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# The antimicrobial activity of enhanced virgin coconut oil (EVCO) on growth of mastitis pathogens

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**Aims:** The effect of EVCO, containing about 57.48% triglycerides, 26.88% diglycerides, 1.51% monoglycerides and 14.13% free fatty acids, against clinical mastitis pathogens, bought from American Type Cultures Collection (ATCC), was investigated. The present study aims to determine the efficacy of EVCO against three potent mastitis causal agents, namely *S. aureus* (ATCC 31885), *S. agalactiae* (ATCC 12927) and *S. dysagalactiae* (ATCC 27957).
**Methodology and results:** The *In-vitro* study showed that EVCO can act as a potent agent to inhibit the growth of *Staphylococcus aureus* (ATCC 31885), *Streptococcus agalactiae* (ATCC 12927) and *Streptococcus dysgalactiae (*ATCC 27957). A time kill study showed that EVCO at 2.5% is able to kill both *Streptococcus* spp. (ATCC 12927 & ATCC 27957) in 5 min of incubation time. Among three mastitis pathogens, *S. aureus* was the most difficult to eradicate and required at least 5% EVCO for 100% inhibition after 30 min of treatment. The *In-vivo* study on mastitis-induced lactating cows showed the amount of EVCO introduced into the infected mammary gland was considered over dosage; 50% concentration of EVCO was over dosage, even though it reduced the growth of pathogens from $10^4$ to $10^0$. The acidic characteristics of EVCO caused the protein in milk to clot and the udders to swell, even after several days of treatment.
**Conclusion, significance and impact study:** The EVCO had shown its antimicrobial efficacy against three potent mastitis causal agents. The amount of EVCO used to treat mastitis-induced cows and the number of treatments applied needed to be reduced to avoid the milk clotting and udders swelling as a result of the acidic characteristics of EVCO.

*Keywords:* Dairy cow, enhanced virgin coconut oil (EVCO), mastitis

## INTRODUCTION

Mastitis is defined as an inflammation of the mammary glands or udders that occurs as a result of a bacterial infection that gains entry to the udders of a mammal *via* a damaged teat duct, which usually occurs during milking. In a dairy herd's environment, there are large numbers of gram-positive and gram-negative species present in the surrounding area. These cause clinical or subclinical infections in the udders that are known as environmental mastitis pathogens (Anderson, 2008). Both subclinical and clinical mastitis decrease the cow's milk production and reduce the quality of the milk. If the cow has clinical mastitis, its milk must be discarded, and in untreatable or serious case, the cows might have to be destroyed.

Numerous microorganisms are known to cause mastitis, but the major mastitis causal agents are *S. aureus*, *S. agalactiae*, *S. dysagalactiae*, *Streptococcus uberis* and *Mycoplasma bovis* (Leitner *et al.*, 2003; Grőhn *et al*., 2004; Filioussis *et al.,* 2007; Fadlelmula *et al.,* 2009). Among these pathogens, *S. aureus* is one of the most frequent causes of subclinical and clinical bovine mastitis (Leitner *et al.*, 2003; Brouillette and Malouin, 2005). Presently, a few antibiotics, like Amoxicillin, Mastivac, Tretra-Delta LC$^{TM}$ and Penikan, are used as therapeutic drugs for the treatment of bovine mastitis (Leitner *et al.*, 2003; Roberson *et al.*, 2004; Karimuribo *et al*., 2006; Fadlelmula *et al*., 2009). Even though many technological advances have been made, mastitis continues to cause a major economic issue for dairy producers. Moreover, the incidence of antibiotic residue in milk has raised issues regarding food safety. Therefore, there is regulatory pressure to justify the use of therapeutic drugs in dairy cows.

For the past few decades, medium-chain fatty acids, MCFA (caprylic acid, capric acid and lauric acid) and its corresponding monoglycerides (monocaprylin, monocaprin and monolaurin) have been discovered to have antifungal and bactericidal properties (Kabara *et al*., 1972). In fact, this group of fatty acids is used in postmilking teat germicide products and has been found useful for bovine mastitis treatment in the dairy farming industry (Boddie and Nickerson, 1992; Wei *et al*., 2003; Dee and Gradle, 2004). Between MCFA and their corresponding monoester, lauric acid and monolaurin are the most potent and widely employed as antimicrobial agents in food, cosmetics, and pharmaceutical products

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(Garfinkel *et al*., 1992; Enig, 1998; Carpo *et al.,* 2007; Long, 2009).

EVCO is a name given to a partially hydrolyzed virgin coconut oil derived from an enzymatic reaction between the VCO and 1, 3-positional specific lipase (Long, 2009). This oil contains a mixture of lipid classes: triglycerides, diglycerides, monoglycerides and free fatty acids (FFA), i.e. medium-chain free fatty acids (14.13%) and their corresponding monoglycerides (1.51%), in its lipid profile. EVCO has proven to have a broad spectrum of antimicrobial activities against pathogenic microorganisms, including Gram positive bacteria, i.e. *S. aureus*, *Listeria monocytogenes*, *Streptococcus pyogenes* and *Bacillus cereus*; Gram negative bacteria, i.e., *Escherichia coli, Vibrio cholera* and *Salmonella* Typhi and yeast, i.e. *Candida albicans, C. krusei* and *Pityrosporum ovale* (Long, 2009). In addition, a recent study on the effect of EVCO against coccidiosis in broiler chicken also showed promising results and can be used as an alternative way to control coccidiosis in the poultry industry (Tan and Long, 2012). The present study aims to determine the efficacy of EVCO against three potent mastitis causal agents, namely *S. aureus* (ATCC 31885), *S. agalactiae* (ATCC 12927) and S. dysagalactiae (ATCC 27957). A field study was also conducted to determine the potential of EVCO in controlling and preventing bovine mastitis disease.

## MATERIALS AND METHODS

### Microorganisms and culture maintenance

Three potent mastitis pathogens strains, *S. aureus* (ATCC 31885), *S. agalactiae* (ATCC 12927) and *S. dysgalactiae* (ATCC 27957), were purchased from ATCC. The EVCO product was obtained from Wawasan Tebrau Group (Johor, Malaysia). Lactating cows were contributed by the Faculty of Veterinary Medicine at Universiti Putra Malaysia (Selangor, Malaysia).

### Preparation of suspension culture

Cultures were specifically grown in each respective broth, for example: a) *S. aureus* (ATCC 31885) was grown in tryptone soy broth; b) *S. agalactiae* (ATCC 12927) and *S. dysgalactiae* (ATCC 27957) were grown in brain heart infusion broth, and c) mix of cultures *S. aureus* (ATCC 31885), *S. agalactiae* (ATCC 12927) and *S. dysgalactiae* (ATCC 27957) was grown in a broth consisting of brain heart infusion and tryptone soy in a ratio of 50:50. A loopful of cultures were inoculated in a 20 mL sterilized broth containing 0.1% Tween 80 in 100 mL bottle and incubated at 37 °C for 16 h with the agitation speed set at 160 rpm. A serial dilution was carried out to estimate the colony forming unit (CFU/mL) and each culture was grown on specified agar with the same type of broth used. Each suspension culture was fixed at $10^8$-$10^9$ CFU/mL before proceeding with the next experiment.

### Minimum bactericidal concentration (MBC) of EVCO against mastitis pathogens: broth microdilution method

A broth microdilution susceptibility assay was used to determine the minimum bactericidal concentration (MBC) for EVCO. The MBC of EVCO against each mastitis pathogens was confirmed by the viable count method. The MBC is identified by determining the lowest concentration of EVCO that reduces the viability of the initial bacterial inoculum by ≥99.9%. Each suspension culture contained an initial bacteria count of $10^8$-$10^9$ CFU/mL. A total of 120 µL of broth containing 0.1% Tween 80 was added into all mixtures. Thirty percent and five percent of EVCO were used as initial working concentrations to determine MBC on three mastitis pathogens strains. A respective amount of EVCO was added into the first well and mixed thoroughly by pipetting up and down about 30 times. Then, 120 µL of mixture solution from the first well was transferred into the second well, and the same procedure was repeated with nine consecutive wells using a multichannel pipette. Each well contained 120 µL of the test material in descending concentrations. Well 11 was a growth control and well 12 was a control for sterility. Finally, 20 µL of inoculums containing bacterial suspension ($10^8$-$10^9$ CFU/mL) were added into each well from well 1 to 11. After that, the plate was incubated at 37 °C for 24 h. Serial dilutions were performed in eppendoft tubes using micropipettes. All plates were incubated at 37 °C for 24 h and the plate counts for each dilution were performed in triplicate.

### Effect of EVCO against mastitis pathogens: Time kill study

In this study, two different concentrations of EVCO were used to determine the optimum time for inhibiting the growth of ATCC's mastitis strains, which were 5% and 2.5% (v/v), respectively. The blends of specified broth and EVCO were thoroughly mixed with a homogenizer (Ultra Turrax ® T25 basic, IKA Labortechnik) at a speed of 11,000 $min^{-1}$ for 2 min. Then, a 100 µL of suspension cultures were inoculated into the mixture. This gave a final density of $10^8$-$10^9$ CFU/mL in the test sample. The mixture was vigorously shaken at 200 rpm under an optimum temperature of 37 °C. At the incubation times of 5, 10, 30 and 60 min, a 100 µL sample was transferred into an eppendoft tube containing 900 µL of Ringer solution to obtain $10^{-1}$ dilution and serial dilution was continued until $10^{-7}$. From each dilution, a 100 µL diluted sample was spread on the specified agar and incubated for 24 h at 37 °C before counting the colony. All data were expressed as the means ± standard deviations of triplicate measurements. A one-way analysis of variance (ANOVA) at the 5% significance level was used to determine significant differences ($p < 0.05$) between the means (Minitab software, version 14.1).

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**Effect of EVCO against mastitis-induced lactating cows: *in vivo* study**

This experiment was conducted in one of the dairy farms located at Universiti Putra Malaysia (Selangor, Malaysia). A total of six lactating cows were used, out of which two cows would be challenged with one type of mastitis pathogens: a) *S. agalactiae* (ATCC 12927); b) *S. dysgalactiae* (ATCC 27957) and c) *S. aureus* (ATCC 31885). A total of $10^1$ bacterial colonies were inserted into an udder. Every 2 h, the milk of the udders were examined using Draminski mastitis detector and California Mastitis Test (CMT) to detect symptoms of subclinical mastitis. Once subclinical mastitis was detected, the milk samples were collected to do a total plate count (TPC). Then 10 mL of EVCO at the concentration of 50% (v/v) was injected into the udder. The treatment was carried out twice a day, at 4 a.m. and 4 p.m. The milk quality of the infected cows was examined using a Draminski mastitis detector, CMT and TPC to assess the progression.

**Effect of EVCO in the formation of milk clotting**

Ten EVCO in the concentrations of 0.1%, 0.5%, 1%, 5%, 10% and 20% (v/v) in milk were prepared in duplicate. All the milk mixtures were subjected to heating temperature of 100 °C with the agitation speed of 200 rpm. The heating
was stopped immediately after boiling to accelerate the milk clotting formation. The pH of each solution was analyzed also (modified method from Mortenson *et al.*, 1935).

**RESULTS**

*In-vitro* assessment to determine the effectiveness of EVCO as an antimicrobial agent against these three pathogens was done to determine the minimum bactericidal concentrations (MBC). In this study, the mastitis causal pathogens used were *S. agalactiae* (ATCC 12927), *S. dysgalactiae* (ATCC 27957) and *S. aureus* (ATCC 31885), all known to cause clinical mastitis. In a broth microdilution susceptibility assay, each pathogen was tested individually with EVCO. A mixture of three mastitis pathogens was conducted as well to determine its MBC value, as summarized in Table 1. Overall*, S. agalactiae* (ATCC 12927) was noted to be the most susceptible to EVCO, with an MBC value of 0.625%. On the other hand, the mixed culture of *S. agalactiae* (ATCC 12927), *S. dysgalactiae* (ATCC 27957) and *S. aureus* (ATCC 31885) was slightly difficult and required a higher MBC value of EVCO (7.5%) than the individual pathogens. The concentration of EVCO required to inhibit the growth of both *S. dysgalactiae* (ATCC 27957) and *S.aureus* (ATCC 31885) was 5% (v/v); at this concentration, more than 99.9% pathogens were inhibited.

The time kill study was conducted to determine the optimum time of the respective concentrations of EVCO to inhibit the growth of the mastitis pathogens, as presented in Figure 1. In this study, two concentration of EVCO were conducted, 2.5% and 5% (v/v). Overall, both *Streptococcus* spp. (ATCC 12927 and 27957) were easily inhibited within 5 min incubation time when using 2.5% (v/v) of EVCO. The presence of *S. aureus* (ATCC 31885) in the mix culture of mastitis pathogens or *S. aureus* (ATCC 31885) alone required a longer time and a higher dose of EVCO compared to both *Streptococcus* spp. (ATCC 12927 & 27957), the same phenomenon observed in the MBC microdilution study.

The efficacy of EVCO against mastitis-induced lactating cows was conducted to assess its antimicrobial capability as an agent for intramammary therapy. In this study, every other lactating cow was challenged with one type of mastitis pathogen individually, including: *S. agalactiae* (ATCC 12927), *S. dysgalactiae* (ATCC 27957) and *S. aureus* (ATCC 31885). A Draminski mastitis detector and CMT were used to check the milk quality and subclinical mastitis stage. If the number of the Draminski mastitis detector showed 300 or above, the milk was considered in good condition. If the number was below 250, the lactating cows were considered to have a mastitis problem. Although the udder of lactating cows was induced with 1 mL of $10^1$ colonies bacteria, it took only 24 to 36 h for lactating cows to reach the subclinical mastitis stage, which was shown in the Draminski mastitis detector and CMT reading.

**Table 1:** Minimum bactericidal concentration (MBC) of EVCO against *Streptococcus agalactiae*, *Streptococcus dysgalactiae, Staphylococcus aureus* and mix culture of three mastitis pathogens.

| Mastitis pathogens | MBC of EVCO |
|---|---|
| *Streptococcus agalactiae* (ATCC 12927) | 0.625 |
| *Streptococcus dysgalactiae* (ATCC 27957) | 5.0 |
| *Staphylococcus aureus* (ATCC 31885) | 5.0 |
| Mix culture of the above three pathogens | 7.5 |

The MBC is identified by determining the lowest concentration of EVCO that reduces the viability of the initial bacterial inoculum by ≥99.9%.

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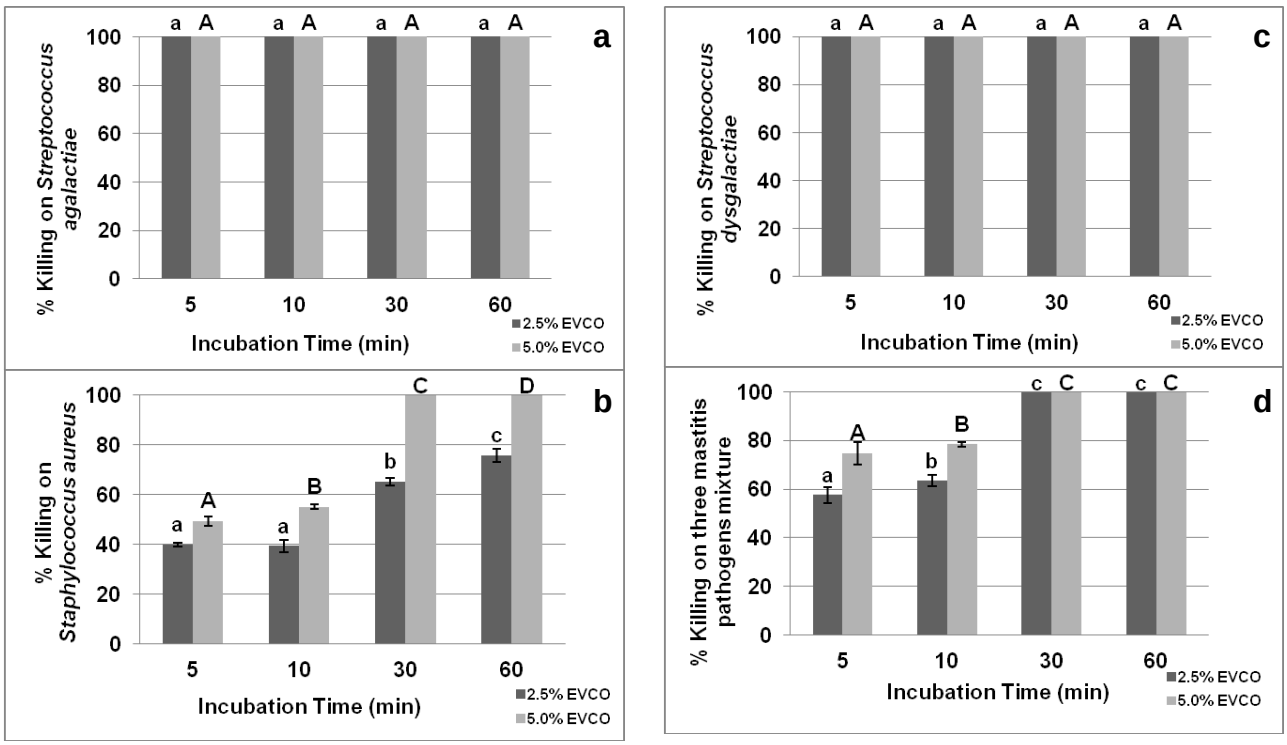


**Figure 1**: Time kill study of EVCO against *Staphylococcus aureus, Streptococcus agalactiae* and *Streptococcus dysgalactiae*. (Mean values with different superscripts are significantly different at $p < 0.05$).

Table 2 shows that all milk samples contained a total plate count (TPC) of $10^4$ when the cows were confirmed in the subclinical stage with the CMT value of 3+. During the intramammary treatment with EVCO, the milk quality did not change from the subclinical mastitis stage to then on-mastitis stage, as the Draminski reading showed below 300. Although the milk was observed clotting after seven days of treatment with EVCO, the TPC after a one-week treatment was zero, indicating that EVCO has antimicrobial capabilities to eradicate mastitis pathogen.

## DISCUSSION

Among three mastitis pathogens, *S. agalactiae* (ATCC 12927) was observed to be the most susceptible to EVCO and was easily inhibited when treated with 0.625% (v/v) EVCO, followed by *S. dysgalatiae* (ATCC 27957) and *S. aureus (*ATCC 31885). As expected, the combination of three mastitis pathogens required a higher concentration of EVCO compared to individual pathogens, with evidence shown in the MBC value of EVCO at 7.5%, mainly due to the presence of *S. aureus* (ATCC 31885) known to be more difficult to eradicate than other mastitis pathogens, as reported earlier (Brouillette and Malouin, 2005). Results from the time kill study showed the growth of both *S. agalactiae* (ATCC 12927) and *S. dysgalactiae* (ATCC 27957) were inhibited within 5 min of incubation time when using 2.5% (v/v) EVCO. Among the three mastitis pathogens, *S. aureus* (ATCC 31885) required the highest dosage of EVCO, of at least 5% (v/v) for 100% growth inhibition after 30 min of incubation time. However, the combination of three mastitis pathogens was observed to require 2.5% (v/v) EVCO with the same incubation time for 100% growth inhibition. Under agitation conditions, both *Streptococcus* spp. (ATCC 12927 and 27957) grew well in the broths, which may have caused an antagonistic effect on the growth of *S. aureus* (ATCC 31885) in the mixed culture. Therefore, the survival rate of mixed cultures was lower when treated with EVCO compared to *S. aureus* (ATCC 31885) alone since the growth of both *Streptococcus* spp. (ATCC 12927 and 27957) were susceptible to EVCO treatment.

*In-vitro* assessments on three potent mastitis pathogens had shown that EVCO has antimicrobial properties against mastitis causal agents. To further examine the antimicrobial capability of EVCO in intramammary therapy, an *in vivo* study on the efficacy of EVCO against six mastitis-induced lactating cows was carried out. The cows were infected with a single dose of mastitis pathogen individually with the concentration of bacterial colonies of $10^1$. Within 24 to 36 h, both the Draminski mastitis detector and the CMT confirmed that all infected cows with mastitis causal agents were in a subclinical mastitis stage with an increase in somatic cell count with a CMT value of 3+ observed in mastitis-infected cows.


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**Table 2:** Effect of EVCO against mastitis-induced lactating cows during an *in-vivo* assessment

| Mastitis Pathogen | Day | Draminski Reading | | CMT Reading | | Milk and Udder Appearance |
|---|---|---|---|---|---|---|
| | | 4 a.m. | 4 p.m. | 4 a.m. | 4 p.m. | |
| *Staphylococcus* | 1 | 280 | 290 | ++ | ++ | All normal |
| *aureus* | 2 | 280 | 250 | ++ | +++ (TPC=$10^4$) | Udder normal except milks were warmer |
| (ATCC 31885) | 3 | 260 | 270 | - | - | Udder swollen and harden slightly, milks normal |
| | 4 | 270 | 280 | - | - | Same as Day 3 |
| | 5 | 260 | 250 | - | - | Udder harden slightly, milks started to clot |
| | 6 | 210 | 240 | - | - | Udder harden slightly, milks were clotting |
| | 7 | 260 | 260 | - | - | Same as Day 6 |
| | 8 | 260 | 250 | - | - | Same as Day 6 |
| | 9 | 250 | 260 | - | - (TPC = 0) | Same as Day 6 |
| *Streptococcus* | 1 | 335 | 375 | ++ | ++ | All normal |
| *dysgalactiae* | 2 | 325 | 260 | ++ | +++ (TPC=$10^4$) | Udder normal except milks were warmer |
| (ATCC 27957) | 3 | 250 | 215 | - | - | Udder swollen and harden slightly, milks normal |
| | 4 | 245 | 225 | - | - | Udder swollen and harden slightly, milks were clotting |
| | 5 | 255 | 245 | - | - | Udder swollen slightly, milks were thick & clotting |
| | 6 | 245 | 230 | - | - | Same as Day 5 |
| | 7 | 230 | 245 | - | - | Same as Day 5 |
| | 8 | 245 | 250 | - | - | Udder swollen and harden slightly, milks more dilute but clotting |
| | 9 | 260 | 265 | - | - (TPC = 0) | Udder swollen and harden slightly, milks whitish but clotting |
| *Streptococcus* | 1 | 330 | 320 | ++ | ++ | All normal |
| *agalactiae* | 2 | 300 | 260 | ++ | +++ (TPC=$10^4$) | Udder normal except milks were warmer |
| (ATCC 12927) | 3 | 215 | 240 | - | - | Udder swollen and harden slightly, milk normal |
| | 4 | 235 | 225 | - | - | Udder normal, milks started to clot |
| | 5 | 255 | 260 | - | - | Udder normal, milks were clotting |
| | 6 | 220 | 240 | - | - | Udder swollen and harden slightly, milks were clotting |
| | 7 | 265 | 270 | - | - | Udder normal, milks were clotting |
| | 8 | 275 | 280 | - | - | Same as Day 7 |
| | 9 | 280 | 270 | - | - (TPC = 0) | Same as Day 7 |

Abbreviations: CMT will only show changes in cell counts above 300,000. Score of CMT: negative,milk remain watery; +,mild; ++,moderate; +++,heavy, almost solidify.


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A total plate count analysis indicated that the presence of $10^4$ bacterial colonies was enough to cause subclinical mastitis symptoms. At the subclinical stage, each cow was given intramammary therapy with 10 mL of 50% EVCO twice a day. After being treated with EVCO, the udders were observed becoming slightly enlarged and hardened within 24 to 36 h. The degree of udder enlargement was different for each cow, indicating different individual inflammatory responses. In general, all mastitis-induced cows showed the same symptoms with the milk becoming clotted after being infected; some turned yellowish in color and very thick in consistency.

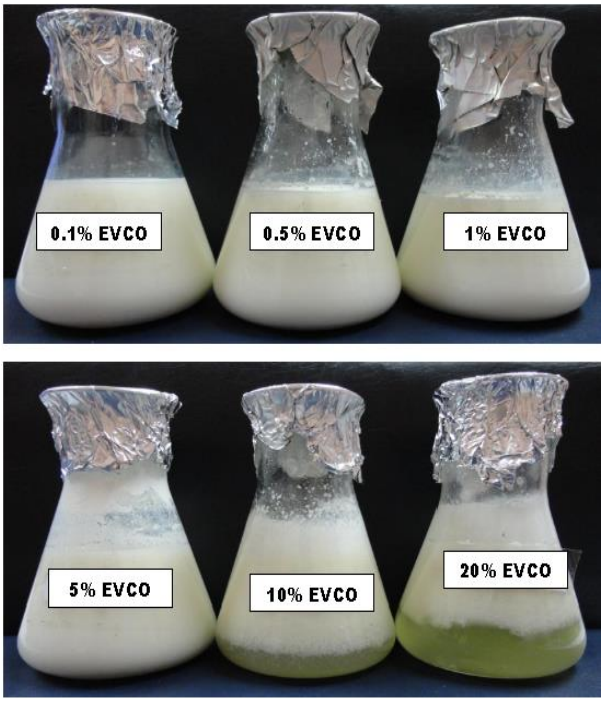


**Figure 2:** Effect of different percentages of EVCO on the clotting formation in milk.

Throughout seven days of treatment with EVCO, the yellowish milk of the infected cows turned back to a normal white milk color. However, the issue of milk clotting continued. As a consequence, swelling in the udders occurred. Milk production was lower each day. These changes were possibly due to the acidic properties of EVCO (pH 3.78). As shown in Figure 2, a clotting formation was observed in the milk when the concentration of EVCO was 10% (v/v) or above (pH < 5.5). The degree of milk clotting was greatly affected by the pH value (Kugelmass, 1937). The protein in milk is normally suspended in a colloidal solution, but when the pH of their solution changes, they can attract one another and form clumps. As the pH of milk drops and become more acidic with the increment of EVCO content, the protein (casein) molecules attract one another and become curdles floating in a solution (Mortenson *et al.,* 1935). The acidic behavior of EVCO might have also caused internal tissue injuries or irritated the cell walls of the udders, triggering inflammatory responses to fight back against the irritants. This problem might have been caused by an overdose of EVCO injected into the udders when high concentrations (50%, v/v) were used and caused milk clotting as a side effect. All of the udders of the tested cows swelled after being injected with EVCO twice a day. When the tissue was injured, it could have caused fibrosis, explaining why the udders hardened. Nonetheless, the TPC after one week of treatment with EVCO showed a zero reading, indicating that the EVCO had successfully eradicated all mastitis-causing bacteria inside the udders. Based on the *in vivo* observation and problems encountered, the current dosage of EVCO used is not suitable for intramammary treatment in mastitis-affected cows. Therefore, it is recommended that a reduced amount of EVCO, 5%, is used for intramammary therapy, which could help avoid the presence of acidic conditions that resulting in milk clotting, subsequently having the issues of udder hardening and swelling.

## CONCLUSION

Both *in vitro* and *in vivo* studies have shown evidence that EVCO has the antimicrobial capability to kill mastitis pathogens. Even though the intramammary therapy using EVCO on the mastitis-induced lactating cow had unwanted side effects on the udders, with swelling and milk clotting problems, the effectiveness of EVCO against mastitis pathogens was proven. The acidic conditions caused by EVCO treatment needs to be considered in order to avoid clotting as a result of intramammary therapy. In future work, it is recommended that a solution of 5% EVCO is used in postmilking to treat infections rather than 50%. Overall, EVCO has shown a potential application in protecting the teats of dairy animals from microbial infections. In fact, EVCO is an effective antimicrobial agent with GRAS status and can be applied as teat dip germicides to eradicate any possible mastitis infection from surrounding areas.

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