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Comparable Efficacy of Clove Leaf Oil Nanoemulsion and Povidone-Iodine on Reducing Microbial and Somatic Cell Counts in Subclinical Mastitis

A. N. Syamsi[1,7], S. Suyadi[2], L. E. Radiati[3], P. Surjowardojo[4], H. Sulistyarti[5], O. Sjofjan[6], & T. E. Susilorini[4,*]

[1] Doctoral Student in Animal Science, Faculty of Animal Science and Technology, Universitas Brawijaya, Malang, Indonesia
[2] Department of Animal Reproduction and Breeding, Faculty of Animal Science and Technology, Universitas Brawijaya, Malang, Indonesia
[3] Department of Animal Products Technology, Faculty of Animal Science and Technology, Universitas Brawijaya, Malang, Indonesia
[4] Department of Animal Production, Faculty of Animal Science and Technology, Universitas Brawijaya, Malang, Indonesia
[5] Department of Chemistry, Faculty of Science, Technology, and Mathematics, Universitas Brawijaya, Malang, Indonesia
[6] Department of Feed and Animal Nutrition, Faculty of Animal Science and Technology, Universitas Brawijaya, Malang, Indonesia
[7] Department of Animal Production, Faculty of Animal Science, Universitas Jenderal Soedirman, Purwokerto, Indonesia
*Corresponding author: triekos@ub.ac.id
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ABSTRACT

Effective and sustainable teat dipping strategies are essential to treat persistent mastitis in smallholder dairy farming. This study compared the efficacy of 25% clove leaf oil nanoemulsion (CLON), a natural alternative to post-milking teat dipping, with 1% povidone-iodine (PV-I) in field conditions in Banyumas, Indonesia. We applied the California Mastitis Test to 138 dairy cows, found 40 mastitis-positive udder quarters, and assigned them to two teat dipping treatments (25% CLON and 1% PV-I) in a completely randomized 2×4 factorial design with five replicates. Teat dipping was applied twice daily for 30 days to measure the total plate count (TPC) and somatic cell count (SCC) on days 0, 10, 20, and 30. Reduction kinetics were estimated using the first-order models and compared using two-way ANOVA and equivalence testing. The results showed that over 30 days, the 25% CLON treatment reduced TPC by 31.65%–64.03% and SCC by 17.52%–45.05% across all mastitis severities, with the highest percentage on trace level. Total reduction or first-order reduction constants were not significantly different between 25% CLON and 1% PV-I, ±10 percentage points, as evidenced by the equivalence testing. Dermal irritation assessment showed no erythema or edema after the 24-h application. Exploratory 16S rRNA sequencing of paired samples revealed a decrease in pathogenic abundance (*Epilithonimonas bovis*: from 65.04% to 1.51%; *Lactococcus garvieae*: from 10.03% to 0.09%) and an increase in microbial diversity. Conclusively, 25% CLON was comparable to 1% PV-I, suggesting a feasible natural substitute for post-milking teat dipping in smallholder dairy farming.

Keywords: clove leaf oil; dairy cattle; mastitis; nanoemulsion; teat dipping

INTRODUCTION

Mastitis is one of the diseases with the highest economic burden on dairy cattle production worldwide, recording an annual loss up to USD 22 billion (Rasmussen *et al.*, 2024). It disproportionately affects Indonesian smallholder farms, which constitute 80% of national dairy production. Conventional farming practices contribute to a high prevalence (52.39%-66.49%), predominantly subclinical mastitis (Nuraini *et al.*, 2023), which often goes undetected due to a lack of visible clinical signs. This highlights the need for effective and practical prevention strategies in smallholder dairy systems.

Post-milking teat dipping with 1% povidone-iodine (PV-I) solution is a standard mastitis control measure (NMC, 2017). Although iodine-based teat disinfectants are relatively popular among Indonesian dairy farmers, their inconsistent use and lack of farmers' awareness (40%-60% according to a survey by Akzar *et al.*, 2023) were mostly due to complicated implementation and limited access to required inputs. This hinders the effectiveness of this control measure. The drawback of teat dipping includes the potential

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transfer of iodide residues to milk, although the extent varies among iodine-based teat disinfectants (French *et al.*, 2016). In addition, low iodine concentration (0.2%) has been reported to induce morphological changes in keratinocytes and fibroblasts, consistent with the teat skin dryness and irritation observed after repeated application (Kekül *et al.*, 2021; Steins *et al.*, 2023). These effects are likely to be aggravated in smallholder systems lacking standardized dilution and solution management. Inconsistent disinfectant management may also increase the likelihood of sublethal exposure. Behiry *et al.* (2012) reported that repeated passage of *Staphylococcus aureus* through sublethal concentrations of an iodine-based teat disinfectant, when exposed below optimal biocidal concentrations, reduced susceptibility in nine of ten strains. Together, these limitations have increased interest in plant-derived alternatives.

Essential oils are among the most extensively studied groups of bioactive compounds for this purpose, and their multi-target mode of action is considered less likely to select for resistant strains than single-target antimicrobial agents. Clinical evidence supporting the use of essential oil-based teat dipping formulations remains limited, creating a substantial gap between promising in vitro findings and practical field application (Ucella-Filho *et al.*, 2024).

Clove leaf oil (*Syzygium aromaticum*) is one of the abundant agricultural by-products with high eugenol content (>70%) and broad-spectrum antimicrobial activity, making it the highest-potential essential oil for mastitis prevention. Clove leaf oil has been reported to effectively disrupt the bacterial cytoplasmic membrane and fight major mastitis-associated pathogens, including *Staphylococcus aureus*, *Streptococcus* spp., and *Escherichia coli* (Maggini *et al.*, 2024). However, the efficacy of essential oils is constrained by their high volatility and poor water solubility. To address this, nanoemulsion technology can improve the stability, increase the surface area, and enhance the bioavailability of clove leaf oil (Singh *et al.*, 2017).

This study builds upon previous work that optimized and validated the *in vitro* antimicrobial activity of a 25% (v/v) clove leaf oil nanoemulsion (CLON) against mastitis-associated pathogens (Syamsi *et al.*, 2026). The novelty of this study lies in translating these laboratory findings into on-farm practical application through the first field trial of 25% CLON as a post-milking teat dipping solution under smallholder dairy farm conditions. Therefore, this study aimed to evaluate whether 25% CLON has comparable efficacy to 1% povidone-iodine (PV-I) in reducing total plate count (TPC) and somatic cell count (SCC) in the milk of dairy cow quarters with different mastitis statuses, based on reduction kinetics and California Mastitis Test (CMT) status.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Research Ethics Commission (Animal Care and Use Committee) of the Faculty of Veterinary Medicine, Brawijaya University, under ethical clearance certificate No. 2-KEP-FKHUB-2025, issued on May 16, 2025. All animal procedures were conducted in accordance with the ethical guidelines and regulations established by the committee.

Materials

Milk samples were procured from the lactating dairy cows in a study conducted at the Peternak Satria (PESAT) Cooperative in Banyumas Regency, Central Java. Indonesia. Materials included clove leaf oil (local commercial distiller, Cilacap, Indonesia), Tween 80 (≥95%; Iberchem, Jakarta, Indonesia), polyethylene glycol (PEG) 400 (≥95%; Green Pharma, Selangor, Malaysia), commercial povidone-iodine (Kimia Farma, Jakarta, Indonesia), California Mastitis Test (CMT) solution (DeLaval International AB, Tumba, Sweden), plate count agar (HiMedia, Mumbai, India), and Loeffler's methylene blue (Merck Sigma-Aldrich, Burlington, MA, USA). Major equipment used were a high-speed homogenizer (Hangzhou Jingfei Instrument Technology Co., Ltd., Hangzhou, China), ultrasonic bath, laminar airflow cabinet, electric autoclave, and digital microscope (BioBase, Shandong, China), a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), an Opentrons Flex Thermal Cycler (Opentrons, New York, NY, USA), and an Oxford Nanopore PromethION sequencer (Oxford Nanopore Technologies, Oxford, UK).

Study Population and Experimental Design

The study involved 388 lactating cows from a total of 781 dairy cattle owned by 17 farmer groups across six subdistricts. The cattle population was distributed across Pekuncen (215), Sumbang (181), Cilongok (160), Baturraden (125), Karanglewas (82), and Kedungbanteng (18) subdistricts. We purposively selected four subdistricts with the largest cattle populations as the study area.

All lactating cows in these subdistricts (n=138) were owned by 57 cooperative members, in their fourth or later lactation, and considered eligible for the study. All 552 mammary quarters from these cows were screened using the California Mastitis Test (CMT). Quarters with a CMT score of +++, clinical mastitis, or teat lesions were excluded. The remaining quarters were classified according to CMT score and grouped into four mastitis status groups (−, Trace, +, and ++). Ten quarters were randomly selected from each group, totaling 40 experimental units.

The field trial was conducted as a 2 × 4 factorial experiment in a completely randomized design (CRD). The first factor was teat dipping treatment: 1% povidone-iodine (PV-I) as the positive control and 25% clove leaf oil nanoemulsion as the CLON. The second factor was mastitis status. Individual mammary quarters served as the experimental units due to their independent teat canal, blood supply, and infection status, which may influence their response to the treatment. Within each mastitis-status category, selected quarters were

randomly assigned to the two teat dipping treatments, resulting in five replicate quarters per treatment × status combination (2 × 4 × 5 = 40). Treatments were applied twice daily immediately after milking for 30 consecutive days. Samples were collected from each quarter on days 0, 10, 20, and 30. The measurements on day 0 became the baseline for each quarter, resulting in a repeated-measures longitudinal dataset.

California Mastitis Test (CMT) Assessment

Mastitis was determined using the California Mastitis Test (CMT) according to the NMC (2017) guidelines. Before evaluation, barns and udders were cleaned, and the first streams of milk were discarded. Milk samples were mixed 1:1 with CMT reagent in paddle cups and scored: negative (−) for no precipitation, trace for slight precipitation, positive (+) for distinct precipitation without gel formation, positive (++) for mixture thickening with early gel formation, and positive (+++) for gel formation creating a convex surface.

Formulation and Application of Teat Dipping

Formulation. This study used a clove leaf oil nanoemulsion (CLON) concentrate, optimized with a self-nanoemulsifying drug delivery system (SNEDDS) by Syamsi *et al.* (2026). The formulation contained 14.29% clove leaf oil, 68.56% Tween 80, and 17.15% PEG 400 (v/v), while the optimal concentration for antimicrobial application against mastitis-associated pathogens was 25% (v/v) dilution. Therefore, the same concentration was selected for field evaluation and is referred to as 25% CLON. For field application, the formulation was prepared using the previously established procedure and scaled from a 10 mL laboratory batch to a 500 mL production batch. Clove leaf oil, Tween 80, and PEG 400 were homogenized at 8,000 rpm for 12 min while maintaining the temperature below 40 °C. The mixing speed and time were adjusted to maintain an equivalent energy dissipation rate at the larger production volume. The coarse emulsion resulting from this step was sonicated at 40 kHz for 600 s at 40 °C, similar to the original protocol. Finally, the concentrate was dispersed in deionized water at a 25:75 (v/v) ratio and stirred at 350 rpm for 3 min, consistent with the spontaneous emulsification characteristics of SNEDDS formulations.

Irritation testing. A preliminary dermal irritation test was conducted before field application on three clinically healthy lactating dairy cows with intact skin and no lesions or history of dermatological treatment. A patch containing 0.5 mL of 25% CLON was applied to a cleaned area of the udder skin (approximately 6 cm^2) and let attached for 24 h, as per the acute dermal irritation patch-test procedure described by Hemmati *et al.* (2016). The udder skin, instead of the teat, was chosen to allow for uninterrupted occlusive exposure on a flat surface for 24 h without interfering with the microbial or somatic cell status. The test site was evaluated for erythema and edema after patch removal.

Application. Each quarter received teat-dipping treatment (25% CLON or 1% PV-I) immediately after milking twice daily for 30 consecutive days. Teats were dipped into a teat cup containing 10 mL of the solution for approximately 15 s.

Efficacy Assessment

Total plate count (TPC) and somatic cell count (SCC). TPC was determined using the pour plate method on Plate Count Agar, incubated at 37 °C for 48 hours (AOAC, 2016). The results were expressed as colony-forming units per milliliter (CFU/mL). Somatic cell count (SCC) was determined by the direct microscopic method with methylene blue staining and examined under oil immersion microscopy at 1000× magnification (AOAC, 2002). Cells were counted in 20 randomly selected microscopic fields.

16S rRNA gene sequencing analysis. The grade (+) category was selected for exploratory microbiome profiling because it represents prevalent subclinical mastitis, which post-milking teat disinfection aims to prevent. Two composite milk samples (100 mL each) were collected from the four quarters of a cow diagnosed with grade (+) subclinical mastitis before and after teat dipping treatment. Samples were transported under sterile, refrigerated conditions and processed within 24 h. Total bacterial DNA was extracted with a Favorgen Bacterial DNA Extraction Kit and quantified with a Qubit 4.0 Fluorometer using an Equalbit 1× dsDNA HS Assay Kit. The V1–V9 hypervariable region of the 16S rRNA gene (~1,500 bp) was amplified using multiplexed primers (forward: 16S_mix_F, 16S_Bor_F, 16S_Chl_F, and 16S_Ent_F; reverse: 16S_mix_R, 16S_Bor_R, and 16S_Chl_R) supplied with the SQK-MAB114.24 kit (Oxford Nanopore Technologies, n.d.), based on the degenerate 27F primer formulation of Frank *et al*. (2008). Amplicon quality was verified by agarose gel electrophoresis. Sequencing libraries were prepared using the SQK-MAB114.24 kit and sequenced on an Oxford Nanopore PromethION platform (Aja-Macaya *et al*., 2025).

Data Analysis

TPC and SCC measurements from each quarter on days 0, 10, 20, and 30 were fitted to a first-order kinetic model by linear regression of ln-transformed counts against time. Four kinetic parameters were derived from the fitted model: total reduction (R, %), first-order rate constant (k, day^{-1}), daily reduction rate (r, % day^{-1}), and half-time ($T_{0.5}$, days). These parameters describe the rate and extent of decline within each quarter and reduce the influence of variation in baseline TPC and SCC values among quarters. Repeated measurements were used to estimate a single kinetic profile for each quarter and were not considered independent replicates (Matthews *et al*., 1990). The derived parameters (n = 5 quarters per treatment × mastitis status combination) were analyzed using two-way ANOVA in a 2 × 4 factorial design, with fixed effects including treatment, mastitis status, and their interaction ($\alpha = 0.05$).

The R and the k were subjected to statistical analysis, whereas the r and $T_{0.5}$ were reported descriptively because they are deterministic functions of k. The normality of residuals and homogeneity of variances were examined using the Shapiro–Wilk and Levene's tests. Both assumptions were satisfied for the TPC parameters, whereas the SCC parameters showed departures from normality and heterogeneity of variance, reflecting a greater dispersion of reduction values among CMT-negative quarters. As the design was balanced (n = 5 per cell), the analysis was repeated on rank-transformed data using a permutation test stratified by mastitis status, and reproduced the conclusions of the parametric analysis. A linear mixed-effects model was additionally fitted to $\log_{10}$-transformed counts by restricted maximum likelihood to assess robustness, including treatment, mastitis status, day of treatment, and all interaction terms as fixed effects, with quarter-specific random intercepts. The treatment × day interaction, which tested whether the rate of decline differed between treatments, was evaluated using a likelihood-ratio test on models refitted by maximum likelihood. Treatment equivalence was further assessed using two one-sided tests (TOST) with a prespecified equivalence margin of ±10 percentage points for R. All analyses were performed using Jamovi version 2.7.38, with mixed models fitted through the GAMLj module ($\alpha = 0.05$).

The 16S rRNA gene sequence data were processed as follows. Basecalling was performed using the Dorado basecall server v7.11.2 with a minimum Q-score of 10, followed by read quality assessment using NanoPlot (De Coster & Rademakers, 2023). Reads of 1,300–1,600 bp were taxonomically classified using the EPI2ME wf-16S workflow v1.6.1 (Oxford Nanopore Technologies, 2024) with Kraken2 (Wood *et al*., 2019) against the NCBI RefSeq 16S/18S/28S/ITS database (O'Leary *et al*., 2016). Relative abundance was calculated from the classified read counts, and alpha diversity was assessed using the Shannon index, Berger–Parker dominance index, and effective number of species within the same workflow. Taxonomic composition was visualized using Krona charts (Ondov *et al*., 2011). Because the analysis was based on a single paired sample, microbiome data were reported descriptively without statistical testing.

RESULTS

Irritation Testing

The dermal irritation testing revealed no signs of erythema or edema in any of the three cows after a 24-h patch application of 25% CLON. These findings suggest the formulation was well tolerated under the test conditions, supporting its subsequent evaluation in the 30-day field trial.

Total Plate Count (TPC) and Somatic Cell Count (SCC) Reduction

Total plate count (TPC) consistently decreased across all treatments and mastitis status categories throughout the study (Table 1). Baseline TPC values increased with mastitis severity, followed by progressive reductions through day 30. The greatest reduction was observed in the trace category, reaching 64.03 ± 2.77% with 25% CLON and 62.46 ± 1.24% with 1% PV-I (Table 3). Generally, the magnitude of TPC reduction

Table 1. Total plate count (CFU/mL) of dairy cow quarters across different mastitis statuses and observation days

Observation day	Mastitis status	Treatments		Mean
		1% PV-I	25% CLON	
Day 0	−	79,400 ± 1,517	77,600 ± 1,817	78,500 ± 1,841
	Trace	159,800 ± 2,588	152,000 ± 5,745	155,900 ± 5,878
	+	452,000 ± 19,235	510,000 ± 25,495	481,000 ± 37,253
	++	1,104,000 ± 32,094	1,110,000 ± 29,155	1,107,000 ± 29,079
	Mean	448,800 ± 413,713	462,400 ± 419,053	455,600 ± 411,076
Day 10	−	65,400 ± 5,459	66,000 ± 4,472	65,700 ± 4,715
	Trace	129,600 ± 5,030	129,000 ± 2,828	129,300 ± 3,860
	+	404,000 ± 27,019	446,000 ± 15,166	425,000 ± 30,277
	++	992,000 ± 36,332	1,002,000 ± 61,806	997,000 ± 48,086
	Mean	397,750 ± 375,996	410,750 ± 381,244	404,250 ± 373,801
Day 20	−	62,800 ± 1,643	59,000 ± 1,581	60,900 ± 2,514
	Trace	103,000 ± 3,742	103,200 ± 5,891	103,100 ± 4,654
	+	364,000 ± 11,402	374,000 ± 18,166	369,000 ± 15,239
	++	866,000 ± 51,284	862,000 ± 13,038	864,000 ± 35,340
	Mean	348,950 ± 329,338	349,550 ± 327,968	349,250 ± 324,413
Day 30	−	54,400 ± 3,782	53,000 ± 1,581	53,700 ± 2,831
	Trace	60,000 ± 2,449	54,600 ± 3,578	57,300 ± 4,057
	+	284,000 ± 11,402	304,000 ± 18,166	294,000 ± 17,764
	++	786,000 ± 16,733	776,000 ± 11,402	781,000 ± 14,491
	Mean	296,100 ± 305,500	296,900 ± 302,691	296,500 ± 300,175

Note: PV-I: povidone-iodine; CLON: clove leaf oil nanoemulsion; TPC values expressed in CFU/mL. Mastitis status based on the California Mastitis Test (CMT): (−) negative, no precipitation; trace, slight precipitation; (+) distinct precipitation without gel formation; (++) thickening with early gel formation.

declined as mastitis severity increased. An exception was observed in the negative (−) category, where reductions were similar to those recorded in the ++ category (31.48%–31.65% vs. 28.73%–30.05%). The k and the r exhibited trends similar to the R values. The $T_{0.5}$ decreased as the reduction magnitude increased. The shortest $T_{0.5}$ values were recorded in the Trace category, reaching 21.11 days for 25% CLON and 21.88 days for 1% PV-I.

The SCC followed a pattern similar to TPC across all mastitis categories and sampling times (Tables 2 and 3), although the reductions were consistently smaller. The largest SCC reduction was also observed in the Trace category, reaching 45.05 ± 1.37% with 25% CLON and 44.15 ± 2.81% with 1% PV-I. The corresponding $T_{0.5}$ values were 37.24 and 37.08 days, respectively. The longest $T_{0.5}$ values occurred in the negative (−) category, reaching 118.15 and 132.88 days.

A two-way ANOVA of the quarter-level kinetic parameters revealed a significant effect of mastitis status on both TPC and SCC reduction ($p<0.001$; Table 4). Neither teat dipping treatment ($p \geq 0.055$) nor the treatment × status interaction ($p \geq 0.78$) was significant, indicating similar performance of 25% CLON and 1% PV-I across all mastitis categories. Mean TPC reduction was 41.50 ± 14.25% for 25% CLON and 39.94 ± 14.01% for 1% PV-I ($F(1,32) = 2.26$, $p = 0.1426$). The corresponding k values were 0.0188 ± 0.0089 and 0.0175 ± 0.0087 day^{-1}, respectively ($F(1,32) = 3.96$, $p = 0.0553$). For SCC, the R values reached 26.78 ± 11.40% with 25% CLON and 25.92 ± 11.46% with 1% PV-I ($F(1,32) = 1.05$, $p = 0.3131$). The corresponding k values were 0.0104 ± 0.0051 and 0.0103 ± 0.0054 day^{-1} ($F(1,32) = 0.12$, $p = 0.7357$). The relatively large standard deviations primarily reflected differences among mastitis categories, while variation among quarters within the same treatment × status combination was substantially smaller (Table 3).

The confirmatory linear mixed-effects model produced results consistent with the ANOVA. The treatment × day interaction, which assessed differences in the rate of decline between treatments, was not significant for either TPC ($\chi^2(1) = 1.46$, $p = 0.227$) or SCC ($\chi^2(1) = 0.04$, $p = 0.846$). Equivalence testing further demonstrated that treatment differences remained within the prespecified equivalence margin of ±10 percentage points (Table 5). The estimated difference was +1.56 percentage points for TPC (90% CI, −0.20 to +3.31; $p<0.001$) and +0.87 percentage points for SCC (90% CI, −0.57 to +2.30; $p<0.001$). These results indicate that 25% CLON achieved kinetic performance comparable to 1% PV-I in reducing both TPC and SCC during the 30-day treatment period.

California Mastitis Test (CMT) results demonstrated qualitative improvement in udder health status (Table 6). Quarters classified as negative (−) at baseline remained negative throughout the study. Quarters with Trace status improved to negative by day 10. Quarters classified as + improved to Trace by day 30, whereas those classified as ++ improved to + over the same period. Both teat dipping treatments produced identical patterns of CMT improvement.

16S rRNA gene sequencing analysis

An exploratory analysis of the udder microbial community was performed using full-length 16S rRNA gene sequencing to compare milk samples collected before (day 0) and after (day 30) treatment with 25% CLON. The analysis was based on a single paired sample without biological replication. Therefore,

Table 2. Somatic cell count (cells/mL) of dairy cow quarters across different mastitis statuses and observation days

Observation day	Mastitis status	Treatments		Mean
		1% PV-I	25% CLON	
Day 0	−	136,000 ± 3,162	138,800 ± 3,033	137,400 ± 3,273
	Trace	330,000 ± 13,565	318,800 ± 6,723	324,400 ± 11,693
	+	628,000 ± 2,449	638,400 ± 3,578	633,200 ± 6,197
	++	973,200 ± 10,826	987,600 ± 9,529	980,400 ± 12,249
	Mean	516,800 ± 324,776	520,900 ± 331,895	518,850 ± 324,125
Day 10	−	132,400 ± 3,286	131,600 ± 1,673	132,000 ± 2,494
	Trace	303,200 ± 4,147	245,600 ± 2,608	274,400 ± 30,533
	+	562,400 ± 8,173	549,200 ± 3,899	555,800 ± 9,211
	++	910,800 ± 5,404	902,800 ± 12,458	906,800 ± 9,987
	Mean	477,200 ± 301,108	457,300 ± 306,919	467,250 ± 300,273
Day 20	−	129,600 ± 3,847	125,600 ± 2,608	127,600 ± 3,748
	Trace	266,400 ± 2,608	229,600 ± 4,561	248,000 ± 19,709
	+	493,200 ± 14,464	498,000 ± 9,055	495,600 ± 11,654
	++	846,000 ± 8,124	858,400 ± 7,797	852,200 ± 9,953
	Mean	433,800 ± 278,263	427,900 ± 290,684	430,850 ± 280,885
Day 30	−	113,200 ± 5,215	114,400 ± 4,336	113,800 ± 4,565
	Trace	184,000 ± 2,000	175,200 ± 5,933	179,600 ± 6,240
	+	478,000 ± 3,162	478,000 ± 6,782	478,000 ± 4,989
	++	788,800 ± 4,147	795,600 ± 4,980	792,200 ± 5,613
	Mean	391,000 ± 274,285	390,800 ± 278,363	390,900 ± 272,766

Note: PV-I: povidone-iodine; CLON: clove leaf oil nanoemulsion; SCC values expressed in cells/mL. Mastitis status based on the California Mastitis Test (CMT): (−) negative, no precipitation; trace, slight precipitation; (+) distinct precipitation without gel formation; (++) thickening with early gel formation.

Table 3. Kinetics of total plate count (TPC) and somatic cell count (SCC) reduction in dairy cow quarters with different mastitis statuses following 30 days of teat dipping treatment

Variables	Mastitis status	Treatments		Mean
		1% PV-I	25% CLON	
Total plate count				
R (%)	−	31.48 ± 4.68	31.65 ± 3.16	31.57 ± 3.77[c]
	Trace	62.46 ± 1.24	64.03 ± 2.77	63.24 ± 2.19[a]
	+	37.11 ± 2.98	40.28 ± 4.53	38.69 ± 3.99[b]
	++	28.73 ± 3.22	30.05 ± 2.24	29.39 ± 2.70[c]
	Mean	39.94 ± 14.01	41.50 ± 14.25	40.72 ± 13.97
k (day^{-1})	−	0.0118 ± 0.0025	0.0125 ± 0.0014	0.0122 ± 0.0019[c]
	Trace	0.0317 ± 0.0009	0.0330 ± 0.0025	0.0323 ± 0.0019[a]
	+	0.0150 ± 0.0017	0.0173 ± 0.0028	0.0161 ± 0.0025[b]
	++	0.0116 ± 0.0021	0.0122 ± 0.0015	0.0119 ± 0.0017[c]
	Mean	0.0175 ± 0.0087	0.0188 ± 0.0089	0.0181 ± 0.0087
r (% day^{-1})	−	1.17 ± 0.24	1.25 ± 0.14	1.21 ± 0.19
	Trace	3.12 ± 0.09	3.25 ± 0.25	3.18 ± 0.19
	+	1.49 ± 0.17	1.71 ± 0.27	1.60 ± 0.25
	++	1.15 ± 0.20	1.21 ± 0.15	1.18 ± 0.17
	Mean	1.73 ± 0.85	1.86 ± 0.87	1.79 ± 0.85
$T_{0.5}$ (day)	−	60.67 ± 11.06	55.78 ± 6.13	58.22 ± 8.82
	Trace	21.88 ± 0.64	21.11 ± 1.61	21.49 ± 1.22
	+	46.76 ± 4.86	40.95 ± 6.91	43.86 ± 6.41
	++	61.43 ± 10.19	57.37 ± 6.85	59.40 ± 8.46
	Mean	47.68 ± 17.95	43.80 ± 15.88	45.74 ± 16.84
Somatic cell count				
R (%)	−	16.69 ± 5.18	17.52 ± 4.29	17.10 ± 4.51[c]
	Trace	44.15 ± 2.81	45.05 ± 1.37	44.60 ± 2.14[a]
	+	23.89 ± 0.27	25.13 ± 1.00	24.51 ± 0.95[b]
	++	18.94 ± 0.68	19.44 ± 0.73	19.19 ± 0.71[c]
	Mean	25.92 ± 11.46	26.78 ± 11.40	26.35 ± 11.29
k (day^{-1})	−	0.0057 ± 0.0021	0.0063 ± 0.0016	0.0060 ± 0.0018[c]
	Trace	0.0188 ± 0.0015	0.0186 ± 0.0008	0.0187 ± 0.0012[a]
	+	0.0095 ± 0.0003	0.0097 ± 0.0003	0.0096 ± 0.0003[b]
	++	0.0070 ± 0.0003	0.0070 ± 0.0003	0.0070 ± 0.0003[c]
	Mean	0.0103 ± 0.0054	0.0104 ± 0.0051	0.0103 ± 0.0052
r (% day^{-1})	−	0.57 ± 0.21	0.63 ± 0.16	0.60 ± 0.18
	Trace	1.86 ± 0.15	1.85 ± 0.08	1.85 ± 0.11
	+	0.95 ± 0.03	0.96 ± 0.03	0.95 ± 0.03
	++	0.70 ± 0.03	0.70 ± 0.03	0.70 ± 0.03
	Mean	1.02 ± 0.53	1.03 ± 0.51	1.03 ± 0.51
$T_{0.5}$ (day)	−	132.88 ± 42.91	118.15 ± 38.39	125.52 ± 39.16
	Trace	37.08 ± 3.21	37.24 ± 1.74	37.16 ± 2.44
	+	72.99 ± 2.34	71.79 ± 2.31	72.39 ± 2.28
	++	98.57 ± 3.55	99.36 ± 4.84	98.97 ± 4.02
	Mean	85.38 ± 41.08	81.64 ± 35.98	83.51 ± 38.16

Note: PV-I: povidone-iodine; CLON: clove leaf oil nanoemulsion; R: total reduction; k: first-order reduction constant; r: reduction rate; $T_{0.5}$: half-time, defined as the time required to reduce 50% of the initial population. Mastitis status based on the California Mastitis Test (CMT): (−) negative, no precipitation; trace, slight precipitation; (+) distinct precipitation without gel formation; (++) thickening with early gel formation.

Table 4. Two-way ANOVA of total plate count (TPC) and somatic cell count (SCC) reduction parameters in dairy cow quarters following 25% CLON and 1% PV-I teat dipping treatment

Source of variation	df	TPC	p	TPC	p	SCC	p	SCC	p
		R (%): F		k (day^{-1}): F		R (%): F		k (day^{-1}): F	
Treatment (T)	1	2.26	0.143	3.96	0.055	1.05	0.313	0.12	0.736
Mastitis status (S)	3	224.53	<0.001	231.27	<0.001	220.68	<0.001	257.88	<0.001
T × S	3	0.36	0.784	0.36	0.783	0.03	0.992	0.18	0.908
Error	32								
Total	39								

Note: PV-I = povidone-iodine; CLON = clove leaf oil nanoemulsion; R = total reduction (%); k = first-order reduction constant (day^{-1}). Values are mean ± SD (n = 20 quarters per treatment). Degrees of freedom for treatment, mastitis status, and treatment × status interaction were 1, 3, and 3, respectively. Statistical significance was set at $p<0.05$.

Table 5. Equivalence testing (two one-sided tests) of total plate count (TPC) and somatic cell count (SCC) reduction in dairy cow quarters between 25% CLON and 1% PV-I teat dipping treatments

Variables	Mean R		Difference (pp)	SE	df	90% CI		p (TOST)	Conclusion
	1% PV-I	25% CLON				Low	High		
TPC	39.94 ± 14.01	41.50 ± 14.25	+1.56	1.04	32	-0.20	3.31	<0.001	Equivalent
SCC	25.92 ± 11.46	26.78 ± 11.40	+0.87	0.85	32	-0.57	2.30	<0.001	Equivalent

Note: TPC = total plate count; SCC = somatic cell count; PV-I = povidone-iodine; CLON = clove leaf oil nanoemulsion; R = total reduction; pp = percentage points; TOST = two one-sided tests. Equivalence was evaluated using an a priori equivalence margin of ±10 percentage points. Treatments were considered equivalent when p (TOST) < 0.05.

Table 6. Progression of mastitis status reduction in dairy cow quarters during teat dipping treatment based on the California Mastitis Test (CMT)

Treatments	Observation day			
	0	10	20	30
1% Povidone-Iodine	-	-	-	-
	Trace	-	-	-
	+	+	+	Trace
	++	++	++	+
25% Clove leaf oil nanoemulsion	-	-	-	-
	Trace	-	-	-
	+	+	+	Trace
	++	++	++	+

Note: Mastitis status based on the CMT: (−) negative, no precipitation; trace, slight precipitation; (+) distinct precipitation without gel formation; (++) thickening with early gel formation.

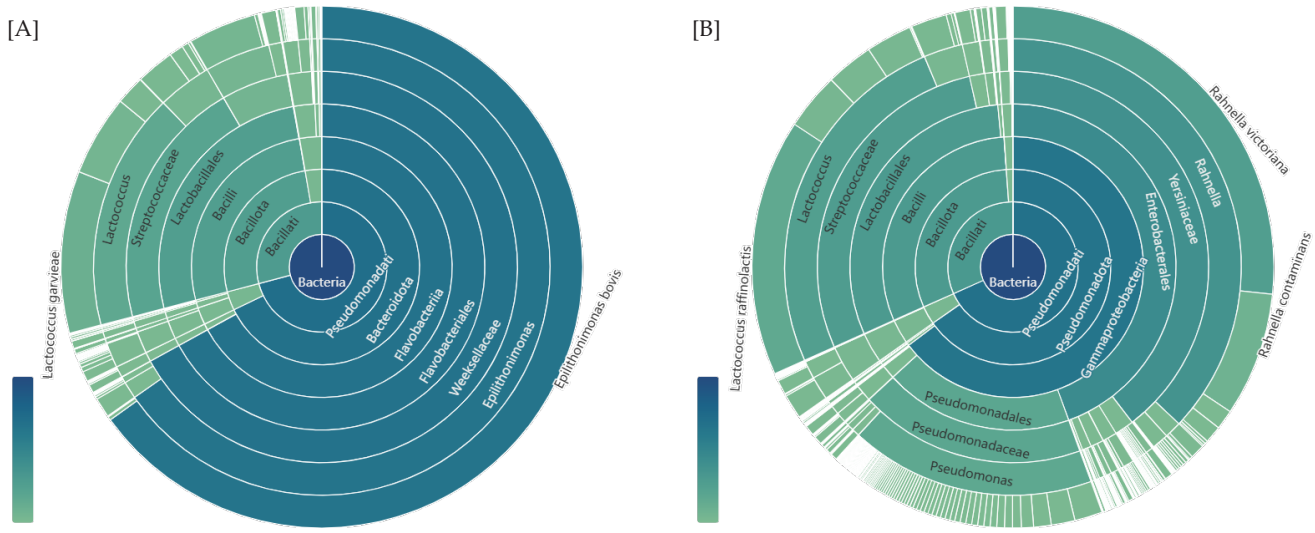


Figure 1. Sunburst visualization of udder microbial community composition of a dairy cow with subclinical mastitis before (A) and after (B) 25% CLON teat dipping treatment

the results should be interpreted as exploratory and hypothesis-generating rather than confirmatory.

At the phylum level, Bacteroidota dominated the pre-treatment community, accounting for 67.79% of total relative abundance (Figure 1). Flavobacteriales represented the predominant order within this phylum, comprising 67.01% of the community. Following treatment, the relative abundances of Bacteroidota and Flavobacteriales declined to 3.03% and 2.97%, respectively. In contrast, Pseudomonadota dramatically increased from 3.12% to 65.26%, primarily due to the emergence of Enterobacterales (44.46%) and Pseudomonadales (18.74%), which were absent before treatment. Bacillota and Actinomycota showed minor changes in their abundance.

At the species level (Figure 2), *Epilithonimonas bovis*, the dominant pre-treatment species, decreased from 65.04% to 1.51% after treatment. Other mastitis-associated pathogens, *Lactococcus garvieae* and *Streptococcus agalactiae*, also declined from 10.03% to 0.09% and 2.35% to 0.31%, respectively. Post-treatment, the relative abundances of *Rahnella victoriana* (26.66%) and *Lactococcus raffinolactis* (15.92%) increased, while *Streptococcus uberis* increased from 0.88% to 2.21%.

Alpha-diversity metrics (Table 7) showed a notable increase in community diversity post-treatment. The Shannon index increased from 1.65 to 3.51, the Berger–Parker index dropped from 0.65 to 0.27, and the effective number of species increased from 5.19 to 33.48. Disparities in sequencing depth (119,931 vs.

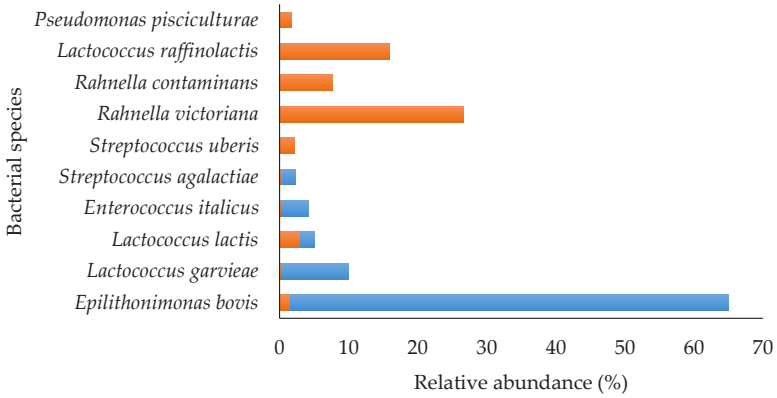


Figure 2. Relative abundance (%) of the ten most dominant bacterial species in dairy cow milk samples before and after 25% CLON teat dipping treatment. Note : Before, After.

Table 7. Alpha diversity indices of the udder microbial community of a dairy cow with subclinical mastitis before and after 25% CLON teat dipping treatment

Diversity indexes	Before	After
Shannon index	1.65	3.51
Simpson index	0.56	0.89
Berger-Parker index	0.65	0.27
Richness (OTU)	132	654
Pielou's Evenness	0.34	0.54
Effective species	5.19	33.48
Total reads	119,931	1,017,500

Note: OTU = Operational taxonomic unit; Diversity indices were computed on non-rarefied data; the difference in sequencing depth between samples precludes formal comparison.

1,017,500 reads) might have influenced the observed richness differences, reflected in the varied number of operational taxonomic units (132 vs. 654 OTUs).

DISCUSSION

Irritation Testing

The lack of erythema and edema, and its near-neutral pH (7.13 ± 0.01), which closely matches bovine teat skin physiology (pH 7.00–7.18), confirmed that 25% CLON is a safe, topical solution for bovine teats (Syamsi *et al.*, 2026). This contrasts with conventional acidic 1% PV-I (pH < 7), which can cause tissue dehydration and protein denaturation (Kekül *et al.*, 2021). Also, iodine concentrations ≥0.2% can induce morphological changes in keratinocytes and fibroblasts (Steins *et al.*, 2023). The dermal compatibility of 25% CLON is attributable to the synergistic effects of eugenol (72.90%; antimicrobial, antioxidant), β-caryophyllene (12.00%; CB2 receptor ligand modulating inflammation) (Jha *et al.*, 2021), and emollients (Tween 80, and PEG 400) to maintain skin barrier integrity. Its nanoemulsion formulation further reduces eugenol cytotoxicity through controlled release (Haro-González *et al.*, 2023). Therefore, 25% CLON offers a safer profile for repeated post-milking application, although long-term tolerance studies are necessary.

Total Plate Count (TPC) Reduction

CLON significantly reduces TPC, especially in the trace status (R = 64.03%; $T_{0.5}$ = 21.11 days in 25% CLON). This is because teat dipping primarily prevents environmental microbial invasion. In the trace stage (mild infection), the combination of contamination prevention and the cow's immune capacity results in optimal TPC reduction. Conversely, in + and ++ mastitis, microorganisms have proliferated extensively within the mammary gland, so teat dipping can only prevent additional bacterial load from external sources without eliminating internal bacteria. The lower TPC reduction in the negative status (healthy teats) compared to trace is due to already low initial TPC, so the kinetic model indicates a smaller reduction despite the ongoing biological reduction (Sharun *et al.*, 2021).

The CMT-negative quarters were included to evaluate the preventive function of teat dipping. The baseline TPC values of these quarters (77,600–79,400 CFU/mL) were well below the Indonesian national standard (<1,000,000 CFU/mL), confirming healthy udder status. Although TPC reduction was lower than in trace status (R ≈ 31% vs. 64%), microbial counts remained stable in healthy quarters. CLON effectively maintains udder hygiene by preventing bacterial proliferation.

While two-way ANOVA showed no significant treatment effect on TPC reduction, equivalence testing confirmed comparable efficacy between treatments with a ±10 percentage-point margin. These field results validated the previous in vitro findings of CLON's broad-spectrum antimicrobial activity against mastitis pathogens (Syamsi *et al.*, 2026). The study reported a >3 log10 CFU/mL reduction within 60 minutes against mastitis-associated microorganisms, confirming tangible evidence in farm conditions, where the teat surface is exposed to a complex polymicrobial environment rather than single reference strains. These field data were obtained at a single cooperative with a limited number of quarters per treatment × status combination. Further confirmation across multiple farms and production systems is advised.

The antimicrobial efficacy of 25% CLON stems from the synergistic action of multiple bioactive compounds, such as eugenol (72.90%), β-caryophyllene

(12.00%), eugenyl acetate (3.84%), and caryophyllene oxide (2.12%), which disrupt bacterial membranes, increase permeability, inhibit membrane-bound ATPase, and trigger ROS production (Syamsi *et al.*, 2026; Pilong *et al.*, 2022). This multi-target mechanism reduces the potential for resistance compared with single-compound disinfectants (Syamsi *et al.*, 2026). The nanoemulsion's ultra-small droplet (25.19 ± 2.31 nm) enhances contact with teat skin microflora and sustains release during the critical 30–60 minute post-milking window (Rainard *et al.*, 2022; Singh *et al.*, 2017). Further studies of 90–180 days are needed to confirm long-term efficacy and shelf-life stability.

Somatic Cell Count (SCC) Reduction

Somatic Cell Count (SCC) reduction is slower than TPC reduction because SCC comprises leukocytes (75%) and epithelial cells (25%) that migrate to the mammary gland to combat pathogens. Teat disinfection indirectly reduces SCC by forming a residual antimicrobial barrier during the post-milking window, when the teat canal remains dilated, thereby reducing new bacterial challenges and allowing the mammary immune system to downregulate cellular defense through decreased chemotactic signaling (IL-8, complement C5a) and reduced neutrophil migration (Alhussien *et al.*, 2021).

Over time, accumulated somatic cells undergo apoptosis and are cleared through phagocytosis by resident macrophages, while damaged mammary epithelial cells regenerate (Kirkeby *et al.*, 2021). This cascade explains why SCC $T_{0.5}$ is consistently longer than TPC $T_{0.5}$ across mastitis statuses. The trace status showed the fastest SCC reduction ($T_{0.5}$ = 37.24 days vs. TPC $T_{0.5}$ = 21.11 days), which may be due to the combination of low pre-existing bacterial burden and intact immune resolution capacity. The ++ status required substantially longer time ($T_{0.5}$ = 99.36 days) due to extensive tissue inflammation. Two-way ANOVA confirmed no significant difference between treatments in either R values or k values for SCC, with equivalence within the prespecified margin.

The CMT improvement pattern corroborated the quantitative data. The transition from trace to negative status by day 10 is consistent with the rapid TPC reduction kinetics, while the slower improvement in the + and ++ statuses (requiring 30 days to shift by one grade) aligns with the longer $T_{0.5}$ at these stages. Both 25% CLON and 1% PV-I produced identical CMT progression patterns, complementing the in vitro bactericidal classification (MBC/MIC ratio = 4:1) reported by Syamsi *et al.* (2026). However, while effective in reducing TPC, SCC, and CMT (NMC, 2017), 25% CLON's preventive efficacy against new infections requires further evaluation.

Beyond comparable efficacy, 25% CLON offers practical advantages as a local, plant-derived alternative. Its near-neutral pH (Syamsi *et al.*, 2026) is compatible with bovine teat skin physiology, and preliminary tests showed no irritation. However, cumulative tolerance and direct skin condition comparison were not evaluated. Its multi-target mechanism of essential oils may reduce resistance development compared with single-target antimicrobial agents.

Early intervention with teat dipping is most effective, as evidenced by the greatest TPC and SCC reductions in the trace category. This observation was consistent with the CMT results, in which trace quarters reverted to negative status earlier than quarters with higher initial CMT scores. Routine CMT screening is crucial for timely intervention. Milk residue analysis for eugenol and its effects on organoleptic properties is needed before commercial application.

16S rRNA gene sequencing analysis

This pilot assessment, based on a single pair of 16S rRNA without biological replication or untreated controls, offers hypothesis-generating observations rather than definitive conclusions regarding 25% CLON treatment effects without a concurrent 1% PV-I. The observed microbial shifts could reflect normal temporal variation in the udder microbiota.

The pre-treatment profile was notably dominated by *Epilithonimonas bovis* (65.04%), a Gram-negative bacterium commonly associated with raw cow's milk and moist environments surrounding cattle housing (Laviad-Shitrit *et al.*, 2017; Nicholson *et al.*, 2020). The dominance of this species, rather than the conventionally reported *Staphylococcus aureus* or *Streptococcus agalactiae*, suggests that culture-independent approaches such as NGS may reveal non-classical microbial profiles missed by conventional diagnostic methods. The detection of a complex microbial composition is consistent with the elevated TPC and SCC values at day 0.

After 30 days of 25% CLON treatment, a significant decline was evident in *E. bovis* (from 65.04% to 1.51%), *L. garvieae* (from 10.03% to 0.09%), and *S. agalactiae* (from 2.35% to 0.31%). These reductions are broadly consistent with the TPC data and the broad-spectrum antimicrobial activity of 25% CLON demonstrated in vitro against Gram-positive (*S. aureus*), Gram-negative (*E. coli*), and yeast (*C. albicans*) species (Syamsi *et al.*, 2026), and consistent with a membrane-directed mechanism acting independently of cell wall structure (Pilong *et al.*, 2022). Alpha diversity increased (Shannon index from 1.65 to 3.51), and dominance decreased (Berger–Parker index from 0.65 to 0.27), suggesting suppression of dominant pathogenic species. However, substantial differences in sequencing depth (119,931 vs. 1,017,500 reads) limit the interpretation of all diversity metrics, without rarefaction.

A minor increase in *S. uberis* (0.88% to 2.21%) may indicate competitive release (Jung *et al.*, 2024) or natural variability. The emergence of *Pseudomonas pisciculturae* likely reflects environmental exposure rather than active infection (Vidal *et al.*, 2017). This pilot study provides preliminary evidence that 25% CLON teat dipping may alter the udder microbial community, complementing the TPC and SCC data. Future investigations with biological replicates, standardized sequencing depth, and immunological parameters are needed to confirm these observations.

CONCLUSION

Post-milking teat dipping with 25% clove leaf oil nanoemulsion (CLON) showed comparable efficacy to 1% povidone-iodine in reducing total plate count and somatic cell count and improving California mastitis test status in dairy cow quarters across different mastitis statuses, with the greatest reduction at trace status, and caused no dermal irritation. Exploratory 16S rRNA gene sequencing further indicated increased udder microbiota diversity following treatment. Despite limitations of a single-site trial, limited replicates, and unreplicated microbiome analysis, these findings warrant multi-farm, longer-term studies assessing new intramammary infection rates, milk residues, and immune responses. Overall, 25% CLON can serve as a safe, plant-derived alternative to iodine-based teat disinfectants for mastitis control in smallholder dairy farms, particularly when applied at an early stage of subclinical mastitis.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used Claude.ai to facilitate academic discussion and review the language used in the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the publication.

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