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Sumário

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Antimicrobial activity of **Desplac®** oralgel in the subgingival multispecies biofilm formation

Antimicrobial activity of **Desplac®** oral gel in the subgingival multispecies biofilm formation

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Natural products are well-known due to their antimicrobial properties. This study aimed to evaluate the antimicrobial effect of Desplac® product (composed of Aloe Vera, Propolis Extract, Green Tea, Cranberry, and Calendula) on the subgingival biofilm. Two different protocols were used to treat the 33-species biofilms: (A) 2×/day (12/12 h) for 1 min with Desplac® or Noplak Toothpaste (Chlorhexidine + Cetylpyridinium Chloride) or Oral B ProGengiva (stannous Fluoride) or a placebo gel; (B) a 12-h use of the Desplac® product or 0.12% chlorhexidine gel or a placebo gel. After 7 days of biofilm formation, the metabolic activity (MA) and biofilm profile were determined by 2,3,5-triphenyltetrazolium chloride and Checker-board DNA–DNA hybridization, respectively. Statistical analysis used the Kruskal-Wallis test followed by Dunn's post-hoc. In protocol A, all treatments presented reduced MA compared to the placebo ($p \leq 0.05$). The Desplac®-treated biofilm showed a similar microbial profile to other antimicrobials, although with higher bacterial total counts. In protocol B, MA of Desplac®-treated biofilms was lower than the placebo's MA but higher than chlorhexidine-treated biofilms ($p \leq 0.05$). Pathogen levels in Desplac®-treated biofilms were lower than in placebo-treated biofilms and elevated compared to the chlorhexidine-treated biofilms ($p \leq 0.05$). Desplac® inhibited the biofilm development and disrupted the mature subgingival biofilm, highlighting its effect on Tannerella forsythia counts.

1. Introduction

The mechanical removal of biofilm is necessary for the prevention, treatment, and post-therapy maintenance of periodontal diseases, either professionally or through manual control by the individual (Axelsson et al., 2004). However, satisfactory cleanliness levels are not always achieved with manual brushing alone. Furthermore, tooth surfaces only represent a small percentage of the total mouth area (Kerr et al., 1991). Therefore, the use of antimicrobial agents can help control the supragingival biofilm because they are able to reach other oral niches and can delay the accumulation on the tooth surface (Teles and Teles, 2009).

One of the ways to use chemical agents in oral health is through mouthwashes. These agents can act by promoting cell death, inhibiting bacterial reproduction, or inhibiting cell metabolism (Tartaglia et al., 2017). A wide range of antimicrobial chemical agents are being studied as active principles to control dental biofilm formation, such as bisbiguanides (chlorhexidine), quaternary ammonium compounds (cetylpyridinium chloride), essential oils, enzymes (mutanase/glucanase, amyloglucosidase/glucose oxidase), metal ions (zinc, copper, tin), and plant extracts (Jones, 1997; Radford et al., 1997; Faveri et al., 2006; Feres and Figueiredo, 2009; Kumar et al., 2013; James et al., 2017).

In this context, previous research (Newman and Cragg, 2020) reported that among all new drugs approved by the US's Food and Drug Administration (FDA), or other equivalent entities in other countries, 30% are directly derived from natural products, 44% are from derivatives of these natural products, and only 26% have synthetic origins. Natural products have been a more sustainable and ecological therapeutic alternative for different clinical situations. Some of the main benefits arising from the use of natural products are formulas that are not aggressive to the human body, they do not present polluting agents in nature, and they decrease the risk of allergies and inflammatory diseases. The search for natural cosmetic products for dental applications (dentifrices and mouthwashes) has been growing constantly. There is intense research in the literature to find new antimicrobials that lead to the rupture of the subgingival multispecies biofilm and one of the main sources to discover novel compounds are the natural products (Freires et al., 2015; Lazar et al., 2016; Slobodnikova et al.,

2016; Arbia et al., 2017; Lee et al., 2017; Miranda et al., 2019; Bim-Júnior et al., 2020; de Figueiredo et al., 2020; de Faveri et al., 2022).

The initial studies to prove the antimicrobial effect of a novel agent usually adopt the biofilms model. The common monospecies biofilms were inappropriate for the periodontal disease since bacteria organize themselves as dynamic multispecies biofilms in the subgingival environment (Prado et al., 2022). Hence, the literature looks for innovative biofilm models to reproduce what happens in vivo. Recently, our research group developed a multispecies biofilm composed of 33 distinct bacterial species using the Calgary Biofilm Device, which includes a cover with 96 polystyrene pegs mounted up into a 96-well plate (Miranda et al., 2019; de Figueiredo et al., 2020). This model's advantages include the number of health- and disease-associated species, encompassing most of the species studied in Socransky's complexes (Socransky et al., 1998). To our knowledge, no biofilm model quantifies so many species as the present one. It better simulates what happens in vivo when compared to a model with fewer species due to the number of representative bacteria species involved in the periodontal disease initiation and progression included in the model. In addition, bacteria must actively adhere to pegs instead of being deposited at the bottom of the wells in order to form the biofilm. Recently, the natural product Desplac® (Premium Oral Gel), composed of propolis, Aloe vera, green tea, cranberry, and calendula, became available in the Brazilian market. This oral product has lawful approval from the responsible government departments in the country (Brazilian Health Regulatory Agency—ANVISA) and recommendations for dental use. The main biological constituents of natural products can act as antioxidants, anti-inflammatories, and antimicrobials, in addition to other properties. Several studies have already been carried out to better understand the use of propolis (Bueno-Silva et al., 2013, 2015, 2017a,b,c, 2020; Lima Cavendish et al., 2015; Kiani et al., 2022), Aloe vera (Sujatha et al., 2014; Ali and Wahbi, 2017; Pattnaik et al., 2022), green tea (Yang et al., 2016; Miyoshi et al., 2020; Kong et al., 2022), cranberry (Ben Lagha et al., 2020; Galarraga-Vinueza et al., 2020; Mizutani et al., 2021; Nawrot-Hadzik et al., 2021a,b; Nemzer et al., 2022), and calendula (Alexandre et al., 2018; Tanideh et al., 2020; Yin et al., 2021).

However, it is necessary to carry out scientific investigations that prove and support the commercial recommendations of these products. Thus, the objective of this study was to evaluate the antimicrobial effect of the Desplac® product (Premium Oral Gel) on the metabolic activity and the profile of multispecies subgingival in vitro biofilm model.

2. Materials and methods

2.1. Experimental design

The design of this study (Figure 1) involved two laboratory experiments that aimed to reproduce the clinical indications of the Desplac® product (Premium Oral Gel): as a dentifrice (A) and as a night gel on acrylic plates (B). The (in vitro) multispecies bacterial biofilm was exposed to the respective products according to the test or control groups.

Experiment A (simulation of use as a toothpaste, 2×/day, 12/12 h, for 1 min).

• Test Group: Desplac® (Premium Oral Gel);

• Negative Control Group: Placebo Gel;

• Positive Control Group 1: Noplak Toothpaste (Chlorhexidine + Cetylpyridine Chloride);

• Positive Control Group 2: Oral B ProGengiva (Stannous Fluoride).

For experiment A, the pins with attached biofilm were removed from the culture media, placed in another 96-well plate with the treatments each time, and later returned to the same media.

Experiment B (simulation of use as an overnight gel on acrylic plates, for 12 h on day 6).

• Test Group: Desplac® (Oral Gel Premium);

• Negative Control Group: Placebo Gel;

• Positive Control Group: 0.12% Chlorhexidine Gel.

The cover with the pins was placed in another 96-well plate containing culture media BHI mixed with the treatments for experiment B. Noplak Toothpaste and Oral B ProGengiva products are commercially available and were purchased locally. The Desplac® and the placebo gels were provided by the company responsible for the former's manufacturing (Sysplac). The placebo gel was formulated with the same physical characteristics as the Desplac® product but without the active ingredients. Considering that there is no other commercially available dental product in the national market with the same recommendation for overnight use during 12 h, a 0.12% Chlorhexidine Gel, purchased from a compounding pharmacy, was chosen for its recognized gold standard antimicrobial activity.

2.2. In vitro multispecies biofilm model

In vitro multispecies biofilm cultures were prepared with 33 bacterial species (Table 1) as described by Miranda et al. (2020), with some modifications. Tryptone soy agar with 5% sheep blood (Probac, São Paulo, Brazil) was used to grow most species under anaerobic conditions, 85% nitrogen, 10% carbon dioxide, and 5% hydrogen. Porphyromonas gingivalis was grown on tryptone soy agar containing yeast extract enriched with 1% hemin, 5% menadione, and 5% sheep blood. Tannerella forsythia was grown on tryptone soy agar containing yeast extract enriched with 1% hemin, 5% menadione, 5% sheep blood, and 1% N-acetylmuramic acid. All species were allowed to grow on agar plates for 24 h and then transferred to glass tubes containing Brain Heart Infusion (BHI) culture medium (Becton Dickinson, Sparks, MD, United States) supplemented with 1% hemin. After 24 h growing on conical tubes, the optical density was adjusted for the inoculum to have about 108 cells/mL of each species. A dilution of individual cell suspensions was performed and 100 µL aliquots containing 106 cells from each species were added to 11,700 µL of BHI broth complemented with 1% hemin and 5% sheep blood to obtain an inoculum of 15 mL (Miranda et al., 2019, 2020; Pingueiro et al., 2019; Shibli et al., 2021).

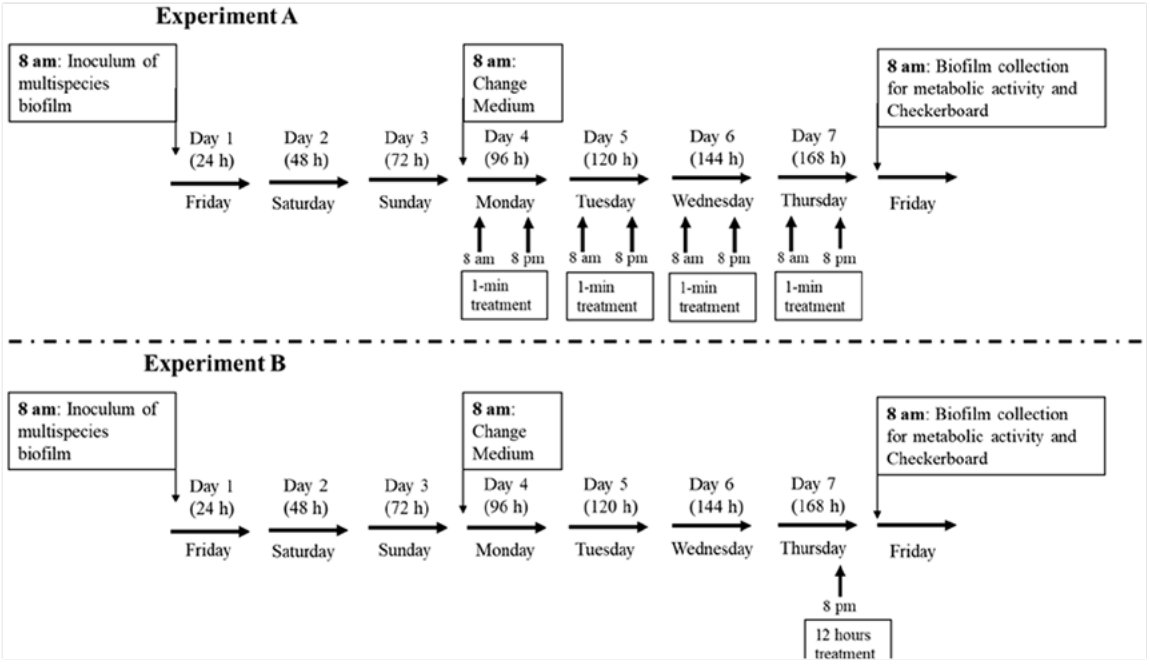

FIGURE 1
Scheme of the therapeutic approaches of experiments A and B.

The multispecies biofilm model was developed using a Calgary biofilm device (CBD) in a 96-well plate (Nunc; Thermo Scientific, Roskilde, Denmark; Ceri et al., 1999). A 150 µL aliquot of each inoculum was added to the wells and corresponded to ~1×104cells of each bacterial strain—except for P. gingivalis and Prevotella intermedia, whose inocula were adjusted to 2×104cells. A lid containing polystyrene pins was used to seal the 96-well plate (Nunc TSP system; Thermo Scientific, Roskilde, Denmark). Coated plates were incubated at 37°C under anaerobic conditions. After 72 hours, the used medium (BHI broth with 1% hemin and 5% sheep blood) was replaced and biofilm cultures were kept at 37°C under anaerobic conditions for an additional 4 days to obtain 7-day-old biofilms (Miranda et al., 2019). In the middle of the seventh day, the biofilms were transferred to a culture medium mixed with the different treatments according to the description of Experiments A and B. All products used in the experiments (Desplac®—Premium Oral Gel; Placebo Gel; Noplak Dentifrice; Oral B

ProGengiva; Chlorhexidine Gel 0.12%) were diluted (1 part of the product for 2 parts of BHI) to obtain a more fluid solution that could act on the biofilm for its biological properties and not for a merely mechanical effect. After 7 days of biofilm formation, the pins were collected for microbiological processing. The experiments were performed in triplicate for each of the groups (Miranda et al., 2019; Faveri et al., 2022).

2.2.1. Quantification of biofilm bacterial metabolic activity

The effects of Desplac® and other products used as positive and negative controls on the metabolic activity of multispecies biofilm cells were measured in a spectrophotometric assay with 2,3,5-triphenyltetrazolium chloride (TTC; catalog No. 17779; Fluka analytical). TTC is used to differentiate between metabolically active and inactive cells. TTC white substrate is enzymatically reduced to red formazan by live cells due to the activity of several dehydrogenases. The change in substrate color is an indirect measure of bacterial metabolic activity.

To mensurate the metabolic activity of biofilm cells, the pins were transferred to 96-well plates with 200 µL/well of fresh BHI medium supplemented with 1% hemin and 0.1% TTC solution. The plates were incubated under anaerobic conditions for 8 h at 37°C. TTC reduction to red formazan was read at 485 nm in a spectrophotometer (Miranda et al., 2019).

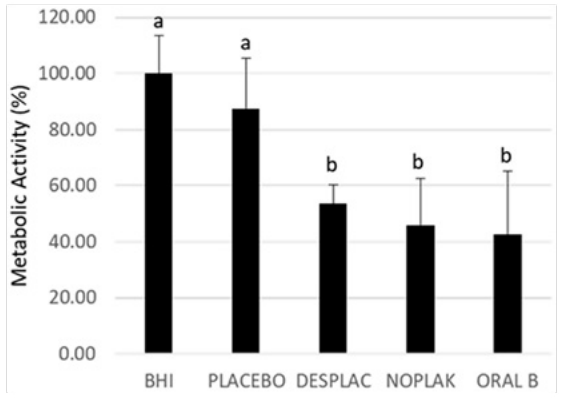

FIGURE 2
Mean and standard deviation of the mean of the biofilms' metabolic activities treated with the different agents in experiment A. The metabolic activity of the biofilm treated with the culture medium was considered 100%. Different letters mean a statistically significant difference using ANOVA, followed by Tukey's test (p 0.05).

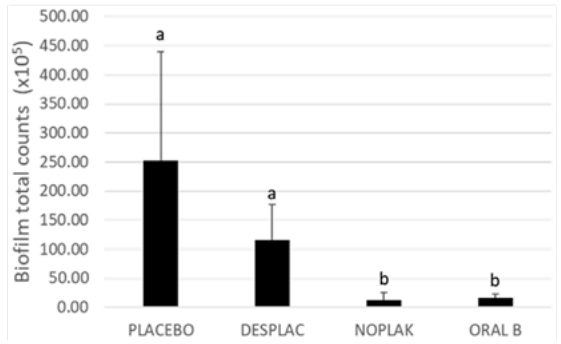

FIGURE 3
Mean and standard deviation of total counts of all bacterial species in experiment A, analyzed using Checkerboard DNA-DNA Hybridization. Different letters mean a statistically significant difference performed using the Kruskal-Wallis test followed by Dunn's *post hoc* test (p 0.05).

TABLE 1 List of bacterial species cultured in multispecies biofilms.

Species	ATCC
***Actinomyces* sp.**	
Actinomyces naeslundii	12104
Actinomyces oris	43146
Actinomyces gerencseriae	23840
Actinomyces israelii	12102
Purple complex	
Veillonella parvula	10790
Actinomyces odontolyticus	17929
Ydlow complex	
Streptococcus sanguinis	10556
Streptococcus oralis	35037
Streptococcus intermedius	27335
Streptococcus gordonii	10558
Streptococcus mitis	49456
Green complex	
Aggregatibacter actinomycetemcomitans	29523
Capnocytophaga ochracea	33596
Capnocytophaga gingivalis	33624
Eikenella corrodens	23834
Capnocytophaga sputigena	33612
Orange complex	
Campylobacter showae	51146
Campylobacter gracilis	33236
Eubacterium nodatum	33099
Fusobacterium nucleatum vincentii	49256
Parvimonas micra	33270
Fusobacterium nucleatum polymorphum	10953
Fusobacterium periodonticum	33693
Prevotella intermedia	25611
Streptococcus constellatus	27823
Red complex	
Porphyromonas gingivalis	33277
Tannerella forsythia	43037
Others species	
Eubacterium saburreum	33271
Streptococcus anginosus	33397
Streptococcus mutans	25175
Selenomonas noxia	43541
Propionibacterium acnes	11827
Gemella morbillorum	27824

The strains were categorized in the microbial complexes described by Socransky et al. (1998).

2.2.2. Checkerboard DNA–DNA hybridization

The pins coated with 7-day-old biofilms from each group were transferred to Eppendorf tubes containing 100 µL of TE buffer (10 mM Tris–HCl, 1 mM EDTA [pH 7.6]); then, 100 µL of 0.5 M NaOH was added to each tube. The tubes containing the pins and the final solution were boiled for 10 min and the solution was neutralized by adding 0.8 mL of 5 M ammonium acetate. The samples were individually analyzed for the presence and counting of the 33 bacterial species using the DNA–DNA hybridization technique, as previously described (Socransky et al., 1994; Mestnik et al., 2010). Briefly, following sample lysis, the DNA was placed onto a nylon membrane using a Minislot device (Immunetics, Cambridge, United States) and fixed onto the membrane at 120°C for 20 min. Next, the membrane was placed in a Miniblotter 45 (Immunetics). Digoxigenin-labeled whole genomic DNA probes of the 33 bacterial species were hybridized in each lane of the Miniblotter. Following hybridization, the membranes were washed, and DNA probes were detected using a specific antibody to digoxigenin conjugated with alkaline phosphatase. The signals were detected using the AttoPhos substrate (Amersham Life Sciences, Arlington Heights, United States), and the data were obtained in the Typhoon Trio Plus program (Molecular Dynamics, Sunnyvale, United States). Two lanes in each membrane contained the standards with 1 × 105 and 1 × 106 cells of each strain. The signals were converted into absolute counts via comparison with the standards on the same membrane. The measurements of the experimental groups were compared against those of the negative and positive controls. Counts below the method detection limit (1 × 104) were considered zero (Socransky et al., 1994; Miranda et al., 2019).

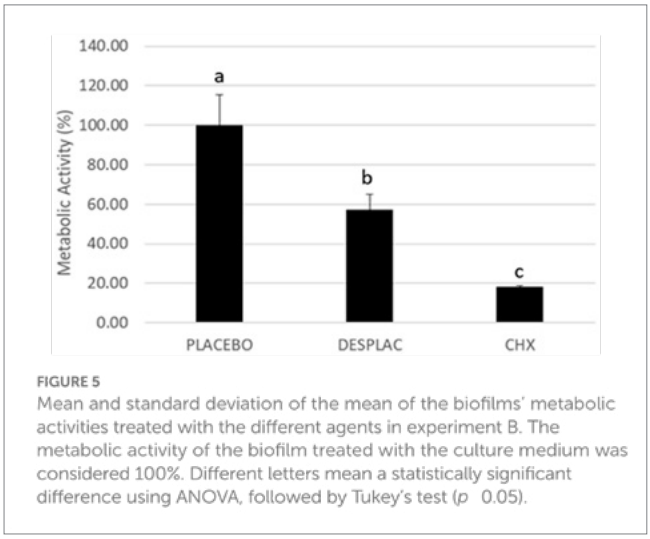

FIGURE 5
Mean and standard deviation of the mean of the biofilms' metabolic activities treated with the different agents in experiment B. The metabolic activity of the biofilm treated with the culture medium was considered 100%. Different letters mean a statistically significant difference using ANOVA, followed by Tukey's test (p 0.05).

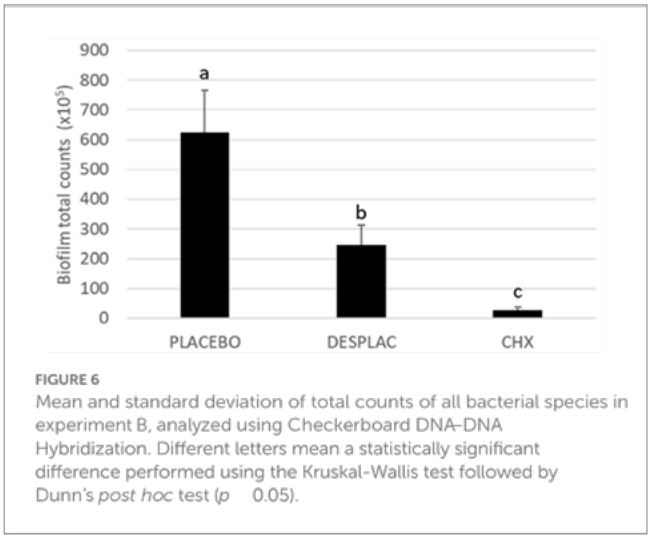

FIGURE 6
Mean and standard deviation of total counts of all bacterial species in experiment B, analyzed using Checkerboard DNA-DNA Hybridization. Different letters mean a statistically significant difference performed using the Kruskal-Wallis test followed by Dunn's *post hoc* test (p 0.05).

2.2.3. Statistical analysis

Data from the biofilm's metabolic activity test were statistically analyzed using Analysis of Variance (ANOVA) followed by Tukey's test. The results of the Checkerboard DNA–DNA Hybridization were statistically analyzed using Kruskal-Wallis followed by Dunn's post hoc ($p \leq 0.05$).

3. Results

The analysis of data from Experiment A is shown in Figures 2–4. Figure 2 shows that the metabolic activity of the Desplac® product was statistically similar to Noplak (chlorhexidine + cetylpyridinium chloride) and the Oral B toothpaste (Stannous Fluoride), while the three treatments were statistically better than the placebo group.

Figure 3 shows the total count of all species present in the biofilm subgingival model. Noplak and Oral B reduced total biofilm counts by more than 90% when compared to placebo and Desplac® treated biofilms ($p \leq 0.05$). In addition, Desplac® and placebo behaved similarly in reducing the total count of bacteria present in biofilms ($p = 0.07$).

Figure 4 shows the individual mean count of each bacterial species included in the biofilm formation evaluated by Checkerboard DNA–DNA Hybridization. The Noplak product reduced the count of 23 bacterial species, the Oral B dentifrice of 25 species, and Desplac® of two species when compared to the placebo-treated biofilms ($p \leq 0.05$). It is noteworthy that Noplak, Oral B toothpaste, and Desplac® reduced the P. gingivalis count

demonstrating specific action on key bacteria for the development and progression of periodontal disease.

Regarding Experiment B, Figure 5 shows that Desplac® statistically reduced biofilm metabolic activity when compared to placebo by about 45% ($p \leq 0.05$), but Chlorhexidine Gel (0.12%) showed the best inhibition of metabolic activity reducing it by more than 80% ($p \leq 0.05$).

Figure 6 presents the total count of all bacterial species present in the biofilm model. Desplac® statically reduced the total biofilm count when compared to the placebo by about 59%. However, the chlorhexidine gel (0.12%) showed the best reduction in the total biofilm count, about 89% ($p \leq 0.05$).

Figure 7 shows the individual mean count of each bacterial species included in the biofilm formation evaluated by Checkerboard DNA–DNA Hybridization. Chlorhexidine Gel (0.12%) reduced the count of 27 species while Desplac® reduced the count of 24 different bacteria in relation to the placebo group ($p \leq 0.05$), highlighting Fusobacterium nucleatum polymorphum, Prevotella intermedia, and P. gingivalis, all recognized periodontal pathogens. Only Desplac® was able to reduce T. forsythia counts (Figure 7).

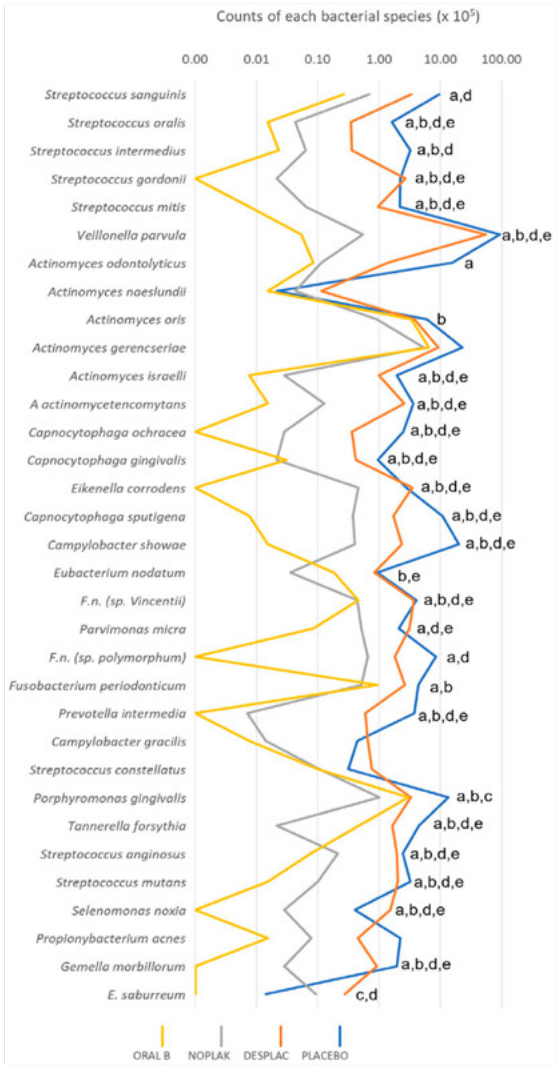

FIGURE 4
Mean counts of each of the bacterial species present in the biofilms of experiment A. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn'*spost hoc* test (*p* 0.05). The letter "a" represents the statistical difference between placebo and Oral B; letter "b" represents the statistical difference between placebo and Noplak; letter "c" represents the statistical difference between placebo and Desplac®; letter "d" represents the statistical difference between Desplac® and Oral B and letter "e" represents the statistical difference between Desplac® and Noplak.

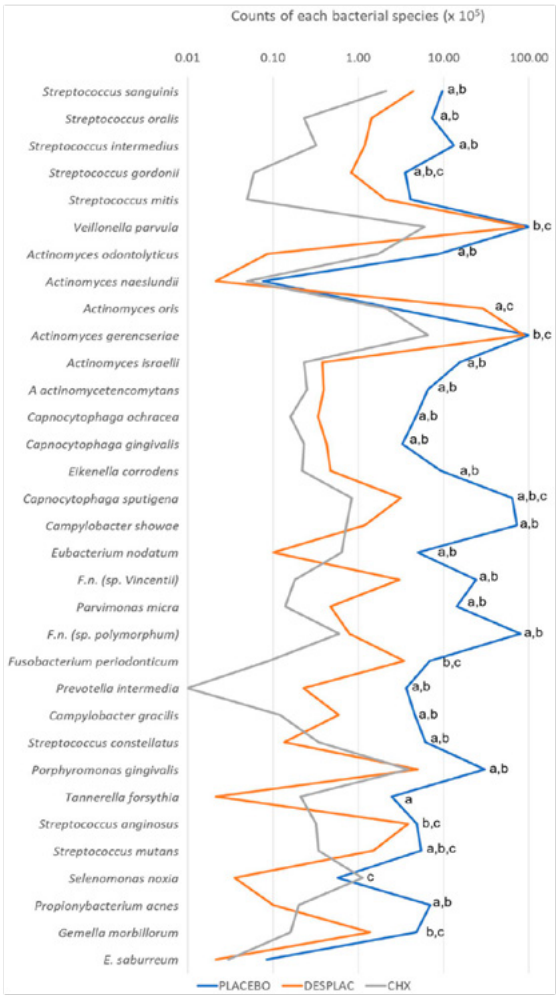

FIGURE 7
Mean counts of each of the bacterial species present in the biofilm of experiment B. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn'*spost hoc* test (*p* 0.05). Letter "a" represents the statistical difference between Placebo and Desplac®; letter "b" represents the statistical difference between Placebo and Chlorhexidine Gel; letter "c" represents the statistical difference between Desplac® and Chlorhexidine Gel.

4. Discussion

The success of periodontal treatment is directly related to an ecological change in the biofilm, making its microbial profile more compatible with periodontal health. This leads to an improvement in periodontal clinical parameters (Cugini et al., 2000; Feres et al., 2015). The therapy known as the gold standard is scaling and root planing. However, not all individuals are able to maintain the benefits achieved with such treatment in the long term (Cugini et al., 2000; Carvalho et al., 2005; Feres et al., 2015). The fact that the standard therapeutic proposal does not reach the periodontopathogens found throughout the mouth—including supragingival biofilm and those in other oral niches, such as the tongue, oral mucosa, and saliva—is one of the reasons for therapeutic failure (Socransky and Haffajee, 2002).

According to the current concept of periodontitis, a dysbiotic microbial community is pointed out as responsible for disease initiation (Hajishengallis and Diaz, 2020). Thus, the red complex members still possess a crucial role in the development of the disease. Among them, P. gingivalis and T. forsythia are the most studied microorganisms. Both have been proposed as targets to prevent oral microbiome dysbiosis since its incidence may contribute to the shift from healthy to diseased-associated biofilm (Hoare et al., 2021). Therefore, Desplac's® effects on these bugs are outstanding.

Porphyromonas gingivalis has been indicated as the keystone pathogen in periodontal disease since this bacterium produces several virulence factors (for example, gingipains and FimA) with properties to subvert the human immune response, including neutrophils, macrophages, and complement system (Hajishengallis, 2021). Besides its role in periodontitis, T. forsythia may be relevant in peri-implantitis pathogenesis. This microorganism was found at more elevated levels in dental implant replacements in contrast with the adjacent tooth, and its presence is correlated with the increase in severity of peri-implantitis (Eckert et al., 2018; Eick et al., 2019).

Healthy sites in individuals with periodontal disease have higher proportions of pathogens when compared to those without the disease (Feres et al., 2015). Hence, the search for anti-infective therapies capable of enhancing the clinical results achieved is constant. In this context, the benefits attained with the use of chlorhexidine stand out among several scientific studies (Carvalho et al., 2005; Mestnik et al., 2010; Feres et al., 2012), and for this reason, it was the chemical agent of choice to represent the positive control group in the experiments of this study.

The problem with the continued use of chlorhexidine mouth rinses is the possibility of developing adverse effects. The most reported in the literature are extrinsic pigmentation of teeth, tongue, mucous membranes and restorations, taste alteration, burning sensation, supragingival calculus formation, and less frequent cases of allergy (Keni et al., 2012; James et al., 2017). For this reason, formulations with other active ingredients have been described in the literature in an attempt to show similar benefits, but with less frequent adverse effects associated with the use of chlorhexidine. Special emphasis can be given to the potential of natural products. Currently, there is a relevant proportion of the world population that searches for cosmetic oral hygiene products (toothpaste and mouthwash) with this profile. The antimicrobial activity of the natural agents propolis, Aloe vera, green tea, cranberry, and calendula is already evidenced in the scientific literature. However, to date, this activity evaluated in a combined way as in the commercial product Desplac® is unprecedented in the literature.

In this direction, the green propolis produced in the South region of Brazil was the first to be recognized for its antimicrobial potential. Recently, it was found to impair gut microbiota dysbiosis by enhancing the Bacteroidetes/Firmicutes proportion in an animal study (Okamura et al., 2022). In addition, the baccharin, one of its biocompounds, has shown a possible antimicrobial mechanism of action on P. gingivalis. As an antimicrobial mechanism, this compound induces membrane depolarization so to increase membrane permeability leading to bacterial death (Yoshimasu et al., 2018). The apigenin, found in green propolis, has been shown to inhibit the development of Candida albicans (Cheah et al., 2014) and Streptococcus mutans (Jeon et al., 2011).

Another natural agent is Aloe vera or Aloe barbadensis. It is also an option in toothpaste considering its antimicrobial potential on oral microorganisms, such as S. mutans and C. albicans, and improvement in plaque index comparable to those obtained with products with triclosan in their composition (Lee et al., 2004; Pradeep et al., 2012; Vajrabhaya et al., 2022). The Aloe vera main components are aloin A, aloin B, aloesin, aloe-emodin, aloeresin D, orientin, cinnamic acid, and chlorogenic acid (Solaberrieta et al., 2022). Among them, the antibacterial mechanism of aloe-emodin was determined on Staphylococcus epidermidis. The compound provokes abnormalities in S. epidermidis morphology and ruins membrane permeability (Li et al., 2021).

Several components of green tea can also promote health benefits. Mazur et al. (2021) demonstrated through a systematic review that clinical periodontal parameters were found to be positively affected by green tea. Chemical analysis of green tea revealed the presence of some phenolic compounds (rutin, quercetin, and chlorophyll) and four main catechins: epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG); the latter being the most active and abundant among them (Ku et al., 2010; Reygaert, 2018; Kolackova et al., 2020). More recently, Kong et al. (2022) published a literature review showing the antimicrobial activity of epigallocatechin-3-gallate, one of the green tea's compounds as mentioned, in the microbiota associated with oral diseases. The antimicrobial effect was evident for P. gingivalis, A. actinomycetemcomitans, P. intermedia, and F. nucleatum. EGCG damages the P. gingivalis membrane and cellular wall preventing biofilm formation and ruining the pre-formed biofilm. Regarding A. actinomycetemcomitans, EGCG inhibits a relevant virulence factor, the leukotoxin that is associated with the impairment of human macrophages.

Cranberry has bioactive agents such as proanthocyanidins (propelargonidin, procyanidin, and prodelphinidin) that characterize this natural product as beneficial for health (Zhao et al., 2020). In dentistry, Polak et al. (2013) showed the potential protective and/or preventive effect of cranberry on P. gingivalis and F. nucleatum-induced periodontitis in mice. Galarraga-Vinueza et al. (2020) demonstrated that proanthocyanidins, known to inhibit oral biofilm adherence and for their anti-inflammatory effect, could potentially neutralize the destructive inflammatory response of macrophages. These compounds do not interfere with P. gingivalis growth; however, they inhibit many virulence factors related to P. gingivalis adhesion, such as collagenases, proteinases, and other proteins associated with P. gingivalis's attachment to periodontal tissue, with subsequently smaller bacterial biofilm formation (Nawrot-Hadzik et al., 2021a). In recent years, the medicinal potential of Calendula officinalis has encouraged scientific studies in dentistry especially on topics involved in the treatment of periodontitis and peri-implantitis (Lima et al., 2017; Alexandre et al., 2018; Tanideh et al., 2020). Although calendula presents distinct classes of well-known antimicrobial compounds in its composition, such as triterpenoids, flavonoids, quinones, tannins, coumarins, and phenolic acids, the literature on calendula's antimicrobial activity is scarce., The only report found demonstrated that a calendula-based dentifrice did not present an antimicrobial effect on A. viscosus, C. albicans, L. casei, S. mitis, S. mutans, S. oralis, S. sanguis, S. sobrinus, and clinically isolated C. albicans, S. mitis, S. mutans, S. oralis, S. sanguis, S. sobrinus, and Lactobacillus spp. (Modesto et al., 2000).

It is interesting to observe how the agent's contact time with the biofilm improved the antibacterial effect. The unique 12-h treatment of an established biofilm reduced a more significant number of species than two daily treatments of 1 min during the biofilm formation. Usually, an established biofilm is a more complex challenge for antimicrobial agents than a biofilm in formation. However, probably due to the time of contact, Desplac® reduced a larger number of species in experiment B (12-h treatment of an established biofilm).

Considering the design of this study, it is important to point out that experiments A and B correspond to the uses recommended by the manufacturer in accordance with ANVISA's authorization for the commercialization of Desplac®. It is important to note that chlorhexidine gel does not have an indication to be used overnight as Desplac®. However, due to the absence of a positive control with this kind of indication, chlorhexidine gel was kept as a positive control of experiment B due to its excellent antimicrobial properties. In addition, limitations of the biofilm model include the semi-quantitative characteristic of the checkerboard and the absence of Treponema denticola since this bug is also a member of the red complex (Socransky et al., 1998). Moreover, a possible improvement of the present biofilm model may include further examination, such as confocal microscopy, that would allow the assessment of the biofilm portion structure, bacteria biomass, and exopolysaccharide amount. Currently, confocal microscopy analysis is prevalent for caries-related monospecies biofilms but not periodontal ones. Therefore, future studies should consider improving the existing knowledge by evaluating dyes for confocal analysis of periodontitis-related multispecies biofilms (Torrez et al., 2023). The analysis of the data obtained in this laboratory research showed promising results related to antimicrobial activity in a multispecies subgingival biofilm. Thus, it was possible to conclude that the combination of natural agents present in the commercial product Desplac® was able to inhibit the biofilm development and disrupt the mature subgingival biofilm, highlighting its effect on T. forsythia counts. Although the present subgingival multispecies biofilm was revealed as a good model for the initial analysis of novel antibacterial agents, it is still necessary to carry out randomized controlled clinical studies in order to confirm whether the microbiological benefits observed here will be able to support the periodontal clinical condition associated with health.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

LF, BB-S, EO, and AB: conceptualization. LF, BB-S, MM, and MF: methodology. BB-S, MM, and KK: data analysis. BB-S, LF, and FG: resources. LF, BB-S, KK, and FG: writing—original draft preparation. LF, BB-S, and MF: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

EO was the owner of Sysplac Company that produces Desplac product.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DESPLAC

Atividade antimicrobiana do produto **Desplac®** (Gel Oral Premium)

DESPLAC

Atividade antimicrobiana do produto **DESPLAC** (Gel Oral Premium)

Luciene Figueiredo e Bruno Bueno Silva

Objetivo

Avaliar a atividade antimicrobiana do produto DESPLAC (Gel Oral Premium) em modelo de biofilme bacteriano in vitro.

Metodologia

Os procedimentos laboratoriais foram realizados em três fases individuais para os três grupos experimentais.

A) Grupos Experimentais:

Experimento I (simulação do uso como dentifrício)

O biofilme bacteriano in vitro em formação foi exposto aos respectivos produtos dos grupos teste e controles por 2x/dia (12/12h) durante 1 minuto.
- Grupo T: Desplac
- Grupo C - : Gel placebo
- Grupo C +1: Novak dentifrício (CLX)
- Grupo C +2: Oral B ProGengiva (Fluoreto de Estanho)

Experimento II (simulação do uso do gel na placa noturna)

O biofilme bacteriano in vitro de 7 dias de formação foi exposto aos respectivos produtos dos grupos teste e controles durantes 12 horas.
- Grupo T: Desplac
- Grupo C +: Gel de clorexidina 0,12%
- Grupo C -: Gel placebo

Experimento III (simulação de uma possível aplicação após raspagem e alisamento radicular com aplicações contínuas na bolsa por 1 semana para manter o produto o maior tempo possível na bolsa periodontal)

O biofilme bacteriano in vitro foi exposto aos produtos Desplac e Placebo desde o momento inicial de sua formação.
- Grupo T: Desplac
- Grupo C -: Gel placebo

B) Fases Laboratoriais:

Atividade antimicrobiana em modelo de biofilme

Inicialmente todas as espécies bacterianas foram cultivadas individualmente em meio de cultura sólido em

placas de Petri durante 7 dias em câmara de anaerobiose. Em seguida, todas foram transferidas para meio líquido. Após 48 horas de crescimento, todas as espécies foram transferidas para tubos cônicos de 15 mL (Tubos Falcon®) com meio de cultura BHI (Brain Heart Infusion, Becton Dickinson, Sparks, MD) suplementado com 1% de hemina. Após 24 horas de crescimento, a densidade ótica (DO) a 600 nm foi ajustada para 0,1, correspondendo a cerca de 10^8 células/ml de cada uma das espécies. As suspensões de células individuais de cada espécie foram diluídas para 10^7 células/ml, com ajustamento para os seus respectivos tamanhos de células. Alíquotas de 100 ul contendo 10^6 células de cada uma das espécies foram misturadas para se obter um inóculo final de biofilme. Onze ml de caldo BHI com 1% de hemina e 5% de sangue de ovelha foram adicionados para se obter um inóculo final de biofilme no volume de 15 ml para cada dos experimentos descritos acima.

O modelo de múltiplas espécies de biofilme foi desenvolvido utilizando o Dispositivo de Calgary Biofilme (DCB). Uma placa de 96 poços (sistema Nunc; Thermo Scientific, Roskilde, Dinamarca) foi utilizada para adição de 150 ul de inóculo por poço, contendo 10^4 células de cada uma das espécies, exceto a Porphyromonas gingivalis que continha $2x10^4$ e também uma cobertura de placa contendo os pinos de poliestireno, também conhecida como Dispositivo de Calgary (sistema Nunc TSP; Thermo Scientific, Roskilde, Dinamarca). As placas foram então incubadas a 37°C, sob condições anaeróbias. Após 72 h de incubação, o meio de cultura foi trocado e as coberturas das placas foram transferidas para novas placas de 96 poços com caldo fresco (caldo BHI com 1% de hemina e 5% de sangue de ovelha) por mais 4 dias. Os tratamentos do biofilme com os tratamentos ou controles foram realizados de acordo com os três grupos experimentais. Após 7 dias de formação do biofilme, os pinos foram coletados e a análise microbiológica foi realizada em seguida.

Atividade metabólica do biofilme (TTC)

A redução percentual da atividade metabólica do biofilme bacteriano in vitro foi determinada com o uso de cloreto de 2,3,5-trifeniltetrazólio (TTC) e espectrofotometria. TTC é usado para diferenciação entre células metabolicamente ativas e inativas. O substrato branco é enzimaticamente reduzido a formazan vermelho 1,3,5-trifenil (TPHP) por células vivas bacterianas, devido à atividade de várias desidrogenases. Alíquota de 90 µL de caldo BHI (Brain Heart Infusion) foi adicionada em placa de 96 poços com 10 µL de solução de TTC a 1%. As placas foram incubadas sob condições anaeróbias por 8 horas à 37°C. Em seguida, a mudança na cor do substrato, ou seja, a conversão de TTC foi lida a 485nm usando um espectrofotômetro para determinar a taxa de redução da atividade metabólica.

Composição microbiana (Checkerboard DNA-DNA Hybridization)

As amostras de biofilme bacteriano provenientes dos três grupos experimentais foram colocadas em tubos Eppendorf contendo 0,15 mL de TE (10 mM Tris-HCl, 1 mM EDTA, pH 7,6), e 100 mL de 0,5 M NaOH foram adicionados a cada tubo. Subsequentemente, as amostras foram fervidas durante 10 min e neutralizadas usando 0,8 mL de acetato de amónio 5M. O DNA liberado foi então colocado nos slots de um aparelho Minislot 30 (Immunetics), concentrado em uma membrana de nylon de carga positiva de 15/15 cm (Boehringer Mannheim), e fixado à membrana por aquecimento a 120°C por 20 minutos. A membrana foi então colocada em um Miniblotter 45 (Immunetics) com as linhas de DNA posicionadas em 90° às canaletas do dispositivo. Sondas de DNA genômico total marcado com digoxigenina para 33 espécies bacterianas foram depositadas em cada canaleta individual do Miniblotter. Após a hibridação, as membranas foram lavadas e as sondas de DNA foram detectadas usando o anticorpo anti-digoxigenina conjugado à fosfatase alcalina e quimiluminescência. Os sinais foram avaliados visualmente por comparação com os padrões de 10^5 e 10^6 células bacterianas, dispostos nas duas últimas canaletas de cada membrana, por um único examinador calibrado (teste k = 93%). Os dados foram registrados da seguinte forma: escore 0, não detectado; escore 1, $<10^5$ células; escore 2, ~ 10^5 células; escore 3, entre 10^5 e 10^6 células; escore 4, ~ 10^6 células; e escore 5, > 10^6 células. As contagens médias (10^5 células) das espécies bacterianas individuais foram calculadas em cada repetição e depois dentro de cada grupo experimental. As contagens de cada espécie foram somadas para obtenção da contagem total do biofilme e as respectivas médias foram calculadas.

RESULTADOS

Experimento I

A figura 1 mostra os resultados da atividade metabólica do experimento 1. O Desplac foi estatisticamente igual ao Novak (clorexidina + cloreto de cetilpiridínio) e o dentifrício Oral B (Fluoreto Estanhoso). Em relação à atividade antimicrobiana, os três tratamentos foram estatisticamente melhores que o Placebo e o biofilme tratado com meio de cultura. Não houve diferença estatística entre Placebo e meio de cultura.

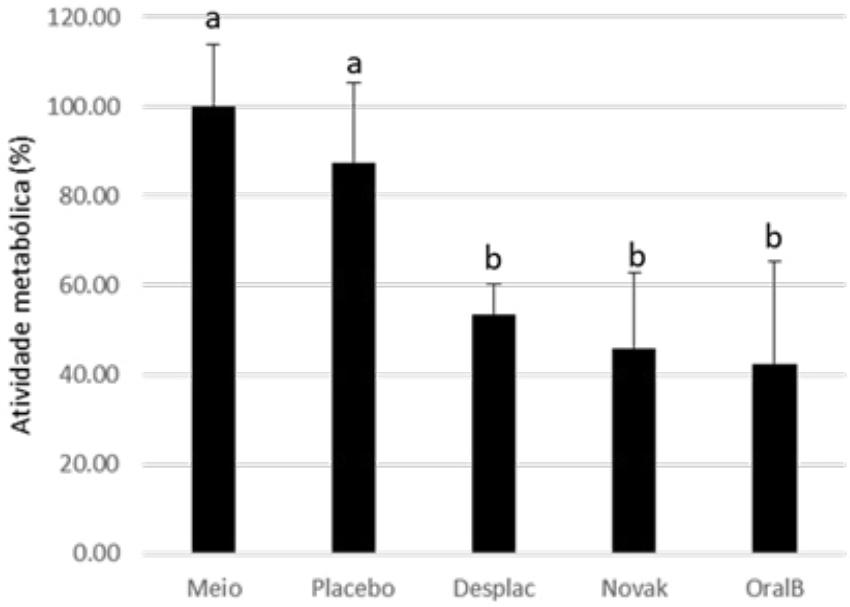

Figura 1: Média e desvio padrão da média das atividades metabólicas dos biofilmes tratados com os diferentes agentes. A atividade metabólica do biofilme tratado com meio de cultura foi considerada 100%. Letras diferentes significam diferença estatisticamente significante por meio de ANOVA, seguida do teste de Tukey (p≤ 0,05).

A figura 2 mostra a contagem total de todas as espécies presentes no modelo de biofilme do experimento I. Novak e Oral B reduziram as contagens totais dos biofilmes em mais de 90% de maneira significante estatisticamente quando comparado com os biofilmes tratados com Placebo e Desplac (p≤0,05). O biofilme tratado com o Desplac não apresentou diferença estatística para o biofilme tratado com Placebo (p= 0,07).

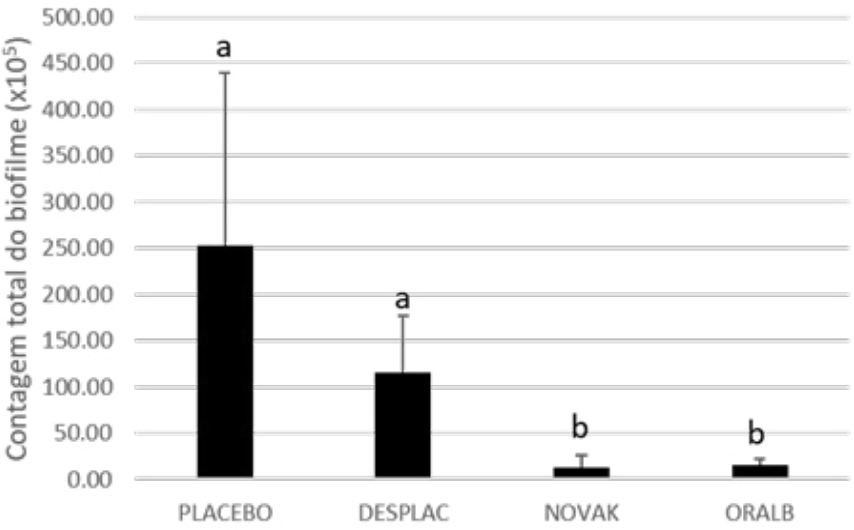

Figura 2: Média e desvio padrão das contagens totais de todas as espécies bacterianas analisadas por meio do Checkerboard DNA-DNA Hybridization. Letras diferentes significam diferença estatisticamente significante realizada por meio do teste de Kruskal-Wallis seguido do post-hoc de Dunn (p ≤ 0.05).

A figura 3 mostra a média individual da contagem de cada espécie bacteriana incluída na formação do biofilme do Experimento I avaliada pelo Checkerboard DNA- DNA Hybridization. Novak reduziu a contagem de 23 espécies bacterianas, o dentifrício da Oral B de 25 espécies e o Desplac de 02 espécies quando comprados com os biofilmes tratados com o placebo. Destaca-se que Novak, dentifrício da Oral B e o Desplac reduziram a contagem da Porphyromonas gingivalis, demonstrando ação específica em bactérias chave para o desenvolvimento e progressão da doença periodontal.

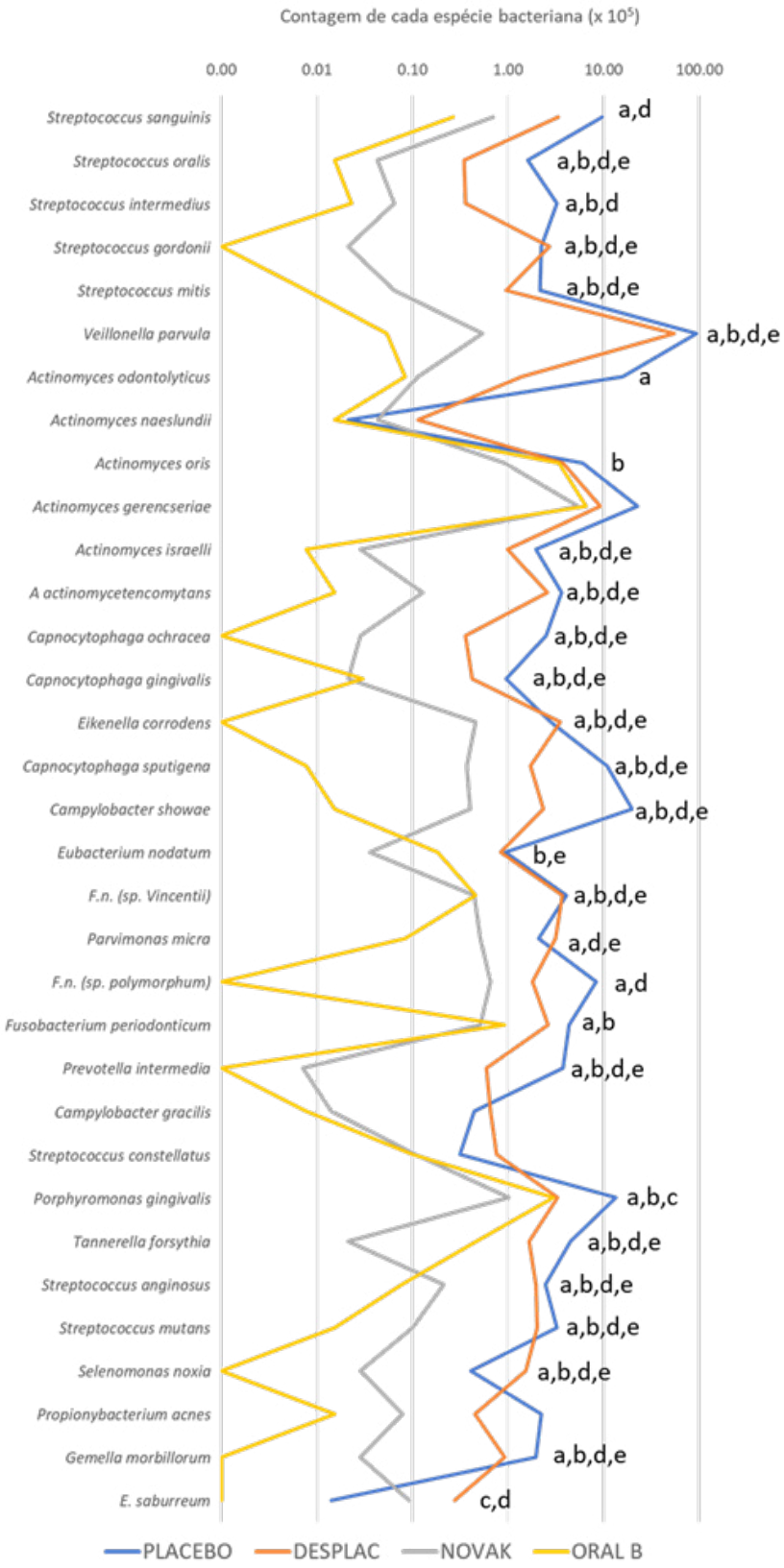

Figura 3: Média das contagens de cada uma das espécies bacterianas presentes no biofilmes. Análise estatística realizada por meio do teste de Kruskal-Wallis seguido do post-hoc de Dunn ($p \leq 0,05$). Letra "a" significa diferença estatística entre Placebo e Oral B; letra "b" significa diferença estatística entre Placebo e Novak; letra "c" significa diferença estatística entre Placebo e Desplac; letra "d" significa diferença estatística entre Desplac e Oral B e letra "e" significa diferença estatística entre Desplac e Novak.

Experimento II

A figura 4 mostra os resultados da atividade metabólica do experimento II. O Desplac reduziu estaticamente a atividade metabólica do biofilme quando comparado com o Placebo em cerca de 45%, porém o Gel de Clorexidina (CHX) apresentou a melhor inibição da atividade metabólica, reduzindo-a em mais de 80%.

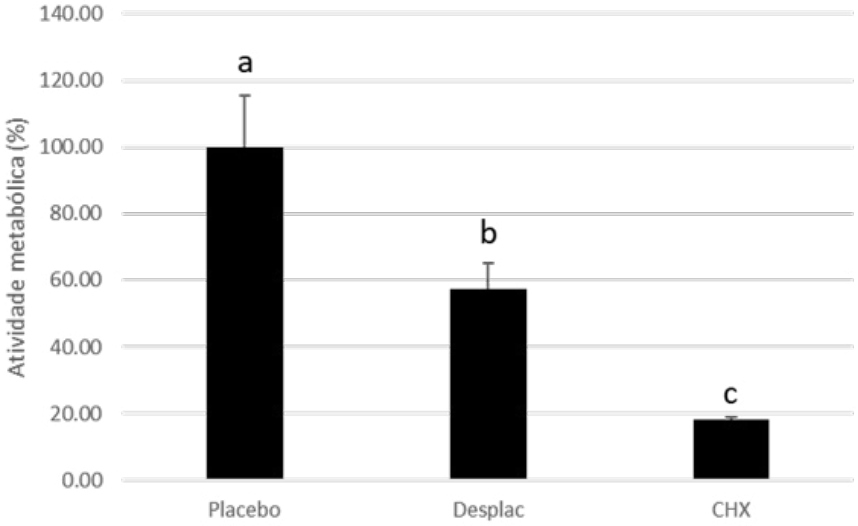

Figura 4: Média e desvio padrão da média das atividades metabólicas dos biofilmes tratados com os diferentes agentes. Letras diferentes significam diferença estatisticamente significante por meio de ANOVA, seguida do teste de Tukey (p≤ 0,05).

A figura 5 mostra a contagem total de todas as espécies presentes no modelo de biofilme do experimento II. O Desplac reduziu estaticamente a contagem total do biofilme quando comparado com o Placebo em cerca de 59%, porém o Gel de Clorexidina (CHX) apresentou a melhor redução da contagem total do biofilme, cerca de 89%.

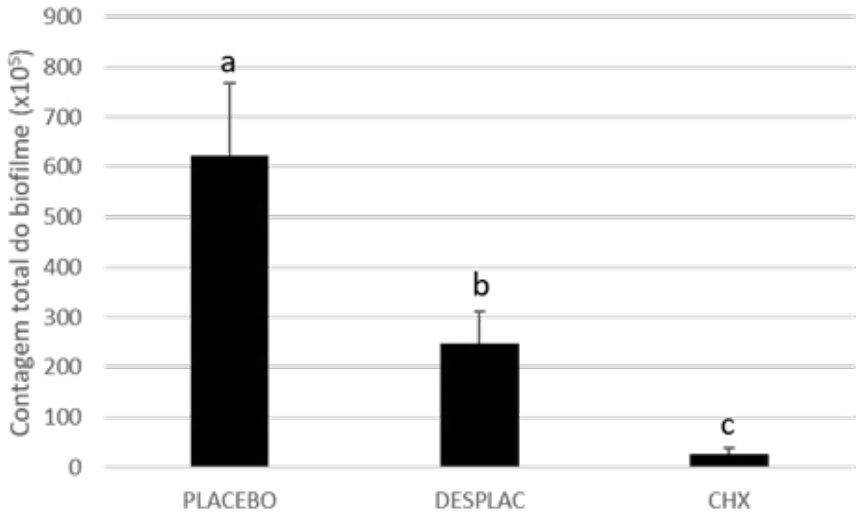

Figura 5: Média e desvio padrão das contagens totais de todas as espécies bacterianas por meio do Checkerboard DNA-DNA Hybridization. Letras diferentes significam diferença estatisticamente significante realizada por meio do teste de Kruskal-Wallis seguido do post-hoc de Dunn (p ≤ 0,05).

A figura 6 mostra a média individual da contagem de cada espécie bacteriana incluída na formação do biofilme do Experimento II avaliada pelo Checkerboard DNA-DNA Hybridization. O Gel de Clorexidina (CHX) reduziu a contagem de 27 espécies enquanto o Desplac reduziram a contagem de 24 bactérias diferentes em relação ao grupo tratado com Placebo, destacando Fusobacterium nucleatum polymorphum, Prevotella intermedia e P. gingivalis, reconhecidos patógenos periodontais. Somente o Desplac foi capaz de reduzir as contagens de Tannerella forsythia. O Gel de Clorexidina apresentou o melhor resultado na contagem de 8 bactérias com destaque para o patógeno periodontal Fusobacterium periodonticum.

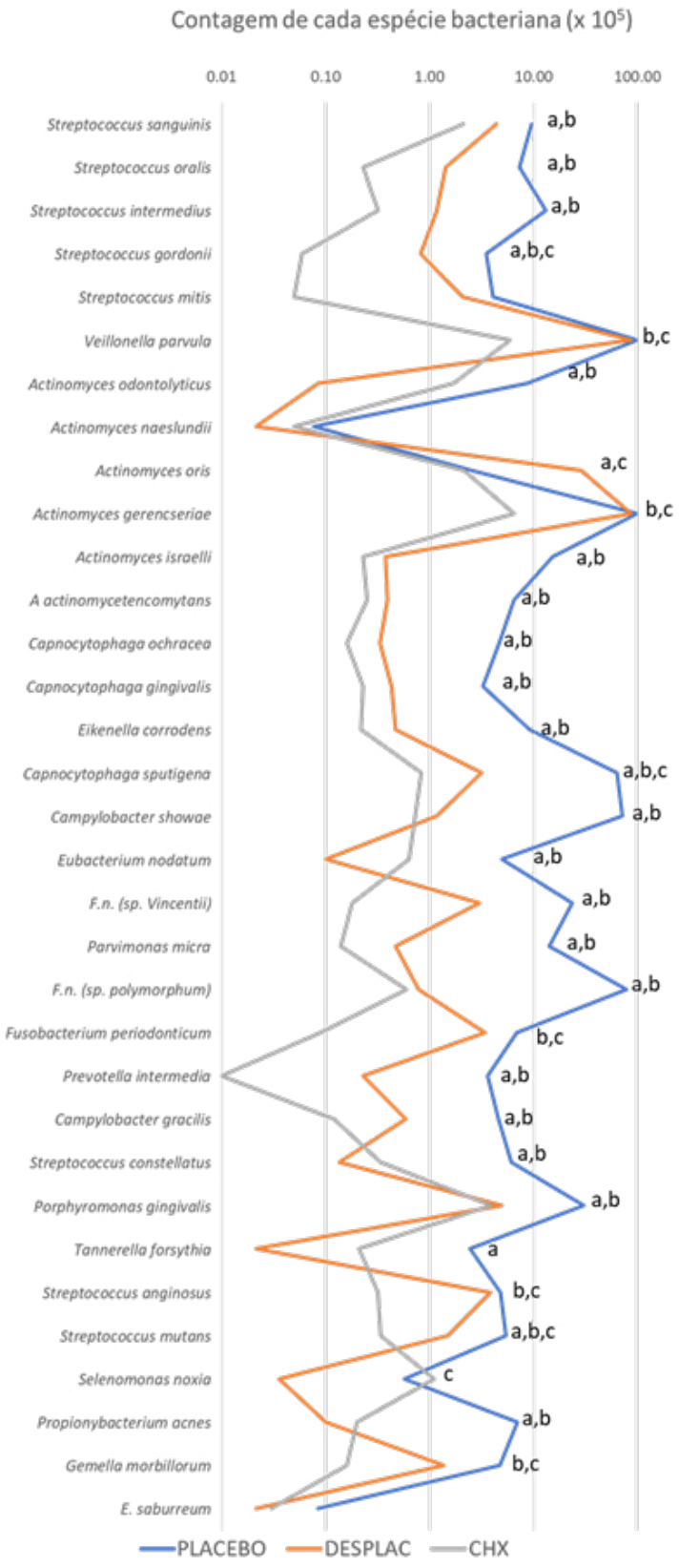

Figura 6: Média das contagens de cada uma das espécies bacterianas presentes no biofilme. Análise estatística realizada por meio do teste de Kruskal-Wallis seguido do post-hoc de Dunn ($p \leq 0,05$). Letra "a" significa diferença estatística entre Placebo e Desplac; letra "b" significa diferença estatística entre Placebo e Gel de Clorexidina (CHX); letra "c" significa diferença estatística entre Desplac e Gel de Clorexidina.

Experimento III

A figura 7 mostra os resultados da atividade metabólica do experimento III. A redução da atividade metabólica do biofilme pelo Desplac foi aproximadamente 43% superior em comparação ao Placebo.

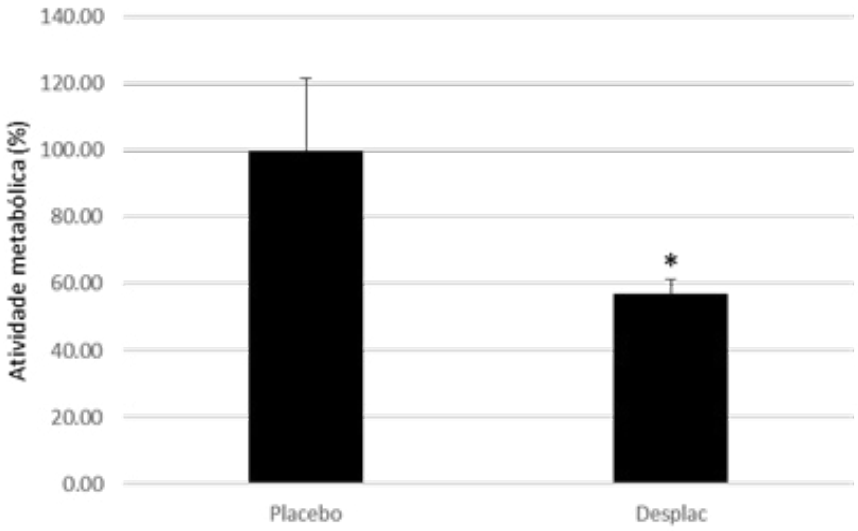

Figura 7: Média e desvio padrão da média das atividades metabólicas dos biofilmes tratados com os diferentes agentes. *Significa diferença estatisticamente significante por meio de ANOVA, seguida do teste de Tukey (p≤ 0,05).

A figura 8 mostra a contagem total de todas as espécies presentes no modelo de biofilme do experimento II. O Desplac reduziu estaticamente a contagem total do biofilme quando comparado com o Placebo em cerca de 40%.

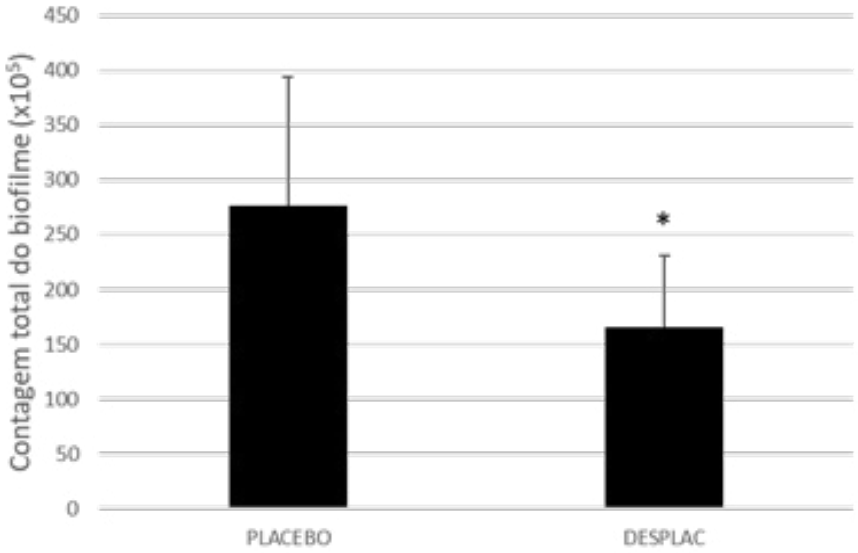

Figura 8: Média e desvio padrão das contagens totais de todas as espécies bacterianas por meio do checkerboard. *Significa diferença estatisticamente significante por meio do teste de Kruskal-Wallis seguido do post-hoc de Dunn (p ≤ 0,05).

A figura 9 mostra a média individual da contagem de cada espécie bacteriana incluída na formação do biofilme do Experimento III avaliada pelo Checkerboard DNA- DNA Hybridization. O Desplac reduziu a contagem de 14 bactérias diferentes em relação ao grupo tratado com Placebo, destacando 3 espécies de Fusobacterium, Parvimonas micra, P. gingivalis e T. forsythia.

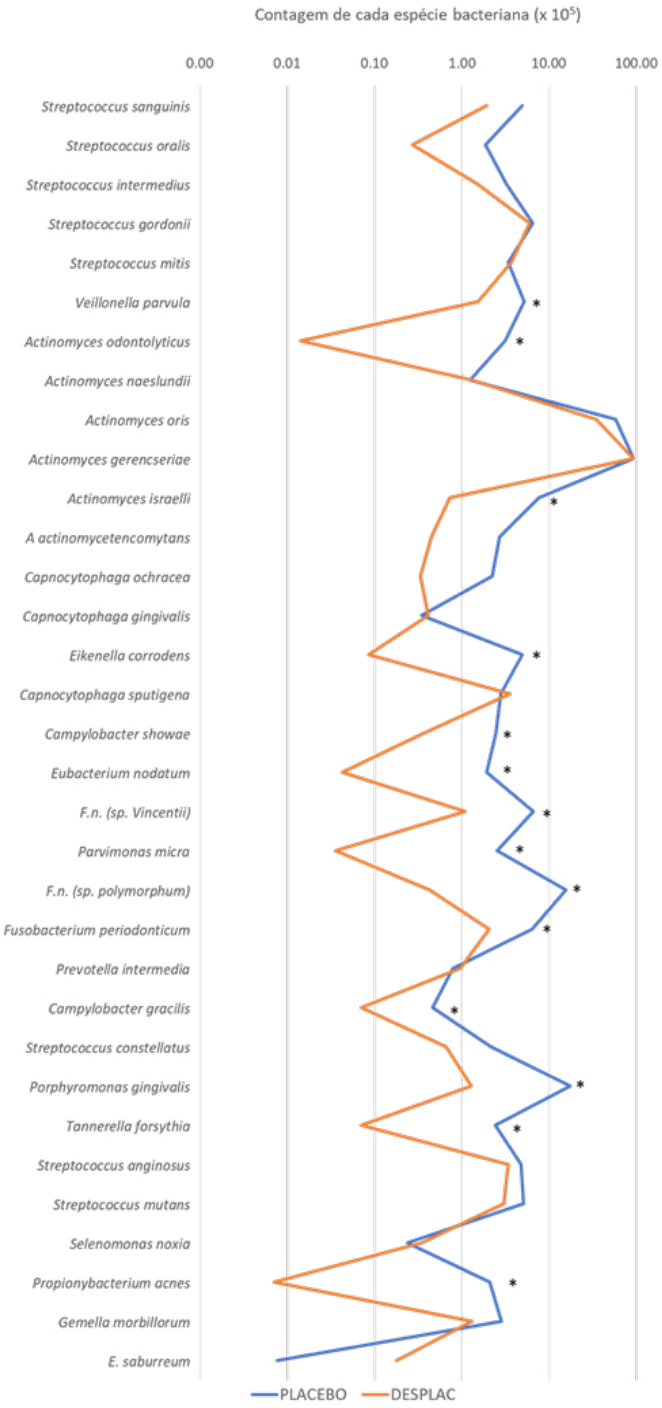

Figura 9: Média das contagens de cada uma das espécies bacterianas presentes nos biofilmes formados no experimento III. Análise estatística realizada por meio do teste de Kruskal-Wallis seguido do post-hoc de Dunn ($p \leq 0,05$). *Significa diferença estatística entre Placebo e Desplac.

CONSIDERAÇÕES FINAIS

De modo geral, o produto Desplac apresentou forte efeito antimicrobiano no modelo in vitro de formação de biofilme subgengival multiespécie. A simulação do uso como dentifrício (2x/dia), demonstrou que os produtos já disponibilizados no comércio (Novak e dentifrício Oral B) apresentaram melhor atividade antimicrobiana geral, porém o Desplac apresentou efeito específico contra P. gingivalis. Novak e dentifrício Oral B também reduziram a contagem de P. gingivalis.

A simulação de uso noturno em placa (12h contínuas) demonstrou que o Gel de Clorexidina teve atividade antimicrobiana contra um maior número de espécies bacterianas em relação ao Desplac. O Desplac apresentou efeito específico em periodontopatógenoss, como P. gingivalis e T. forsythia. É importante ressaltar que a clorexidina não possui indicação específica para este protocolo clínico.

A simulação com o uso após raspagem e alisamento radicular demonstrou um efeito seletivo do Desplac em patógenos periodontais, destacando novamente seu efeito inibidor em Fusobacterium, P. gingivalis e T. forsythia. Por esse modelo, notou-se um grande efeito em patógenos periodontais e um pequeno efeito em bactérias associadas à saúde periodontal.

DESPLAC

SAFETY DATA SHEET

DESPLAC

1. IDENTIFICATION

Product identifier
Product Name: EverSmile WhiteFoam

Other means of identification
OProduct Code(s): 1422909

Recommended use of the chemical and restrictions on use
Recommended Use: Mouthwash, Dental Care
Restrictions on Use: No information available

Details of the supplier of the safety data sheet
Supplier Identification: EverSmile, Inc.
Address: 10547 W. Pico Blvd. Los Angeles CA. 90064 US
Telephone: 8555952999
E-mail: info@eversmilewhite.com

Emergency telephone number
Company Emergency Phone Number
8555952999

2. HAZARDS IDENTIFICATION

Classification
Skin corrosion/irritation: Category 2
Serious eye damage/eye irritation: Category 1
Appearance: Clear
Physical state: Foam Liquid
Odor: Mint-like

GHS Label elements, including precautionary statements
Danger
Hazard statements
Causes skin irritation
Causes serious eye damage

Precautionary Statements - Prevention
Wash face, hands and any exposed skin thoroughly after handling
Wear protective gloves/eye protection/face protection

Precautionary Statements - Response
Specific treatment (see supplemental first aid instructions on this label)

Eyes
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing Immediately call a POISON CENTER or doctor

Skin
IF ON SKIN: Wash with plenty of water and soap
If skin irritation occurs: Get medical advice/attention
Take off contaminated clothing and wash it before reuse

Other information
Harmful to aquatic life with long lasting effects.

Unknown acute toxicity
6% of the mixture consists of ingredient(s) of unknown toxicity
2% of the mixture consists of ingredient(s) of unknown acute oral toxicity
6% of the mixture consists of ingredient(s) of unknown acute dermal toxicity
6% of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (gas)
6% of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (vapor)
6% of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (dust/mist)

3. COMPOSITION/INFORMATION ON INGREDIENTS

Substance
Not applicable.

Mixture

Chemical name	CAS-No	Percent	Hazardous Material Information Review Act registry number (HMIRA registry #)	Date HMIRA filed and date exemption granted (if applicable)
Hydrogen peroxide	7722-84-1	3.7875	-	-
Glycerin	56-81-5	2	-	-

4. FIRST AID MEASURES

First aid measures
General advice: Immediate medical attention is required. Show this safety data sheet to the doctor in attendance.

Inhalation: Remove to fresh air.

Eye contact: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Keep eye wide open while rinsing. Do not rub affected area. Get immediate medical advice/attention.

Skin contact: Wash off immediately with soap and plenty of water for at least 15 minutes. Get medical attention if irritation develops and persists.

Ingestion: Clean mouth with water and drink afterwards plenty of water. Never give anything by mouth to an unconscious person. Do NOT induce vomiting. Call a physician.

Self-protection of the first aider: Avoid contact with skin, eyes or clothing. Wear personal protective clothing (see section 8).

Most important symptoms and effects, both acute and delayed
Symptoms: Burning sensation.

Indication of any immediate medical attention and special treatment needed
Note to physicians: Treat symptomatically.

5. FIRE-FIGHTING MEASURES

First aid measures

Suitable Extinguishing Media: Use extinguishing measures that are appropriate to local circumstances and the surrounding environment.

Unsuitable extinguishing media: CAUTION: Use of water spray when fighting fire may be inefficient.

Specific hazards arising from the chemical: No information available.

Hazardous Combustion Products: Carbon oxides.

Explosion Data
Sensitivity to Mechanical Impact: None.
Sensitivity to Static Discharge: None.

Special protective equipment for fire-fighters: Firefighters should wear self-contained breathing apparatus and full firefighting turnout gear. Use personal protection equipment.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions, protective equipment and emergency procedures

Personal precautions: Avoid contact with skin, eyes or clothing. Ensure adequate ventilation. Use personal protective equipment as required.

Other Information: Refer to protective measures listed in Sections 7 and 8.

Methods and material for containment and cleaning up

Methods for containment: Prevent further leakage or spillage if safe to do so.

Methods for cleaning up: Dam up. Soak up with inert absorbent material. Pick up and transfer to properly labeled containers.

7. HANDLING AND STORAGE

Precautions for safe handling

Advice on safe handling: Handle in accordance with good industrial hygiene and safety practice. Avoid contact with skin, eyes or clothing. Do not eat, drink or smoke when using this product. Take off contaminated clothing and wash before reuse.

Conditions for safe storage, including any incompatibilities

Storage Conditions: Keep containers tightly closed in a dry, cool and well-ventilated place. Store locked up. Keep out of the reach of children.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Control parameters

Exposure Limits

Chemical name	ACGIH TLV	OSHA PEL	NIOSH IDLH
Hydrogen peroxide 7722-84-1	TWA: 1 ppm	TWA: 1 ppm TWA: 1.4 mg/m3 (vacated) TWA: 1 ppm (vacated) TWA: 1.4 mg/m3	IDLH: 75 ppm TWA: 1 ppm TWA: 1.4 mg/m3
Glycerin 56-81-5	TWA: 10 mg/m3 mist	TWA: 15 mg/m3 mist, total particulate TWA: 5 mg/m3 mist, respirable fraction (vacated) TWA: 10 mg/m3 mist, total particulate (vacated) TWA: 5 mg/m3 mist, respirable fraction	

Chemical name	Alberta	British Columbia	Ontario TWAEV	Quebec
Hydrogen peroxide 7722-84-1	TWA: 1 ppm TWA: 1.4 mg/m3	TWA: 1 ppm	TWA: 1 ppm	TWA: 1 ppm TWA: 1.4 mg/m3
Glycerin 56-81-5	TWA: 10 mg/m3	TWA: 10 mg/m3 TWA: 3 mg/m3		TWA: 10 mg/m3

Other Exposure Guidelines: Vacated limits revoked by the Court of Appeals decision in AFL-CIO v. OSHA, 965 F.2d 962 (11th Cir., 1992). See section 15 for national exposure control parameters.

Appropriate engineering controls

Engineering controls: Showers. Eyewash stations. Ventilation systems.

Individual protection measures, such as personal protective equipment

Eye/face protection: Tight sealing safety goggles.

Hand protection; Wear suitable gloves. Impervious gloves.

Skin and body protection; Wear suitable protective clothing. Long sleeved clothing.

Respiratory protection: No protective equipment is needed under normal use conditions. If exposure limits are exceeded or irritation is experienced, ventilation and evacuation may be required.

General hygiene considerations: Handle in accordance with good industrial hygiene and safety practice. Avoid contact with skin, eyes or clothing. Wear suitable gloves and eye/face protection. Do not eat, drink or smoke when using this product.

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical and Chemical Properties

Physical state: Foam; Liquid
Appearance: Clear
Odor: Mint-like
Color: No information available
Odor: Threshold: No data available

Property	Values	Remarks Method
pH	5.0	
Melting / freezing point	No data available	None known
Boiling point / boiling range	No data available	None known
Flash Point	No data available	None known
Evaporation Rate	No data available	None known
Flammability (solid, gas)	No data available	None known
Flammability Limit in Air	None known	
Upper flammability limit	No data available	
Lower flammability limit	No data available	
Vapor pressure	No data available	None known

Vapor density	No data available	None known
Relative density	1	
Water Solubility	Completely soluble	
Solubility(ies)	No data available	None known
Partition coefficient: n-octanol/waterNot Applicable		
Autoignition temperature	No data available	None known
Decomposition temperature	No data available	None known
Kinematic viscosity	No data available	None known
Dynamic viscosity	No data available	None known

Other Information

Explosive properties: No information available
Oxidizing properties: No information available
Softening Point: No information available
Molecular Weight: No information available
VOC Content (%): No information available
Liquid Density: No information available
Bulk Density: No information available
Particle Size: No information available
Particle Size Distribution: No information available.

10. STABILITY AND REACTIVITY

Reactivity: No information available.

Chemical stability: Stable under normal conditions.

Possibility of Hazardous Reactions: None under normal processing.

Hazardous Polymerization: Hazardous polymerization does not occur.

Conditions to avoid: None known based on information supplied.

Incompatible materials: Strong acids. Strong bases. Strong oxidizing agents.

Hazardous Decomposition Products Carbon oxides.

11. TOXICOLOGICAL INFORMATION

Information on likely routes of exposure

Product Information

Inhalation: Specific test data for the substance or mixture is not available. May cause irritation of respiratory tract.

Eye contact: Specific test data for the substance or mixture is not available. Causes serious eye damage. (based on components). Severely irritating to eyes. May cause burns. May cause irreversible damage to eyes.

Skin contact: Specific test data for the substance or mixture is not available. Causes skin irritation. (based on components).

Ingestion: Specific test data for the substance or mixture is not available. Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea.

Information on toxicological effects

Symptoms: Redness. Burning. May cause blindness. May cause redness and tearing of the eyes.

Numerical measures of toxicity

Acute Toxicity

The following values are calculated based on chapter 3.1 of the GHS document .

ATEmix (oral) 12,897.00 mg/kg
ATEmix (dermal) 52,805.00 mg/kg
ATEmix (inhalation-dust/mist) 39.60 mg/L
ATEmix (inhalation-vapor) 290.00 mg/L

Unknown acute toxicity

6% of the mixture consists of ingredient(s) of unknown toxicity
2% of the mixture consists of ingredient(s) of unknown acute oral toxicity
6% of the mixture consists of ingredient(s) of unknown acute dermal toxicity
6% of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (gas)
6% of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (vapor)
6% of the mixture consists of ingredient(s) of unknown acute inhalation toxicity (dust/mist)

Component Information

Chemical name	Oral LD50	Dermal LD50	Inhalation LC50
Hydrogen peroxide	= 1518 mg/kg (Rat)	= 2000 mg/kg (Rabbit) = 4060 mg/kg (Rat)	= 2 g/m3 (Rat) 4 h
Glycerin	= 12600 mg/kg (Rat)	> 10 g/kg (Rabbit)	> 570 mg/m3 (Rat) 1 h

Delayed and immediate effects as well as chronic effects from short and long-term exposure

Skin corrosion/irritation: Classification based on data available for ingredients. Irritating to skin.

Serious eye damage/eye irritation: Classification based on data available for ingredients. Causes burns. Risk of serious damage to eyes.

Respiratory or skin sensitization: No information available.

Germ cell mutagenicity: No information available.

Carcinogenicity: No information available.

The table below indicates whether each agency has listed any ingredient as a carcinogen.

Chemical name	ACGIH	IARC	NTP	OSHA
Hydrogen peroxide 7722-84-1	A3	Group 3	-	-

Legend

ACGIH (American Conference of Governmental Industrial Hygienists)
A3 - Animal Carcinogen
IARC (International Agency for Research on Cancer)
Group 3 - Not Classifiable as to Carcinogenicity in Humans

Reproductive toxicity: No information available.

STOT - single exposure: No information available.

STOT - repeated exposure: No information available.

Aspiration hazard: No information available.

12. ECOLOGICAL INFORMATION

Ecotoxicity: Harmful to aquatic life with long lasting effects.

Chemical name	Toxicity to Algae	Toxicity to Fish	Toxicity to Microorganisms	Daphnia Magna (Water Flea)
Hydrogen peroxide	72h EC50: = 2.5 mg/L (Chlorella vulgaris)	96h LC50: 10.0 - 32.0 mg/L (Oncorhynchus mykiss) 96h LC50: 18 - 56 mg/L (Lepomis macrochirus) 96h LC50: = 16.4 mg/L (Pimephales promelas)	-	24h EC50: = 7.7 mg/L 48h EC50: 18 - 32 mg/L
Glycerin	-	Group 3	-	24h EC50: > 500 mg/L

Persistence and Degradability: No information available.

Bioaccumulation

Chemical name	Log Pow
Glycerin	-1.76

Mobility: No information available.
Other adverse effects: No information available.

13. DISPOSAL CONSIDERATIONS

Waste treatment methods

Waste from residues/unused products: Dispose of in accordance with local regulations. Dispose of waste in accordance with environmental legislation.
Contaminated packaging: Do not reuse empty containers.
California Waste Codes: 331

This product contains one or more substances that are listed with the State of California as a hazardous waste

Chemical name	California Hazardous Waste
Hydrogen peroxide 7722-84-1	Toxic Corrosive Ignitable Reactive

14. TRANSPORT INFORMATION

DOT

Not regulated
Proper Shipping Name: NON-REGULATED
Hazard Class: N/A

TDG

Not regulated

MEX

Not regulated

ICAO

Not regulated

IATA

Not regulated
Proper Shipping Name: NON REGULATED
Hazard Class: N/A

IMDG/IMO

Not regulated
Hazard Class: N/A

RID

Not regulated

ADR

Not regulated

ADN

Not regulated

15. REGULATORY INFORMATION

Safety, health and environmental regulations/legislation specific for the substance or mixture

International Regulations

Ozone-depleting substances (ODS) Not applicable Persistent Organic Pollutants Not applicable Export Notification requirements Not applicable

International Inventories

TSCA: Contact supplier for inventory compliance status.
DSL/NDSL: Contact supplier for inventory compliance status.
EINECS/ELINCS: Contact supplier for inventory compliance status.
ENCS: Contact supplier for inventory compliance status.
KECL: Contact supplier for inventory compliance status.
PICCS: Contact supplier for inventory compliance status.
AICS: Contact supplier for inventory compliance status.

Legend

TSCA - United States Toxic Substances Control Act Section 8(b) Inventory
DSL/NDSL - Canadian Domestic Substances List/Non-Domestic Substances List
EINECS/ELINCS - European Inventory of Existing Chemical Substances/European List of Notified Chemical Substances
ENCS - Japan Existing and New Chemical Substances
KECL - Korean Existing and Evaluated Chemical Substances
PICCS - Philippines Inventory of Chemicals and Chemical Substances
AICS - Australian Inventory of Chemical Substances

US Federal Regulations

SARA 313

Section 313 of Title III of the Superfund Amendments and Reauthorization Act of 1986 (SARA). This product does not contain any chemicals which are subject to the reporting requirements of the Act and Title 40 of the Code of Federal Regulations, Part 372

Acute Health Hazard: Yes
Chronic Health Hazard: No
Fire Hazard: No
Sudden release of pressure hazard: No
Reactive Hazard: No

CWA (Clean Water Act)

This product contains the following substances which are regulated pollutants pursuant to the Clean Water Act (40 CFR 122.21 and 40 CFR 122.42)

Chemical name	CWA - Reportable Quantities	CWA - Toxic Pollutants	CWA - Priority Pollutants	CWA - Hazardous Substances
Hydrogen peroxide 7722-84-1	x	x	x	x

CERCLA

This material, as supplied, contains one or more substances regulated as a hazardous substance under the Comprehensive Environmental Response Compensation and Liability Act (CERCLA) (40 CFR 302)

Chemical name	Hazardous Substances RQs	Extremely Hazardous Substances RQs	RQ
Hydrogen peroxide 7722-84-1	x	1000 lb	x

US State Regulations

California Proposition 65
This product does not contain any Proposition 65 chemicals.

U.S. State Right-to-Know Regulations
This product may contain substances regulated by state right-to-know regulations.

Chemical name	New Jersey	Massachusett s	Pennsylvania	Rhode Island	Illinois
Hydrogen peroxide 7722-84-1	x	x	x	x	
Glycerin 56-81-5	x	x	x	x	

16. OTHER INFORMATION

NFPA
Health hazards 3 Flammability 0 Instability 0 Physical and Chemical Properties

HMIS
Health hazards 3 Flammability 0Physical hazards 0 Personal Protection X

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1-800-572-6501

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Revision Note
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DESPLAC

The Effect of a Nature-Based Gel on Gingival Inflammation and the Proteomic Profile of Crevicular Fluid: **A Randomized Clinical Trial**

DESPLAC

The Effect of a Nature-Based Gel on Gingival Inflammation and the Proteomic Profile of Crevicular Fluid: A Randomized Clinical Trial

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Abstract: Evidence has shown the clear positive effects of nature-based products on biofilm control and improved gingival health. However, most studies have used in vitro models, have tested single natural components, or have not evaluated proteomic changes after treatment. This double-blind, parallel, randomized, and controlled clinical trial evaluated the benefits of a nature-based gel in controlling gingival inflammation and its effects on the proteomic gingival crevicular fluid (GCF) profile. Gingivitis patients were distributed into the following groups: (1) nature-based gel containing propolis, aloe vera, green tea, cranberry, and calendula (n = 10); (2) control—conventional toothpaste (n = 10). GCF was collected and evaluated by means of liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). At 3 months, the groups showed similar clinical benefits ($p < 0.05$). A total of 480 proteins were identified across all groups. In a pooled comparison of both groups at both time points, exclusive proteins were identified in the nature-based gel (78) and the control (21) groups. The exclusive proteins identified for the toothpaste mainly acted in wound healing, and those for the nature-based gel mainly acted on immune system processes. The nature-based gel achieved similar clinical outcomes to conventional toothpaste. However, the nature-based gel markedly changed the proteomic profile of GCF after treatment, showing a profile associated with a host response.
Keywords: toothpaste; gingivitis; proteomic

1. Introduction

Polymicrobial dysbiotic oral biofilms are the main etiologic factor triggering inflamma- tory chronic diseases on tooth-supporting tissues, known as periodontal diseases [1]. These prevalent conditions affect millions of people worldwide, showing increasing rates with age and negatively impacting people's lives [2]. The biofilm's biomass and its microbial composition act as chemical and physical "stress" factors on tooth-supporting tissues, triggering inflammatory responses and subsequent tissue damage [3]. This initial process is characterized by clinical gingival inflammation, known as gingivitis, which often pro- gresses in severity and tissue damage [4]. Since it is a multifactorial condition, different risk, genetic, and systemic factors, as well as the intensity and duration of microbial challenges and host susceptibility, may promote the destruction of periodontal supporting tissue [5]. Oral hygiene procedures, such as toothbrushing and other mechanical cleansing methods, are effective in controlling biofilm accumulation and achieving gingival health [6]. Differ- ent antimicrobial agents and adjunctive therapies have been tested to control oral biofilm accumulation and reduce gingival inflammation [7], and the composition of toothpaste also plays an important role in achieving these outcomes [6].

Previous evidence suggests that specific chemical agents in toothpaste may present enhanced benefits in controlling gingival inflammation [6]. In fact, toothpaste composition may also affect local molecular parameters, such as microbial pathways at the metatran- scriptomic level and the levels of pro-inflammatory components in gingival crevicular fluid (GCF) [8]. These new molecular and omics approaches provide new insights into the modulation of the abilities of new products and explain the effects of different agents on oral sites. However, although the proteomic profile is responsible for mediating molecular functions and biological outcomes, it has not been tested in new toothpastes, especially at the GCF level [9]. An understanding of the proteins involved in health and disease states provides significant potential in identifying new biomarkers, given the close relationship between molecular activity and biological/clinical outcomes [10].

In this sense, changes in toothpaste formulation should consider not only the clin- ical evaluation of gingival

conditions and biofilm control but also the local molecular parameters that drive one toward a health-associated state. Incorporating new agents to enhance oral biofilm control and address its pathogenicity is a logical development in improving clinical outcomes [11]. Although traditional components have been used in most commercially available toothpastes, new formulations have been explored as agents, such as nature-based compounds. Thus, over the past decade, there has been increasing interest in nature-based products for oral health [12]. In the United States of America (USA), a study identified that 35% of the investigated population reported using some form of herbal medicine [13]. Furthermore, a high proportion of new drugs approved by the Food and Drug Administration (FDA) are natural or naturally derived products [14]. These products have shown high antimicrobial abilities, combined with additional benefits, such as anti-inflammatory and antioxidant properties [12]. Evidence shows the clear positive effects of nature-based products on biofilm control and improved gingival health, but most of this evidence comes from in vitro models [12]. Moreover, most studies have only tested products with single natural components [12], and combinations in a single product have not been widely explored, especially in terms of proteomic changes after treatments.

Therefore, this clinical trial aims to evaluate and compare the clinical benefits of a nature-based gel in controlling gingivitis and its effects on the proteomic GCF profile compared with a conventional toothpaste.

2. Results and Discussion

2.1. Clinical Outcomes

Natural products have shown promising antimicrobial effects against periopathogens, but most evidence has emerged from in vitro models [15]. These outcomes demonstrate the anti-inflammatory and antioxidant abilities of these agents, making them effective com- pounds in the manufacturing of nature-based products to control gingival inflammation. It is important to note that most of the clinical evidence has come from tests of nature-based products for oral biofilm control using mouthrinses as the vehicle [12]. This clinical study included 20 subjects (DESPLAC® group: 8 women and 2 men, 30–47 years of age; and Oral-B group: 7 women and 3 men, 31–48 years of age). There were no dropouts during the experimental period. All subjects who finished the study reported full adherence to the prescribed oral hygiene protocol. Throughout the study, no adverse effects on the soft or hard tissues of the oral cavity were detected by the examiner or reported by the subjects. Table 1 presents the mean (± SD) of full-mouth clinical parameters evaluated at baseline and 3 months after using the products. At the beginning, there were no clinical differences between the groups for any of the evaluated parameters ($p > 0.05$), demonstrating the ho- mogeneity of the sample groups. At 3 months, no statistically significant differences were observed between the two groups ($p > 0.05$); however, a reduction in the gingival index was noted in both the nature-based gel and control toothpaste groups. Moreover, when evaluating the proportion of each score on the plaque index, there was a clear reduction trend after 3 months, with an approximately 20% increase in the number of sites/teeth categorized with a score of 0 on the plaque index (Table S1). Bleeding on probing, expressed as the percentage of sites bleeding, also did not show any differences between the groups or time points (Table S2). Therefore, the clinical findings showed similar outcomes for both tested products (nature-based gel and control toothpaste) after 3 months, with both being effective in reducing gingival inflammation.

Table 1. Full-mouth clinical parameter evaluation of the study population at baseline and after 3 months. Average (± SD).

		Groups (Average and SD)	
Parameters	Time Point	DESPLAC® (n = 10)	ORALB (n = 10)
Plaque index	Baseline	0.78 ± 0.59 a	0.82 ± 0.61 a
	3 months	0.82 ± 0.6 a	0.86 ± 0.72 a
Gingival index	Baseline	1.52 + 0.57 aA	1.50 + 0.57 aA
	3 months	0.81 ± 0.58 aB	0.73 ± 0.53 aB
Probing depth (mm)	Baseline	2.34 ± 0.62 a	2.33 ± 0.60 a
	3 months	2.33 ± 0.60 a	2.28 ± 0.67 a
Clinical attachment level (mm)	Baseline	2.41 ± 0.65 a	2.39 ± 0.67 a
	3 months	2.39 ± 0.67 a	2.37 ± 0.68 a
Bleeding on probing	Baseline	0.28 ± 0.45 a	0.34 ± 0.47 a
	3 months	0.34 ± 0.47 a	0.20 ± 0.40 a

Plaque index and gingival index are expressed as averages of scores. Bleeding on probing is expressed as the average number of affected sites. The significance of differences in clinical parameters between groups at baseline and 3 months were compared using the t-test ($p > 0.05$—the same lowercase letters in the lines indicate no statistical differences). Different capital letters in the columns indicate significant differences over time based on repeated measures ANOVA (Tukey's test, $p < 0.05$). SD—standard deviation.

DESPLAC® gel (Premium Oral Gel) includes a combination of different natural agents in its formula: propolis, aloe vera, green tea, cranberry, and calendula. Although the individual antimicrobial, anti-inflammatory, and/or antioxidant benefits of these natural agents are well known, their combination in a single product has not been widely explored [16–19]. Previous in vitro studies have explored the antimicrobial ability of DESPLAC® in a multispecies biofilm model [16–19]. The results demonstrated its potential to reduce bacterial metabolic activity and the levels of two important periopathogens: Tannerella forsythia and Porphyromonas gingivalis [20,21]. In our clinical setting with gingivitis patients, the nature-based gel was clinically effective, controlling gingival inflammation over 3 months of use. However, the clinical outcomes of the two tested products were similar, with the nature-based gel showing no additional benefit compared with conventional toothpaste. In fact, a systematic review demonstrated that mechanically removing biofilm through toothbrushing is more important than the presence of toothpaste [22]. However, toothpaste may contain other important chemical agents crucial for oral health, such as fluoride, which is well known for its ability to control dental caries but is absent in the DESPLAC® product [23]. Importantly, clinical trials like the one conducted here, comparing different gel/toothpaste formulations for oral biofilm and gingival inflammation control, may be helpful in further developing clinical guidelines, particularly regarding the use of nature-based products for periodontal health and respecting patients' preferences and characteristics [12]. Moreover, although a 3-month follow-up period is commonly used in clinical trials to identify significant changes in gingival health recovery post-treatment, aligning with the gingival healing timeline and the recommended interval for maintenance visits, longer periods should be considered in future studies to evaluate the maintenance of outcomes [24,25]. Furthermore, the reduction in gingival inflammation was not accompanied by a reduced plaque index after 3 months. However, the plaque index was expressed as an average of scores, and when evaluating the proportion of each score, there was a clear improvement in plaque control, with an increased proportion of sites scoring 0 after 3 months (Supplementary Materials). The lack of differences in the average values for this outcome may be attributable to the sample size, necessitating further investigation.

2.2. Composition of Toothpaste Modulated the Proteomic Profile in GFC

Our pioneering study explored the effect of a nature-based product on the GCF proteomic profile of gingivitis patients. A total of 480 proteins were identified across all groups at both time points. The conventional toothpaste group presented 143 proteins at baseline and 141 after 3 months (Figure 1A). For the nature-based gel group, 352 proteins were identified at baseline and 206 after 3 months (Figure 1A). Although the number of proteins somewhat decreased after 3 months, their intensities were similar for both groups, with no significant differences over time (Figure 1B). Importantly, both groups showed notable differences in the proteomic profile of GCF after 3 months compared with baseline (Figure 1C). Exclusive proteins were identified in the conventional toothpaste group (99 proteins) and the nature-based gel group (101 proteins) after 3 months within the same-group comparison. When comparing the two groups at each time point, our findings showed a lower level of similarity, with approximately 36% of proteins being shared at baseline and only 15.6% after 3 months (Figure 1C). In the pooled comparison of the groups at each time point, exclusive proteins were identified in both, indicating differences in proteomic profile according to time and treatment. These results demonstrate important differences in the proteomic profile of GCF during gingivitis treatment/control, which were also modulated by the toothpaste/gel applied. Despite differences in proteomic composition and the presence of exclusive proteins in each group, the heatmap shows that even among shared proteins, some differences in intensities were identified between both groups (Figure 2).

Periodontal disease progression markedly changes the proteomic profile of GCF [26]. Moreover, changes in GCF protein levels caused by inflammation and disease resolution have been reported [27]. However, an important level of overlap in proteomic profile between healthy individuals and those with periodontal disease has been found [28], as reported in our study comparing scores at baseline and after 3 months. Our findings show that the nature-based gel increased the number of proteins identified in GCF, and compared with conventional toothpaste, it showed only 15.6% similarity after 3 months. Importantly, the pooled comparison (including both groups and time points) revealed that specific proteins could be uniquely identified for each group: 78 proteins were exclusive to the nature-based gel group, while 21 proteins were unique to the conventional toothpaste group at 3 months. This shows that the composition of the products highly affected the proteomic composition of GCF after 3 months of use. Notably, GCF is a complex mixture with various components, and its composition is affected by factors such as aging and biofilm composition [29]. Although all patients were standardized in terms of clinical parameters, molecular factors may have contributed to increased protein content in the nature-based gel group at baseline. However, after 3 months, the groups showed close protein quantities, with differences primarily driven by composition, which should be further investigated at the screening stage. Moreover, there was a clear change in the proteomic profile within the nature-based gel group when comparing the baseline and the 3-month follow-up, suggesting that the identified proteomes are a result of the treatment rather than the initial composition.

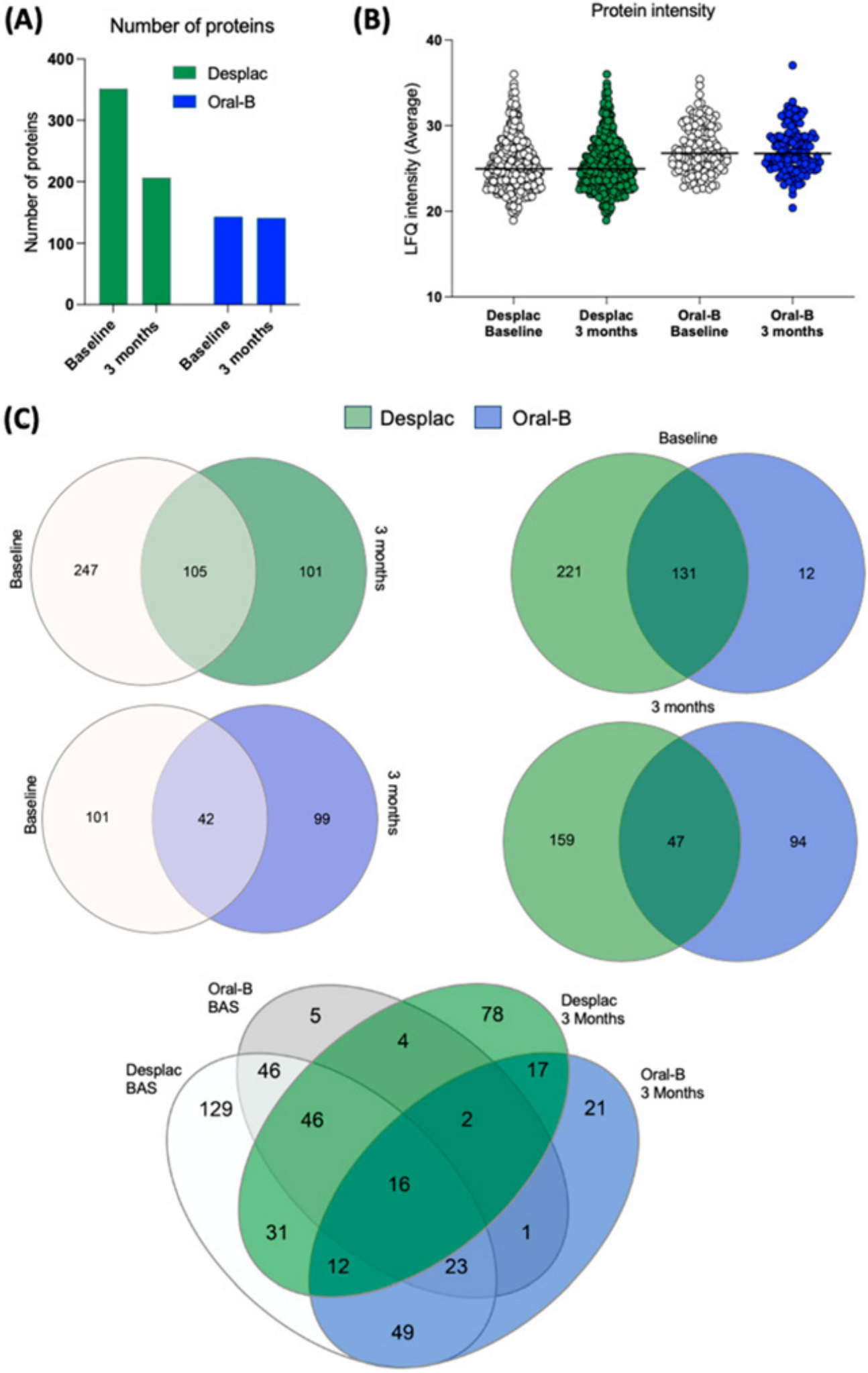
(A)
Number of proteins
Number of proteins
400
300
200
100
0
Desplac
Oral-B
Baseline
3 months
Baseline
3 months
(B)
Protein intensity
LFQ intensity (Average)
40
30
20
10
Desplac Baseline
Desplac 3 months
Oral-B Baseline
Oral-B 3 months
(C)
Desplac
Oral-B
Baseline
3 months
247
105
101
Baseline
3 months
221
131
12
Baseline
3 months
101
42
99
3 months
159
47
94
Oral-B BAS
Desplac BAS
Desplac 3 Months
Oral-B 3 Months
5
78
4
46
17
129
46
2
21
16
31
1
12
23
49

Figure 1. Proteomic profile of gingival crevicular fluid of patients treated with nature-based gel (DESPLAC®) or conventional toothpaste (Oral-B). (A) Total proteins identified for each group and time point (baseline and after 3 months). Proteomic profile was evaluated using liquid chromatogra- phy coupled with tandem mass spectrometry. (B) Average LFQ intensity of proteins identified for each group and time point. (C) Venn diagrams comparing the groups and time points.

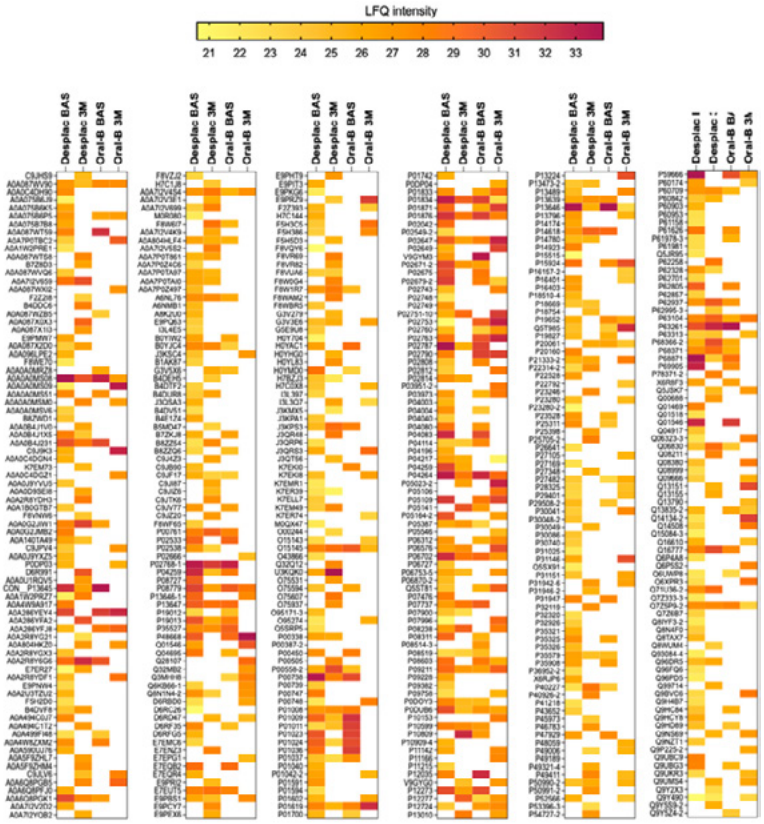

Figure 2. Proteomic profile of gingival crevicular fluid of patients treated with nature-based gel (DESPLAC®) or conventional toothpaste (Oral-B) at different time points (BAS—baseline; or 3M—3 months). Heatmap of LFQ intensity of proteins identified for each group and time point.

The exclusive proteins identified for each group in the pooled comparison (all groups and time points—Figure 1C, bottom diagram) are described in Table 2. After 3 months, the exclusive proteins identified for the conventional toothpaste mainly acted in wound healing, the most common biological process identified. In contrast, for the nature-based gel group, the exclusive proteins identified after 3 months primarily mediated immune system processes (Table 2). Therefore, considering that each protein has a specific molec- ular function and mediates specific biological processes, the profile for all proteins (not only exclusive ones) identified in each group and at each time point was also explored (Figure 3A,B). Although there is a high level of similarity between the groups at both time points in terms of the top 10 most common molecular functions and biological processes related to the identified proteins, some slight differences were observed. The inflammatory response process was among the top three biological processes for the nature-based gel group after 3 months but not for the conventional toothpaste group. Furthermore, cell dif- ferentiation was a common biological process identified for the nature-based gel but not for the conventional toothpaste. However, wound healing was only found among the common biological processes for the conventional toothpaste (Figure 3B). Therefore, the differences in proteomic profile led to slight differences in the main molecular functions and biological processes mediated by the identified proteins. The immune response process plays a key role in restoring homeostasis in injured tissues [30]. Therefore, the nature-based product and its composition may enhance the host response, and some of its components—such as propolis, with its well-recognized anti-inflammatory properties—may also contribute to faster inflammation resolution [30,31]. Although immune-response-related proteins were highly associated with the nature-based gel, clinical parameters, as measured by the gingival index, suggest the resolution of the inflammatory process, similar to what was observed with the conventional toothpaste, indicating no delay in the healing process for this group. Notably, the same protein can exhibit a wide range of molecular functions and be involved in different biological processes. While proteins may have primary functions, they can play roles in various processes. Moreover, the presence of a specific protein does not necessarily indicate a high concentration; some components of GCF may show similar levels immediately after therapy compared with the baseline but then exhibit reduced levels during the maintenance phase [32].

Table 2. Protein ID, name, molecular function, and biological process of the exclusive proteins in each group and at each time when comparing the four groups' proteomic profiles (data extracted from the UniProt database).

Protein ID	Name	Molecular Function	Biological Process
Oral-B—Baseline			
A0A087WZB5	Parvin beta	cytoskeletal protein binding	cytoskeleton organization
A0A0A0MRZ8	Immunoglobulin kappa variable 3D-11		immune system process
K7EKI0	Envoplakin	structural molecule activity	cytoskeleton organization
P08514-3	Integrin alpha-IIb	molecular adaptor activity	immune system process
Q9Y5Z4-2	Heme-binding protein 2		
Oral-B—3 months			
A0A087WXI2	Fc gamma binding protein		
A0A0C4DGZ1			
P02666	Beta-casein	molecular function regulator activity	inflammatory response
Q28107	Coagulation factor V	molecular adaptor activity	circulatory system process
Q32MB2	Keratin, type II cytoskeletal 73	structural molecule activity	cytoskeleton organization
E7EPG1	Multimerin 1		
K7ER74	Apolipoprotein C-II	molecular function regulator activity	lipid metabolic process
P0DP04	Immunoglobulin heavy variable 3-43D		immune system process
P03951-2	Coagulation factor XI	catalytic activity, acting on a protein	wound healing
P05106	Integrin beta-3	catalytic activity, acting on a protein	wound healing
P05546	Heparin cofactor 2	molecular function regulator activity	wound healing
P10153	Non-secretory ribonuclease	catalytic activity, acting on rna	defense response to other organism
P11166	Solute carrier family 2, facilitated glucose transporter member 1	transporter activity	transmembrane transport
P13224	Platelet glycoprotein Ib beta chain	molecular transducer activity	wound healing
P16157-2	Ankyrin-1	structural molecule activity	vesicle-mediated transport
P22792	Carboxypeptidase N subunit 2	catalytic activity	
P27105	Stomatin	molecular function regulator activity	transmembrane transport
P48059	LIM and senescent cell antigen-like-containing domain protein 1	gtpase activity	cell adhesion
Q13790	Apolipoprotein F	transporter activity	lipid metabolic process
Q6P5S2	Protein LEG1 homolog		
Q9P225-2	Dynein axonemal heavy chain 2	cytoskeletal motor activity	cell motility
Desplac—Baseline			
A0A0C4DH90	Immunoglobulin kappa variable 3/OR2-268 (non-functional)		immune system process
A0A075B6J9	Immunoglobulin lambda variable 2-18		immune system process
A0A075B6K5	Immunoglobulin lambda variable 3-9		immune system process
A0A075B7B8	Immunoglobulin heavy variable 3/OR16-12 (non-functional)		immune system process
A0A1W2PRE1	Guanine nucleotide-binding protein G(o) subunit alpha	gtpase activity	signaling
A0A087WVQ6	Clathrin heavy chain	structural molecule activity	intracellular protein transport
E9PMW7	Eukaryotic translation elongation factor 1 delta		
A0A096LPE2	SAA2-SAA4 readthrough		inflammatory response
F8WE70	Serpin family B member 13	hydrolase activity	
A0A0A0MSV6	Complement C1q B chain		
B8ZWD1	Acyl-CoA-binding protein	lipid binding	lipid metabolic process
A0A0C4DGN4	Zymogen granule protein 16B		
A0A0J9YVU5	Immunoglobulin heavy variable 2–70		immune system process
A0A1B0GTB7	Spectrin alpha, non-erythrocytic 1		
A0A0G2JMB2	Immunoglobulin heavy constant alpha 2		immune system process
A0A0J9YXZ5	IQ motif containing GTPase activating protein	molecular function regulator activity	cytoskeleton organization
A0A4W9A917	Immunoglobulin heavy constant gamma 3		immune system process
A0A2R8YGX3	Tropomyosin 4	cytoskeletal protein binding	cytoskeleton organization
E9PNW4	CD59 glycoprotein		immune system process
A0A2U3TZU2	Glucose-6-phosphate isomerase	isomerase activity	nucleobase-containing small molecule metabolic process
F5H2D0	complement subcomponent C1r	hydrolase activity	immune system process
A0A494C0J7	Transglutaminase-like domain-containing protein	transferase activity	
A0A590UJ76	Deleted in malignant brain tumors 1 protein		cell differentiation

Protein ID	Name	Molecular Function	Biological Process
A0A6Q8PFJ0	Lamin A/C	structural molecule activity	cytoskeleton organization
A0A7I2V2D2	Serpin family G member 1	molecular function regulator activity	
F8VZJ2	Nascent polypeptide associated complex subunit alpha		
M0R080	DnaJ homolog subfamily B member 1	molecular adaptor activity	regulation of dna-templated transcription
A0A7P0Z4C6	Transitional endoplasmic reticulum ATPase		
A0A7P0Z497	Peptidyl-prolyl cis-trans isomerase	catalytic activity, acting on a protein	protein folding
A6NMB1	Sialic acid-binding Ig-like lectin 16		defense response to other organism
A8K2U0	Alpha-2-macroglobulin-like protein 1	molecular function regulator activity	
E9PQ63	Carbonyl reductase 1	oxidoreductase activity	
I3L4E5	Aldehyde dehydrogenase, dimeric NADP-preferring	oxidoreductase activity	
B1AK87	F-actin-capping protein subunit beta	cytoskeletal protein binding	cytoskeleton organization
B4DTF2	Annexin	lipid binding	vesicle-mediated transport
J3QSA3	Ubiquitin B	protein tag activity	protein catabolic process
B4DV51	GTP-binding nuclear protein Ran	gtpase activity	nucleocytoplasmic transport
B4E1Z4	Complement C2	catalytic activity, acting on a protein	immune system process
C9JB90	RAB6B, member RAS oncogene family	gtpase activity	vesicle-mediated transport
F8WF65	Elongation factor 1-beta		
CON__P08727	Keratin, type I cytoskeletal 19	structural molecule activity	anatomical structure development
D6RC26	Mitogen-activated protein kinase 10		
D6RFG5	Annexin	lipid binding	
E7EMC6	Annexin	lipid binding	anatomical structure development
E7EQR4			
E9PIT3	Prothrombin	hydrolase activity	wound healing
E9PKG6	Nucleobindin 2		
H7C144	Alpha-actinin-4	cytoskeletal protein binding	cytoskeleton organization
F8VQY6	Large ribosomal subunit protein uL10	rna binding	cytoplasmic translation
F8WBR5	Calmodulin 2		muscle system process
G5E9U8	Poly (ADP-ribose) polymerase family, member 9, isoform CRA_b	transferase activity	immune system process
H0Y704	Zinc finger protein 185 with LIM domain		
I3L397	Eukaryotic translation initiation factor 5A	translation regulator activity	
J3KPA1	Cysteine rich secretory protein 3		
J3QRP6	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	molecular adaptor activity	protein localization to plasma membrane
K7EMR1	Granulin precursor		inflammatory response
K7ELL7	Glucosidase 2 subunit beta	rna binding	anatomical structure development
M0QX47	Glia maturation factor gamma		cytoskeleton organization
O43866	CD5 antigen-like	hydrolase activity	immune system process
O95171-3	Sciellin		anatomical structure development
Q5SRP5	Apolipoprotein M	transporter activity	protein-containing complex assembly
P00739	Haptoglobin-related protein	catalytic activity, acting on a protein	
P00747	Plasminogen	catalytic activity, acting on a protein	wound healing
P01040	Cystatin-A	catalytic activity, acting on a protein	cell adhesion
P01591	Immunoglobulin J chain	molecular adaptor activity	immune system process
P01594	Immunoglobulin kappa variable 1–33		immune system process
P01742	Immunoglobulin heavy variable 1–69		immune system process
P02042	Hemoglobin subunit delta	oxidoreductase activity	
P02549-2	Spectrin alpha chain, erythrocytic 1	structural molecule activity	cytoskeleton organization
P02748	Complement component C9		immune system process
P02749	Beta-2-glycoprotein 1	lipid binding	lipid metabolic process
P04003	C4b-binding protein alpha chain	rna binding	immune system process
P04040	Catalase	antioxidant activity	programmed cell death
P04196	Histidine-rich glycoprotein	molecular function regulator activity	wound healing
P06727	Apolipoprotein A-IV	lipid binding	lipid metabolic process
P06870-2	Kallikrein-1	catalytic activity, acting on a protein	circulatory system process
P07476	Involucrin		cell differentiation
P09228	Cystatin-SA	hydrolase activity	nervous system process
P09382	Galectin-1	rna binding	immune system process

Protein ID	Name	Molecular Function	Biological Process
P09382	Galectin-1	rna binding	immune system process
P10909-4	Clusterin	cytoskeletal protein binding	immune system process
P11215	Integrin alpha-M	isomerase activity	immune system process
P14174	Macrophage migration inhibitory factor	isomerase activity	immune system process
P14923	Junction plakoglobin	molecular adaptor activity	anatomical structure development
P15515	Histatin-1		anatomical structure development
P16403	Histone H1.2	dna binding	dna recombination
P18510-4	Interleukin-1 receptor antagonist protein	molecular transducer activity	inflammatory response
P20160	Azurocidin	catalytic activity, acting on a protein	immune system process
P22528	Cornifin-B	structural molecule activity	anatomical structure development
P23280-2	Carbonic anhydrase 6	lyase activity	nervous system process
P26641	Elongation factor 1-gamma	translation regulator activity	
P27169	Serum paraoxonase/arylesterase 1	hydrolase activity	lipid metabolic process
P30048-2	Thioredoxin-dependent peroxide reductase, mitochondrial	oxidoreductase activity	programmed cell death
P30086	Phosphatidylethanolamine-binding protein 1	molecular function regulator activity	signaling
P30740	Leukocyte elastase inhibitor	hydrolase activity	
P31025	Lipocalin-1	hydrolase activity	nervous system process
Q5SX91	Rab GDP dissociation inhibitor	gtpase activity	signaling
P32320	Cytidine deaminase	hydrolase activity	nucleobase-containing small molecule metabolic process
P32926	Desmoglein-3		cell adhesion
P35321	Cornifin-A	structural molecule activity	anatomical structure development
P35326	Small proline-rich protein 2A	lipid binding	immune system process
P36952-2	Serpin B5	molecular function regulator activity	anatomical structure development
X6RJP6	Transgelin 2	cytoskeletal protein binding	cytoskeleton organization
P41218	Myeloid cell nuclear differentiation antigen	dna binding	immune system process
P43652	Afamin		
P60903	Protein S100-A10	gtpase activity	anatomical structure development
P60953	Cell division control protein 42 homolog	gtpase activity	anatomical structure development
P61158	Actin-related protein 3	structural molecule activity	defense response to other organism
P61981	14-3-3 protein gamma	transferase activity	signaling
Q5JR95	Small ribosomal subunit protein eS8	structural molecule activity	ribosome biogenesis
P62701	Small ribosomal subunit protein eS4, X isoform	rna binding	cytoplasmic translation
P62857	Small ribosomal subunit protein eS28	structural molecule activity	ribosome biogenesis
Q01469	Fatty acid-binding protein 5	lipid binding	signaling
Q01518	Adenylyl cyclase-associated protein 1	cytoskeletal protein binding	cytoskeleton organization
Q04917	14-3-3 protein eta	transporter activity	signaling
Q08999	Retinoblastoma-like protein 2	transferase activity	regulation of dna-templated transcription
Q15084-3	Protein disulfide-isomerase A6	catalytic activity, acting on a protein	protein folding
Q6P4A8	Phospholipase B-like 1	hydrolase activity	lipid metabolic process
Q7Z333-3	Probable helicase senataxin	dna binding	regulation of dna-templated transcription
Q7Z6B7	SLIT-ROBO Rho GTPase-activating protein 1	gtpase activity	signaling
Q8IYF3-2	Testis-expressed protein 11		programmed cell death
Q8N4F0	BPI fold-containing family B member 2	lipid binding	
Q8TAX7	Mucin-7	immune system process	
Q93084-4	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	molecular function regulator activity	transmembrane transport
Q96FQ6	Protein S100-A16	rna binding	
Q96PD5	N-acetylmuramoyl-L-alanine amidase	hydrolase activity	immune system process
Q9H4B7	Tubulin beta-1 chain	gtpase activity	cytoskeleton organization
Q9HCY8	Protein S100-A14		immune system process
Q9NZT1	Calmodulin-like protein 5	catalytic activity	anatomical structure development
Q9UBC9	Small proline-rich protein 3	structural molecule activity	anatomical structure development

Protein ID	Name	Molecular Function	Biological Process
Desplac—3 months			
C9JHS9	High density lipoprotein binding protein		
A0A087WTS8			
B7Z8D3	Proteasome activator subunit 3	molecular function regulator activity	protein catabolic process
F2Z2I8	Stomatin like 2	mitochondrion organization	
B4DDC6	Prostaglandin E synthase 3	isomerase activity	lipid metabolic process
A0A087X0X3			
A0A087X1I3			
A0A0B4J1V0	Immunoglobulin heavy variable 3–15		immune system process
K7EM73	Calpain small subunit 1		
A0A0D9SEI8	Y-box binding protein 3		
A0A2R8YDH3	RNA helicase	hydrolase activity	reproductive process
F8VNW6	Prefoldin subunit 5		
D6R991	Matrin 3		
A0A0U1RQV5	Eukaryotic translation initiation factor 6	translation regulator activity	ribosome biogenesis
A0A286YFA2	Phosphoglycerate dehydrogenase	oxidoreductase activity	amino acid metabolic process
A0A2R8YG21	Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha	catalytic activity	lipid metabolic process
A0A494C1T2	C-1-tetrahydrofolate synthase, cytoplasmic	oxidoreductase activity	cellular modified amino acid metabolic process
A0A5F9ZHL7	Acetyl-CoA acetyltransferase 1	transferase activity	lipid metabolic process
A0A5F9ZHM4	L-lactate dehydrogenase	oxidoreductase activity	
A0A7I2YQB2	Stress-70 protein, mitochondrial	Protein folding chaperone	
H7C1J8	Heterogeneous nuclear ribonucleoprotein A3	rna binding	mrna metabolic process
A0A7I2V3E1	Poly [ADP-ribose] polymerase	transferase activity	immune system process
A0A7I2V4K9	Non-POU domain containing octamer-binding	rna binding	regulation of dna-templated transcription
A0A7I2V5S2	Nucleophosmin	rna binding	ribosome biogenesis
B4DEH5	Leukotriene A4 hydrolase	hydrolase activity	
B5MD47	Septin 2	gtpase activity	vesicle-mediated transport
C9J4Z3	Ribosomal protein L37a	structural molecule activity	
C9JI87	Voltage-dependent anion-selective channel protein 1	transporter activity	transmembrane transport
C9JIZ6			
C9JTK6	Obg like ATPase 1	hydrolase activity	
C9JZ20	Prohibitin		mitochondrion organization
E7ENZ3	Chaperonin containing TCP1 subunit 5	protein folding chaperone	
E9PRI2	Eukaryotic translation initiation factor 3 subunit M		
E9PCY7	Heterogeneous nuclear ribonucleoprotein H1	rna binding	
E9PEX6	Dihydrolipoyl dehydrogenase	oxidoreductase activity	
E9PHT9	Annexin	lipid binding	
F5H5D3	Tubulin alpha chain	structural molecule activity	cytoskeleton organization
F8VR69	Ribosomal protein L6	structural molecule activity	cytoplasmic translation
F8VR82	Serine/threonine-protein phosphatase	hydrolase activity	generation of precursor metabolites and energy
F8W0G4	Poly(rC) binding protein 2	dna binding	
F8WAM2	T-complex protein 1 subunit eta	hydrolase activity	
G3V279	Enhancer of rudimentary homolog		
H0YHG0	DnaJ homolog subfamily C member 14		
H0YL83	Electron transfer flavoprotein subunit alpha		lipid metabolic process
J3KMX5	Small ribosomal subunit protein uS15	Small ribosomal subunit protein uS15	
J3QR48	Importin subunit beta-1	molecular carrier activity	nucleocytoplasmic transport
J3QT56	Huntingtin interacting protein K		programmed cell death
O00244	Copper transport protein ATOX1	molecular carrier activity	
Q32Q12	Nucleoside diphosphate kinase	transferase activity	nucleobase-containing small molecule metabolic process
O75531	Barrier-to-autointegration factor	dna binding	chromatin organization
O75607	Nucleoplasmin-3	histone binding	chromatin organization
O75937	DnaJ homolog subfamily C member 8		

Protein ID	Name	Molecular Function	Biological Process
P00505	Aspartate aminotransferase, mitochondrial	transferase activity	amino acid metabolic process
P18754	Regulator of chromosome condensation	molecular function regulator activity	mitotic cell cycle
P23246	Splicing factor, proline- and glutamine-rich	dna binding	regulation of dna-templated transcription
P25398	Small ribosomal subunit protein eS12	structural molecule activity	cytoplasmic translation
P25705-2	ATP synthase subunit alpha, mitochondrial	atp-dependent activity	generation of precursor metabolites and energy
P27348	14-3-3 protein theta		signaling
P30049	ATP synthase subunit delta, mitochondrial	transporter activity	generation of precursor metabolites and energy
P31946-2	14-3-3 protein beta/alpha	molecular function regulator activity	signaling
P32119	Peroxiredoxin-2	antioxidant activity	immune system process
P40926-2	Malate dehydrogenase, mitochondrial	oxidoreductase activity	oxidoreductase activity
P45973	Chromobox protein homolog 5	histone binding	regulation of dna-templated transcription
P46783	Small ribosomal subunit protein eS10	structural molecule activity	cytoplasmic translation
P49321-4	Nuclear autoantigenic sperm protein	histone binding	chromatin organization
P50991-2	T-complex protein 1 subunit delta	protein folding chaperone	protein folding
P53396-3	ATP-citrate synthase	transferase activity	lipid metabolic process
P54727-2	UV excision repair protein RAD23 homolog B	dna binding	protein catabolic process
P62258	14-3-3 protein epsilon	transporter activity	signaling
P62995-3	Transformer-2 protein homolog beta	rna binding	mrna metabolic process
P78371-2	T-complex protein 1 subunit beta	protein folding chaperone	protein folding
Q00688	Peptidyl-prolyl cis-trans isomerase FKBP3	rna binding	
Q08211	ATP-dependent RNA helicase A	atp-dependent activity	regulation of dna-templated transcription
Q13155	Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	molecular adaptor activity	programmed cell death
Q8WUM4	Programmed cell death 6-interacting protein		programmed cell death
Q99714	3-hydroxyacyl-CoA dehydrogenase type-2	oxidoreductase activity	lipid metabolic process
Q9Y2X3	Nucleolar protein 58	rna binding	ribosome biogenesis
Q9Y5S9-2	RNA-binding protein 8A	rna binding	mrna metabolic process

The exclusive proteins identified in each group also need to be explored. For example, the conventional toothpaste group showed the exclusive protein integrin beta-3 at 3 months, which is part of a protein group associated with stimulating growth factors, forming granulation tissue, and modulating inflammatory processes [33]. The nature-based gel group also presented an important exclusive proteomic profile at 3 months, including the presence of Peroxiredoxin-2, which plays a role in immune system processes and has well-recognized antioxidant abilities [34]. These findings may provide important information on clinical changes at the molecular level and reveal new biomarkers for testing the effectiveness of therapeutic products or disease resolution.

Although these findings are promising in demonstrating the effectiveness of a nature- based product in controlling biofilm accumulation and gingival inflammation—and al- though 3 months of evaluation is commonly used for this type of trial [12]—further clinical trials should evaluate the maintenance of outcomes over longer periods. Moreover, the use of high-throughput techniques focusing on the oral microbiome should also be fur- ther explored and aligned with evaluating immune response pathways. Furthermore, the sample size used here may have reduced the chance of identifying differences between the groups, and further trials should consider larger samples. Clinical trials testing these new nature-based products are essential to elucidating the clinical efficacy of these formulations. However, patient profiles, the occurrence of other oral diseases, adverse effects, and the therapeutic protocols applied need to be considered before recommending these products. Moreover, patients need to be well informed about oral hygiene procedures and the importance of mechanical biofilm control through toothbrushing, regardless of toothpaste formulation.

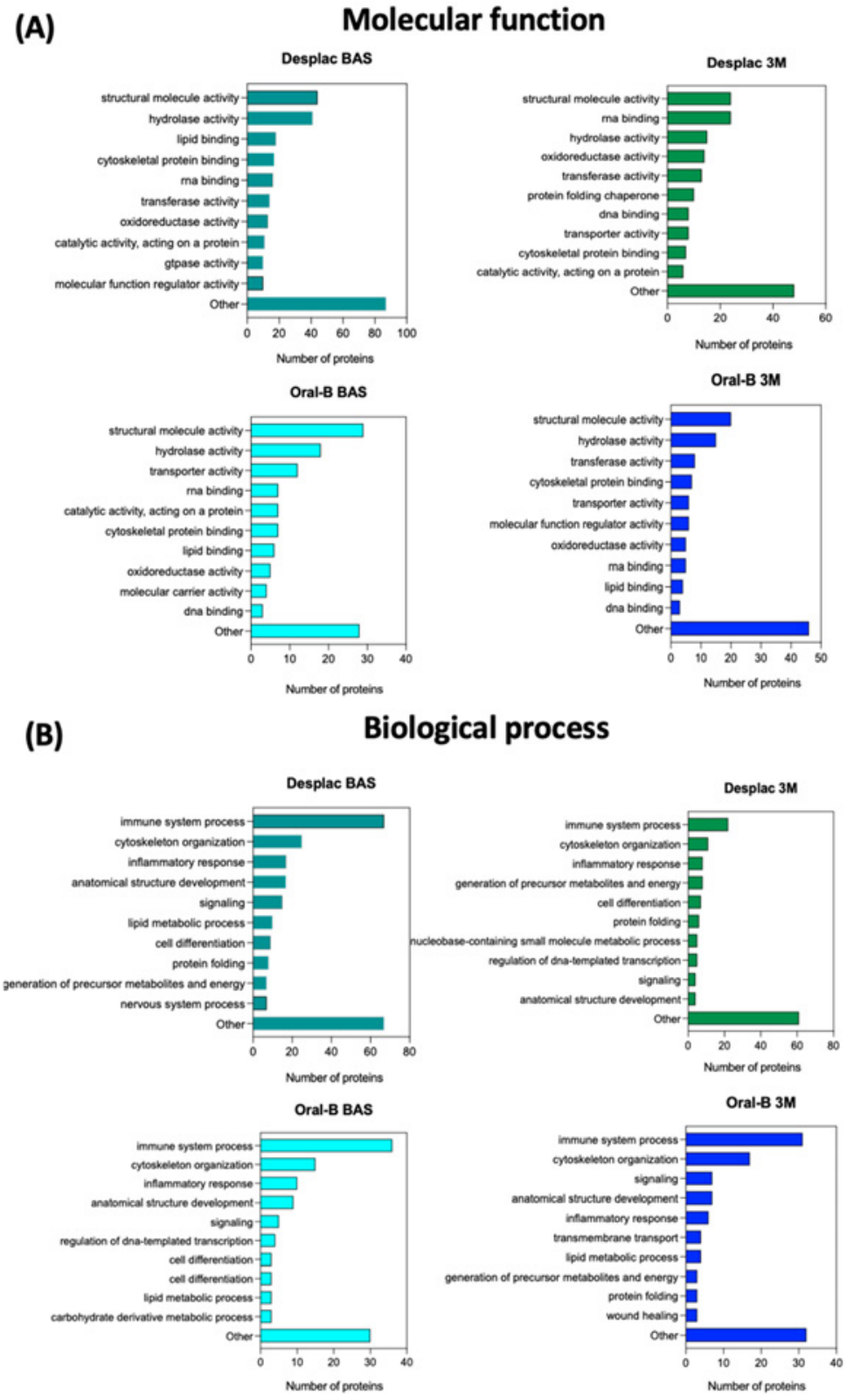

Figure 3. Molecular functions and biological processes mediated by the proteins identified in each group and at each time point. Top 10 molecular functions (A) and biological processes (B) with the highest number of proteins adsorbed onto each substrate. Nature-based gel (DESPLAC®) or conventional toothpaste (Oral-B) at different time points (BA—baseline; or 3M—3 months).

3. Conclusions

We demonstrated that a nature-based gel achieved similar clinical outcomes to a conventional toothpaste in controlling gingival inflammation among gingivitis patients. However, the nature-based gel markedly changed the proteomic profile of GCF after treatment compared with the conventional toothpaste, increasing the number of proteins and showing a profile associated with a host response.

4. Materials and Methods

4.1. Study Design

This study protocol was developed following SPIRIT (Standard Protocol Items: Rec- ommendations for Interventional Studies), and the manuscript was prepared according to the CONSORT checklist [35]. This was a double-blind, parallel, randomized, and controlled study. All subjects received and signed a form of informed consent. The study protocol was approved by the local Ethics Committee (IRB approval #51781621.9.0000.5506), and the clinical trial was registered (RBR-7dz5y9x—REBEC platform).

4.2. Treatment Groups

Using a table of equiprobable numbers, all selected individuals were randomly dis- tributed to the following treatment groups: (1) test group (n = 10): nature-based gel (DESPLAC®, São Paulo, São Paulo, Brazil)—applied 2x/day during toothbrushing in the morning and evening; (2) control group (n = 10): Oral-B ProGengiva (Cincinnati, OH, EUA)—applied 2x/day during toothbrushing in the morning and evening. The DESPLAC® product (Premium Oral Gel) is the only toothpaste available on the Brazilian national market that includes a combination of natural agents in its formula: propolis, aloe vera, green tea, cranberry, and calendula. Considering the outcomes found for the average and standard deviation values of the nature-based gel group's gingival index at baseline and after 90 days, the sample power was estimated using an α of 5% and an effect size of 1.23, resulting in a power of 90%.

4.3. Subject Population and Inclusion/Exclusion Criteria

Participants were selected from the population referred to the Dental Clinic of Guarul- hos University (Guarulhos, SP, Brazil). The inclusion criteria were as follows: availability for the duration of the study; at least 15 natural teeth with minimal tooth restorations; good general health or health well controlled under a physician's care; aged 18—65 years; and an average initial gingivitis index of at least 1.5 [36]. The exclusion criteria were as follows: a medical condition requiring premedication before dental visits/procedures; use of any medication that may affect salivary flow; xerostomia; carious lesions; sites with probing depths of >4 mm; use of orthodontic appliances; use of antibiotics within 6 months before or during the study; use of any over-the-counter medications that would affect the results, other than analgesics (i.e., aspirin, ibuprofen, acetaminophen, or naproxen) at the time of informed consent; pregnant or breastfeeding individuals; immune-compromised individuals; history of allergies to oral-care/personal-care consumer products or their ingredients; and a history of alcohol abuse, smoking, or drug abuse.

4.4. Procedures

All participants received the same oral hygiene instructions in terms of products to be used, a toothbrush with soft bristles, and the respective tube of toothpaste according to their assigned group. The study coordinator, who was uninvolved in the clinical evaluations, distributed the oral hygiene products to the participants. Allocation concealment was achieved using numbered, opaque, and sealed envelopes handled by the study coordinator. All products were stored in a sealed bag to eliminate any differences in product aesthetics and packaging between the study groups. Label information included a study group code, instructions for at-home use, and safety information (including emergency contact details). Participants were instructed to apply 1 cm of toothpaste to the brush and brush their teeth for 2 min twice a day (morning and evening) and to return their assigned products to the study site before receiving new products. They were instructed to follow these oral hygiene regimens for 90 days and to exclusively use the provided products. There were no specific instructions related to toothbrushing technique. They were not instructed to use dental floss to avoid introducing additional factors that could affect outcomes related to the toothpaste/gel composition and use. At the end of the 90 days, all participants returned to the dental clinic for a clinical evaluation and for the collection of gingival crevicular fluid samples. They were also instructed to refrain from performing any oral hygiene procedures for 4 h before the clinical evaluation at 90 days. The clinical evaluations and sample collections were conducted by calibrated examiners. Calibration was conducted according to a previously established protocol for periodontal studies [37]. The calibration phase was conducted before the study began, with examiners assessing specific clinical conditions in patients after familiarizing themselves with the protocol measures. At the first clinical appointment, an examiner assessed up to 42 sites per patient, followed by a second examiner who repeated the measurements in the same quadrant during the same appointment. Seven days later, at the second appointment, one of the original examiners and a third examiner repeated the measurements. The standard error of measurement was calculated, with intra-examiner variability at 0.26 mm and 0.28 mm for probing depth and clinical attachment level, respectively; inter-examiner values were 0.25 mm and 0.29 mm for probing depth and clinical attachment level, respectively, showing adequate reproducibility for periodontal evaluations. For categorical variables, such as plaque index and bleeding on probing, the level of agreement was estimated using Kappa, showing a 91% agreement rate.

4.5. Clinical Evaluation

At baseline and at 90 days, the presence or absence of visible plaque, marginal bleed- ing, and bleeding on probing, as well as probing depth and clinical attachment level, were assessed at six sites per tooth (excluding third molars) using a manual periodontal probe (North Carolina—Hu-Friedy, Chicago, IL, USA). Plaque accumulation and gingival inflam- mation were assessed for each tooth using standardized methods: the gingival index [36] and plaque index [38]. Each tooth was categorized by score, and an average score was calculated. The percentage of teeth assigned to each score was also calculated. Bleeding on probing was expressed as the average number of bleeding sites [39]. Probing depth and clinical attachment level were estimated in millimeters and expressed as an average.

4.6. Monitoring Compliance and Adverse Events

At the end of the study, participants were asked to return to the dental clinic and bring their tube(s) of toothpaste (empty or not), which were checked for any remaining toothpaste. The oral soft tissues of all subjects were evaluated during the clinical assessment at 90 days, and subjects were queried about any self-perceived side effects.

4.7. Gingival Crevicular Fluid (GCF) Sampling

The same GCF sampling procedure was performed for all patients. GCF was collected approximately one week after the clinical evaluation to avoid changes in the GCF due to blood contamination. Two non-adjacent shallow sites with a PD and CAL of ≤3 mm were randomly selected per patient. Supragingival biofilm was removed, and the sites were isolated and dried to avoid saliva contamination. GCF was sampled by inserting a standard absorbent paper cone approximately 2 mm into the sulcus/pocket for 60 s. After 20 s, a second absorbent paper cone was inserted in the same site for another 60 s. The two paper cones from the same site were pooled in a single dry microcentrifuge tube and stored at -80°C. Strips contaminated with blood were discarded.

4.8. Protein Extraction

The absorbent papers containing the samples were placed in tubes with 150 µL of protein extraction solution (58% acetonitrile, 40% purified water, and 2% acetic acid). Each tube was agitated for 1 min, and the extraction procedure was performed in duplicate for each sample. After extraction, the solutions were pooled by group to increase protein concentration to a detectable level for the technique. The samples were then dried for 2 h, resuspended in ammonium bicarbonate (50 mM; pH 7.8), and dried again for an additional 1 h.

4.9. Liquid Chromatography Coupled with Tandem Mass Spectrometry

The proteomic profile was evaluated using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), following a previous protocol [40–42]. Pooled samples were resuspended in urea (8 M). The Bradford method was used to quantify the total protein content. Then, the proteins were reduced, alkylated, digested with trypsin (1:50 w w-1), and subjected to LC–MS/MS. Samples were dried in a vacuum concentrator and immersed in 22.5 µL of 0.1% formic acid. An aliquot of 2 µL (0.88 µg) was analyzed on an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) connected to an EASY-nLC system (Proxeon Biosystem, West Palm Beach, FL, USA) through a Proxeon nanoelectrospray ion source. Peptides were separated by applying a 2–90% acetonitrile gradient in 0.1% formic acid in an analytical PicoFrit Column (20 cm × ID75 µm, 5 µm particle size) (New Objective Inc., Woburn, MA, USA) over 80 min [43]. The instrument methods were set up in the data-dependent acquisition mode. After accumula- tion to a target value of 1 × 106, full-scan MS spectra (m/z 300–1600) were made from an Orbitrap analyzer with a resolution of r = 60,000. A thousand (1000) counts was the signal threshold for triggering an MS/MS event. A size list of 500, a duration of 60 s, and a repeat count of 1 were enabled for dynamic exclusion.

Peptide sequences acquired were identified using MaxQuant (v.1.3.0.3—Martinsried, Munich, Germany), and MS/MS spectra were searched against the Human UniProt database. A maximum of 1%FDR was set for both protein and peptide identification. Protein quantification was performed using the LFQ algorithm implemented in MaxQuant, with a minimum ratio count of 2 and a window of 2 min for matching between runs. The list of peptides identified was filtered by a minimum 0.75 localization probability of containing at least one peptide with Perseus v.1.5. Reverse and contaminant entries were excluded from further analysis.

Exclusive proteins identified in each group were depicted using Venn diagrams con- structed using a free web-based tool [44]. The name, molecular function, and biological process of each protein were checked using the UniProt database. Protein ID was used to search manually on UniProt for all information. Heatmaps were constructed using GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, CA, USA) to show LFQ intensity (protein intensity/expression) for each group and time.

4.10. Statistics

Prism (GraphPad) 8.0 and SPSS (20.0) were used to generate graphs and perform statistical analysis. Group

comparisons, when necessary, were conducted using the t-test or repeated measures ANOVA (Tukey's test). A significance level of 5% was adopted.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/gels10120772/s1, Table S1: Full-mouth gingival and plaque index evaluation of the study population at baseline and after 3 months according to scores. Proportion (%); Table S2: Percentage (%) of sites with bleeding on probing.

Author Contributions: Conceptualization: L.C.F., J.A.S. and B.B.-S.; data collection and analysis: L.C.F., J.G.S.S., G.D., N.F.F., D.F.d.C., M.H.R.B., D.H. and T.A.; manuscript writing: L.C.F., J.G.S.S. and V.A.R.B.; critical review: L.C.F., J.G.S.S., J.A.S., V.A.R.B., D.H. and T.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the local Ethics Committee (protocol code #51781621.9.0000.5506 and date of approval) for studies involving humans.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

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Conflicts of Interest: Authors Doron Haim and Thabet Asbi were employed by the company Maccabi- Dent Research Department. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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