ORIGINAL RESEARCH

Biological Activities of Virgin Coconut and Virgin Olive Oil Mixture against Oral Primary Colonizers: An *In Vitro* Study

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Abstract

Aim and background: This study aimed to explore the potential synergistic interaction of virgin coconut oil (VCO) and virgin olive oil (VOO) mixture against *Streptococcus sanguinis*, *Streptococcus mutans*, and *Lactobacillus casei* in a single and mixture species through the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), antiadherence, and antibiofilm activities.

Materials and methods: The broth microdilution technique was used to individually determine the MIC of both oils and an oil mixture (in the ratio of 1:1) in a 96-well microtiter plate. As for the MBC, the subcultured method was used. The fractional inhibitory concentration index (ΣFIC) was determined to identify the interaction types between both oils. The oil mixture at its MIC was then tested on its antibiofilm and antiadherence effect.

Results: The MIC of the oil mixture against the tested microbiota was 50–100%. The oil mixture was bactericidal at 100% concentration for all the mentioned microbes except *S. mutans*. The ΣFIC value was 2 to 4, indicating that the VCO and VOO acted additively against the microbiota. Meanwhile, the oil mixture at MIC (50% for *S. sanguinis* and *L. casei*; 100% for *S. mutans* and mixture species) exhibited antiadherence and antibiofilm activity toward the microbiota in mixture species.

Conclusion: The oil mixture possesses antibacterial, antibiofilm, and antiadherence properties toward the tested microbiota, mainly at 50–100% concentration of oil mixture. There was no synergistic interaction found between VCO and VOO.

Clinical significance: Children and individuals with special care may benefit from using the oil mixture, primarily to regulate the biofilm formation and colonization of the bacteria. Furthermore, the oil mixture is natural and nontoxic compared to chemical-based oral healthcare products.

Keywords: Antiadherence, Antibiofilm, Edible oils, *Lactobacillus casei*, Minimum bactericidal concentration, Minimum inhibitory concentration, *Streptococcus mutans*, *Streptococcus sanguinis*.

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Introduction

World Health Organization data shows dental caries is the most common oral health condition, affecting over 530 million children worldwide.[1] The high prevalence of this disease is because of its complex relationship among the biological and social variables.[2] One of the ways of caries prevention is the use of mouthwash due to its antibiofilm properties.[3] Chemical control of plaque is achievable with therapeutic mouthwash ingredients such as chlorhexidine, essential oil, and cetylpyridinium chloride.[4] However, the risk of swallowing mouthwash with potential adverse effects has limited its use among children under six. Besides, some chemical-based mouthwash ingredients can cause adverse effects such as tooth staining, transience taste disturbance, and desquamation of the oral mucosa.[5] Therefore, there is an inclination to use natural products as the main mouthwash ingredients.[6]

Oil-pulling therapy is essential in Ayurvedic medicine, and its benefits in dental care are gaining popularity. Edible oils are natural and safe to consume.[7] Some edible oils, such as sunflower, sesame, coconut, and olive oils, have also been used for oil-pulling therapy.[8] Incorporating oils into mouthwashes as an ecological approach in caries prevention might help preserve the commensal bacteria and eliminate the cariogenic virulence factors responsible for plaque biofilm dysbiosis. Virgin coconut oil (VCO) has always garnered attention in healthcare industries because of its bactericidal, antioxidant, and anti-inflammatory properties.[9] In recent years, virgin olive oil (VOO) has also proven its anti-inflammatory and antimicrobial properties in health aspects.[10] From an *in vitro* study by Hazmi et al., VCO and VOO have demonstrated their antibacterial, antiadherence, and antibiofilm properties against *Streptococcus sobrinus* and *Lactobacillus casei* with the effectiveness of VOO during the initial stage of plaque formation and superior antibiofilm activity of VCO.[11]

Given these oils' antibacterial, antiadherence, and antibiofilm properties, combining VCO and VOO might work synergistically

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against oral microbiota. Synergistic interaction of plant derivative products has always been explored to assess whether positive synergistic interactions or antagonisms exist between the tested products.[12] To the best of our knowledge, no study is found in literature utilizing VCO and VOO mixture against oral microorganisms. This study aimed to investigate the biological activities of VCO and VOO mixture against oral primary colonizers in a single and mixture species.

Materials and Methods

Ethical approval was obtained from the National University of Malaysia Research Ethics Committee before study commencement (UKM PP1/111/8/JEP-2021-647). The entire study lasted 10 months, from March 2022 to February 2023.

Preparation of Oil Mixture

The VCO and VOO used in the current study were of similar identical trade brands used in the study conducted by Hazmi et al.[11] The VCO trade brand name was ItWorks!™ from the Malaysian Development Institute (MARDI, Malaysia). Meanwhile, the VOO was a commercial product, "Extra VOO," by BorgesTM (Spain). To increase the solubility of the oils in broths, brain heart infusion (BHI, Oxoid, UK) and de Man, Rogosa, and Sharpe (MRS, Oxoid, UK), 1% of ethanol (Fisher Scientific, USA) were added.[11] The concentrations for both oils used in this study were 100%, respectively, and both oils were added in the ratio of 1:1 (v/v) to obtain the oil mixture. Nine concentrations of test oil mixture were prepared through 2-fold serial dilution ranging from 100 to 0.39%.

Preparation of Bacterial Suspension

Three strains of bacteria were used in the current study, namely, *Streptococcus sanguinis* ATCC BAA-1455™ (ATCC, USA), *Streptococcus mutans* ATCC®25175™ (ATCC, USA), *L. casei* ATCC®393™ (ATCC, USA). The medium for *Streptococcus* species was BHI agar/broth, while the medium for *L. casei* was MRS agar/broth. Bacterial inoculation procedures were conducted per the American Type Culture Collection (ATCC)'s manual instructions. Their turbidity was standardized for *S. mutans* and *S. sanguinis* by adjusting the absorbance to 0.144 at 550 nm, equivalent to 1.53×10^7 CFU/mL and 1.53×10^8 colony forming units (CFU)/mL, respectively.[12,13] Meanwhile, the turbidity for *L. casei* was standardized by adjusting the absorbance to 0.032 at 590 nm, equivalent to 10^6 CFU/mL.[11] Prior to the experiment, the standardized bacterial suspension was then prepared to a dilution of 10^6 CFU/mL to ensure the concentration of the tested bacterium in the respective well achieves 10^5 CFU/mL following Clinical and Laboratory Standards Institute (CLSI) protocol.[14]

Determination of Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, and Fractional Inhibitory Concentration Index

The broth microdilution technique was implemented to determine the minimum inhibitory concentration (MIC) value of the oil mixture using a 96-well microtiter plate. A 570 µL of the respective oil mixture and 30 µL of standardized respective bacterial suspension were added into a 2 mL centrifuge tube, contributing to 600 µL of oil mixture containing bacterium suspension. The tubes were mixed, and 100 µL of the respective mixture oils–bacteria suspension were transferred to the respective well in triplicate (from A1 to A9; from B1 to B9; from C1 to C9) of the 96-well microtiter plate. The corresponding blank controls in triplicate (from E1 to E9; from F1 to F9; from G1 to G9) were also prepared, minus the color of the oil suspension. Meanwhile, 0.12% (v/v) chlorhexidine and broth without the oils were used as positive and negative controls, respectively. The BHI and MRS broths with or without bacteria were also prepared to detect the growth and sterility of the fresh broth used. Later, the microtiter plates were covered with the lid and incubated anaerobically at 37°C for 24 hours. Similar procedures were repeated for respective oils. A day after incubation, the MIC was determined at 590 nm based on the turbidity with enzyme-linked immunosorbent assay (ELISA) reader (Varioskan Flash® Multimode Reader Thermo, USA) by comparing the absorbance of the suspension in wells of test extract in triplicate (from A1 to A9; from B1 to B9; from C1 to C8) with the corresponding blank control in triplicate (from E1 to E9; from F1 to F9; from G1 to G9).[11] The lowest concentration of respective oil or oil mixture that showed almost similar turbidity with the blank control comprising broth medium without bacterium was recorded as MIC value.[15] The test was run in triplicate to ensure the reproducibility of the results and was repeated three times, representing the three biological replicates.

The minimum bactericidal concentration (MBC) was determined by subculturing 3 µL from each well content with no turbidity as the MIC value. Each MIC well content was inoculated on the agar plate, streaked, and incubated at 37°C for 24 hours. The well contents from positive and negative controls were also spread onto the agar plate to check the growth of bacteria. The lowest concentration that yielded no growth after the subculturing was considered as the MBC value.

The fractional inhibitory concentration index (ΣFIC) sum determined the synergistic interaction between VCO and VOO. It was calculated using the MIC values of the respective oil and oil mixture. The ΣFIC was expressed as the interaction of two agents where the concentration of each test agent in combination was expressed as a fraction of the concentration that would produce the same effect when used independently.[16] Thus, the ΣFIC was calculated for each test sample independently, as specified in the following equations:

$$FIC^{VCO\ (I)} = \frac{\text{MIC (I) in combination with (II)}}{\text{MIC (I) independently}}$$

$$FIC^{VOO\ (II)} = \frac{\text{MIC (II) in combination with (I)}}{\text{MIC (II) independently}}$$

Then ΣFIC was calculated as follows:

$$\Sigma FIC = FIC^{VCO} + FIC^{VOO}$$

According to the interpretation of the ΣFIC value reported by van Vuuren and Viljoen, $\Sigma FIC \leq 0.5$ suggests synergistic interaction; $0.5 < \Sigma FIC \leq 1.0$ suggests additivity; $1.0 < \Sigma FIC \leq 4.0$ suggests indifference, and $\Sigma FIC > 4.0$ suggests antagonism.[17]

Transmission Electron Microscopy Analysis of Bacteria under Oil Mixture at Minimum Inhibitory Concentration Value

The samples with the MIC value of the oil mixture against *S. sanguinis* and *L. casei* with the respective negative control were visualized under TEM (Zeiss TEM Libra 120, Germany) with a magnification of 17000. Meanwhile, the samples with the MIC value of oil mixture against mixture species were not determined because the MIC value was similar to the MBC value. The bacteria colonies present in the sample of MIC value of the oil mixture against *S. mutans* were not sufficient to be viewed under TEM.

Antiadherence Effect of Oil Mixture for Single- and Mixture-species Biofilms

The stimulated whole saliva (SWS) was collected from a volunteer who was fit and healthy after consent was taken. The volunteer was instructed to chew on the sugar-free chewing gum and approximately 150 mL of saliva was collected on separate days. The sterilized saliva was then prepared according to the ways described by Shafiei et al. with some modifications.[13] The antiadherence method was adapted from Azeredo et al., Hazmi et al., and Kwasny and Opperman with some modifications.[11,18,19] A 96-well microtiter plate was utilized in this experiment to determine the antiadherence effect of oil mixture for the single and mixture species biofilms. Initially, the sterilized SWS prepared earlier was thawed at room temperature, and 200 µL of sterilized SWS was placed into the 96-well microtiter plate at allocated wells. The plate was covered and incubated at 37°C for 15 minutes. After that, the content of each well was aspirated and rinsed once with 250 µL of sterile phosphate buffer saline (PBS, pH 7.2) to remove the excess saliva. The individual wells were then added in triplicate with 200 µL of oil mixture at concentrations of 100, 50, and 25% through 2-fold serial dilution to represent the sub-MIC of the oil mixture. A 200 µL of 0.12% (v/v) chlorhexidine and sterilized distilled water served as positive and negative controls. The plate was covered and incubated for 15 minutes at 37°C to simulate the gargling period of mouthrinse in the oral cavity. Following that, the content of each well was aspirated and rinsed once with 250 µL of sterile PBS to remove the excess oils. Subsequently, 200 µL of standardized respective bacterial suspension (10^6 CFU/mL) in a single or mixture species was placed into the allocated wells, and the plate was incubated at 37°C for 24 hours. The next day, the content of the wells was aspirated gently to leave the biofilm intact. A 250 µL of PBS was used to remove the free-floating planktonic bacteria. After that, the 24-hour biofilm was being quantified. The antiadherence test was conducted in triplicate on the 96-well microtiter plate and repeated in three times to ensure reproducibility biologically.

Antibiofilm Effect of Oil Mixture for Single- and Mixture-species Biofilms

The antibiofilm method was adapted from Azeredo et al., Hazmi et al. and Kwasny and Opperman with some modifications.[11,18,19] A 200 µL of sterilized saliva was first placed into each allocated well and incubated for 15 min at 37°C. The content of the well was aspirated and rinsed once with sterilized PBS. Following that, 200 µL of standardized bacteria suspension (10^6 CFU/mL) in a single or mixture species was placed into the allocated wells and incubated at 37°C for 24 hours to form a 24-hour biofilm. After 24 hours of incubation, the bacterial suspension in the wells was aspirated and rinsed once with sterile PBS to remove the unattached biofilm. Then, the biofilm was treated with 200 µL of the prepared oil mixture at a concentration of 100, 50, and 25% in triplicates. Meanwhile, 200 µL of 0.12% chlorhexidine and sterile distilled water were placed in triplicate into the allocated wells to serve as the positive and negative controls. After that, the microtiter plate was incubated for 15 minutes at 37°C, followed by removal of all the contents and rinsed once with sterile PBS. The treated biofilm was subjected to quantification.

Biofilm Quantification for Antiadherence and Antibiofilm Tests

The procedures to quantify the biofilm started with placing 200 µL of 100% methanol (Sigma–Aldrich, USA) into the respective wells for 45 minutes or until there was a sign of more than half of the methanol being evaporated. The remaining absolute methanol was removed, and then the biofilm was stained with 200 µL of 0.06% (v/v) crystal violet dye (Sigma–Aldrich, USA) for 30 minutes at room temperature. Then, the excess dye was removed and gently rinsed three times with 250 µL of sterile PBS to remove the excess dye. The content of the well plate was allowed to dry. After drying, 200 µL of 33% (v/v) acetic acid (Sigma–Aldrich, USA) was added into the respective well of antiadherence and antibiofilm test plates to release the bound dye. The turbidity of the biofilm suspension in each well was then measured at an optical density (OD) of 590 nm using an ELISA reader (Thermo Scientific, USA). The formula to calculate the inhibition on biofilm formation and biofilm adherence is shown as below:[11]

$$1 - \frac{\text{OD590 (compound)}}{\text{Average OD590 (negative control)}} \times 100$$

Statistical Analysis

The data collected were in OD units and expressed as mean ± standard deviation (SD) where the number of determinations, $n = 9$, considering each test was carried out in triplicate and repeated three times. The data were analyzed with statistical package for the social science (IBM-SPSS®) statistical software, version 23.0. A normality test was carried out to test the assumptions of normality with the Shapiro–Wilk test. Due to the normal data distribution, results were analyzed with one-way between-groups analysis of variance (ANOVA) for MIC, antiadherence, and antibiofilm assays, comparing the values with the positive control (0.12% chlorhexidine). The results were considered significant at $p < 0.05$. Further analysis was done with *post hoc* Tukey's honestly significant difference (HSD) for multiple group comparisons.

Results

Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, and Fractional Inhibitory Concentration of the Oil Mixture

Table 1 shows the MIC, MBC and ΣFIC index of the respective oil and oil mixture against *S. sanguinis*, *S. mutans*, and *L. casei* in their single and mixture species. Virgin coconut oil effectively inhibited *S. sanguinis* at a concentration of 25%, followed by *S. mutans* and *L. casei* at 50% and their mixture species at 100% concentration. Meanwhile, for VOO, the MIC for all tested species occurred at 50% concentration. The oil mixture effectively inhibited *S. sanguinis* and *L. casei* at a concentration of 50%, followed by *S. mutans* and mixture species at 100% concentration. Regarding the bactericidal effect, no MBC was detected against the tested species, regardless of a single or mixture species for VCO. The MBC for VOO and oil mixture for all tested species occurred at a 100% concentration except *S. mutans*, with no bactericidal activity of the oil mixture against it.

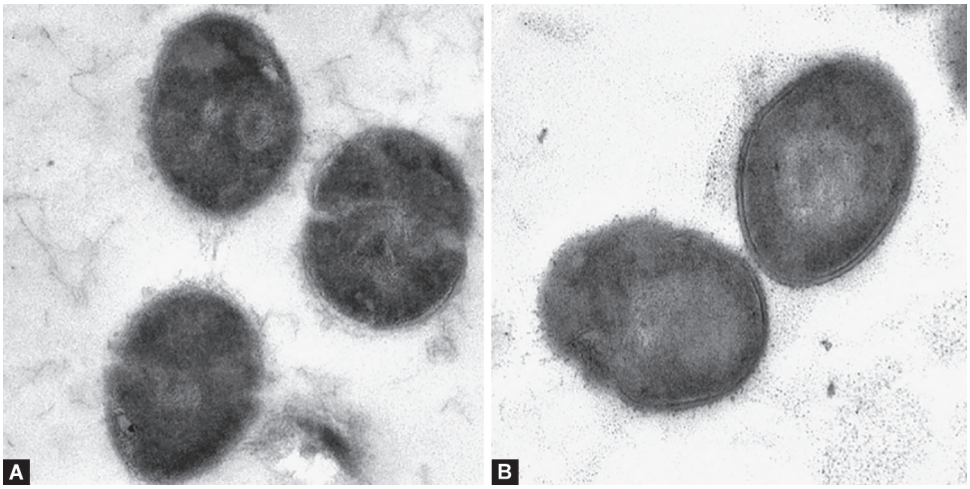
Morphological Changes under Transmission Electron Microscopy

Upon examination of the negative control group under transmission electron microscopy (TEM), the surface of the cell walls of *S. sanguinis* and *L. casei* colonies appeared smooth with intact cell membranes and complete cell content (Fig. 1; Fig. 2A). Under the effect of the oil mixture, significant cytological differences were observed in both bacteria under TEM. *S. sanguinis* showed structural changes in the cell wall (Fig. 1B). Secondary to the porous cell wall,

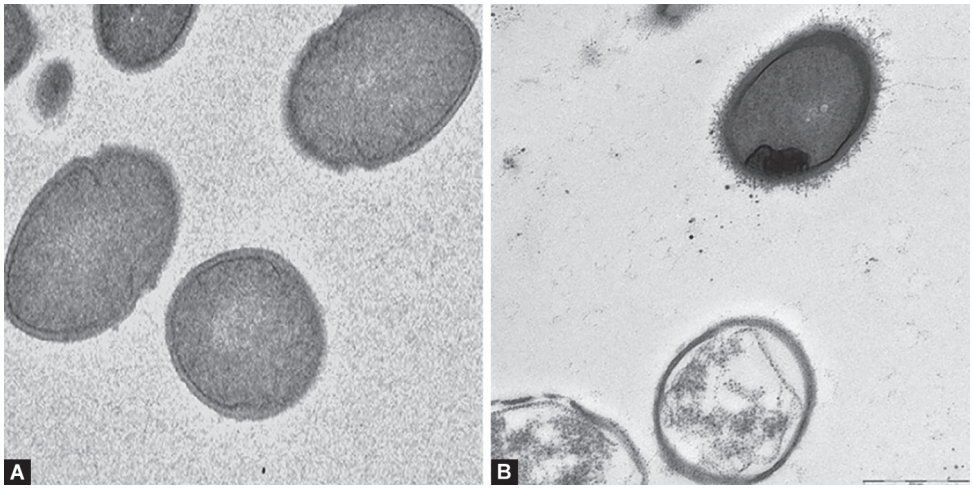
Table 1: The MIC, MBC, and ΣFIC of respective oil and oil mixture against the tested microbiota

	MIC (MBC) (% concentration)			*FIC*			
	VCO	*VOO*	*Oil mixture*	FIC_I	FIC_{II}	*ΣFIC*	*Interaction*
S. sanguinis	25% (No MBC)	50% (100%)	50% (100%)	2	1	3	Indifference
S. mutans	50% (No MBC)	50% (100%)	100% (No MBC)	2	2	4	Indifference
L. casei	50% (No MBC)	50% (100%)	50% (100%)	1	1	2	Indifference
Mixture species	100% (No MBC)	50% (100%)	100% (100%)	1	2	3	Indifference

ΣFIC ≤ 0.5 = Synergy; 0.5 < ΣFIC ≤ 1.0 = Additivity; 1.0 < ΣFIC ≤ 4.0 = Indifference; 4.0 > ΣFIC = Antagonism



Figs 1A and B: Ultrastructure of *S. sanguinis*: (A) Control without oil mixture treatment; (B) Treatment with 100% oil mixture



Figs 2A and B: Ultrastructure of *L. casei*: (A) Control without oil mixture treatment; (B) Treatment with 100% oil mixture

cellular content leakage occurred, resulting in cell lysis. Apart from the structural changes in the cell wall of *L. casei* after treatment with a 100% oil mixture, there was disrupted structural integrity and disordered structural architecture of the cytoplasmic membrane (Fig. 2B). Loss of homogeneity in cytoplasm was evident when compared to negative control. Multiple cystic spaces were visible in the cytoplasm of the damaged bacterial cells.

Antiadherence Effect of Oil Mixture for Single- and Mixture-species Biofilms

For antiadherence activities of the oil mixture against the microbiota, the oil mixture at the MIC value displayed similar antiadherence activities with the positive control, 0.12% chlorhexidine ($p > 0.05$) against *S. sanguinis*, *S. mutans*, and mixture species except for *L. casei*. The antiadherence activities of the oil mixture against

Table 2: Antiadherence activity of oil mixture toward the microbiota in a single and mixture species

	Percentage of antiadherence (mean + SD) (%)			
	S. sanguinis	*S. mutans*	*L. casei*	*Mixture species biofilm*
100% oil mixture	80.87 ± 0.78	*72.84 ± 0.65	79.30 ± 0.49	*77.74 ± 3.82
50% oil mixture	*80.16 ± 0.67	9.98 ± 2.23	*11.13 + 11.18	71.13 ± 5.76
25% oil mixture	79.64 ± 0.54	–	1.51 + 0.93	–
Positive control: 0.12% CHX	80.41 ± 0.49	73.9 ± 0.76	80.25 + 3.79	79.58 ± 3.77
ANOVA				
F	5.97	6046.69	465.15	8.61
p-value	0.002	<0.001	<0.001	0.002

*Minimum inhibitory concentration value of oil mixture

Table 3: Antibiofilm activity of oil mixture toward the microbiota in a single and mixture species

	Percentage of biofilm formation inhibition (Mean + SD) (%)			
	S. sanguinis	*S. mutans*	*L. casei*	*Mixture-species biofilm*
100% oil mixture	68.31 ± 2.15	*69.01 ± 3.95	63.78 ± 2.83	*70.49 + 6.39
50% oil mixture	*66.94 ± 2	11.72 ± 8.96	*62.64 ± 5.21	62.79 ± 6.36
25% oil mixture	3.65 ± 2.61	–	16.43 ± 7.11	–
Positive control: 0.12% CHX	70.33 ± 2.35	66.88 ± 4.7	58.77 ± 6.37	70.33 ± 4
ANOVA				
F	1813.17	241.47	147.64	5.38
p-value	<0.001	<0.001	<0.001	0.012

*Minimum inhibitory concentration value of oil mixture

L. casei occurred at 100% oil mixture. On the contrary, all the sub-MIC values of the oil mixture for respective microbiota did not possess similar antiadherence activities with the positive control, 0.12% chlorhexidine, except 25% oil mixture exhibited similar antiadherence activities against *S. sanguinis* ($p > 0.05$). Table 2 shows the antiadherence effect of oil mixture toward *S. sanguinis*, *S. mutans*, and *L. casei* in single- and mixture-species biofilms.

Antibiofilm Effect of Oil Mixture for Single- and Mixture-species Biofilms

The oil mixture at MIC value for respective microbiota presented a similar antibiofilm effect with the positive control, 0.12% chlorhexidine toward all the microbiota except *S. sanguinis* in which 50% concentration of oil mixture did not possess a similar antibiofilm effect with the positive control ($p < 0.05$). All the sub-MIC values of the oil mixture for respective microbiota did not exhibit a similar antibiofilm effect with the positive control, 0.12% chlorhexidine ($p < 0.05$). Table 3 depicts the antibiofilm activity of oil mixture toward the microbiota in single and mixture species.

Discussion

The present study was the preliminary study on the antibacterial, antiadherence, and antibiofilm activities of VCO and VOO mixture against oral microbiota. The oil mixture exhibited antibacterial activities toward *S. sanguinis*, *S. mutans*, and *L. casei* in their single and mixture species at higher MIC values compared to respective VCO and VOO. The MIC of the oil mixture against *S. mutans* and mixture species was 100%, while against *S. sanguinis* and *L. casei* was 50%. In addition to that, the oil mixture was bactericidal at 100% concentration toward all microbiota except *S. mutans*, where no bactericidal activity was observed. This suggests that a lower concentration of respective oil, either VCO or VOO, is preferred over a higher oil mixture concentration in inhibiting bacterial growth.

The antimicrobial effect of VCO is mainly due to the presence of medium-chain fatty acids, especially lauric acid, which enhances its antibacterial activity. The antimicrobial mechanism of lauric acid disrupts the cell membrane of bacteria and initiates the production of reactive oxygen species that subsequently destroy the susceptible microorganisms.[20] In addition, VCO has higher antimicrobial activities toward Gram-positive bacteria than Gram-negative bacteria because Gram-positive bacteria are more sensitive to lauric acid than Gram-negative bacteria.[21] This rationalizes the antibacterial effect of VCO on *S. sanguinis*, *S. mutans*, and *L. casei* in the current study. Meanwhile, the antimicrobial effect of VOO against oral microorganisms was credited to the phenolic compounds.[22] Among the active ingredients within VOO, malonic acid was found to be the most effective antimicrobial compound against oral streptococci.[23]

The ΣFIC determined the reactive relationship between VCO and VOO.[12] The ΣFIC was calculated from the MIC of the respective oil and oil mixture. When the MIC value of the oil mixture appeared to be higher than the MIC value of the respective oil, the interaction between respective oils would be either additive, indifferent, or antagonistic. The ΣFIC of oil mixture toward *S. sanguinis*, *S. mutans*, and *L. casei* in a single and mixture species was 2–4, interpreted as indifference interaction. Even though the active ingredients of VCO and VOO differ, we postulated that both oils have overlapping mechanisms toward the bacterial cell wall and cell membrane. Due to the overlapping mechanism of action, the combined activity of the oil mixture was inadequate to exceed the summative effects of individual oils; thus, the interaction between both oils was confined to indifference.[24]

In the assessment of the antiadherence activity of the oil mixture at their MIC value, it was found that the oil mixture at MIC exhibited comparable antiadherence activity with positive control 0.12% chlorhexidine against all the microbiota in a single and mixture species, except *L. casei* where 100% of oil

mixture was needed to exhibit similar effect. The antiadherence of oil mixture against oral microorganisms was not reported in the literature previously. Hazmi et al. found that VOO possessed superior antiadherence activities toward *L. casei*, but this result was not reproducible when the oil mixture was used. Perhaps the antiadherence effect of the oil mixture has been masked by the presence of VCO.[11] All the sub-MIC of the oil mixture did not exhibit similar antiadherence activity compared to 0.12% chlorhexidine, except the 25% oil mixture exhibited similar antiadherence activities against *S. sanguinis* ($p > 0.05$). This result reflected that a lower concentration of oil mixture was required to inhibit the adherence of *S. sanguinis* as the pioneer species onto the tooth surface in the oral environment. It was hypothesized that the antiadherence property of the oil mixture is attributable to its superhydrophobic nature when the water contact angle exceeds 150°.[11] Removal of bacteria will be easier when superhydrophobic material can reduce the adhesion force between bacteria and tooth surface. When using an oil mixture as mouthwash, it is possible to generate an easy-to-clean superhydrophobic environment, thus facilitating the removal of biofilm that has formed on the tooth surface.

The present study showed that the oil mixture at MIC value had comparable antibiofilm activity with 0.12% chlorhexidine toward all the tested microbiota, except for *S. sanguinis* (MIC value of 50% concentration of oil mixture) in which 100% concentration of oil mixture was required to exhibit comparable antibiofilm activity. We hypothesized that the oil mixture at MIC value could not exert antibiofilm activity toward *S. sanguinis* probably because of the strong affinity and attachment of *S. sanguinis* onto the tooth surface, which was not able to be disrupted by the oil mixture.[25] All the sub-MIC values of the oil mixture in our study portrayed lower antibiofilm activities toward *S. sanguinis*, *S. mutans*, *L. casei*, and mixture species compared with 0.12% chlorhexidine. This result was consistent with a study by Hazmi et al. where VCO and VOO at their sub-MIC displayed significantly lower antibiofilm activity against *S. sobrinus* and *L. casei* compared to 0.12% chlorhexidine.[11] The oil mixture at MIC value achieved the highest antibiofilm activity toward mixture species compared to other single species. This may be due to the oil mixture disrupting the composition and structure of the biofilm.[26] As Sari et al. reported, the lauric acid in VCO also contributes to its ability to prevent aggregation of dental biofilm formation.[27]

Given the involvement of a mixture of *S. sanguinis*, *S. mutans*, and *L. casei* species in this study, the interaction and relationship among them need to be explored. *S. sanguinis,* as the pioneer colonizer in dental biofilm formation, can produce only one type of glucosyltransferase (Gtf), *gtfP*. At the same time, *S. mutans* can synthesize three Gtfs (*gtfB*, *gtfC*, and *gtfD*) associated with cariogenic biofilms. Apart from that, *S. sanguinis* produces hydrogen peroxide (H_2O_2) in which the H_2O_2 production will interfere colonization of *S. mutans* on the teeth surface. Therefore, an inverse relationship exists between *S. sanguinis* and *S. mutans*.[28] Furthermore, *S. sanguinis* is an acid-sensitive microorganism. At the same time, *S. mutans* and *L. casei* are highly aciduric microorganisms that can endure in acidic environments. *S. mutans* contains bacteriocins known as antibacterial peptides and are synthesized by ribosomes. A study by Watanabe et al. shows that the bacteriocin gene with a positive strain enables the *S. mutans* to alter the bacterial composition within dental biofilm.[29] A recent study has shown that *Lactobacillus* sp. alone has poor adherence to the tooth surface and requires the presence of *S. mutans* and other primary colonizers to enhance their adherence.[30] The complex relationship among *S. sanguinis*, *S. mutans*, and *L. casei* explains the rationale of the effect of oil mixture against mixture species in antibacterial, antibiofilm, and antiadherence activities in which disruption of one species will directly affect another species.

Nevertheless, the limitation of the current study is that the antibiofilm and antiadherence assay were not tested against the individual VCO and VOO. The microbiota involved in this study was only limited to three individual species of *S. sanguinis*, *S. mutans*, *L. casei*, and their mixture species. It may be more important to describe what the bacteria are doing in the biofilm community rather than identifying which bacteria are present. Since no synergistic interaction was detected between both oils through the MIC value of the respective oil, further study can be carried out to modify the ratio of 1:1 combination to obtain the golden ratio of the oil mixture. The amount of VOO can probably be increased in the oil mixture whilst reducing the amount of VCO due to the superior antimicrobial effect of VOO over VCO.

Conclusion

The VCO and VOO mixture possesses antibacterial, antibiofilm, and antiadherence properties toward *S. sanguinis, S. mutans*, and *L. casei* in a single and mixture species, mainly at 50–100% concentration of oil mixture. Even though no synergistic interaction was found between VCO and VOO, the oil mixture exhibits all the desired properties of both oils when applied at 100% concentration. Thus, we concluded that the VCO and VOO mixture is helpful to be incorporated in oral healthcare products especially in children and individuals with special needs, to regulate the colonization of the bacteria, in view that it is natural and not toxic compared to chemical-based oral healthcare products.

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References

1. World Health Organization. Fact sheet: Oral health 2020. Available at: https://www.who.int/news-room/fact-sheets/detail/oral-health. Accessed on: 12 May 2023.
2. Pitts NB, Twetman S, Fisher J, et al. Understanding dental caries as a non-communicable disease. Br Dent J 2021;231(12):749–753. DOI: 10.1038/s41415-021-3775-4.
3. Takenaka S, Ohsumi T, Noiri Y. Evidence-based strategy for dental biofilms: Current evidence of mouthwashes on dental biofilm and gingivitis. Jpn Dent Sci Rev 2019;55(1):33–40. DOI: 10.1016/j.jdsr.2018.07.001.
4. Jafer M, Patil S, Hosmani J, et al. Chemical plaque control strategies in the prevention of biofilm-associated oral diseases. J Contemp Dent Pract 2016;17(4),337–343. DOI: 10.5005/jp-journals-10024-1851.
5. James P, Worthington HV, Parnell C, et al. Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. Cochrane Database Syst Rev 2017;3(3):CD008676. DOI: 10.1002/14651858.CD008676.pub2.
6. Farias JM, Stamford TCM, Resende AHM, et al. Mouthwash containing a biosurfactant and chitosan: An eco-sustainable option for the

control of cariogenic microorganisms. Int J Biol Macromolecules 2019;129,853–860. DOI: 10.1016/j.ijbiomac.2019.02.090.
7. King A. Bad science: Oil pulling. Br Dent J 2018;224(7):470. DOI: 10.1038/sj.bdj.2018.281.
8. Naseem M, Khiyani MF, Nauman H, et al. Oil pulling and importance of traditional medicine in oral health maintenance. Int J Health Sci (Qassim) 2017;11(4):65–70. DOI: PMID: 29085271.
9. Deen A, Visvanathan R, Wickramarachchi D, et al. Chemical composition and health benefits of coconut oil: An overview. J Sci Food Agric 2021;101(6):2182–2193. DOI: 10.1002/jsfa.10870.
10. Pietrocola G, Ceci M, Preda F, et al. Evaluation of the antibacterial activity of a new ozonized olive oil against oral and periodontal pathogens. J Clin Exp Dent 2018;10(11):e1103–e1108. DOI: 10.4317/jced.54929.
11. Hazmi NH, Shafiei Z, Zakaria ASI, et al. Antibacterial and antibiofilm effects of virgin coconut and virgin olive oils on *Streptococcus sobrinus* and *Lactobacillus casei*. J Res Med Dent Sci 2020;8(3):141–152. eISSN: 2347-2367; pISSN: 2347-2545.
12. Caesar LK, Cech NB. Synergy and antagonism in natural product extracts: When 1 + 1 does not equal 2. Nat Prod Rep 2019;36(6):869–888. DOI: 10.1039/c9np00011a.
13. Shafiei Z, Haji ARZ, Philip K, et al. Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium* sp., *Mangifera* sp., and *Mentha* sp.) on single- and dual-species biofilms. PeerJ 2016;4:e2519. DOI: 10.7717/peerj.2519.
14. Clinical Laboratory Standard Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 10th edition. CLSI document M07-A10; 2015, pp. 1–267.
15. Kowalska–Krochmal B, Dudek–Wicher R. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. Pathogens 2021;10(2):1–21. DOI: 10.3390/pathogens10020165.
16. Berenbaum MC. A method for testing for synergy with any number of agents. J Infect Dis 1978;137(2):122–130. DOI: 10.1093/infdis/137.2.122.
17. van Vuuren S, Viljoen A. Erratum. Plant-based antimicrobial studies methods and approaches to study the interaction between natural products. Planta Med 2011;77(11):1168–1182. DOI: 10.1055/s-0031-1298216.
18. Azeredo J, Azevedo NF, Briandet R, et al. Critical review on biofilm methods. Crit Rev Microbiol 2017;43(3):313–351. DOI: 10.1080/1040841X.2016.1208146.
19. Kwasny SM, Opperman TJ. Static biofilm cultures of Gram positive pathogens grown in a microtiter format used for anti-biofilm drug discovery. Curr Protoc Pharmacol 2010;50:13A.8.1–13A.8.23. DOI: 10.1002/0471141755.ph13a08s50.
20. Casillas–Vargas G, Ocasio–Malave C, Medina S, et al. Antibacterial fatty acids: An update of possible mechanisms of action and implications in the development of the next-generation of antibacterial agents. Porg Lipid Res 2021;82:101093. DOI: 10.1016/j.plipres.2021.101093.
21. Hussain MS, Al-Alaq FT, Al-Khafaji NS, et al. Antibacterial effect of virgin and refined coconut oils on pathogenic bacteria: A review. Indian J Forensic Med Toxicol 2020;14(4):6042–6048. DOI: 10.37506/ijfmt.v14i4.12548.
22. Nazzaro F, Fratianni F, Cozzolino R, et al. Antibacterial activity of three extra virgin olive oils of the Campania region, southern Italy, related to their polyphenol content and composition. Microorganisms 2019;7(9):1–10. DOI: 10.3390/microorganisms7090321.
23. Karygianni L, Cecere M, Argyropoulou A, et al. Compounds from *Olea europaea* and *Pistacia lentiscus* inhibit oral microbial growth. BMC Complement Altern Med 2019;19(1):1–10. DOI: 10.1186/s12906-019-2461-4.
24. Noel DJ, Keevil CW, Wilks SA. Synergism versus additivity: Defining the interactions between common disinfectants. mBio 2021;12(5):e0228121. DOI: 10.1128/mBio.02281-21.
25. Zhu B, Macleod LC, Kitten T, et al. *Streptococcus sanguinis* biofilm formation & interaction with oral pathogens. Future Microbiol 2018;13(8):915–932. DOI: 10.2217/fmb-2018-0043.
26. Bertolini M, Costa RC, Barão VAR, et al. Oral microorganisms and biofilms: new insights to defeat the main etiologic factor of oral diseases. Microorganisms 2022;10(12):1–9. DOI: 10.3390/microorganisms10122413.
27. Sari LNI, Fauziah E, Budiardjo SB, et al. Antibacterial and antifungal effectiveness of virgin coconut oil mousse against *Streptococcus mutans* and *Candida albicans* biofilms. J Int Dent Med Res 2019;12(3):917–922. ISSN: 1309-100X.
28. Lozano CP, Díaz–Garrido N, Kreth J, et al. *Streptococcus mutans* and *Streptococcus sanguinis* expression of competition-related genes, under sucrose. Caries Res 2019;53(2):194–203. DOI: 10.1159/000490950.
29. Watanabe A, Kawada–Matsuo M, Le MNT, et al. Comprehensive analysis of bacteriocins in *Streptococcus mutans*. Sci Rep 2021;11(1):1–13. DOI: 10.1038/s41598-021-92370-1.
30. Wen ZT, Huang X, Ellepola K, et al. *Lactobacilli* and human dental caries: More than mechanical retention. Microbiology (Reading) 2022;168(6):1–11. DOI: 10.1099/mic.0.001196.