Dental black stain poses two main challenges to dentists:Identifying the etiology and managing dental black stain. Few studies address dental black stain because the etiology has not been identified. Common dental black stain treatments include scaling and polishing the teeth [6,9]. However, black stain tends to recur regardless of the patient’s good oral hygiene [10]. This recurrence may be attributed to above normal quantities of chromogenic bacteria on the plaque and saliva of the teeth in children with dental black stain [11]. Which suggests that an antibacterial agent is required to inhibit the bacterial growth that causes a black stain.

These antibacterial agents may be derived from chemical compounds or natural ingredients, such as coconuts [12]. Processed coconuts can be made into therapeutic compounds, including coconut oil or virgin coconut oil (VCO), due to the high content of lauric acid, which has properties that kill a variety of microorganisms whose cell membranes contain lipid acid, such as Gram-positive or Gram-negative bacteria. The nonpolar nature of lauric acid enables it to penetrate into the bacteria’s cell membrane, damaging the phospholipid bilayers, and causing cell lysis [13,14].

Studies on the dental benefits of VCO are limited, especially with regard to the prevention of dental black stain. However, previous studies on the effect of VCO in concentrations of 12.5%, 25%, and 50% on the growth and protein profile of Streptococcus mutans revealed a decline in the colony counts and a significant change in the protein profile with the increasing concentrations of VCO [15] which motivated a deeper investigation into the antibacterial effect of VCO on the viability of Actinomyces spp. and Prevotella spp., the primary chromogenic bacteria that cause dental black stain in children.

METHODS

This study is an in vitro laboratory experiment that tested the viability of Actinomyces spp. and Prevotella spp. after the administration of VCO in various concentrations. With the approval from Ethical Committee of the Faculty of dentistry Universitas Indonesia, the subject, a child diagnosed with dental black stain, was identified. The inclusion criteria were as follows: A child aged 4-11 years with a good oral condition, the presence of black stain on at least 10 tooth enamel surfaces, and the participant’s parental consent. The exclusion criteria included: Poor oral hygiene, high incidence of caries (DMFT >5), subjects under medical care or taking medicines, and subjects with plaque samples that were difficult to harvest.

Plaque sampling was conducted on a child diagnosed with black stain. Then, the sample was cultured in a selective medium, and a
colony assessment was conducted. The experimental and laboratory study with sample measurement used the Federer Formula to obtain three samples. Then, Duplo was conducted, so each treatment was six samples. The next step was to visually confirm with Gram staining method. Following identification, 200 ul of bacteria was suspended on a 96-well plate and incubated in an anaerobic setting for 20 hrs at 37°C. While the bacteria were in the incubator, a VCO testing solution was created in the following concentrations: 12.5%, 25%, 50%, and 100%. Dilution of the VCO was completed using phosphate buffer solution. Then, the VCO solutions were homogenized to obtain a homogenous solution.

After 20 hrs, 200 ul of Actinomyces spp. and Prevotella spp. suspended on 96-well plates were exposed to 100 ul VCO in various concentrations. Bacteria exposed to the positive control (0.2% chlorhexidine gluconate) and the negative control (without testing material) were also prepared. Then, bacteria were incubated for 60 minutes in an anaerobic setting at 37°C. Finally, a methyl-thiazolyl-tetrazolium (MTT) solution was poured into each well of tested material, and the tested material solutions were incubated for 3 hrs. The results of the MTT testing were read using an Enzyme-linked-immunosorbent-assay reader with the wavelength 490 nm. The absorption score or optical density (OD) of the treatment and control groups were used in the following formula to calculate the cell viability percentage score.

Table 1: Differences in bacterial viability of Actinomyces spp. toward administration of VCO in various concentrations

| Treatment group | n  | Viability means±SD (%) | p       |
|-----------------|----|------------------------|---------|
| Negative control| 3  | 100.00                 | <0.001  |
| Positive control| 3  | 29.97±3.51             |         |
| 100% VCO        | 3  | 32.58±2.75             |         |
| 50% VCO         | 3  | 48.02±2.64             |         |
| 25% VCO         | 3  | 63.84±4.01             |         |
| 12.5% VCO       | 3  | 83.54±7.14             |         |

One-way ANOVA testing, *significant score based on p<0.05. VCO: Virgin coconut oil, SD: Standard deviation

Table 2: Post hoc analysis of differences in bacterial viability of Actinomyces spp. in treatment intergroups

| Treatment group | Difference in viability means (%) | p       |
|-----------------|----------------------------------|---------|
| Negative control versus 25% VCO | 36.16 | <0.001 |
| Negative control versus 12.5% VCO | 16.47 | 0.637 |
| Positive control (0.2% CHX) versus 100% VCO | 2.62 | 0.952 |
| Positive control (0.2% CHX) versus 50% VCO | 10.05 | 0.004 |
| 50% VCO versus 100% VCO | 7.44 | 0.011 |
| 100% versus 25% VCO | 31.27 | <0.001 |
| 100% versus 12.5% VCO | 50.96 | 0.010 |
| 50% versus 25% VCO | 23.82 | <0.001 |
| 50% versus 12.5% VCO | 43.52 | 0.021 |
| 25% versus 12.5% VCO | 19.69 | 0.428 |

Post hoc Tamhane’s testing, *significant score based on p<0.05. VCO: Virgin coconut oil

Table 3: Difference in bacterial viability of Prevotella sp. with administration of VCO in various concentrations

| Treatment group | n  | Viability means±SD (%) | p       |
|-----------------|----|------------------------|---------|
| Negative control| 3  | 100.00                 | <0.001  |
| Positive control (0.2% CHX) | 3  | 37.24±4.23             |         |
| 100% VCO        | 3  | 65.35±11.02            |         |
| 50% VCO         | 3  | 84.15±13.19            |         |
| 25% VCO         | 3  | 89.04±4.28             |         |
| 12.5% VCO       | 3  | 96.83±5.74             |         |

One-way ANOVA testing, *significant value based on p<0.05. VCO: Virgin coconut oil, SD: Standard deviation

Table 4: Post hoc analysis of difference in bacterial viability of Prevotella spp. in treatment intergroups

| Treatment group | Difference in viability means (%) | p       |
|-----------------|----------------------------------|---------|
| Negative control versus 100% VCO | 34.65 | 0.009 |
| Negative control versus 50% VCO | 15.85 | 0.388 |
| Negative control versus 25% VCO | 10.96 | 0.406 |
| Negative control versus 12.5% VCO | 3.17 | 0.982 |
| Positive control (0.2% CHX) versus 100% VCO | 28.11 | 0.013 |
| 100% VCO        | 18.80 |         |
| 100% versus 50% VCO | 23.69 | 0.038 |
| 100% versus 25% VCO | 31.48 | 0.005 |
| 100% versus 12.5% VCO | 4.89 | 1.000 |
| 50% versus 25% VCO | 12.68 | 0.657 |
| 50% versus 12.5% VCO | 7.78 | 0.846 |

Post hoc Tamhane’s testing, *significant value based on p<0.05. VCO: Virgin coconut oil
To identify the difference in the viability values of *Actinomyces* spp. and *Prevotella* spp. after the administration of VCO in various concentrations, unpaired *t*-tests were conducted.

Table 5 summarizes a significant difference between the bacterial viability of *Actinomyces* spp. and *Prevotella* spp. after the administration of the 100%, 50%, and 25% VCO concentrations (*p*<0.05).

### DISCUSSION

This study was conducted to identify the antibacterial effect of VCO on the bacterial viability of *Actinomyces* spp. and *Prevotella* spp., which cause dental black stain in children. *Actinomyces* spp. and *Prevotella* spp. were cultured in a selective medium.

The results of the bacterial culture were supported by the literature, which states that *Actinomyces* spp. and *Prevotella* spp. are primary chromogenic bacteria that play a role in the formation of dental black stain in children [1,11,16].

The antibacterial agent used in this study was VCO made using the fermentation method as it is a natural antibacterial agent due to its abundance of lauric acid (up to 61.07%). Lauric acid is a natural antibacterial agent that can kill microorganisms whose cell membranes contain lipid acids [13,17]. The VCO used in the study was in the following concentrations: 100%, 50%, 25%, and 12.5%. Previous studies showed that VCO in concentrations of 12.5%, 25%, and 50% has had positive effects on the growth and protein profile of S. mutans [15].

One-way to test the antibacterial effect of an agent is cytotoxicity testing. The cytotoxicity testing used in this study was an MTT assay, which is a standard colorimetric laboratory test that measures viable cells and is stated in OD. The OD value is comparable to the number of viable cells after exposure to an antibacterial agent [18,19].

In this study, exposure to VCO was completed during the 20-hr biofilm formation phase or the active accumulative phase, which is supported by previous studies that state that in the active accumulative phase, active growth occurs, enabling antibacterial agents to kill bacteria. In the 4-hr biofilm formation phase (adhesive phase), exposure to antibacterial agents did not reveal bacterial growth, and bacterial viability was not detected. In the 24-hr biofilm formation phase (maturation phase), bacterial growth slows, increasing the formation of extracellular polysaccharides, and the bacteria’s resistance to antibacterial agents increases [20]. The exposure of *Actinomyces* spp. and *Prevotella* spp. to VCO was followed by a 60-minute incubation period. The length of exposure was based on previous studies that stated that the maximal bacteria inhibition effect of antibacterial agents requires exposure to the antibacterial agent for 60 minutes [21].

The *in vitro* administration of VCO resulted in a decline in the bacterial viability of *Actinomyces* spp. and *Prevotella* spp. The decline in bacterial viability after the administration of VCO is due to its antibacterial content, namely, the lauric acid that kills a wide variety of microorganisms whose cell membranes contain lipid acids, such as Gram-positive and Gram-negative bacteria [15,17]. Lauric acid is a non-polar, saturated lipid acid. The non-polar properties enable lauric acid to penetrate the bacteria’s cell membrane, destroying the phospholipid bilayer, at which point bacterial cell lysis ensues [22,23]. The ability of VCO to kill bacteria is supported by previous studies that use a novel two-color fluorescent assay to identify bacterial viability and electron microscopy to show that lipids kill bacteria by disintegrating the cell membrane [14].

The statistical analysis (Tables 1 and 3) shows that each VCO concentration causes a decline in the bacterial viability value. The lowest viability occurred when the bacteria were exposed to the 100% VCO concentration, whereas the highest viability value occurred when the bacteria were exposed to the 12.5% VCO concentration [24].

These findings support the literature that states that the effectiveness of antibacterial agents in killing bacteria has a correlation with the effect caused. This correlation can be explained using the hyperbolic curve. If the concentration is increased, the maximal effect of the agent will increase. However, after exceeding the maximal value, an increase in the dose will not augment the effectiveness of the antibacterial [25].

The statistical analysis in Table 2 summarizes no significant difference in the bacterial viability of *Actinomyces* spp. between the negative control and the treatment group with the 12.5% VCO, which may mean that although 12.5% VCO decreases the bacterial viability of *Actinomyces* spp., the concentration is not high enough kill *Actinomyces* spp. However, a significant difference was found in the bacterial viability of *Actinomyces* spp. between the 25% VCO treatment group and the negative control group. Therefore, in this study, the minimum concentration of VCO necessary to kill *Actinomyces* spp. is a 25% [24].

The statistical analysis in Table 4 summarizes no significant difference in the bacterial viability of *Prevotella* spp. between the negative control; the treatment control; and the 50%, 25%, and 12.5% VCO groups. Lower concentrations of VCO are not adequate as antibacterial agents in killing *Prevotella* spp. Thus, this study concludes that a 100% VCO concentration is necessary to kill *Prevotella* spp. [24].

This study found no significant difference in the bacterial viability of *Actinomyces* spp. between the positive control group (0.2% chlorhexidine gluconate, the best broad-spectrum antibacterial agent against Gram-positive and Gram-negative bacteria) and the treatment group with 100% VCO. Therefore, 100% VCO has an antibacterial effect comparable to that of 0.2% chlorhexidine gluconate on *Actinomyces* spp. The VCO testing on *Prevotella* spp. showed a significant difference in viability between the positive control group and the treatment group with 100% VCO. Therefore, the administration of 100% VCO is not as effective as 0.2% chlorhexidine gluconate in killing *Prevotella* spp. [24,26].

The statistical analysis in Table 5 summarizes that the decline in the bacterial viability of *Actinomyces* spp. is higher than *Prevotella* spp. after the administration of various concentrations of VCO. This finding is supported by the literature, which states that Gram-positive bacteria are more susceptible to antibacterial agents than Gram-negative bacteria [22,23].

The structure of the simple cell lining of Gram-positive bacteria enables the antibacterial VCO to penetrate the cell membrane, where
in intracellular material leakage and cell biosynthesis impairment causes bacterial cell lysis [23,27].

Gram-negative bacteria, such as *Prevotella* spp., have cell membranes composed of lipopolysaccharides and lipoprotein, which are bound to peptidoglycan linings. Lipopolysaccharides and peptidoglycan function as the bacteria cell's defense system by selecting the foreign substances that enter the cell. The penetration of the antibacterial VCO into the Gram-negative bacteria may be inhibited due to the presence of lipoprotein, which contains a hydrophilic protein at the outer membrane of the bacterial cells, commonly referred to as porins, preventing the antibacterial agent from entering the cell [27].

This study is preliminary and involves direct contact between the antibacterial solutions and the *Actinomyces* spp. and *Prevotella* spp., so it does not describe the real situation in the oral cavity. Therefore, further studies that use saliva biofilm harvested from healthy volunteers are necessary to identify the actual oral environment. The results of this study can serve as the foundation for further in vivo studies that assess the antibacterial effect of VCO on the dental black stain.

**CONCLUSIONS**

Based on the in vitro study results, the following conclusions can be made:

1. There is a difference in the bacterial viability of *Actinomyces* spp. and *Prevotella* spp. after the administration of VCO in various concentrations;
2. Each augmentation of VCO concentration causes a reduction in the bacterial viability of *Actinomyces* spp. and *Prevotella* spp.;
3. The antibacterial effect of VCO on the decline in the viability value of *Actinomyces* spp. is higher than that of *Prevotella* spp.;
4. The administration of 12.5% VCO reduced bacterial viability; however, the reduction of the bacterial viability of *Actinomyces* spp. is significant after the administration of 25% VCO, and the reduction of the bacterial viability of *Prevotella* spp. is significant after the administration of 100% VCO.

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