



Research Article

Antibacterial and immunomodulator activities of virgin coconut oil (VCO) against *Staphylococcus aureus*



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ABSTRACT

Antibiotics have components to inhibit infections against *Staphylococcus aureus*, but they depend on judicious use to minimize the incidence of resistance forms. Strategies to improve the current situation include research in finding a new antimicrobial from virgin coconut oil (VCO). The saturated fatty acid, lauric acid (LA) (C12) contain in VCO was reported to have antibacterial activities. This study developed antimicrobial of VCO as an antimicrobial and immunomodulatory agent. *Staphylococcus aureus* used in this study had been isolated and identified from the mastitis milk crossbreed Etawa goat from Riau, Indonesia. The susceptibility of *S. aureus* to VCO was tested using the broth dilution method. The inhibition mechanisms of *S. aureus* had been studied by scanning electron microscopy (SEM) after treatment with VCO, and potential of VCO, which is known in phagocytosis macrophage. *In vitro* test confirmed the inhibitory effect of VCO on the growth of *S. aureus* at the concentration of 200 μ l (equal to 0.102 % LA). Based on the result of the phagocytosing assay, VCO could increase the ability of the macrophage cells to phagocyte *S. aureus* significantly at a concentration of 200 μ L (equal to 0.102% LA). This study concluded that the VCO could inhibit the growth of *S. aureus* with destructive mechanisms of bacterial cell walls and increase the ability of the phagocytic immune cells.

1. Introduction

Intramammary infections among small ruminants raised in Indonesia are not well documented in spite of research overseas reported that describe the susceptibility of cows and goats to Staphylococcal infections. *Staphylococcus aureus* is one of the bacteria caused clinical mastitis or subclinical mastitis in small ruminants as well as sheep and goats [1, 2, 3]. In dairy goats, *S. aureus*, as well as coagulase-negative staphylococci, are usually considered to have the higher pathogenicity of subclinical mastitis agents [4]. The subclinical mastitis in dairy cow and goat farms, causing significant economic clauses due to the reduction in milk production and poor milk quality [5].

Staphylococcus aureus has an inadequate response to various antibiotic therapy, reflected a severe problem in the world. This problem because of the far-ranging use of lactam drugs, multidrug-resistant *S. aureus* (especially the methicillin-resistant *Staphylococcus aureus*, MRSA) became a frightening new threat in the past decades [6, 7]. Methicillin-resistant

Staphylococcus aureus can hydrolyze almost all kinds of lactams, and the drug-resistant strains spread quickly, causing high mortality rate in infected patients and the severe overdraft of medical resources due to its broad drug-resistant spectrum [8].

The risk of antibiotic residues in milk is also becoming a threat to consumer health. Negative consequences of antibiotics residues in dairy products and antibiotics resistance genes developed in bacteria following exposure to antimicrobial agents are well documented. Data on antibiotic resistance could also be used to characterize these opportunistic pathogens, which may further limit the risks associated with the consumption of contaminated milk and its products [9, 10].

As many natural products that possess antibacterial activity can resolve the problems of chemical and antibiotic resistance, they have the potential to be a choice [11, 12, 13, 14, 15]. The developments of alternatives to the existing antibiotics against MRSA infections are still of interest and need to be actualized [8, 16]. *Chuzhou chrysanthemum* oil (CCO) has been reported by Cui et al. [17] has antibac-

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terial activity. The antibacterial mechanism of CCO affects through the Embden-Meyerhof-Parnas Pathway of *S. aureus*. Virgin coconut oil (VCO) has a functional ingredient such as caprylic acid (C₈) 8%, capric acid (C₁₀) 10%, lauric acid (LA) 48% and meristic acid 17%. Medium-chain fatty acids (MCFAs) were found to be a potential antibacterial functional food [18]. Many recent studies indicate that exposure to LA was the most inhibitory to growth bacteria. Capric acid and caprylic acid can also inhibit the growth of bacteria [19, 20, 21].

Although there are many studies reported the potential of virgin coconut oil as antibacterial activity, there is need more investigations to support that the virgin coconut oil has the ability to inhibit the *S. aureus* isolated from mastitis milk goat and its potential as an immunomodulator.

2. Materials and methods

2.1. Bacterial isolates

In this research, we used *S. aureus* isolated from subclinical mastitis milk's Peranakan Etawa (PE) or crossbred Etawa goats in Riau, Indonesia. Peranakan Etawa goat is descended originally from crossings between the Kacang goat (an indigenous breed of goat in Indonesia) with Etawa (Jamnapari India) goat. All samples were identified based on the Gram staining, fermentation on mannitol salt agar (MSA, Oxoid, England UK), catalase, and coagulase tests as described previously [22, 23]. The bacterial isolate was genetically confirmed as *S. aureus* by using the Polymerase Chain Reaction (PCR) amplification of the 23S rRNA gene [24], thermonuclease *nuc* gene [25], and coagulase (*coa*) gene [26].

The bacterial isolates were cultured on the Base Agar (BA; Oxoid, England UK) supplemented with 5% sheep blood. After incubation at 37 °C for 18–24 h, colonies were picked and resolved in 2 ml volume of sterile Todd Hewitt Broth (THB; Oxoid, England UK). The bacterial cell density was adjusted to 4 McFarland standard or approximately 10⁹ colony-forming units CFU ml⁻¹. The inoculate were then diluted ten-fold in sterile normal saline solution (NSS), giving a final cell density of approximately 10⁸ CFU ml⁻¹.

2.2. Agar dilution method

The procedure followed the international recommendations provided by the Clinical and Laboratory Standards Institute (CLSI) [27] in a modified tube dilution assay. Dilution tubes containing 1% Müller Hinton Agar (MHA; Oxoid, England UK) were adjusted at a final volume of 7.5 mL. *Staphylococcus aureus* were streaked on the MHA tubes containing VCO (50, 100, 150, 200, 250, 300, 350 and 400 µL, which 1 µL VCO equal to 0.00051 LA) and incubated for 16–18 h. After incubation, the growth of the bacteria on the tubes was recorded as densities of media with no visible growth of *S. aureus* in compare to the positive control tube.

2.3. Assessment of morphological alteration by scanning electron microscopy (SEM)

The alteration *S. aureus* after treatment with VCO was examined by SEM. The bacteria were cultured in THB and incubated at 37 °C for 24 h. The suspensions were spun at 3000 rpm for 15 min. A total of 250 µL VCO as a recommended dose was added to the pellet, centrifuged and incubated for 24 h at 37 °C. After incubation, the suspension was spun at 3000 rpm for 15 min. Pellet was dried according to Duke [28] at the critical point by using a Polaroid CPD 7501 (VG Microtech, E Sussex, UK). The specimens were coated with a thin layer of gold by a Sputter Coater (SPI suppliers, PA, USA) prior to examination with SEM (JSM-6510LA, Japan).

2.4. Phagocytosing activity

In this study, we used male Balb/c mice with bodyweight 20 g of the pathogen-free strain, maintained at the UHPH LPPT UGM. This study meets the requirements of ethics for research in experimental animals and approved by the ethical committee clearance for preclinical research Gadjah Mada University with number: 333/KEC-LPPT/X/2015.

Mice were euthanized with pentobarbital 125 mg kg⁻¹ intravenous. The skin over the abdomen was cleaned up, and 1 ml of Hanks Balanced Salt Solution (HBSS, Lonza, USA) was injected into the peritoneal cavity. The abdomen was massaged gently, and after about 1 min, the cell-rich fluid was removed with a capillary pipette and placed in a tube (Biologic, China). These fluid contain macrophage cells. The cells were centrifuged for 8 min, at 150 g, at room temperature. Total 2 ml HBSS was added to the pellet. The cells were thoroughly mixed by repeated pipetting [29].

A total of 10⁸ bacteria mL⁻¹ was suspended with macrophage cells (10⁵ cells mL⁻¹) and VCO with a ratio of 1:1:1 into 2 mL microtube. The suspension containing bacteria was stained with Safranin 10% (Merck, Germany) and incubated for 1 h at 37 °C. Phagocytosing activity were assayed by placing a drop of suspension on an object-glass. The slides were examined with immersion objective at a magnification of 100x, by using a Zeiss binocular microscope. Phagocytosing result was evaluated by counting the number of bacteria phagocytosed by 20 macrophages cells [30].

2.5. Data analysis

The susceptibility test was recorded descriptively as densities of media with no visible growth of *S. aureus* in compare to the control. The result of phagocytosing activity was analyzed using ANOVA single factor with considered significant at $p = 0.05$, and followed by the honestly significant difference (HSD) test (SPSS statistical software version 21.0; SPSS Inc., Chicago, IL, USA).

3. Results

Staphylococcus aureus used in this study was isolated from the sub-clinical mastitis milk's Peranakan Etawa (PE) and confirmed based on biochemical and genotypic identification according to the previous study [22, 23].

Virgin coconut oil was evaluated at different concentration levels (0, 50, 100, 150, 200, 250, 300, 350, 400 µL) against *S. aureus* on the MHA dilution test. The result showed that VCO could inhibit the growth of *S. aureus* on the agar dilution media with a minimal concentration of 200 µL (equal to 0.102% LA) of media. Amoxicillin was used as an antibiotic control. The inhibition growth of *S. aureus* in the agar dilution media containing VCO was summarized in Fig. 1.

Scanning electron microscopy is a well-established basic method to observe the morphology of the material surface. Virgin coconut oil could damage the surface of the *S. aureus* cell walls. In this study, there were some leakage and change of the bacteria cell walls following exposure to VCO. Treatment by VCO yielded bacteria with surface depressions and visualized the holes in the bacteria cell walls (Fig. 2a). This finding was different from the control of *S. aureus* without treatment (Fig. 2b).

Table 1 shows the results of the phagocytic activities of mice peritoneal macrophage cells against *S. aureus* after treatment with VCO. The number of phagocytosed *S. aureus* in the macrophage cells at any given 50, 100, 150, 200 µL VCO were 24.7 ± 14.14 , 26.1 ± 12.94 , 42.7 ± 18.31 , 49.1 ± 32.29 bacteria cell⁻¹, respectively. There were significant ($P < 0.05$) increased comparing to untreated VCO (23.1 ± 7.52 bacteria cell⁻¹).

4. Discussion

Our finding resulted that the addition of VCO to MHA could inhibit

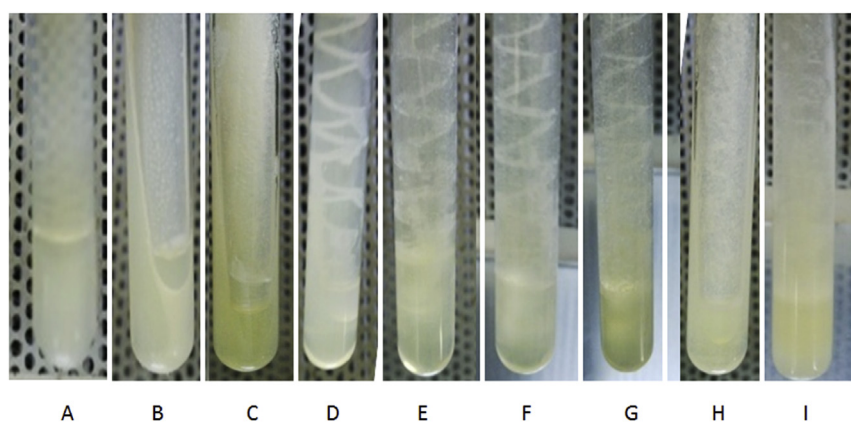


Fig. 1. Müller Hinton Agar dilution test results of *Staphylococcus aureus* on the MHA containing VCO with different concentrations. A = MHA without VCO (clear), B = MHA containing VCO 100 μ L (clear), C = MHA containing ampicillin after inoculation with *S. aureus* (no bacterial growth), D = MHA after inoculation with *S. aureus* (bacterial growth), E–G = MHA containing VCO 50 μ L, 100 μ L and 150 μ L after inoculation with *S. aureus* (bacterial growth), H = MHA containing VCO 200 μ L after inoculation with *S. aureus* (less bacterial growth), I = MHA containing VCO 400 μ L after inoculation with *S. aureus* (no bacterial growth).

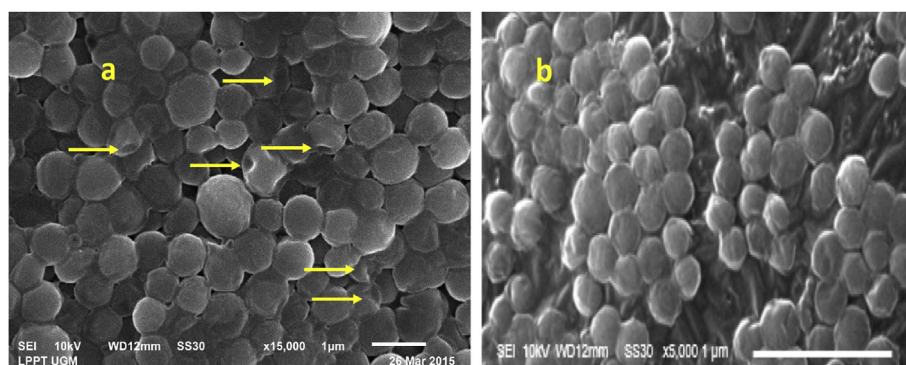


Fig. 2. Morphology of *Staphylococcus aureus* after treatment with 250 μ L VCO (equal to 0.1275% LA) (a), compare to control of *Staphylococcus aureus* without treatment (b), examined with Scanning electron Microscopy (SEM) 15000 $\times$ magnification. Yellow arrows indicate holes in the bacterial wall.

Table 1

The phagocytic activities of mice peritoneal macrophage cells against *S. aureus* after treatment with VCO.

Concentration VCO (μ L) (1 μ L equal to 0.00051% LA)	Number of phagocytosed <i>Staphylococcus aureus</i> (bacteria cell ⁻¹)
0	23.1 $\pm$ 7.52 ^b
50	24.7 $\pm$ 14.14 ^{ab}
100	26.1 $\pm$ 12.94 ^{ab}
150	42.7 $\pm$ 18.31 ^{ab}
200	49.1 $\pm$ 32.29 ^a
250	19.4 $\pm$ 16.19 ^b

Note: Different notations indicate significant differences at the level of 5% ($p < 0.05$).

the growth of *S. aureus*. The mechanism of killing or inhibition of organisms had many variable aspects and complete process [19]. Sihombing et al. [31] reported VCO with a dose equal to 0.5% LA inhibited the growth of *S. aureus*. The amount of high LA (40–60%) mainly in the form of free fatty acids and monoglycerides in coconut oil has antibacterial activity, antiviral, antifungal, antiprotazoal, and also can enhance the immune system. Lauric acid is an MCFA, which has the additional beneficial function of being formed into monolaurin in the human or animal body [32]. Some studies had also shown some antimicrobial effects of free LA. This component did not cause resistance problem, so far increased the use of coconut oil as an alternative treatment in humans. Several health benefits have been attributed to this oil. These include benefits in skincare, haircare, stress relief, weight loss, cholesterol level maintenance, immunomodulatory effects, cardiovascular uses, and more recently, in Alzheimer's disease [33]. Huang et al. [34] evaluated the antimicrobial property of LA against *Propionibacterium acnes* both *in vitro* and *in vivo*. Incubation of the skin bacteria *P. acnes*, *S.*

aureus and *Staphylococcus epidermidis* with LA yielded minimal inhibitory concentration (MIC) values against the bacterial growth over 15 times lower than those of benzoyl peroxide (BPO). The lower MIC values of LA indicated stronger antimicrobial properties than that of BPO.

Observations by using SEM, revealed that VCO could damage the *S. aureus* through the mechanisms of the bacterial cell wall surface. Medium-chain fatty acids (MCFA) of VCO, especially LA, has a composition similar to bacterial peptidoglycan cell wall constituent. LA was initially covered the entire surface of the cell wall, penetrate and destroyed the bacteria. As seen in Fig. 2a, the *S. aureus* visualized to be damaged and there were some holes in the surface of cell walls after treatment with VCO. Monoglycerides and MCFA in VCO break up the bacterial membrane with the aid of gastrointestinal lipase enzyme. The working system of the MCFA, especially LA, has been changing the permeability of the bacterial cell wall, disrupting metabolism, inhibiting the essential nutrients needed by bacteria or linkage with carbohydrate metabolism. Monoglyceride monolaurin has been functioning as an antiviral, antibacterial and antiprotzoa. Essential oils and their components have activity against the bacteria with a variety of targets, particularly the membrane and cytoplasm, and in some cases, they completely change the morphology of the cells [35, 36].

Shilling et al. [20] reported 2 mg mL⁻¹ of LA in the VCO could disturb of both the cell membrane and cytoplasm of bacterial cells, under the transmission electron microscopy (TEM) examination. *In vitro* study activities of VCO against bacterial showed that LA and monolaurin in combination with lactic acid could damage the cytoplasm and membrane cells of the bacterium by using SEM and TEM as well [19]. The antibacterial activity of essential oils from *S. officinalis* has been reported by several authors. Four essential oil samples from this plant were tested against *Pseudomonas aeruginosa*, *Escherichia coli*, *Listeria monocytogenes*, and *S. aureus* [11, 15].

Adhesions between bacteria due administration of VCO, facilitating the process of phagocytosis, in which the bacterial cells are closer to become more phagocyte by macrophage cells. Lauric acid might show antimicrobial activity by disruption of the cell membrane, inactivation of enzymes, and denaturation of cell proteins similar to other long-chain fatty acids. Monoglycerides and the free fatty acid act as an antibacterial. Lauric acid has a role either as bacteriostatic or bacteriocidal [19, 36].

Cells evolved a whole host of mechanisms for ingesting particles and fluids. These vary from receptor-mediated endocytosis (absorption of small particles into clathrin-coated vesicles) to pinocytosis (the uptake of soluble material), to phagocytosis. Phagocytosis is the mechanism by which relatively large (>0.5 μ m) particles, such as bacteria, dead cells, or polystyrene beads [34]. The mechanisms that lead to high numbers of *S. aureus* phagocytosed by macrophages possibly because of LA contained in the VCO helps macrophages in recognizing bacteria. *Staphylococcus aureus* infection activated the most highly conserved unfolded protein response sensor, inositol-requiring enzyme 1 α (IRE1 α). The macrophage IRE1-dependent bactericidal activity required reactive oxygen species (ROS). Response sensor, inositol-requiring enzyme (IRE1)-mediated persistent ROS generation might act as a fail-safe mechanism to kill bacterial pathogens that evade the initial macrophage oxidative burst [37]. Macrophages contain bactericidal ingredients to kill bacteria. Phagosome membrane secreted enzymes that form oxidants such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻) and hydroxyl ions (OH⁻). Myeloperoxidase has been one of the lysosomal enzymes that can catalyze the reaction between H₂O₂ and chloride ions to form hypochlorite is bactericidal [38].

Medium-chain fatty acids contained in VCO might have a role in enhancing phagocytic function. Essential fatty acids contained in the VCO thought to play a role in improving the ability of macrophage cell phagocytosis. Phagocytosis process has been influenced by the physical and chemical state of phagocytes and foreign particles. Called humoral components of the complement opsonin is major factor phagocytosis. Opsonin is a component of blood serum, blanketing the surface of the foreign agent. Several factors promote avoidance of phagocytosis by PMN leucocytes. Clumping factor A (ClfA) was a fibrinogen-binding surface protein of *S. aureus* that is an important virulence factor in several infection models. Structural and functional analysis has identified four distinct classes of surface proteins, of which microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) are the largest class. These surface proteins have numerous functions, including adhesion to and invasion of host cells and tissues, evasion of immune responses and biofilm formation. Thus, cell wall-anchored proteins are essential virulence factors for the survival of *S. aureus* in the commensal state and during invasive infections, and targeting them with vaccines could combat *S. aureus* infections [39].

5. Conclusion

The results presented in this study concluded that the VCO can inhibit the growth of *S. aureus* and increase the ability of phagocytic immune cells against *S. aureus* originated from PE goat milk. Virgin coconut oil might be used as an alternative to antibiotics and used as a modulator of the cellular immune system.

Declarations

Author contribution statement

Desy Cahya Widianingrum: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Cuk Tri Noviandi: Analyzed and interpreted the data.

Siti Isrina Oktavia Salasia: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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