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Investigating the Antibacterial and Antibiofilm Potential of Honey on Plaque Bacteria.

A Thesis

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ABSTRACT

Aim and Hypothesis: The aim of the study was to compare the potential antibacterial and antibiofilm formation of Revamil® and manuka honey solutions alone as well as in combination. The hypothesis being tested is that the combination of Revamil® with manuka will have more antibacterial and antibiofilm formation than Revamil® and manuka used alone.

Materials and method: The antimicrobial properties of Revamil®, manuka, a 1:1 mixture of both as well as a sugar control were tested at 50, 25, 12.5, 6.25, 3.12, 1.56 % (w/v) concentrations against four plaque-associated bacteria: *Streptococcus mutans (S. mutans), Streptococcus salivarious, Streptococcus sanguinis, and Aggregatibacter actinomycetemcomitans.* The minimum bacteriostatic concentrations (MIC) and the half maximal inhibitory concentration (IC50) were determined for each testing solution. Methylglyoxal (MGO) titration assay with 6.25% Revamil® was used to recreate the synergy of the combined honey. A biofilm disruption assay was performed using different concentrations of honey on *S. mutans, S. sanguinis, A. actinomycetemcomitans* and a mixture of all the bacteria including *S. salivarius.* Finally, the pH of the honey solutions at all the tested concentrations was determined. Kruskal-Wallis test with pair wise Mann-Whitney U test was utilized to compare between the groups.

Results: The results indicated that manuka and the combined honey solution had more efficacy than Revamil® on all tested organisms. *A. actinomycetemcomitans* was more sensitive to all tested honey solutions with MIC of 6.25% compared to the other bacteria that had MICs ranging from 12.5 to 25%. The synergy analysis revealed that experimental additivity exceeded theoretical additivity (p <0.001). The MGO analysis showed a

potentiating effect when MGO was mixed with 6.25% Revamil® compared to MGO alone. The biofilm disruption assay yielded insignificant results except for the multi-species biofilm in which manuka had more effect on biofilm disruption than the combined honey (p =0.08). The pH analysis revealed that all used solutions aside from 50 and 25% (pH 4-6) were on the neutral side with a pH =7.

Conclusion: Although the sugar control had antibacterial effect, this study highlights the bactericidal properties of both Revamil® and manuka, which is enhanced when both honeys are combined regardless of the sugar content and pH. Similarly, MGO bactericidal activity is enhanced when combined with Revamil®.

DEDICATION

This work is dedicated to the love of my life Aya, for her kindness and devotion, and for her endless support throughout all the hard times, her selflessness and sacrifice will always be remembered.

And to my son Kamal his smile lights up my world.

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Introduction

1. DENTAL PLAQUE AND ANTIBIOTIC THERAPY

It is well documented that the transformation of "healthy" oral biofilms into pathological ones is strongly associated with major dental diseases; dental caries, periodontal diseases, and peri-implantitis [1]. Dental caries is defined as a destructive process that affects dental hard tissues and if not treated, may progress to pulpitis and its sequala. Increased numbers of *Streptococcus mutans, Streptococcus sobrinus,* and *Lactobacillus* species are strongly implicated in the initiation and progression of carious lesions [2]. Plaque bacteria is considered the primary etiologic factor for periodontal diseases pathogenesis [3]. The inflammatory process combined with the immune response may lead to loss of attachment, and that may ultimately lead to losing the supportive structures around the affected teeth, and eventual loss of those teeth. The accumulation of dental plaque species *Porphyromonas gingivalis, Prevotella intermedia,* and *Aggregatibacter actinomycetemcomitans* are strongly implicated in advanced periodontal disease. The subsequent inflammatory response combined with immune system contribute to the loss of attachment [4].

Many reviews have stated that antimicrobial agents used alone or in combination can improve the outcomes of mechanical therapy [5-7] Several systemic antibiotics have been proven to be effective in specific types of periodontitis; tetracyclines, metronidazole, clindamycin, and Augmentin can be used with success in the treatment of aggressive periodontitis [8]. Systemic antibiotic therapy though has some success, also has some limitations; the fact that periodontal infection arises from bacteria in biofilms is the reason behind most of these limitations [9]. Biofilms are nearly 1000 times more resistant to antibiotics and the immune system than planktonic bacteria [10, 11]. Another limitation is

microbial resistance, which is a serious concern for the health care community [12, 13]. The speed of occurrence of antimicrobial resistance cannot be predicted, and, and every time a new antimicrobial agent is invented, resistance eventually occur [14]. New antimicrobial agents are needed, yet there is always the chance resistance might emerge to those agents [15]. innovative scientific efforts to investigate and create novel compounds to be used in addition to conventional antibiotic therapy [16].

2. COMPLEMENTARY AND ALTERNATIVE MEDICINE

By definition, complementary and alternative medicine is a various group of medical and healthcare practices, therapies, and products that are not considered as a part of traditional western medicine [17]. Complementary and alternative medicine has gained popularity in recent years among the public, encouraging dental and other healthcare researchers to explore the existing science of complementary and alternative medicine [18]. Complementary and alternative medicine includes : aromatherapy, acupuncture, hydrotherapy, massage therapy, meditation, herbal therapy, and apitherapy [19]. Apitherapy is the art and science of utilizing honeybee products such as : honey, bee pollen, bee venom, propolis, and royal jelly for sustaining health and wellness [20]. The term apitherapy comes from the word ‘apis’ which means bee in Latin language [21]. Apitherapy was tracked back more than 6000 years in ancient Egypt. Also, the use of bee products was described by Hippocrates, Aristotle, and Galen, indicating that it was used by The Romans and Greeks [22].

3. THERAPEUTIC POTENTIALS OF HONEY

Honey is a supersaturated sugar solution with high viscosity which is derived from the nectar that is gathered and modified by the honeybee, Apis mellifera [23]. The medicinal potential of honey as a wound dressing has been recorded in the world's ancient literature, including the Holly Quran, circa 590 AD. "the Lord taught the bee to build its cells on trees and in men's habitations, then to eat all the products of earth and find with skill the spacious paths of its Lord, there issues from within their bodies a drink of varying colors, wherein is healing for men, verily in this is a sign for those who give thought" [24, 25]. The contemporary interest in the therapeutic use of honey, and especially manuka honey as an antibacterial agent owes much to the pioneering efforts of the New Zealand researcher, Peter C. Molan. His contributions extending from 1991 to 2015 - some 163 publications - provided much of the interest and guidance for what is known today. Honey has been known to have antimicrobial as well as wound-healing properties [26]. A case report by Natarajan et al. showed a healing of a leg ulcer in a 47-year-old immunocompromised Caucasian woman using wound dressing that was made with manuka honey [6]. In a systematic review by Jull et al. 19 trials with cumulative sample size of 2554 patients were reviewed and concluded that healing times in mild to moderate superficial and partial thickness burns might be improved using honey in comparison to some traditional dressings [23].

Recently, honey has been 'rediscovered' by the medical research and clinical community especially where modern conventional therapeutic agents fail to work as intended [27]. Honey has been reported to contain about 200 substances. Carbohydrates, in forms of sugars, constitute 70 to 80%, and are the major component. Honey contains approximately 18%

water and trace amounts of amino and organic acids, proteins, enzymes, pigments, minerals, flavonoids and antibacterial factors [28, 29].

It has been proved that honey has antimicrobial potential against many bacteria such as *Staphylococcus aureus* [30, 31], *Pseudomonas aeruginosa* [32] and *Helicobacter pylori* (*H. pylori*) [33],viruses like *Influenza viruses* [34, 35] and fungi like *Candida albicans* [30-36]. The honey's antibacterial effect was first described in by Dustmann in 1979. Dustmann examined various types of honey composition and tested them on *Staphylococcus aureus*, *Streptococcus* species, *Salmonella pullorum*, *S. gallinarum*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Sarcina lutea*, and *Proteus vulgaris*. He concluded that antibacterial activity of honey differs substantially according to the honey type [37].

To date, there are several publications reporting antibacterial properties of honey in vitro with a focus on pathogenic bacteria; Cooper et al. examined honey effect on coagulase-positive *Staphylococcus aureus*, that were isolated from infected wounds, using manuka and pasture honey. The results indicate that both have similar inhibitory values [38]. Schneider et al. examined the antibacterial effect of manuka honey and portobello honey on *S. aureus, P. aeruginosa*, and *E. coli* by agar disc diffusion assay and broth culture assays. Both kinds of honey showed antibacterial properties [16]. Alandejani et al. assessed the effect of manuka honey from New Zealand and Sidr honey from Yemen on *P. aeruginosa* and *S. aureus*. They concluded both were effective against these bacteria in vitro. *H. pylori* sensitivity to honey was also tested [39]. Al Somal et al. demonstrated that *H. pylori* isolates from biopsies of gastric ulcers. were more sensitive to manuka honey than other mono-floral honey solutions with only hydrogen peroxide activity. A concentration of 5% (v/v) of manuka honey,

exhibited complete inhibition of growth within 72 hours using the agar well diffusion assay [33].

Studies were also conducted on viruses and fungi. Ansari et al. evaluated the antifungal properties of Jujube honey (a local honey from Al-Baha, Saudi Arabia) on *Candida albicans.* They concluded that Jujube honey has antifungal activity against *Candida albicans* at a concentration of 40% (w/v) [36]. On the other hand, Lusby et al. found that honey had no effect on *Candida albicans* and one bacteria *Serratia marcescens.* The authors suggest that this result might be due to the concentration used in the experiment; five concentrations ranging from 0.1 to 20% [40].

Watanabe et al. examined the antiviral effect of manuka honey alone or combined with zanamivir/oseltamivir, known antiviral agents, on *Influenza viruses*. In their study honey had a viricidal effect on Influenza viruses and showed a synergetic effect when combined with antiviral agents thus reducing their dosage [34].

This interest in manuka honey has attracted the interest of the dental research community Aparna et al. examined the antimicrobial effect of honey in vitro and in vivo. The in vitro study tested honey, chlorohexidine and saline on six oral bacteria *Eubacterium nodatum, S. mutans, Campylobacter rectus, S. sangiunis, A. actinomycetemcomitans,* and *P. gingivalis*. The result showed that although honey inhibited all six strains, chlorohexidine had the lowest MIC. The in vivo study was a double-blinded clinical trial, in which 66 subjects (20-24 years of age) were screened and baseline plaque score was recorded. After four days of using either chlorhexidine, honey or saline mouthwash another plaque score was recorded and compared with the baseline score. They found that honey mouthwash did inhibit/ reduce plaque formation [18]. Schmidlin et al. tested seven honey products on *S. mutans, P. gingivalis* and

A. actionmycetemcontains in vitro. The results showed that manuka honey that had an NPA (non-peroxide activity) value higher than 15 exhibited a significantly higher antibacterial effect. Also, all manuka honey products were more effective in inhibiting the growth of *P. gingivalis* than *S. mutans* and *A. actinomycetemcomitans* [41]. Eick et al. used manuka and domestic beekeeper honey to assess the antimicrobial effect on *P. gingivalis* in a planktonic growth and in a mono-species biofilm. They found that 2% manuka honey inhibited 50% of *P. gingivalis* compared to domestic beekeeper honey which was 5%. Both kinds of honey at a concentration of 10% inhibited the formation of biofilm and reduced the numbers of viable bacteria in 42-hour biofilms. However, complete inhibition and eradication of bacteria from 42-hour biofilms were not established [42].

4. HONEY AND QUORUM-SENSING AND BIOFILM FORMATION

The ability of honey to counter bacterial infections stems from two main components: bactericidal compounds and inhibition of quorum-sensing [43]. Quorum sensing is an intercellular communication among bacteria that controls gene expression in response to population density [44, 45]. In quorum-sensing bacteria release autoinducers, for example, N-acyl homoserine lactones (AHLs) in Gram-negative bacteria that enables a single bacterial cell to sense the surrounding population density [46]. When the cellular density reaches a certain level, expression of target genes encoding virulence factors and biofilm initiation is triggered [47].

Truchado et al, evaluated quorum-sensing inhibition of 29 uni-floral kinds of honey using the bacterial model *Cromobacterium violaceum*. The evaluation tested honey's ability to inhibit AHLs production. The results showed that all honey solutions tested showed significant

quorum-sensing inhibition at very low concentrations of 0.1g/ml. They found out that quorum-sensing inhibition potential varied according to the floral origin [48]. Wang et al. [43], used sub-inhibitory concentrations of honey on *Pseudomonas aeruginosa*. Their assays revealed that the expression of MvfR, las and rhl regulons, which are the two-main quorum-sensing systems for *Pseudomonas aeruginosa,* were inhibited by low concentrations of honey. Additionally, this study showed that sugar content also plays an inhibitory role [43].

5. HONEY AND DENTAL CARIES

Honey is viscous solution with a high sugar content with pH of 3.1 to 4.5, these qualities render honey to be considered cariogenic [23, 49, 50]. This can be problematic and may hinder the usage of honey in oral preparations. However, honey was proven to inhibit the growth and viability of *S. mutans*, the main implicated organism in dental caries [51-53]. Many studies attempted demineralization assays using manuka honey, natural beekeepers honey [54, 55]. Since the oral environment is complicated by the presence of the teeth in addition to the cariogenic properties of honey, a fear rose that honey preparations might cause dental caries. Several studies tackled this point by trying to study the antimicrobial effect of honey on *S. mutans* and assess the demineralization power of honey on the tooth or tooth-like structures. Nassar et al. compared natural honey and artificial honey effect on *S.mutans'* growth, viability, and biofilm formation. They found out that natural honey had more antimicrobial properties in terms of inhibiting effects than artificial honey [52]. Ghabanchi et al. tested commercialized honey (Khomein Honey, Iran) on *S. mutans* strains isolated from unstimulated saliva from seventy participants. A carbohydrate solution with sugar content mimicking the composition of natural honey was used as a negative control.

The study concluded that honey is bacteriostatic against *S. mutans*. The minimum inhibitory concentration (MIC) value was 75% (v/v) [53]. Badet and Quero explored the effect of manuka honey on growth and adherence of *S. mutans*. The effect on growth was assessed via MIC and minimum bactericidal concentration (MBC) using macro-dilution, while the effect on the adherence was evaluated on growing *S. mutans* alone or in a multi-species model on an angulated glass surface and saliva-coated hydroxyapatite discs. The results for the MIC and MBC were comparable with other studies [56]. As for the adhesion, the results showed that honey had a weak effect on the adherence to the MIC value, however, increasing the concentration above MIC yielded better effect on the adherence of *S. mutans*. However, Safii et al. tested manuka honey and white clover honey on various plaque bacteria. All the tested strains were sensitive to honey except for *S. mutans* which showed some resistance [55].

The literature on the demineralizing effect of honey on enamel is somewhat scarce. Safii et al. examined the demineralization effect of honey on hydroxyapatite beads (HA), with and without *S. mutans* for 24 hours. The results showed that honey alone caused demineralization on HA beads when compared to the negative control (saline). According to the authors this might be due to the low pH rather than to a biological process. The addition of *S. mutans* potentiated the demineralizing effect of honey at lower concentrations when compared to adding honey alone [55]. In Motamayel et al. study, 36 extracted caries- free pre-molars were used to assess the demineralizing effect of natural honey in comparison to two different sugars (glucose and fructose). Teeth were placed in tubes containing different solutions of either honey, glucose or fructose mixed with *S. mutans*. After 21 days, teeth were sectioned and examined. Honey had the lowest mean value of demineralization depth

when it was compared to glucose and fructose but the combination of the two was not used in such assays [54].

6. ANTIMICROBIAL ACTIVITY OF HONEY

The antimicrobial activities of honey can be attributed to its high osmolarity, acidic pH, hydrogen peroxide (H_2O_2), phenolic acids, flavonoids, methylglyoxal (MGO) and derived antibacterial peptide defensin-1(def-1) [30, 34, 35, 57, 58]. These antimicrobial mechanisms cannot be found in a single type of honey [59]. Among the spectrum of different honey types, manuka and Revamil® stand out to have different antimicrobial compounds and action. Also, the only licensed honey preparations are manuka and Revamil® which are sterilized with γ-irradiation for medical use.

6.1. Manuka honey

Manuka honey is mainly found in New Zealand and Australia, produced from *Leptospermum scoparium*, also known as the manuka bush. Manuka honey has high MGO content with no def-1 [59, 60]. MGO is formed during the conversion of dihydroxyacetone (DHA), a compound thatg is deived from nectar, during the ripening of honey [61]. DHA is found at very high concentrations in the nectar of *L. scoparium* flowers, however, the reason behind this high concentration is yet to be known. MGO is found in great amounts in manuka honey (828 mg/kg) when compared to other honey types (24 mg/kg) that did not come from the manuka bush [62]. MGO can react with DNA, RNA, and proteins, and in theory it could be toxic to mammalian cells. However, there is no evidence of toxicity to mammalian cells when manuka honey is consumed orally or used topically [63]. Manuka honey is infamously

known for its variability, the number of antimicrobial compounds and activity varies from batch to batch [59, 64].

The antimicrobial potential of manuka is commonly known as (Unique Manuka Factor) UMF™. The UMF ™ is based on the antimicrobial activity of manuka against *S. aureus* [59].

6.2. Revamil® honey

Revamil® honey, made in a controlled production process in greenhouses, contain relatively high levels of bee def-1 and H_2O_2 and only a trace amounts of MGO [59]. Compared to manuka, Revamil® is more consistent in terms of composition from batch to batch which is a wanted quality in clinical usage [65]. Bee def-1 (formerly known as a royalisin) is produced from the hypopharyngeal gland of the bee and mainly used in honey and royal jelly production, however, its concentration varies considerably among the different types of honey [66, 67]. Bee def-1 is a broad acting peptide that was thought to be part of bees immune system [64]. A study by Bucekova et al. found that def-1 in royal jelly triggered the secretion of Matrix metallopeptidase-9 by human keratinocytes and promoted keratinocyte migration in vitro [68].

Thus, the idea of creating a honey solution that has the combined effect of MGO and def-1 can be beneficial to have a broader spectrum of antimicrobial and anti-inflammatory properties.

Aim and Hypothesis:

1. Aim

The objective of the study was to compare the potential antibacterial and biofilm disruption of Revamil® and manuka honey used alone as well as in combination.

2. Hypothesis

The combination of Revamil® with manuka will have more antibacterial and antibiofilm potential than Revamil® and manuka used alone.

Materials and Methods

Bacterial strains and inoculum preparation:

Bacterial strains were purchased from American Type Culture Collection (ATCC), (Manassas/ Virginia). Four bacterial strains were used: *Streptococcus salivarius* (ATCC® 13419™), *Streptococcus sanguinis* (ATCC® BAA-1455™), *A. actinomycetemcomitans* (ATCC® 33384™) and *S. mutans* (ATCC® 25175™). *S. mutans* and *S. sanguinis* were grown on Brain Heart Infusion agar, while *A. actinomycetemcomitans* and *S. sanguinis* were grown on Tryptic soy agar with 5% defibrinated sheep blood. All bacteria were grown at $37^{\circ 0}$C aerobically except *A. actinomycetemcomitans* was grown aerobically with 5% CO_2. The quadrant streak plate method was utilized to obtain discrete bacterial colonies. After 24-48 hours of cultivation, the final inoculum was made by adjusting the optical density at 600 nm. A bacterial stock was made by inoculating 2ml of 50% glycerol solution and stored at -80 ˚C for future use.

Honey samples

Medical grade sterile honeys were obtained for this study; Medihoney Paste (100% manuka honey) was purchased from The Betty Mills Company, Inc. (San Mateo, California). Revamil® gel was acquired from Farmacia Loreto (Napoli, Italy). The combined honey was made by mixing equal amounts of 50% manuka and Revamil®. A honey stock solution of 50 % (w/v), was prepared by dissolving 5 g of honey in 10 mL of Brain Heart Infusion (BHI) broth. Honey solutions were stored at 4°C in a dark environment until use. Honey solutions were placed at 37 ˚ C one hour before use, to reduce their viscosity.

A solution mimicking the sugar composition of 50% honey was prepared and used as a control. The solution was made by dissolving 1.665g glucose, 1.925g fructose, 0.365g sucrose and 0.310g maltose in 10 mL BHI broth. Then the solution was filter sterilized using 0.22 µm syringe filter (Nalgene Nylon Sterile Syringe, 0.22 µm Filter, MedLab Supply, Pompano Beach, FL).

Agar diffusion assay

Agar plates were prepared and standardized to a uniform thickness. An overnight liquid culture was adjusted to an OD_{600} of 0.08 using a spectrophotometer. The agar plates were inoculated with 100 µL of the adjusted inoculum and placed in an incubator for 30 min. A cork borer was used to punch-out 5 mm wells on each agar plate. Each well received 100 µL of honey solutions at 25, 50 and a 100% concentration. For positive and negative controls, a 100 µL of 0.12% chlorhexidine (CHX) and phosphate buffered saline (PBS), were added into separate wells in each plate. The plates were stored for 3 hours at 4^{o}C, and +then were incubated at 37° ^{0}C. After 24 -48 hours, zones of inhibition were measured using a spring caliper (Miltex Iwanson spring caliper, Net32, Cary, NC).

Minimum inhibitory concentration (MIC) and IC50 determination

Micro-dilution was used to determine MIC. A 96-well plate (Fisherbrand™ 96 well plates sterile, Fisher Scientific, Leicestershire, UK) was used for each strain. Each strain (6 replicates) was tested with the four honey solutions. Honey solutions were prepared the day of the experiment by serial dilution from the stock solution and the following concentrations were used 50, 25, 12.5, 6.25, 3.12, 1.56 and 0%. Each well received 200 µL of honey

solution. The bacterial inoculum was made from an overnight liquid culture that was adjusted to an OD_{600} of 0.005 using spectrophotometer, which is the equivalent to 0.5 McFarland standards (approximately 1.5 X 10^8 colony-forming units per milliliter [CFU/mL]). Then 10 µL of the adjusted inoculum were added to the wells. The plates were incubated at 37ºC. After 24 hours, the microplates were read using an ELISA reader (Thermo Labsystems Multiskan EX Microplate Reader, Beverly, MA) to determine bacterial growth. The MIC90 values, which are the lowest concentration of a compound that can inhibit the growth of 90% of the tested organisms were determined [67].

Role of MGO

MGO was obtained from Sigma-Aldrich (St. Louis, MO) and diluted to 40, 20, 10, 2.5, 1.25, 0.625 and 0.312% and mixed with 6.25% Revamil®. Two plates for each bacterium were made, one with Revamil® and the other without. Then 200 µL of diluted MGO solution. were added to the wells followed by 10 µL of the adjusted inoculum OD_{600} 0.005. The plates were incubated at 37 ˚C. After 24 hours, the microplates were read using an ELISA reader.

Biofilm disruption assay

The qualitative determination of biofilm disruption was made according to the method described by Stepanovic´ et al. [69]. For this part, only three bacteria were used *S. mutan, S. sanguinis,* and *A. actinomycetemcomitans* as mono-species biofilm assay. A combination of these bacteria was also used including *S. salivarious* as a multi-species biofilm assay. Each bacterium was tested in six replicates. A 96-well microplate was used, each well received 200 µL of overnight bacterial inoculum and 20 µL of 2.5% sucrose solution except

for the control wells that received bacteria alone. The plates were covered with breathable plate seals (Breathe-Easy® sealing membrane, Sigma-Aldrich) and incubated at 37 ˚C anaerobically. Plates that had *A. actinomycetemcomitans* and multiple bacteria were incubated aerobically with 5% CO_2. After 48 hours, the content of each well was carefully aspirated and washed twice with 250 µL sterile deionized distilled water to remove any planktonic bacteria. Afterwards the wells received 200 µL of 25%, the calculated IC50, or 1.56% of Revamil®, manuka, or the combined honeys. Sugar control was tested at 50, 25, 12.5, 6.25, 3.12 and 1.56%. Then the plates were re-sealed and placed back in the corresponding incubators. After 24 hours, the content of each well was carefully aspirated and washed three times with 250 µL of sterile deionized distilled water; the plates were shaken with washes to remove nonadherent bacteria. Each well received 200 µL of 99% methanol to fix the remaining attached bacteria, and after 15 min, the plates were emptied and left to dry. Afterwards, each well was stained for 10 min with 50 µL of 2% Hucker crystal violet. The excess stain was aspirated, and the wells were washed six times with PBS. The plates were air dried and 200 µL of 33% (v/v) glacial acetic acid was added to each well to solubilize the adherent cells. The OD was measured at 600 nm using an ELISA reader.

pH determination of honey solutions

The pH of each solution was determined using pH strips (Universal pH Indicator Strips, Range 0-14, Carolina Biological Supply Company, Burlington, NC). The pH was measured three times for all the testing solutions at these following concentrations 50, 25, 12.5, 6.25, 3.12 and1.56% w/v.

Statistical Analyses

Data were expressed as means, standard deviations, medians and interquartile ranges. The outcome variables were optical density (OD) and percent (%) inhibition which were measured on a continuous scale. Normality of the data was assessed using histograms; homogeneity of variance was assessed using Levene's test. Both assumptions of homogeneity of variance and normality of distribution were violated.

Therefore, comparisons of OD and % inhibition between the groups were made using Kruskal-Wallis test after splitting the data into groups by concentrations. Pairwise Mann-Whitney U tests for statistically significant results found following Kruskal-Wallis test. For MGO titration Mann -Whitney U test was utilized. All p-values less than 0.05 were considered statistically significant except for the pairwise tests, which considered results to be statistically significant for p-values < 0.017. All analyses were performed using SPSS Version 24. All graphs were made using GraphPad Prism Version 7.

Results

Agar Diffusion assay

The bactericidal effect of manuka was tested against *S. mutans* and *S. sanguinis* using two concentrations of manuka honey 50 and 100% (w/v) as a preliminary testing. Figure 1 shows that at 50% (w/v) manuka did not inhibit the bacterial growth, mean ± SD zone of inhibition (0.0 ± 0.0) for both bacteria. The 100% (w/v), however, created zone of bacterial inhibition of 15.0 ± 0.0 and 13.73 ± 0.37 for *S. mutans* and *S. sanguinis,* respectively. Since low concentrations of honey did not show any zone of inhibition and therefore not useful in determining MIC and IC50, a different assay was used. The micro-dilution assay was chosen for MIC and IC50 determination.

Minimum inhibitory concentration (MIC_{90}) and IC50 determination

The microdilution assay using Revamil®, manuka, and the sugar control was performed with *S. mutans, S. sanguinis, S. Salivarious,* and *A. actinomycetemcomitans*. Microdilution was the best option since it did not require large amounts of honey and it covered a wide concentration range. Figure 2 shows that the curves of Revamil, manuka and sugar controls followed a similar pattern. The MIC and IC50 were determined for each bacterium (Table 1, 2). The data for *S. mutans* show that there was a statistically significant difference between Revamil® and manuka ($p = 0.004$) at the 12.5% concentration and between Revamil® and the sugar control ($p = 0.004$) at the 12.5% and 50% concentrations (Table 3). Additionally, there was a statically significant difference between manuka and the sugar control ($p = 0.004$) at the 12.5% concentration (Table 3). For *S. sanguinis*, there was a statistically significant difference between Revamil® and manuka at concentrations of 6.25 and 12.5% (p

= 0.004). Furthermore, there was a significant difference at 3.12, 6.25 and 25% (p =0.004), 12.5 (p = 0.016) between Revamil® and the sugar control, also there was a statically significant difference between manuka and the sugar control (p = 0.004) at 3.12, 6.25 and 12.5% (Table 4). As for *S. salivarious*, there was a statistical significance difference between Revamil® and manuka (p = 0.004) at the 12.5% concentration and between Revamil® and the sugar control at 1.56 (p =0.004), 3.12 (p = 0.004), 12.5 (p = 0.006), 25 (p = 0.004) and 50% (p = 0.003). There was a statistically significant difference between manuka and the sugar control (p = 0.004) at the 1.56, 3.12, 12.5 and 50% concentrations (Table 5). For *A. actinomycetemcomitans*, there was a statistical significance between Revamil® and manuka at 6.25% concentration (p = 0.004) and between Revamil® and the sugar control for all tested concentrations (p = 0.004). Similarly, there was a statistically significant difference between manuka and the sugar control (p =0.005) for 6.25% and (p =0.004) for the rest of the concentrations tested (Table 6). The MIC and IC50 data suggest that the combined honey had the most antibacterial potential followed by manuka. Revamil® and the sugar control had the least effect.

Synergystic effect of mixing Manuka with Revamil®

In order to evaluate the synergy of combining manuka and Revamil®, the theoretical additivity of the combined honey was extracted from the data obtained from Revamil and manuka in microdilution assays. The theoretical additivity values were calculated by summing the inhibitory values of manuka and Revamil® at a given concentration. The obtained values were compared with experimental additivity values observed form the combined honey testing. The comparison was made at a chosen concentration (3.12% for *A.*

actinomycetemcomitans and 6.25% for the rest of the test bacteria) the reason behind selecting these concentrations is any higher concentration the synergy will not appreciated as all tested solutions had above 80% of growth inhibition. Additionally, the experimental additivity of the combined honey was compared with the sugar control at the same sugar concentration (6.25% of combined honey was compared with 12.5% sugar control) (Figure 3). The data shows that the experimental additivity of the combined honey was statistically significantly higher than the theoretical additivity for all bacteria ($p < 0.001$). The experimental additivity of the combined honey was statistically significantly higher than the sugar control for *S. sanguinis, A. actinomycetemcomitans,* and *S. sanguinis*. ($p = 0.002$, 0.009, 0.009; respectively). However, it was not statistically significant for *S. mutans* ($p = 0.65$). The difference between the theoretical additivity and the sugar control was significant for *S. mutans* ($p = 0.002$) and *S. sanguinis* ($p = 0.002$), but not significant for *S. salivarious* ($p = 0.589$) and *A. actinomycetemcomitans* (p =0.818) (Table 7).

These data show that there is a true synergy when both honeys are combined, which is believed to be due to other factors, not just the added sugar content.

Role of MGO

To further investigate the synergy shown in the microdilution assay, an MGO titration assay was made. MGO is a natural compound found in greater amounts in manuka honey and is produced as a side-product of many metabolic pathways [59]. After the synergy was noted in the previous assay, we hypothesized that MGO might play a pivotal role in that synergy when it is mixed with Revamil®. A titration curve of MGO was used either with or without Revamil® to test this hypothesis. Figure 4 shows that the curves of MGO without Revamil®

and the curves of MGO with Revamil® follow a similar dose dependent curves. The IC50 was calculated (Table 8). The cutoff for inhibition (>95% inhibition) for all tested bacteria was lower by one concentration when MGO was mixed with Revamil compared to MGO alone. The cutoff for *S. mutans* was 5 mM of MGO alone and was 2.5 mM of MGO + Revamil®. For *S. sanguinis* and *S. salivarious,* the cutoff of MGO without Revamil® was 10 mM and 5 mM of MGO with Revamil®. Also, *A. actinomycetemcomitans* had a similar response in which MGO alone inhibited the bacteria at 2.5 mM cutoff and when it was mixed with Revamil® inhibited the organism at 1.25 mM. The difference between MGO alone and MGO with 6.25% Revamil® was tested using Mann-Whitney U test, and the data was statistically significant ($p = 0.013$) for *S. sanguinis* and not statistically significant for the rest of the tested bacteria (Tables 9-12).

The data suggest that MGO contained in manuka honey might be in part responsible for the experimental synergy obtained when manuka was mixed with Revamil®.

Biofilm disruption assay

This assay was used to investigate the disrupting effect of honey on a pre-formed biofilm. Figure 5 display the percent inhibition of Revamil®, manuka, combined honey and the sugar control on 48 hours old biofilms. Manuka had the strongest inhibitory effect, at 25% all mono-species biofilms were disrupted with more than 75% inhibited (Figure 5). Although the multi-species biofilm was not disturbed as the mono-species biofilm only 20% inhibited, 25% (w/v) manuka inhibited the multi-species biofilm the most when compared to the other tested solutions. Revamil® had a lesser inhibitory effect than manuka at 25% (w/v) with less than 70% inhibition in all mono-species biofilms and 10% inhibition in the multispecies

biofilm. On the other hand, 25% (w/v) of the sugar control was far less effective than manuka and Revamil® with percent inhibition less than 50% for mono-species biofilm and less than 5% for multi-species biofilms. The combined honey solution at 25% had percent inhibition values that lies between manuka and Revamil® in the mono-species biofilms, however, it had no effect on multi-species biofilms. The IC50 used each solution (in the multi-species biofilm 10% was used) showed that manuka was also more potent at biofilm inhibition (50-40% inhibition) followed by Revamil® (45-30% inhibition) and then combined honey solution (45-5% inhibition). For the sugar control only 25 and 1.56% were used in the assay. For 1.56% concentration all three honey solutions (manuka, Revamil and the combined honey) had a similar range of % inhibition which was less than 20%, however, the sugar control was the most inhibitory at 1.56% against *S. mutans* (40% inhibition*)* and *S. sanguinis* (25% inhibition). For *A. actinomycetemcomitans* and multi-species biofilms the sugar control had no effect (Figure 5). The difference between all the tested solutions yielded statistically not significant results ($p > 0.05$) for the mono-species biofilm. However, there was a statistically significant difference ($p = 0.08$) for the tested solutions in the assay with multispecies biofilm ($p > 0.05$). *Post-hoc* pairwise Mann-Whitney U test revealed a statistically significant difference between manuka and the combined honey solution in which manuka had more effect on biofilm disruption (Tables 13-16).

These data show that all tested honey had the ability to disrupt biofilms at 25% (w/v) concentration.

pH of honey solutions

The pH was measured to determine if pH had a role in the antimicrobial properties shown by our testing solutions. The recorded pH is shown in Table 17. Most honey preparations were on the neutral side with pH 7.0 readings. The 50% concentrations had slightly lower pH at 4.0 for manuka, 5.0 for the combined honey and 6.0 for both Revamil® and the sugar control.

These findings suggest that pH had a limited role in the antimicrobial effects of honey.

Discussion

This study was conducted to investigate the antibacterial and antibiofilm potential of two major types of honey each having a different mechanism of action. The well diffusion method failed to show any zone of inhibition at the 25 and 50% concentrations. However, at 100% (w/v) a zone of inhibition can be easily detected and measured. One possible explanation is that the 25 and 50% solution might evaporate before diffusing into the agar producing no inhibition of bacterial growth. This is in agreement with the work of Ghabanchi et al. who reported similar results on *S. mutans* using 100% (v/v) Khomein honey. They reported a mean zone of inhibition of 13 mm [53]. Furthermore another study by Patel et al. evaluating the antimicrobial effect of honey on *S. mutans* reported no inhibition at 5%, 10%, 20% and 40% concentrations of the tested honey solutions and only the 60% concentration exhibited a zone of inhibition (10.0 mm) [70]. However, this result contradicts a study by Mathai et al. [71], which showed a minimal zone of inhibition (0.5 ± 0.6 mm) using 100% of commercially available honey on *S. mutans* [71]. Beena et al., on the other hand, had some success using 25% of manuka on *S. mutans* and *Lactobacillus* with mean zone of inhibition of 14.4 and 15.8 respectively [72].

Other studies using different bacteria showed some significant results using lower dilutions; Al Somal et al. tested 20 and 40% (v/v) manuka honey on *H. pylori* and reported zones of inhibition of 22.3 - 25.6 mm [33]. Badawy et al. examined different honey samples (5%–20%) and compared their abilities to inhibit the growth *E. coli* (12 mm – 24 mm) and *Salmonella typhimurium* (0 mm – 20 mm) [73]. These variations might be attributed to the type of honey, the water content of the honey used, type of bacteria being tested and cultivating conditions.

Overall, the agar diffusion assay was not practical, since MIC and IC50 could not be determined using lower concentrations of honey. Therefore, it was decided to perfom a microdilution assay instead.

The MIC and IC50 values obtained in our study indicated that all tested honey solutions have potential bactericidal activities against all tested species. This was similar to other studies. In a study by Basson et al. in which MIC was determined using seven oral streptococci species. Eucalyptus honey used in that study had a MIC of 25 % (v/v) on *S. mutans* and *S. sanguinis* [74]. Another study by Safii et al. showed that medical grade manuka honey had a MIC of 25% on *S. mutnas* [55]. Schmidlin et al. tested various manuka honey products on three oral bacteria. The MIC for *S. mutans* was 20% and for *A. actinomycetemcomitans* was 4% [41]. These values are comparable to the ones obtained in the present study.

Manuka honey displayed slightly greater inhibitory efficacy at certain concentrations when compared to Revamil®. Kwakman et al. reported similar results comparing manuka to Revamil. They showed that Revamil® was more potent than manuka after two hours, however, after 24 hours, manuka was the most potent [59]. In our study, Revamil® showed a lower inhibitory efficacy when compared to the sugar control in all tested bacteria except for *A. actinomycetemcomitans* which Revamil had a better efficacy than the sugar control (Table 3-6). *A. actinomycetemcomitans* had the lowest MIC and IC50 values among all tested bacteria. *A.actinomycetemcomitans* is a gram-negative bacterium while the other bacteria are all gram-positive (*streptococcus species*) which might explain this effect in agreement with findings from other studies [55, 59].

The difference between manuka and Revamil® is mainly due to the difference in antimicrobial compounds that exert completely different bactericidal pathways. The bactericidal effect of manuka is substantially attributed to MGO content. Bee defensin-1 and H_2O_2 are responsible for of the rapid activity of Revamil® honey. Sugar solution, which is considered hypertonic, plays an important role in this activity explaining the similarity in the bactericidal properties of the sugar control to manuka and Revamil® (Figure 2).

The analysis of synergy obtained with the combined honey revealed that there is a significant synergy between manuka and Revamil® on all tested bacteria. The comparison was made at 3.12% (w/v) for *A. actinomycetemcomitans* and 6.25% for the other bacteria. The reason behind choosing this concentration is to be able to detect additive effects. All the concentrations above these had very close percent inhibition. Since the sugar content is doubled in the combined honey, these concentrations were compared to the double concentration of the sugar control. Our synergy suggests that the antimicrobial properties of each honey work very well when combined together and is not due to the increased sugar content.

To further investigate this synergy, MGO titration curve was made with or without 6.25% Revamil®. The reason for choosing 6.25% of Revamil® is because we wanted to eliminate possible antimicrobial effect due to the sugar content that might be evident at higher concentrations and the antimicrobial would be too diluted at lower concentrations. It is established that MGO, which is derived from its precursor DHA, plays a major role in the antimicrobial effect of medical-grade manuka honey [61]. It was documented that MGO concentrations higher than 0.15 mg/g are directly related to the antibacterial properties of manuka. Other types of honey have lower amounts of MGO ranging from 0.0004 to 0.0054

mg/g [75]. It was shown in this study that MGO performed better when combined with Revamil ®. This suggest that the synergy between Revamil and manuka is partly due to the presence of MGO in the manuka honey. This result is in agreement with the work of Bardy et al. who reported an augmented effect when mixing MGO with non-MGO containing honey [76].

Many studies demonstrating honey's efficacy in the disruption of bacterial biofilms have emerged [39, 77]. The biofilm disruption assay was carried out on mono-species and multi-species biofilm with all four bacteria. *S. Salivarious* was excluded from mono-species assay because it did not form a strong biofilm but was included in the multi-species biofilm to mimic the oral environment [78]. The data showed a dose dependent effect of the tested concentrations (50%, IC50 and 1.56%,) please refer to Table 2 for all IC50 values (Figure 5). At 25% and IC50, all the tested solutions were able to disrupt the mono-species biofilms (55-85% inhibition) with the sugar control being the least effective (25-45% inhibition, $p > 0.05$). However, they were less successful in disrupting the multi-species biofilms, with manuka being the best (20% inhibition) and the combined being the least inhibitory with <1%. These results indicate that the biofilm disruption might have occurred due to the antimicrobial compounds of the honey other than sugar content evident by the fact that the sugar control was the least inhibitory. The effect obtained in the multi-species biofilm can be explained by the fact that the biofilm made by mixing all bacteria was more robust and stable than the one made by individual bacteria. The interactions among oral bacteria are key to the development of a strong biofilm [79]. It was reported that dextran production, a vital component in biofilm formation of *S. mutans* was inhibited by honey [56]. In a study by Wang et al. it was suggested that anti-quorum sensing properties might be partially due to the

sugar content, since their results were reproducible by using glucose and fructose. Although the concentration used in the sugar control in their study did not inhibit bacterial growth, the exact mechanism is still unknown [43]. A possible explanation to their result is that quorum-sensing responses to environmental nutritional factors [80], and that might be the reason, in which abundance of sugar molecules might inhibit or alter quorum-sensing pathways. At 1.56% the opposite was noticed for *S. mutans* and *S. sanguinis*, at which the sugar control was more inhibitory than the rest of the tested honey solutions, however, the percent inhibition of 1.56% sugar control was within the same range as 25% for both bacteria. For *A. actinomycetemcomitans* and the multi-species biofilm, 1.56% of the sugar control was the least inhibitory. A possible explanation is that at this concentration the sugar molecules themselves weakened the biofilm. Lerrer et al. demonstrated that fructose found in honey inhibits *Pseudomonas aeruginosa* infection by interfering with the bacterial sugar-binding protein [81].

The pH reading indicated that at high concentrations, the low pH might play a role in the antimicrobial effect shown by the tested solutions. However, it is on the neutral side at a concentration where the bactericidal effects can be recorded as well (12.5 and 6.25%). This is a good indication that at these low concentrations, other properties of honey were responsible for the bactericidal effect.

Limitations of this study include the in-vitro design that did not evaluate the influence of the oral environment and the effect of saliva and other organisms. Also, this study did not include anaerobic bacteria which are major pathogens in many periodontal conditions [82]. Furthermore, the experiments testing role of MGO did not include a sugar control and the effect of combining manuka with pure defensin-1 was also not investigated.

Future studies testing the effect of combining manuka and Revamil® on anaerobic bacteria, fungi, and viruses are warranted. Furthermore, the effect of MGO combined with purified defensin-1 is worth investigating.

Conclusion

Within the limitations of this in vitro study, we conclude that both Revamil® and manuka have bactericidal properties, which are enhanced when both kinds of honey are combined. MGO present in manuka is partly responsible for this synergistic action. A combination of Revamil and manuka honey could be considered for clinical use as an antimicrobial agent with fewer side effects, resistance, and cost than the conventional antibiotics most commonly prescribed today.

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APPENDICES

Appendix A: Tables

Appendix B: Figures

Appendix A: Tables

Table 1: Minimum inhibitory concentration (MIC) of each tested bacterium

	MIC (w/v) %			
Bacteria	Revamil®	Manuka	Combined	Sugar Control
S. mutans	25	12.5	12.5	25
S. sanguinis	25	12.5	12.5	25
S. salivarious	25	25	12.5	25
A. actinomycetemcomitans	6.25	6.25	6.25	25

Table 2: IC50 for each tested bacterium

IC50 (w/v) %				
Bacteria	Revamil®	Manuka	Combined	Sugar Control
S. mutans	11.0	7.0	4.0	9.08
S. sanguinis	12.0	7.0	3.7	10
S. Salivarious	~14.0	8.6	6.8	~ 19.4
A. actinomycetemcomitans	4.0	4.1	2.0	3.08

Table 3: Means, SD, medians and IQR for *S. mutans* optical density treated with the testing solutions at various concentrations.

		S. mutans		
Concentration (w/v) %	OD	Revamil®	Manuka	Sugar control
50	Mean (SD)	0.007 (0.007) [a]	0.005(0.01) [ab]	- 0.005(0.0008) [b]
	Median (IQR)	0.006 (0.01) [a]	- 0.001(0.02) [ab]	- 0.005(0.001) [b]
25	Mean (SD)	0.005 (0.006) [a]	- 0.005(0.005) [ab]	- 0.0002 (-0.003) [a]
	Median (IQR)	0.002 (0.01) [a]	0.006(0.01) [a]	0.001(0.009) [a]
12.50	Mean (SD)	0.25 (0.09)	- 0.003(0.004)	0.1(0.03)
	Median (IQR)	0.26 (0.16)	0.005(0.007)	0.11(0.04)
6.25	Mean (SD)	0.5 (0.06) [a]	0.4 (0.12) [a]	0.49 (0.12) [a]
	Median (IQR)	0.5 (0.12) [a]	0.38(0.23) [a]	0.52(0.15) [a]
3.12	Mean (SD)	0.48 (0.24) [a]	0.57(0.045) [a]	0.59(0.11) [a]
	Median (IQR)	0.54 (0.23) [a]	0.57 (0.08) [a]	0.63 (0.10) [a]
1.56	Mean (SD)	0.63 (0.05) [a]	0.68(0.05) [a]	0.62(0.06) [a]
	Median (IQR)	0.62 (0.08) [a]	0.68 (0.09) [a]	0.61(0.1) [a]

-Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 4: Means, SD, medians and IQR for *S. sanguinis* optical density treated with the testing solutions at various concentrations.

		S. sanguinis		
Concentration (w/v) %	OD	Revamil®	Manuka	Sugar control
50%	Mean (SD)	0.0003(0.002) [a]	0.003(0.009) [a]	- 0.002(0.001) [a]
	Median (IQR)	0.001(0.003) [a]	0.003(0.02) [a]	- 0.002(0.001) [a]
25%	Mean (SD)	- 0.002(0.001) [a]	- 0.002(0.005) [ab]	- 0.0002(0.0008) [b]
	Median (IQR)	- 0.002(0.002) [a]	- 0.001(0.01) [ab]	- 0.0003(0.001) [b]
12.50%	Mean (SD)	0.27(0.09)	- 0.001(0.009)	0.09(0.02)
	Median (IQR)	0.29(0.13)	- 0.003(0.01)	0.09(0.03)
6.25%	Mean (SD)	0.52 (0.02)	0.39(0.04)	0.62(0.04)
	Median (IQR)	0.53(0.05)	0.39(0.07)	0.6(0.06)
3.12%	Mean (SD)	0.56 (0.02) [a]	0.55 (0.04) [a]	0.68 (0.02)
	Median (IQR)	0.56 (0.04) [a]	0.57 (0.05) [a]	0.68(0.03)
1.56%	Mean (SD)	0.62 (0.06) [a]	0.61 (0.05) [a]	0.67 (0.04) [a]
	Median (IQR)	0.63 (0.09) [a]	0.63 (0.1) [a]	0.67(0.07) [a]

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 5: Means, SD, medians and IQR for *S. salivarious* optical density treated with the testing solutions at various concentrations.

		S. Salivarious		
Concentration (w/v) %	OD	Revamil®	Manuka	Sugar control
50	Mean (SD)	0.002 (0.002) [a]	- 0.0001 (0.005) [a]	-0.009(0.0008)
	Median (IQR)	0.003 (0.004) [a]	-0.0005(0.006) [a]	-0.009 (0.002)
25	Mean (SD)	0.001 (0.001) [a]	0.003 (0.006) [ab]	- 0.006 (0.002) [b]
	Median (IQR)	0.001 (0.002) [a]	0.004 (0.01) [ab]	-0.007 (0.002) [b]
12.50	Mean (SD)	0.51 (0.05)	0.08 (0.05)	0.37 (0.07)
	Median (IQR)	0.5 (0.08)	0.06 (0.1)	0.37 (0.11)
6.25	Mean (SD)	0.55 (0.09) [a]	0.49 (0.06) [a]	0.5 (0.08) [a]
	Median (IQR)	0.56 (0.13) [a]	0.47 (0.13) [a]	0.53 (0.11) [a]
3.12	Mean (SD)	0.6 (0.03) [a]	0.59 (0.03) [a]	0.4 (0.06)
	Median (IQR)	0.59 (0.04) [a]	0.59 (0.05) [a]	0.4 (0.09)
1.56	Mean (SD)	0.59 (0.008) [a]	0.59 (0.03) [a]	0.47 (0.06)
	Median (IQR)	0.59 (0.011) [a]	0.594 (0.03) [a]	0.5 (0.12)

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 6: Means, SD, medians and IQR for *A. actinomycetemcomitans* optical density treated with the testing solutions at various concentrations.

		A. actinomycetemcomitans		
Concentration (w/v) %	*OD*	Revamil®	Manuka	Sugar control
50	*Mean (SD)*	0.006 (0.001) [a]	0.00 (0.007) [a]	0.11 (0.02)
	Median (IQR)	0.007 (0.001) [a]	0.001 (0.01) [a]	0.11 (0.04)
25	*Mean (SD)*	0.003 (0.002) [a]	0.006 (0.003) [a]	0.25 (0.07)
	Median (IQR)	0.004 (0.005) [a]	0.008 (0.006) [a]	0.24 (0.08)
12.5	*Mean (SD)*	0.003 (0.001) [a]	0.001 (0.001) [a]	0.35 (0.09)
	Median (IQR)	0.003 (0.001) [a]	0.001 (0.003) [a]	0.35 (0.12)
6.25	*Mean (SD)*	0.01 (0.004)	0.008 (0.0008)	0.64 (0.03)
	Median (IQR)	0.01 (0.008)	0.009 (0.001)	0.63 (0.06)
3.12	*Mean (SD)*	0.22 (0.05) [a]	0.24 (0.05) [a]	0.68 (0.008)
	Median (IQR)	0.22 (0.1) [a]	0.22 (0.072) [a]	0.69 (0.012)
1.56	*Mean (SD)*	0.27 (0.033) [a]	0.26 (0.02) [a]	0.6 (0.04)
	Median (IQR)	0.28 (0.05) [a]	0.26 (0.03) [a]	0.59 (0.07)

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 7: Means, SD, medians and IQR for % inhibition of all tested bacteria treated with the combined honey solution and compared to the expected % inhibition and the sugar control.

Bacteria	% inhibition	Observed Combined honey	Expected combined honey	Sugar control
S. mutans	Mean (SD)	90.79 (10.1) [a]	48.35 (20.39)	79.64 (5.24) [a]
	Median (IQR)	91.94 (18.1) [a]	45.39 (42.28)	78.73 (7.51) [a]
S. sanguinis	Mean (SD)	95.18 (4.71)	39.01 (6.06)	82.41 (4.83)
	Median (IQR)	96.81 (9.30)	39.81 (10.21)	84.45 (8.57)
S. salivarious	Mean (SD)	33.95 (8.83)	9.32 (13.66) [a]	17.97 (8.02) [a]
	Median (IQR)	34.26 (17.0)	15.0 (21.08) [a]	15.76 (13.88) [a]
A. actinomycetemcomitans	Mean (SD)	96.11 (3.58)	60.71 (24.2) [a]	30.94 (89.70) [a]
	Median (IQR)	96.81 (4.53)	72.88 (29.09) [a]	55.55 (103.85) [a]

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 8: IC50 for each tested bacterium for the MGO titration assay

	IC50 (w/v) %	
Bacteria	MGO + Revamil®	MGO
S. mutans	3.07	1.5
S. sanguinis	3.6	3.5
S. salivarious	1.2	2.7
A. actinomycetemcomitans	1.09	1.2

Table 9: Means, SD, medians and IQR for *S. mutans* optical density treated with MGO ± Revamil solutions.

		S. mutans	
MGO Concentration (w/v) %	OD	MGO	MGO + Revamil® (6.25%)
40	Mean (SD)	0.0006 (0.0008)	0.005 (0.01)
	Median (IQR)	0 (0.001)	0.001 (0.014)
20	Mean (SD)	0.0004 (0.001)	0.004 (0.012)
	Median (IQR)	0 (0.002)	0 (0.019)
10	Mean (SD)	0.001 (0.002)	0.005 (0.01)
	Median (IQR)	0 (0.004)	0.002 (0.015)
5	Mean (SD)	0.026 (0.01)	0.004 (0.01)
	Median (IQR)	0.023 (0.02)	0 (0.013)
2.5	Mean (SD)	0.352 (0.05)	0.018 (0.015)
	Median (IQR)	0.364 (0.092)	0.012 (0.027)
1.25	Mean (SD)	0.432 (0.036)	0.357 (0.026)
	Median (IQR)	0.424 (0.059)	0.351 (0.046)
0.625	Mean (SD)	0.47 (0.042)	0.453 (0.022)
	Median (IQR)	0.461 (0.076)	0.455 (0.034)
0.3125	Mean (SD)	0.479 (0.057)	0.476 (0.029)
	Median (IQR)	0.496 (0.107)	0.48 (0.053)

-Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.05$.

Table 10: Means, SD, medians and IQR for *S. sanguinis* optical density treated with MGO ± Revamil solutions.

		S. sanguinis	
MGO Concentration (w/v) %	OD	MGO	MGO + Revamil® (6.25%)
40	Mean (SD)	- 0.006 (0.001)	0.003 (0.006)
	Median (IQR)	- 0.006 (0.002)	0.0005 (0.005)
20	Mean (SD)	- 0.001 (0.002)	0.004 (0.009)
	Median (IQR)	- 0.001 (0.004)	0.001 (0.008)
10	Mean (SD)	- 0.001 (0.001)	0.006 (0.01)
	Median (IQR)	- 0.002 (0.002)	0.003 (0.01)
5	Mean (SD)	0.122 (0.033) [a]	0.006 (0.01) [b]
	Median (IQR)	0.123 (0.058) [a]	0.002 (0.008) [b]
2.5	Mean (SD)	0.255 (0.035)	0.291 (0.06)
	Median (IQR)	0.249 (0.071)	0.282 (0.08)
1.25	Mean (SD)	0.336 (0.027)	0.449 (0.07)
	Median (IQR)	0.343 (0.046)	0.442 (0.101)
0.625	Mean (SD)	0.384 (0.056)	0.510 (0.04)
	Median (IQR)	0.38 (0.059)	0.498 (0.06)
0.3125	Mean (SD)	0.381 (0.026)	0.528(0.04)
	Median (IQR)	0.381 (0.044)	0.526(0.07)

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.05$.

Table 11: Means, SD, medians and IQR for *S. salivarious* optical density treated with MGO ± Revamil solutions.

		S. Salivarius	
MGO Concentration (w/v) %	OD	MGO	MGO + Revamil® (6.25%)
40	Mean (SD)	0.002 (0.014)	0.004 (0.006)
	Median (IQR)	0 (0.015)	0.002 (0.006)
20	Mean (SD)	0.004 (0.01)	0.003 (0.007)
	Median (IQR)	0.001 (0.01)	0 (0.006)
10	Mean (SD)	0.004 (0.011)	0.003 (0.007)
	Median (IQR)	0.001 (0.011)	0.001 (0.006)
5	Mean (SD)	0.066 (0.073)	0.009 (0.011)
	Median (IQR)	0.025 (0.121)	0.004 (0.013)
2.5	Mean (SD)	0.103(0.065)	0.072 (0.056)
	Median (IQR)	0.095 (0.105)	0.05 (0.082)
1.25	Mean (SD)	0.212 (0.112)	0.104 (0.053)
	Median (IQR)	0.163 (0.168)	0.087 (0.091)
0.625	Mean (SD)	0.169 (0.048)	0.238 (0.033)
	Median (IQR)	0.17 (0.083)	0.238 (0.05)
0.3125	Mean (SD)	0.244 (0.074)	0.225 (0.072)
	Median (IQR)	0.242 (0.119)	0.215 (0.13)

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.05$.

Table 12: Means, SD, medians and IQR for *A. actinomycetemcomitans* optical density treated with MGO ± Revamil solutions.

	A. actinomycetemcomitans		
MGO Concentration (w/v) %	OD	MGO	MGO + Revamil® (6.25%)
40	Mean (SD)	- 0.005 (0.003)	0.008 (0.002)
	Median (IQR)	- 0.006 (0.006)	0.007 (0.003)
20	Mean (SD)	- 0.003 (0.001)	0.004 (0.003)
	Median (IQR)	- 0.004 (0.003)	0.003 (0.004)
10	Mean (SD)	0.001 (0.001)	0.005 (0.003)
	Median (IQR)	0.001 (0.002)	0.005 (0.005)
5	Mean (SD)	0.011 (0.002)	0.004 (0.002)
	Median (IQR)	0.01 (0.005)	0.004 (0.003)
2.5	Mean (SD)	0.02 (0.002)	0.003 (0.001)
	Median (IQR)	0.02 (0.003)	0.003 (0.001)
1.25	Mean (SD)	0.111 (0.003)	0.006 (0.003)
	Median (IQR)	0.112 (0.004)	0.005 (0.004)
0.625	Mean (SD)	0.194 (0.016)	0.267 (0.079)
	Median (IQR)	0.197 (0.033)	0.246 (0.086)
0.3125	Mean (SD)	0.225 (0.007)	0.22 (0.026)
	Median (IQR)	0.226 (0.014)	0.222 (0.032)

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.05$.

Table 13: Means, SD, medians and IQR for *S. mutans* biofilm optical density disrupted by the testing solutions at 25%, IC50 and 1.56%.

		S.mutans			
Concentration (w/v)	OD	Revamil®	Manuka	Combined	Sugar control
25%	Mean (SD)	1.857 (0.577)	1.035 (0.17)	1.056 (0.204)	2.52 (0.46)
	Median (IQR)	1.519 (1.022)	1.038 (0.322)	1.046 (0.301)	2.35 (0.88)
IC50	Mean (SD)	2.789 (0.431)	2.157 (0.136)	2.738 (0.389)	
	Median (IQR)	2.605 (0.742)	2.106 (0.25)	2.605 (0.55)	
1.56%	Mean (SD)	3.667 (0.117)	3.648 (0.086)	3.609 (0.118)	1.604 (0.121)
	Median (IQR)	3.709 (0.219)	3.649 (0.162)	3.625 (0.221)	1.568 (0.220)

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 14: Means, SD, medians and IQR for *S. sanguinis* biofilm optical density disrupted by the testing solutions at 25%, IC50 and 1.56%.

		S. sanguinis			
Concentration (w/v)	OD	Revamil®	Manuka	Combined	Sugar control
25%	Mean (SD)	1.224 (0.680)	0.611 (0.092)	0.9118 (0.070)	2.560 (0.704)
	Median (IQR)	0.932 (0.929)	0.599 (0.141)	0.895 (0.139)	2.506 (1.221)
IC50	Mean (SD)	2.491 (0.609)	2.184 (0.855)	2.846 (0.142)	
	Median (IQR)	2.190 (1.208)	1.700 (1.413)	2.846	
1.56%	Mean (SD)	3.288 (0.268)	3.275 (0.194)	3.257 (0.039)	2.129 (0.555)
	Median (IQR)	3.288	3.217	3.276	2.023(1.016)

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 15: Means, SD, medians and IQR for *A. actinomycetemcomitans* biofilm optical density disrupted by the testing solutions at 25%, IC50 and 1.56%.

		A. actinomycetemcomitans			
Concentration (w/v)	OD	Revamil®	Manuka	Combined	Sugar control
25%	Mean (SD)	1.029 (1.090)	0.660 (0.618)	0.619 (0.498)	0.691 (0.061)
	Median (IQR)	0.339 (2.051)	0.332 (0.751)	0.322 (0.914)	0.696 (0.115)
IC50	Mean (SD)	1.303 (1.478)	1.065 (0.914)	1.120 (1.309)	
	Median (IQR)	0.586 (2.261)	0.524 (1.768)	0.546 (1.502)	
1.56%	Mean (SD)	1.862 (0.639)	2.099 (1.290)	1.168 (0.044)	
	Median (IQR)	1.548 (0.972)	1.401	1.167 (0.085)	

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 16: Means, SD, medians and IQR for multi-species biofilm optical density disrupted by the testing solutions at 25%, IC50 and 1.56%.

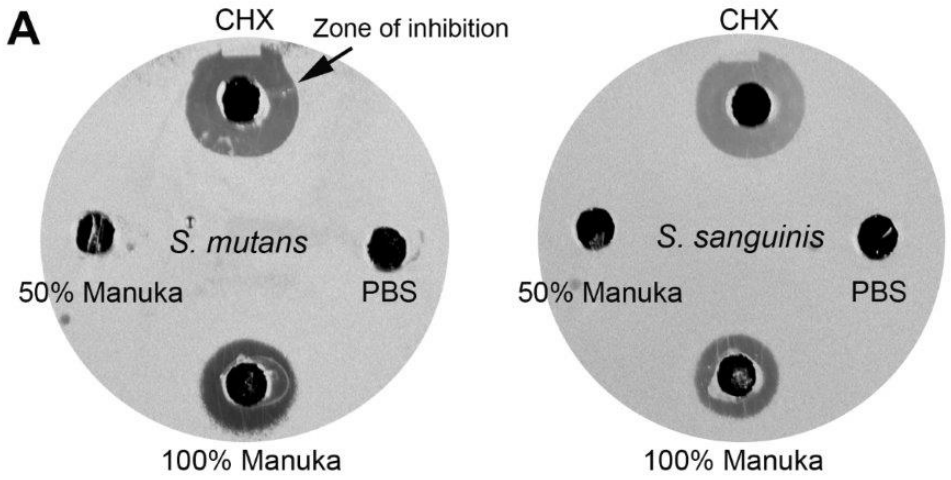
		All Bacteria			
Concentration (w/v)	OD	Revamil®	Manuka	Combined	Sugar control
25%	Mean (SD)	3.190 (0.251)	2.847 (0.429)		3.822 (0.206)
	Median (IQR)	3.066 (0.367)	3.014 (0.745)		3.86
IC50	Mean (SD)	3.579 (0.299) [ab]	3.011 (0.558) [a]	3.544 (0.389) [b]	
	Median (IQR)	3.523 (0.565) [ab]	3.19 (1.004) [a]	3.594 (0.745) [b]	
1.56%	Mean (SD)	3.553 (0.354)		3.616 (0.344)	
	Median (IQR)	3.354		3.441	

- Shared letters correspond to a lack of statistically significant difference between tested solutions at each concentration at $p < 0.017$.

Table 17: Recorded pH values for testing solutions

Concentration %	Revamil®	Manuka	Combined	Sugar Control
50	6	4	5	6
25	6	6	6	7
12.5	6	7	6	7
6.25	7	7	7	7
3.12	7	7	7	7
1.56	7	7	7	7

Appendix B: Figures



B

	Zone of inhibition: Mean (S.D.)	
	Chlorhexidine	Manuka (100%)
S. mutans	18.00 (0.41)	15.00 (0.00)
S. sanguinis	17.47 (0.40)	13.73 (0.37)

Figure 1: Well diffusion assay for *S. mutans* and *S. sanguinis*. (A) Zones of growth inhibition of 50, 100% manuka, chlorhexidine (CHX, positive control) and phosphate buffer saline (PBS, negative control). (B) means ± SD of zones of inhibition of 100% (w/v) manuka and CHX, n =3 per group.

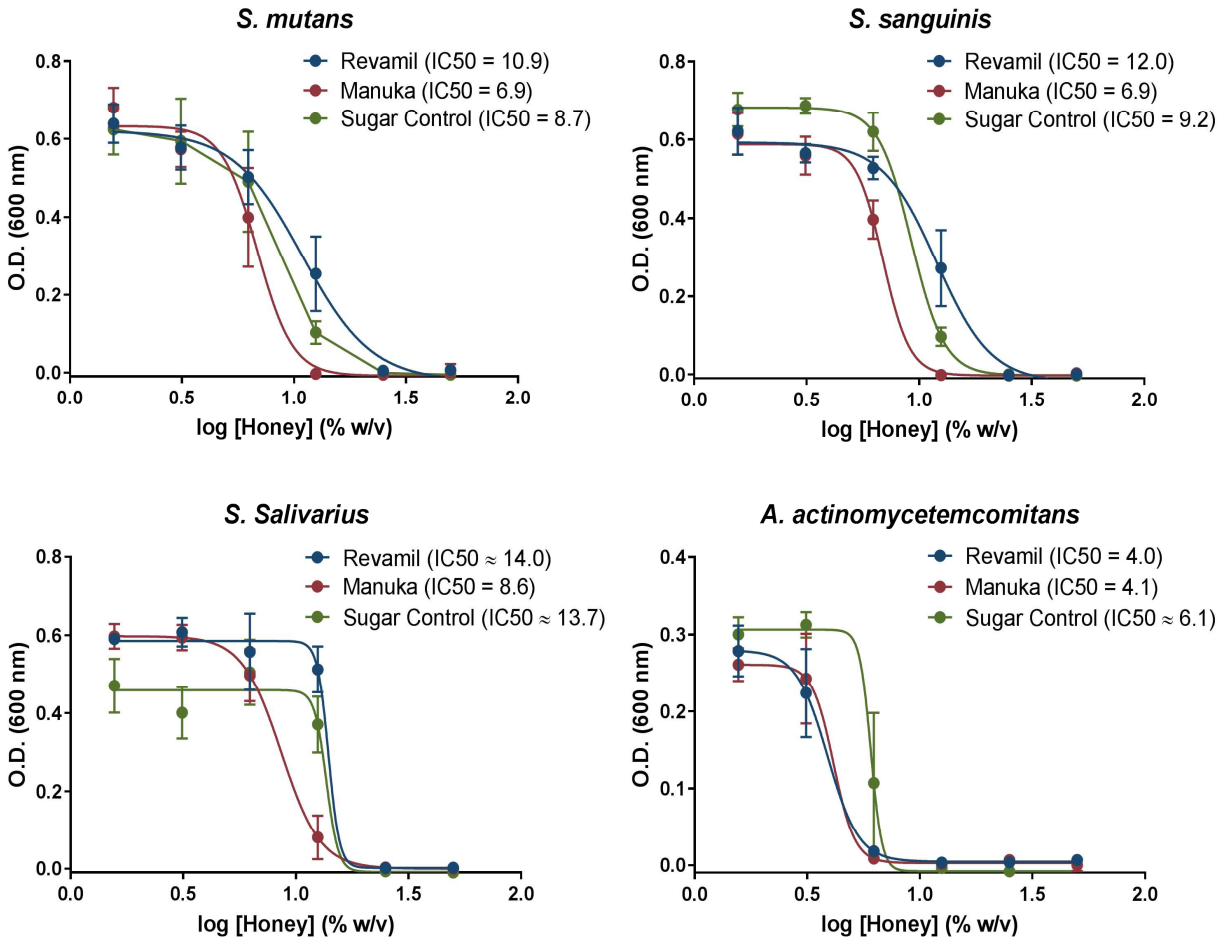


Figure 2: Effect of different concentration of Revamil®, manuka and sugar control on the growth of *S. mutans, S. sanguinis, S. Salivarious,* and *A. actinomycetemcomitans*. IC50 values were presented for each tested honey. The data was presented as means ±SD, n=6 per group.

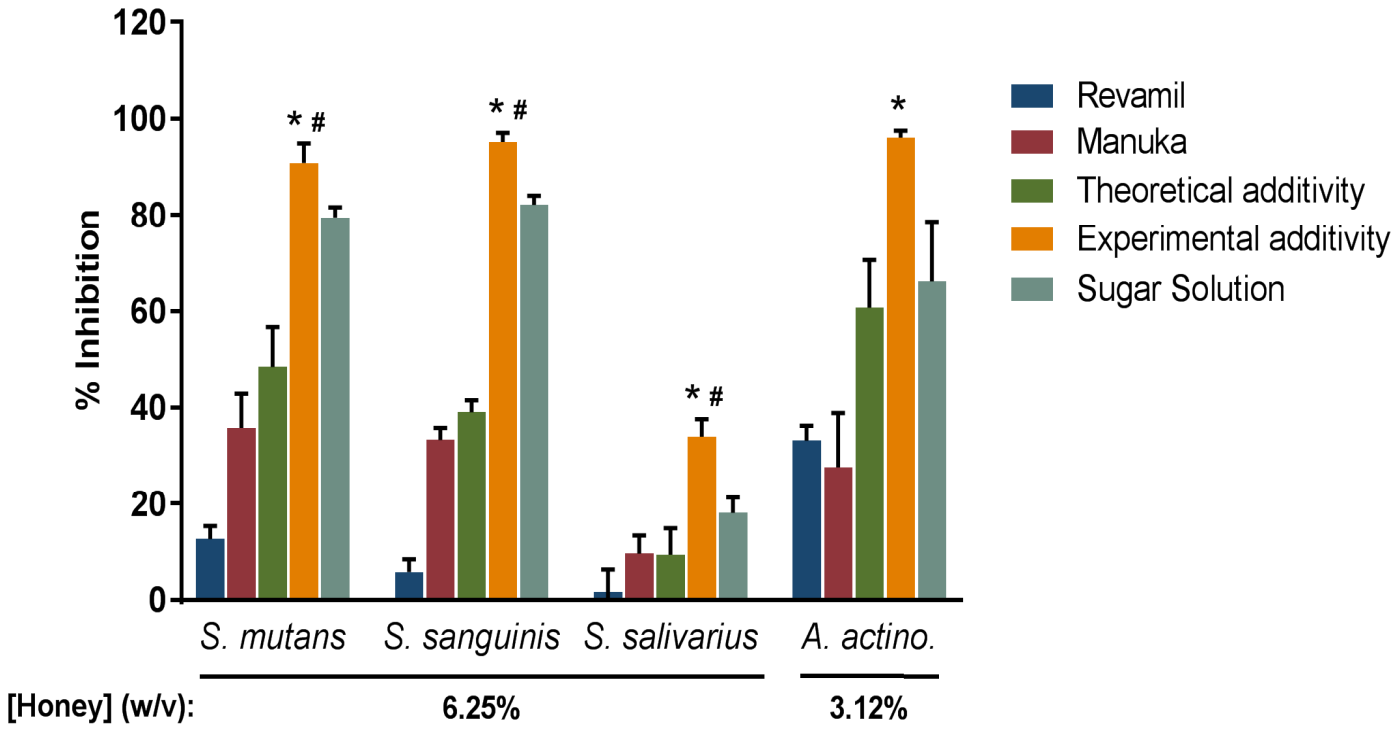


Figure 3: The additivity of combining Revamil® and Manuka compared to theoretical additivity and sugar control. The data was presented as means ±SD, n=6 per group. Star denotes statistically significant difference compared to theoretical additivity. Pound sign indicates statistically significant difference compared to the sugar solution.

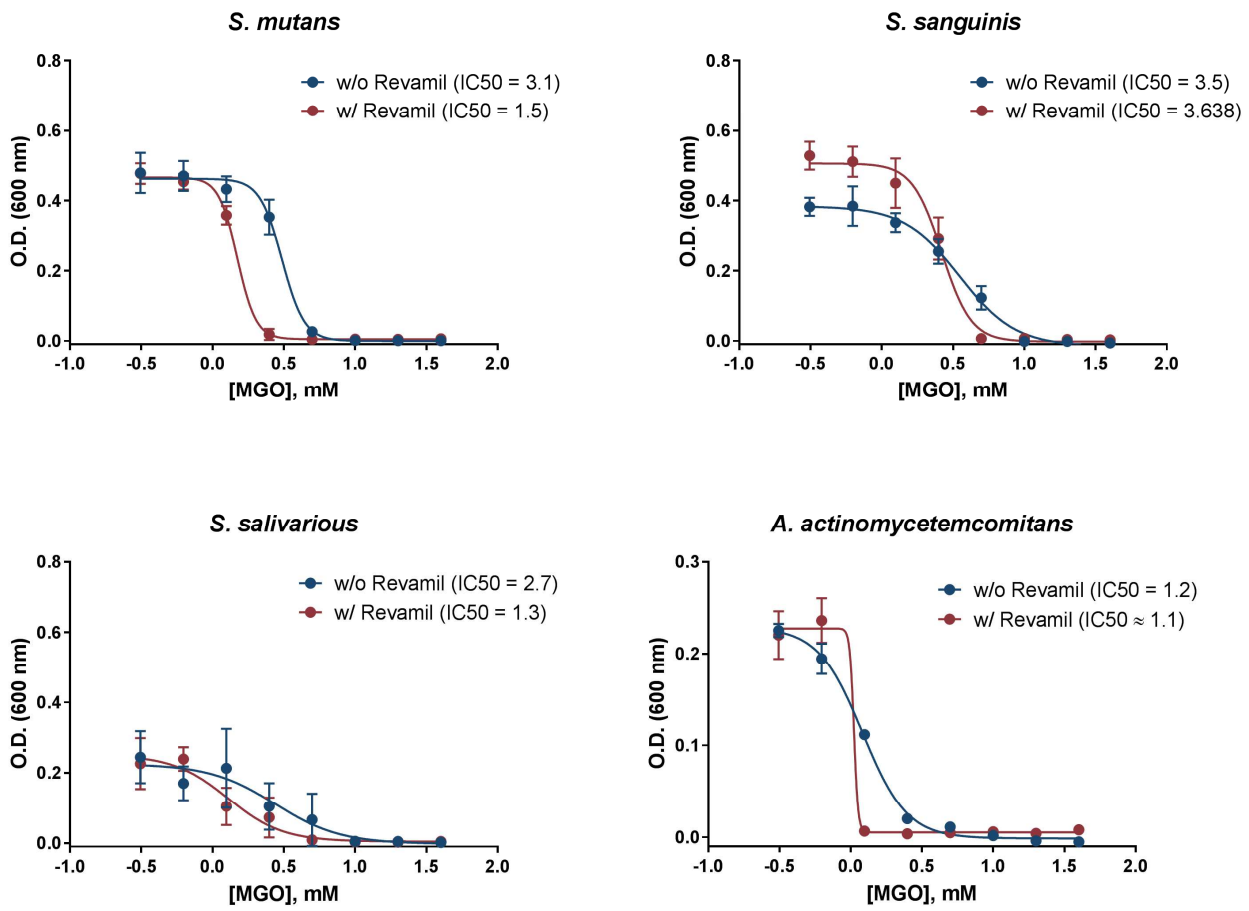


Figure 4: Effect of combining various MGO concentrations with or without 6.25% Revamil® on the growth of *S. mutans, S. sanguinis, S. Salivarious,* and *A. actinomycetemcomitans*. IC50 values were presented for each tested honey. The data was presented as means ±SD, n=6 per group.

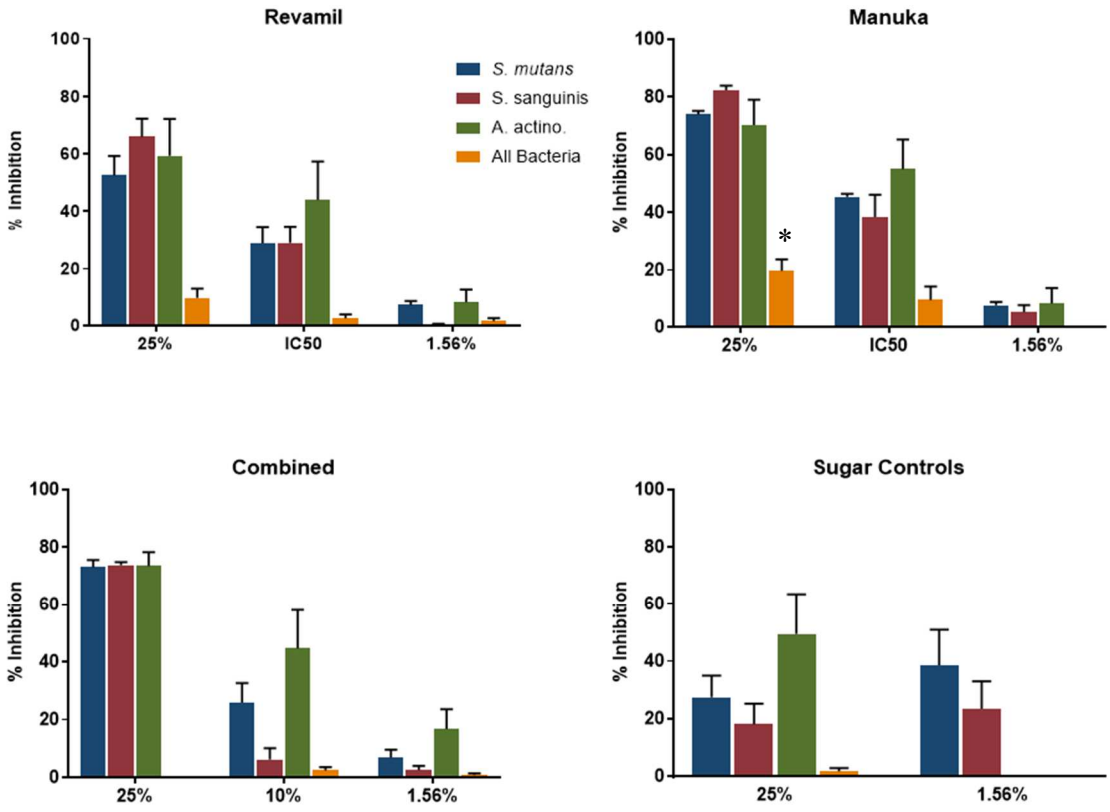


Figure 5: Biofilm disruption assay showing the effect of various concentrations of Revamil, manuka, combined honey and the sugar control on the tested bacteria. The bars show the average values of percent inhibition. The data was presented as means ±SD, n=6 per group. Star indicates statistically significant difference compared to combined honey group.