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Manuka Honey as an Adjunct to Nonsurgical Periodontal Treatment: A Pilot Study

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KEYWORDS

root surface debridement, antimicrobial, Porphyromonas gingivalis, polymerase chain reaction

ABSTRACT

This pilot study evaluated the effect of manuka honey as a subgingival adjunct to scaling and root surface debridement in the treatment of periodontitis. This study used a split-mouth design with a 3-month follow-up in seven participants diagnosed with periodontitis Stage III Grade B or C. Root surface debridement was performed on one side of the mouth (control); the other side received debridement plus manuka honey application (test). Clinical parameters were recorded at baseline, 6- and 12-weeks. Gingival crevicular fluid and subgingival plaque were sampled. Microbiological outcomes were analysed using benzoylarginine *p*-nitroanilide assay and polymerase chain reaction assay. Single application of manuka honey to periodontal pockets did not result in additional reduction of pocket depth, improvement of attachment levels or changes in *p*-nitroaniline enzymes when compared with root surface debridement alone. However, test sites exhibited greater reduction in bleeding than control sites, mean differences 1.3 (95%CI 1.2-1.5) and 1.7 (95%CI 1.5-1.9) at 6-weeks and 12-weeks, respectively. The proportion of mutans streptococci decreased at 6-weeks in test sites but increased at 12-weeks in control sites. Adjunctive application of manuka honey to periodontal pockets improved gingival inflammation but did not demonstrate significant clinical benefits compared with root surface debridement alone.

INTRODUCTION

Periodontal disease is one of the most common oral diseases in the world [1, 2]. It is a non-resolving inflammation affecting periodontal tissues with irreversible tissue loss manifested as gingival recession, deep pockets, tooth mobility and alveolar bone resorption [3, 4]. Established disease, characterised by the presence of deep pockets of ≥6 mm, affects 10% to 15% of adults worldwide [5].

Root surface debridement (RSD) is the accepted conventional therapy for treatment of periodontitis; microbial biofilm is removed from the root surfaces, allowing favourable healing manifested as reduction of probing pocket depths (PPD) and improvement of clinical attachment levels (CAL) [6]. However, the efficacy of RSD may be compromised in deep pockets, in areas inaccessible to instrumentation (narrow pockets, furcations) and due to the anatomical topography of root surfaces (root concavities) [7]. This leads to a risk of incomplete removal of biofilm and subgingival deposits which may compromise treatment outcomes. Locally-delivered pharmacological interventions may support scaling and root surface debridement by suppressing or eliminating pathogenic microorganisms [8] and modulating the inflammatory response [9] to facilitate healing of periodontal tissues. Thus, adjunctive treatment may provide additional clinical benefits to RSD and reduce the need for periodontal surgery.

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Locally-delivered antimicrobials reach the target site in a predictable manner, whereas systemic antimicrobials are dependent on factors such as the absorption and distribution of the drug, its susceptibility to metabolic degradation, and eventual excretion from the body [10]. Nevertheless, for local antimicrobials to be successful, an effective local delivery system must be developed in order to maintain therapeutic concentrations for a sufficient duration of time [11]. Local antimicrobials (antiseptics and antibiotics) such as chlorhexidine, azithromycin, doxycycline, metronidazole, minocycline, and tetracycline have demonstrated additional clinical improvements of -0.407 mm (95%CI -0.48, -0.33) in PPD and -0.310 mm (95%CI -0.40, -0.22) in CAL [8]. The clinical benefits of adjunctive local antimicrobial agents were considered inconclusive previously, but more recent evidence suggests significant beneficial outcomes [12].

Whilst local antibiotic therapy is effective, there is increasing concern about the overuse of antibiotics and the development of bacterial resistance [13]. Resistance by microorganisms associated with periodontal diseases, such as *Porphyromonas gingivalis*, *Prevotella spp.*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans,* against antibiotics such as amoxicillin, clindamycin and metronidazole, has been reported [14, 15]. There is growing interest in the development of alternative antibacterial substances, such as products derived from plants; one such alternative is manuka honey.

Manuka honey contains various compounds with accepted antimicrobial and tissue healing properties [16, 17]. In addition, the mechanisms of action responsible for the antibacterial properties of manuka honey [18], hypothetically reduces the risk of bacterial resistance. The dominant antimicrobial compound found in manuka honey is methylglyoxal, which is present in high concentration relative to other medicinal honeys, and positively correlated to the level of non-peroxide antibacterial activity (NPA) [19, 20]. Methylglyoxal has antibacterial activity against planktonic gram-positive and gram-negative bacteria at 1.1 to 1.8 mM and against multi-drug-resistant bacteria at 7 mM [20, 21].

Manuka honey with NPA>20 is active *in vitro* against a variety of dental biofilm-associated microorganisms. Gram-negative bacteria associated with periodontal diseases are more sensitive than the gram-positive bacteria implicated in gingival health [22, 23]. Clinical studies in humans have shown encouraging outcomes in reduction of plaque accumulation and gingival inflammation [24, 25]. Therefore, the aim of this study was to evaluate the effect of manuka honey administered as subgingival adjunct to scaling and root surface debridement in the treatment of periodontitis.

MATERIALS AND METHODS

Experimental design

This pilot study used a split-mouth design with a three-month follow-up interval. Participants were recruited from a pool of patients referred to the Periodontology Postgraduate Clinic, School of Dentistry, University of Otago from 2014 to 2015. Ethical approval was obtained from the Southern Health and Disability Ethics Committee (14/STH/18). The study was conducted according to the guidelines of the Declaration of Helsinki 1975 and registered with The Australian New Zealand Clinical Trial Registry (ACTRN) UTN U1111-1153-4716. Participants gave informed consent following a detailed explanation of the study that described the purpose, the benefits and the associated possible risks.

Inclusion criteria for participants were: (i) age ≥ 18 years old; (ii) diagnosed with periodontitis [26] (iii) two non-molar teeth in contralateral quadrants with ≥ 2 periodontal pockets of ≥5 mm. Exclusion criteria were: (i) had taken antibiotics within the last 6 months; (ii) were pregnant or breastfeeding; (iii) had been diagnosed with a medical condition that would affect treatment, including uncontrolled diabetes mellitus, being immunocompromised, needing antibiotic prophylaxis, or undergoing bisphosphonate therapy; (iv) moderate to heavy smokers(those who smoke >10 cigarettes per day); (v) were allergic to pollen or bee products.

Clinical examinations were recorded at baseline, 6 weeks, and 12 weeks. Samples of gingival crevicular fluid (GCF) and subgingival plaque were collected at baseline and at 1, 6 and 12 weeks (Figure 1).

Data collection

Demographic information (age, ethnicity, gender and smoking status), medical, dental and social history were obtained from dental records and patient questionnaires. Clinical measurements were recorded on a form customised for the study. All teeth, except third molars, were examined but molars were excluded from the analysis comparing clinical outcomes of test and control teeth.

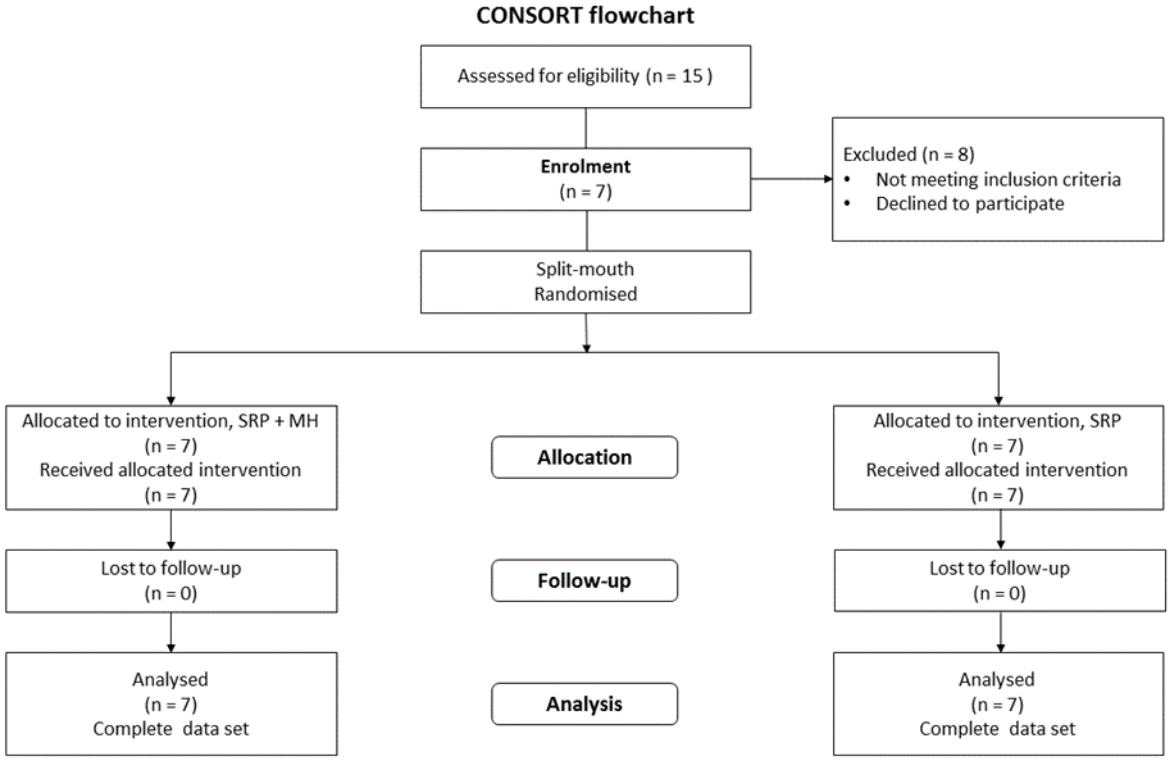

Figure 1 Study flowchart

Examiner calibration

Prior to the start of the study, examiner alignment was undertaken between a junior periodontist (SHS) and a senior periodontist (WD) [27]. Clinical examiners were aligned for evaluating probing pocket depth (PPD) and clinical attachment level (CAL). The discrepancies of measurements were discussed and standard criteria were agreed upon.

Examiner alignments were performed prior to the study and before the 6 weeks follow-up. Duplicate measurements were collected with at least one-hour intervals between the recordings. Analysis using kappa scores indicated examiner reproducibility for PD (κ = 0.941), and CAL (κ = 0.910) at ±1mm precision. Reproducibility at "perfect precision" were κ = 0.418 and κ = 0.449 for PD and CAL, respectively. Participants involved in the examiner calibration were not included in the main study.

Clinical examination

All clinical examinations were performed by one periodontist (SHS). The periodontal parameters recorded were plaque scores (PS), modified gingival index (MGI), bleeding on probing (BOP), PPD and CAL. Teeth were stained with plaque disclosing agent (GC Tri Plaque ID Gel, GC Corporation, Tokyo, Japan), visually examined and plaque was scored dichotomously. BOP was assessed as bleeding from the bottom of the pocket within 20 sec after probing, and calculated as the percentage of sites. All teeth except third molars had PS recorded at four sites (mesial, distal, buccal and palatal/ lingual) and all other parameters at six sites (mesial, mid and distal; bucally and lingually) using a UNC 15 periodontal probe (Hu-Friedy, Chicago, IL, USA).

PPD was measured from the gingival margin to the base of the pocket. Gingival recession (REC) was measured from gingival margin to either the cemento-enamel junction or the cervical margin of a restoration. Both measurements were rounded to the nearest millimetres. CAL was calculated as PPD added to REC. MGI was recorded only on test and control teeth. The grading criteria for MGI were: (0) absence of inflammation; (1) mild inflammation; slight change in colour and little change in texture of any portion of, but not entire marginal or papillary gingival unit; (2) mild inflammation; criteria as for (1) but involving the entire marginal or papillary gingival unit; (3) moderate inflammation; glazing, redness, oedema, and/ or hypertrophy of the marginal or papillary gingival unit and; (4) severe inflammation; marked redness, oedema and/ or hypertrophy of the marginal or papillary gingival unit, spontaneous bleeding, congestion or ulceration.

Sampling of gingival crevicular fluid (GCF) and subgingival plaque

Sample teeth were carefully cleaned to remove supragingival plaque prior to sampling. Only one site per tooth was sampled. When a tooth had more than one site with PD ≥5 mm, the deepest site was sampled for the microbiological evaluation.

A sterile endodontic paper point (size #30) was inserted into the selected periodontal pocket for GCF sampling and kept in place for 30 sec, then transferred into a microcentrifuge tube. Sampling was repeated after 90 sec if the first sample was contaminated with blood [28].

Subgingival biofilms were sampled from periodontal sites using a Gracey curette. A curette was gently inserted into the periodontal pocket and three strokes were employed at each site and wiped on a sterile endodontic paper point, which was then placed in a microcentrifuge tube. Immediately after collection, the samples were kept on ice and transported for storage within two hours. Samples were collected before root instrumentation at baseline, and at one week, six weeks and 12 weeks. All samples were stored in a freezer at -80°C until assayed.

Periodontal treatment

Oral hygiene instruction was provided at baseline and consisted of guidance on toothbrushing, flossing and the use of interdental brushes. Participants were instructed not to use mouthwash for the duration of the study. Appropriate interdental cleaning aids were supplied to all participants. Oral hygiene instructions were reinforced at follow-up appointments according to individual requirements.

RSD was performed under local anaesthesia and completed within one or two appointments on two consecutive days. Scaling was performed using an ultrasonic scaler (EMS™ Piezon® Mini-Master, Nyon, Switzerland) and root surface debridement was carried out using Gracey curettes (LM Instruments TM Oy, Parainen, Finland) until smooth root surfaces were achieved. Test and control quadrants were randomly assigned by a computer-generated number sequence following RSD. Test sites received RSD followed by subgingival application of manuka honey whereas the control sites received only RSD. Manuka honey NPA25+ (Watson & Son, New Zealand) was slowly administered using a 5 mL syringe with an 18G blunt-ended needle to the base of the pocket until it overflowed. Participants were asked to refrain from rinsing and drinking for at least 30 minutes after honey application and were instructed not perform interdental cleaning for one week, after which time they resumed interdental cleaning.

At six weeks follow-up, RSD was repeated at sites with PD ≥5 mm but without repeat application of manuka honey at test quadrants. Professional mechanical plaque removal (PMPR) was performed as necessary. All treatments were performed by the same clinician who performed the clinical measurement. The study timeline is depicted in Figure 2.

Adverse conditions

Information on side effects was solicited at each follow-up appointment. Participants were asked whether they developed either sensitivity or discomfort/ pain in the treated sites. All treated sites were examined for disease pathology (caries and root surface sensitivity).

Level of trypsin-like enzymes in gingival crevicular fluid

A trypsin-like enzyme assay was performed using *N*α-benzoyl-L-arginine *p*-nitroanilide (BAPNA, Sigma Aldrich, St. Louis, MO, USA) as a colorimetric substrate [29]. Trypsin-like enzymes cleave BAPNA substrate and release the chromophore, *p*-nitroaniline which is measured by spectroscopy.
Tris HCl (50 mM), pH 7.4 (150 µL) was added to each tube containing a GCF sample and incubated at 37ºC for 10 min. The tubes were then vortexed for three minutes and the paper points were removed. The reaction was initiated by adding 50 µL of BAPNA (10 mM) and incubated for 1 hour at 37° C. The absorbance was measured at 412 nm using a plate reader (Biotek Instruments Synergy 2, Vermont, NE, USA). The concentration of *p*-nitroaniline was calculated using the Beer-Lambert law equation, $A = \varepsilon lc$. The molar extinction coefficient for *p*-nitroaniline is 8,800 $M^{-1}cm^{-1}$. The concentration of *p*-nitroaniline represents levels of trypsin-like enzymes.

DNA extraction

Sterile phosphate-buffered saline (500 µL) was added to each microcentrifuge tube containing subgingival plaque and maintained at room temperature to elute for 1 h. The tubes were then vortexed for three minutes and the paper points were removed. Bacterial DNA was extracted using InstaGene™ matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. The concentration and purity of DNA was determined spectrophotometrically (Nano-Vue™ UV, GE Healthcare, Little Chalfont, Buckinghamshire, UK).

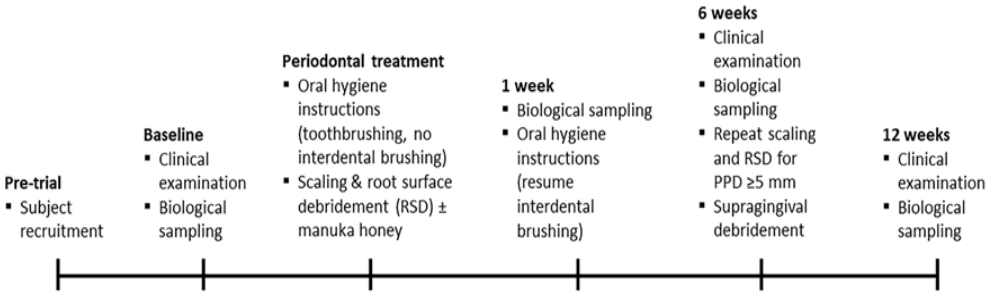

Figure 2 Study timeline

DNA extraction

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Polymerase chain reaction (PCR)

The universal 16S rRNA target region was used to estimate the total bacteria in each sample [30]. The glucosyltransferase (gtf) genes, specific for *Streptococcus mutans* (gtf-S) and *Streptococcus sobrinus* (gtf-T) were amplified using primers (Sigma-Aldrich) previously published [31]. All primers were cross-examined against all sequenced nucleotide collection using Nucleotide BLAST (National Center for Biotechnology Information) to ensure specificity (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

PCR reaction master mix was prepared according to the manufacturer's instructions (Invitrogen™; Thermo Fisher Scientific, North Shore City, New Zealand). A 20 µL reaction was made up of 1 µL extracted bacterial DNA in a 19 µL reaction mix. Amplification of the DNA was carried out in a thermal cycler (T100) following a pre-defined programme recommended for the PCR reaction (Invitrogen). The PCR products (10 µL) were loaded onto a 2.0% agarose gel using loading buffer and electrophoresis was carried out for 40 min at 100V. DNA Ladder (100 bp/1000bp; Thermo Fisher Scientific) was used as a gene ruler to estimate the size of products. The specificity of the primers to detect *S. mutans* and *S. sobrinus* was validated using purified DNA from a variety of oral streptococci. For the 16SRNA gene, the PCR products were resolved on a 0.7% agarose gel.

Gels were scanned (Image Lab Viewer, Bio-Rad, Auckland, New Zealand) and analysed using ImageJ software (National Institutes of Health). The relative proportions (%) of mutans streptococci were calculated as the ratio DNA concentrations of mutans streptococci to that of the concentrations of total bacterial DNA (16S RNA) from the subgingival plaque samples.

TaqMan Duplex qPCR assay

A qPCR assay using TaqMan duplex (Life Technologies, New Zealand Limited, Auckland, New Zealand) was used for the detection and quantification of single-copy gene of 16S RNA for bacteria and arginin-specific cystein-proteinase gene (rgp) for *P. gingivalis* [32, 33]. The analysis of 16S RNA gene was to quantify bacterial genomic DNA (gDNA) in the samples. The assay has been validated for analysis of plaque samples.

The master mix contained 2x of TaqMan master mix, 20 µM of each forward and reverse primers (Sigma-Aldrich), 50 µM of DNA probes (Sigma-Aldrich) and RNase-free water to make up a 20 µL reaction mix per well. Two positive controls were prepared: (1) gDNA, a cocktail of DNA purified from *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4, (2) *P. gingivalis*, DNA prepared from an overnight culture of *P. gingivalis* ATCC 33277. The assay was performed in a 96-well PCR array plate (Life Technologies), containing 5 µL gDNA template in a 15 µL reaction mix. All samples were diluted to a final concentration of 10 ng/µL and were analysed in duplicates. The quantification was performed using a thermal cycler (Quant Studio™ 6 Flex PCR machine, Applied Biosystems by Life Technologies) with a pre-defined programme; 10 min at 95°C (hot start), and 40 cycles of 15 s at 95°C and 1 min 15 s at 60°C. Threshold cycles (Cq)

were calculated using Quant Studio™ 6 and 7 Flex Real-Time PCR system software.

Statistical analysis

Data analysis was performed using GraphPad Prism Version 8.0.0 (GraphPad Software, San Diego, California USA). Continuous variables were presented as mean and standard deviation and categorical data as percentages. Normality of sample distribution was examined using the Shapiro-Wilk test. Clinical data were analysed using student's t-test for intragroup comparisons and repeated measures ANOVA for intergroup comparisons. Microbiological data were analysed using Wilcoxon Signed-Rank test for intra-group comparisons. For inter-group group comparisons, Mann-Whitney U test was employed. The level of significance of 0.05 was set for this study.

RESULTS

All participants (n=7) completed the study. Participants were aged between 33 and 69 years (mean age= 54.4 ± 12.2 years). Demographic details and baseline clinical characteristics of the participants are presented in Table 1. The participants were diagnosed with Stage III Grade B or C periodontitis as indicated by mean full-mouth CAL of 4.1 (± 1.7), PD of 3.6 (± 1.6). Of the total of n = 173 teeth, 83 teeth had 185 sites with periodontal pockets ≥5 mm (Table 1). Full-mouth BOP (FMBOP) and Full-mouth PS (FMPS) were 35.9% (±18.9) and 53.6% (±14.3), respectively. No adverse condition was reported throughout the study.

Table 1 Demographic and baseline clinical characteristics of participants

Patient characteristics	n = 7
Mean age (±SD) years	54.4 ±12.2
Age range (years)	33 – 69
Male, female (n)	M:2, F:5
Ethnicity (n)	
European	5
Maori	1
Asian	1
Non-smoker (n)	6
Current smoker (n)	1
Clinical measurements	
Total teeth	173
Avg no of teeth/patient, mean±SD	24.7±1.6
No of teeth with PPD >4mm (%)	83 (48)
Plaque score, mean±SD	53.6±14.3
Bleeding on probing, mean±SD	35.9±18.9

Clinical outcomes

A total of 24 teeth with 144 sites representative of the test sites (RSD+MH) (n=12 teeth) and control sites (RSD) (n=12 teeth) were analysed. Mean clinical outcomes of the test and control sites and the differences between baseline and follow-ups (six weeks and 12 weeks) for MGI are displayed in Table 2. The test sites demonstrated greater reduction in MGI than the control sites (p< 0.001) with mean differences of 1.3 (95% CI 1.2-1.5) and 1.7 (95% CI 1.5-1.9) at six weeks and 12 weeks, respectively.

Table 3 shows mean (±SD) and frequency distributions of PPD and the changes over 12 weeks. The test and control sites exhibited significant reductions of PPD over time at 6 weeks and 12 weeks. However, there was no difference between the treatments (p= 0.804).

Table 4 shows mean (±SD) and frequency distributions of CAL and the changes over 12 weeks. Both treatments demonstrated significant CAL improvement at 6 weeks and 12 weeks but there was no difference between the two treatments (p= 0.615).

Tooth level and subject level analyses

PPD and CAL were further analysed at tooth and subject levels for teeth that demonstrated pocket measurements ≥5 mm at baseline. Changes in PPD and CAL that were ≥2mm were compared between test and control groups after 6 weeks and 12 weeks (Table 5). At tooth level and subject level, the outcomes for PPD and CAL between RSD+MH and RSD were not statistically different at 6 weeks (p> 0.05) or at 12 weeks (p> 0.05) (Table 5).

Microbiological outcomes

Samples were collected from 24 sites representing 12 test (RSD+MH) and 12 control (RSD) sites at each follow-up.

Trypsin-like enzyme activity

Figure 3 presents the concentrations of *p*-nitroaniline (nM) generated by each GCF sample indicating the relative levels of trypsin-like enzyme before and after treatments. The enzyme levels in the test sites were significantly decreased at 6 weeks (p= 0.002) and 12 weeks (p= 0.004) following treatment whereas the levels in the control sites were significantly reduced after 1 week (p= 0.005), 6 weeks (p= 0.005) and 12 weeks (p= 0.002). Comparing the two groups, the test and control treatments did not differ at any time point (p> 0.05) (Figure 3).

Table 2 Mean modified gingival index (MGI) at baseline (BL), 6 and 12 weeks and the difference between the treatment sites (teeth = 48)

Clinical outcome	RSD Mean (±SD)	RSD + MH Mean (±SD)	Mean difference (95% CI)	*p*-value
MGI				
Baseline	2.9 (±1.0)	2.9 (±1.0)		
6 weeks	1.7 (±0.9)	1.5 (±0.9)	1.3 (1.2, 1.5)	< 0.001
12 weeks	1.3 (±0.8)	1.1 (±0.7)	1.7 (1.5, 1.9)	< 0.001

	RSD			RSD + MH		
	Mean difference	95% CI	*p*-value	Mean difference	95% CI	*p*-value
Δ BL-6 weeks	1.2	1.0, 1.4	< 0.001	1.4	1.3, 1.6	< 0.001
Δ BL-12 weeks	1.6	1.4, 1.8	< 0.001	1.8	1.7, 2.0	< 0.001
Δ 6-12 weeks	0.4	0.2, 0.5	< 0.001	0.4	0.2, 0.5	< 0.001

Table 3 Comparison of probing depth (PPD) and proportion of sites (%) of various PPD categories at baseline, 6 and 12 weeks and the differences between the treatments

Clinical outcome	RSD	RSD + MH	*p*-value (Between groups)
PPD (mm)	Mean±SD	Mean±SD	
Baseline	3.6±1.7	3.7±1.5	0.666
6 weeks	3.0±1.4*	2.9±1.4*	0.541
12 weeks	2.8±1.4*	2.7±1.3*	0.758
	No of sites (%)	No of sites (%)	
Baseline (n= 144)			
PPD ≤3mm	79 (54.9)	83 (57.6)	
PPD 4-6mm	57 (39.6)	54 (37.5)	
PPD >6mm	8 (5.5)	7 (4.9)	
6 weeks			
PPD ≤3mm	108 (75.0)	112 (77.8)	
PPD 4-6mm	32 (22.2)	29 (20.1)	
PPD >6mm	4 (2.8)	3 (2.1)	
12 weeks			
PPD ≤3mm	109 (75.7)	120 (83.3)	
PPD 4-6mm	32 (22.2)	20 (13.9)	
PPD >6mm	3 (2.1)	4 (2.8)	

Table 4 Changes of clinical attachment level (CAL) before and after periodontal treatments in mean (±SD) and frequency distributions of CAL as percentage of sites

Clinical outcomes	RSD	RSD + MH	*p*-value (Between groups)
CAL (mm)	Mean±SD	Mean±SD	
Baseline	4.1±1.8	4.2±1.7	0.538
6 weeks	3.8±1.5*	3.8±1.5*	0.845
12 weeks	3.6±1.5*	3.8±1.5*	0.442
	No. of sites (%)	No. of sites (%)	
Baseline (n= 144)			
CAL ≤3mm	78 (54.2)	84 (58.3)	
CAL 4-6mm	53 (36.8)	43 (29.9)	
CAL >6mm	13 (9.0)	17 (11.8)	
6 weeks			
CAL ≤3mm	103 (71.5)	109 (75.7)	
CAL 4-6mm	33 (22.9)	25 (17.3)	
CAL >6mm	8 (5.6)	10 (7.0)	
12 weeks			
CAL ≤3mm	106 (73.6)	107 (74.3)	
CAL 4-6mm	29 (20.1)	30 (20.8)	
CAL >6mm	9 (6.3)	7 (4.9)	

*****indicates statistically significant different for changes between baseline and different follow-ups (within group comparison)

Detection of P. gingivalis in subgingival plaque

A subgroup analysis was performed to determine the proportions of *P. gingivalis* in the subgingival plaque and to correlate the proportions to the concentrations of *p*-nitroaniline (Figure 4). Only two (of seven) participants tested positive for *P. gingivalis* at baseline. Both treatments eliminated *P. gingivalis* at 1-week post-treatment and the microorganism remained undetected at 6 weeks and 12 weeks follow-up. *P. gingivalis* was not detected in the pockets of other participants at any time points.

Baseline data were included in the correlation analysis. The concentrations of *p*-nitroaniline indicating levels of trypsin-like enzymes, did not correlate with the relative proportions of *P. gingivalis* (r_s = -0.286, p>0.05).

Table 5 Percentage of sites with clinical improvement of ≥2mm in PPD and CAL at tooth and subject levels

Clinical outcomes	0-6 weeks			0-12 weeks		
	RSD	RSD + MH	*p*-value	RSD	RSD + MH	*p*-value
% (±SD) of sites with ≥2mm of PPD reduction						
Tooth level analysis						
Initial PPD 5-6mm	55.2 (46.3)	62.0 (40.0)	0.662	54.8 (47.8)	66.4 (41.2)	0.467
Initial PPD ≥7mm	44.4 (50.9)	41.7 (58.9)	0.334	55.6 (50.9)	50.0 (70.7)	0.527
Subject level analysis						
Initial PPD 5-6mm	60.0 (39.8)	58.5 (40.5)	0.948	52.3 (43.7)	59.9 (45.5)	0.742
Initial PPD ≥7mm	44.4 (50.9)	41.7 (58.9)	0.767	55.6 (50.9)	50.0 (70.7)	1.000
% (±SD) of sites with ≥2mm of CAL gain						
Tooth level analysis						
Initial CAL 5-6mm	24.6 (32.4)	40.6 (42.3)	0.225	33.6 (35.2)	31.9 (37.6)	0.838
Initial CAL ≥7mm	29.2 (45.2)	60.0 (46.0)	0.163	29.2 (45.2)	68.3 (43.4)	0.075
Subject level analysis						
Initial CAL 5-6mm	23.5 (26.6)	35.8 (28.5)	0.432	37.3 (12.0)	26.2 (25.8)	0.651
Initial CAL ≥7mm	28.9 (34.2)	55.0 (51.2)	0.442	28.9 (34.2)	62.5 (46.8)	0.227

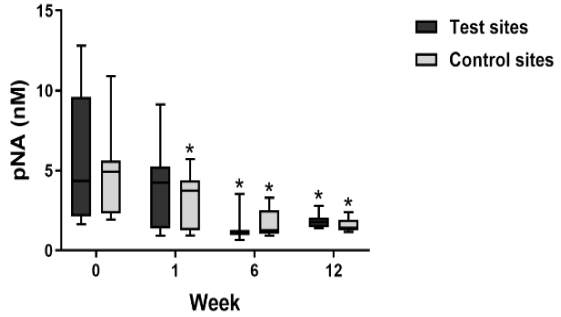

Figure 3 Comparison of trypsin-like enzyme activity (generation of *p*-nitroaniline (*p*NA) in the test (RSD+MH) and control sites (RSD) at 0 (baseline), 1, 6 and 12 weeks. *indicates statistically significant different for changes between baseline and different follow-ups (within group comparison).

Prevalence of mutans streptococci

Percentages of mutans streptococci in periodontal pockets at different time points following periodontal treatments are depicted in Figure 5. There was an increase in mean proportions of mutans streptococci in test sites at one week, however, the difference was not significant due to high variation across the sites being sampled (p> 0.05). In the test sites, a decrease in the proportions of mutans streptococci was observed at 6 weeks (p< 0.01). However, the proportions of mutans streptococci did not differ statistically between baseline and 12 weeks (p> 0.05). By 12 weeks the proportions of mutans streptococci had returned to pre-treatment levels in test sites.

There were no differences for control sites between baseline and 1 week (p> 0.05) or baseline and 6 weeks (p> 0.05). However, proportions of mutans streptococci were significantly increased after 12 weeks (p< 0.01). The differences between groups (RSD compared with RSD+MH) were not significant at 1 week (p> 0.05) but were significantly decreased in the test sites at 6 weeks (p< 0.01) and 12 weeks (p< 0.01).

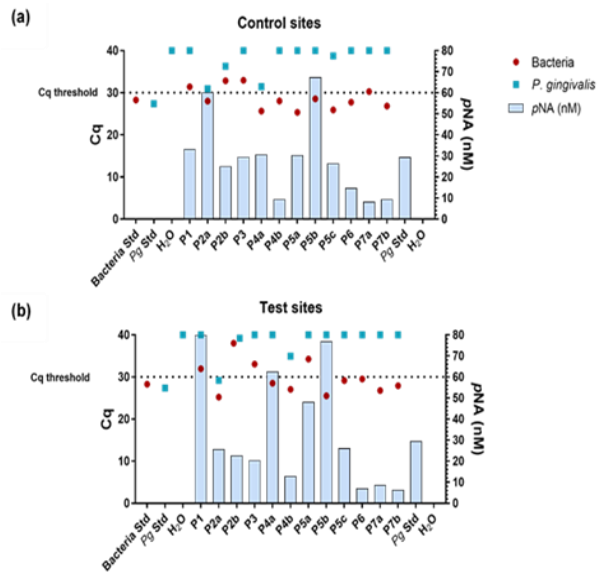

Figure 4 Relative levels of *P. gingivalis* (*Pg*, determined by qPCR) in periodontal pockets and concentrations of *p*-nitroaniline (*p*NA, vertical bar) at baseline: (a) Control sites, (b) Test sites. Cq value of more than 30 was considered as no detection, labelled as Cq threshold.

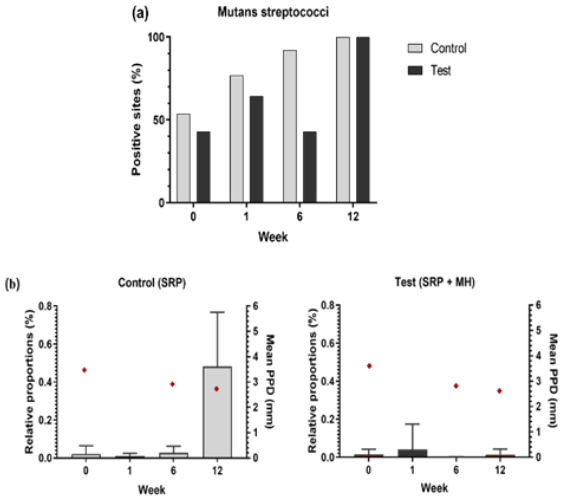

Figure 5 Occurrence of mutans streptococci in periodontal pockets following periodontal treatments. (a) Percentage of mutans streptococci in test and control sites before and after treatments. (b) Relative proportions of mutans streptococci from the positive sites before and after treatments.

◆ indicates mean probing pocket depth

DISCUSSION

To the best of our knowledge, this is the first study to evaluate the adjunctive effect of pure manuka honey as an antimicrobial agent delivered subgingivally following RSD in the treatment of periodontitis. Honey is highly viscous and may provide a natural sustained-release mechanism when administered to periodontal pockets. In addition, there is support for the antibacterial efficacy of manuka honey against plaque-associated bacteria [22, 23] and for the use of manuka honey in the intra-oral environment [24].

Even though manuka honey is highly viscous, its substantivity in periodontal pockets may be compromised by continuous flushing of gingival crevicular fluid, rendering it ineffective. Our findings demonstrate that a single application of manuka honey NPA>25 to periodontal pocket as an adjunct to RSD did not provide a clinically significant benefit to reduction of pocket depths or improvement of attachment levels when compared with RSD alone.

Restricting analysis to a threshold for clinical improvement of ≥2 mm in PPD revealed a similar effect for both treatment groups. For CAL, there was a trend of improvement in the test group, especially for sites with advanced attachment loss (initial CAL ≥7 mm). However, the difference was not significant due to high variation across samples at tooth and subject levels analysis. This effect might be attributable to the potential healing properties of manuka honey [34]. On the other hand, meta-analyses of various locally-delivered antimicrobials, including chlorhexidine (chip, varnish), doxycycline, metronidazole, minocycline and tetracycline fibres reported significant additional benefit with weighted mean differences of -0.328, -0.413, -0.573, -0.157, -0.472 and -0.727, respectively [8]. Even though manuka honey demonstrated antibacterial effect *in vitro* [34], results from the present study indicated that manuka honey is not as effective as other local antimicrobial agents when applied to periodontal pockets as an adjunct to RSD.

Conversely, the present findings demonstrated significant improvement of marginal gingival inflammation in the test sites (RSD+MH) compared to the control sites (RSD). This result is in agreement with the outcomes reported by English et al. (2004) [24]. However, this study employed an alternative mode of delivery. English and co-workers (2004) used manuka honey NPA>15 prepared as "honey leather" and the participants were instructed to chew the honey leather for 10 minutes three times a day for 21 days [24]. In the present study, manuka honey NPA>25 was administered directly to periodontal pocket following RSD. Furthermore, the present study included subjects with periodontitis Stage III Grade B or C, whilst English et al. (2004) tested subjects with gingivitis [24]. Therefore, the findings are not directly comparable and cannot be generalised. A recent study by Opšivač and co-workers (2023) investigated the effects of commercially available manuka honey mixed with hydrogen peroxide (Pocket Protect™, CleverCool®, Lijnden) as an adjunct to PMPR and RSD in subjects with generalised periodontitis Stage III [35]. They exhibited significant reduction of PPD and improvement of CAL in the test sites (PMPR+RSD+MH+H_2O_2) after 3, 6 and 12 months, however the differences were not significant when compared to the control sites (PMPR+RSD) [35]. These findings and the findings from the present study exhibit similar trend of clinical outcomes following application of manuka honey to deep periodontal pockets.

A microbiological parameter was chosen to estimate the substantivity of manuka honey in periodontal pockets. Concentration of *p*-nitroaniline chromophore generated by gingival crevicular fluid was measured to evaluate the effect of manuka honey on suppression/ elimination of trypsin-like enzyme-producing periodontal microorganisms. The concentration of *p*-nitroaniline was within the moderate to weak range, possibly due to the dilute nature of the

samples. Even after RSD, the concentration of *p*-nitroaniline did not significantly decrease after 1 week but did after 6 weeks. The level reached a plateau after 6 weeks with no further reduction at 12 weeks. It is possible that the effect of the active ingredient(s) within manuka honey was achieved even after the manuka honey was no longer present. The present clinical finding corroborates the *in vitro* investigation that demonstrated the effectiveness of MH against microorganisms associated with periodontitis such as *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* [22].

It is important to note a trend of higher proportions of mutans streptococci observed at test sites (RSD+MH) after one week. During this time, participants were asked to refrain from performing interdental brushing, so that manuka honey was not immediately removed from the periodontal pockets. These findings suggest that manuka honey selectively alters the subgingival flora to favour mutans streptococci, when placed into sites with compromised plaque control. The *in vitro* investigation demonstrated that *Streptococcus mutans* is relatively resistant to manuka honey (NPA>20). Furthermore, the acidic environment resulting from manuka honey has been shown to cause demineralisation of hydroxyapatite beads [23]. Another factor to consider is the expected half-life of a pharmacological agent in the gingival crevice which is about one minute [36]. Goodson (1989) estimated that the fluid present in a 5 mm periodontal pocket is replaced about 40 times per hour [36]. When the concentration of manuka honey is exponentially diluted, the antimicrobial activity will also reduce to subinhibitory levels, thus promoting bacterial carbohydrate metabolism and consequent acid production by bacteria, resulting in further demineralisation. Manuka honey is effective at higher concentration but loses its antimicrobial effect at lower concentrations (<13% w/v) [23]. As a result of the diminishing concentration, the antimicrobial effect will be reduced whilst the remaining sugars will provide substrates for metabolic activity of the plaque-associated microorganisms, posing a risk to root integrity. Even though no side-effects implying occurrence of demineralisation were reported in the present study, the risk of demineralisation with honey application into periodontal pocket cannot be completely ruled out in the light of the *in vitro* findings [23].

Relative proportions of mutans streptococci from the sites positive for these microorganisms were significantly higher in the pockets of the control sites than the test sites, at 12 weeks post-treatment. Parallel to this outcome was a reduction of pocket depth measurement from a mean of 3.6 mm before periodontal treatment, to a mean of 2.8 mm after treatment at 12 weeks. Even though the results demonstrated increased level of cariogenic species, this may also suggest that shallow pockets provide a favourable environment for recolonization by gram-positive streptococci associated with gingival health [37].

The present study was conducted using a split-mouth design. The advantage of a split-mouth study was that it reduced inter-individual variability over comparison of two groups of individuals. However, there is a concern that the treatment performed in the treated sites may have affected the control sites within the same mouth, termed as “carry-across effects”. Carry-across effects to the control sites have been reported [38, 39]. Although carry-across effects are possible, the determination whether it occurred or did not is difficult [40]. We believe that this effect might be possible around supragingival area. However, since the periodontal pocket is a concealed environment [41] it may not directly influence the healing process subgingivally.

A three-month duration of follow-up was considered sufficient to observe changes of clinical outcomes based on the effect of single application of an antimicrobial agent. The expected improvement should be seen between 6 weeks and 12 weeks [42]. Further clinical improvements due to the application of adjunctive materials were not expected beyond 12 weeks. The steady improvement in plaque scores that we observed have a strong influence on positive clinical outcomes, independent of any putative adjunctive agent. Dahlén et al. (1992) have shown that consistent plaque control practice of <20% improved periodontal outcomes; markedly reduced gingivitis scores, maintained PD of the shallow pockets and improved PD of 5-7 mm [43].

The present study had small sample size, however the findings from these *in vitro* investigations and this clinical study collectively provide evidence that subgingival application of honey may provide some clinical benefits but may also pose an increased risk of demineralisation of root surfaces. Therefore, the results from the present study do not support the use of manuka honey as an adjunctive local antimicrobial to scaling and root surface debridement.

CONCLUSIONS

Adjunctive application of manuka honey to periodontal pockets improved gingival

inflammation but did not demonstrate significant clinical benefits for reduction of pocket depth or improvement of attachment level, in comparison to scaling and root surface debridement alone. Therefore, the findings do not support the use of manuka honey as a local delivery device to treat periodontitis.

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DECLARATION OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Petersen PE, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C. The global burden of oral diseases and risks to oral health. Bull World Health Organ. 2005; 83:661-669.
2. World Health Organization. Oral health. Geneva: WHO; 2012. Available from: http://www.who.int/mediacentre/factsheets/fs318/en/. Accessed March 2017.
3. Lindhe J, Ranney R, Lamster I, Charles A, et al. Consensus report: chronic periodontitis. Ann Periodontol. 1999; 4:38-38.
4. Page RC, Offenbacher S, Schroeder HE, Seymour GJ, Kornman KS. Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. Periodontol 2000. 1997; 14:216-248.
5. Petersen PE, Ogawa H. The global burden of periodontal disease: towards integration with chronic disease prevention and control. Periodontol 2000. 2012; 60:15-39.
6. Cobb CM. Clinical significance of non-surgical periodontal therapy: an evidence-based perspective of scaling and root planning. J Clin Periodontol. 2002; 29:s22-32.
7. Jepsen S, Deschner J, Braun A, Schwarz F, Eberhard J. Calculus removal and the prevention of its formation. Periodontol 2000. 2011; 55:167-188.
8. Matesanz-Pérez P, García-Gargallo M, Figuero E, Bascones-Martínez A, Sanz M, Herrera D. A systematic review on the effects of local antimicrobials as adjuncts to subgingival debridement, compared with subgingival debridement alone, in the treatment of chronic periodontitis. J Clin Periodontol. 2013; 40(3):227–241.
9. Oringer RJ. Modulation of the host response in periodontal therapy. J Periodontol. 2002; 73:460-470.
10. Genco RJ. Antibiotics in the treatment of human periodontal diseases. J Periodontol. 1981; 52:545-558.
11. Needleman IG, Martin GP, Smales FC. Characterisation of bioadhesives for periodontal and oral mucosal drug delivery. J Clin Periodontol. 1998; 25:74-82.
12. Tan OL, Safii SH, Razali M. Clinical efficacy of single application local drug delivery and adjunctive agents in nonsurgical periodontal therapy: A systematic review and network meta-analysis. Pharmaceutics. 2020; 12:1086.
13. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010; 74:417-433.
14. Almaguer-Flores A, Moreno-Borjas JY, Salgado-Martinez A, Sanchez-Reyes MA, Alcantara-Maruri E, Ximenez-Fyvie LA. Proportion of antibiotic resistance in subgingival plaque samples from Mexican subjects. J Clin Periodontol. 2006; 33:743-748.
15. Ardila CM, Granada MI, Guzmán IC. Antibiotic resistance of subgingival species in chronic periodontitis patients. J Periodontal Res. 2010; 45:557-563.
16. Alvarez-Suarez JM, Gasparrini M, Forbes-Hernández TY, Mazzoni L, Giampieri F. The composition and biological activity of honey: a focus on Manuka honey. Foods. 2014; 3:420-432.
17. Oryan A, Alemzadeh E, Moshiri A. Biological properties and therapeutic activities of honey in wound healing: a narrative review and meta-analysis. J Tissue Viability. 2016; 25:98-118.

18. Kwakman PH, de Boer L, Ruyter-Spira CP, Creemers-Molenaar T, et al. Medical-grade honey enriched with antimicrobial peptides has enhanced activity against antibiotic-resistant pathogens. European J Clin Microbiol Infect Dis. 2011; 30:251-257.
19. Adams CJ, Manley-Harris M, Molan PC. The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey. Carbohydr Res. 2009; 344:1050-1053.
20. Mavric E, Wittmann S, Barth G, Henle T. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium) honeys from New Zealand. Mol Nutr Food Res. 2008; 52:483-489.
21. Hayashi K, Fukushima A, Hayashi-Nishino M, Nishino K. Effect of methylglyoxal on multidrug-resistant Pseudomonas aeruginosa. Front Microbiol. 2014; 5:180.
22. Schmidlin PR, English H, Duncan W, Belibasakis GN, Thurnheer T. Antibacterial potential of Manuka honey against three oral bacteria in vitro. Swiss Dent J. 2014; 124:922-924.
23. Safii SH, Tompkins GR, Duncan WJ. Periodontal application of manuka honey: Antimicrobial and demineralising effects in vitro. Int J Dent. 2017; ID9874535.
24. English HK, Pack AR, Molan PC. The effects of manuka honey on plaque and gingivitis: a pilot study. J Int Acad Periodontol. 2004; 6:63-67.
25. Aparna S, Srirangarajan S, Malgi V, Setlur KP, Shashidhar R, Setty S, Thakur S. A comparative evaluation of the antibacterial efficacy of honey in vitro and antiplaque efficacy in a 4-day plaque regrowth model in vivo: preliminary results. J Periodontol. 2012; 83:1116-1121.
26. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, Flemmig TF, Garcia R, Giannobile WV, Graziani F, Greenwell H. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. J Periodontol. 2018; 89:S173-82.
27. Hefti AF, Preshaw PM. Examiner alignment and assessment in clinical periodontal research. Periodontol 2000. 2012; 59:41-60.
28. Chapple ILC, Brock GR, Milward MR, Ling N, Matthews JB. Compromised GCF total antioxidant capacity in periodontitis: cause or effect? J Clin Periodontol. 2007; 34:103-110.
29. Shah HN, Gharbia SE. Lysis of erythrocytes by the secreted cysteine proteinase of Porphyromonas gingivalis W83. FEMS Microbiol Lett. 1989; 61:213-217.
30. Baker GC, Smith JJ, Cowan DA. Review and re-analysis of domain specific 16S primers. J Microbiol Methods. 2003; 55:541-555.
31. Hoshino T, Kawaguchi M, Shimizu N, Hoshino N, Ooshima T, Fujiwara T. PCR detection and identification of oral streptococci in saliva samples using gtf genes. Diagn Microbiol Infect Dis. 2004; 48:195-199.
32. Morillo JM, Lau L, Sanz M, Herrera D, Martín C, Silva A. Quantitative real-time polymerase chain reaction based on single copy gene sequence for detection of periodontal pathogens. J Clin Periodontol. 2004; 31:1054-1060.
33. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiol. 2002; 148:257-266.
34. Vandamme L, Heyneman A, Hoeksema H, Verbelen J, Monstrey S. Honey in modern wound care: A systematic review. Burns. 2013; 39:1514-1525.
35. Opšivač D, Musić L, Badovinac A, Šekelja A, Božić D. Therapeutic Manuka Honey as an Adjunct to Non-Surgical Periodontal Therapy: A 12-Month Follow-Up, Split-Mouth Pilot Study. Mater. 2023; 16(3):1248.
36. Goodson JM. Pharmatokinetic principles controlling efficacy of oral therapy. J Dent Res. 1989; 68:1625-1632.
37. Kumar PS, Leys EJ, Bryk JM, Martinez FJ, et al. Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. J Clin Microbiol. 2006; 44:3665-3673.
38. Goodson JM, Offenbacher S, Farr DH, Hogan PE. Periodontal disease treatment by local drug delivery. J Periodontol. 1985; 56:265-272.
39. Kaldahl WB, Kalkwarf KL, Patil KD, Dyer JK, Bates Jr RE. Evaluation of four modalities of periodontal therapy: mean probing depth, probing attachment level and recession changes. J Periodontol. 1988; 59:783-793.
40. Hujoel PP, DeRouen TA. Validity issues in split-mouth trials. J Clin Periodontol. 1992; 19:625-627.

41. Schroeder HE, Listgarten MA. The gingival tissues: the architecture of periodontal protection. Periodontol 2000. 1997; 13:91-120.
42. Van der Weijden GA, Timmerman MF. A systematic review on the clinical efficacy of subgingival debridement in the treatment of chronic periodontitis. J Clin Periodontol. 2002; 29(Suppl 3):55-71.
43. Dahlén G, Lindhe J, Sato K, Hanamura H, Okamoto H. The effect of supragingival plaque control on the subgingival microbiota in subjects with periodontal disease. J Clin Periodontol. 1992; 19:802-809.

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