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## **SnF<sub>4</sub> Antimicrobial Effects**

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**3.B. New microbiology studies demonstrate that SnF<sub>2</sub> has antimicrobial activity against representative organisms responsible for, or associated with, gingivitis. Based on these data, we request that FDA include in the monograph labeling a provision entitling SnF<sub>2</sub> to use the additional indication of “helps (select one) control, inhibit, or kill plaque bacteria that contribute to the development of (select one or more) gingivitis; gingivitis, an early form of gum disease; or bleeding gums” that is identical to that currently recommended for essential oils**

During the Subcommittee proceedings, Warner-Lambert (now Pfizer) presented data demonstrating the antimicrobial properties of the fixed combination of essential oil active ingredients. These studies included:

1. Time kill studies, 30 sec., with and without serum present using representative organisms
2. Time kill in saliva
3. Salivary bacteria reductions, single 30 sec., 20 ml rinse
4. Bacterial suppression
5. *In situ* cidal activity on plaque bacteria
6. Vital staining – *in situ* killing of plaque bacteria

During the Subcommittee deliberations focused on these antimicrobials studies, Dr. Bill Bowen commented that plaque must be regarded as a biofilm, and that “if you do indeed disrupt the matrix, that in fact, is antibacterial by any reasonable definition.”<sup>55</sup> No Subcommittee members disagreed.

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<sup>55</sup> Plaque Subcommittee Deliberations. Transcript from Miller Reporting Company, Washington, DC, Vol. XIX, October 22, 1998, at page 68.

Based on these data the Subcommittee voted in favor of the proposed indication for essential oils, “*aids or helps in the control (killing, inhibition) of plaque bacteria that contribute to the development of gingivitis (gingivitis, an early form of gum disease, bleeding gums)*”, allowing for the indication of “kills bacteria”.

To generate the same type of data that Warner Lambert provided to the Subcommittee, Procter & Gamble conducted a series of microbial studies to establish the antimicrobial activity of a 0.454 percent SnF<sub>2</sub> dentifrice and to demonstrate that SnF<sub>2</sub> can provide antimicrobial benefits similar to those associated with the fixed combination of essential oils. Specific details of the study designs, results, and statistical analysis of these data are found in Appendices 7 through 9 of this document. These studies demonstrate that SnF<sub>2</sub> dentifrice meets, and often exceeds, the antibacterial data that the Subcommittee relied on to recommend the optional indication for the essential oils. These studies are briefly summarized below:

### **3.B.1. Time Kill Study (Appendix 8)**

The objective of the time kill study was to evaluate the *in vitro* antimicrobial activity of stannous fluoride dentifrice against a battery of organisms responsible for or associated with gingivitis. The study included organisms cited by the Subcommittee as being associated with gingivitis, as well as other organisms presented during the Subcommittee deliberations by Warner Lambert for essential oils, some of which are not necessarily implicated in gingivitis. The assay is based on the time taken by the test product to kill the representative microorganisms, with a faster kill time indicating greater efficacy.

The table below summarizes the time kill study results for organisms cited by the Subcommittee as plaque-associated bacteria as well as those reported in the Warner Lambert studies. Results of the time kill study demonstrate a substantial antimicrobial kill of plaque organisms when exposed to a 1:4 slurry [slurry dilution ratio identical to that utilized by essential oils in their testing] of a 0.454 % SnF<sub>2</sub>

dentifrice for 30 seconds. Stannous fluoride demonstrated approximately a 3 to 5 log reduction in the majority of plaque organisms tested. The percent reductions are calculated from the raw data: to give an example, if an inoculum count of  $3 \times 10^8$  cfu/mL is reduced to  $3 \times 10^7$  cfu/mL the percent reduction or kill is 90%. This means a 1-log reduction corresponds to a 90% germ kill. A 2-log reduction corresponds to 99%, 3-log reduction corresponds to 99.9%.

**Time Kill Kinetic Study Data after 30 Seconds Exposure of 0.454% SnF<sub>2</sub> to Representative Plaque Bacteria**

<b>Representative Organisms</b>	<b>0.454% SnF<sub>2</sub>, Log cfu/ml</b>	<b>Water, Log cfu/ml</b>	<b>% kill relative to water</b>	<b>Log reduction relative to water</b>
<i>A. viscosus</i> ATCC19246	3.6	7.22	99.97	3.62
<i>C. albicans</i> ATCC 10231	3.6	6.47	99.86	2.87
<i>C. rectus</i> ATCC 33238	3.6	4.84	93.08	1.24
<i>F. nucleatum</i> ATCC 10953	3.6	7.65	99.99	4.05
<i>H. actinomycetem-comitans</i> ATCC 29522	4.1	7.90	99.97	3.80
<i>L. casei</i> ATCC 393	3.6	7.94	99.99	4.34
<i>P. intermedia</i> ATCC 25611	3.6	8.76	99.99	5.16
<i>P. aeruginosa</i> ATCC 27853	7.55	8.29	74.6	0.74
<i>S. sanguinis</i> ATCC 10556	3.6	6.12	99.69	2.52

**Time Kill Kinetic Study Data after 30 Seconds Exposure of 0.454% SnF<sub>2</sub> to Representative Plaque Bacteria**

*Continued*

<b>Representative Organisms</b>	<b>0.454% SnF<sub>2</sub>, Log cfu/ml</b>	<b>Water, Log cfu/ml</b>	<b>% kill relative to water</b>	<b>Log reduction relative to water</b>
<i>P. gingivalis</i> ATCC 33277	3.6	8.25	99.99	4.65
<i>S. mutans</i> ATCC 35668	3.6	7.77	99.99	4.17
<i>Eikenella corrodens</i> ATCC 23834 (GC agar)	3.6	6.82	99.89	3.22
<i>Eikenella corrodens</i> ATCC 23834 (BBA agar)	3.6	6.83	99.90	3.23
<i>Salmonella typhimurium</i> ATCC13311	3.6	7.65	99.99	4.05
<b>Stimulated Whole Saliva Total Aerobes</b>	3.6	5.74	99.27	2.14
<b>Stimulated Whole Saliva Total Facultative Anaerobes</b>	3.6	5.16	96.80	1.56
<b>Stimulated Whole Saliva Total GNAs</b>	3.6	6.63	99.90	3.03

**3.B.2. Minimum Bactericidal Dilution (MBD) Analysis (Appendix 9)**

The objective of the MBD study was to evaluate the *in vitro* antimicrobial activity of a 0.454% stannous fluoride dentifrice against a battery of organisms responsible for or associated with gingivitis. The study included the same battery of organisms cited

by the Subcommittee as those organisms associated with gingivitis, as well as those organisms cited by Warner Lambert in their evaluation of essential oils (MBD data were not reported by Warner Lambert). The assay is based on the concentration of the test product required to kill the representative microorganism, with a lower kill concentration indicating greater efficacy.

The table below summarizes results of the Minimum Bacterial Dilution study for these organisms. Results of the Minimum Bactericidal Dilution test indicate substantial antimicrobial kill of plaque organisms from dilutions of 0.454% SnF<sub>2</sub> dentifrice. The cidal dilutions reported for SnF<sub>2</sub> are considerably less concentrated than typically associated with resultant dentifrice slurry resulting from oral brushing when considering effects of salivary dilution (1:3 dilution).

**Minimum Bactericidal Dilution of 0.454% SnF<sub>2</sub> Dentifrice and Essential Oils Required to Kill Representative Plaque Organisms**

	<b>0.454% SnF<sub>2</sub></b>	<b>Listerine</b>
<b>Representative Organisms</b>	<b>MBD</b>	<b>MBD</b>
<i>A. viscosus</i> ATCC19246	1:20	<1:10
<i>C. albicans</i> ATCC 10231	1:50	<1:10
<i>C. rectus</i> ATCC 33238	1:200	<1:10
<i>E. corrodens</i> ATCC 23834	>1:50	1:10
<i>F. nucleatum</i> ATCC 10953	1:10	<1:10
<i>H. actinomycetem-</i> <i>comitans</i> ATCC 29522	1:20	<1:10
<i>L. casei</i> ATCC 393	<1:10	<1:10

**Minimum Bactericidal Dilution of 0.454% SnF<sub>2</sub> Dentifrice and Essential Oils Required to Kill Representative Plaque Organisms**

Continued

	<b>0.454% SnF<sub>2</sub></b>	<b>Listerine</b>
<i>P. intermedia</i> ATCC 25611	NR	NR
<i>P. aeruginosa</i> ATCC 27853	1:10	<1:10
<i>P. gingivalis</i> ATCC 33277	1:350	1:10
<i>S. sanguinis</i> ATCC 10556	1:450	<1:10
<i>S. mutans</i> ATCC 35668	1:150	<1:10
<i>Salmonella</i> <i>typhimurium</i>	1:20	<1:10
<b>Stimulated Whole Saliva</b>	1:50	<1:10

<1:10 means the product did not inhibit the growth of the microorganism even at 1:10 dilution. Lower dilutions have not been performed here.

>1:1000 means the product inhibited the growth of the test organism at the highest dilution tested in this experiment.

NR not reported – *P. intermedia* did not grow on BBA plates

The water control did not show any inhibition of the test organisms.

The results of this MBD testing, without exception, demonstrate superior cidal activity for stannous fluoride as compared to the essential oils.

**3.B.3. Oral Biofilm Inhibitory Properties (Appendix 10)**

Oral biofilms, consisting of bacterial organisms, can colonize on the teeth and produce an extracellular matrix that protects it from substances that could be detrimental to the organisms. This matrix provides the organisms with an

environment conducive to producing virulence factors involved in gum disease.<sup>56</sup> The current study assessed the ability of a 0.454% SnF<sub>2</sub> dentifrice to inhibit the growth of an oral biofilm. The plaque chip model is an *in vitro* assay that uses a synthetic enamel chip as a surface on which a saliva-based biofilm is grown. Sucrose is added to human saliva to yield a 0.1% sucrose concentration, and this is used as the inoculum for the enamel chips. After incubation, the chips are treated with the test solutions for one minute twice a day. On the fourth day, the plaque is collected and plated on agar plates. The resulting total facultative anaerobes and total facultative gram-negative anaerobic colonies are counted. Two separate assays were performed using this test method.

Results demonstrated a significant reduction in both total facultative anaerobes and total facultative gram-negative anaerobes from the SnF<sub>2</sub> dentifrice compared to a negative control (water).

**SnF<sub>2</sub> Dentifrice Effects on Bacterial Populations contained within an *In Vitro* Oral Biofilm**

**Mean Log<sub>10</sub> CFU/ml Difference vs. Water**

<b>Oral Flora Subgroup</b>	<b>Assay #1*</b>	<b>Assay #2*</b>	<b>Average</b>
<b>Total Facultative Anaerobes</b>	0.44 <sup>b</sup>	0.61 <sup>b</sup>	0.52
<b>Total Facultative Gram-Negative Anaerobes</b>	0.43 <sup>b</sup>	0.56 <sup>b</sup>	0.49

\* All data shown here were calculated by subtracting the mean log CFU/ml for the SnF<sub>2</sub> group from that of the water group.

<sup>b</sup> For all treatments, the mean differences from water were significant with p<0.05.

The mechanism by which stannous fluoride inhibits the growth of bacteria involves the inhibition of two key enzymes, fructose-1,6 bisphosphate aldolase and

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<sup>56</sup> Marsh, Philip and Martin, Michael: Oral Biology, 3<sup>rd</sup> Ed., pp 167 – 197, 1992.

triosephosphate dehydrogenase.<sup>57</sup> Inhibition of these two enzymes in the glycolytic pathway results in decreased bacterial metabolism, and a subsequent reduction in bacterial acid production and other virulence factors in the oral biofilm. Furthermore, data from this study show a definitive bacterial mass reduction in gram-negative facultative anaerobes, the organisms implicated in plaque and gingivitis formation, in the oral biofilm.

#### **3.B.4. Vital Staining of *In Vitro* Stannous Fluoride-Treated Saliva Samples (Appendix 11)**

The final study submitted herein to assess the microbial activity of a 0.454% SnF<sub>2</sub> dentifrice utilized a live/dead staining technique to provide a visual representation of the effect. Bacterial organisms obtained from human saliva samples were incubated for one minute with the treatment solution, i.e. 0.454% stannous fluoride or water. After centrifugation and washing the pellet, the pellet slurry was exposed to a solution of two fluorescent dyes, SYTO9 dye and propidium iodide. The dye/pellet was then forced through a filter containing a polycarbonate black filter membrane, which fixed the bacteria onto the membrane. The membrane was covered with a cover slip and sealed, and the sample was viewed under the epifluorescent microscope.

As is evidenced from the digital color photographs from this study [Appendix 9], the saliva samples treated with SnF<sub>2</sub> resulted in a >90% red color (denoting kill) compared to a water treated sample which was >90% green color (denoting live cells). This provides visual confirmation of the bactericidal effects of SnF<sub>2</sub> dentifrice against plaque associated with gingivitis.

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<sup>57</sup> Rolla G and Ellingsen JE, Chemical Effects and Possible Mechanism of Action of Stannous Fluoride, J. Clin Dent; 44: 99-105, 1994.

### 3.B.5. Summary of SnF<sub>2</sub> Antibacterial Results and Recommendations

The data submitted herein from four *in vitro* microbiological studies provide substantial evidence that SnF<sub>2</sub> formulated in a dentifrice is bacteriocidal to plaque organisms that are associated with or responsible for gingivitis. These data include:

- A time kill study demonstrating approximately 3 to 5 log reduction of plaque organisms exposure to SnF<sub>2</sub> dentifrice for 30 seconds,
- A study showing that dilutions of SnF<sub>2</sub> dentifrice in ratios of 1:10 or greater are bactericidal against the majority of representative plaque organisms. Data show that SnF<sub>2</sub> is equal to few and superior to most of the bactericidal effects demonstrated by essential oils.
- Plaque organisms within oral biofilms derived from human saliva are significantly reduced in a 1-minute exposure to SnF<sub>2</sub> dentifrice.
- Fluorescent staining techniques which discriminate live vs dead cells demonstrate a >90% kill rate of plaque bacteria following exposure to SnF<sub>2</sub> dentifrice.

Based on these data, Procter & Gamble requests that the Agency provide for the optional indication (“uses”) in the labeling for stannous fluoride, identical to that currently specified for essential oils, viz, “helps (select one of the following: ‘control,’ ‘inhibit’ or ‘kill’) plaque bacteria that contribute to the development of (select one or more of the following): ‘gingivitis,’ ‘gingivitis, and early form of gum disease,’ or ‘bleeding gums’.”