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Combination of neovestitol and vestitol impair the subgingival multispecies biofilm development

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ABSTRACT

The aim of this study was to evaluate effects of neovestitol-vestitol fraction (NVF) on an *in vitro* subgingival multispecies biofilm. The 33-species biofilm was formed for seven days using a Calgary device. Starting on day 3, treatments for applied twice daily for 1 min each: NV (400–1,600 μg ml^{-1}), chlorhexidine 0.12% (CHX; positive control) or vehicle (negative control). After seven days, metabolic activity and microbial composition were accessed through colorimetric reaction and DNA–DNA hybridization, respectively. ANOVA/Tukey's and Kruskal–Wallis/Dunn's were performed ($p < 0.05$). NV1,600 and NV800 and CHX significantly reduced biofilm metabolic activity by 67%, 48% and 64% respectively, compared to vehicle-treatment. NV1,600, NV800 and CHX reduced red complex proportions versus vehicle-treatment. NV1,600 also reduced orange complex and increased healthy-associated purple complex compared to negative control ($p < 0.05$). NV1,600, NV800 and CHX reduced nine species, including *Fusobacterium periodonticum* and *Porphyromonas gingivalis*. NV1,600 also reduced *Fusobacterium nucleatum polymorphum*. NV seems to be a good candidate to control biofilm formation and pathogenicity in dental practice.

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Subgingival biofilm; periodontitis; red propolis; antimicrobial; neovestitol; vestitol

Introduction

Periodontitis is one of the most common oral diseases, affecting approximately 20–50% of the world's population (Durham et al. 2013). It is defined as a chronic multifactorial inflammatory disease with the main etiological factor being a highly organized biofilm, composed of specific microorganisms and their products. Contact with periodontal tissues by this biofilm may lead to tissue destruction (Papapanou et al. 2018). Bacterial species related to periodontitis have been grouped into six complexes. The first four complexes consist of species related to periodontal health (purple, yellow, green and *Actinomyces* complexes) while the last two complexes are formed by bacterial species associated with periodontitis (orange and red complexes) (Socransky et al. 1998; Socransky and Haffajee 2002). When the host's immunoinflammatory response comes into contact with these periodontopathogenic bacteria, it becomes exacerbated, resulting in the subsequent destruction of periodontal tissue (Hajishengallis 2015).

The traditional therapy for periodontitis consists of mechanically removing the biofilm, through scaling and root planing (SRP). Reducing or eliminating pathogens is essential at all stages of disease progression. However, due to the limitations of mechanical SRP alone in reversing the dysbiotic biofilms, systemic antibiotics and other antimicrobial agents are often used as adjuncts to periodontal treatment (Teughels et al. 2020). Since recolonization of periodontal pathogens in recently scaled pockets and other oral niches can lead to recurrent infections, antimicrobials that are effective in controlling the growth of dental biofilms during active periodontal treatment or the maintenance phase are essential for long-term periodontal health.

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Several products with antimicrobial properties have been used as adjuvants to periodontal therapy (da Costa et al. 2017; Teughels et al. 2020). Among them, chlorhexidine (CHX) is the preferred chemical agent for controlling dental biofilm (Chye et al. 2019; Thangavelu et al. 2020). Due to its broad-spectrum activity, CHX eliminates both Gram-positive and Gram-negative bacteria, fungi, and viruses. As a mouthwash CHX can have bactericidal or bacteriostatic action depending on the concentration. Several studies have shown satisfactory outcomes in preventing and controlling the formation of biofilm when using CHX as an adjuvant to periodontal treatment (Sgolastra et al. 2021; Zhao et al. 2021). However, CHX use in the medium and long term is limited by some side effects, such as staining of teeth and mucous membranes and changes in taste sensitivity (Jones 1997; Brookes et al. 2020).

In an attempt to find alternatives to CHX and avoid its side effects, many studies have targeted the use of natural products with known therapeutic properties (Vanni et al. 2015; Kaymaz et al. 2019; Jassoma et al. 2019; Newman and Cragg 2020). Among these products, Brazilian red propolis (BRP) stands out for its unique composition and several significant pharmacological effects, including antimicrobial (Silva et al. 2008; Bueno-Silva et al. 2017a), anti-inflammatory (Bueno-Silva et al. 2015, 2017b) and antioxidant properties (Dantas Silva et al. 2017). However, due to the complex chemical profile and seasonal variations of BRP (Bueno-Silva et al. 2017c), propolis quality control may be a problematic task. Therefore, Bueno-Silva et al. isolated and identified two propolis compounds, vestitol and neovestitol. These compounds affect *Streptococcus mutans* biofilm, exhibit anti-inflammatory activities *in vitro* and have anticaries properties (Bueno-Silva et al. 2013a, 2013b).

Recently, our research group showed the effectiveness of red propolis ethanolic extract in reducing multi-species subgingival colonization in both immature (Miranda et al. 2019) and mature biofilms (de Figueiredo et al. 2020). Thus, the objective of the current study was to evaluate the alterations on the levels of species on a subgingival biofilm in formation under treatment with the neovestitol-vestitol fraction (NVF), obtained from the Brazilian red propolis (BRP).

Material and methods

Red propolis collection and isolation of neovestitol-vestitol fraction

BRP samples were obtained from a private farm in Maceio city (Alagoas state, Brazil), under authorization of the owner. Research on red propolis was recorded at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge of Brazilian federal government (SISGEN - register Number A305815). BRP samples were obtained from boxes containing *Apis mellifera* bees. Any possible residue of beeswax and/or other substances was separated. The BRP ethanolic extract was produced through mixing 25 g of propolis with 200 ml of 80% ethanol (v/v) and blending constantly for 45 min. Then, the suspension was filtered with the aid of a paper filter and rotary evaporator equipment was used to evaporate the solvent. The BRP ethanolic extract was obtained with an efficiency of 73%. To prevent stability loss, the BRP extract was stored at 4 °C and protected from light. The samples containing BRP were submitted to liquid–liquid fractionation with hexane and chloroform. The chloroform fraction underwent two distinct chromatographic separations: dry-column and Sephadex LH-20 column. The NVF was obtained after Sephadex LH-20 and a previous study chemically characterized it by GC-MS and showed its effect on *S. mutans* biofilm (Silva et al. 2008; Bueno-Silva et al. 2013a)

High performance liquid chromatography (HPLC)

The chemical profile of the NVF was obtained using reversed phase HPLC equipment with a Shimadzu ODS-A column (RP-18, 5 µm particle size and 4.6 × 250 mm column size) and a photodiode array sensor (SPD-M10AVp, Shimadzu Co., Kyoto, Japan). A filter of 0.22 µm diameter (Millipore, San Jose, CA) were used to clean the extracts before injecting 20 µl of each sample into the HPLC equipment. A gradient of water and methanol was used as the eluent of the column. Initially, the gradient was 40%, increasing to 60% at 45 min. Then, the proportion was kept at 90% from 45–75 min before reducing to 30% for solvent B (75-85 min), with solvent rate of 1 ml min^{-1} and a sensor of a diode array. As Silva et al. (2008) reported, the chromatograms were documented at 260 nm. The authentic standards of phenolic acids and flavonoids were formononetin, daidzein, biochanin A, neovestitol, vestitol, catechin, epicatechin, rutin, propyl gallate, ferulic acid and p-coumaric acid (Extrasynthese Co., Genay Cedex France).

Formation of multispecies subgingival biofilm

An *in vitro* multispecies biofilm was developed as explained by Miranda et al. (2019) and Pingueiro et al. (2019), with a few alterations. Table 1 shows the

Table 1. Species cultivated in multispecies biofilms grouped into the bacterial complexes (Socransky et al. 1998).

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

bacterial strains used in the multispecies biofilm model.

Most of the species were grown in tryptone soy agar plus 5% sheep blood agar (Probac, São Paulo, Brazil) under anaerobic conditions (85% nitrogen, 10% carbon dioxide and 5% hydrogen). *Porphyromonas gingivalis* was cultured on tryptone soy agar medium plus yeast extract and supplemented with hemin at 1%, menadione and sheep blood, both at 5%. *Tannerella forsythia* was cultivated on tryptone soy agar plus yeast extract, supplemented with hemin and *N*-acetylmuramic acid at 1% and menadione and sheep blood at 5%. All bacterial species were cultured on agar plates for 24 h. Subsequently, they were transferred to glass tubes containing brain heart infusion (BHI) culture medium (from Becton Dickinson, Sparks, MD, USA) enriched with 1% hemin. After growing in the conical tubes for 24 h, the optical density (OD) was adjusted to approximately 10^8 cells ml^{-1} for each bacterial species. Individual bacterial cell suspensions were diluted, and 100 µl samples containing 10^6 cells from each species were combined with 11,700 µl of BHI broth supplemented with 1% hemin and 5% sheep blood to create a 15 ml inoculum.

To establish the multispecies biofilm model, a Calgary biofilm device (CBD) was positioned in a 96-well plate from Nunc (Thermo Scientific, Roskilde, Denmark). In each well, 150 µl of the inoculum were introduced, representing roughly 1×10^4 cells for each bacterial species. However, for *Prevotella intermedia* and *P. gingivalis*, 2×10^4 cells were used. A lid with polystyrene pins (Nunc TSP system) was employed to cover the 96-well plate. These plates were subsequently incubated at 37 °C under anaerobic conditions. After a three-day incubation period, the medium was substituted with fresh BHI broth enriched with 1% hemin and 5% sheep blood. The biofilms were then allowed to mature at 37 °C under anaerobic conditions for an additional four days, resulting in the formation of seven-day-old biofilms. This procedure was similar to that used in previous studies (Miranda et al. 2019; Pingueiro et al. 2019).

Treatments with NVF

The biofilms underwent treatment with NVF after maturing for 72 h, commencing on day 3 following the replacement of the spent medium. The treatment regimen involved two sessions per day, each lasting for 1 min, for the subsequent four days. The time interval between consecutive treatments was set at 8 h. Subsequently, pins coated with biofilms were carefully transferred to 96-well plates, each containing varying concentrations of NVF, according to:

1. NVF 1,600 µg ml^{-1} (5% of ethanol);
2. NVF 800 µg ml^{-1} (5% of ethanol);
3. NVF 400 µg ml^{-1}(5% of ethanol);
4. Positive control: CHX 0.12% (a commercial formulation from OralB®, Cincinnati, OH);
5. Negative control: Vehicle (5% ethanol)

The pins were exposed to the treatments for a duration of 1 min. Subsequently, they were thoroughly rinsed with phosphate-buffered saline (PBS) and reintroduced into the same culture medium. The experimental procedures were carried out in triplicate across three separate trials, encompassing both the test and control groups. This resulted in a total of nine samples for each group, forming the basis of subsequent analyses (Bueno-Silva et al. 2013b; Miranda et al. 2019).

Biofilm metabolic activity

The impact of NVF and the control groups on the metabolic activity of the multispecies biofilm was

evaluated through the use of 2,3,5-triphenyltetrazolium chloride (TTC) (catalog no. 17779; Fluka Analytical, Darmstadt, Germany) in a spectrophotometric analysis. TTC serves as a distinguishing agent between metabolically active and inactive cells. Its initial white substrate undergoes enzymatic conversion to red formazan (1,3,5-triphenyl) in the presence of active bacterial cells, driven by the action of various dehydrogenases. The change in substrate color serves as an indirect indicator of bacterial metabolic activity.

To quantify the metabolic activity of the biofilms, following the seven-day experimental period, three pins from each experimental group were transferred to plates containing 200 µl/well of fresh BHI medium supplemented with 1% hemin and 0.1% TTC solution. These plates were then incubated under anaerobic conditions at 37 °C for 6–8 h. The measurement of TTC reduction to red formazan was conducted using a spectrophotometer (Fred and Knight 1949; Miranda et al. 2019). The absorbance values of the negative control group (biofilm treated with the vehicle) were considered as representing 100% metabolic activity, and the percentages of metabolic activity for the other groups were calculated proportionally.

Biofilm dry weight

Three pins of each treatment group were used to calculate the biomass (dry weight) of the biofilms. Briefly, the biofilms were dissolved into a saline solution (500 µl), placed in pre-weighted aluminum papers inside a stove to evaporate the saline solution, and then the aluminum papers were weighed again and the biomass of biofilm calculated.

DNA–DNA hybridization (checkerboard)

Three pins coated with seven-day biofilms, representing each experimental group, were subjected to a series of steps. Initially, they were washed with PBS and then transferred to microcentrifuge tubes containing 150 µl of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.6)]. Subsequently, 100 µl of 0.5 M NaOH was added to each tube. These tubes, containing the pins and the solution, were heated and boiled for 10 min. The resulting solution was neutralized by adding 0.8 ml of 5 M ammonium acetate. Individual sample analysis was conducted to identify and quantify the presence of 33 bacterial species utilizing the DNA–DNA hybridization technique. In brief, the biofilm samples were lysed through boiling and the ammonium acetate treatment as previously described. The corresponding DNA was then applied onto a nylon membrane using a Minislot device (Immunetics, Cambridge, MA, USA). After membrane attachment, DNA samples were placed into a Miniblotter 45 (Immunetics). Digoxigenin-labeled DNA probes, representing the entire genome of subgingival species, were hybridized to individual lanes on the Miniblotter 45. Following hybridization, the membranes were washed, and DNA probes were detected using a specific antibody against digoxigenin conjugated to alkaline phosphatase. Signal detection was achieved using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL, USA), and results were acquired using Typhoon Trio Plus (Molecular Dynamics, Sunnyvale, CA, USA). Each run included two lanes with standards containing 10^5 and 10^6 cells of each strain. Signals obtained *via* Typhoon Trio were quantified into absolute counts by comparing them to the standards on the same membrane. In cases where no signal was detected, it was recorded as zero. The values obtained following NVF treatment were compared to those of both negative and positive control groups (Socransky et al. 1994).

Statistical analysis

The statistical analysis was carried out using BioEstat® software (https://www.mamiraua.org.br/downloads/programas/). Data normality was checked *via* the Shapiro–Wilk and Kolmogorov–Smirnov tests and was found to be not normal. For comparisons between three or more groups, the Kruskal–Wallis test followed by Dunn's post hoc test was used at a 5% significance level ($p < 0.05$).

Results

Neovestitol and vestitol were the main compounds demonstrating similarity to the same fraction used in a previous manuscript from our research group (Bueno-Silva et al. 2013b) and their chemical composition are shown in Figure 1.

Figure 2 reveals the effect of NVF and CHX on the metabolic activity of multispecies biofilm. Treatment with NVF at 1,600 and 800 µg ml^{-1} and also CHX at 0.12% significantly decreased biofilm metabolic activity by 67%, 48% and 64% respectively, in comparison to a negative control ($p \leq 0.05$). However, no significant difference was detected in metabolic activity between cultures treated with NVF 1,600 and 800 µg ml^{-1} and CHX 0.12% ($p \geq 0.05$). NVF 400 µg ml^{-1} treated biofilms did not present a

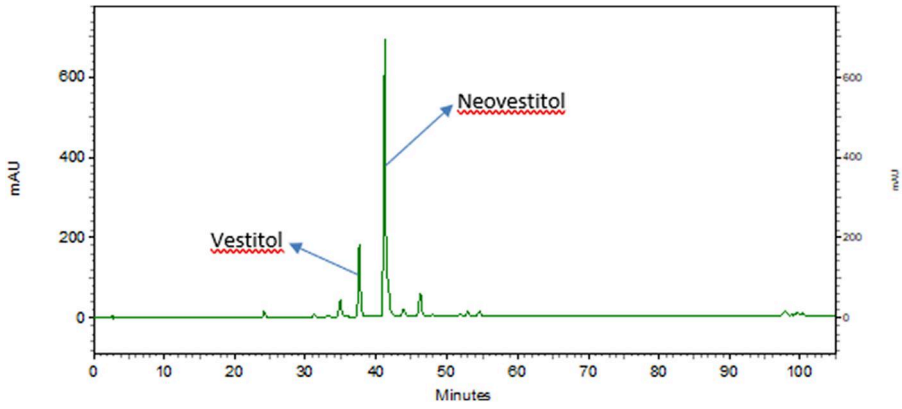

Figure 1. NVF chemical profile through HPLC analysis. Neovestitol (66%) and vestitol (30%) were the compounds identified.

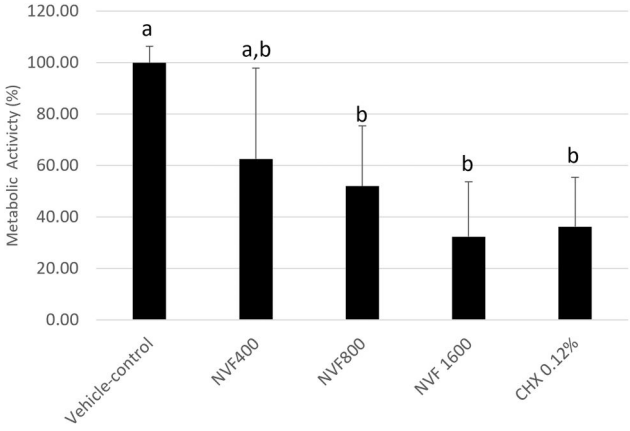

Figure 2. Biofilm metabolic activity after treatments with the vehicle-control, Neovestitol-vestitol fraction (NVF) 400, 800 and 1,600 μg ml^{-1} and chlorhexidine (CHX) 0.12%. Vehicle-treated biofilms were considered as 100% metabolic activity. Different letters show statistical significance between groups by Kruskal–Wallis followed by Dunn's post-hoc test ($p \leq 0.05$).

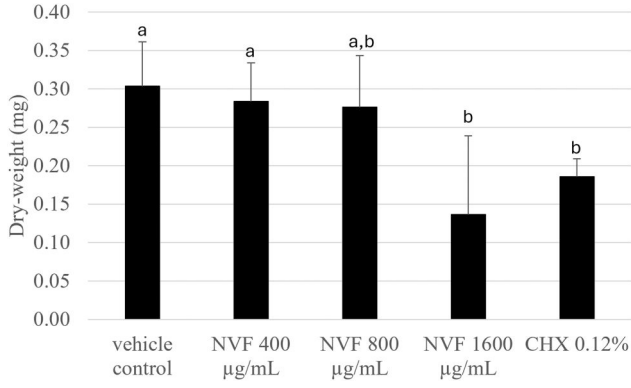

Figure 3. Biofilm dry weight after treatments with the vehicle-control, neovestitol-vestitol fraction (NVF) 400, 800 and 1,600 μg ml^{-1} and chlorhexidine (CHX) 0.12%. Different letters show statistical significance between groups by Kruskal–Wallis followed by Dunn's post-hoc test ($p \leq 0.05$).

statistically significant effect compared to any other treatment group ($p \leq 0.05$).

Treatments with NVF at 1,600 μg ml^{-1} and CHX significantly reduced the dry weight of biofilms by approximately 55% and 41% respectively when compared to a vehicle control group, and there was not a statistically significance difference in the result obtained from these two treatments. Treatment with 400 μg ml^{-1} corresponded to the vehicle-control while treatment, while 800 μg ml^{-1} did not differ from any other treatment group or the vehicle control (Figure 3).

Figure 4 demonstrates the total number of the microorganisms contained within the biofilms. NVF at 1,600 and 800 μg ml^{-1} and also CHX 0.12% significantly reduced the number of the bacteria in the biofilm when compared to the negative control ($p \leq 0.05$). The three treatment groups did not present statistically significant differences among each other ($p \geq 0.05$). The total number of bacteria associated with biofilms treated with NVF at 400 μg ml^{-1} did not statistically differ from the number associated with the vehicle-control or NVF 800 μg ml^{-1} treated biofilms ($p \geq 0.05$).

Since there were no statistically significant differences in metabolic activity, dry weight, or number of microorganisms between the NVF at 400 μg ml^{-1} and the negative control, only the results of the NVF at 1,600 μg ml^{-1} and 800 μg ml^{-1} were included in the next evaluations.

Figure 5 shows NVF effects on bacterial complexes. Both NVF concentrations and CHX treatments were more effective in reducing the proportions of periodontopathogens from the red complex species than the vehicle-control. Although the percentage of the red complex in vehicle-treated biofilms was small, NVF at 1,600 μg ml^{-1} and CHX reduced it by more than half. Furthermore, NVF at 1,600 μg ml^{-1} was the only treatment that reduced the proportion of the putative orange complex and increased the health-associated species belonging to the purple complex, in comparison to the negative control ($p \leq 0.05$).

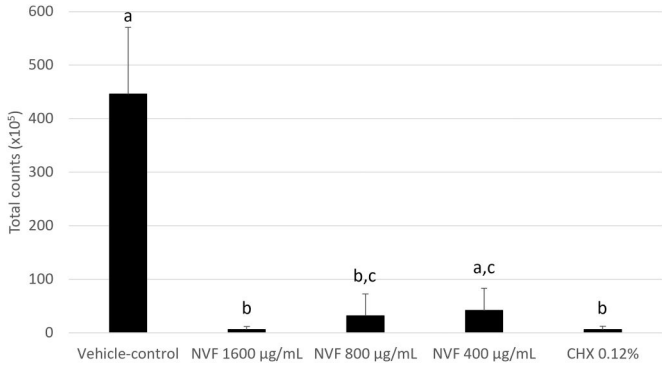

Figure 4. Total counts of multispecies biofilm treated with the dilution vehicle (vehicle-control), neovestitol-vestitol fraction (NVF) 400, 800 and 1,600 µg ml^{-1} and chlorhexidine (CHX) 0.12%. Different letters indicate statistical significance between groups by Kruskal–Wallis followed by Dunn's post-hoc test ($p \leq 0.05$).

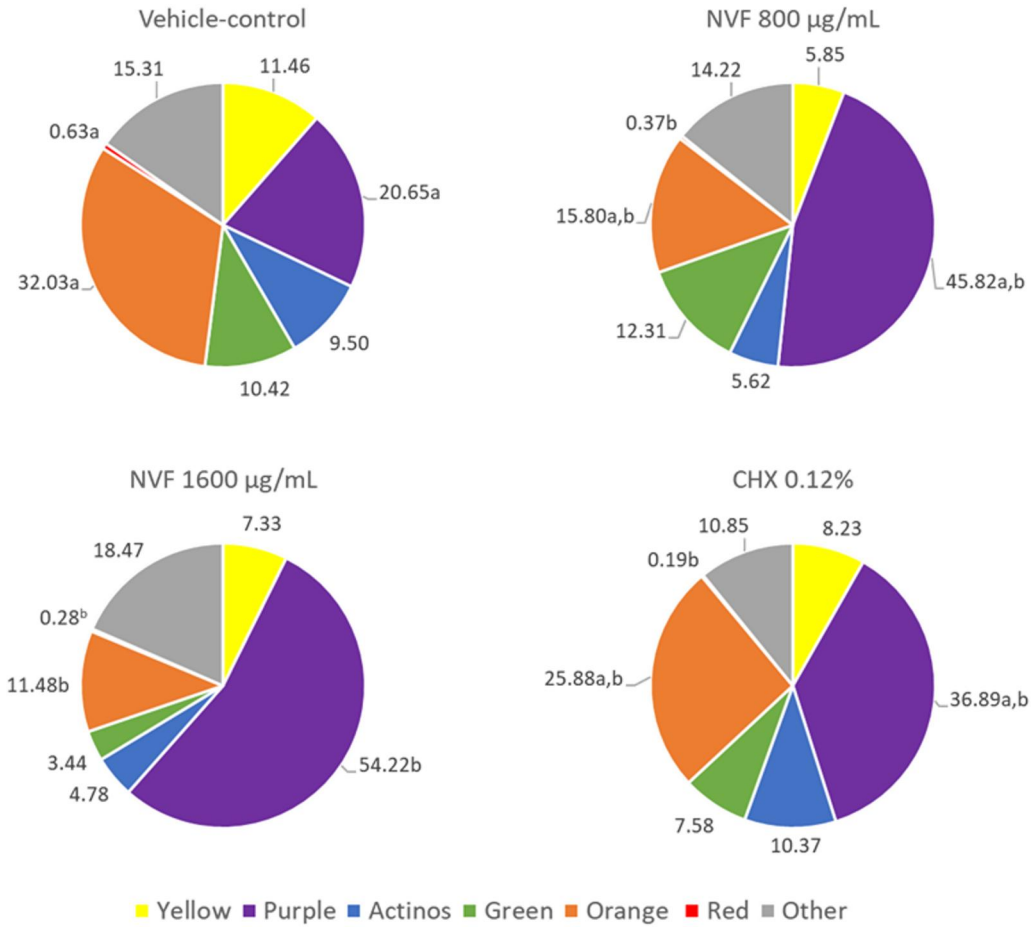

Figure 5. Mean proportions of bacterial complexes in the four groups after the treatments. The colors represent different microbial complexes described by Socransky et al. (1998). Different letters means statistical significance among groups by Kruskal–Wallis followed by Dunn post-hoc ($p \leq 0.05$).

Figure 6 and Supplemental Table 2 demonstrate the total number (in mean) of the respective bacterial strains used in the biofilm. No statistically significant differences were detected between the treatment groups NVF and CHX ($p > 0.05$). When compared to negative control, treatments with NVF (1,600 and 800 µg ml^{-1}) and CHX (0.12%) were effective in decreasing the number of five distinct species (*Actinomyces naeslundii*, *Actinomyces gerencseriae*, *Fusobacterium nucleatum vincentii*, *Parvimonas micra* and *P. gingivalis*) ($p < 0.05$). The number of *Actinomyces odontolyticus*, *Streptococcus oralis*, *Streptococcus gordonii*, *Aggregatibacter actinomycetemcomitans*, *Capnocytophaga gingivalis*, *Fusobacterium*

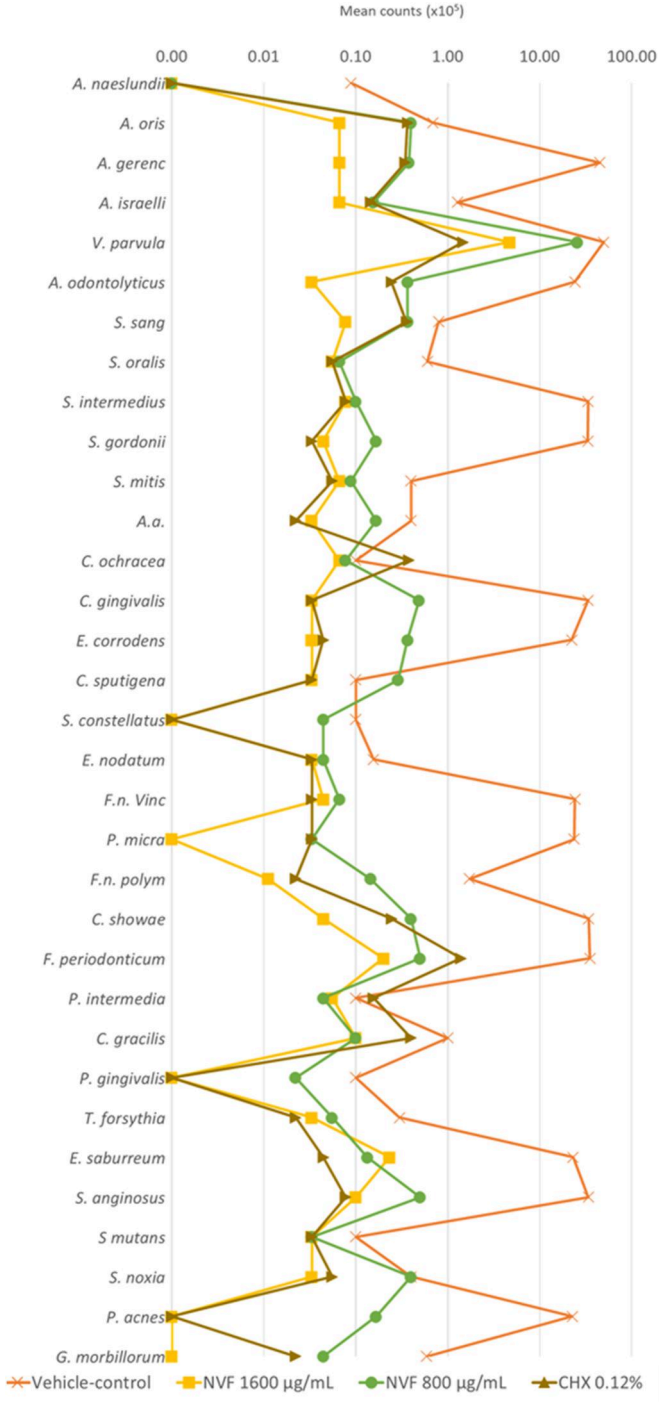

Figure 6. Mean total counts of the species included in the vehicle control, NVF (1,600 and 800 µg ml^{-1}) and CHX (0.12%) treated biofilms. Statistical analysis was performed by the Kruskal–Wallis test followed by Dunn post-hoc ($p \leq 0.05$) and is described in Supplementary Table 2.

nucleatum polymorphum, *Campylobacter showae*, *Fusobacterium periodonticum*, *T. forsythia* and *Gemella morbillorum* were reduced in the NVF 1,600 and CHX groups ($p < 0.05$). *C. gracilis* was significantly reduced only in the two NVF groups (1,600 and 800 µg ml^{-1}), while *Streptococcus anginosus* was reduced only in the CHX treatment ($p < 0.05$).

Discussion

The present manuscript evaluated the impacts of a NVF fraction, derived from Brazilian red propolis on the most well-known bacterial species involved in the etiology of periodontal disease and that compose the subgingival biofilm. Two concentrations of NVF (1,600 and 800 µg ml^{-1}) were as effective as CHX

0.12% in reducing the overall biofilm (Figures 2–4). Also, the highest concentration of NVF (1,600 μg ml^{-1}) and CHX 0.12% reduced 15 and 14 distinct bacterial species, respectively, while NVF 800 reduced nine species compared to the biofilms treated with vehicle-control (Figure 6 and Supplemental Table 2).

At first glance, reducing a greater number of species may appear to be a better result. However, the subgingival biofilm has special characteristics. The classical work of Socransky et al. (1998) suggested that the subgingival microbiota present different groups (named complexes) of microorganisms that are intrinsically correlated and may be associated with periodontal health or disease. Therefore, it is necessary to take a closer look at which species or groups of species are affected by different therapies or antimicrobial substances. In this regard, analyzing microbial complexes appears to be an interesting approach.

As seen in Figure 5, the three treatments (NVF 1,600 and 800 and CHX) statistically reduced the red complex proportion (which is strongly associated with periodontitis) when compared to vehicle-control treated group. Interestingly, the effects of the two concentrations of NVF in changing the biofilm composition were very similar to that observed with the gold standard CHX. NVF at 1,600 was the only treatment that statistically reduced the putative orange complex and improved the health-associated purple complex. This effect is an advantage of this NVF 1,600 over NVF 800 and CHX. However, although not statistically significant, NVF 800 reduced orange complex by more than 50% and elevated purple complex by more than 50% when compared to vehicle-control treated biofilm. These changes are relevant and suggest that future studies should test concentrations between 800 and 1,600 of NVF, to establish the most effective concentration for clinical practice.

A previous study by our research group using a very similar design to the present one (Miranda et al. 2019) pointed out that the crude extract of Brazilian red propolis at 1,600 μg ml^{-1} caused a striking decrease in both red and orange complexes, and a reduction in the levels of 14 individual bacterial species. Similar to the present study's findings, this concentration of Brazilian red propolis was the only one to decrease the orange complex. Therefore, the concentrations used in this study were determined based on this previous manuscript. Although using different substances (crude extract and an isolated purified fraction of propolis), the results for the higher concentration of the products (1,600 μg ml^{-1}) were similar in that they reduced a larger number of species than the lower concentration (800 μg ml^{-1}). However, here the NVF 800 was able to reduce the red complex and a greater number of bacterial species. The lower concentration tested, 400 μg ml^{-1}, did not have a statistically significant impact on the overall biofilm. Interestingly, the same occurred in a paper with the crude extract of red propolis (Miranda et al. 2019). Nevertheless, this concentration seems to be noteworthy when evaluating the possible future systemic use as a medicine taken orally (de Figueiredo et al. 2020). In addition, futures studies should also evaluate its cytotoxicity on eukaryotic cells to guarantee the security of clinical uses of this natural product.

The present experimental design was developed considering the possible future use of the NVF in a mouthwash that is normally used two or three times a day, for 1 min each time. These two daily 1-minute treatments of the biofilms have been widely described in the literature as a common protocol for several mouthwashes (Bueno-Silva et al. 2013b; Hwang et al. 2017; Castillo Pedraza et al. 2020). Thus, this may be a valid protocol to test novel antimicrobial substances against mono- and multi-species biofilms. Although mouthwashes alone are not effective in treating severe periodontitis, they have a key role as adjuvants during the maintenance phase of periodontitis treatment (Quirynen et al. 2005; Zhang et al. 2021) and in treating gingivitis after mechanical removal of biofilm (Halboub et al. 2020).

Porphyromonas gingivalis along with *T. forsythia* and *S. gordonii* (and probably with other oral bacteria) are considered to be important players in dysbiosis of the subgingival biofilm, leading to periodontal disease. The overgrowth of these microorganisms stimulates the shift from a health-associated to a pathogenic biofilm, provoking tissue destruction triggered by an increased immunoinflammatory reaction (Hajishengallis 2015). In this scenario, the reduction of the three species mentioned above in biofilms treated with NVF 1,600 and CHX 0.12% should be highlighted. Still, NVF 800 only led to a statistically significant reduction of *P. gingivalis*. NVF 800 decreased the levels of *S. gordonii* and *T. forsythia* to values similar of those obtained with NVF 1,600 and CHX, but without statistical significance in comparison to biofilms treated with vehicle control.

Reduction of *F. nucleatum vincentii* in all treatment groups, of *F. nucleatum polymorphum* in the NVF 1,600 and CHX groups, and of *F. periodonticum* only in the NVF 1,600 group are other relevant findings. The genus *Fusobacterium* has an important role in the transition from periodontal health to disease

(Kolenbrander 2000). *F. nucleatum* is a Gram-negative species, considered the most prevalent anaerobic bacteria in the late stages of the disease and it has been considered to be a possible periodontal pathogen (Socransky and Haffajee 2002). Some authors (Colombo et al. 2002; Socransky and Haffajee 2002) have reported that the presence of *F. nucleatum* is mainly associated with individuals with periodontitis and periodontal abscesses, and its numbers are reduced after effective periodontal therapy. As an intermediate colonizer of dental biofilm and one of the first Gram-negative species to be stable in the subgingival biofilm, *Fusobacterium* species play a prominent role in the interactions between Gram-positive and Gram-negative species, contributing to the colonization of other anaerobic species, including the pathogens of the red complex (Kolenbrander 2000).

The inflammatory response has a critical role in the tissue destruction occurring during periodontal disease. Neovestitol and vestitol both present an anti-inflammatory activity. Their effects on lipopolysaccharide-activated macrophages has been demonstrated through modulation of the factor nuclear kappa B (NF-κB) pathway, resulting in decreased expression of several pro-inflammatory cytokines. They were also shown to reduce neutrophil rolling, adhesion and migration to the inflammatory focus by diminishing Intercellular Adhesion Molecule 1 (ICAM-1) mRNA levels through an *in vivo* acute peritonitis experiment (Franchin et al. 2016; Bueno-Silva et al. 2017c; Bueno-Silva et al. 2020). Moreover, the crude extract of Brazilian red propolis also down-regulated the NF-κB pathway and disfavored the release of pro-inflammatory cytokines in lipopolysaccharide-activated macrophages (Bueno-Silva et al. 2015; Bueno-Silva et al. 2017a).

It is necessary to remember that the scientific evidence supporting the clinical treatment of periodontal disease is derived from randomized clinical trials and systematic reviews. Thus, the present results cannot still support the clinical use of these compounds, but they may endorse future *in vivo* studies. Besides, our experimental design did not allow us to verify whether NVF can act on a mature biofilm, which is usually found in periodontitis patients. Therefore, future studies evaluating the possible *in vivo* use of NVF are preferable to be done with gingivitis than periodontitis. Additionally, future clinical trials should evaluate whether NVF causes less tooth staining and taste alteration than chlorhexidine.

Although NVF is derived from a natural product, that does not guarantee that it is completely safe. In line with this, a recent manuscript reported a 5% incidence of contact allergy to Brazilian propolis (Antelmi et al. 2025). The literature does not specify which Brazilian propolis was evaluated, but they all present a complex chemical profile with several distinct molecules that could act as allergens. Thus, fractionating crude extracts may reduce the risk of such reactions as purified or isolated compounds have fewer possible allergens. Nevertheless, cytotoxic studies of NVF would be beneficial.

A limitation of our study lies in the semi-quantitative aspect and the limit of detection of the checkerboard assay. On the other hand, this methodology allows the concomitant evaluation of multiple species of microorganisms in numerous samples. Finally, the fact that the number of colony formation units (CFUs) was not estimated may be considered as a limitation. The procedure to count CFUs turns out to be a challenging task since our model uses different species. When these species grow on an agar plate, the colonies with their different formats overlap with each other, turning the counting into an uncertain result, even to verify number of bacteria in general, without determining which species it belongs to. To overcome this limitation, we performed a metabolic activity test to confirm that the bacteria were viable. This allows for an indirect measurement of total biofilm.

In conclusion, NVF presented inhibitory effects on the formation of the subgingival multispecies biofilm related to periodontal disease. Moreover, NVF reduced the levels of classic periodontopathogens similarly to the levels of the gold standard CHX. Therefore, NVF seems to be a good candidate to control biofilm formation and pathogenicity in clinical practice. However, further clinical studies are necessary to confirm these results.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author, BBS, upon reasonable request.

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