

Optimization of Green Betel Leaf-Essential Oil Encapsulation as an Antibacterial against *Staphylococcus epidermidis* and *Klebsiella pneumoniae* Isolated from Dairy-Goat Mastitis

(OPTIMISASI ENKAPSULASI MINYAK ATSIRI DAUN SIRIH HIJAU
SEBAGAI ANTIBAKTERI TERHADAP *STAPHYLOCOCCUS EPIDERMIDIS*
DAN *KLEBSIELLA PNEUMONIAE* PENYEBAB MASTITIS PADA KAMBING)

**Clara Ajeng Artdita^{1,2*}, Morsid Andityas¹, Muhammad Fatan Hidayatullah¹,
Desyah Rahmayanti¹, Achmad Fauzi¹, Anis Shidqi Rosyadah¹**

¹Department of Bioresources Technology
and Veterinary Vocational College, Universitas Gadjah Mada
Jl. Yacarana, Sekip Unit II, Depok, Sleman, Yogyakarta, Indonesia 55281

²Center of Excellence Dairy, Field Research Center,
Universitas Gadjah Mada, Yogyakarta

*Email: clara.ajeng@ugm.ac.id

ABSTRACT

Green betel leaves (*Piper betle* L.) essential oil (PBEO) exhibits antibacterial activity; however, volatility limits its stability and effectiveness. Chitosan, as a biopolymer-based drug delivery system, can enhance the stability and controlled release of essential oils. This study was aimed to optimize the concentration of PBEO when encapsulated with chitosan (PEC) to achieve antibacterial activity against two common mastitis-associated bacteria in dairy goats, *Staphylococcus epidermidis* and *Klebsiella pneumoniae*. The PBEO was extracted through steam distillation, followed by an ionic-gelation process to encapsulate it using chitosan with varying PBEO concentrations (PEC 7.5%, 15%, and 30%). Antibacterial activity was first assessed *in vitro* using disc diffusion method, with amoxicillin and distilled water as positive and negative controls, respectively. *In vivo* assay was conducted on eight lactating Jamnapari or Ettawa crossbred goats that confirmed positive mastitis, which were then randomly divided into five groups treated by post-milking dipping with three PEC concentration, 70% ethanol (positive control), and distilled water (negative control) for 35 days, and bacterial count was determined using total plate count (TPC) assay every seven days. Concentrations 7.5%, 15% and 30% of PEC produced inhibition zones against *S. epidermidis* (15.0, 17.5 and 18.5 mm, respectively) and *K. pneumoniae* (12.0, 14.0 and 14.0 mm, respectively). Moreover, TPC revealed that PEC 15% ($R=-1$, $P<0.01$) and 30% ($R=-0.94$, $P<0.05$) reduced the total number of milk bacteria ($P<0.05$). Collectively, PEC at 15% and 30% demonstrated *in vitro* antibacterial activity and were associated with reduced milk bacterial counts in goats.

Keywords: bacterial infection; herb medicine; *Piper betle* L.; udder inflammation; Jamnapari or Ettawa crossbred goat

ABSTRAK

Minyak atsiri daun sirih hijau (*Piper betle* L. *essential oil*; PBEO) dikenal memiliki aktivitas antibakteri yang potensial, namun sifatnya yang *volatile* (tidak stabil) menyebabkan efektivitasnya cepat menurun. *Chitosan*, adalah suatu bahan pembawa zat aktif (*drug delivery system*), berpotensi menstabilkan dan mengontrol pelepasan berbagai jenis minyak atsiri. Penelitian ini bertujuan untuk mengoptimasi konsentrasi *Piper Betle Essential Oil* (PBEO) hasil Enkapsulasi *Chitosan* (PEC) agar memperoleh aktivitas antibakteri terhadap dua bakteri penyebab mastitis kambing perah yaitu *Staphylococcus epidermidis* dan *Klebsiella pneumoniae*. Daun sirih hijau dikoleksi, kemudian dilakukan proses distilasi uap air hingga diperoleh minyak atsiri dan dilanjutkan proses gelasi-ionik antara PBEO dan kitosan dengan konsentrasi PBEO berbeda (PEC 7,5%, 15%, dan 30%). Uji *in vitro* dilakukan dengan metode difusi cakram untuk menilai daya hambat ketiga konsentrasi PBEO terhadap isolat bakteri mastitis, dibandingkan kontrol positif (*amoxicillin*) dan kontrol negatif. Uji *in vivo* dilakukan pada delapan kambing peranakan ettawa (PE) betina, masa laktasi, dengan status positif mastitis yang dibagi acak ke kelompok perlakuan *dipping* PEC, kontrol positif (*dipping* ethanol 70%), dan kontrol negatif selama 35 hari. Pengamatan jumlah bakteri dalam susu dilakukan tujuh hari sekali dengan metode *Total Plate Count* (TPC). Hasil uji zona hambat menunjukkan bahwa PEC 7,5%, 15% dan 30% efektif menghambat pertumbuhan *S. epidermidis* (masing-masing 15,00; 17,50 dan 18,55 mm) dan *K. pneumoniae* (masing-masing 12,0; 14,0 dan 14,7 mm). Hasil uji TPC menunjukkan bahwa konsentrasi 15% ($R=-1$, $P<0.01$) dan 30% ($R=-0.94$, $P<0.05$) mampu menurunkan total jumlah bakteri susu pada kambing dengan status mastitis ($P<0.05$). Konsentrasi PEC 15% dan 30% menunjukkan aktivitas antibakteri *in vitro* dan berhubungan dengan penurunan jumlah bakteri dalam susu.

Kata-kata kunci: infeksi bakterial; pengobatan herbal; *Piper betle* L.; radang ambing; kambing Peranakan Ettawa atau Jamnapari

INTRODUCTION

Mastitis is an infectious disease that causes a significant economic impact in the dairy industry. It is characterized by inflammation of the udder glands in female goats, which impairs the physiological function of the udder (Herlina *et al.*, 2015). Mastitis is divided into two types based on its clinical symptoms: clinical mastitis and subclinical mastitis (Nurhayati and Martindah, 2015). Clinical mastitis can be identified through clinical symptoms such as udder swelling, fever, the presence of clots in the milk and decreased milk production. In contrast, sub-clinical mastitis does not present obvious clinical symptoms but leads to reduced milk production, making it economically detrimental for farmers (Raj *et al.*, 2021; Chu *et al.*, 2024; Chu *et al.*, 2025). Moreover, this type of mastitis is difficult to detect, can

persist for long periods, and acts as a reservoir of microorganisms capable of infecting other livestock that come into direct contact with affected cows (Herlina, 2015). The major pathogens associated with mastitis are predominantly bacteria, including *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus* sp., *Klebsiella pneumoniae* and *Escherichia coli* (Artdita *et al.*, 2020; Chu *et al.*, 2024).

A meta-analysis study by Nuraini *et al.* (2023) on the prevalence of mastitis in various regions of Indonesia reported that the prevalence of mastitis in dairy livestock was 59.44%, with the majority of cases being subclinical mastitis (58.24%) and the remainder being clinical mastitis (3.31%). This condition indicates that more than half of the cattle in Indonesia have been exposed to mastitis. The same study also noted differences in prevalence between provinces on

the island of Java, with Central Java at 66.62% having the highest number of mastitis cases, followed by West Java at 64.68%, East Java at 60.52%, and Yogyakarta at 41.77%, which had the lowest number of mastitis cases. This pattern of mastitis cases is unlikely to have changed significantly between 2003 and 2022, given that good dairy farming practices (GDFP) have not been fully adopted by many Indonesian farmers, and treatment is typically provided only when mastitis occurs during lactation (Nurhayati and Martindah, 2015).

Mastitis is commonly treated with antibiotics, such as β -lactams, aminoglycosides, fluoroquinolones, lincosamides and macrolides (Lestari *et al.*, 2025). However, the use of these drugs presents challenges, as antibiotic residues can remain in milk, compromising product quality (Meutia *et al.*, 2016) and posing risks to food safety. Furthermore, inappropriate or prolonged antibiotic use contributes to the development of antimicrobial resistance in both animals and humans (Chowdhury *et al.*, 2021). Therefore, alternative treatment approaches are needed to reduce antibiotic resistance, including the use of herbal medicine such as Green betel (*Piper betle* L.) leaves, which are valued for their essential oil content (Kusuma *et al.*, 2017). Green betel leaf essential oil contains active phenolic compounds and their derivatives with antibacterial properties that are capable of inhibiting bacterial growth (Almasyhuri and Sundari, 2019).

Green betel leaves are non-toxic even when given in high concentrations (Sadiah *et al.*, 2022). The essential oil extracted from Green betel leaf has been shown to inhibit the growth of bacteria, including *Staphylococcus* sp., which belong to the Gram-positive group (Almasyhuri and Sundari, 2019; Sujono *et al.*, 2019) and several Gram-negative bacteria, including *K. pneumoniae* (Nouri *et al.*, 2014). However, pure Green betel leaf essential oil has a weakness due to its volatile nature; hence, it is necessary to carry out an encapsulation process to decrease volatility, increase stability, and protect

from external factors (Madhumita *et al.*, 2019; Zabot *et al.*, 2022). Along with the importance of essential oils encapsulation, it is necessary to optimize the essential oil concentration when encapsulated with chitosan as well as observe the antibacterial activity against *S. epidermidis* and *K. pneumoniae* bacteria, which are the common pathogens causing mastitis in dairy goats.

RESEARCH METHODS

All animal experiment procedures were approved by the Animal Experimental Ethics Committee of Universitas Sebelas Maret (UNS) with approval number 644/U27.14/TA.00.03/2025.

Encapsulation of Green Betel Leaf Essential Oil with Chitosan (PEC)

The deep Green betel leaves measured 11 to 13 cm in length to ensure uniform size, were collected from several locations in Sleman, Yogyakarta, cleaned with distilled water and air-dried at room temperature (25 to 30°C) for 24 hours. The Green betel leaves were then distilled using steam distillation. The *P. betle* L. essential oil (PBEO) was heated to produce steam, then cooled and 1 g of Sodium Sulfate (Na_2SO_4 , Merck, Darmstadt, Germany) was added and left overnight to obtain the essential oil liquid that separated from water (Rahmiyani *et al.*, 2020). The Green betel leaf essential oil was collected in a separatory funnel and diluted into three concentration groups, i.e. 7.5%, 15% and 30% with polyethylene glycol 400 (PEG400; Merck, Darmstadt, Germany). Each dilution was transferred into a dark glass bottle and covered with aluminum foil for further use. Green betel leaf essential oil was encapsulated with chitosan (PEC; Merck, Darmstadt, Germany). The PBEO at each concentration was mixed with chitosan by slowly adding drops and homogenizing with a magnetic stirrer at 1,500 rpm for 4 to 5 hours. The solution was then stored at room temperature and covered with aluminum foil

for further use.

Preparation for Mastitis-Causing Bacteria

The mastitis pathogens used in this study, *S. epidermidis* and *K. pneumoniae*, were obtained from the Veterinary Development and Innovation Laboratory, Veterinary Technology Study Program, Vocational College, Universitas Gadjah Mada, Yogyakarta and originated from mastitis-positive dairy goats on a farm in Sleman, Yogyakarta. These bacteria had previously been screened for mastitis using the Californian Mastitis Test (Kruuse Bovivet CMT Liquid®, Kruuse, Langeskov, Denmark) and identified through a series of microbiological and biochemical tests. The two isolates were then employed as sample units in the *in vitro* assays.

Zone of Inhibition Assay (*In Vitro* Assay)

The inhibition zone assay of Green betel leaf essential oil against *S. epidermidis* and *K. pneumoniae* was conducted using the Kirby-Bauer disc diffusion method (CLSI M02, 2024). Overnight bacterial cultures in Todd-Hewitt Broth (THB) media were then mixed in tubes containing sterile physiological NaCl 0.9% (Sodium Chloride 0.9%, Widatra Bhakti, Jakarta, Indonesia), homogenized and the turbidity level was observed. The turbidity level was standardized to a concentration of 0.5 McFarland to ensure the bacterial count met the sensitivity test requirement, equivalent to $1-2 \times 10^8$ CFU/mL (Bellik, 2014). The bacterial solution was cultured using the pour plate method on Mueller-Hinton Agar (MHA, Oxoid, Hampshire, England) media. The plates were incubated at 37°C overnight, and the resulting inhibition zone was measured in millimeters (mm).

The study employed a completely randomized design (CRD) with five treatments, each replicated twice on two plates, consisting of three concentrations of PEC selected from preliminary screening (7.5%, 15% and 30%), a positive control (penicillin 30 µg, C+), and a negative control (PEG400, C-). For all three PEC concentrations, blank test discs were soaked in each PEC concen-

tration before use. All discs were placed aseptically on the surface of MHA using a laminar air flow cabinet to maintain sterile conditions. After incubation at 37°C for 18-24 hours, the diameter of the inhibition zone around the disc was measured to assess the inhibitory power against both bacteria (Hulankova, 2024). The sample units in this *in vitro* assay were bacterial isolates obtained from mastitis-positive cases, with each isolate replicated twice.

Effectiveness of PEC Product (*In Vivo* Assay)

The *in vivo* experiment was performed using a CRD with animals randomly assigned to treatment and control groups. The effectiveness test of PEC was conducted on eight lactating Ettawa crossbreed goats (Kambing Peranakan Ettawa, PE), resulting in a positive mastitis test based on the CMT test on a farm in District of Turi, Sleman, Yogyakarta. These eight goats were divided into five groups: a negative control group (without dipping treatment, C-), a positive control group (with 70% ethanol dipping, C+), and three PEC treatment groups (7.5%, 15%, and 30%), with two goats assigned to each treatment. Treatment was administered daily after milking by dipping the teats in either 70% ethanol or each concentration of the PEC product for 35 days of milking period. Total bacterial count (Total Plate Count, TPC) in the milk was evaluated on day 0 (before treatment), and subsequently on days 7, 14, 21, 28 and 35.

Total Plate Count assay

The bacterial enumeration using TPC method was performed following a previous study (Kusumah *et al.*, 2023), with slight modifications to the agar medium and dilution procedures. These modifications were necessary because the previous study used fresh milk, whereas the present study involved mastitis-positive milk, which naturally contains a much higher bacterial count. Consequently, the dilution range was expanded. One mL of mastitis goat milk sample was diluted with 0.9% NaCl to obtain dilution factors from 10^{-1} to 10^{-7} . Milk samples at

diluteons of 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} were then poured into a plate and followed by the addition of 15 mL of sterile plate count agar medium (Oxoid, Hampshire, England). The plates were gently shaken to evenly distribute the bacteria. Colony counting was performed manually on plates containing 30-300 colonies, with merged colonies were counted as a single colony. The results were recorded to one decimal place and multiplied by the corresponding dilution factor. The number of colonies in each sample was calculated using this following formula: colonies per mL = (number of colony per plate $\times$ 1 mL) $\times$ (dilution factor) $^{-1}$.

Data Analysis

Data were analyzed using R software version 4.3.1 (Comprehensive R Archive Network, Vienna, Austria). Normality was assessed using the Shapiro-Wilk method and supported by Q-Q plot visualization. Homogeneity of variance between groups was evaluated using Levene's test. For the *in vitro* assay, inhibition zone diameters from five groups (the PEC group at 7.5%, 15%, 30%, the negative control and the positive control) were assessed against *S. epidermidis* and *K. pneumoniae*. When the data met the assumptions of normality and homogeneity, the parametric one-way Analysis of variance was performed. When these assumptions were not met, the non-parametric Kruskal-Wallis test was applied. Post hoc analyses were performed to identify significant differences between groups, using Tukey's Honestly Significant Difference (HSD) (after Anova test) or Dunn's test with Bonferroni correction (after the Kruskal-Wallis test). Statistical significance was set at $P < 0.05$. Furthermore, the interpretation of inhibition zone categories for herbal products followed Davis and Stout (1971); Suryanah and Nurjanah (2021) as follows: very strong for zone diameters greater than 20 mm (>20 mm), strong for zone diameters of 10-20 mm, moderate for zone diameters of 5-10 mm, and weak for zone diameters less than 5 mm (<5 mm). For the *in vivo* analysis, the correlation between total bacteria in milk

(TPC data) based on the dipping treatment and concentration was analyzed using the Pearson correlation coefficient test for normally distributed data, or Spearman's rho for non-normal data. The correlation coefficient is denoted by R.

RESULTS AND DISCUSSION

In this study, PBEO was obtained by steam distillation. This method was chosen because it was effective in extracting essential oils from leaves, resulting in higher quality and percentage of essential oil produced compared to hydro-distillation. The higher percentage of essential oil is due to the steam produced in this method being able to spread widely and evenly throughout the leaf tissue (Nirwana and Zamrudu, 2021; Guna-wan and Kurnianty, 2021). Several studies have shown that choosing the appropriate extraction method is a crucial factor in the essential oil production process. Steam distillation accounts for 93% of the volume of essential oils produced worldwide (Machado *et al.*, 2020; Machado *et al.*, 2024). The active compounds with antibacterial properties in Green betel leaf essential oil, obtained through steam distillation, can reach concentrations of up to 4.2%. These compounds act by inhibiting the bacterial cell walls formation (Khwaza and Aderibigbe, 2025). However, the green betel leaf essential oil also has limitations due to its highly volatile nature. Therefore, encapsulation serves as an effective approach to preserve its active compounds by enhancing their stability (Madhumita *et al.*, 2019; Zabot *et al.*, 2022).

The inhibition zone analysis of the treatment groups (PEC) against *S. epidermidis* showed that the Shapiro-Wilk normality test and visualization through Q-Q plots indicated that most groups had non-normal data distribution ($P < 0.05$). In addition, the homogeneity of variance test using Levene's test showed that the variance between groups was not homogeneous ($P < 0.05$). Similar results were also obtained in the PEC inhibition zone against *K. pneumoniae* analysis,

where the Shapiro-Wilk test and visualization through Q-Q plots showed normal

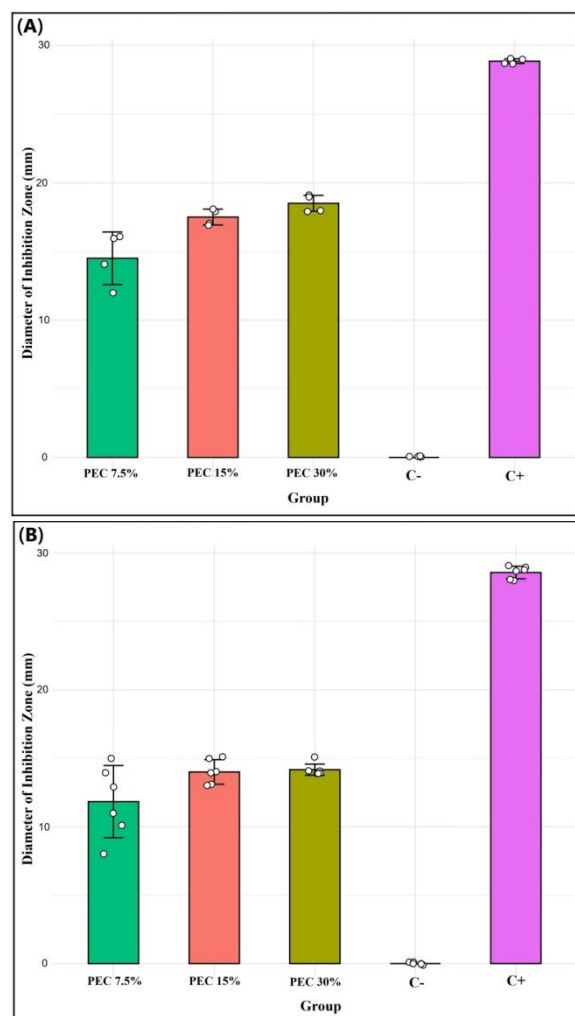


Figure 1. Bar plot of the inhibition zone result. (A) Inhibition zone of PEC against *Staphylococcus epidermidis*. (B) Inhibition zone of PEC against *Klebsiella pneumoniae*. Notes: PEC= *Piper betle* L. leaf essential oil encapsulated with chitosan; C+ = positive control; C- = negative control.

data distribution in most groups ($P < 0.05$), and Levene's test indicated non-homogeneous variance between groups ($P < 0.05$). According to these results, since the assumptions of normality and homogeneity of variance were not met, the Kruskal-Wallis non-parametric analysis method was chosen as the most appropriate approach to evaluate the differences in effects between treatment groups against these two bacteria (Figure 1). In detail, Table 1 shows the Kruskal-Wallis

analysis of the treatment groups against the pathogen *S. epidermidis*, which revealed a statistically significant difference between groups ($P < 0.05$). Further analysis using Dunn's test and Bonferroni correction identified a significant difference between the negative and positive control ($P < 0.05$). Furthermore, the PEC 15% and 30% groups had a zone of antibacterial activity compared to the negative control ($P < 0.05$). This result indicated that these PEC concentrations had a potential effect in inhibiting the growth of *S. epidermidis*. Meanwhile, analysis of the effectiveness of the treatment against *K. pneumoniae* also showed a significant difference between groups based on the Kruskal-Wallis test ($P < 0.05$). Further Dunn's test with Bonferroni correction revealed that the positive control group was significantly different from the negative control group ($P < 0.05$) and the PEC 7.5% group ($P < 0.05$); while the PEC 15% and 30% groups were significantly different from the negative control ($P < 0.05$). However, no significant differences were found between the three extract concentrations (PEC 7.5%, 15% and 30%). These results indicated that PEC showed a potency against *K. pneumoniae*, with no significant differences between concentrations.

Referring to the antibacterial level category of herbal products according to Davis and Stout (1971), the overall PEC concentration was in the 10-20 mm diameter range, meaning it falls into the strong category. The PEG400, as a negative control, showed no inhibition zone. In contrast, penicillin, as a positive control, showed a very strong inhibition zone (Table 1). Encapsulation can enhance the chemical stability and solubility of essential oils, reduce evaporation and toxicity rate, and minimize the degradation of their active components. When combined with essential oils, encapsulation also provides antimicrobial potential against bacteria exhibiting multidrug resistance (Mukurumbira et al., 2022). The antibacterial activity of green betel leaf essential oil is due to its active compounds, including chavicol, eugenol, germacrene D, caryophyllene and several other phenol derivatives (Sujono et al., 2019).

Table 1. Zone of inhibition (disc-difussion method) result.

Groups	Median (<i>range</i> Q1-Q3) diameter zone (mm)		Zone category
	<i>S. epidermidis</i> (n=4)	<i>K. pneumoniae</i> (n=6)	
PEC 7.5%	15.0 (13.5-16) ^{ab}	12.0 (10.2-13.8) ^{ac}	Strong
PEC 15%	17.5 (17-18) ^b	14.0 (13.3-14.8) ^{bc}	Strong
PEC 30%	18.5 (18-19) ^b	14.0 (14.0-14.0) ^{bc}	Strong
C+	28.8 (28.7-29) ^b	28.7 (28.2-28.9) ^b	Very strong
C-	0.0 (0.0-0.0) ^a	0.0 (0.0-0.0) ^a	No inhibition

Different superscript^(abc) showed significant different ($P < 0.05$); *Davis and Stout (1971); Suryanah and Nurjanah, 2021; Abbreviation: PEC, *Piper betle* L. leaf essential oil encapsulated with chitosan; C+, positive control; C-, negative control; n, sample unit.

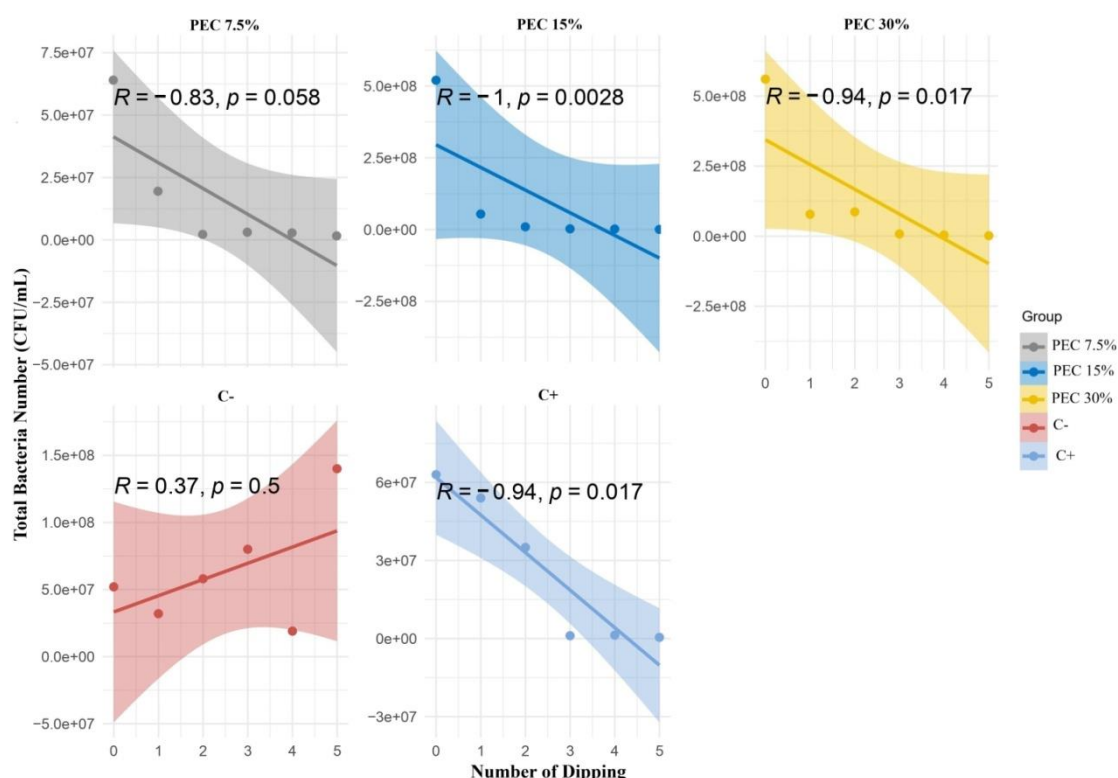


Figure 2. Scatter plot of TPC assay between the total bacteria number in each dipping against the concentration of group PEC 7.5%, 15%, 30%, negative control, and positive control. Abbreviation: PEC, *Piper betle* L. leaf essential oil encapsulated with chitosan; C+, positive control; C-, negative control.

These compounds play a role in damaging bacterial cell walls and membranes, inducing protein de-naturation and inhibiting the growth of harmful microorganisms, including Gram-negative and Gram-positive bacteria (Nisyak *et al.*, 2022). The TPC assay in mastitis milk during the dipping treatment

was analyzed using the Shapiro-Wilk normality test. It showed that the data in the PEC 7.5%, 15% and 30% groups were not normally distributed ($P < 0.05$). In contrast, the data in the negative control and positive control showed a normal distribution as indicated by P-values of 0.44 and 0.09,

respectively. Therefore, correlation analysis using the non-parametric Spearman's rho test was more appropriate. The results of the Spearman's rho correlation test in Figure 2 shows that there was a significant negative correlation between the concentration and the dipping effect in the group of PEC 15% ($R=-0.94$, $P=0.0028$) and PEC 30% ($R=-0.94$, $P=0.017$). Meanwhile, in the PEC 7.5% group, there was a strong however did not meet a statistically significant negative relationship ($R=-0.83$, $P=0.058$). No significant relationship was found in the negative control ($P=0.5$), while in the positive control, there was a significant negative relationship ($R=-0.94$, $P=0.017$). Therefore, these results indicated that the concentration of PEC 15% and 30% encapsulation treatment of Green betel leaves essential oil-chitosan tends to have a negative correlation, meaning that bacterial count (TPC) decreases as the dipping duration increases.

The encapsulated products exhibited clear inhibition zones against *S. epidermidis* and *K. pneumoniae* isolated from mastitis goat milk, indicating their antibacterial potential. All three concentrations showed inhibitory effects, confirming that the encapsulation process preserved the antibacterial properties of the active compounds. Consistently, the reduction in bacterial counts in milk further supported these results, demonstrating that the encapsulated products effectively suppressed bacterial growth within the milk. In this study, the decrease in the TPC value could be interpreted as a decrease in the total number of bacteria in milk treated with encapsulated products containing the active ingredient of Green betel leaf essential oil. As previously mentioned, all three concentrations, especially PEC 15% and PEC 30%, had the potential to have antibacterial properties against *S. epidermidis* and *K. pneumoniae*, which are common bacteria that cause mastitis in dairy goats. Green betel leaf essential oil is often used in the pharmaceutical field because it has antibacterial properties that can inhibit the growth of various pathogenic bacteria simultaneously. The antibacterial action mechanism of Green betel leaf essential oil is by disrupting the

process of bacterial cell wall and cell protein synthesis, inhibiting metabolic processes and cell permeability and damaging bacterial nucleic acids (Sadiah *et al.*, 2022). The results of the Gas Chromatography-Mass Spectrometry (GC-MS) assay in the previous study (Sujono *et al.*, 2019) also confirmed that PBEO consisted 38 compounds with 10 compounds are the highest concentration including chavicol (21.27%), eugenol (13.3%), eugenol acetate (8.34%), 2-allyl-phenol (7%), terpineol (2.87%), germa-crene-D (9.08%), caryophyllene (8.37%), β -chamigrene (4.62%), α -cadinene (3.88%), and α -humulene (2.42%), most of which have antibacterial properties.

Encapsulation with polymers is necessary to protect the bioactive compounds of essential oils, thereby increasing compound stability, reducing oxidation and maintaining organoleptic properties under extreme temperature, radiation and pH conditions (Zabot *et al.*, 2022). Chitosan, as a natural biopolymer for encapsulation, also has the advantages of being antibacterial, antifungal and anti-inflammatory, non-toxic, biodegradable, biocompatible and water-soluble (Islam *et al.*, 2020).

CONCLUSION

The encapsulation of Green betel leaf essential oil with chitosan demonstrated the ability to inhibit the growth of *S. epidermidis* and *K. pneumoniae*, two common mastitis-causing bacteria in dairy goats. In detail, the 15% and 30% PEC formulations showed *in vitro* antibacterial activity, as shown by their inhibition zones, and both concentrations exhibited a strong positive correlation in their capability to decrease the total bacteria number in milk. These results indicated its strong potential as an effective herbal alternative for the prevention and treatment of mastitis.

ACKNOWLEDGEMENT

We extend our gratitude to the Veterinary Development and Innovation Laboratory, Veterinary Technology Study Pro-

gram, Vocational College, Universitas Gadjah Mada, for providing the bacterial isolates used in this study.

REFERENCES

- Almasyhuri, Sundari D. 2019. Antiseptic Activity Test Extract of Betel Leaf (*Piper betle* Linn.) in Mouthwash Against *Staphylococcus aureus* in Vitro. *Jurnal Kefarmasian Indonesia* 9(1): 10–18. DOI: [10.22435/jki.v9i1.351](https://doi.org/10.22435/jki.v9i1.351)
- Artdita CA, Andityas M, Prihanani NI, Budi-yanto YW. 2020. Bacterial detection causing subclinical mastitis on Etawah crossbreed goat in Kokap, Kulon Progo, Yogyakarta Province. *Jurnal Sain Veteriner* 38(1): 37-44. DOI: <https://doi.org/10.22146/jsv.39818>
- Bellik Y. 2014. Total antioxidant activity and antimicrobial potency of the essential oil and oleoresin of *Zingiber officinale* Roscoe. *Asian Pacific Journal of Tropical Disease*. 4(1): 40–44. DOI: [https://doi.org/10.1016/S2222-1808\(14\)60311-X](https://doi.org/10.1016/S2222-1808(14)60311-X)
- Chowdhury S, Ghosh S, Aleem MA, Parveen S, Islam MA, Rashid MM, Akhtar Z, Chowdhury F. 2021. Antibiotic usage and resistance in food animal production: What have we learned from Bangladesh?. *Antibiotics* 10(9): 1-8. DOI: <https://doi.org/10.3390/antibiotics10091032>
- Chu M, Si Y, Li Q, Liu X, Liu G. 2025. Deep learning-based model to classify mastitis in Holstein dairy cows. *Biosystem Engineering* 252: 92-104. DOI: <https://doi.org/10.1016/j.biosystemseng.2025.02.013>
- Chu S, Zhao T, Li M, Sun Y, Yang Y, Yang Z. 2024. Long non-coding RNA (CMR) involved in autoprotection in *S. aureus* mastitis in dairy cows by regulating miR-877/FOXO1. *Ecotoxicology and Environmental Safety* 278: 1-11. DOI: <https://doi.org/10.1016/j.ecoenv.2024.116456>
- CLSI (Clinical and Laboratory Standards Institute). 2024. Performance standards for antimicrobial disc susceptibility tests; approved standard - 14th edition (CLSI M02). Wayne, PA: CLSI.
- Davis WW, Stout TR. 1971. Disc plate method of microbiological antibiotic assay. *Applied Microbiology* 22(4): 659–665.
- Gunawan D, Kunianty R. 2021. Utilization of Essential Oil from Betel Leaf (*Piper betle* Linn) as Mosquito Repellent. *Journal of Pharmaceutical and Health Research* 2(2): 46-49. DOI: <https://doi.org/10.47065/jharma.v2i2.862>
- Herlina N, Afiati F, Cahyo AD, Herdiyani PD, Qurotunnada Q, Tappa B. 2015. Isolation and identification of *Staphylococcus aureus* from subclinical infection dairy cattle in Tasikmalaya, West Java. *Proceeding. National Seminar of the Indonesian Biodiversity Society* Institut Teknologi Bandung-Indonesian Biodiversity Society. Sumedang. 15 Juni 2015. Pp. 413-417. DOI: <https://doi.org/10.13057/psnmbi.m010305>
- Hulankova, R. 2024. Methods for Determination of Antimicrobial Activity of Essential Oils *In Vitro*—A Review. *Plants*, 13 (19), 2784. DOI: <https://doi.org/10.3390/plants13192784>
- Islam MM, Shahruzzaman M, Biswas S, Nurus-Sakib M, Rashid TU. 2020. Chitosan based bioactive materials in tissue engineering applications-A review. *Bioactive Materials* 5(1): 164–183. DOI: <https://doi.org/10.1016/j.bioactmat.2020.01.012>
- Khwaza V, Aderibigbe BA. 2025. Antibacterial Activity of Selected Essential Oil Components and Their Derivatives: A Review. *Antibiotics* 14(1): 68. DOI: <https://doi.org/10.3390/antibiotics14010068>
- Kusuma MS, Susilorini TE, Surjowardojo P. 2017. Effect of Storage Duration and Temperature of Green *Piper betle*

- Linn Extract with Distilled Water on the Inhibitory Activity Against *Streptococcus agalactiae*, a Causative Agent of Mastitis in Dairy Cows. *Ternak Tropika* 18(2): 9–16. DOI: <https://doi.org/10.21776/ub.jtapro.2017.018.02.3>
- Kusumah NAF, Widjiati W, Puspitasari Y, Tyasningsih W, Wardhana D, Permatasari DA, Luqman EM. 2023. Comparison of Total Number of Bacteria in Raw Milk Friesian Holstein Cow Based on Milking Techniques Using Total Plate Count (TPC) Test. *International Journal of Scientific Advances* 4:37-40. DOI: [10.51542/ijscia.v4i1.9](https://doi.org/10.51542/ijscia.v4i1.9)
- Lestari FB, Artdita CA, Wasissa M, Hidayah N. 2025. Antimicrobial Resistance Character Variation of Bacterial Causes Subclinical Mastitis in Ettawa Crossbreed Goat from Yogyakarta. *Jurnal Sains Veteriner* 43(1): 78-83. DOI: 10.22146/jsv.52529
- Machado CAT, De Andrade MAN, Lepikson H.A. 2020. Manufacturing 4.0: Discussion on Application in the Extractive Industry of Essential Oils. *Journal of Bioengineering and Technology Applied to Health* 2(3): 86–91. DOI: <https://doi.org/10.34178/jbth.v2i3.76>
- Machado CAT, Hodel KVS, Lepikson HA, Machado BAS. 2024. Distillation of essential oils: An innovative technological approach focused on productivity, quality and sustainability. *Plos ONE*. 19(2): 1-20. <https://doi.org/10.1371/journal.pone.0299502>
- Madhumita M, Guha P, Nag A. 2019. Extraction of Betel Leaves (*Piper betle* L.) Essential Oil and Its Bio-actives Identification: Process Optimization, GC-MS Analysis and Anti-microbial Activity. *Industrial Crops and Products* 138(1): 1-12. DOI: <https://doi.org/10.1016/j.indcrop.2019.111578>
- Meutia N, Rizalsyah T, Ridha S, Sari MK. 2016. Antibiotic Residues in Water Fresh Milk Derivat From Farms in The Territory of Aceh Besar. *Jurnal Ilmu Ternak* 16(1): 1-5. DOI: <https://doi.org/10.24198/JIT.V16I1.9817>
- Mukurumbira AR, Shellie RA, Keast R, Palombo EA, Jadhav SR. 2022. Encapsulation of essential oils and their application in antimicrobial active packaging. *Food Control* 136: 108883. DOI: <https://doi.org/10.1016/j.foodcont.2022.108883>
- Nirwana CH, Zamrudy W. 2021. A Literature Review on the Characteristics of Clove Oil from Various Distillation Methods. *Jurnal Teknologi Separasi* 7(2): 561–569. DOI: <https://doi.org/10.33795/distilat.v7i2.271>
- Nisyak K, Hisbiyah A, Haqqo A. 2022. Antibacterial Activity of Ethanolic Extract and Green Piper Betle Leaf Essential Oil Against Methicillin Resistant *Staphylococcus aureus*. *Journal of Pharmaceutical Care Anwar Medika* 5(1): 1-14. DOI: <http://dx.doi.org/10.36932/jpcam.v5i1.82>
- Nouri L, Nafchi AM, Karim AA. 2014. Phytochemical, antioxidant, antibacterial, and α -amylase inhibitory properties of different extracts from Betel leaves. *Industrial Crops and Products*. 62: 47-52. DOI: <https://doi.org/10.1016/j.indcrop.2014.08.015>
- Nuraini DM, Andityas M, Sukon P, Phuektes P. 2023. Prevalence of mastitis in dairy animals in Indonesia A systematic review and meta-analysis. *Veterinary World* 16(7): 1380-1389. DOI: www.doi.org/10.14202/vetworld.2023.1380-1389
- Nurhayati IS, Martindah E. 2015. Controlling Subclinical Mastitis by Antibiotic Application during Dry Period of Dairy Cow. *Wartazoa* 25(2): 65-74. DOI: <http://dx.doi.org/10.14334/wartazoa.v25i2.1143>
- Rahmiyani I, Rizki TR, Nurlaili DH, Yuliana A. 2020. Isolation and Identification of Essential Oil Compounds from Gamal Leaves (*Gliricidia sepium* [Jacq] Walp). *Jurnal Farmasi Udayana* 134–143. DOI: <https://doi.org/10.24843/JFU.2020.v09.i03.p01>

- Suryanah S, Nurjannah S. 2021. The effectiveness of Herbal Antibacterial Formulations Against Mastitis-Causing Bacteria in Dairy Cattle. *Animal Production* 23(3): 151-159. DOI: <https://doi.org/10.20884/1.jap.2021.23.3.78>
- Raj A, Chattothayil L, Kulangara V, Vareed TP, Melepat DP, Chullipparambil S. 2021. Variations in the levels of acute-phase proteins and lactoferrin in serum and milk during bovine subclinical mastitis. *Journal of Dairy Research* 88(3): 321–325. DOI: <https://doi.org/10.1017/S002202992100056X>
- Sadiah HH, Cahyadi AI, Windria S. 2022. A Review of Green Betel Leaf (*Piper betle* L.) Potency as Antibacterial. *Jurnal Sain Veteriner* 40(2): 128–138. DOI: <https://doi.org/10.22146/jsv.58745>
- Sujono H, Rizal S, Purbaya S, Jasmansyah. 2019. Antibacterial Activity of the Essential Oil from Betel leaf (*Piper betle* L.) against *Streptococcus pyogenes* and *Staphylococcus aureus*. *Jurnal Kartika Kimia* 2(1): 30–36. DOI: <https://doi.org/10.26874/jkk.v2i1.27>
- Zabot GL, Rodrigues FS, Ody PL, Tres MF, Herrera E, Palacin H, Córdova-Ramos JS, Best I, Olivera-Montenegro L. 2022. Encapsulation of Bioactive Compounds for Food and Agricultural Applications. *Polymers* 14(1): 1–37. DOI: <https://doi.org/10.3390/polym14194194>