glucose uptake (38) and acid production (26) by the bacteria, this effect was not observed in the case of the toothpaste A. On the other hand this effect was observed in the case of the toothpaste B (Table 2).

There are two mechanisms to inhibit cariogenic bacteria. The first is by $HOSCN/OSCN^-$ ions. Under acidic conditions (pH 5.0) these ions inhibit the growth of the mutans streptococci and lactobacilli in whole saliva, but no effect is seen at a pH > 6.0 - pH of the toothpaste A.
The second mechanism is the activity of lactoferrin, lysozyme and immunoglobulins. All these molecules have an activity against mutans streptococci and lactobacilli at a pH between 5.0 and 8.0. Toothpaste B was demonstrated to have a significantly greater effectiveness against such microorganisms, independent of the pH. The lack of evidence of such activity in toothpaste A resulted in the conclusion that the total antimicrobial capacity of saliva is not dependent on any single agent (39). The increased levels of other antimicrobial factors in the saliva of the subjects using toothpaste B supports the

significance of the entire repertoire of
antimicrobial components for oral health (40).

Table 1a . Effects of a 2-week daily use of the experimental toothpastes on the flow rate and the different active components in saliva.

	Toothpaste A		Toothpaste B	
	60 min mean+/-SD	2 weeks mean +/-SD	60 min mean+/-SD	2 weeks mean +/-SD
Flow rate (ml/min)	1.8 +/- 1.0	2.0 +/- 1.0	1.6 +/- 1.0	1.7 +/- 1.0
OSCN- (µM)	48,9 +/- 12,1	56,8+/- 17,2	34,5 +/- 22,1	38,7+/- 12,2
Immunoglobulins				
IgA (µg/ml)	2,5 +/- 0,3	1,4 +/- 0,6	38, 4 +/- 3,7	37,4 +/- 2,5
IgG (µg/ml)	6,5 +/- 0,5	6,8 +/- 0,9	15,4 +/- 3,4	14,3 +/- 4,5
IgM (µg/ml)	1,2 +/- 1,1	1,4 +/- 2,3	5,2 +/- 2,1	6,5 +/- 2,3
Lactoferrin (µg/ml)	6,98 +/-2,34	5,65 +/- 3,12	14,56 +/- 3,87	35,51 +/- 7,8
Lysozyme (µg/ml)	10,4 +/- 1,09	11,23 +/- 2,34	32,5 +/- 2,8	34,5 +/- 8,9
Growth factors (ng/ml)				
TGF	0,1 +/- 0,6	0,06 +/- 0,2	9,7 +/- 0,4	10,2 +/- 0,6
EGF	0,05 +/- 0,8	0,06 +/- 0,4	5,7 +/- 0,5	5,8 +/- 0,7_

Table 1b . Effects of a 2-week daily use of the experimental toothpastes on the flow rate and the different active component in saliva.

	Toothpaste A		Toothpaste B	
	2 weeks mean+/-SD	4 weeks mean +/-SD	2 weeks mean+/-SD	4 weeks mean +/-SD
Flow rate (ml/min)	2.0 +/- 1.0	1.8 +/- 1.0	1.7 +/- 1.0	1,8+/-1.0
OSCN- (µM)	56,8+/- 17,2	54,5 +/- 12,1	38,7+/- 12,2	112,5+/- 12,6
Immunoglobulins				
IgA (µg/ml)	1,4 +/- 0,6	1,2 +/- 0,5	37,4 +/- 2,5	35,6 + - 6,5
IgG (µg/ml)	6,8 +/- 0,9	5,7 +/- 0,8	14,3 +/- 4,5	15,7 +/- 3,2
IgM (µg/ml)	1,4 +/- 2,3	1,7 +/- 3,5	6,5 +/- 2,3	6,8 +/- 1,4
Lactoferrin (µg/ml)	5,65 +/- 3,12	4,25 +/- 2,3	35,51 +/- 3,4	37,6 +/- 4,5
Lysozyme (µg/ml)	11,23 +/-,34	10,8 +/- 3,4	34,5 +/- 8,9	36,8 +/- 3,5
Growth factors (ng/ml)				
TGF	0,06 +/- 0,2	0,07 +/- 0,3	10,2 +/- 0,6	10,5 +/- 0,7
EGF	0,06 +/- 0,4	0,07 +/- 0,3	5,8 +/- 0,7	4,7 +/- 0,8_

Table 2. Effects of a 4-week daily use of the experimental toothpastes on plaque accumulation and lactic acid production.

	Toothpaste A		Toothpaste B	
	Base line mean+/-SD	4 weeks mean +/-SD	Base line mean+/-SD	4 weeks mean +/-SD
Lactic acid (µgr/mg)	1,65+/-0,34	1,87+/-0,98	1,55+/-0,62	0,34+/-0,12
Quant.of plaque (mgr)	32,8+/-14,1	30,6+/-12,4	30,7+/-12,7	17,5+/-13,6

Table 3. Glucose (15%)-induced pH drop in dental plaque with and without using the experimental toothpastes.

	Plaque pH			
	Base line		4 weeks	
	0 min mean+/-SD	60 min mean+ /-SD	0 min mean+/-SD	60 min mean+ /-SD
Toothpaste A				
Toothpaste + glucose	6,5+/-0,2	5,1+/-0,3	6,6+/-0,5	4,9+/-0,3
Glucose	6,5+/-0,2	5,2+/-0,3	6,6+/-0,4	5,0+/-0,4
Toothpaste B				
Toothpaste + glucose	6,5+/-0,2	6,3+/-0,2	6,6+/-0,5	6,5+/-0,2
Glucose	6,5+/-0,2	5,1+/-0,3	6,6+/-0,3	5,2+/-0,3

Table 4a. Effects of a 4-week daily use of the experimental toothpastes on numbers of bacteria in saliva and dental paque.

	Toothpaste A		Toothpaste B	
	Base line mean+/-SD	2 weeks mean +/-SD	Base line mean+/-SD	2 weeks mean +/-SD
Total count number	1,0 +/-0,03	0,7+/-0,04	1,0+/-0,04	0,01+/-0,002
S.mutans	1,0 +/-0,03	0,65+/-0,03	1,0+/-0,04	0,013+/-0,002
Lactobacilli	1,0 +/-0,03	0,71+/-0,04	1,0+/-0,04	0,017+/-0,002

Table 4b. Effects of a 4-week daily use of the experimental toothpastes on numbers of bacteria in saliva and dental plaque.

	Toothpaste A		Toothpaste B	
	2 weeks mean+/-SD	4 weeks mean +/-SD	2 weeks mean+/-SD	4 weeks mean +/-SD
Total count number	0,7 +/-0,04	0,65+/-0,3	0,01+/- 0,002	0,018+ /-0,004
S.mutans	0,65+/-0,03	0,71+/-0,04	0,013+/-0,002	0,012+/-0 ,002
Lactobacilli	0,71+/-0,04	0,69+/-0,04	0,017+/-0,002	0,015+/-0,002

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Novel Methods for the prevention of Infectious Diseases involving Microbial Biofilms
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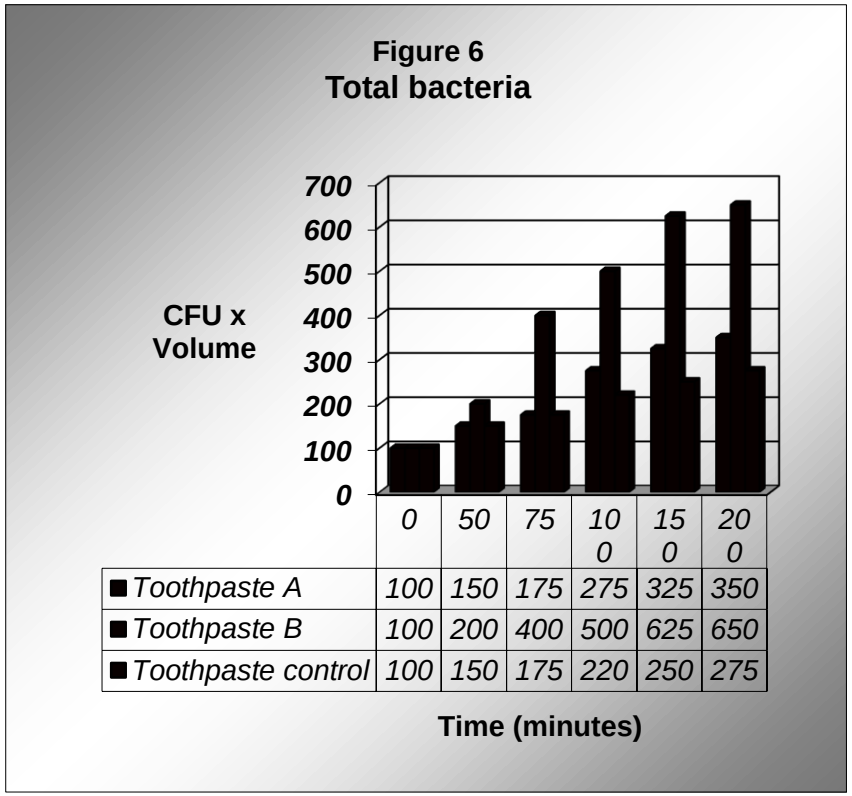
Total bacteria CFU x volume



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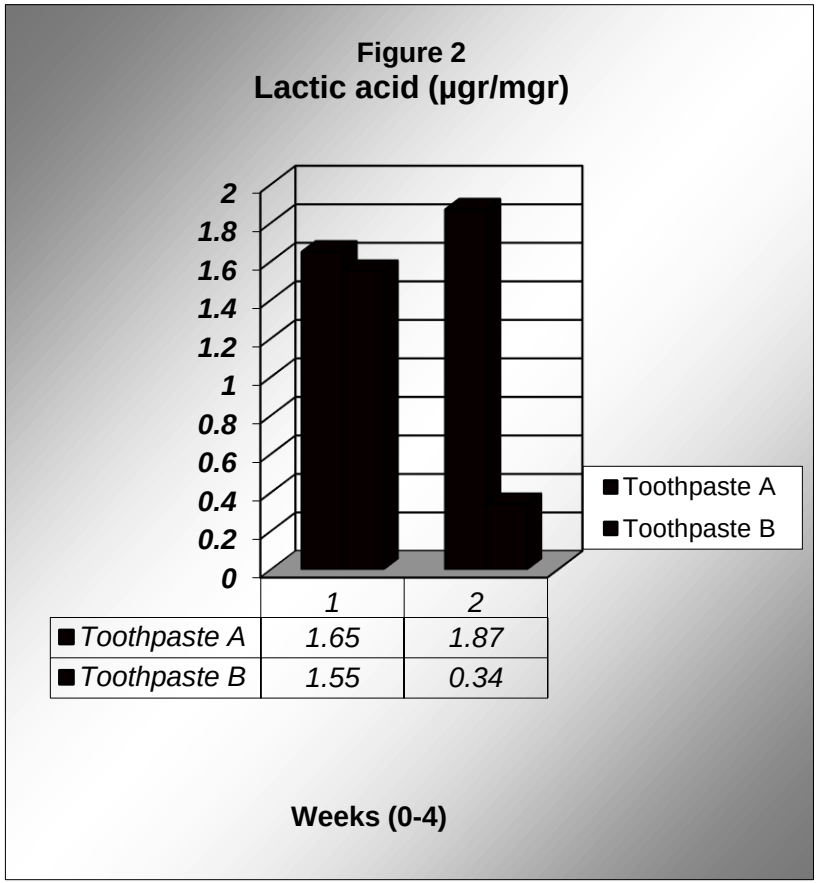
Lactic Acid reduction



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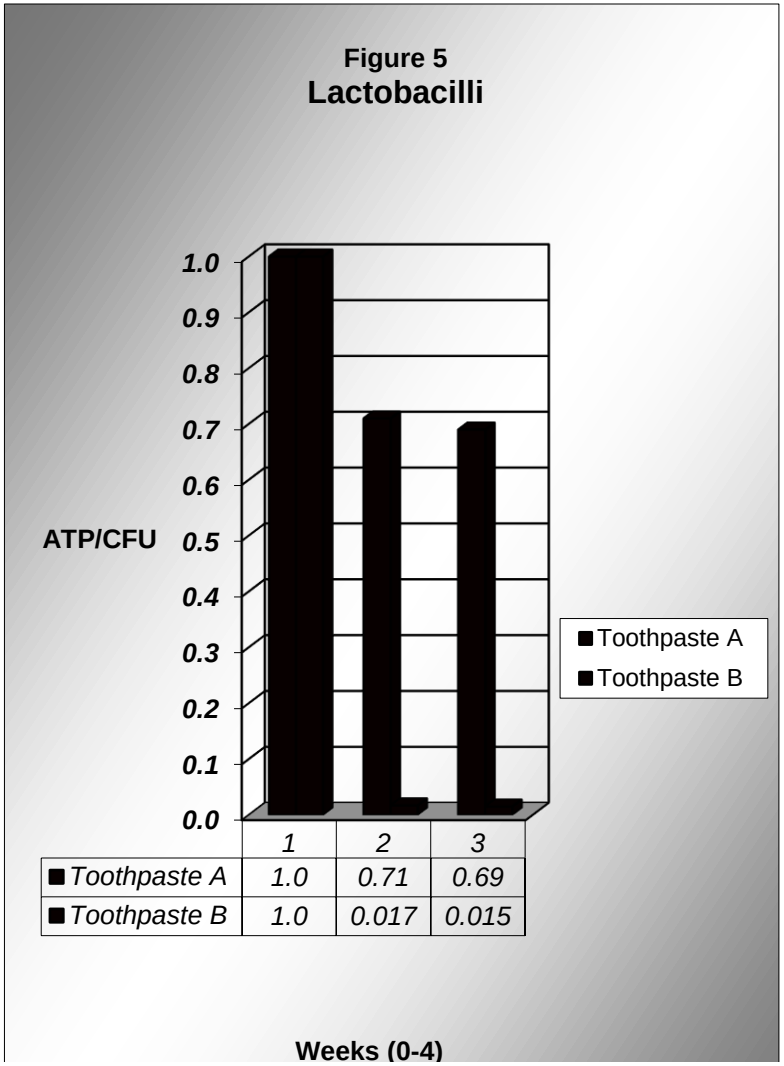
Lactobacillus reduction



	1	2	3
Toothpaste A	1.0	0.71	0.69
Toothpaste B	1.0	0.017	0.015

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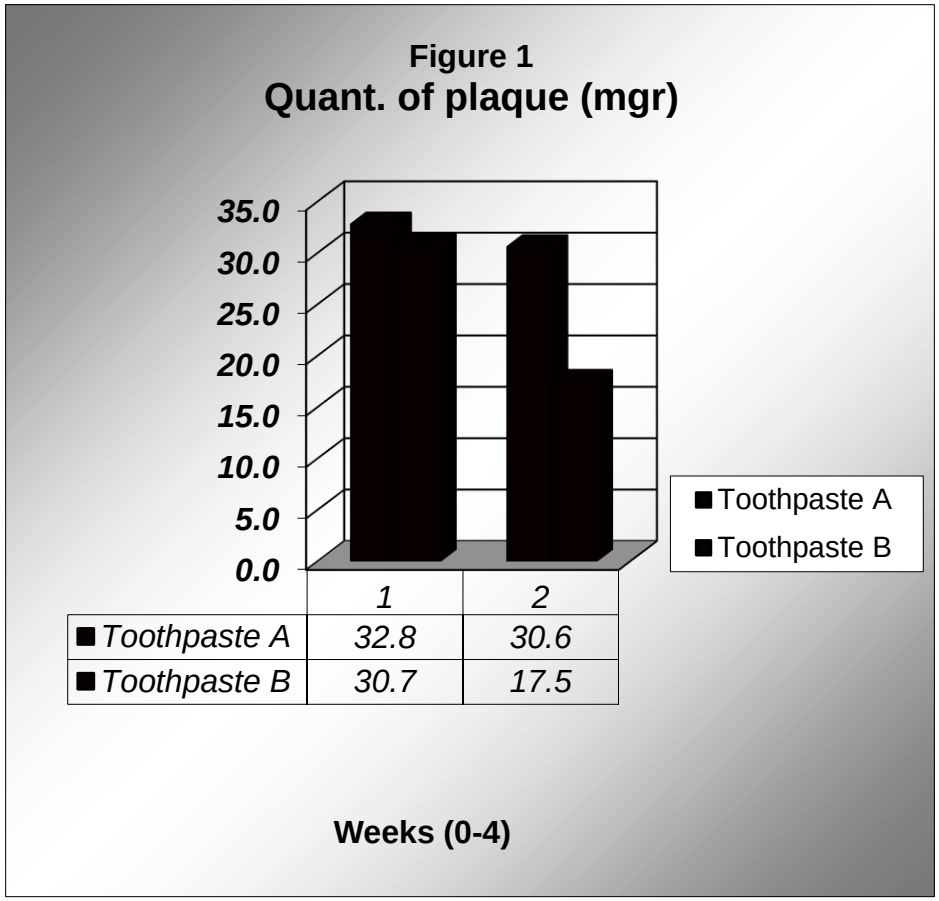
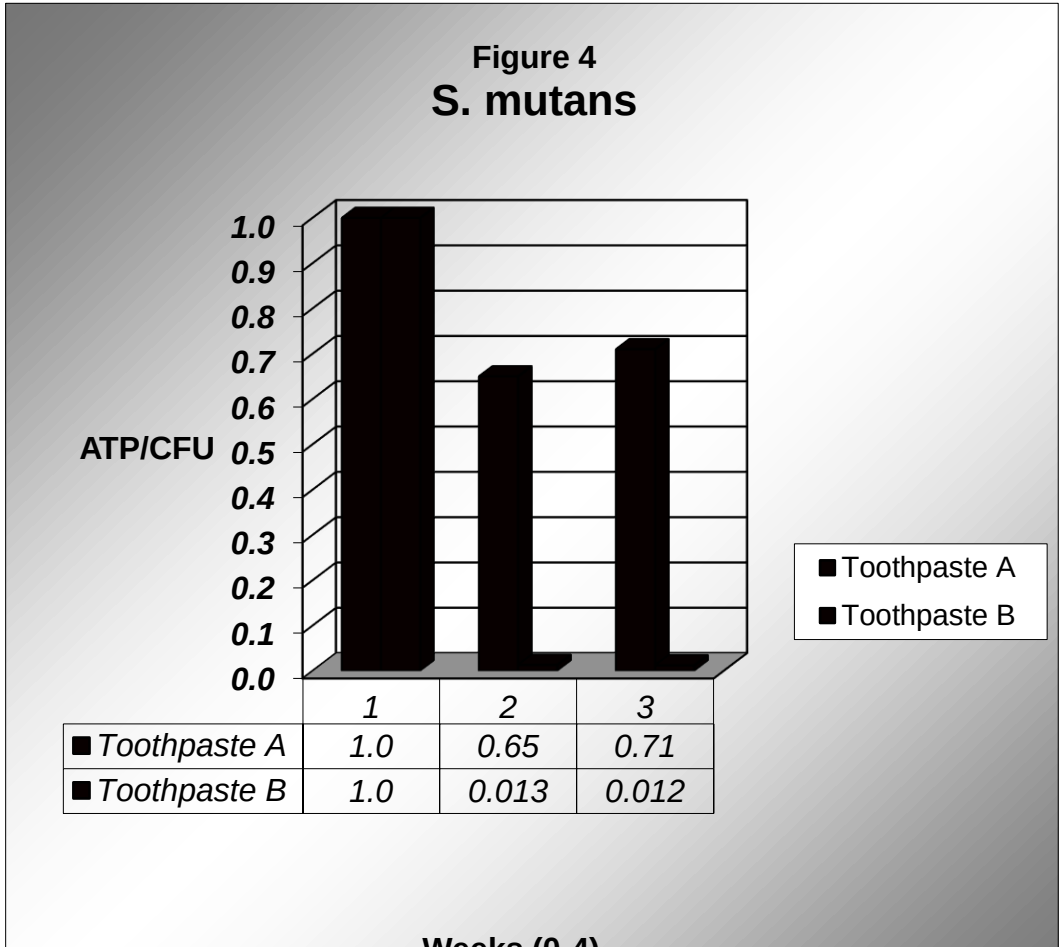
Plaque reduction



S. Mutans reduction

Figure 4



Weeks (0-4)

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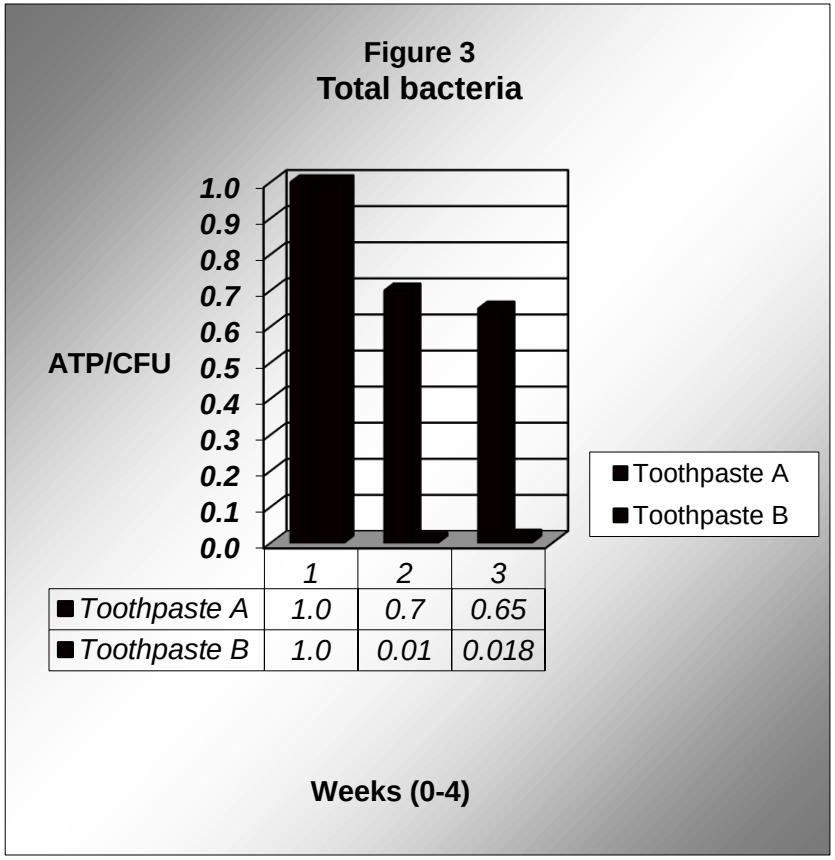
Total bacteria reduction



	1	2	3
Toothpaste A	1.0	0.7	0.65
Toothpaste B	1.0	0.01	0.018