Propolis Fluoride Inhibits Streptococcus mutans and Veillonella parvula Dual- species Biofilm Growth and alters Gene Expression

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Abstract

Background

Propolis fluoride 10% (PPF) was developed to arrest and prevent caries, with inhibitory effects on a single species of bacteria. However, no study has been conducted on the effectiveness of PPF in inhibiting multispecies biofilms, particularly early colonizers. This study aimed to investigate the effects of PPF on *Streptococcus mutans* and *Veillonella parvula* (Sm-Vp) dual-species biofilm growth and gene expression.

Methods

Biofilms were prepared using the 96-well method with 1, 3, and 6 h incubation, and 30% silver diamine fluoride was used as the positive control. Crystal violet assay and total plate counting (TPC) were performed to analyze inhibitory effects. An inverted microscope was used to analyze the formed structure. RNA was extracted from the samples for cDNA synthesis and standardized using a Qubit fluorometer for reverse-transcription quantitative-polymerase chain reaction. The target genes used in this study were *NRAMP* and *SloR*, and 16 srRNA was used as an internal control. Consequently, alterations of gene expression were quantified using Livak's method ($2^{-\Delta\Delta Ct}$). Statistical analyses were performed using the Statistical Package for the Social Sciences software. Statistical significance was set at $P < 0.05$.

Results

Inverted microscopic observation showed lower aggregate formation in the treated and positive control groups, whereas large aggregates were observed in the negative control groups. The TPC and crystal violet results showed a significant difference between the control and treatment groups in the monospecies group ($P < 0.05$), and the lower mean was statistically significant ($P < 0.05$). The *NRAMP* and *SloR/Dlg* gene expressions were downregulated in PPF-treated samples.

Conclusion

The PPF-treated Sm-Vp biofilms showed lower biofilm formation and altered virulence and symbiotic-related gene expression. Therefore, PPF effectively inhibits Sm-Vp biofilm growth and prevents caries.

BACKGROUND

A dental biofilm is a functional, multispecies, and complex bacterial community containing viable and nonviable bacteria and their metabolites encapsulated in a saliva-derived polymer matrix [1]. A mature dental biofilm with dysbiotic bacterial composition is known to be the cause of most oral diseases, including gingivitis, periodontitis, and dental caries [1, 2]. Prevention of oral diseases is crucial and

cheaper than curative oral and dental treatment, making it a sustainable target as an oral health intervention.

Mouthwashes, toothpaste, and fluoride-containing agents are commonly used as caries-preventing agent [3]. Fluoride varnish is one of the caries-preventing agents commonly used in dentistry [3, 4]. Silver diamine fluoride (SDF) is the gold standard of this preparation, effective in preventing and arresting carious lesion [5]; however, its use for arresting caries causes black staining, influencing its acceptability as a treatment [6]. Other preparations, such as acidulated phosphate fluoride (APF) and ammonium hexafluorosilicate (AHF), are developed to overcome the limitations of SDF; however, they are considered expensive in developing countries [4]. Therefore, another alternative is needed to provide readily accessible and affordable caries preventing agents with better aesthetic properties.

Propolis fluoride 10% (PPF) was developed in Indonesia as an alternative caries-preventing agent to SDF. Previous studies on the effects of PPF treatment have shown improvement in enamel and dentin defects *in vitro* [7], prevention of enamel demineralization *in vitro* [8], and prevention of *Streptococcus mutans* (*S. mutans*) monospecies biofilm formation *in vitro* [4]. However, to determine its clinically relevant preventive effects, the impact of PPF on multispecies biofilms needs to be studied.

Streptococcus mutans and *Veillonella parvula* (*V. parvula*) are two early colonizers of dental biofilms that play an essential role in the initial attachment phase [9, 10]. They have a mutualistic symbiotic relationship, where oral *Veillonella* metabolizes lactic acid, a metabolic waste of oral *Streptococci*. In turn, this interaction maintains an optimum pH level and lactic acid concentration for oral *Streptococci* growth [11]. Therefore, the inhibitory effects of PPF on *S. mutans* and *V. parvula* (Sm-Vp) dual-species biofilm growth is needed to be assessed. Moreover, a transcriptional profiling study showed that oxidative stress response-related gene expression of *V. parvula* is altered in *Streptococcus gordonii* and *V. parvula* coaggregation and plays a role in their symbiotic behavior [12]. This study analyzed the changes in this pattern under PPF treatment by studying the *SloR* gene expression of *S. mutans* and NRAMP gene expression in *V. parvula*.

This study aimed to investigate the effects of PPF on Sm-Vp dual-species biofilm growth and gene expression using a 96-well plate biofilm assay and reverse-transcription quantitative-polymerase chain reaction (RT-qPCR).

METHODS

Bacterial Strain and Culture Conditions

S. mutans American Type Culture Collection (ATCC) 25175^T and *V. parvula* ATCC 10790^T were used in this study. *Streptococcus mutans* was cultured in brain heart infusion (BHI) agar (Thermo Fisher Scientific, Waltham, MA, USA) under anaerobic conditions for 3 days. *V. parvula* was cultured on BHI blood agar (Thermo Fisher Scientific, Waltham, MA, USA) with 5% defibrinated sheep blood and 2%

sodium lactate, with the addition of hemin and menadione and incubated anaerobically (dinitrogen: 80%, carbon dioxide: 10%, and hydrogen: 10%) for 5 days.

Biofilm Samples on 96-well Plate

The 96-well plate method is used to prepare the biofilm samples in this study, according to the protocol published by Merritt et al. [13]. We innoculated 200 μL bacteria suspension (100 μL each species for dual-species samples) with a turbidity of optical density (OD)$_{600}$: 0.1 (1.0 × 10^8 colony forming unit [CFU]/mL bacterial concentration) was inoculated into each well of the 96-well U-shaped microtiter plates (Greiner Bio-One, Frickenhausen, Germany) and was then incubated anaerobically for 1 h. After the first incubation, the medium was removed to eliminate the planktonic bacteria.

Then 100 μL of BHI broth (Thermo Fisher Scientific, Waltham, MA, USA) and 100 μL treating agents were inoculated into each well. PPF (Universitas Indonesia, Jakarta, Indonesia) was used as the experimental treatment, 30% SDF (Biodinamica, Brazil) as the positive control, and BHI broth (Thermo Fisher Scientific, Waltham, MA, USA) as the negative control. The samples were then incubated under anaerobic conditions for 1 and 3 h. After incubation, the medium was carefully aspirated and then discarded. The samples were then washed using 200 μL phosphate buffer saline (PBS). Biological replicates were used for each experiment.

Crystal Violet Biofilm Mass Observation and Total Plate Counting Viability Testing

After washing, 200 μL of crystal violet at a concentration of 0.1% was seeded into each well. The samples were then incubated at room temperature for 15 min and covered with aluminum foil. The crystal violet was then removed, and 100 μL of 100% ethanol was seeded into each well. The samples were observed using an Accureader M965 (Metertech, Taipei, Taiwan) at a wavelength of 600 nm to determine the turbidity of the biofilm at OD$_{600}$.

From a separate sample, 100 μL of PBS was seeded into each well, and the bottom was scraped using a pipette tip. The samples were serially diluted to a dilution factor of 1−0.001 and spread onto agar plates. The samples were incubated anaerobically for 24 h, and total plate counting (TPC) was performed.

NRAMP and SloR Gene Expression Analysis on RT-qPCR

RNA was extracted from biofilm samples using GENEzol Reagent (Geneaid, Taipei, Taiwan). The RNA samples were reverse transcribed using the SensiFAST cDNA Synthesis Kit (Bioline, Meridian, USA) and a THERM-1001 Thermal Cycler (Axygen, USA). Copy DNA concentration was then determined using Qubit dsDNA HS Assay Kit on Qubit® 1.0 Fluorometer (Invitrogen life technologies). The samples were then diluted to 5 ng/mL for standardization.

All RT-qPCR reactions were performed in a volume consisting of 5μL template cDNA, forward/reverse primers each at 1 μmol/L, 10 μL Power SyBr Green PCR mix (Bioline), and nuclease-free water.

Thermocycling was performed on their optimal annealing temperature (Table 1) in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The 16S rRNA gene 27F-1392R was used to normalize all samples.

Table 1
Primer and thermal cycling

Primer		Thermal cycling temperature
16srRNA	Forward	94°C for 5 min (1 x), 94°C for 30 s, 55°C for 30 s, 72°C for 90 s (24 x)
	5'-AGAGTTTGATCCTGGCTCAG-3'	
	Reverse	
	5'-AAGGAGGTGATCCAGCCGCA-3'	
SloR/Dlg [14]	Forward	67°C for 5 min (1 x), 98°C for 10 s, 67°C for 30 s (35 x)
	5'-CGTCATCTCTTTATCGCAAGCATC-3'	
	Reverse	
	5'-ACTCCCATCTCAGTTACACCCT-3'	
NRAMP [12]	Forward	94°C for 5 min (1 x), 94°C for 10 s, 51.1°C for 30 s, 68°C for 30 s (35 x)
	5'-TTGAACAGGCTGAGGAGTTG-3'	
	Reverse	
	5'-TACCTGCCGTAGCAGATGAG-3'	

RT-qPCR Data Analysis

The data were quantified using Livak's $2^{-\Delta\Delta CT}$ method [15]. The negative control of each incubation time and bacteria were used as the calibrator sample. Log-fold changes are then presented.

Statistics

Statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical analyses were performed using Statistical Package for the Social Sciences software version 26.0 (IBM Corp.) for Mac [16]. The α value or significant level was set at 0.05. If ANOVA yielded significant results, Bonferroni post-hoc tests were performed to identify specific group differences. Statistical significance was set at $P < 0.05$.

RESULTS

Biofilm Growth in 1, 3, and 6 h Incubation

The OD of each sample was measured before and after incubation, and the increase in the value was recorded as the biofilm mass. At all incubation times (1, 3, and 6 h) and in all bacterial groups (dual-species, *S. mutans*, and *V. parvula*), the PPF groups consistently exhibited lower OD values than both the negative control groups. The differences between the groups were statistically significant ($P < 0.05$) at all the incubation times. Furthermore, the differences between the PPF and positive control groups were not significant ($P > 0.05$). The full OD data are presented in Table 2 and Fig. 1.

Table 2
Biofilm mass of each groups.

Bacteria	Incubation time	Negative Control (OD ± SD)	Positive Control (OD ± SD)	10% PPF (OD ± SD)	*P*-value
Dual-species	1 h	0.226 ± 0.020	0.093 ± 0.032	0.055 ± 0.001	0.000
	3 h	0.322 ± 0.021	0.100 ± 0.012	0.075 ± 0.008	0.000
	6 h	0.403 ± 0.016	0.117 ± 0.015	0.082 ± 0.010	0.000
S. mutans	1 h	0.209 ± 0.039	0.063 ± 0.003	0.048 ± 0.002	0.000
	3 h	0.262 ± 0.070	0.082 ± 0.018	0.073 ± 0.017	0.003
	6 h	0.299 ± 0.088	0.075 ± 0.037	0.064 ± 0.009	0.003
V. parvula	1 h	0.201 ± 0.051	0.079 ± 0.006	0.052 ± 0.002	0.002
	3 h	0.354 ± 0.036	0.083 ± 0.009	0.064 ± 0.047	0.000
	6 h	0.380 ± 0.053	0.114 ± 0.011	0.098 ± 0.005	0.000

OD: optical density; SD: standard deviation; PPF, propolis fluoride

Viability Testing by Total Plate Counting is Lower in PPF-treated Samples

TPC was performed on samples to compare the viability of each sample. On 1 h incubation, dual species samples showed that negative control (4.86 ± 0.49) had slightly higher bacterial viability compared with positive control (4.26 ± 0.08) and PPF (4.38 ± 0.69), but the difference was not statistically significant ($P > 0.05$). However, significant differences were observed at 3 and 6 h incubation of the dual-species samples. Moreover, no significant difference was found between the positive control and 10% PPF samples in the dual-species group after 1 and 3 h incubation. However, the difference between positive control samples and PPF samples in the 6 h incubation dual-species group was significant ($P = 0.016$).

Significant differences were observed between the groups in *S. mutans* and *V. parvula* samples. Lower bacterial viability was observed in all the PPF and positive control samples at all incubation times. In addition, the difference between the negative control and treated groups was greater in *V. parvula*

samples. Moreover, the difference between the PPF and positive control groups was not significant. The complete bacterial viability results are presented in Table 3 and Fig. 2.

Table 3
Bacterial viability based on treatments.

Bacteria	Incubation time	Negative Control (LogCFU/mL ± SD)	Positive Control (LogCFU/mL ± SD)	10% PPF (LogCFU/mL ± SD)	P-value
Dual-species	1 h	4.86 ± 0.49	4.26 ± 0.08	4.38 ± 0.69	0.117
	3 h	5.63 ± 0.05	4.45 ± 0.32	4.57 ± 0.18	0.001
	6 h	6.37 ± 0.25	4.59 ± 0.16	5.20 ± 0.47	0.000
S. mutans	1 h	5.93 ± 0.43	4.35 ± 0.08	4.87 ± 0.65	0.001
	3 h	6.38 ± 0.13	4.61 ± 0.24	4.98 ± 0.24	0.000
	6 h	6.96 ± 0.40	4.59 ± 0.16	5.12 ± 0.40	0.000
V. parvula	1 h	6.21 ± 0.46	4.26 ± 0.52	4.40 ± 0.46	0.000
	3 h	6.51 ± 0.14	3.69 ± 0.42	4.07 ± 0.23	0.000
	6 h	6.88 ± 0.07	3.50 ± 0.49	4.24 ± 0.62	0.000

SD: standard deviation; CFU, colony forming unit; PPF, propolis fluoride

NRAMP and SloR Expression is Downregulated in PPF-Treated Samples

In the dual-species groups, significant differences were found between the positive and negative control samples ($P < 0.05$). At 1-hour incubation, the positive control and PPF samples showed significant downregulation of NRAMP levels, but greater in the PPF group (-3.15 vs. -21.26 log-fold change). Similarly, both treatments significantly downregulated the NRAMP levels after 3 and 6 h of incubation.

V. parvula biofilms treated with positive control and 10% PPF showed a significant downregulation of NRAMP levels. At 1 h incubation, both treatments showed significant downregulation, with log-fold changes of approximately − 15.65 for the positive control and − 28.04 for the 10% PPF treatment. Similarly, after 3 h incubation, a significant downregulation was observed in both treatments. The log-fold change was approximately − 5.41 for the positive control and a more substantial downregulation of -49.04 for the 10% PPF treatment. Interestingly, after 6 h incubation, the positive control treatment showed a positive log-fold change, indicating the upregulation of NRAMP levels by approximately 2.28. However, despite this upregulation, 10% PPF treatment still exhibited significant downregulation, with a log-fold change of approximately − 39.50.

In the dual-species bacteria, SloR/Dlg expression was consistently downregulated across all incubation periods. The downregulation was more pronounced with the PPF treatment, indicated by larger negative log-fold change values in 1 (-1.40 vs. -24.64 log-fold change), 3 (-4.96 vs. 24.00 log-fold change), and 6 h (-5.80 vs. -37.58 log-fold change) incubation.

In contrast, the expression of SloR/Dlg in *S. mutans* exhibited a different pattern from that in the dual-species samples. At 1 and 3 h incubation periods, the positive control treatment resulted in the upregulation of SloR/Dlg, as indicated by positive log-fold change values. However, 10% PPF treatment still exhibited downregulation, though to a lesser extent. At the 6 h incubation period, both treatments showed downregulation of SloR/Dlg, with the positive control treatment resulting in further increase in expression compared to earlier time points. The results are shown in Figs. 3 and 4.

DISCUSSION

PPF 10% is a dental varnish used as a therapeutic or preventive agent. The early colonization stage occurs over a period of 6 h, making the observation of biofilm formation within this time frame important in testing preventive dental agents [17]. The inhibition of biofilm growth by PPF was investigated using the 96-well plate method and TPC. Moreover, the preventive efficacy of PPF was further investigated through the relationship of oxidative response gene expression in two early colonizing bacteria, *S. mutans* and *V. parvula*.

The biofilm mass and viability of bacteria treated with PPF decreased during the incubation period. Biofilm mass, as observed in the crystal violet assay, showed significant differences between the groups. The SDF-treated samples had the lowest mass, followed by the SDF-treated and negative control groups. Moreover, viability testing revealed significant differences in bacterial viability between the PPF-treated and negative control biofilms ($P < 0.05$), with no significant differences between the 10% PPF-treated and SDF-treated biofilm samples. This emphasizes the efficacy of PPF in inhibiting biofilm growth at a level similar to that of SDF.

Previous studies demonstrated the biofilm-inhibiting ability of PPF in *S. mutans*. Satyanegara et al. in their study showed no significant differences between PPF and SDF in terms of their ability to inhibit *S. mutans* biofilm [7]. Marpaung et al. showed similar results for the ability of PPF to inhibit *S. mutans* biofilm formation [4]. These results were confirmed in our study, in addition to the ability of PPF to inhibit *S. mutans* and *V. parvula* dual-species biofilm formation.

According to the optimum oral environment, genus *Veillonella* live in association with lactic acid-producing species, such as *S. mutans*, as early colonizer of dental biofilms, which causes the continuation of colonization by dental biofilm bacteria and decreases the pH of the oral environment to become acidic [2]. Oxidative response is necessary for prokaryotic organisms to survive. The ability of bacteria to respond to oxidative stress is one of the determinants of aerobic, facultative anaerobic, or obligate anaerobic properties of bacteria [18]. The lower expression of the NRAMP gene in dual-species biofilms of *Streptococcus gordonii* and *V. parvula* in a previous study suggests that the oxidative

relationship may be one of the symbioses of the two bacteria [12]. The metalloregulator gene was also studied in the present study due to its significant change in expression in that previous study.

The results of this study showed differences in NRAMP gene expression patterns between dual-species and mono-species samples. Gene expression was lower in 10% PPF-treated samples than in the positive control. Furthermore, a more significant change in gene expression was observed in *V. parvula* samples treated with 10% PPF than that in the dual-species samples. This indicated that 10% PPF was more influential on monospecies biofilms than on dual-species biofilms.

The decrease in NRAMP gene expression indicates another mechanism by which fluoride in PPF interferes with bacterial activity. Metal ions are required for cellular activities such as enzyme cofactors and oxidative responses. Fluoride is known to inhibit numerous aspects of bacterial activity such as glycolysis, nutrient transport, and cellular respiration due to the inhibition of metalloproteins [19]. NRAMP is a metalloregulator that functions in the regulation of Fe^{2+} and Mn^{2+} ions in oxidative responses and other cellular activities in some bacteria [19]. Downregulation of metalloregulatory gene expression may be translated to increased susceptibility of bacteria to hydrogen peroxidase-induced damage [19]. The downregulation of NRAMP genes may indicate NRAMP dysfunction that may occur due to fluoride or propolis exposure.

The oxidative response of *S. mutans* through the gene SloR/Dlg, known as the SloR gene, plays an important role in the virulence of *S. mutans*. SloR/Dlg can be located in the upstream and downstream regions of a number of important operons, so that a decrease in SloR/Dlg gene expression is known to occur along with a decrease in gene expression (pleiotropic) related to *S. mutans* virulence [20, 21]. One of the genes that underwent decreased expression along with decreased SloR/Dlg expression is the spaP gene, which plays a role in the attachment of *S. mutans* surface proteins to the pellicle [22]. In addition, the expression of the gtfB gene (which plays a role in extracellular matrix regulation) decreased with decreasing SloR/Dlg expression [20]. Furthermore, the decrease in SloR/Dlg may be related to the expression of fimA, an adhesin-encoding gene. Thus, the downregulation of SloR/Dlg expression in this study may indicate a downregulation of virulence gene expression of *S. mutans* along with the downregulation of SloR/Dlg gene expression [21]. In this study, SloR/Dlg gene expression was observed to be lower in PPF-treated biofilms than in the positive control.

The survivability of *S. mutans* in the oral cavity depends on its resistance to overcoming the toxic effects of reactive oxygen species (ROS) [23]. According to Johnston et al., fluoride exposure results in increased oxidative stress caused by impaired bacterial metabolic work, impaired respiration function, and increased intracellular pH which can result in cell damage [19]. Aerotolerance of *Streptococcus* is one of the mechanisms known to play a role in the defense of *V. parvula* in dual-species biofilms [12]. In this study, it was found that the SloR/Dlg expression of *S. mutans* decreased after 1 h PPF administration and was lower in 3 and 6 h administration. This phenomenon not only affects *S. mutans*, which is unable to counterbalance oxidative stress, but also damages *V. parvula*, which has weaker resistance to ROS, in a multispecies environment.

The metalloregulatory damage mechanism of these two bacteria may be an additional preventive mechanism of PPF against biofilm formation, especially during the early colonization stage. However, further investigation of the parameters of the two systems is required. The present study did not test this ability for each PPF component. Therefore, further research on the effects of PPF, especially the 10 new compounds found in this propolis, is needed [24].

CONCLUSION

Based on our findings, PPF causes changes in the gene expression patterns of *S. mutans* and *V. parvula* dual-species biofilms, thereby reducing bacterial aerotolerance and increasing biofilm susceptibility to oxidative stress, resulting in cellular damage in *S. mutans* and *V. parvula*.

Abbreviations

PPF, propolis fluoride; OD: optical density; TPC, total plate counting; SDF, silver diamine fluoride; RT-qPCR, reverse-transcription quantitative-polymerase chain reaction; ATCC, American Type Culture Collection; CFU, colony forming unit; PBS, phosphate buffer saline; ANOVA, analysis of variance; ROS, reactive oxygen species

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPANTS

The study protocol was reviewed by the Research Ethics Committee of the Faculty of Dentistry, Universitas Indonesia (KEPKG FKG UI) and was exempted from ethical approval under protocol number 19/*Ethical Exempted*/FKGUI/IX/2021.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING

AUTHORS' CONTRIBUTION

ACKNOWLEDGMENTS

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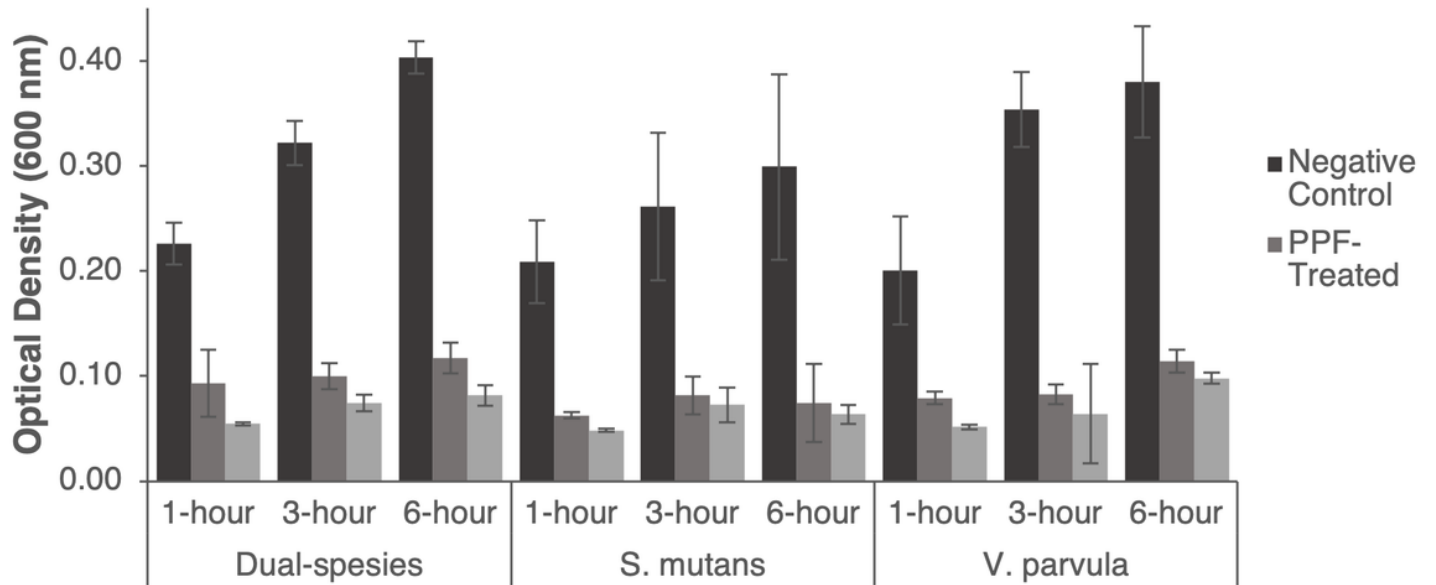
Figures

Effect of Treatments on Biofilm Mass

Figure 1

Biofilm mass of each groups

S. mutans, Streptococcus mutans; V. parvula, Veillonella parvula; CFU, colony forming unit; PPF, propolis fluoride
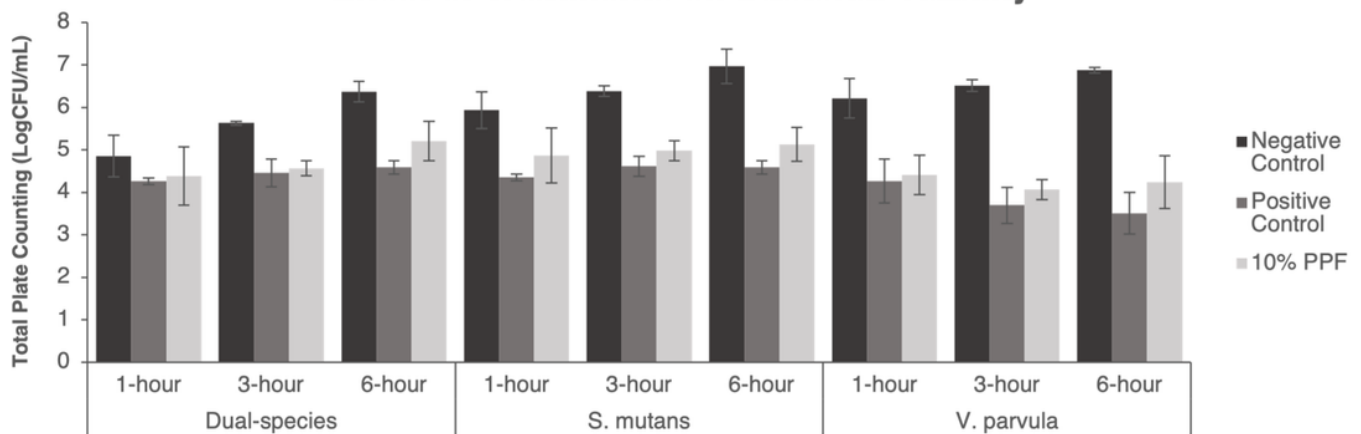
Effect of Treatments on Bacterial Viability

Figure 2

Effect of treatments on bacterial viability

S. mutans, Streptococcus mutans; V. parvula, Veillonella parvula; CFU, colony forming unit; PPF, propolis fluoride
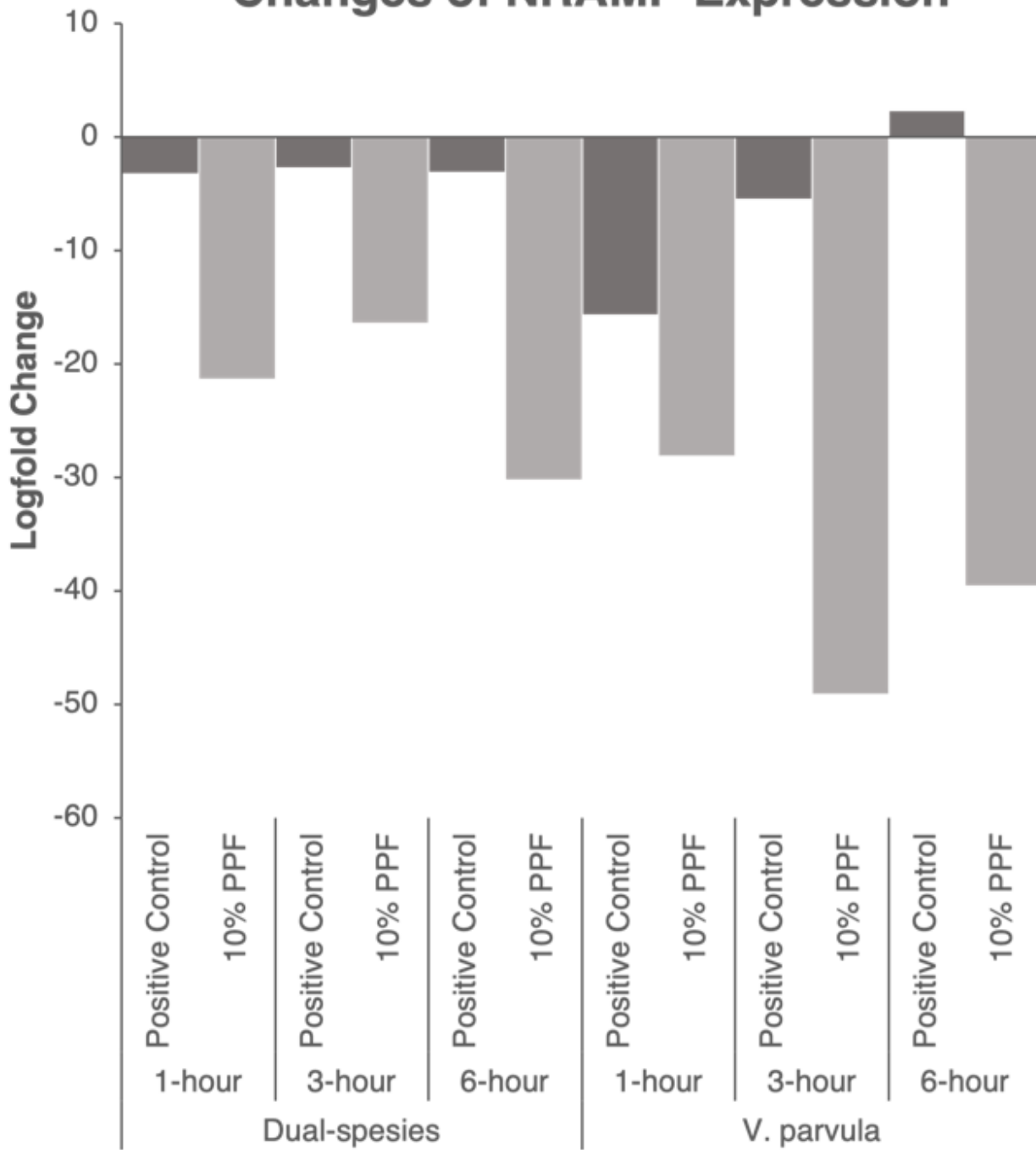
Figure 3

NRAMP relative expression across samples calibrated to negative controls

S. mutans, Streptococcus mutans; V. parvula, Veillonella parvula; CFU, colony forming unit; PPF, propolis fluoride
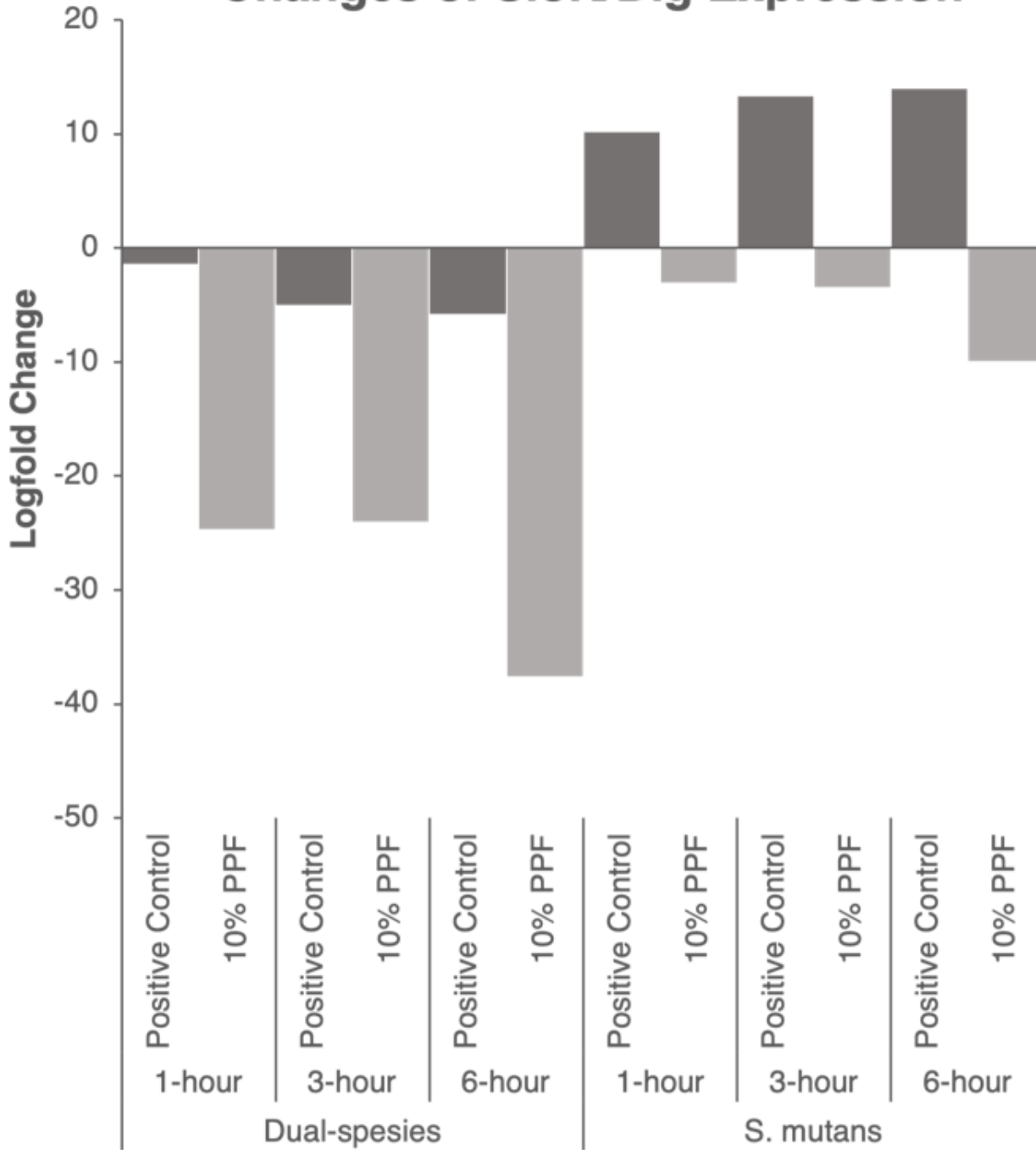
Changes of SloR/Dlg Expression

Figure 4

SloR/Dlg relative expression across samples calibrated to negative controls

S. mutans, Streptococcus mutans; V. parvula, Veillonella parvula; CFU, colony forming unit; PPF, propolis fluoride