

RESEARCH ARTICLE

In vitro antibacterial activity of carvacrol and thymol against clinical bacterial isolates associated with bovine metritis



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ABSTRACT

Background and Aim: Bovine metritis is an important postpartum disease that compromises reproductive performance, milk production, and profitability in dairy herds. Increasing antimicrobial resistance among uterine pathogens highlights the need for alternative antimicrobial strategies. Carvacrol and thymol, major phenolic monoterpenes in oregano essential oil, have broad antibacterial activity; however, evidence of their activity against diverse clinical isolates associated with bovine metritis remains limited. This study evaluated the *in vitro* antibacterial activity of purified carvacrol and thymol against clinical bacterial isolates associated with bovine metritis.

Materials and Methods: Forty-three clinical bacterial isolates recovered from bovine metritis cases were evaluated, comprising four *Staphylococcus aureus*, six coagulase-negative staphylococci, ten *Streptococcus* spp., ten *Trueperella pyogenes*, ten *Escherichia coli*, and three *Klebsiella pneumoniae* isolates. Five American Type Culture Collection reference strains were included exclusively for quality control and excluded from statistical analyses. Antibacterial activity was evaluated using disk diffusion and broth microdilution assays to determine inhibition zones, minimum inhibitory concentrations (MICs), and minimum bactericidal concentrations (MBCs). Ceftiofur and ethanol served as positive and negative controls, respectively. The clinical isolate was considered the experimental unit, and assays were performed in technical triplicate.

Results: Carvacrol and thymol inhibited all bacterial groups evaluated. Gram-positive isolates exhibited significantly larger inhibition zones and lower MIC and MBC values than Gram-negative isolates ($p < 0.05$). Mean MIC values ranged from 0.0062 ± 0.0004 to 0.0065 ± 0.0004 mg/mL for Gram-positive isolates and from 0.0101 ± 0.0019 to 0.0143 ± 0.0042 mg/mL for Gram-negative isolates. Corresponding mean MBC values ranged from 0.0143 ± 0.0010 to 0.0166 ± 0.0012 mg/mL and from 0.0209 ± 0.0040 to 0.0292 ± 0.0084 mg/mL, respectively. Coagulase-negative staphylococci, *Streptococcus* spp., and *T. pyogenes* showed high susceptibility, whereas *K. pneumoniae* was comparatively less susceptible.

Conclusion: Purified carvacrol and thymol demonstrated antibacterial activity against clinically relevant bacteria associated with bovine metritis, particularly Gram-positive isolates. These findings support further investigation of these naturally derived antimicrobial candidates. Cytotoxicity, antibiofilm activity, pharmaceutical formulation, pharmacokinetics, and controlled *in vivo* efficacy should be evaluated before clinical application in dairy cattle.

Keywords: antimicrobial alternatives, bovine metritis, carvacrol, clinical bacterial isolates, minimum bactericidal concentration, minimum inhibitory concentration, phenolic monoterpenes, thymol.

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INTRODUCTION

Reproductive efficiency is essential for dairy herd productivity; however, hormonal imbalances, nutritional deficiencies, inadequate management, environmental stress, and reproductive diseases can compromise uterine health and fertility in cattle [1–3]. Among postpartum reproductive disorders, metritis is particularly important because of its high prevalence and substantial economic impact. In Mexico, reported metritis incidence ranges from 10% to 30%, particularly in northern regions. Postpartum metritis increases culling risk, prolongs days open, reduces milk production, increases treatment costs, and decreases herd profitability, making it one of the most economically important postpartum diseases in dairy cattle [4].

After calving, bacterial contamination and tissue damage from fetal and placental expulsion trigger a physiological inflammatory response that contributes to uterine involution. Under normal conditions, bacterial clearance, endometrial regeneration, and reduction in uterine size occur within 4–6 weeks postpartum [4, 5]. However, transient immunosuppression and negative energy balance during this period can impair immune function, including neutrophil activity, thereby promoting bacterial persistence and increasing susceptibility to postpartum uterine disease [6, 7]. The major bacterial pathogens associated with clinical metritis include *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella melaninogenica*, although their prevalence varies throughout the postpartum period [8]. *Escherichia coli* often colonizes the uterus early, promoting inflammation and facilitating subsequent infection by *T. pyogenes*, which can increase tissue damage. Therefore, timely diagnosis and appropriate management are important for preserving uterine health and reproductive performance in dairy cows [9].

Antimicrobial therapy is widely used to manage postpartum uterine infections; however, inappropriate or excessive antimicrobial use can promote the selection of resistant bacteria among uterine pathogens and the resident microbiota. Ideally, therapeutic decisions should be supported by etiological identification and antimicrobial susceptibility testing, although these approaches are not always feasible under field conditions [7, 10]. Consequently, alternative strategies that can reduce reliance on conventional antimicrobials while supporting sustainable animal health and production are increasingly important [11, 12].

Among naturally derived antimicrobial candidates, oregano essential oil has attracted attention because it contains phenolic monoterpenes, particularly carvacrol and thymol, with documented bactericidal and fungicidal activities [13–15]. Researchers have also investigated these compounds for antimicrobial, antioxidant, and anticancer properties, and they may have therapeutic potential at appropriate concentrations [14, 16–20]. Their antibacterial properties make them promising candidates for alternative approaches to bacterial infections in food-producing animals, particularly in efforts to reduce conventional antimicrobial use [2, 21, 22].

Despite increasing interest in plant-derived antimicrobial compounds, important evidence gaps remain regarding their activity against bacteria associated with bovine uterine disease. Previous investigations have evaluated essential oils against bovine reproductive pathogens; however, essential oils are complex mixtures whose chemical composition and biological activity can vary considerably. Comparatively little information exists on the antibacterial activity of purified carvacrol and thymol against a diverse collection of clinical bacterial isolates recovered specifically from cases of bovine metritis. In particular, comparative evidence regarding their inhibitory and bactericidal activity against both Gram-positive and Gram-negative clinical isolates remains limited. Determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), together with inhibition-zone measurements, may therefore provide quantitative evidence regarding the susceptibility of clinically relevant bovine metritis-associated bacteria to these purified phenolic monoterpenes.

This study therefore aimed to evaluate the *in vitro* antibacterial activity of purified carvacrol and thymol against clinical bacterial isolates associated with bovine metritis. Their inhibitory and bactericidal activities were assessed using inhibition-zone diameters, MIC, and MBC and compared between Gram-positive and Gram-negative bacterial isolates. The study hypothesized that carvacrol and thymol would exhibit antibacterial activity against the evaluated isolates, with greater activity against Gram-positive than Gram-negative bacteria. These findings were intended to provide an experimental basis for further investigation of carvacrol and thymol as naturally derived antimicrobial candidates for bovine uterine infections.

MATERIALS AND METHODS

Ethical approval

The bacterial isolates evaluated in this study were obtained from samples collected during routine veterinary

diagnostic procedures from dairy cows with clinical bovine metritis. This study did not involve additional animal handling, experimental interventions, or collection of biological samples specifically for this investigation. The study was conducted using previously isolated and cryopreserved bacterial strains. Therefore, formal approval by an Institutional Animal Care and Use Committee was not required.

Study period and location

The study was conducted at the Bacteriology Laboratory of the National Center for Disciplinary Research in Animal Health and Safety (CENID-SAI), National Institute for Forestry, Agriculture, and Livestock Research (INIFAP), Mexico, from January to June 2023. The clinical bacterial isolates were originally recovered between June to September 2018 from 30 multiparous dairy cows diagnosed with clinical bovine metritis. The isolates originated came from 10 dairy herds in the Tizayuca dairy basin, Hidalgo, central Mexico. Thirty cows exhibiting clinical signs consistent with metritis were selected during the first 21 days postpartum. Diagnosis was based on clinical assessment, taking into account the presence and characteristics of uterine discharge (watery, reddish-brown, or purulent, often with a foul odor), the state of uterine involution, and the animal's general condition. Data recorded for each cow included identification, days since calving, reproductive history, rectal temperature, and the presence of reduced appetite or other clinical abnormalities. Materials used for sample collection included rectal palpation gloves, sterile gloves, sterile swabs, water-based lubricant, external cleaning solution, sterile gauze, sterile tubes containing Stuart transport medium, and a refrigerated container. Each cow was restrained in a handling chute to facilitate collection and minimize the risk of injury. The perineal region, vulva, and adjacent areas were carefully cleaned to remove feces, secretions, and other contaminants. Excess cleaning solution was removed, and the area was dried using sterile gauze. Transrectal palpation was performed to locate the cervix and uterus and to guide the sampling device. The swab was carefully inserted into the vagina, shielded during advancement, and guided via transrectal manipulation through the cervical canal into the uterine cavity. Once in place, the sample was collected, and the swab was withdrawn while avoiding contact with the vaginal walls. The swab was then placed in a sterile tube containing Stuart transport medium. Each tube was labeled with the animal's identification number, collection date, and days postpartum. Transcervical sampling was performed by trained veterinary personnel without forcing passage through the cervix, particularly in animals with inflammation or reproductive abnormalities. Samples were kept refrigerated at 2–4°C and sent to the CENID-SAI laboratory within 12 hours of collection. Upon arrival at the laboratory, they were transferred to a maintenance medium (BHI broth/glycerol 20% (v/v)) and stored at –80°C.

Study design

This *in vitro* experimental study evaluated the antibacterial activity of purified carvacrol and thymol against clinical bacterial isolates associated with bovine metritis. A total of 43 clinical isolates were evaluated, and each individual bacterial isolate was considered the experimental unit. Four American Type Culture Collection (ATCC) reference strains were included exclusively as quality control strains and were excluded from inferential statistical analyses. The antibacterial activity of carvacrol and thymol was evaluated using disk diffusion and broth microdilution assays to determine inhibition-zone diameters, MICs, and MBCs. All assays were performed in technical triplicate.

Oregano oil metabolites

Analytical-grade carvacrol (99%) and thymol (98.5%), the principal phenolic monoterpenes of oregano essential oil, were used (Sigma-Aldrich, St. Louis, MO, USA; lots MKBT0467V and SLBT5627, respectively). The compounds were stored in amber glass vials at 4°C and protected from light. Stock solutions were freshly prepared in absolute ethanol before each experiment. Working solutions were prepared by serial dilution in the appropriate culture medium, maintaining a final ethanol concentration of ≤1% (v/v). Ethanol at the corresponding concentration was used as the negative control.

Bacterial strains

Five reference strains were included: *Staphylococcus aureus* ATCC®BAA976, *Trueperella pyogenes* ATCC®10308, *Klebsiella pneumoniae* ATCC®BAA-1705, and *Escherichia coli* ATCC®35218.

In addition, 43 clinical isolates recovered from the 30 multiparous dairy cows diagnosed with clinical bovine metritis were evaluated. These comprised four *S. aureus*, six coagulase-negative staphylococci (CoNS), ten *Streptococcus* spp., ten *T. pyogenes*, ten *E. coli*, and three *K. pneumoniae* isolates. Following primary isolation and identification, the clinical isolates were cryopreserved at –80°C until use. Before antibacterial susceptibility

testing, the isolates were reactivated by subculture on blood agar and incubated at 37°C for 24 h to confirm viability and purity. Bacterial identification was performed using conventional microbiological methods and the API identification system (bioMérieux, Marcy-l'Étoile, France). The ATCC reference strains were used exclusively for quality control to verify assay performance and were excluded from statistical analyses.

Antimicrobial susceptibility testing

The antimicrobial susceptibility profiles of the clinical bacterial isolates were determined using the disk diffusion method on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [23]. Reactivated bacterial isolates were suspended and adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 colony-forming units [CFU]/mL). The inoculum was spread uniformly over the agar surface using sterile swabs, and commercial antimicrobial disks were subsequently placed on the inoculated plates.

The antimicrobial agents evaluated were dicloxacillin (DC, 1 µg), vancomycin (VA, 30 µg), clindamycin (CLM, 2 µg), erythromycin (E, 15 µg), penicillin G (PE, 10 U), tetracycline (TE, 30 µg), cephalothin (CF, 30 µg), gentamicin (GE, 10 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), ampicillin (AM, 10 µg), cefoxitin (CFX, 30 µg), ciprofloxacin (CPF, 5 µg), ceftiofur (XNL, 30 µg), carbenicillin (CB, 100 µg), chloramphenicol (CL, 30 µg), nitrofurantoin (NF, 300 µg), amikacin (AK, 30 µg), netilmicin (NET, 30 µg), and norfloxacin (NOF, 10 µg) (BD BBL™, Sensi Disc™, Becton, Dickinson and Company, Franklin lakes, NJ, USA). The plates were incubated at 37°C for 18–24 h under aerobic conditions. After incubation, inhibition-zone diameters were measured, and isolates were classified as susceptible (S), intermediate (I), or resistant (R). Because organism-specific interpretive criteria for bovine uterine isolates were unavailable for some organism–antimicrobial combinations, inhibition-zone diameters were interpreted using human clinical CLSI criteria [23] where applicable. These criteria have not been validated for bovine metritis; therefore, the resulting susceptibility and resistance frequencies are exploratory and should be interpreted cautiously.

Disk diffusion assay of carvacrol and thymol

The antibacterial activities of carvacrol and thymol were evaluated using the Kirby–Bauer disk diffusion procedure, following the general disk diffusion methodology described in CLSI guidelines [23]. Ceftiofur was included as a comparator. Inhibition-zone diameters were measured and compared among treatments as experimental outcomes. Because CLSI clinical breakpoints are not established for carvacrol or thymol, inhibition zones produced by these compounds were not categorized as susceptible, intermediate, or resistant. Mueller-Hinton agar (BD Bioxon (Becton Dickinson)) was used for *CoNS*, *S. aureus*, *K. pneumoniae*, and *E. coli*, whereas Mueller-Hinton agar supplemented with 5% blood was used for *Streptococcus* spp. and *T. pyogenes*.

Sterile 6-mm filter-paper disks were impregnated with 20 µL of carvacrol or thymol at 50 mg/mL, corresponding to 1 mg of compound per disk. Based on previous studies evaluating carvacrol and thymol at concentrations of up to 1000 µg/disk, a concentration of 1 mg/disk was selected to enable comparison of inhibitory activity among clinical isolates with potentially heterogeneous susceptibility [24]. Carvacrol and thymol were dissolved in ethanol to ensure complete impregnation of the disks because the other diluents evaluated (Tween (Droguería Cosmopolita, s. f., Mexico) and phosphate-buffered saline [PBS] (Meyer Corporation, California, EE.UU.)) did not fully dissolve the compounds. Disks impregnated with 20 µL of ethanol alone served as the negative solvent control, whereas commercial ceftiofur disks (30 µg; Becton Dickinson, Franklin Lakes, NJ, USA) served as the positive control.

Bacterial suspensions were prepared in sterile saline and adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL), corresponding to an optical density at 600 nm (OD₆₀₀) of 0.1. The inocula were spread uniformly over the agar surface using sterile swabs, after which disks containing carvacrol, thymol, ceftiofur, or the solvent control were placed on the inoculated plates. Plates inoculated with *S. aureus*, *K. pneumoniae*, *E. coli*, and *Streptococcus* spp. were incubated aerobically at 37°C for 24 h. Plates inoculated with *T. pyogenes* were incubated at 37°C for 24 h in an atmosphere supplemented with 5% CO₂.

After incubation, inhibition-zone diameters were measured in millimeters using a digital caliper. All assays were performed in technical triplicate. For each clinical isolate, replicate measurements were averaged before statistical analysis. The ATCC reference strains were included exclusively for quality control and were not included in statistical comparisons.

Determination of MIC and MBC

MIC and MBC were determined using the broth microdilution method in sterile 96-well round-bottom

microplates following CLSI M07 with modifications [25]. Each well initially received 100 µL of brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA; pH 7.4 ± 0.2). Stock solutions of carvacrol and thymol were prepared at 10 mg/mL in analytical-grade ethanol. Subsequently, 100 µL of each stock solution was added to the first well, followed by two-fold serial dilution to obtain final concentrations ranging from 5.0 to 0.0049 mg/mL.

Bacterial suspensions were prepared in sterile saline and adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL; $OD_{600} = 0.1$). Microdilution plates (Corning Life Sciences, NY, USA) were inoculated with 20 µL of the bacterial suspension for *S. aureus*, *K. pneumoniae*, and *E. coli*. For *Streptococcus* spp. and *T. pyogenes*, 50 µL was inoculated because of their higher nutritional requirements. Before inoculation, the suspensions were adjusted to obtain a final bacterial density of approximately 5×10^5 CFU/mL in all wells, irrespective of the inoculum volume.

Each microplate included (i) growth-control wells containing inoculated BHI broth without carvacrol or thymol; (ii) sterility-control wells containing uninoculated BHI broth; (iii) solvent control wells containing ethanol (Merck KGaA, Darmstadt, Alemania) at the highest concentration used in the assay; and (iv) positive control wells containing ceftiofur. ATCC reference strains were included as quality control strains to verify assay performance but were excluded from statistical analyses. All assays were performed in technical triplicate.

MIC was defined as the lowest concentration of carvacrol or thymol that completely inhibited visible bacterial growth, as indicated by the absence of turbidity and a bacterial pellet at the bottom of the well. To determine MBC, 15 µL from each well showing no visible bacterial growth was subcultured onto BHI agar (BD Bioxon) or BHI agar supplemented with 5% defibrinated sheep blood, depending on the nutritional requirements of each bacterial species. Plates were incubated at 37°C for 24 h under aerobic conditions or, for *Streptococcus* spp. and *T. pyogenes*, in an atmosphere supplemented with 5% CO₂. MBC was defined as the lowest concentration of carvacrol or thymol resulting in the complete absence of visible bacterial colonies on solid medium, indicating loss of bacterial viability [23].

Statistical analysis

The bacterial isolate was considered the experimental unit, and technical replicates were not treated as independent observations. For each bacterial group (*Staphylococcus* spp. n=10, *Corynebacterium* spp. n=10, *Streptococcus* spp. n=10, *Escherichia coli* n=10, *Klebsiella pneumoniae* n=3), a separate one-way ANOVA was performed. The fixed factor was compound with three levels: Carvacrol, Thymol, and Ceftiofur (positive control). The dependent variable was the mean inhibition zone diameter (mm) per isolate (average of three technical replicates). Normality of residuals and homogeneity of variances were assessed using Shapiro-Wilk and Levene's tests, respectively. Residuals were approximately normally distributed (Shapiro-Wilk $p > 0.05$ for 4/5 groups) and variances were homogeneous (Levene $p > 0.05$) except for *Corynebacterium* (Levene $p = 0.008$). As ANOVA is robust to mild heteroscedasticity with balanced designs, and Welch's ANOVA confirmed the same outcome ($p > 0.05$), parametric results are reported. Post hoc comparisons were performed with Tukey's HSD test. Significance was set at $\alpha=0.05$. Analyses were performed in IBM SPSS V.32 (IBM Corp., Armonk, NY, USA).

RESULTS

Antimicrobial susceptibility of clinical isolates to antibiotics

The antimicrobial susceptibility profiles of the bacterial isolates recovered from clinical cases of bovine metritis are shown in Figure 1. Overall, Gram-positive isolates showed high susceptibility to several β -lactam antimicrobials, particularly dicloxacillin, cephalothin, and ceftiofur, as well as to vancomycin. Among Gram-positive bacteria, *T. pyogenes* showed the highest resistance frequencies, including clindamycin (80%), gentamicin (80%), trimethoprim-sulfamethoxazole (70%), and tetracycline (60%).

Among the Gram-negative isolates, all *E. coli* and *K. pneumoniae* isolates were resistant to ampicillin, whereas they were highly susceptible to ceftiofur, ciprofloxacin, nitrofurantoin, and amikacin. All *E. coli* isolates were resistant to carbenicillin, whereas 66.7% of *K. pneumoniae* isolates were susceptible to this antimicrobial (Figure 1).

Disk diffusion assay

Mean inhibition-zone diameters differed significantly between Gram-positive and Gram-negative clinical isolates ($p < 0.05$). Carvacrol, thymol, and ceftiofur produced larger inhibition zones against Gram-positive than Gram-negative isolates. Mean inhibition-zone diameters for Gram-positive isolates ranged from 28.3 ± 0.86 to 31.06 ± 0.60 mm, whereas those for Gram-negative isolates ranged from 20.4 ± 0.77 to 22.3 ± 0.63 mm (Figure

2). Significant differences in inhibition-zone diameters were also observed among the bacterial groups evaluated ($p < 0.05$). Among Gram-positive bacteria, CoNS showed the largest inhibition zones with both carvacrol and thymol, whereas *S. aureus* showed the largest inhibition zones with ceftiofur. Among Gram-negative bacteria, *E. coli* and *K. pneumoniae* showed comparable inhibition-zone diameters with carvacrol and thymol, whereas ceftiofur generally produced slightly larger inhibition zones.

Antimicrobial	Gram-positive bacteria (n = 30)			Gram-negative bacteria (n = 13)		% susceptible isolates
	<i>Staphylococcus</i> spp. (n = 10)	<i>Streptococcus</i> spp. (n = 10)	<i>T. pyogenes</i> (n = 10)	<i>E. coli</i> (n = 10)	<i>K. pneumoniae</i> (n = 3)	
Dicloxacillin	100	100	100	ND	ND	100%
Vancomycin	100	100	100	N/A	N/A	70–99%
Clindamycin	70	90	20	N/A	33.3	50–69%
Erythromycin	80	90	50	N/A	0	<50%
Penicillin	70	100	90	N/A	0	ND (Not determined)
Tetracycline	70	90	40	N/A	0	N/A (Not applicable)
Cephalothin	70	100	100	50	66.7	
Gentamicin	80	90	20	100	66.7	
Trimethoprim-sulfamethoxazole	90	100	30	90	66.7	
Ampicillin	70	100	100	0	0	
Ceftiofur	100	100	70	100	100	
Cefotaxime (CFX)	70	90	100	60	100	
Ceftiofur sodium (XNL)	70	90	100	60	100	
Carbenicillin	ND	ND	ND	0	33.3	
Chloramphenicol	N/A	N/A	N/A	100	66.7	
Nitrofurantoin	N/A	ND	ND	100	100	
Amikacin	N/A	N/A	N/A	100	100	
Netilmicin	N/A	ND	ND	90	66.7	
Norfloxacin	N/A	ND	ND	90	100	

Figure 1: Antimicrobial susceptibility profiles of bacterial isolates recovered from clinical cases of bovine metritis. The heatmap shows the percentage of susceptible clinical isolates for each antimicrobial agent according to bacterial group. A total of 43 clinical isolates were evaluated: *Staphylococcus* spp. (n = 10), *Streptococcus* spp. (n = 10), *Trueperella pyogenes* (n = 10), *Escherichia coli* (n = 10), and *Klebsiella pneumoniae* (n = 3). Colors indicate the percentage of susceptible isolates: dark green = 100%; light green = 70–99%; yellow = 50–69%; and red = <50%. ND = Not determined; N/A = Not applicable. American Type Culture Collection reference strains were used exclusively as quality control strains and were excluded from the susceptibility percentages.

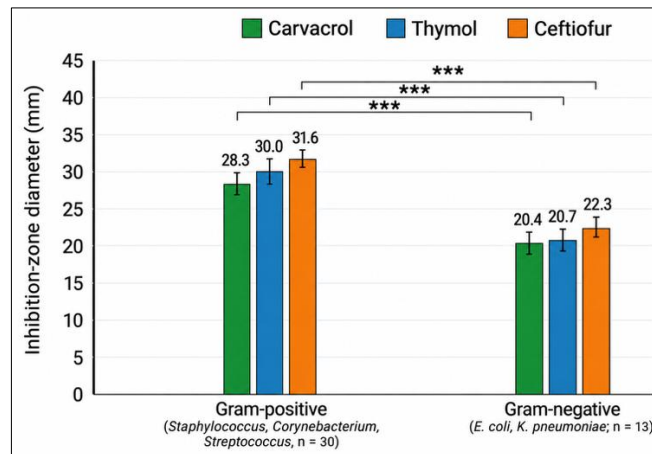


Figure 2: Inhibition-zone diameters for Gram-positive (*Staphylococcus*, *Corynebacterium*, and *Streptococcus*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) clinical isolates treated with carvacrol, thymol, or ceftiofur. The numbers of isolates evaluated are shown below each group. Bars represent the mean $\pm$ standard error of the mean. For each treatment, Gram-positive and Gram-negative isolates were compared using Welch's *t*-test with Holm correction. *** indicates $p < 0.001$.

Minimum inhibitory and MBCs

MIC values differed significantly between Gram-positive and Gram-negative clinical isolates ($p < 0.05$). Both carvacrol and thymol showed lower MIC values against Gram-positive than Gram-negative isolates. Mean MIC values ranged from 0.0062 ± 0.0004 to 0.0065 ± 0.0004 mg/mL for Gram-positive isolates and from 0.0101 ± 0.0019 to 0.0143 ± 0.0042 mg/mL for Gram-negative isolates (Figure 3).

MBC values were also significantly lower for Gram-positive than for Gram-negative clinical isolates ($p < 0.05$). Mean MBC values ranged from 0.0143 ± 0.0010 to 0.0166 ± 0.0012 mg/mL for Gram-positive isolates and from 0.0209 ± 0.0040 to 0.0292 ± 0.0084 mg/mL for Gram-negative isolates. For both carvacrol and thymol, MBC values were consistently higher than their corresponding MIC values (Figure 3).

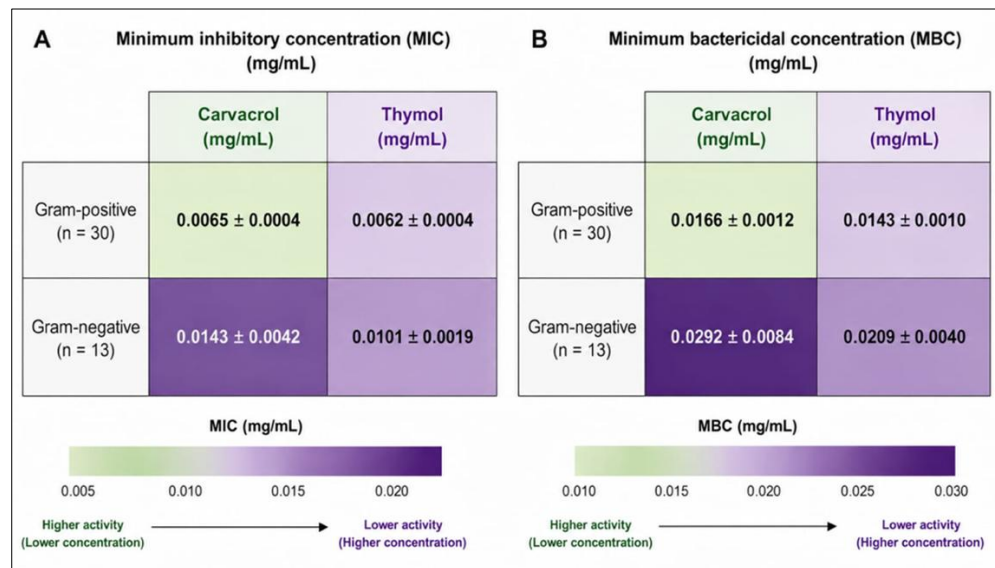


Figure 3: MIC and MBC of carvacrol and thymol against Gram-positive (n = 30) and Gram-negative (n = 13) bacterial isolates recovered from clinical cases of bovine metritis. (A) Mean MIC values. (B) Mean MBC values. Values are expressed as mean $\pm$ standard deviation. MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration.

DISCUSSION

Principal findings

This study showed that carvacrol and thymol exhibited antibacterial activity *in vitro* against all bacterial groups evaluated. This activity was consistently demonstrated using disk diffusion and broth microdilution assays, with both compounds showing lower MIC and MBC values against Gram-positive than Gram-negative isolates. Collectively, these findings support the antibacterial potential of these phenolic monoterpenes against bacterial pathogens associated with bovine metritis and justify their further investigation as naturally derived antimicrobial candidates [22–26].

Antimicrobial resistance among bovine metritis-associated isolates

The antimicrobial susceptibility profiles of the clinical isolates revealed heterogeneous resistance patterns among the bacterial groups evaluated. Gram-positive isolates generally showed high susceptibility to β -lactam antimicrobials, particularly dicloxacillin, cephalothin, and ceftiofur, whereas variable resistance was observed to clindamycin, erythromycin, tetracycline, and penicillin. Among these pathogens, *T. pyogenes* showed the highest resistance frequencies, particularly to clindamycin (80%), gentamicin (80%), trimethoprim-sulfamethoxazole (70%), and tetracycline (60%). This finding is relevant because *T. pyogenes* is an important opportunistic pathogen associated with postpartum uterine disease and may act synergistically with anaerobic bacteria such as *F. necrophorum* and *P. melaninogenica*, contributing to more severe uterine lesions and delayed reproductive recovery [2, 9].

Among Gram-negative isolates, all *E. coli* and *K. pneumoniae* isolates were resistant to ampicillin, whereas ceftiofur, ciprofloxacin, nitrofurantoin, and amikacin retained comparatively high activity. All *E. coli* isolates were also resistant to carbenicillin. These findings are consistent with reports describing antimicrobial resistance among bacterial pathogens of dairy cattle [27–30]. Similar resistance patterns have been reported in dairy herds across different geographic regions and are associated with prolonged or empirical antimicrobial use during the postpartum period [28]. The resistance profiles observed in the present study, particularly among *T. pyogenes* isolates, reinforce the importance of investigating alternative or complementary antibacterial strategies [11, 16, 30, 31].

Antibacterial activity of carvacrol and thymol

Disk diffusion testing showed that carvacrol and thymol inhibited all bacterial groups evaluated. Gram-

positive isolates showed significantly larger inhibition zones than Gram-negative isolates, indicating greater susceptibility to both phenolic compounds. CoNS and *Streptococcus* spp. exhibited the largest inhibition zones, whereas *E. coli* and *K. pneumoniae* showed smaller, but measurable, zones of inhibition.

These findings align with previous studies showing that oregano- and thyme-derived phenolic compounds have greater antibacterial activity against Gram-positive bacteria while retaining activity against clinically relevant Gram-negative pathogens [32–35]. Paiano *et al.* [2] reported that oregano and thyme essential oils inhibited *T. pyogenes* and *E. coli* isolated from cows with endometritis, although the inhibition zones were generally smaller than those observed here. Similarly, Ratajac *et al.* [36] reported that *Satureja montana* essential oil, composed mainly of p-cymene (42.8%) and carvacrol (28.1%), showed antibacterial activity against pathogens associated with reproductive disorders in cattle and produced mean MIC values below 1 mg/mL against the aerobic bacterial groups evaluated.

Differences among studies may reflect variation in botanical source, chemical composition, purity of the active compounds, bacterial species and strain origin, and experimental methodology. Unlike essential oils, which are complex mixtures of volatile constituents with variable composition, this study evaluated highly purified carvacrol (99%) and thymol (98.5%), allowing a more direct assessment of the antibacterial activity of these individual compounds.

Interpretation of disk diffusion and broth microdilution findings

Although disk diffusion is widely used as a screening method for antibacterial activity, inhibition-zone measurements obtained with hydrophobic compounds should be interpreted cautiously. The movement of carvacrol and thymol from impregnated disks through the agar matrix may be influenced by their limited aqueous solubility, restricted agar diffusion, and volatilization during incubation [37–39]. Consequently, inhibition-zone diameters may not fully reflect the antibacterial activity of poorly diffusible compounds.

Broth microdilution therefore provided a complementary quantitative approach by reducing the influence of agar diffusion and allowing direct determination of MIC and MBC. The broadly concordant susceptibility patterns obtained using disk diffusion and broth microdilution support the antibacterial activity of carvacrol and thymol, whereas MIC and MBC provide quantitative measures of the concentrations required for growth inhibition and bacterial killing.

Differential susceptibility of Gram-positive and Gram-negative isolates

Broth microdilution showed significantly lower MIC and MBC values for Gram-positive than Gram-negative isolates, indicating that lower concentrations of carvacrol and thymol were required to inhibit and kill Gram-positive bacteria. This pattern is consistent with the reported membrane-targeting activity of these lipophilic phenolic monoterpenes. Carvacrol and thymol can disrupt bacterial membranes, increase membrane permeability, impair proton gradients, and promote leakage of intracellular components [34].

The comparatively lower susceptibility of *E. coli* and *K. pneumoniae* may be related to structural characteristics of Gram-negative bacteria, particularly the outer membrane and associated porins, which can restrict penetration of hydrophobic compounds. Reported effects of carvacrol and thymol on energy metabolism, intracellular pH, ion gradients, and biofilm-associated processes further indicate that these compounds can act through multiple bacterial targets [13, 34, 38]. However, whether such multitarget activity reduces the likelihood of resistance development requires further investigation.

Minimum inhibitory and bactericidal concentrations

The MIC values obtained in the present study were low, particularly for Gram-positive isolates. Previous studies evaluating carvacrol- or thymol-rich essential oils have generally reported MIC values of 0.03–0.50 mg/mL against *S. aureus*, *E. coli*, and *T. pyogenes*, depending on the botanical source and chemical composition [26, 32, 35, 39]. In comparison, the present study found mean MIC values of 0.0062–0.0065 mg/mL for Gram-positive isolates and 0.0101–0.0143 mg/mL for Gram-negative isolates.

The lower MIC values observed in this study may partly reflect the use of highly purified, analytical-grade carvacrol and thymol rather than crude essential oils. The biological activity of crude essential oils can vary according to extraction method, geographic origin, plant chemotype, and synergistic or antagonistic interactions among individual constituents. Corona Gómez *et al.* [34] reported increasing thymol MIC values from *Staphylococcus* spp. (0.430 mg/mL) to *Streptococcus* spp. (0.664 mg/mL) and *E. coli* (0.977 mg/mL), also suggesting lower susceptibility among Gram-negative bacteria. Nevertheless, the markedly lower MIC values obtained in the present study may also reflect differences in bacterial species, strain origin, compound purity, and experimental

conditions. Therefore, direct quantitative comparisons among studies should be interpreted cautiously (Table 1).

Table 1: Comparative antimicrobial activity of carvacrol, thymol, and carvacrol-rich essential oils against bacterial pathogens of veterinary relevance in previous studies and the present study.

Study	Antimicrobial agent	Bacterial species and origin	Method	MIC (mg/mL)	MBC (mg/mL)	Main findings
Paiano et al. [11]	Oregano and thyme EOs	<i>Escherichia coli</i> , <i>Trueperella pyogenes</i> , <i>Fusobacterium necrophorum</i> , and <i>Staphylococcus aureus</i> associated with bovine endometritis (reference strains)	Disk diffusion	NR	NR	Oregano and thyme EOs showed marked antibacterial activity against pathogens associated with bovine endometritis.
Paiano et al. [27]	Cinnamon, oregano, and thyme EOs	<i>T. pyogenes</i> and <i>E. coli</i> isolated from dairy cows with clinical endometritis	MIC, MBC, and FIC assays	Cinnamon EO: 0.51 (<i>T. pyogenes</i>); (<i>E. coli</i>)	Cinnamon EO: 16.38 (<i>T. pyogenes</i>); 32.77 (<i>E. coli</i>)	Cinnamon EO showed the greatest antibacterial activity; oregano and thyme EOs were also active, whereas EO combinations showed variable interactions.
Corona-Gómez et al. [31]	TTO, thymol, and carvacrol	<i>Staphylococcus</i> spp., <i>Streptococcus</i> spp., <i>E. coli</i> , and <i>Klebsiella pneumoniae</i> isolated from bovine clinical mastitis	Agar diffusion and broth microdilution	TTO: 1.56–25; thymol: 0.05–0.40; carvacrol: 0.02–0.20	0.39–0.78†	Carvacrol and thymol exhibited greater antimicrobial activity than TTO, and selected combinations showed additive interactions.
Corona-Gómez et al. [32]	Thymol, tilmicosin, and thymol–tilmicosin combinations	<i>Staphylococcus</i> spp., <i>Streptococcus</i> spp., and <i>E. coli</i> isolated from bovine mastitis	Broth microdilution and checkerboard assay	Thymol: 0.430–0.977‡	Thymol: 0.859–1.953‡	Thymol exhibited antibacterial activity against all bacterial groups, with lower MIC and MBC values against Gram-positive bacteria. Its combination with tilmicosin predominantly produced additive interactions.
Ratajac et al. [35]	<i>Satureja montana</i> EO (p-cymene 42.8%, carvacrol 28.1%, and γ-terpinene 14.6%)	<i>T. pyogenes</i> , <i>E. coli</i> , <i>Pasteurella</i> spp., <i>S. aureus</i> , <i>Streptococcus</i> spp., and <i>F. necrophorum</i> of bovine reproductive origin	Disk diffusion, agar dilution, and broth microdilution	0.47–0.68§; <i>F. necrophorum</i> : 0.125	NR	<i>S. montana</i> EO exhibited broad-spectrum antibacterial activity, with mean MIC values below 1 mg/mL against all aerobic bacterial groups evaluated.
Present study	Carvacrol (99%) and thymol (98.5%)	Bacterial isolates associated with bovine metritis	Disk diffusion, MIC, and MBC assays	0.0043–0.0101	0.0078–0.0235	Both compounds exhibited potent broad-spectrum antibacterial activity, with particularly low MIC and MBC values and greater susceptibility among Gram-positive isolates.

EO = essential oil; TTO = tea tree oil; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; FIC = fractional inhibitory concentration; NR = not reported or not determined in the cited study. All MIC and MBC values are expressed in mg/mL. † MBC range reported for the bacterial groups evaluated by Corona-Gómez et al. [31]. ‡ MIC and MBC ranges reported by Corona-Gómez et al. [32] represent the mean values for thymol against *Staphylococcus* spp., *Streptococcus* spp., and *Escherichia coli*, respectively. The corresponding MIC/MBC values were 0.430/0.859, 0.664/1.328, and 0.977/1.953 mg/mL. § The MIC range of 0.47–0.68 mg/mL reported by Ratajac et al. [35] represents mean values for aerobic bacterial groups determined by agar dilution; *Fusobacterium necrophorum* was evaluated separately by broth microdilution (MIC = 0.125 mg/mL). Antimicrobial susceptibility results should be compared cautiously across studies because of differences in antimicrobial formulations (pure compounds versus essential oils), chemical composition, bacterial species and isolate origins, inoculum concentrations, solvents, and testing methods. Lower MIC or MBC values do not necessarily indicate superior antibacterial efficacy across studies conducted under different experimental conditions.

The MBC values obtained in the present study were also lower than those reported for several essential oil

preparations evaluated against veterinary pathogens. Gram-positive isolates showed mean MBC values of 0.0143–0.0166 mg/mL, whereas Gram-negative isolates required 0.0209–0.0292 mg/mL for bacterial killing. MBC values were consistently higher than the corresponding MIC values, as expected because bactericidal activity generally requires higher concentrations than inhibitory activity. The relatively small differences between MIC and MBC in the present study are consistent with bactericidal activity under the experimental conditions; however, this interpretation should be considered within the limits of the *in vitro* assay.

Translational considerations and formulation strategies

Despite the antibacterial activity observed *in vitro*, several physicochemical and biological factors may limit the direct clinical application of purified carvacrol and thymol. These compounds have limited aqueous solubility, are volatile, may degrade chemically, and could be rapidly diluted or cleared from the uterine lumen. In addition, the postpartum uterus contains inflammatory exudate, cellular debris, and organic material that may affect compound diffusion, stability, and local bioavailability.

These limitations highlight the potential value of pharmaceutical delivery systems designed to protect the active compounds, improve dispersion, prolong uterine residence time, and provide sustained local concentrations. Researchers have investigated nanoformulations, including nanoemulsions, liposomes, polymeric nanoparticles, and chitosan-based nanocapsules, to improve delivery of essential oil constituents [14, 25]. Encapsulation of carvacrol and thymol has been reported to improve chemical stability, reduce volatility, enhance aqueous dispersibility, facilitate tissue penetration, and provide controlled release. Such delivery systems may also help reduce local irritation while maintaining antibacterial concentrations for longer periods [25, 29]. However, their suitability for intrauterine administration in dairy cattle requires specific formulation, safety, pharmacokinetic, and efficacy studies.

One Health implications

From a One Health perspective, identifying effective naturally derived antibacterial compounds may support efforts to reduce unnecessary conventional antimicrobial use in dairy production systems [40]. Antimicrobial use in livestock can drive the selection and spread of resistant bacteria among animals, humans, and the environment. Therefore, developing effective nonconventional antibacterial strategies may complement antimicrobial stewardship programs by preserving the efficacy of medically important antimicrobial agents while supporting sustainable livestock production [41].

Nevertheless, these findings do not show that carvacrol or thymol can currently replace conventional antimicrobial therapy. Their potential contribution to antimicrobial stewardship depends on demonstrating safety, efficacy, appropriate dosing, formulation stability, and responsible clinical use.

CONCLUSION

This study demonstrated that carvacrol and thymol exhibited antibacterial activity *in vitro* against clinically relevant bacterial isolates associated with bovine metritis. Both compounds showed greater activity against Gram-positive than Gram-negative isolates, as demonstrated by significantly larger inhibition zones and lower MIC and MBC values. Among Gram-positive bacteria, CoNS, *Streptococcus* spp., and *T. pyogenes* showed comparatively high susceptibility to carvacrol and thymol, whereas Gram-negative bacteria, *E. coli* and *K. pneumoniae*, required higher concentrations to achieve inhibitory and bactericidal activity. In addition, the clinical isolates showed heterogeneous resistance to several conventional antimicrobials, including substantial resistance in *T. pyogenes* isolates and resistance to ampicillin in all *E. coli* and *K. pneumoniae* isolates.

The antibacterial activity of purified carvacrol and thymol against both Gram-positive and Gram-negative clinical isolates provides an experimental basis for their continued investigation as naturally derived antibacterial candidates for bovine uterine infections. The low MIC and MBC values observed, particularly among Gram-positive isolates, indicate that these phenolic monoterpenes warrant further evaluation as potential components of complementary strategies for managing bacterial infections associated with bovine metritis. However, these findings show antibacterial activity only under *in vitro* conditions and do not establish that carvacrol or thymol can replace conventional antimicrobial therapy in dairy cattle.

A key strength of this study was evaluating purified, analytical-grade carvacrol and thymol rather than complex essential oil preparations, enabling a more direct assessment of the antibacterial activity of the individual phenolic compounds. The study included 43 clinical isolates of Gram-positive and Gram-negative bacteria associated with bovine metritis, and used ATCC reference strains exclusively for quality control. Furthermore,

combining disk diffusion and broth microdilution assays provided complementary measures of antibacterial activity through inhibition-zone diameters, MIC, and MBC. The bacterial isolate was considered the experimental unit, and technical replicates were averaged before inferential statistical analysis, avoiding treatment of technical replicates as independent observations.

The principal limitation of this study is that the experiments were conducted exclusively *in vitro*; therefore, the antibacterial activity observed under controlled laboratory conditions may not directly translate to the postpartum uterine environment. All clinical isolates originated from dairy herds in the Tizayuca dairy basin, Hidalgo, Mexico, which may limit the geographic representativeness and generalizability of the findings. In addition, carvacrol and thymol were evaluated individually, and potential synergistic or antagonistic interactions between these compounds, conventional antimicrobials, or other essential oil constituents were not investigated. The study also did not evaluate activity against mature bacterial biofilms, cytotoxicity in bovine endometrial cells, pharmaceutical formulation and stability, pharmacokinetics, or safety and therapeutic efficacy in dairy cows.

Further investigations should evaluate the antibacterial activity of carvacrol and thymol against mature biofilms and determine their cytotoxicity in bovine endometrial cells. Developing and evaluating suitable pharmaceutical delivery systems, including controlled-release formulations, is also required to address their physicochemical limitations and determine whether they can maintain therapeutically relevant concentrations in the uterine environment. Pharmacokinetic studies should establish local exposure, persistence, and appropriate routes of administration. Controlled *in vivo* studies in dairy cows with naturally occurring metritis will then be required to determine effective dosage, clinical efficacy, safety, withdrawal periods, and cost-effectiveness.

Carvacrol and thymol showed *in vitro* antibacterial activity against clinical bacterial isolates associated with bovine metritis, with Gram-positive isolates more susceptible than Gram-negative isolates. These findings, together with the resistance to several conventional antimicrobials observed among the clinical isolates, support continued investigation of these purified phenolic monoterpenes as naturally derived antibacterial candidates. Nevertheless, their potential veterinary application remains preliminary and requires validation through cytotoxicity, formulation, pharmacokinetic, and controlled *in vivo* studies. These investigations are necessary to determine whether the antibacterial activity demonstrated under laboratory conditions can translate into safe, effective, and practical strategies for managing bovine metritis.

DATA AVAILABILITY

All data generated during this study are included in the manuscript.

GENERATIVE AI DECLARATION

Generative AI was used to assist in the design and formatting of the figures. The authors prepared the figures from their original data, which they checked. Generative AI was not used to generate, alter, or interpret the underlying data. The authors reviewed and approved the final figures and take responsibility for their accuracy.

AUTHORS' CONTRIBUTIONS

LFEC: Conceptualized the study, designed the methodology and experimental procedures, conducted the experiments, collected and analyzed the data, and prepared the original draft of the manuscript. LCG: Performed and validated the bacteriological diagnostic procedures, contributed to the interpretation of the microbiological findings, and critically reviewed the manuscript. IDRG: Performed statistical analysis, processed and validated the data, and contributed to interpreting the statistical findings. LHA: Performed bacteriological diagnosis, contributed to validating and interpreting the microbiological findings, and provided specialized resources and equipment required for the experimental work. HJS: Contributed to the analysis and interpretation of findings related to pathogens associated with the reproductive diseases evaluated in the study and participated in the scientific discussion and contextualization of the results. SEM: Provided specialized resources and equipment, contributed complementary diagnostic procedures, and helped interpret and validate the corresponding findings. DQG: Contributed to the study conception and design, supervised the research activities, and critically reviewed the manuscript. All authors critically reviewed the manuscript, read and approved the final manuscript, and agreed to be accountable for all aspects of the work.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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