Antibacterial Effects of Fermented and Cold Press VCO against Aggregatibacter Actinomycetemcomitans and Porphyromonas Gingivalis

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Abstract

The objective of this study is to investigate the antibacterial effects of fermented and cold press Virgin Coconut Oils against Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis.

We analyze the antibacterial activity of two types of virgin coconut oil (VCO) at different concentrations of 12.5%, 25%, and 50%, and chlorhexidine 0.2% (CHX) was used as a positive control. The antibacterial activity of A.actinomycetemcomitans and P.gingivalis were investigated using disc diffusion agar test for determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Scanning Electron Microscope (SEM) of A.actinomycetemcomitans and P.gingivalis after tested with fermented and cold press VCO was taken for the assessment of bacterial morphology.

In this study, 6.25% fermented VCO and 3.13% cold press VCO were found to have antibacterial activity against A.actinomycetemcomitans. In comparison, 3.125% fermented VCO and 1.5% cold press VCO were found to have antibacterial activity against P.gingivalis (p>0.05). These findings were also found to be lesser than the action of CHX. SEM shows that the morphologic configurations of bacteria were affected after exposed to fermented and cold press VCO.

Fermented and cold press VCO were found to have notable antibacterial activity against A.actinomycetemcomitans and P.gingivalis.

Experimental article (J Int Dent Med Res 2020; 13(3): 969-974)

Keywords: Coconut Oil, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Microbial Sensitivity Tests, Anti-Bacterial Agents.

Received date: 14 April 2020   **Accept date:** 10 May 2020

Introduction

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* are two common types of gram-negative bacteria associated with periodontal diseases, including other types of bacteria[1,2]. Periodontal disease is often denoted as a complex disease and is characterized by the host response destruction of soft tissues and hard tissues supporting the tooth. Many studies have shown the extent and severity of periodontal disease are linked to the presence of these two bacterias. *A.actinomycetemcomitans* is a facultative anaerobic bacteria that produce a virulence factor known as leukotoxin. It has seven serotypes, being serotypes a, b, and c are the most common[3]. Serotype b has higher pathogenicity due to its ability to produce more leukotoxin. Leukotoxin is responsible for stopping the activity of neutrophil and macrophages, resulting in diminishes host immune response and leads to possible periodontal breakdown[4]. *P.gingivalis* is an obligate anaerobic bacteria, non-motile and often found in coccus or rod-shaped. It is from the Bacteroides family and forms a black colony on blood agar plates[5,6]. It produces multiple virulence factors such as collagenase, hemolysins, proteases, and endotoxin. It also used its fimbria to attack the epithelial and endothelial cells. *P.gingivalis* is also capable of inhibiting the migration of polymorphonuclear cells.

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Scaling and root debridement (SRD) are the gold standards to treat periodontal disease[7]. However, SRD unable to achieve the complete elimination of bacteria due to tooth anatomical factors such as involvement of furcation areas. Several studies revealed less response of probing depth seen in multirooted teeth after SRD[8–10]. This finding has directed the application of antimicrobial agents act as an adjunctive to SRD. Minocycline, tetracycline, and doxycycline are well-known examples of antimicrobial drugs that have shown to increase bacterial resistance over some time[11–15]. On the other side, interestingly, many studies involving end products of plants exhibit antimicrobial activity against several microorganisms, particularly periodontal pathogens[16–24]. It offers a safe and natural alternative in the treatment of periodontal disease.

Coconut (*Cocos nucifera Linn*) belongs to the plant in the Palmae family. It is widely grown in Southeast Asia, mainly in Indonesia, the Philippines, and Malaysia. Coconut oil has two types, primarily copra oil and virgin coconut oil (VCO)[25,26]. VCO is extracted from the matured kernel of the coconut palm, either with or without heat[27]. Different methods of processing VCO have been used, and it will determine the quality of the end products, by ensuring no alterations of its quality and compositions. Generally, the processing of VCO is divided into dry or wet methods, which the latter without the involvement of any drying process. Cold press is a dry method used controlled heating and being pressed mechanically to acquire the oil, whereas fermented is a wet method where the kernel heated to remove moisture in it[28]. VCO is composed of 92% saturated fatty acids with lauric acid making up 50% of this saturated acids[25]. Due to that, it is also usually known as lauric oils because of the significant constituents is lauric acid. The health benefits of its active ingredients, lauric acid make a gain in reputation as the comestible oil. Monolaurin of lauric acid has antimicrobial activity against the range of microorganisms[29]. In Malaysia, the usage of virgin coconut oil has got attention as functional food oil by the layperson, and also the researchers in the treatment of various diseases. There are many available studies using coconut oil as an in vitro study[30–32]. A study in 2017 has tested VCO on *A.actinomycetemcomitans* and *P.gingivalis* with only single dilution techniques[33].

However, to our knowledge, there is an absence of research that compares the effect of two commonly producing coconut oil, which is fermented and cold press VCO tested on *A.actinomycetemcomitans* and *P.gingivalis* with three repetitions of the dilution method. This study aimed to assess the antibacterial action of two types of commonly used VCO against *A.actinomycetemcomitans* and *P.gingivalis.*

Materials and methods

Preparation of VCO

The study was conducted in Research Laboratory at Faculty of Dentistry UiTM Sungai Buloh and Faculty of Dentistry UKM. This study is an in-vitro study of two periodontal pathogenic bacterias, *A.Actinomycetemcomitans* and *P.gingivalis,* after administrations of fermented and cold press coconut oil at various concentrations (12.5%, 25%, and 50%). The VCO was supplied by Umaty Industries Sdn Bhd. Each stock solution of VCO was solubilized with dimethyl sulfoxide DMSO (12.5% VCO with 87.5% DMSO, 25% VCO with 75% DMSO, 50% VCO with 50% DMSO).

Bacterial strains culture and isolation

A.Actinomycetemcomitans (ATCC 43718) and *P.gingivalis* (ATCC 33277) were taken from the collection of Professor Fouad Hussain Al-Bayaty at Level 4, Research Laboratory, Faculty of Dentistry, UiTM Sungai Buloh. *A.actinomycetemcomitans* were cultured in Tryptic Soy Broth (TSB) agar and broth. At the same time, *P.gingivalis* was cultured in Brain Heart Infusion (BHI) agar and broth with additional additives for *P.gingivalis* which is 0.5g/ml cysteine, 5mg/ml haemin and 5 mg/ml vitamin K, followed by incubation period for three to seven days in anaerobic condition at 37 ^{0}C. *A.actinomycetemcomitans* were kept in an anaerobic jar in the presence of 5% carbon dioxide, while *P.gingivalis* was assessed in the anaerobic chamber in the presence of nitrogen and mixed gas. The bacteria were kept in glycerol stock at -80 ^{0}C.

Preparation of McFarland Standard and disc diffusion assay

The sterilized loop was used to streak the bacteria into the normal saline in the test tube, and the solution was stirred until it looks homogenous and free of clumps. Approximately 1 X 10^8 CFU/mL of bacteria densities

corresponding to a 0.5 McFarland turbidity standard was used to inoculate bacteria into fresh BHI and further incubated at 37 ^{0}C for three to seven days. The turbidity (0.5) will be compared and measured using a spectrometer (at 625nm, with absorbance, 0.08-0.1). A sterile cotton swab was used to streak the bacteria culture on BHI agar followed by application of 100 ul stock solution of VCO and DMSO with three concentrations, 12.5%, 25% and 50%, 100 ul of DMSO 10% as negative control and chlorhexidine 0.12% as a positive control. All tested plates were kept in an anaerobic jar with the presence of anaerobic gas. The steps were conducted twice for both fermented VCO and cold press VCO and were repeated for two times on different occasions. Following incubation, the zone of inhibition (clear zone with an absence of growth) was measured using caliper in millimeters by using the center of the disc as the point of reference.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC were performed using serial microdilution techniques in 96 wells microtiter plates. 100ul of TSB for _A.actinomycetemcomitans_ or BHI broth for _P.gingivalis_ was added into the first well until the twelve well. Subsequently, 100 ul of VCO was added into the first well, stirred, and was considered as 10^{-1} dilution. 100ul of VCO was transferred from the first well into the second well to make 10^{-2} dilution. The serial dilution was repeated up to 10^{-11} for each VCO. 100 ul of bacteria were taken from the test tube, which has been determined of its bacteria densities (1×10^6 CFU/mL) and added into the first well until the twelve well. The twelve well served as a growth control that contains only broth and bacteria. Another row of well (eight, ten, and twelve) contain only broth (negative control) while another well from the first until the last well contains only broth and VCO. This process was repeated for three times in the same 96 wells microtiter plate and was repeated with another two 96 wells microtiter plate, to achieve triplicate, for fermented and cold press VCO. The plate was incubated for three to seven days in the anaerobic jar at 37 ^{0}C and observed for turbidity. From the MIC dilution wells, the first four of five wells were plated (which was sensitive in MIC) and incubated for another three to seven days to observe any bacteriostatic or bactericidal effect of VCO against the bacteria.

Scanning electron Microscope

The effect of VCO on _A.actinomycetemcomitans_ and _P.gingivalis_ was observed using scanning electron microscopy (SEM). The dilution of MIC that exhibits inhibitory action towards the bacteria at the lowest concentration of VCO was taken into a tube then centrifuge to get a pellet. The supernatant was discarded while the pellet was dissolved with 500ul of glutaraldehyde. It was incubated overnight at four ^{0}C and send in an icebox to Integrative Pharmacogenomics Institute (iPROMISE), UiTM Puncak Alam, Selangor for image processing.

Data analysis

The data was collected and analyzed using a two-way ANOVA test where the p-value is less than 0.05 ($p < 0.05$).

Results

The zone of inhibitions was observed from table 1, representing the effect of fermented and cold press VCO against _A.actinomycetemcomitans_ by the disc diffusion agar method.

VCO	Concentration (%)			DMSO 10%	CHX 0.12%
	12.5	25	50		
Fermented	0.00±0.00	0.00±0.00	13.56±1.67*	0.00±0.00	34.44±4.64*
Cold Press	0.00±0.00	12.78±1.79	14.56±0.88*	0.00±0.00	35.00±7.50*

Table 1. The zone of inhibition of VCO against _A.actinomycetemcomitans_.

Values are represented as mean ±SD. Values on the same row, followed by *asterisk, differ significantly ($p < 0.05$). Groups: positive control (CHX 0.2%), negative control (DMSO 10%), 12.5%, 25%, 50% of fermented and cold press VCO.

The values are represented in millimeters. 25% and 50% cold press VCO while only 50% of fermented VCO shows antibacterial action against _A.actinomycetemcomitans_. The 50% cold press VCO has a zone of inhibition slightly higher than 50% fermented VCO, whereas 25% cold press VCO is the least. 50% fermented VCO and 25% cold press VCO are used to find the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) values, where 6.25% fermented VCO is

bacteriostatic against *A.actinomycetemcomitans* while 50% fermented VCO is bactericidal against *A.actinomycetemcomitans*. 1.56% cold press VCO shows inhibitory action, while 3.13% cold press VCO shows bactericidal action against *A.actinomycetemcomitans* (Table 2).

VCO	MIC (%)	MBC (%)
Fermented	6.25	50
Cold Press	1.56	3.13

Table 2. The MIC and MBC values for fermented and cold press VCO against *A.actinomycetemcomitans*. Values are represented in percentage.

The methods for determining the antibacterial activity of different concentrations of fermented and cold press VCO was similar for *P.Gingivalis* using disc diffusion agar, minimum inhibitory concentrations (MIC), and minimum bactericidal concentrations (MBC). The zone of inhibitions shows in table 3 by using disc diffusion agar, where a similar pattern was observed. Only 50% of fermented VCO exhibit antibacterial activity followed by 25% and 50% cold press VCO. However, the other samples have no antibacterial activity against *P.gingivalis* as no zone of inhibition seen. 50% fermented VCO and 25% cold press VCO are used to find the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) values, where 3.13% fermented VCO is bacteriostatic against *P.gingivalis* while no samples exhibit bacteriostatic activity. 1.56% cold press VCO shows inhibitory action, while 12.5% cold press VCO shows bactericidal action against *P.gingivalis* (Table 4).

VCO	Concentration (%)			DMSO 10%	CHX 0.12%
	12.5	25	50		
Fermented	0.00±0.00	0.00±0.00	12.7±2.64*	0.00±0.00	25.00±4.33*
Cold Press	0.00±0.00	10.56±1.67	14.78±2.59*	0.00±0.00	25.56±3.90

Table 3. The zone of inhibition of VCO against *P.gingivalis*. Values are represented as mean ± SD. Values on the same row, followed by *asterisk, differ significantly (p<0.05). Groups: positive control (CHX 0.2%), negative control (DMSO 10%), 12.5%, 25%, 50% of fermented and cold press VCO.

VCO	MIC (%)	MBC (%)
Fermented	3.125	-
Cold Press	1.56	12.5

Table 4. The MIC and MBC values for fermented and cold press VCO against P.gingivalis values are represented in percentage.

SEM photomicrographs of *A. actinomycetemcomitans* and *P. gingivalis* before and after treatment with several concentrations of fermented and cold press VCO were displayed, as shown in Figure 1 and 2. Micrographs were taken at lower magnification, 5000 X, to assess the general structures of bacteria and up to 20000 X magnifications for specific morphological patterns.
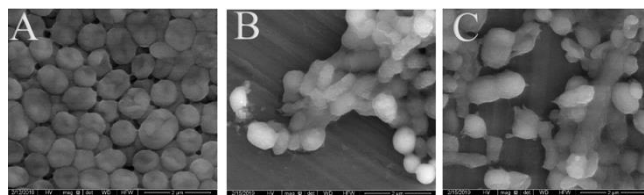

Figure 1. SEM images of *A. actinomycetemcomitans* (original magnifications 20000 X). (A) The bacterium was grown and subjected to three days of incubation. The cell wall is preserved with confined structures. (B) After treatment with 6.25% of fermented VCO, note that configurations and morphology of bacteria were altered. (C) After treatment with 1.56% cold press VCO, similar alterations were present.
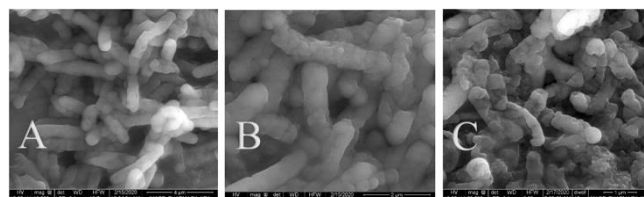

Figure 2. SEM images of *P. gingivalis* (original magnification 10000 X and 20000 X). (A) The bacterium was grown and subjected to five days of incubations in the anaerobic chamber. The cell wall is preserved with confined structures. (B) After treatment with 3.125% of fermented VCO, note that configurations and morphology of bacteria were altered. (C) After treatment with 1.56% cold press VCO, similar alterations were identified.

Discussion

We have identified that both VCOs exhibits antibacterial activity at 50%, the highest concentration. This finding is comparable with another study by Lili Nur Indah Sari et al. in 2019. They have found that VCO was capable at 80%, the highest concentration, which indicates the presence of more components of lauric acid that contributes to greater antibacterial effect[31].

Coconut oil is composed of 92% saturated acids, with lauric acid making up for 50% of these saturated acids. Monolaurin and monoglycerides of lauric acid have antimicrobial activity against a wide range of microorganisms [20,34–39]. The mechanism underlying the antibacterial effect of lauric acid is its ability to disrupt the cell membrane of bacteria, as reported by few studies [38,40,41].

In this study, we found the antibacterial activity of fermented and cold press VCO was demonstrated at different concentrations, being the fermented VCO at 50% only while cold press VCO at 25%, and 50% against *A.actinomycetemcomitans* and *P.gingivalis.* Furthermore, the mean zone of inhibition was higher for cold press VCO, although there is no significant difference between fermented and cold press VCO. The difference seen is probably due to the different ways of processing methods whereby cold press is believed to preserve its natural components best. There are two ways of producing coconut oil, which are dry and wet methods. Cold press is a dry method using controlled heating and subsequently pressed mechanically to obtain the oil. Fermented is a wet method without undergone drying process, and the kernel was heated under specific conditions to remove the moisture in it [28]. According to Fife 2003, cold process extraction conserved all of the functional components of coconuts and maintained the structure of its fatty acid as no polymerization takes place [42]. Due to that finding, we found cold press VCO has a better antibacterial effect than fermented VCO. However, both did not show any antibacterial activity at the lowest concentration, 12.5%.

The significant difference was examined between VCO and Chlorhexidine (CHX). CHX still has the highest mean zone of inhibition compared to both VCOs, although one study has reported a slightly superior antibacterial effect of VCO than CHX [43] on *Streptococcus mutans*, a facultative anaerobic gram-positive bacteria. CHX is well-known and has been the gold standard of oral antiseptic for plaque control for the past three decades. The downside of long-term usage can predispose teeth for staining and also perturbation of taste [44].

Several anti-bacterial studies carried out SEM to examine the changes in the shape and structure of the microorganisms before and after treatment with different types of agent [45–47]. The bacteria were in their original structure with a smooth surface and patent cell wall membrane before treatment. However, the loss of their original shape, either cell wall membrane shrunk, swollen, or reduced in size, was observed after treatment.

Many studies have proven the antimicrobial effects of VCO. Nevertheless, this is the first study to report and compare the effect of two common types of VCO against periodontopathogenic bacterias. Microscopic observations of the *A. actinomycetemcomitans* and *P.gingivalis* after treatment with the coconut oil revealed a disturbance in its viability, density, and the total bacterial number [48].

Conclusions

This finding exhibits the growth of *A. actinomycetemcomitans,* and *P.gingivalis* was inhibited by fermented and cold press virgin coconut oils with similar effectiveness. This result is promising to suggest that virgin coconut oil has antibacterial effects against *A. actinomycetemcomitans* and *P. gingivalis*.

Acknowledgements

Declaration of Interest

The authors report no conflict of interest.

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