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ORIGINAL RESEARCH

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Antibacterial effects of dentifrices against *Streptococcus mutans* in children: a comparative *in vitro* study

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Abstract

Fluoridated dentifrices have antibacterial effects on children's teeth. On the other hand, the side effects encountered with the use of them have led researchers to look for safe alternatives. This study aimed to determine the antibacterial effect of different commercially available fluoride-free dentifrices on *Streptococcus mutans* (*S. mutans*) in comparison with different concentrations of fluoridated dentifrices. Study groups comprised of fluoride-free dentifrices, which contain Probiotic (Activated Charcoal Probiotic Dentifrice—Group P), Aloe Vera—Group AV and Salivary Proteins—Group SP. Fluoridated dentifrices containing 1450 ppm fluoride—Control Group 1 and 500 ppm fluoride—Control Group 2 served as control groups. Antibacterial activity was assessed by Minimum Inhibitory Concentrations and agar well diffusion assays on *S. mutans*. Biofilm inhibition assay was performed with dentifrices, which had antibacterial activities, and a negative control phosphate-buffered saline (Group PBS) on sterile hydroxyapatite discs against *S. mutans*. Statistical evaluation was performed. Only group AV showed an antibacterial effect on *S. mutans*, while control groups showed a similar antibacterial effect. The mean number of viable bacteria present in *S. mutans* biofilm in Control Group 1 and 2 and Group AV were statistically significantly lower than that in Group PBS, but there were no statistically significant differences between Control Groups and Group AV. Antibacterial activity of commercial dentifrices against *S. mutans* may be exerted by antibacterial components other than fluoride. Aloe vera-containing toothpaste showed an antibacterial effect on *S. mutans*, although not as much as the fluoride-containing toothpastes in the control groups. However, further *in vivo* and long-term studies are required.

Keywords

Aloe vera; Fluoride; Antibacterial activity; Commercial; Dentifrice

1. Introduction

Dental caries is considered as the most common bacterial illness in children and affects 72% and 84% children aged 5–7 years and 12–15 years, respectively [1]. Antimicrobial products are increasingly being used as an adjuvant for mechanical plaque control to remove biofilm, thereby preventing dental caries [2].

Dental caries is an endogenous, microbial community-based disease that arises from an ecological shift from dynamic stability to metabolic imbalance in a consortium of acidogenic and aciduric bacteria comprising the dental plaque biofilm [3]. Highly acidogenic and acidophilic bacteria, such as *Streptococcus mutans* (*S. mutans*), *Streptococcus sobrinus* (*S. sobrinus*) and *Lactobacillus*, can become highly dominant in severe and long-term acidic plaque environments. The composition of bacterial plaques can change during the progression of caries [4, 5]. Dental biofilms cannot be removed entirely; however, they can be diminished and controlled with regular dental hygiene. Dental products provide a physical method for disrupting dental biofilms. The incorporation of antiplaque/antimicrobial chemical agents into dental products, such as fluoride compounds, surfactants, antiseptics, propolis, probiotics, salivary proteins, flavors, sodium lauryl sulfate (SLS), xylitol, arginine and humectants, may prevent the growth of *S. mutans*; thus, these supplements are considered as viable preventative measures for reducing the incidence of plaque-mediated illnesses [6, 7].

The most popular source of topical fluoride for children are fluoridated dentifrices. Considering that the protective action of fluoridated dentifrices is almost exclusively topical, the potential oral overdose of toxic fluoride from this source in children younger than 3 years deserves significant attention and special care. Children can swallow up to 25%–33% of the fluoridated dentifrice used at each brushing since the development of their swallowing reflex is incomplete at this age [8, 9].

Significant concern has been raised with regards to the

potential overdose of fluoridated dentifrice by ingestion in this body of patients. As such, there is an urgent need to identify active ingredients with an efficacy similar to that of fluoride but without the potential for toxicity. It is essential to develop a safe and effective dentifrice that is appropriate for young children.

With regards to mechanical plaque control, the use of dental products containing various antimicrobial components (lactoferrin, lactoperoxidase, lysozyme, probiotics, aloe vera, propolis and arginine) has gained increasing acceptance as a potential alternative type of oral health therapy over the last 10 years [10, 11].

A salivary defense system containing lactoferrin, lactoperoxidase, lysozyme, immunoglobulin and growth factors, can help to maintain a healthy oral microbiota [12]. The pattern of early microbial colonization and biofilm formation is directly influenced by the salivary enzymes and proteins that are integrated into the salivary pellicle and immobilized in an active form on the tooth surface [13, 14].

Researchers have focused particularly on the relationship between probiotics and the oral microbiota and their potential to reduce the levels of cariogenic bacteria in the oral cavity [15, 16]. Of all probiotic strains, *Lactobacillus* is one of the most commonly used and studied bacteria in humans [17]. *In vitro* and in saliva, *Lactobacillus paracasei* (*L. paracasei*) DSMZ16671 preferentially co-aggregates with *S. mutans*, but not with other commensal bacteria that are present in the oral microbiota [18]. In a previous study, Tanzer *et al.* [19] (2010) reported that *L. paracasei* DSMZ16671 considerably reduced caries in rats with *S. mutans* infection and prevented colonization of the bacterium in their teeth [19].

Aloe vera gel is known to exert anti-inflammatory, antibacterial, antioxidant, immune-stimulating and hypoglycemic characteristics, along with a range of other pharmacological effects [20, 21]. Fani and Kohanteb [22] (2012), reported that aloe vera gel can exhibit a significant bactericidal effect against cariogenic bacteria. Furthermore, these authors demonstrated that *S. mutans* is extremely sensitive to aloe vera gel [22].

Another antimicrobial agent, propolis, is combined with aloe vera and has generated intense interest in dental research on account of its inhibitory effects against pathogenic microorganisms and inflammatory reactions [23, 24].

In a previous study, Bhati *et al.* [25] (2015) conducted an *in vivo* investigation to compare the antibacterial efficacy of fluoridated (Colgate) and herbal (Forever Bright Aloe vera Toothgel and Dabur Meswak toothpaste) dentifrices. In terms of antibacterial efficacy, these authors concluded that dentifrices containing aloe vera and meswak could be safely recommended as an alternative to fluoride dentifrices [25]. Another *in vivo* study evaluated the antibacterial and antiplaque activity of three edible dentifrices: KidScents (containing essential oils and xylitol), Browning B&B (containing medicinal plants, poloxamer 407 and xylitol), and Wysong Probiodent (containing aloe vera and probiotic cultures). Analysis showed that these edible toothpastes exhibited antimicrobial activity but lacked antiplaque activity [26].

In this study, we aimed to compare commercial fluoride-free dentifrices containing probiotics, aloe vera and salivary proteins, with well-known and gold standard "fluoride" dentifrices at different concentrations as controls. We focused on these specific ingredients because of their proven antibacterial efficacy.

2. Materials and methods

The study groups comprised three fluoride-free dentifrices (*i.e.*, probiotic (Activated Charcoal Probiotic dentifrice (Hyperbiotics)); Group P, aloe vera (Aloe Vera (LR)); Group AV, and salivary proteins (Splat Baby (Splat)); Group SP). Fluoridated dentifrices for children containing 1450 ppm fluoride (Barbie (Colgate); Control Group 1) and 500 ppm fluoride (Pro-Expert Stages (Oral B); Control Group 2) served as the control groups. Table 1 shows the dentifrices used in the study and control groups, along with the coding of the groups, the ingredients, brands and manufacturers of the dentifrices.

2.1 Determination of minimum inhibitory concentration (MIC)

The bacterial strain used in this study was *S. mutan* American Type Culture Collection (ATCC) 25175. The inoculum was prepared in sterile saline solution and incorporated a bacterial suspension that had been cultured overnight at 37 °C on brain heart infusion (BHI) agar (Merck KGaA 64271 Darmstadt, Germany). The inoculum was adjusted to a turbidity of 0.5 Mc Farland (10^8 cfu/mL) and diluted to 1:100 (approximately 10^6 cfu/mL). The antibacterial effects of the selected dentifrices on *S. mutans* were then assessed by determining the MIC. Dentifrices were diluted 10% wt/mL with distilled water. The MICs of the dentifrices in the study and control groups were then tested by a microdilution method described by Clinical Laboratory Standards Institute (CLSI) (2006). In brief, serial two-fold dilutions of the extractions were prepared in BHI broth with a final volume of 100 μL in 96-well microplates (TPP, Switzerland). Each well of the stock testing solutions was added to the first test well and mixed. A series of dilutions were then prepared across the plate using a micropipette, and 100 μL of the bacterial suspensions were added and inoculated into all wells. The microplates were then incubated at 37 °C for 24 h. Bacterial growth was determined by optical density (OD) measurements at 650 nm using a Thermo Max microplate spectrophotometer (Uniquely Tecan Freedom EVO 75, Mannedorf, Switzerland). MIC was considered to be the lowest concentration of the treatment that prevented an increase in the OD. Each group of bacteria were tested in triplicate, and the median of three independent replicates was accepted as the MIC.

2.2 *In vitro* antibacterial susceptibility assays

2.2.1 Agar well plate diffusion method

The antimicrobial activity of each group was assessed by the agar well plate diffusion method; this involved measuring the diameter of the inhibition zones of bacterial growth (in mm). This method was performed to assess the antibacterial efficacy of fluoride-free and fluoridated dentifrices, and agar was used

TABLE 1. The ingredients, brand names and manufacturers of dentifrices in the study and control groups.

Study and Control groups	Codes	Ingredients	Brand names and Manufacturers
Probiotic	Group P	Dental-Lac (made with *L. paracasei*), Activated coconut charcoal, Xylitol, Organic coconut oil, Spearmint and tea tree essential oils, Diatomaceous earth, water, Sodium bicarbonate (baking soda), Otric acid, Mentha spicata (mint) oil, Melaleuca alternifolia oil, Xanthan gum	Activated Charcoal Probiotic Toothpaste (Hyperbiotics) Nutraceutix
Aloe Vera	Group AV	Aloe Barbadensis Leaf Juice, Sorbitol, Hydrated Silica, Aqua (Water), Glycerin, Sodium Lauryl Sulfate, Cellulose Gum, Aroma (Flavor), Echinacea Purpurea Extract, Propolis (Propolis Cera), Alcohol, Sodium Saccharin, Sodium Benzoate, Caramel, CI 19140 (Yellow5), CI 42090 (Blue1)	Aloe Vera (LR) LR Health & Beauty
Salivary Proteins	Group SP	Hydrogenated Starch Hydrolysate, Aqua, Dicalcium Phosphate Dihydrate, Hydrated Silica, Glycerin, Calcium Hydroxyapatite, Xanthan Gum, Potassium Thiocyanate, Lactoferrin, Lactoperoxidase, Glucose Oxidase, Glucose Pentaacetate, Aloe Barbadensis Leaf Extract, Apple/Banana Extract, Lonicera Caprifolium Flower Extract, Lonicera Japonica Flower Extract, Dipotassium Glycyrrhizate, Cocamidopropyl Betaine, o-Cymen-5-ol, Glycyrrhiza Glabra (Licorice) Root Extract, Vaccinium Oxycoccos Fruit Extract, Achillea Millefolium Extract, Arginine	Splat Baby (Splat) Splat-Cosmetica company
1450 ppm fluoride	Control Group 1	Sorbitol, Silica, Polyethylene Glycol 600, Sodium carboxymethyl cellulose, Tetrasodium pyrophosphate, Sodium saccharin, Sodium fluoride (1450 ppm), Titanium dioxide-coated mica, Sodium lauryl sulfate, Flavor	Barbie (Colgate) Colgate Palmolive
500 ppm fluoride	Control Group 2	Sorbitol, hydrated silica, Cellulose gum, Sodium saccharin, Trisodium phosphate, Sodium fluoride (500ppm), Sodium lauryl sulfate, Carbomer, Aroma, Aqua, Limonene, Benzyl alcohol, CL 42090	Pro-expert stages Frozen (Oral B) Procter and Gamble

to maintain the culture of *S. mutans*. The inoculum was prepared in sterile saline solution by suspending the bacteria that had been cultured overnight at 37 °C on BHI agar. The inoculum was adjusted to a turbidity of 0.5 McFarland (10^8 cfu/mL) and diluted to 1:100 (approximately 10^6 cfu/mL). Approximately 0.1 mL of this inoculum was spread over Petri plates containing BHI agar, and wells (diameter = 6 mm) were filled with 50 μL of the test samples and incubated in a 5%–7% carbon dioxide (CO_2) atmosphere at 37 °C for 48 h. After incubation, the diameter of the inhibition zone of bacterial growth was measured (in mm). All measurements were performed in triplicate, and the mean and standard deviation were calculated for each group.

2.2.2 Biofilm assays

Round ceramic coated hydroxyapatite discs (HAP) discs (diameter, 12 mm; thickness, 1 mm; 3D Biotek LLC) were used as a substrate to evaluate biofilm formation. The discs were individually packed and sterilized by γ-radiation before testing with the bacteria. The discs were placed in 24-well polystyrene cell culture plates. The discs were then coated with 500 μL of saliva prepared according to Baffone *et al*. [27] (2011) and incubated at 37 °C for 1 h to allow the even formation of a salivary pellicle. All discs were washed gently with phosphate-buffered saline (PBS), placed in new wells containing 160 mL of BHI broth supplemented with 4% saccharose, and inoculated with 200 mL of the *S. mutans* bacterial suspension (32 $\times$ 10^6 cfu/mL). Next, the plates were incubated anaerobically at 37 °C for 24 h. Following incubation, the discs were gently dip-washed three times in physiological saline to remove loose bacteria. Then, 750 μL of the dentifrices were prepared as a slurry, and PBS as the negative control (Group PBS), were placed in the wells of a 24-well polystyrene cell culture plate to immerse the biofilms and evaluate the bactericidal effects of Group AV, Control Group 1 and 2, which exhibited antibacterial activities in the agar well plate diffusion assay. The dentifrice slurries were prepared by dissolving 0.5 g of the dentifrices in 1 mL of physiological saline and glass beads and then vortex mixing for 1 min to harvest the adherent bacteria. Next, the suspensions were sonicated at 30 W for 5 s to disrupt bacterial aggregates, serially diluted (100–106) in sterile physiological saline, and then plated on BHI agar. Then, the plates were incubated anaerobically for 48 h at 37 °C, and colony-forming units (CFUs) were counted. These experiments were repeated seven times.

2.3 Scanning electron microscopy

One Disc sample from each group was analyzed by scanning electron microscopy (SEM). Discs that had been incubated for the same amount of time were selected for SEM observation; these were gently washed three times with PBS to remove non-adherent cells and then placed in a 4% paraformaldehyde fixative solution for 24 h. After the specimens were washed twice with distilled water, they were dehydrated with a series of ethanol rinses (20, 50, 70, 80, 85, 90, 95 and 100%, v/v) and then dried in a desiccator. After sputter coating with gold-palladium, randomly selected positions on the samples were imaged at least five times by a scanning electron microscope (EVO 40, Carl ZEISS, Aalen, Germany) at a magnification of 5000×.

2.4 Statistical analysis

In addition to descriptive statistical methods (mean and standard deviation), statistical analyses were carried out with the Tukey-Kramer *post hoc* test to test the outcomes of agar well plate diffusion assays. To identify differences between groups in the biofilm assays, we applied one-way analysis of variance (ANOVA) with the *post hoc* Tukey HSD (Honestly Significant Difference) Test. $p < 0.05$ was considered as statistically significant.

3. Results

3.1 Minimum inhibitory concentrations

The MICs of the study and control groups against the tested bacterial strain are presented in Table 2. Only Group AV and the control groups exhibited antimicrobial activity against *S. mutans*. Group AV inhibited the growth of *S. mutans* at the lowest concentration tested, and its MIC value for *S. mutans* was found to be 0.0156 mg/mL. Control Groups 1 and 2 inhibited the growth of *S. mutans* with MIC values of 0.0625 and 0.0312 mg/mL, respectively. The MIC of Group AV was lower than those of the control groups.

TABLE 2. Minimum inhibitory concentrations (MIC) of the study and control groups in the study for *S. mutans*.

Groups	MIC values (mg/mL)
Group P	NI
Group AV	0.0156
Group SP	NI
Control Group 1	0.0625
Control Group 2	0.0312

NI: No inhibition.

3.2 Agar well plate diffusion assay

Fig. 1 shows the inhibitory halos of the study and control groups. Of the fluoride-free dentifrices, only Group AV showed a clear inhibition zone (26.33 ± 1.69 mm) for *S. mutans*. The control groups showed similar antibacterial effects (26.33 ± 0.47 mm and 26.33 ± 1.24 mm). No inhibitory zones were evident for Groups P and SP. Statistically significant differences in bacterial growth were identified between Group AV and the other study groups ($p < 0.01$) and between the control groups and Groups P and SP ($p < 0.01$). However, no statistically significant differences in the mean diameter of the inhibition zones between Group AV and the control groups were identified ($p > 0.05$; Table 3).

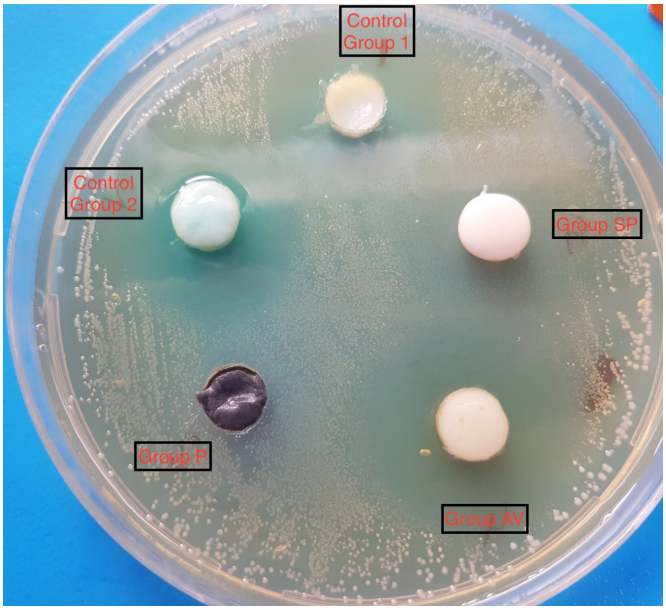

FIGURE 1. A culture plate with wells overlaid with *S. mutans* and inhibitory halo for samples of 1 Group AV, 2 Group P, 3 Control Group 2, and 4 Control Group 1 and 5 Group SP.

TABLE 3. Mean value ± standard deviation (SD) of the zone of inhibition of study and control groups (in mm) against *S. mutans*.

Zones of inhibition of study and control groups	Mean values ± SD (mm)
Group P	0
Group AV	26.33 ± 1.69*
Group SP	0
Control Group 1	26.33 ± 0.47*
Control Group 2	26.33 ± 1.24*

**Tukey-Kramer post hoc test ($p < 0.01$).*
Results represent the mean ± SD of experiments which were repeated at 3 times.

3.3 Inhibition of biofilm formation

The means and standard deviations of the CFUs of *S. mutans* in Control Groups 1 and 2 and Groups AV and PBS are given in Table 4. Statistically significant differences were identified between all groups ($p < 0.05$). Group AV and Control Groups 1 and 2 exhibited similar effects in terms of reducing the number of bacteria in the *S. mutans* biofilm. In contrast, the

abundance of *S. mutans* in Group PBS was higher than that in the other groups.

Table 5 shows an inter-group comparison of the effects of Control Groups 1 and 2 and Groups AV and PBS on the cell viability of *S. mutans* biofilm. According to Tukey's HSD test, inter-group comparisons revealed a statistically significant difference between the control groups and Group PBS ($p < 0.05$). The mean number of viable bacteria present in Control Group 1 was significantly lower than that in Group PBS ($p < 0.05$) although there were no significant differences when compared to Control Group 2 and Group AV ($p > 0.05$). Furthermore, the mean numbers of viable bacteria present in Control Group 2 and Group AV were significantly lower than that in Group PBS ($p < 0.05$). No significant difference was identified between Control Group 2 and Group AV ($p > 0.05$).

TABLE 4. Comparison of the effect of the tested dentifrices on cell vitality of *S. mutans* biofilm ($\times 10^6$ cfu/mL) in a mature biofilm.

Groups	Mean
Control Group 1	0.033 ± 0.087^a
Control Group 2	0^a
Group AV	0^a
Group PBS	38.30 ± 11.48^b

$p < 0.05$; ANOVA; Different letters show statistically significant differences; Control Group 1: containing 1450 ppm fluoride; Control Group 2: containing 500 ppm fluoride; Group AV: containing Aloe Vera; Group PBS: negative control.

TABLE 5. Inter-group comparison of effect of the tested dentifrices on cell vitality of *S. mutans* biofilm according to Tukey HSD Test.

Groups	*p*
Control Group 1/Control Group 2	0.8999947
Control Group 1/Group AV	0.8999947
Control Group 1/Group PBS	0.0010053
Control Group 2/Group AV	0.8999947
Control Group 2/Group PBS	0.0010053
Group AV/Group PBS	0.0010053

$p < 0.05$; Control Group 1: containing 1450 ppm fluoride; Control Group 2: containing 500 ppm fluoride; Group AV: containing aloe vera; Group PBS: negative control.

3.4 Evaluation of biofilm morphology by scanning electron microscopy

SEM micrographs of the biofilms grown on different surfaces after 24 h of incubation are shown in Fig. 2a–d. The SEM images show the *S. mutans* biofilm after the application of the dentifrices on HAP disc surfaces. A large number of bacterial cells, and some bacterial clusters, were observed in Group PBS. The chain length of the cells in this group was also longer than that in other groups (Fig. 2d). Because of the inhibition of *S. mutans* biofilm formation, fewer cells and smaller clusters were observed in the control groups (Fig. 2a,b) and Group AV (Fig. 2c), and the cells seen on the surface were more randomly distributed when compared with those in other groups.

4. Discussion

It is a well-known fact that fluoride is the most efficient and safe method to prevent caries in pediatric patients. This is evidence-based information adapted to international guidelines [28]. Therefore, our intention was to consider the potential for toxic overdose of fluoride, especially in young and vulnerable patient groups and special care patients; new and more appropriate methods of caries protection are required for these groups of patients. The present study aimed to study and compare fluoride-free dentifrices that are available on the dental market with gold standard "fluoride" to identity potential alternative treatments for these patient groups.

In the present study, the antibacterial effects of different fluoride-free dentifrices were evaluated and compared to that of commercially available pediatric dentifrices with low (500 ppm) and high (1450 ppm) concentrations. The study was carried out to identify the antibacterial efficacy of dentifrices against *S. mutans* containing active ingredients other than fluoride.

Dentifrices with antibacterial ingredients have been developed to control the accumulation of dental biofilm and improve the effects of mechanical cleaning. The dentifrice in Group P contained a probiotic that harnesses the power of beneficial bacteria (*L. paracasei*) to eliminate *S. mutans* from the oral cavity. This dentifrice also contains xylitol, which is known to have bacteriostatic effects on *S. mutans* [29]. In a previous study, Maden *et al.* [30] (2018) instructed children to use one of three toothpastes that contained fluoride, xylitol and probiotics to brush their teeth twice a day. Analysis showed that the quantity of *S. mutans* and *Lactobacillus* decreased when the application of xylitol/probiotic and fluoride dentifrice was increased [30]. Caglar *et al.* [31] (2009) conducted an *in vivo* investigation to investigate whether *Lactobacillus reuteri* (*L. reuteri*) can be detected in the oral cavity after discontinuation of the administration of a product prepared with that bacteria and concluded that two weeks of *L. reuteri* consumption did not appear to be adequate for *L. reuteri* to colonize the oral cavity permanently [31]. In our present *in vitro* study, Group P did not exhibit any antibacterial effect on *S. mutans*. It might be anticipated that the regular and prolonged use of the probiotic *L. paracasei* strains found in dentifrices is required to achieve a positive impact. Permanent colonization could be achieved by ongoing use. Further clinical research with the dentifrice used in Group P needs to investigate this possibility.

Gudipaneni *et al.* [32] (2014) compared the antibacterial effects of three different dentifrices (Group I: fluoride-free, Group II: 500 ppm fluoridated, and Group III: salivary proteins containing lactoferrin, lysozyme and lactoperoxidase dentifrices) on *S. mutans* and *Lactobacillus acidophilus* (*L. acidophilus*) in children aged 3–5 years with severe early childhood caries (S-ECC). According to their findings, the dentifrice containing lactoferrin, lysozyme and lactoperoxi-

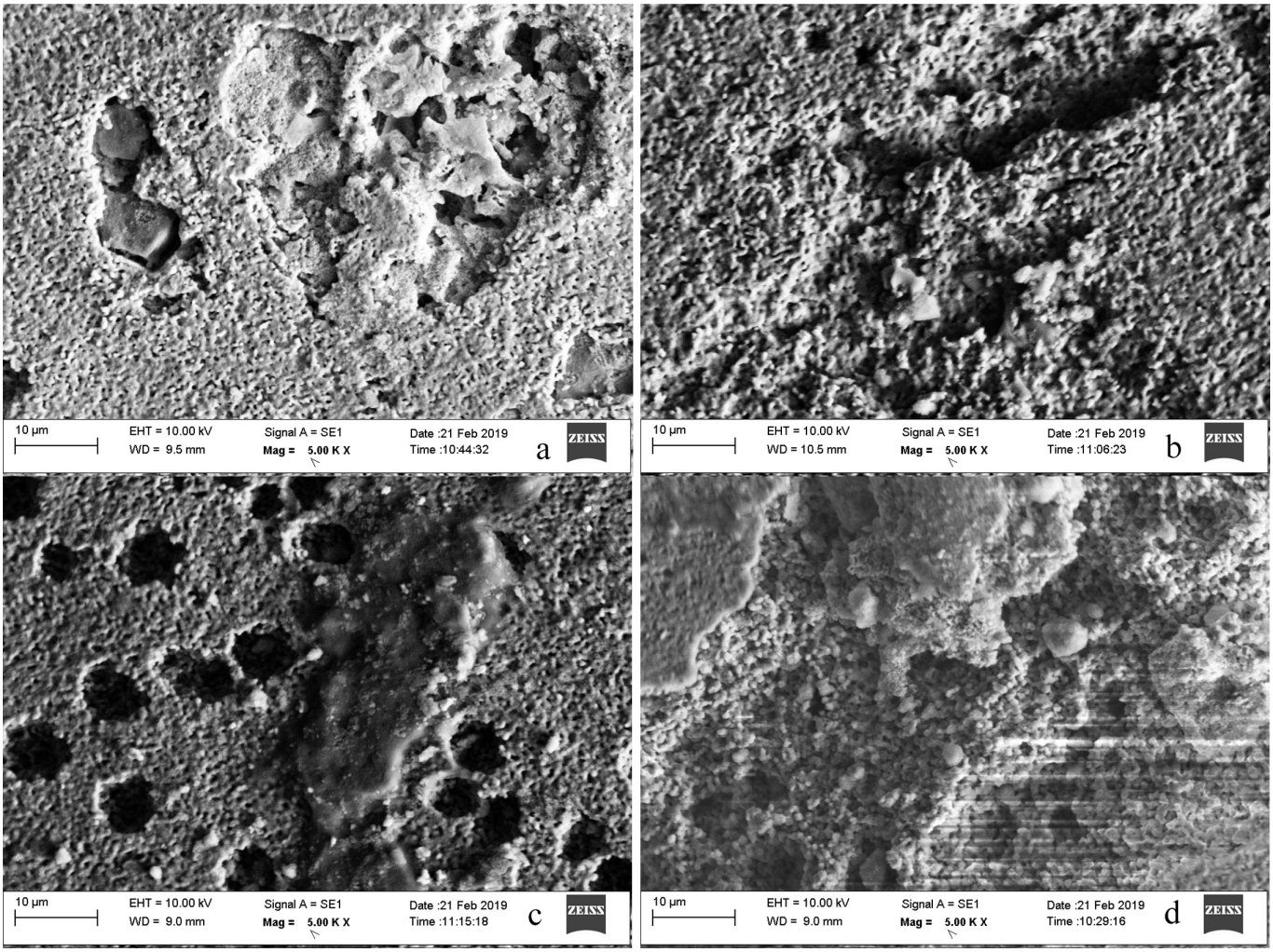

FIGURE 2. Scanning electron microscopy micrograph images of the 24-hour biofilms on the tested specimens. (a) Morphology of *S. mutans* biofilm after the application of fluoridated dentifrice containing 1450 ppm fluoride on the HAP disc surfaces (Control Group 1). (b) Morphology of *S. mutans* biofilm after the application of fluoridated dentifrice containing 500 pmm fluoride on the HAP disc surfaces (Control Group 2). (c) Morphology of *S. mutans* biofilm after the application of the fluoride-free dentifrice containing aloe vera (Group AV). (d) Morphology of *S. mutans* biofilm after the application of negative control group (Group PBS) at a magnification of ×5000.

dase was very effective in terms of reducing levels of bacteria in the saliva of children with S-ECC. In the present study, the dentifrice in Group SP contained lactoperoxidase and lactoferrin as antimicrobial proteins, and also contained arginine, which is known to exert metabolic actions on bacterial biofilms and increase pH. These actions might be linked with the arginine deiminase system (ADS), which several *Streptococcus* species are known to possess. The ADS system is highly active in *Streptococcus gordonii* (*S. gordonii*) and *Streptococcus sanguinis* (*S. sanguinis*), but is absent from *S. sobrinus* and *S. mutans* [32]. Berto *et al.* [33] (2019) assessed the effects of arginine as a dentifrice supplement on *Streptococci* with and without ADS. When 1.5% arginine was present, *S. sanguinis* and *S. gordonii* produced significantly more citrulline, along with an increased pH, than *S. mutans* and *S. sobrinus*. Compared to *S. mutans* and *S. sobrinus*, *S. sanguinis* and *S. gordonii* produced significantly more citrulline with arginine supplementation on all days of biofilm formation on polystyrene surfaces, along with a rise in pH [33]. In the present study, Group SP, containing salivary proteins and arginine, did not exhibit any inhibitory effect on *S. mutans*.

In this study, Group AV was named according to the prime ingredient aloe vera, as given on the brand name of the dentifrice (Aloe Vera (LR) LR Health & Beauty). However, this dentifrice also comprises several other active ingredients, including echinacea purpura extract, propolis, SLS and sorbitol; the potential antibacterial efficacy of these ingredients has yet to be specifically evaluated. It is possible that the antibacterial effect against *S. mutans* may be attributed to the synergistic effect of some of these ingredients with aloe vera. Future research should investigate the antibacterial effects of some of these ingredients against *S. mutans*.

Aloe vera is a medicinal plant that is of great importance and has been used for therapeutic purposes for a very long time [34]. In a previous study, George *et al.* [35] (2009) compared aloe vera dentifrice and two commercially available dentifrices containing 500 ppm and 1000 ppm fluoride in terms of their antimicrobial efficacy against *Candida albicans* (*C. albicans*),

S. mutans, *L. acidophilus*, *Enterococcus faecalis*, *Prevotella intermedia* and *Peptostreptococcus anaerobius*. These authors proved that aloe vera tooth gel was just as successful at removing these bacteria as two commercially available dentifrices [35]. In another study, Fani *et al.* [19] (2012) found that an aloe vera-based dentifrice exhibited antibacterial effects on oral microbes such as *S. mutans*. Without distinguishing between the other chemical agents tested, Bertolini *et al.* [36] (2012) concluded that brushing with a dentifrice comprising aloe vera and propolis reduced the contamination of toothbrush bristles by *S. mutans*. These authors claimed that their findings could be associated with the action of SLS, a common ingredient in the dentifrices used in their study [36].

In a previous study, Chan *et al.* [37] (2020) reported that sorbitol inhibits the production of *S. mutans* biofilms and that this inhibitory effect was suppressed by the presence of sucrose [37]. In another study, Yazdanian *et al.* [38] (2022) evaluated the antibiofilm activities of echinacea purpura extract against *S. mutans*, *Streptococcus mitis*, *Streptococcus salivarius*, *L. acidophilus*, *Escherichia coli*, *Staphylococcus aureus* (*S. aureus*) and *Candida albicans*. These authors observed that this herbal medicine had a bacteriostatic and bactericidal effect against almost all of the samples tested [38]. Randall *et al*., (2015) [39] compared the antibacterial activity of various fluoride- and herb-containing dentifrices and their constituent parts against *S. mutans* and reported that SLS had a potent antibacterial effect on *S. mutans*. However, sodium benzoate failed to prevent bacterial growth [39]. We believe that new studies should further investigate the antibacterial efficacy of these ingredients.

Compared to other oral materials, dentifrices experience prolonged contact with human gingival fibroblasts [40–42]. These factors led some researchers investigating the cytotoxicity of various dentifrices; these studies showed that the toxicity varies according to composition [43–45]. For example, human corneal epithelial cells, human foreskin fibroblasts, vaginal epithelial cells, and HeLa cell lines were previously used in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiment to investigate the effects of extremely porous activated charcoal on cell viability. Analysis showed that all cell types had a cell viability of at least 75% and highly porous activated charcoal was found to be non-cytotoxic [46].

A common remedy for treating either acute or ongoing wounds is aloe vera; this can maintain and enhance fibroblast migration in a non-toxic manner [47].

In a previous study, lysozyme-loaded antibacterial cream was created and tested for use in scald wound healing by Chen *et al.* [48] (2022). These authors concluded that lysozyme-loaded cream had an impact on scalds that prevented wound infection and accelerated wound healing; furthermore, no toxicity was detected in the organs of the animal model utilized in these experiments [48]. Birant *et al.* [45] (2022) investigated the potential harmful effects of pediatric dentifrices containing various detergents on gingival epithelial cells and discovered that toothpaste solutions from Splat, which contained lactoferrin components, had the highest live cell ratios [45].

Most ingredients in dentifrices are detergents. One particular detergent, SLS, has been shown to exhibit some harmful effects [44]. Tabatabaei *et al.* [43] (2019) compared the cytotoxicity of five different pediatric dentifrices and concluded that sodium benzoate was not toxic for human gingival fibroblasts (HGFs) and exhibited the lowest cytotoxicity.

Initially, MIC and agar diffusion assays were used to eliminate dentifrices prior to biofilm assays in the present study. Besides being simple methods, these assays have been used for routine *in vitro* antimicrobial susceptibility testing for many years. We measured the sizes of growth inhibition zones in the agar diffusion test and determined mean values. According to the results of the agar diffusion assay, Group AV produced a wider zone of inhibition for *S. mutans* than the other study groups. However, no inhibition of *S. mutans* was observed in Group P (containing probiotic and xylitol) and Group SP (containing lactoferrin, lactoperoxidase and arginine). Evaluation of the MIC values of the groups showed that Group AV exerted antibacterial effects against *S. mutans* even at very low concentrations.

The use of a single species biofilm could be considered as a drawback from a methodological standpoint. However, in clinical conditions, dental biofilm comprises hundreds of bacterial species and it is difficult to simulate this complexity *in vitro*. Therefore, in our laboratory conditions, the present biofilm model was developed to evaluate the anti-biofilm effect of Group AV, the only fluoride-free dentifrice that was found to have antibacterial effects against *S. mutans*. It was chosen as an early colonizer of dental biofilm because it can attach to pellicle glycoproteins, participate in biofilm accumulation in the presence of sucrose, and as a result, can act on all stages of the molecular pathogenesis of dental caries to form an individual mature cariogenic biofilm. Our oral microbiology laboratory recently validated the *S. mutans* biofilm model we designed for this purpose on hydroxyapatite discs [49, 50].

The fluoridated dentifrices that were investigated in the present study, although containing different concentrations of fluoride, demonstrated similar efficacies against the tested microorganisms. According to the manufacturers, fluoride, SLS and sorbitol were present in the dentifrices used in both control groups. Control Group 2 exhibited slightly greater antibacterial effects than Control Group 1. However, there was no significant difference between these groups in terms of antibacterial effects. This minimal difference may have arisen due to the presence of carbomer and benzyl alcohol in Control Group 2 with greater antibacterial capacity or differences in the concentration of the other active ingredients between Control Group 1 and Control Group 2.

The findings of the present *in vitro* study showed that dentifrices containing aloe vera, propolis, SLS, sorbitol and fluoride effectively inhibited *S. mutans* biofilm formation over 24 h. Further *in vitro* and prospective clinical studies investigating the antibacterial effects of non-fluoridated dentifrices on other oral bacterial strains are now essential to fully validate these observations.

In a previous study, Saddiq and Al-Ghamdi [51] (2018) evaluated the antimicrobial efficacy of aqueous aloe vera leaf extract on the biofilm formation of six *S. aureus* strains *in vitro*. *S. aureus* was incubated for 24 h; then, the authors used SEM to analyze the surface morphology of these bacteria after treatment with aloe vera leaf extract. Treatment of this bacterial strain with the plant extract resulted in abnormal

morphology, as detected by SEM. In contrast to the untreated and unmodified bacteria, which exhibited normal morphology, a reduction in the diameter of the bacterial cells was also noted. In another study, Bijle *et al.* [52] (2019) subjected mono-species biofilms of *S. mutans*, *S. sanguinis* and *S. gordonii* to treatments with test agents containing different concentrations of arginine combined with sodium fluoride (NaF) and NaF alone; cells were evaluated by SEM at a magnification of ×6000. According to biofilm imaging findings, mono-species *S. mutans* and three-species biofilm were both affected by 2% Arginine sodium fluoride (Arg-NaF) and 4% Arg-NaF [52].

In the present study, scanning electron microscopy micrograph (5000×) images of the 24-hour *S. mutans* biofilms treated with Group AV, Control Groups 1 and 2 revealed fewer cells, smaller cell clusters, and a biofilm disrupting effect; these effects were not apparent in the negative control group. In contrast, more bacterial cells and some bacterial clusters, along with a viable *S. mutans* biofilm chain, were observed in Group PBS.

The fact that the cytotoxicity of the substances used in the research and control groups was not investigated in the present study, represents a notable limitation. Further research needs to investigate the cytotoxicity and biocompatibility of current dentifrices.

5. Conclusions

In conclusion, according to MIC and Agar well plate diffusion assay, dentifrices containing probiotic (Group P) and salivary proteins (Group SP) did not show any antibacterial effect against *S. mutans*. In the light of these findings, these may not be considered as alternative dentifrices for pediatric patients with a high risk of caries.

The dentifrices containing aloe vera, propolis, SLS and sorbitol and fluoride (500 and 1450 ppm) showed similar effects in reducing the bacterial counts of *S. mutans* according to the three different microbiological methodologies. Dentifrices containing 500 ppm and 1450 ppm fluoride showed similar effects on the inhibition of *S. mutans* biofilm *in vitro*.

Within the constraints of the current *in vitro* study, ingredients other than fluoride, including sorbitol, propolis, aloe vera and SLS, may exert antibacterial activity in commercial dentifrices against *S. mutans*. Aloe vera-containing toothpaste exerted an antibacterial effect on *S. mutans*, although this was not as extensive as fluoride-containing toothpastes in the control groups. Further *in vivo* and prospective randomized clinical studies are now required to confirm the present findings.

ABBREVIATIONS

Arg-NaF: Arginine sodium fluoride; ATTC: American Type Culture Collection; BHI: Brain Heart Infusion Agar; *C. albicans*: *Candida albicans*; Carbon dioxide: CO_2; CLSI: Clinical Laboratory Standards Institute; Group P: Group Probiotic; Group AV: Group Aloe Vera; Group SP: Group Salivary Proteins; Group PBS: Group phosphate-buffered saline; HAP: hydroxyapatite discs; HGFs: human gingival fibroblasts; *L. paracasei*: *Lactobacillus paracasei*; *L. reuteri*: *Lactobacillus reuteri*; *L. acidophilus*: *Lactobacillus acidophilus*; MIC: Minimum Inhibitory Concentrations; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NI: No inhibition; OD: optical density; post hoc Tukey HSD: Honestly Significant Difference; SEM: Scanning Electron-Microscopy; NaF: sodium fluoride; *S. mutans*: *Streptococcus mutans*; *S. sobrinus*: *Streptococcus sobrinus*; SLS: sodium lauryl sulfate; *S. gordonii*: *Streptococcus gordonii*; *S. sanguinis*: *Streptococcus sanguinis*; *S. aureus*: *Staphylococcus aureus*.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

KTA and SSK—designed the research study. KTA and NT—performed the research. KTA, SSK and NT—analyzed the data. KTA—wrote the manuscript. SSK, NT and GK—revised and edited the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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