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Substantivity of amine fluoride/stannous fluoride following different modes of application: A randomized, investigator-blind, placebo-controlled trial

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Abstract

Objective. Amine fluoride/stannous fluoride (ASF) is proven to be effective against plaque and gingivitis. The purpose of this clinical controlled study was to investigate the influence of different application modes on the substantivity of this formulation. ***Material and Methods.*** Seventeen healthy volunteers received a professional dental prophylaxis. Undisturbed plaque growth was permitted for the next 48 h. In a crossover design, participants received ASF as a single mouthrinse, toothpaste, slurries with high (HA) or low (LA) air content, or a placebo. Vitality of plaque bacteria was investigated before and at 1, 2, 3, 4, 6, and 8 h after application of ASF. ANOVA was applied on a 0.05 significance level. ***Results.*** Highest reduction of plaque vitality resulted after toothpaste application, followed by mouthrinse, LA, and HA slurry. No changes occurred in the placebo group. Compared to baseline and placebo, statistically significant changes were detected up to 4 h in all ASF groups. Toothpaste exerted antibacterial efficacy up to 8 h. Vitality reduction was higher in the LA group than in the HA group. ***Conclusions.*** The concentration of ASF in formulations influences the time course of the antibacterial effect. Contact of ASF formulations with air might reduce their efficacy.

Key Words: *Bacteria, crossover study, mouthrinse, toothpaste, topical fluorides*

Introduction

Caries and periodontal diseases are among the most common diseases in the population. It is generally accepted that dental plaque is important in the etiology of these disorders. Therefore, daily mechanical plaque removal by the patient is the main focus of caries, gingivitis, and periodontitis prevention. In industrialized countries, 90% of the population brush their teeth once or twice a day [1,2]. However, the majority of patients have inadequate oral hygiene. A study has shown that only a third of 72-h old plaque was removed after routine toothbrushing [3]. Therefore, additional devices and chemical formulations have been developed to improve the quality of plaque removal.

Fluorides are important in the promotion of oral health. Incorporated in oral hygiene products, they foster remineralization and inhibit demineralization of the enamel. In addition, fluorides possess an antibacterial activity. This positive effect is enhanced when fluoride is associated with amine or Sn^{++} [4]. It has been proved that amine fluoride/stannous fluoride-containing (ASF) formulations reduce acid production within the dental plaque [5]. In numerous clinical studies, ASF has proved effective against bacteria, plaque, and gingivitis [6–13]. Currently, ASF formulation is available on the market as a mouthrinse (meridol® Mundspül-Lösung) and toothpaste (meridol® Zahnpasta). Whereas testing a mouthrinse is comparatively simple, the evaluation of toothpaste for its plaque and gingivitis-inhibiting properties is more complicated. On the one hand, this is because of the more complex composition of toothpaste [14,15]; on the other hand, it is dependent on the study type selected (with or without toothbrushing).

In an early test stage, so-called substantivity studies can show how long an antibacterial effect

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persists *in vivo* [16]. For this type of study, usually no toothbrushing is performed. The toothpaste can be changed into a slurry that is then applied as a single rinse [17–19]. Alternatively, the toothpaste can be applied unchanged by custom trays [20]. Since retention of a substance and consecutively the substantivity is known to depend on the mode of application [21,22], the aim of this investigation was to determine the influence of different delivery systems, concentrations, and total amounts of ASF on the substantivity of the selected agent.

Material and methods

This randomized, placebo-controlled, investigator-blinded, clinical study was conducted in a crossover design. It was preceded by a recruitment phase to include the participants and to determine the plaque formation rate of each participant. Every cycle of the substantivity test consisted of a 48-h undisturbed plaque growth phase and the parameter recording before introduction of the product (baseline) and again at 1, 2, 3, 4, 6, and 8 h after product use. Between two test cycles, a washout phase of at least 5 days was performed. The study protocol was approved by the Ethics Committee of the Medical Faculty, Dresden, Germany, before the study started.

Study population

The study population consisted of healthy students recruited from the Dental School of the Medical Faculty of the University of Dresden, Germany, with advertisements posted throughout the building. The participants were between 19 and 33 years old, with a mean age of 24.5 (3.4), and were non-smokers. One clinical investigator enrolled the study participants according to specific inclusion and exclusion criteria. Criteria for study inclusion were people: (1) between the ages of 18 and 65, (2) in good general health, (3) having at least 24 existing teeth, and (4) healthy gingiva. Criteria for study exclusion were persons: (1) with periodontal disease or caries, (2) with fillings on the buccal tooth surfaces in the maxilla, (3) with crowns, fixed partial dentures, or orthodontic appliances in the maxilla, (4) presently pregnant or breastfeeding, and (5) medication use during the previous 6 months that could have influenced the test. All suitable study recruits were required to sign statements of informed consent in order to be included as participants in the study.

Study design

During the recruiting phase, the participants brushed their teeth with aronal® toothpaste (GABA, Lörrach, Germany) twice daily for 7 days. After the 7-day time period, the plaque formation rate index (PFRI) [23] was used for study records. Dental plaque was disclosed, plaque index scores recorded and, subsequently, all dental plaque was completely removed. After 24 h of undisturbed plaque growth, the participants returned to the study center for plaque disclosure and an assessment of plaque scores. The percentage of the plaque-covered tooth area was determined and plaque scores from I to V were recorded to represent the plaque formation rate from very low (I) to very high (V).

At baseline, inclusion and exclusion criteria were recorded again. The participants received a dental prophylaxis, after which there followed a 48-h period of undisturbed plaque growth. After the 48-h time period, the participants returned to the study center for parameter recording. Plaque was collected from the maxillary right canine tooth with a dental periodontal probe and then immediately processed for the vital fluorescence technique. Following plaque collection, the study assistant gave the test product to the participant and supervised its use. Again, after 1, 2, 3, 4, 6, and 8 h additional plaque samples were collected at the vestibular surfaces from one maxillary tooth at each time-point in the following order: 1st right premolar, 1st left premolar, 2nd right premolar, 2nd left premolar, 1st right molar, and 1st left molar. After a washout phase of 5 days in which all participants brushed their teeth with aronal® toothpaste (GABA, Lörrach, Germany), a new cycle began. At study completion, each participant received a dental prophylaxis and fluoride application (Duraphat® Fluoridierungslack, Colgate-Palmolive GmbH, Hamburg, Germany).

Treatments

Four treatments containing the active agent ASF and a placebo (calcium carbonate) were tested. ASF was provided as mouthrinse (meridol® Mundspül-Lösung, GABA, Lörrach, Germany), as toothpaste (meridol® Zahnpasta, GABA, Lörrach, Germany), and as two types of slurries of the meridol® toothpaste. The participants rinsed with 10 ml of the ready-to-use mouthrinse for 30 s in accordance with the manufacturer's instructions. The commercially available toothpaste was applied undiluted with a custom tray. A custom tray for the upper jaw was therefore constructed with space for 1 ml toothpaste to be applied for 30 s around selected teeth. During application of the custom tray, the plaque remained untouched. Upon removal of the custom tray, the participants were allowed to rinse with 10 ml of distilled water for 5 s and to expectorate the remaining toothpaste. The toothpaste slurries were produced according to the method described by Addy et al. 1983 [17]. Three grams of meridol® toothpaste and 9 ml distilled water were mixed immediately before use. The slurry with high air content

(HA) was blended at a speed of 6000 revolutions per minute for 1 min. The specially designed blender allowed an effective mixing of the ingredients and incorporation of air. HA slurry was produced 2 h before application. The second slurry with low air content (LA) was mixed immediately before use in two cylindrical plastic cups. The smaller cup containing the ingredients of water and toothpaste was shaken for 1 min within a larger cup of identical shape. The ingredients were mixed so that only a small amount of air contact occurred. To objectively distinguish between the two slurries, the amount of oxygen in both mixtures was measured using a commercial test kit (Tetra O2 Testkit, Tetra Werke Dr. rer. Nat. Ulrich Baensch GmbH, Melle, Germany). For the negative control, a slurry containing 3 g calcium carbonate and 9 ml distilled water was prepared. Subsumed, each mouthrinse sample contained 2.5 mg fluoride, toothpaste samples contained 1.8 mg fluoride, and each slurry contained 4.2 mg fluoride. Accordingly, the fluoride concentration was as follows: 250 mg/kg per mouthrinse sample, 1400 mg/kg per toothpaste sample, and 350 mg/kg per HA and LA slurry sample. After application of the treatments, the participants were not allowed to clean their teeth, to drink, to eat, or to smoke. Non-sparkling water was permitted after 2 h, mashed food after 4 h.

Bacterial vitality

The parameter under investigation was the vitality of dental plaque, which was assessed using the vital fluorescence technique [24]. Plaque samples were collected at the vestibular sites of the selected teeth. The sample was immediately wiped on a slide and disclosed with 5 µl ethidium bromide and fluorescein diacetate solution. After 3 min the disclosing reaction was completed. Vital bacteria cells were dyed green when the fluorescein diacetate was metabolized into fluorescein. In contrast, bacteria were dyed red when ethidium bromide penetrated the cell membrane and accumulated in dead cells. The disclosing solution was freshly prepared for every time interval of investigation. The percentage of dead and vital cells in a plaque sample was estimated by means of a fluorescence microscope (Jenamed 2 histology fluorescence, Carl Zeiss Jena GmbH, Jena, Germany) with 250- fold magnification and a 5×5 square grid applied in 5 different areas of the sample. According to Weiger et al. [25], it distinguished between 100%, 90%, 70%, 50%, 30%, 10%, and 0% vital plaque. The mean of all 125 squares represented the overall vitality of the plaque sample and was used as measurement of the antibacterial effect of the treatment. One trained examiner performed the vital fluorescence technique.

Randomization and statistics

The sample size calculation was based on the results of pre-tests that commenced the clinical study. These tests showed a mean difference of 22% in bacterial vitality between ASF and a negative control and a mean standard deviation of 10% at 6 h. To detect a statistically significant difference at a significance level of 0.05 and a power of 90%, a sample size of ≥ 14 per group was calculated.

Treatment randomization was done as follows: Participants received a consecutive subject number upon recruitment to the study. Each then received a number corresponding randomly to a treatment sequence, which was obtained by means of Latin square tables [26]. The study assistant was the only person who had knowledge of the randomization list. The assistant applied the treatments while the investigator independently took the plaque samples.

All data were entered in an electronic database. A 0.05 error level was set prior to the statistical test procedures. To detect differences between treatment groups and between baseline and any other time-point within a treatment group, analyses of variance (ANOVA) and LSD post-hoc tests were performed. In accordance with previous studies, no Bonferroni adjustments were made [27]. The statistical analysis was based on the full analysis set.

Results

All 17 participants (11 F, 6 M) finished the study. No adverse events were recorded. The plaque formation rate index scores were as follows: Four participants exhibited score I, 10 had score II, and 3 had score III. None of the participants showed PFRI scores IV and V. The low plaque scores showed that the amount of biofilm available after 48 h for treatment application was small. The oxygen content of HA slurries was 14 mg/l and of LA slurries 5 mg/l when measured 10 min after mixing.

Note that application of all active treatments led to a reduction of plaque vitality with time (Table I). The most prominent reduction of plaque vitality compared to baseline appeared 1 h after application. Thereafter, the bacterial vitality increased slowly and almost reached the level of the baseline data in any group after 8 h. The toothpaste application had a greater influence on the bacterial vitality than the HA slurry. Compared to baseline, the reduction of plaque vitality was statistically significant in all treatment groups for at least 4 h. Six hours after

Table I. Plaque vitality (%) before (0 h) and after application of the treatments; means and standard deviations; $n = 17$.

Treatment	0 h	1 h	2 h	3 h	4 h	6 h	8 h
Mouthrinse	86.8 (4.4)	48.2 (17.5)$^{\star,\#}$	51.2 (17.6)$^{\star,\#}$	53.5 (14.1)$^{\star,\#}$	56.9 (11.8)$^{\star,\#}$	72.2 (12.4)$^{\star,\#}$	79.3 (11.8)$^{\#}$
Toothpaste	88.3 (3.6)	46.1 (21.2)$^{\star,\#}$	46.2 (19.0)$^{\star,\#}$	50.8 (18.7)$^{\star,\#}$	54.5 (13.1)$^{\star,\#}$	67.7 (12.5)$^{\star,\#}$	78.9 (9.6)$^{\#}$
Slurry LA[1]	90.0 (2.2)	52.8 (17.6)$^{\star,\#}$	55.8 (14.6)$^{\star,\#}$	53.0 (15.9)$^{\star,\#}$	62.3 (11.6)$^{\star,\#}$	74.8 (7.0)$^{\star,\#}$	85.7 (4.8)
Slurry HA[2]	88.5 (3.0)	60.9 (16.4)$^{\star,\#}$	67.0 (14.3)$^{\star,\#}$	68.8 (16.8)$^{\star,\#}$	79.9 (8.0)$^{\star,\#}$	81.8 (8.6)	87.1 (4.2)
Placebo	87.6 (3.4)	84.9 (9.0)	86.8 (3.7)	86.8 (3.6)	87.8 (3.1)	86.9 (3.1)	88.4 (2.8)

[1]Slurry LA: Toothpaste slurry with low air content.
[2]Slurry HA: Toothpaste slurry with high air content.
$^{\star}p \leq 0.05$ vs baseline.
$^{\#}p \leq 0.05$ vs placebo.

application, the toothpaste, mouthrinse, and the LA slurry still showed a significantly reduced vitality. At 8 h, the effect had disappeared in all groups.

In the placebo group, no changes were detected after rinsing with the slurry. For at least 4 h after rinsing, the reduction of vital plaque bacteria after ASF application was statistically significant compared to the placebo. The toothpaste and mouthrinse exerted an antibacterial effect lasting 8 h.

When the toothpaste and the LA slurry were compared, the higher ASF concentration of the toothpaste reduced the vitality more than the slurry with the lower concentration (Figure 1). Over time, this effect became more prominent, since statistically significant differences were detected between the toothpaste and the LA slurry at 4, 6, and 8 h.

The comparison between the LA and HA slurries demonstrated differences in the vitality reduction. Statistically significant differences were seen between 2 to 6 h after application. The highest difference between the effects of both slurries was calculated at 4 h (Figure 2).

Furthermore, the effect of the amount of plaque accumulated within 48 h on the efficacy of ASF treatments was investigated. Plaque vitality in participants with a PFRI score I was compared to that of participants with PFRI score III. A stronger plaque inhibition was found in the group with less plaque (PFRI score I; Table II). However, a statistical analysis was not possible due to the small sample size.

Discussion

As stated at the Second Workshop on Periodontology [16], substantivity refers to the length of time that a formulation performs with a persistent effect *in vivo*. Because the parameters for measuring this effect are limited, the vital fluorescence technique was established to assess bacterial vitality [24]. Since then, the technique has been used in several studies [10,19,25]. Usually, a single rinse of the treatment under investigation is applied to the biofilm and followed by 1-h or 2-h measurements of bacterial vitality. However, if toothpaste rather than mouthrinse is tested, two kinds of application are considered. Either a toothpaste slurry is produced [17] or the toothpaste is applied undiluted using an acrylic custom tray [20]. Both methods were used in the current investigation to study the influence of the delivery method.

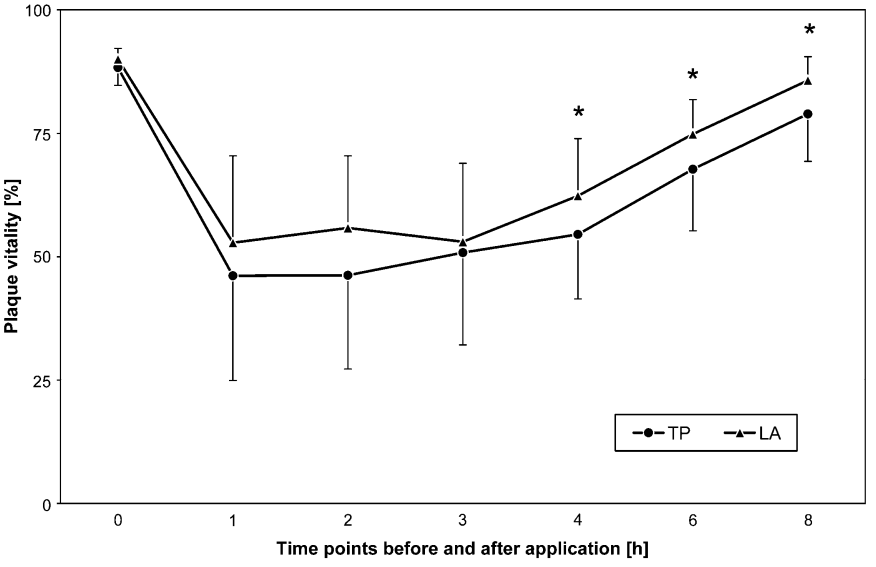

Figure 1. Plaque vitality after application of toothpaste and slurry LA. TP – toothpaste, LA – slurry LA (low air content), *significant difference ($p \leq 0.05$) between TP and LA.

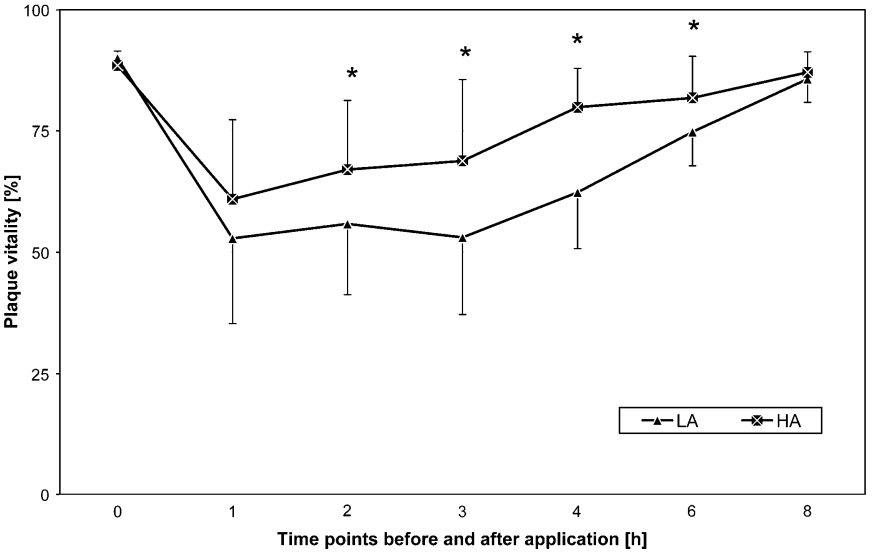

Figure 2. Plaque vitality after application of slurry LA and slurry HA. LA – slurry LA (low air content), HA – slurry HA (high air content), *significant difference ($p \leq 0.05$) between LA and HA.

However, a fundamental question is whether the time profile of vitality reduction of the dental biofilm reflects the parameter of substantivity of ASF or only an initial bactericidal action of the formula. The term "substantivity" also indicates that the prolonged effect of a pharmacological agent is due to its retention in the oral cavity. Retention can occur by binding to all available surfaces, including teeth, gingiva, mucosa, tongue, or dental plaque. The early studies on chlorhexidine directly measured the retention of CHX in the oral cavity by calculating the CHX concentration from the ^{14}C activity of saliva samples. These experiments revealed both a high bactericidal effect as well as a prolonged release of the formulation [28]. For ethical reasons, this study type is no longer in use. Therefore, in the present study on ASF the surrogate parameter of bacterial vitality in plaque was applied. Since this parameter does not measure the actual concentration of ASF in plaque, it can only be hypothesized that the reduction of bacterial vitality for several hours refers to its substantivity. In fact, the experimental design could prove that ASF exerts an antibacterial activity over the time period investigated. Studies investigating fluoride retention and clearance after rinsing have shown that amine fluoride has higher retention rates in the oral cavity than sodium fluoride [29,30]. Another clinical study has shown a reduced acid production in dental plaque up to 8 h after ASF rinses [5]. The reduced plaque metabolism was most pronounced at 30 min after rinsing and diminished slowly thereafter.

The anti-bacterial and anti-plaque effect of ASF toothpaste has been investigated in several studies and a statistically significant decrease in the mean plaque index score was found when participants brushed their teeth over 4 weeks [31] or over 6 months [11]. After a 6-week brushing period, a decrease in total microbiological counts as well as salivary streptococcus mutans counts has shown the potential of ASF to inhibit oral bacteria [32]. However, direct application of the toothpaste on the biofilm followed by substantivity measurements, as demonstrated in this study, has never been done.

Toothpaste slurries have been tested in previous investigations [17,19,33], usually 3 g or 3 ml of

Table II. Plaque vitality (%) for participants with PFRI I and PFRI III before (0 h) and after application of the treatments; means and standard deviations; n PFRI I = 4, n PFRI III = 3.

Treatment	PFRI[+]	0 h	1 h	2 h	3 h	4 h	6 h	8 h
Mouthrinse	I	86.4 (2.0)	43.8 (8.7)	36.5 (9.4)	49.6 (14.9)	47.8 (8.2)	63.5 (9.5)	70.0 (15.3)
	III	89.8 (2.2)	37.2 (8.7)	41.2 (5.5)	44.5 (11.7)	59.1 (13.0)	71.6 (3.9)	86.0 (4.0)
Toothpaste	I	86.6 (2.7)	35.0 (26.4)	32.8 (15.9)	43.7 (24.8)	47.7 (12.8)	61.4 (11.5)	75.1 (9.8)
	III	93.3 (5.5)	55.8 (24.2)	59.5 (23.9)	62.2 (22.9)	65.7 (15.7)	74.4 (7.8)	80.7 (6.9)
Slurry LA[1]	I	89.7 (2.8)	52.5 (3.4)	53.2 (19.7)	49.6 (7.4)	63.6 (17.4)	74.5 (7.9)	82.8 (3.7)
	III	89.8 (0.6)	61.5 (31.0)	70.2 (6.6)	60.4 (25.4)	73.4 (7.3)	80.7 (4.7)	86.1 (0.8)
Slurry HA[2]	I	88.1 (2.4)	55.1 (14.7)	62.9 (16.6)	62.6 (16.1)	76.9 (6.8)	77.4 (9.2)	84.3 (3.8)
	III	92.0 (5.9)	66.1 (19.3)	69.0 (4.1)	63.5 (16.5)	79.6 (12.2)	90.8 (0.5)	92.0 (5.7)

[+] PFRI: Plaque formation rate index (Axelsson 1990).
[1]Slurry LA: Toothpaste slurry with low air content.
[2]Slurry HA: Toothpaste slurry with high air content.

toothpaste diluted in 10 ml of water. An equivalent model was used in this investigation. The slurries consisted of 3 g toothpaste diluted in 9 ml water. The amount of toothpaste in the slurries was twice as much as the amount normally used on a toothbrush, which is on average only 1.45 g. In contrast, the fluoride concentration in the slurry was diluted to 25%, which corresponded to the concentration in an initial brushing phase [34]. During an average brushing cycle of 50 s in the population, toothpastes are diluted up to 10%. Therefore, the results of bacterial inhibition are only valid for the first 30 s in a brushing period and are expected to diminish after that.

Since the best results were shown with the toothpaste that had the highest concentration of ASF, the antibacterial effect seems to depend directly on the concentration and not on the total amount of ASF applied. This is in accordance with the results of Davies et al. concerning the effect of fluorides in general [35]. A study by Jenkins et al. [18] concluded that a higher amount of toothpaste in slurry form compared to a lower amount in slurry, when used during brushing, does not show an additional effect on single bacteria species. However, the higher probability of side effects should be considered when concentrations of a substance are increased in order to enhance clinical performance [35,36].

The differences in efficacy between HA and LA slurries that were observed in this study might indicate that the addition of oxygen to ASF changes its antibacterial properties. There is less evidence in the literature that could support this interesting finding. Only one study has shown less plaque inhibition caused by aged stannous fluoride rinsing solution compared to the fresh stannous fluoride solution [37]. However, it is questionable whether these results can be compared to those of the current investigation.

While the amount of plaque formation differs from individual to individual [38], the reasons for this difference are not fully explained. However, there is the suggestion that the gingival inflammation status as well as salivary secretion rate are possible factors that may influence bacterial attachment and composition [39–43]. In the present study, all dental students showed a low to moderate plaque formation rate (score I to III). None had a plaque formation rate higher than 30% plaque coverage. When the substantivity of subjects who had a PFRI score I was compared to those subjects who had score III, the latter group had the higher percentages at any point of time except at 1 h after application of the mouthrinse. A statistical analysis was not performed because the sample size was too small. These results are in accordance with the statement that the effect of chemical oral hygiene products on plaque is more pronounced when less plaque is present [44].

Conclusions

ASF formulations decrease the vitality of plaque bacteria. Contact with air seems to reduce the antibacterial effect of ASF. Therefore, the delivery method may influence the efficacy of oral hygiene products that contain ASF. The anti-bacterial action of ASF seems to show a more prolonged effect in individuals who have less plaque.

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