UNIVERSIDAD AUTÓNOMA DE ESTADO DE HIDALGO

INSTITUTO DE CIENCIAS AGROPECUARIAS

DOCTORADO EN CIENCIAS AGROPECUARIAS

TESIS DOCTORAL

Efecto antibacteriano del aceite esencial de *Lippia graveolens* contra bacterias multirresistentes aisladas en unidades de producción lechera del Valle del Mezquital, Hidalgo

Para obtener el grado de

Doctor en Ciencias Agropecuarias

PRESENTA

M.C. Jorge Vargas Monter

Director

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Instituto de Ciencias Agropecuarias
Institute of Agricultural Sciences
Área Académica de Medicina Veterinaria y Zootecnia
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DEDICATORIA

A mis hijos

Con amor y cariño

Como constancia de satisfacción

del deber cumplido.

ÍNDICE GENERAL

GLOSARIO

Término	Significado
AELg	Aceite esencial de *Lippia graveolens*
ATCC	Colección americana de cultivos tipo
ATP	Adenosín trifosfato
CLSI	Instituto de estándares clínicos y de laboratorio
CMB	Concentración mínima bactericida
CMI	Concentración mínima inhibitoria
CMT	Prueba de mastitis california
DL50	Dosis letal, 50%
GC-MS	Cromatografía de gases acoplada a espectrometría de masas
IRM	Índice de resistencia múltiple
MALDI-TOF	Matrix-assisted laser desorption/ionization (desorción/ionización láser asistida por matriz)- time-Of-flight (tiempo de vuelo)
MDR	Resistentes a múltiples fármacos
NR	No resistente
OTEM	Extracción de la muestra directamente en la placa objetivo del espectrómetro
PDR	Panresistentes a fármacos
RAM	Resistencia antimicrobiana
ssp	Especies
v/v	Concentración de una solución volumen/volumen
XDR	Extremadamente resistentes a fármacos

1 RESUMEN

El objetivo de la presente investigación fue evaluar el efecto antibacteriano del aceite esencial de *Lippia graveolens* (AELg), contra cepas multirresistentes aisladas de vacas con mastitis subclínica en unidades de producción lechera del Valle del Mezquital del Estado Hidalgo. Se determinó prevalencia de mastitis subclínica por medio de la prueba de california (CMT), en 16 unidades de producción. El aislamiento bacteriano se realizó de muestras con mastitis subclínica en Agar MacConkey y Soya Tripticaseína. Las cepas aisladas se identificaron por espectrometría de masas (MALDI-TOF). Por medio de la técnica de Kirby-Bauer se determinó sensibilidad antimicrobiana y se calculó el Índice de Resistencia Múltiple a Antibacterianos (IRMA). La actividad antibacteriana del AELg se determinó por medio de la concentración mínima inhibitoria (CMI), y bactericida (CMB), y la relación entre estas. Se determinó la citotoxicidad por medio de la prueba de hemolisis indirecta y el daño a la membrana plasmática a través de la liberación de ADN y proteínas. La composición química del aceite se identificó por cromatografía de gases acoplada a masas. La prevalencia de mastitis subclínica fue de entre 24.9 % y 47.0 % por vaca, y entre 17.2 % y 37.4 % por cuarto mamario, asociada a malas prácticas de ordeño. Se identificaron 13 especies bacterianas pertenecientes a 7 géneros, siendo *Staphylococcus* (36.36 %) y *Enterococcus* (27.27 %) los más prevalentes. El 72 % de las cepas presentaron un IRMA igual o superior a 0.5. El AELg mostró actividad antibacteriana con un efecto bactericida en las cepas evaluadas. El mayor efecto fue en bacterias Gram positivas como *Staphylococcus aureus, Macrococcus caseolyticus* y *Enterococcus faecalis*, con CMI y CMB menores a 0.1 mg/mL. Se determinó un efecto hemolítico y daño a la membrada a concentración dependiente. Se identificó al quimiotipo timol (68.9 %) como el compuesto mayoritario en el AELg. El AELg representa una alternativa contra bacterias multirresistentes; sin embargo, se requieren estudios complementarios para validar su uso en el tratamiento clínico de mastitis bovina.

Palabras claves: Mastitis, resistencia antibacteriana, aceite esencial, timol, citotoxicidad.

2 ABSTRACT

The objective of this study was to evaluate the antibacterial effect of *Lippia graveolens* essential oil (AELg) against multigrug-resistant strains isolated from cows with subclinical mastitis in dairy production units in the Mezquital Valley of the State of Hidalgo. The prevalence of subclinical mastitis was determined using the California test (CMT) in 16 production units. Bacterial isolation was performed from samples with subclinical mastitis on MacConkey Agar and Trypticase Soybean. The isolated strains were identified by mass spectrometry (MALDI-TOF). Using the Kirby-Bauer technique, antimicrobial sensitivity was determined and the Multiple Resistance Index to Antibacterials (IRMA) was calculated. The antibacterial activity of AELg was determined by means of the minimum inhibitory concentration (MIC) and bactericidal concentration (MBC) and the relationship between them. Cytotoxicity was determined through the indirect hemolysis test and damage to the plasma membrane through the release of DNA and proteins. The chemical composition of the oil was identified by mass-coupled gas chromatography. The prevalence of subclinical mastitis was between 24.9 % and 47.0 % per cow, and between 17.2 % and 37.4 % per mammary quarter, associated with poor milking practices. 13 bacterial species belonging to 7 genera were identified, with Staphylococcus (36.36 %) and Enterococcus (27.27 %) being the most prevalent. 72 % of the strains had an IRMA equal to or greater than 0.5. AELg showed antibacterial activity with a bactericidal effect in the strains evaluated. The greatest effect was on Gram positive bacteria such as *Staphylococcus aureus*, *Macrococcus caseolyticus* and *Enterococcus faecalis*, with MIC and MBC less than 0.1 mg/mL. A hemolytic effect and concentration-dependent membrane damage were determined. The thymol chemotype (68.9 %) was identified as the major compound in AELg. The findings indicate that AELg represents a potential alternative against multidrug-resistant bacteria; however, further studies are required to validate its clinical application in the treatment of bovine mastitis.

Keywords: Mastitis, antibacterial resistance, essential oil, thymol, cytotoxicity.

3 INTRODUCCIÓN

La mastitis es la inflamación de la glándula mamaria, que afecta el bienestar y la salud de la vaca lechera y ocasiona la mayor pérdida económica en las unidades de producción lechera debido a que reduce la producción, la calidad sanitaria de la leche y requiere costo económico en protocolos de tratamiento y control (Ruegg, 2017). La inflamación está asociada principalmente a infecciones bacterianas (Sharun *et al.,* 2021), clasificadas para su estudio en contagiosas y ambientales, según su medio de trasmisión y por la tinción de Gram en positivas y negativas (Lakew *et al.,* 2019; Cheng *et al.,* 2020).

Las bacterias contagiosas como *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma bovis* y *Corynebacterium*, son parte de la microbiota en la ubre y en la piel del pezón de la vaca; sin embargo, pueden colonizar el canal del pezón, por lo que en procesos deficientes de rutina de ordeño se trasmiten de vaca a vaca dando origen a mastitis subclínicas, con alto recuento de células somáticas y reducción de producción de leche. Las bacterias ambientales se encuentran en corrales, echaderos, fómites y manos de ordeñadores, las más frecuentes son *Streptococcus uberis*, *Streptococcus dysgalactie*, *Eschericha coli, Klebsiella* spp*., Enterobacter* spp*.,* y *Enterococcus faecium* (Ameen *et al.,* 2019).

En los establos especializados en la producción láctea, la prevención y control de la mastitis se realiza con protocolos farmacológicos, diseñados a partir de la identificación de patógenos y estudio de antibiogramas; sin embargo, se reporta una alta incidencia de la enfermedad y un uso descontrolado de fármacos en los tratamientos (Widianingrum *et al.,* 2022; Li *et al.,* 2023). El uso inadecuado de antimicrobianos y el mecanismo de adaptación de las bacterias a las condiciones de la ubre y ambiente, son factores que inciden en el desarrollo de resistencia microbiana (Saini *et al.,* 2012; Sharun *et al.,* 2021). La resistencia antimicrobiana se asocia a la diversidad molecular de los microorganismos y su interacción con el ambiente (Kovačević *et al.,* 2022).

Actualmente los tratamientos contra bacterias multirresistentes a antimicrobianos generan una recuperación parcial, presencia de residuos en la leche y microorganismos resistentes, representando un riesgo para los consumidores. Por tanto, la mastitis bovina es considerada un problema de salud pública que requiere una redefinición científica en la búsqueda de nuevos tratamientos alternativos, para disminuir la contaminación de la leche tanto por patógenos, como por residuos de medicamentos utilizados para el control de esta (Amber *et al.,* 2018).

El desarrollo de resistencia antibacteriana puede reducirse con la aplicación de buenas prácticas de producción en los sistemas lecheros, la restricción de uso de antimicrobianos y el uso de extractos vegetales como alternativas de tratamiento (Kebede *et al.,* 2021). Se ha demostrado que los compuestos del metabolismo secundario de las plantas como flavonoides, alcaloides, saponinas y terpenoides presentan actividad antibacteriana (Harjanti *et al.,* 2020). Se ha reportado la excelente actividad antibacteriana que presentan los aceites esenciales contra bacterias asociadas a mastitis bovina (Harjanti *et al.,* 2020; Cholan *et al.,* 2021). De acuerdo con lo anterior el objetivo de la presente investigación fue determinar la actividad antibacteriana del aceite esencial de *Lippia graveolens*, planta de la flora local de la zona árida del Valle del Mezquital, contra bacterias aisladas de mastitis bovina de los establos de la región.

4 PLANTEAMIENTO DEL PROBLEMA

La producción de leche en el estado de Hidalgo se desarrolla en sistemas de producción heterogéneos complejos, dinámicos con influencia del entorno ecológico, socioeconómico y cultural en el que se originan y se desarrollan. En la región del Valle del Mezquital Hidalgo, la producción de leche a pequeña escala utiliza limitados recursos de tierra y animales para la generación de ingresos de la familia campesina del medio rural. Las problemáticas más importantes que enfrenta la producción lechera a pequeña escala son en el área de la nutrición, reproducción y en el cuidado de la salud animal.

En el área de la salud animal, la mastitis es la enfermedad con mayor prevalencia en las vacas de los hatos lecheros de la región. La mastitis afecta la economía de la unidad de producción, porque reduce la producción de leche, propicia el uso desmedido de antibióticos en el tratamiento de animales enfermos que terminan enviados a rastros. Las malas prácticas en el tratamiento, que realizan los productores a animales enfermos y la usencia de controles de calidad e inocuidad alimentaria en el mercado local de la leche, genera problemas de salud en la población y en los animales, por el desarrollo de resistencia de las bacterias a los antimicrobianos.

En la actualidad no existen reportes de investigaciones de las principales bacterias causales de mastitis en los hatos lecheros del Valle del Mezquital, se desconoce la existencia de resistencia o multirresistencia de las bacterias a los antimicrobianos; sin embargo, en otras regiones del mundo se reporta que las bacterias presentan recombinación genética y desarrollo de factores de resistencia a los fármacos. La presencia de bacterias resistentes en mastitis bovina ocasiona tasas de curación baja en animales enfermos, presencia de residuos de antibiótico y microorganismos resistentes en la leche para los consumidores. La multirresistencia a antimicrobianos de las bacterias asociadas a mastitis bovina es una problemática que demanda la búsqueda de nuevos tratamientos alternativos de control. En la actualidad en el mundo se desarrollan investigaciones de actividad antibacteriana de aceites esenciales de plantas frente a bacterias resistentes asociadas a mastitis bovina.

Por lo anterior dentro de la flora local del Valle del Mezquital, el orégano silvestre conocido también como orégano mexicano (*Lippia graveolens*), es una planta aromática con uso local en la alimentación humana como condimento y en el cuidado de la salud como infusión para el tratamiento de diarreas. La presencia y concentración de metabolitos secundarios en plantas aromáticas determina la actividad antibacteriana, por ello el presente trabajo tuvo como propósito evaluar el efecto antibacteriano del aceite esencial de *Lippia graveolens* sobre cepas bacterianas asociadas a mastitis subclínica en establos de la región del Valle del Mezquital en el Estado de Hidalgo México, como una alternativa de posible uso en el tratamiento clínico de mastitis bovina.

5 JUSTIFICACIÓN

La mastitis es una patología común con alta prevalencia en los hatos lecheros del Valle del Mezquital, origina pérdidas económicas asociadas al descarte de leche y animales, al costo de fármacos para su control y prevención. Es una patología de importancia económica, los ganaderos especializados en conjunto con las farmacéuticas han desarrollado diversos protocolos de prevención y control; sin embargo, en las pequeñas unidades de producción animal el uso de fármacos es inadecuado o nulo. En ambos escenarios el uso continuo de antimicrobianos induce la resistencia de bacterias comensales y patógenas, por lo que en la actualidad se trabaja en la búsqueda de nuevas alternativas terapéuticas para combatir a la mastitis.

El uso de aceites esenciales para el cuidado de la salud en los animales tiene como propósito mitigar los efectos secundarios de algunos antimicrobianos empleados en tratamientos farmacológicos convencionales. La etnoveterinaria aplica los conocimientos, creencias, prácticas y habilidades tradicionales de los pueblos para el cuidado y cría de animales, integrando los conocimientos de la Medicina veterinaria desde un enfoque holístico de la salud considerando la relación entre humanos, animales y ambiente. En la actualidad se investiga los potencializadores de la inmunidad de los animales principalmente los que son a base de extractos de plantas, como tomillo, ajo y orégano, así como de aceites esenciales para la prevención y control de la mastitis. En la búsqueda de alternativas terapéuticas a base de plantas propias de la diversidad florística local Valle del Mezquital Hidalgo, el orégano mexicano (*Lippia graveolens*), por su uso en Medicina tradicional podría ser una alternativa de estudio para su evaluación antibacteriana y su posible uso fitoterapéutico en el tratamiento de la mastitis bovina.

6 HIPÓTESIS

El aceite esencial de *Lippia graveolens* presenta un efecto antibacteriano contra bacterianas multirresistentes aisladas de vacas con mastitis en unidades de producción lechera del Valle del Mezquital, Hidalgo.

7 OBJETIVOS

7.1 Objetivo general

- Evaluar el efecto antibacteriano del aceite esencial *L. graveolens* contra bacterianas multirresistentes aisladas en unidades de producción lechera del Valle del Mezquital, Hidalgo, mediante técnicas bacteriológicas de rutina y espectrometría, para proponerlo como una alternativa fitoterapéutica.

7.2 Objetivos específicos

- Determinar la prevalencia de mastitis subclínica en unidades de producción de leche cooperantes en el Valle del Mezquital.
- Determinar e identificar el perfil de resistencia a antimicrobianos de las bacterias aisladas de vacas con mastitis subclínica.
- Determinar la actividad antibacteriana del aceite esencial de *L. graveolens* contra cepas multirresistentes aisladas de vacas con mastitis.
- Determinar la citotoxicidad indirecta y daño a membrana citoplasmática del aceite de *L. graveolens.*
- Determinar la composición fitoquímica del aceite esencial de *L. graveolens*.

8 CONCLUSIONES GENERALES

La prevalencia de mastitis bovina en las unidades de producción se asoció a la asepsia de las manos del ordeñador, secado de pezón y uso de sellado. Las bacterias con mayor presencia en la mastitis bovina de las unidades de producción pertenecen a los géneros: *Enterococcus, Staphylococcus*, *Escherichia*, y *Macrococcus*. Siendo *Enterococcus faecalis*, *Staphylococcus aur*eus, *Escherichia coli* y *Pseudomonas aeruginosa*, con diferentes grados de sensibilidad a los antimicrobianos evaluados.

El AELg presentó un efecto bactericida contra la mayoría de las bacterias multirresistentes asociadas a mastitis bovina. La actividad bactericida se asocia al quimiotipo timol, compuesto mayoritario del AELg. Se determinó actividad hemolítica y daño a la membrana citoplasmática dependiente a la concentración del AELg. El AELg podría representar una alternativa terapéutica contra la mastitis bovina, sin embargo, se sugiere realizar pruebas de citotoxicidad en líneas celulares que permita garantizar su uso en modelos *in vivo*.

9 Capítulo 1. *Lippia graveolens* y su actividad contra bacterias asociadas a mastitis bovina: Revisión bibliográfica

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Lippia graveolens and its activity against bacteria associated with bovine mastitis: Literature review

Lippia graveolens y su actividad contra bacterias asociadas a mastitis bovina: Revisión bibliográfica

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ABSTRACT

Bovine mastitis is an infectious disease of the mammary gland caused by the invasion of pathogens, among them the bacterial etiology is one of the most important and the treatment of these infections has currently been complicated by the resistance generated by Gram positive bacteria and Gram negative to conventional antimicrobials. The objective of this research was to carry out a bibliographic review of *Lippia graveolens* and its activity against bacteria associated with bovine mastitis. Scientific reports on the phytochemical composition of wild oregano (*L. graveolens*) and the antibacterial activity against bacteria associated with bovine mastitis were consulted. The metabolites identified in *L. graveolens* with the highest reported antibacterial activity were naringenin, quercetin, luteolin as well as the terpenes thymol and carvacrol. *L. graveolens* contains secondary metabolites with reports of antibacterial activity, so it could be an alternative treatment against bacteria associated with bovine mastitis.

Keywords: *Lippia graveolens*, secondary metabolites, antibacterial activity, mastitis.

RESUMEN

La mastitis bovina es una enfermedad infectocontagiosa de la glándula mamaria causada por la invasión de patógenos. La etiología bacteriana de esta enfermedad es una de las más importantes y el tratamiento de estas infecciones actualmente es más complejo por la resistencia que han generado las bacterias a los antimicrobianos convencionales. El objetivo de la presente investigación fue realizar una revisión bibliográfica de *Lippia graveolens* y su actividad contra bacterias asociadas a mastitis bovina. Se consultaron los reportes científicos de composición fitoquímica del orégano silvestre (*L. graveolens)* y la actividad antibacteriana contra bacterias asociadas a mastitis bovina. Los metabolitos identificados en *L. graveolens* con mayor reporte de actividad antibacteriana fueron naringenina, quercetina, luteolina así como los terpenos timol y carvacrol. *L. graveolens* contiene metabolitos secundarios con reportes de

actividad antibacteriana por lo que podría ser una alternativa de tratamiento contra bacterias asociadas a mastitis bovina.
Palabras clave: *Lippia graveolens*, metabolitos secundarios, actividad antibacteriana, mastitis.

INTRODUCTION

Bovine mastitis is inflammation of the mammary gland caused by the invasion of pathogenic microorganisms that destroy milk-secreting tissues. More than 100 species associated with bovine mastitis have been reported in bacterial etiology (Sharun *et al.*, 2021; Morales *et al.*, 2023). The most common bacteria in cases of mastitis are: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and coagulase-negative *Staphylococcus* (Pascu *et al.*, 2022; Morales *et al.*, 2023).

Mastitis is treated with chemical antimicrobials; however, their continuous and excessive use has led to the development of antimicrobial resistance, creating a global problem in animal and human health due to the interaction of bacteria from these two populations and the transfer of intergenic resistance (Galarza *et al.*, 2021; Wang *et al.*, 2021; Li *et al.*, 2023). Bacterial resistance leads to increased treatment costs and premature culling of animals, which has prompted the scientific community to search for new alternatives for the treatment of bovine mastitis (Kovačević *et al.*, 2022; Morales *et al.*, 2023).

Within the flora diversity, Mexican oregano (*Lippia graveolens*) is a plant of interest due to its phytochemical composition. This wild shrub has been used for culinary purposes and in traditional medicine to treat respiratory and digestive diseases, inflammation, headaches, and rheumatism (Bautista *et al.*, 2021). There are reports that demonstrate the antibacterial activity of *L. graveolens* using different methods of secondary metabolite extraction and concentrations at which antibacterial activity has been determined against various bacterial genera, including those associated with bovine mastitis (Bautista *et al.*, 2021; Cortés *et al.*, 2021; Kovačević *et al.*, 2022; Garcia *et al.*, 2022). The objective of this research was to conduct a literature review of *L. graveolens* and its activity against bacteria associated with bovine mastitis.

METHODOLOGY

To conduct this review, an exhaustive search was carried out in the following databases: PubMed, ScienceDirect, and Google Scholar, for studies published up to 2024. The following headings and keywords were used: *L. graveolens*, plant extracts, bovine mastitis, and antibacterial activity. Full-text documents were reviewed, and duplicate documents were removed. The exclusion criteria were inadequate methods and lack of access to the full text.

PLANT BOTANY

L. graveolens is a perennial shrub that grows to a height of two meters. It has exfoliating bark branches with opposite petiolate leaves that are oval-lanceolate in shape, with a rough, scabrous upper surface, glandular striae, densely hairy underside, obtuse apex, and variously crenate margin (Ocampo *et al.*, 2009). Flowering occurs during the rainy season (Bueno, 2014). Its inflorescence is indeterminate, subglobose spike-like, with white, zygomorphic corollas and small, 4 mm hermaphroditic flowers, in quantities of 2 to 20 flowers. It has small indehiscent capsule fruits with seeds without endosperm (Figure 1) (Ocampo *et al.*, 2009).

Figure 1. *Lippia graveolens* in Orizabita, Ixmiquilpan, Hidalgo, Mexico

The plant is wild and is found in the hills of temperate, arid, and semi-arid areas of Mexico. It adapts to altitudes between 1 400 and 2 300 meters, in stony soils with a sandy loam texture. It prefers a dry and semi-dry climate, with temperatures ranging from 20 to 24 °C and precipitation between 182 and 267 mm (Figure 1) (Martínez *et al.*, 2014). It is an aromatic plant used in cooking and herbal medicine for the treatment of digestive disorders. In Chihuahua, Durango, Tamaulipas, Coahuila, Jalisco, Zacatecas, Querétaro, Hidalgo, and Baja California, its foliage is collected for sale in local markets (Bueno, 2014).

COMPOSICIÓN FITOQUÍMICA PHYTOCHEMICAL COMPOSITION

The phytochemical characterization of *L. graveolens* from aqueous extracts, hydroalcoholic extracts, and essential oils shows differences in its phytochemical composition (González *et al.*, 2017; Bernal *et al.*, 2022). Extraction has been carried out using conventional techniques such as maceration with alcohols and water in different proportions, and by current technologies such as ultrasound-assisted extraction using eutectic solvents and supercritical fluid extraction using carbon dioxide as a solvent (Bernal *et al.*, 2023). The presence of metabolites differs depending on the extraction methods, as shown in Table 1, with phenolic compounds and monoterpenes standing out (González *et al.*, 2017).

Table 1. Phytochemical compounds identified in plant extracts of *Lippia graveolens*

Extract	Extraction method	Solvent	Compounds	Reference
Aqueous	Ultrasound and supercritical fluid using CO_2	Choline chloride-ethylene glycol, choline chloride-glycerol, and choline chloride-lactic acid.	Caffeic, protocatechuic, and rosmarinic acids. Quercetin, luteolin, naringenin, eriodictyol, carvacrol	García *et al.* (2022) González *et al.* (2017) Soto *et al.* (2019) Bernal *et al.*(2023)
Hexane	Maceration	Hexane	Thymol, m-cymene-8-ol, methyl salicylate, carvacrol, and linalool.	González *et al.*(2017)
Ethyl acetate	Maceration	Ethyl acetate	p-cymene, thymol, cirsimaritin, naringenin	
Ethanolic	Maceration	Ethanol Supercritical CO_2 modified with ethanol after steam distillation	Naringenin, taxifolin, eriodictyol, caffeic acid, luteolin, coumaric acid, quercetin 3 O-glucoside, 2-hydroxybenzoic acid, apigenin, quercetin, phloridzin, acacetin, sakuranetin, cirsimaritin, chrysoeriol.	Arias *et al.* (2023) Bernal *et al.* (2023) González *et al.* (2017)
Hydroalcoholic	Maceration	Methanol and Chloroform	Loganin, secologanin, secoxiloganin, dimethylsecologanoside, loganic acid, 8-epi-loganic acid, carioptosidic acid and its 6-O-p-coumaroyl and 6-O-caffeoyl derivatives,	Cortes *et al.* (2021) Picos *et al.* (2021) Leyva *et al.* (2016) Rastrelli *et al.* (1998) Lin *et al.* (2007)

		naringenin, pinocembrin, lapachenol and icterogenin, luteolin-7-O-glucoside, apigenin 7-O-glucoside, phloridzin, taxifolin, eriodictyol, scutellarein, luteolin, quercetin, and galangin.	
Essential oil	Hydro-distillation	Carvacrol, α-terpinyl acetate, m-cymene, thymol, β-pinene, and α-thujene, linalool, humulene Sesquiterpene: isocaryophyllene, γ-terpinene.	Hernández *et al.*(2009) Martínez *et al.* (2014) Nonato *et al.* (2022) Castillo *et al.* (2023)

In *L. graveolens*, the most abundant flavonoids are: quercetin, luteolin, naringenin, eriodictyol, luteolin, hesperidin, and phloridzin (Bernal *et al.*, 2023). The metabolites naringenin, quercetin, phloridzin, and cirsimaritin are chemical markers of the Lippia genus (Bernal *et al.*, 2022). The best flavonoid profile is obtained from methanolic leaf extract, in which three major iridoids have been found: carioptosidic acid with two derivatives: 6'-O-p-coumaroyl and 6'-O-caffeoyl, and seven minor iridoids: loganin, secologanin, secoxiloganin, dimethyl, secologanoside, loganic acid, 8-epi-loganic acid, and carioptoside (Rastrelli *et al.*, 1998; Lin *et al.*, 2007). Monoterpenes are the main components of essential oils of the *Lippia* genus (Cortés *et al.*, 2021; Bernal *et al.*, 2023). In water-, hexane-, and methyl acetate-based extracts and in hydrodistillation processes for obtaining essential oil, the presence of the following monoterpenes has been reported: thymol, carvacrol, limonene, b-caryophyllene, r-cymene, camphor, linalool, and a-pinene, which may vary according to the chemotype and extraction method (Calvo *et al.*, 2014; Garcia *et al.*, 2022). In this regard, Vemin (2001) reported that the essential oil of *L. graveolens* from Mexico and Central America has concentrations of 35 to 71% carvacrol and 5 to 7% thymol. Calvo *et al.* (2014) reported the presence of more than 70 compounds in its essential oils and identified three chemotypes of the plant: two phenolic (carvacrol and thymol) and one non-phenolic chemotype of oxygenated sesquiterpenes (β-caryophyllene, α-humulene, and caryophyllene oxide). Plant's habitat determines the oil composition, with the highest concentration of carvacrol obtained from plants growing in a semi-arid climate with thin, rocky soils (Torres *et al.*, 2022). However, there are reports of young plants growing in less arid conditions with deep soils, which provide a higher presence of thymol, and those growing in sub-humid climates have a higher amount of oxygenated sesquiterpenes (Llamas *et al.*, 2022).

METABOLITES AND ANTIBACTERIAL ACTIVITY

Terpenes

Carvacrol and thymol are most prevalent in *L. graveolens* (Calamaco *et al.*, 2023), and their concentration is affected by edaphic and climatic factors in the plant's habitat (Cortés *et al.*, 2021). Carvacrol (2-methyl-5-1-methylethylphenol) provides the characteristic aroma of oregano (Ultee *et al.*, 2000). It is synthesized from cymene via the mevalonate pathway and is a monoterpene that is insoluble in water and soluble in ethanol, carbon tetrachloride, and diethyl ether (Lee *et al.*, 2017). Its stereochemistry (Figure 2) of a single phenolic ring with three substituents of functional groups (Memar *et al.*, 2017) gives it antibacterial, antioxidant, anticancer, and anti-inflammatory properties (Tapia *et al.*, 2017).

Figure 2. Terpenes reported in *Lippia graveolens*

In bacteria, carvacrol induces cell lysis by altering lipophilic compounds and hydrophobic parts of the cytoplasmic membrane, increasing cation permeability (H+ and K+), generating lipopolysaccharide efflux and reactive oxygen species production, inhibiting ATPase activity, microbial DNA replication, and energy synthesis, causing cell death (Gallegos *et al*, 2022). However, Ultee *et al.* (2000) reported that bacteria can adapt to carvacrol and modify the fatty acid composition of the membrane and reduce its permeability.

Thymol is an isomer of carvacrol (Figure 2). It is an aromatic substance with a white crystalline color, low solubility in water, and high solubility in organic solvents. It has a neutral pH but can have alkaline characteristics in aqueous solutions due to the deprotonation of phenol (Chizzola, 2013). It has bactericidal, fungicidal, insecticidal, nematicidal, and varroacidal activity (Gallegos *et al.*, 2022). Its antibacterial effect *in vitro* against *Escherichia coli*, *Salmonella spp.* and *S. aureus* has been reported at a concentration of 0.75 mg/mL (Shapira-Mimran 2007; Gallegos *et al.*, 2019). At concentrations of 1 and 2 % in oregano essential oil, it has greater antimicrobial activity against Gram-positive bacteria and less against Gram-negative bacteria (Du *et al.*, 2015;

Erazo *et al.*, 2017). *In vitro* studies on Gram-negative enterobacteria found greater antibacterial activity of thymol from Lippia berlandieri compared to other antimicrobials (Garcia *et al.*, 2022; Gracia *et al.*, 2022). The antibacterial mechanism of action is similar to that of carvacrol, binding to bacterial membranes in a hydrophobic manner via hydrogen bonds, affecting the outer and inner membranes, increasing permeability and the loss of potassium ions and intracellular ATP, causing cell death (Di Pasqua *et al.*, 2010).

Flavonoids

In *L. graveolens*, the flavonoids (Figure 3) with the highest reported biological activity are naringenin, quercetin, and luteolin (Lin *et al.*, 2007). Naringenin is a bioactive compound with hepatoprotective, antiatherogenic, anti-inflammatory, antimutagenic, anticarcinogenic, and antimicrobial activity (Ke *et al.*, 2017). In reaction with alkyl iodides, it forms O-alkyl compounds of naringenin with antibacterial potential against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* (Kozłowska *et al.*, 2019). A derivative of O-alkyl naringenin is sakuranetin (7-O-methylnaringenin), which has significant antimicrobial activity against Gram-negative and Gram-positive bacteria. Naringenin inhibits the growth of *Staphylococcus aureus* by affecting the cell membrane and fatty acid composition. In *Escherichia coli*, it acts on the genes associated with the biosynthesis of membrane fatty acids (Wang *et al.*, 2018).

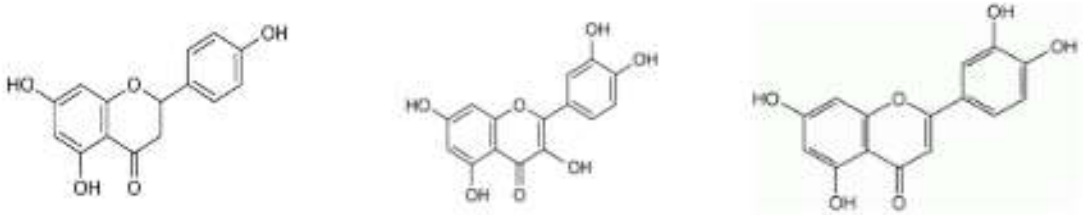

Figure 3. Flavonoids reported in *Lippia graveolens*

Quercetin is a flavonol based on the flavone structure nC6 (ring A)-C3 (ring C)-C6 (ring B). It has broad-spectrum antibacterial activity, breaks down the cell wall of bacteria, inhibits nucleic acid synthesis, and reduces enzyme activity (Wang *et al.*,2018). Specifically, in *Escherichia coli*, it alters the activity of adenosine triphosphate (Plaper *et al.*, 2003). According to Hooda *et al.* (2020), impregnating quercetin with silver nanoparticles inhibits the growth of bacteria: *Klebsiella pneumoniae* (ATCC^{700603}), *Enterococcus faecalis* (ATCC^{51299}), *Proteus vulgaris* (ATCC^{4226}), *Escherichia coli*

(ATCC^{25922}), *Staphylococcus aureus* (ATCC^{4330}), and *Pseudomonas aeruginosa* (ATCC^{27853}).

Luteolin (3',4',5,7-tetrahydroxyflavone) is a polyphenol of the flavone family, with a molecular structure (C6-C3-C6) of two benzene rings and a third ring containing oxygen, and a double bond between carbons C2 and C3 (Figure 3). Its structure favors its biochemical and biological activity (Wu *et al.*, 2019). This flavonoid has various biological activities, such as antioxidant, anti-inflammatory, antimicrobial, anticarcinogenic, and hypoglycemic, hypolipidemic, hypotensive, and immunomodulatory effects (Wu *et al.*, 2019). In bacteria, luteolin affects the integrity of the cell wall and membrane, inhibiting nucleic acid synthesis and protein expression and interfering with energy metabolism (Guo *et al.*, 2022). In a study, Qian *et al.* (2021) found that luteolin impairs cell membrane morphology and affects biofilm formation in *Staphylococcus aureus* and *Escherichia coli*. In studies against *Trueperella pyogenes*, a minimum inhibitory concentration (MIC) of luteolin of 78 µg/mL was reported, and at half the MIC, susceptibility to methicillin- and macrolide-resistant *Staphylococcus increases*, providing an alternative treatment for resistant pathogens (Guo *et al.*, 2022).

ACTIVITY OF *Lippia spp.* AGAINST RESISTANT BACTERIA ASSOCIATED WITH BOVINE MASTITIS

There are reports of the antibacterial activity of *Lippia* spp. extracts on resistant bacteria such as *Streptococcus* spp., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli* (Gupta *et al.*, 2020; Pinheiro *et al.*, 2022; Nonato *et al.*, 2022). The minimum inhibitory concentration in the evaluation of Lippia spp. extracts and metabolites on bacteria varies depending on the species evaluated, extraction methodology, and evaluation (Rani *et al.*, 2022; Nonato *et al.*, 2022; Suarez *et al.*, 2024).

In *Streptococcus* spp., an MIC of 67 mg/mL is reported for supercritical fluid from *L. graveolens*, while for metabolites: thymol, MICs of 0.31 to 8.0 mg/mL are reported, and for carvacrol, 0.31 to 9 mg/mL (Table 2). In *Staphylococcus* spp., the MIC for ethanolic extracts of *Lippia* spp. is reported to be 128 to 512 µg/mL, for essential oil 53.3 to 512 µg/mL, while for thymol it was 0.15 to 0. 75 µg/mL and carvacrol from 0.38 to 1.3 µg/mL (Dal Pozzo *et al.*, 2011; Rani *et al.*, 2022; Gallegos *et al.*, 2019; Nonato *et al.*, 2022).

In *Pseudomonas aeruginosa*, an MIC for ethanolic extracts of 128 to 512 µg/mL is reported, while for essential oils it is 0.37 to 80 µg/mL. In the case of *Escherichia coli*, the following MIC reports were found: ethanolic extracts (74.6 to 256 µg/mL), essential oils (0.37 to 426 µg/mL), thymol (0.15 to 0.38 µg/mL), and carvacrol (0.15 to 0.75 µg/mL). Essential oils and their compounds (carvacrol and thymol) have the highest antibacterial

activity in resistant bacteria isolated from mastitis (Rani *et al.*, 2022; Nonato *et al.*, 2022; Suarez *et al.*, 2024). Pinheiro *et al.* (2022) mention that the essential oil has inhibitory and bactericidal action against strains of *Escherichia coli* and *Klebsiella pneumoniae*, but not against *Pseudomonas aeruginosa*, where its effectiveness is reduced by the formation of biofilm and the action of efflux pumps, intrinsic characteristics of this species. The combination of ethanolic extracts of *Lippia* spp. with antimicrobials reduces the MICs of amikacin, gentamicin, and cephalothin, but has an antagonistic effect with benzylpenicillin and other natural extracts (Nonato *et al.*, 2022). Currently, synergies between extracts and antimicrobials are being investigated to improve efficacy, reduce toxicity, and bacterial resistance (Garcia *et al.*, 2019; Pinheiro *et al.*, 2022; Nonato *et al.*, 2023; Suarez *et al.*, 2024).

Table 2. Antibacterial activity of *Lippia spp* and its metabolites against resistant pathogens associated with bovine mastitis

Bacteria	Extracts and compounds	Minimum Inhibitory Concentration (MIC)	Reference
Streptococcus spp.	Thymol	0.31-0.63 µL/mL	Gupta *et al.* (2020)
	Carvacrol	0.16-0.63 µL/mL	
Streptococcus agalactiae	Supercritical fluid from L. graveolens	67 mg/mL	Garcia *et al.* (2019)
	Thymol	8.0 mg/mL	Gupta *et al.* (2020)
	Carvacrol	9.0 mg/mL	
Staphylococcus aureus	Ethanolic extract of L. alba	853 µg/mL	Nonato *et al.* (2022)
	Ethanolic extract of *L. sidoides*	128 µg/mL	
	Ethanolic extract of *L. gracilis*	512 µg/mL	
	Essential oil of *L. alba*	256 µg/mL	
	Essential oil of *L. sidoides*	53.3 µg/mL	
	Essential oil of *L. gracilis*	512 µg/mL	
	Essential oil of *L. graveolens*	12 µL/mL	Suarez *et al.* (2024)
	Thymol	0.15-0.75 mg/mL	Gallegos *et al.* (2019)
	Carvacrol	0.38-0.45 mg /mL	Rani *et al.* (2022)
Staphylococcus spp.	*L. graveolens* essential oil	1.6 mg /mL	Dal Pozzo *et al.* (2011)
	Thymol	0.4 a 0.5 mg/mL	
	Carvacrol	0.8 a 1.3 mg/mL	
Pseudomonas aeruginosa	Ethanolic extract of *L. alba*	213.3 µg/mL	Nonato *et al.* (2022)
	Ethanolic extract of *L. sidoides*	128 µg/mL	
	Ethanolic extract of *L. gracilis*	512 µg/mL	
	Essential oil of *L. alba*	1024 µg/mL	
	Essential oil of *L. sidoides*	298.6 µg/mL	
	Essential oil of *L. gracilis*	682 µg/mL	

	Essential oil of *L. berlandieri*	80 µg/mL	Reyes *et al.* (2020)
	Essential oil of *L. origanoides*	2500 µg/mL	Pinheiro *et al.* (2022)
	Mixture of essential oils of *L. salvifolia*: *L. sidoides* (9:1)	0.37 µg/mL	Gupta *et al.* (2020)
Escherichia coli	Ethanolic extract of *L. origanoides*	5.0 µL	Castellanos *et al.* (2020)
	Ethanolic extract of *L. alba*	768 µg/mL	Nonato *et al.* (2022)
	Ethanolic extract of *L. sidoides*	74.6 µg/mL	
	Ethanolic extract of *L. gracilis*	256 µg/mL	
	Essential oil of *L. alba*	106.6 µg/mL	
	Essential oil of *L. sidoides*	106.6 µg/mL	
	Essential oil of *L. gracilis*	426.6 µg/mL	
	L. origanoides essential oil	312 µg/mL	Pinheiro *et al.* (2022)
	L. berlandieri essential o	4 µg/mL	Bautista *et al.* (2021)
	Mixture of *L. salvifolia* essential oils: *L. sidoides* (9:1)	0.37 µg/mL	Gupta *et al.* (2020)
	Thymol	0.15-0.38 mg/mL	Gallegos *et al.* (2019)
	Carvacrol	0.15-0.75 mg/mL	Rani *et al.* (2022)
Klebsiella pneumoniae	*L. origanoides* essential oil	312 µg/mL	Pinheiro *et al.* (2022)
	Thymol	0.75 mg/mL	Rani *et al.* (2022)
	Carvacrol	0.75 mg/mL	

CONCLUSIONS

The main secondary metabolites of *L. graveolens* are flavonoids and monoterpenes, and their concentration varies according to the soil and climate conditions of the plant's habitat. Its antibacterial activity has been demonstrated against various bacterial genera of importance in health, including those associated with bovine mastitis. The greatest antibacterial activity of *L. graveolens* has been associated with thymol and carvacrol; however, activity has also been reported due to the presence of naringenin, quercetin, and luteolin. In the search for alternatives to combat resistant or multidrug-resistant bacteria associated with bovine mastitis, the secondary metabolites of *Lippia graveolens* represent an option for studying new treatments.

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Errata Erratum

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10 Capítulo 2. Prevalencia de mastitis bovina y aislamiento de bacterias multirresistentes en granjas lecheras de Hidalgo, México

Veterinary and Animal Science

Prevalence of Bovine Mastitis and Isolation of Multidrug-Resistant Bacteria in Dairy Farms in Hidalgo, Mexico

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Abstract:	Bovine mastitis, mainly caused by bacteria, lowers milk quality and yield, impacting farmers economically. Rising antibacterial resistance complicates treatment and poses a public health risk. The aim of the present study was to determine the prevalence of subclinical bovine mastitis and to isolate multidrug-resistant bacteria associated with this inflammatory disease, in dairy production units from the Mezquital Valley region of Hidalgo, Mexico. Prevalence was determined using the California Mastitis Test in 16 dairy farms. To isolate multidrug-resistant bacteria, samples were collected from mammary quarters with grade 3 subclinical mastitis and inoculated on Tryptic Soy Agar (TSA) and MacConkey agar supplemented with amoxicillin and cefotaxime, supplemented with amoxicillin and cefotaxime. The isolated strains were identified by mass spectrometry (MALDI-TOF), and antibacterial susceptibility was determined using the Kirby-Bauer method with 12 antibacterial ingredients. Based on the generated data, the Multiple Antibiotic Resistance Index (MARI) was calculated. Subclinical mastitis prevalence ranged from 24.9% and 47.0% per cow, and from 17.2% to 37.4% per mammary quarter in study area. Thirteen bacterial species belonging to seven genera were identified, with Staphylococcus (36.36%) and Enterococcus (27.27%) being the most prevalent. One Enterococcus faecium strain, exhibited resistance to 100% of antibacterials evaluated (MARI=1). 72% of strains presented MAR index equal to or greater than 0.5, indicating that isolated strains had been exposed to multiple antibacterials. The strains isolated in the present study represent a risk to both animal health and public health due to their antibacterial resistance and their potential to transmit resistance genes.
Opposed Reviewers:	

Prevalence of Bovine Mastitis and Isolation of Multidrug-Resistant Bacteria in Dairy Farms in Hidalgo, Mexico

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Abstract

Bovine mastitis, mainly caused by bacteria, lowers milk quality and yield, impacting farmers economically. Rising antibacterial resistance complicates treatment and poses a public health risk. The aim of the present study was to determine the prevalence of subclinical bovine mastitis and to isolate multidrug-resistant bacteria associated with this inflammatory disease, in dairy production units from the Mezquital Valley region of Hidalgo, Mexico. Prevalence was determined using the California Mastitis Test in 16 dairy farms. To isolate multidrug-resistant bacteria, samples were collected from mammary quarters with grade 3 subclinical mastitis and inoculated on Tryptic Soy Agar (TSA) and MacConkey agar. The isolated strains were identified by mass spectrometry (MALDI-TOF), and antibacterial susceptibility was determined using the Kirby-Bauer method with 12 antibacterial ingredients. Based on the generated data, the Multiple Antibiotic Resistance Index (MARI) was calculated. Subclinical mastitis prevalence ranged from 24.9% and 47.0% per cow, and from 17.2% to 37.4% per mammary quarter in study area. Thirteen bacterial species belonging to seven genera were identified, with *Staphylococcus* (36.36%) and *Enterococcus* (27.27%) being the most prevalent. One *Enterococcus faecium* strain, exhibited resistance to 100% of antibacterials evaluated (MARI=1). 72% of strains presented MAR index equal to or greater than 0.5, indicating that isolated strains had been exposed to multiple antibacterials. The strains isolated in the present study represent a risk to both animal health and public health due to their antibacterial resistance and their potential to transmit resistance genes.

Keywords: antimicrobial resistance, bovine mastitis, dairy farms, antibiotics

1. Introduction

Bovine mastitis is one of the main diseases affecting productive efficiency in dairy herds, as it induces alterations in the physicochemical composition and decreases milk yield, directly compromising product quality and causing significant economic losses for producers (Ruegg, 2017). This pathology, of a multifactorial etiology, is characterized by an inflammatory response of the mammary gland, generally caused by infectious agents. Among these, bacteria represent the most relevant group, with more than 135 species identified as potential causative agents of the disease (Lakew et al., 2019; Cheng et al., 2020).

Contagious pathogens colonize the mammary gland and are disseminated during the milking process. The most common agents include *Streptococcus agalactiae*, *Staphylococcus aureus* y *Mycoplasma bovis*. In contrast, environmental pathogens are found in soils, water, bedding, and feces, and infect the mammary gland through the teat canal. The most common environmental bacteria include *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp. y *Enterococcus faecium* (Cheng et al., 2020).

The prevalence, distribution and predominance of bacterial agents associated with bovine mastitis are influenced by multiple factors, including seasonality, cattle breed, management practices, and agroecological conditions of each region within specialized production systems. Mastitis control programs are based on the microbiological identification of etiological agents and the assessment of antibacterial susceptibility to select effective treatments (Ruegg et al., 2017; Badawy et al., 2023; Chen et al., 2023).

On the other hand, in small-scale livestock production systems (subsistence/farmer-based), medical treatments are often empirical, characterized by the use of antimicrobials without prior clinical diagnosis, this practice has favored the emergence of multidrug-resistant (MDR) strains, representing an increasing threat to animal and public health herd welfare and food safety (Saini et al., 2012; Li et al., 2023).

Antimicrobial resistance profiles have been associated with the genetic diversity of bacterial species and their exposure to antimicrobial agents (Silva et al., 2023; Sharifi et al., 2023). Multidrug-resistant strains harbor resistance genes that can be transmitted through different

mechanisms, facilitating their dissemination among genera and bacterial species present in both animals and humans (Schabauer et al., 2018; Różańska et al., 2019).

Bacterial resistance constitutes a major threat to the treatment of bovine mastitis, reducing therapeutic efficacy and generating significant implications for public health. The use of antimicrobials against resistant strains often results in ineffective treatments, the presence of drug residues in milk and the selection of multidrug-resistant bacteria (Morales-Ubaldo et al., 2023).

In the Mezquital Valley region, located in the state of Hidalgo, Mexico, small-scale dairy production units predominate and often lack information regarding the main etiological agents of bovine mastitis and their resistance profiles. The aim of the present study was to determine the prevalence of subclinical bovine mastitis and to isolate multidrug-resistant bacteria associated with this inflammatory disease, in dairy production units from the Mezquital Valley region of Hidalgo, Mexico

Materials and methods

2.1. Study area

The study was performed in 16 small-scale dairy production units, located in the Mezquital Valley region, in Hidalgo state, Mexico. Units were distributed in three municipalities: Francisco I. Madero (9 units), San Salvador (3 units) e Ixmiquilpan (4 units).

2.2. Mastitis prevalence and associated factors

Subclinical mastitis was diagnosticated through Mastitis California Test (CMT). Asepsis was performed on the mammary quarters, and the foremilk was discarded, subsequently, an approximate 2 mL sample was collected from each quarter and mixed with 2 mL of the Mastitest® indicator reagent (Mexico) (Adkins and Middleton, 2018). The reaction was interpreted using a gradual ordinal scale: 0, trace, 1, 2, and 3, with values indicating the presence of somatic cells (Fazal et al., 2023).

Prevalence was determined by the cow and for mammary quarters. Associated factors were determined through a verification guide which evaluated the accomplishment of the

following aspects: presence of a milking room, milker training, milker hand hygiene, teat cleaning, use of a pre-sealer, teat trimming, teat drying, and application of a sealer (Mendoza et al., 2017; Medrano et al., 2021).

2.3. Bacterial isolation

Milk samples were collected from the mammary quarters affected by grade 3 subclinical mastitis using 15 mL Falcon sterile tubes. The samples were transported at 4 °C to the Bacteriology Laboratory of the Institute of Agricultural Sciences at the Autonomous University of the State of Hidalgo (Ndahetuye et al., 2020; Fazal et al., 2023).

Samples were inoculated on tryptic soy agar (TSA) (BD Bioxon, Heidelberg, Germany) and MacConkey agar (MCK) (BD Bioxon, Heidelberg, Germany), following the methodology described by Zelaya et al., 2024. Colonies that developed on the media were purified according to their Gram staining: Gram-positive strains were purified on TSA, and Gram-negative strains on MCK. The isolated colonies were cryopreserved and stored at −80 °C until characterization.

2.4. Bacterial identification

The trains were partially classified through Gram staining and basic biochemical tests (Catalase and KOH) (Wanger et al., 2017). Bacterial identification at both the genus and species levels was carried out using matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF), with the Autof MS 1000 analyzer (Wang et al., 2021; Khasapane et al., 2024).

2.5. Antibacterial sensitivity test

Antibacterial sensitivity was determined using the disk diffusion method (Kirby-Bauer) on Mueller-Hinton agar (BD Bioxon, Heidelberg, Germany), in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) for veterinary pathogens (CLSI, 2018). Twelve antibiotics were tested for Gram-positive bacteria: ampicillin (10 µg), cephalothin (30 µg), cefotaxime (30 µg), dicloxacillin (1 µg), penicillin (10 U), clindamycin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), sulfamethoxazole/trimethoprim (25 µg), erythromycin (15 µg), vancomycin (30 µg), and tetracycline (30 µg). For Gram-negative bacteria, the evaluated antimicrobials were amikacin (30 µg), ampicillin (10 µg),

147 carbenicillin (100 µg), cephalothin (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg),
148 chloramphenicol (30 µg), gentamicin (10 µg), netilmicin (30 µg), nitrofurantoin (300 µg),
149 norfloxacin (10 µg), and sulfamethoxazole/trimethoprim (25 µg).

150 After incubation, the diameter of the inhibition zone around each disk was measured, and
151 bacterial strains were classified as susceptible (S), intermediate (I), or resistant (R) according
152 to CLSI specifications. Bacterial strains exhibiting resistance to three or more antibiotics
153 from different families were classified as multidrug-resistant (MDR) strains (Magiorakos et
154 al., 2012; Fazal et al., 2023). The Multiple Antibiotic Resistance Index (MARI) was
155 calculated as a/b, where "a" represents the number of antibiotics to which a strain was
156 resistant, and "b" represents the total number of antibiotics to which it was exposed (Vanegas
157 et al., 2009).

158 **2.6. Statistical analysis**

159 An analysis of variance (ANOVA) was conducted to evaluate the prevalence of mastitis
160 according to municipality, with mean comparisons performed using Tukey's test (SAS v.9).
161 Factors associated with mastitis were identified through Pearson correlations (R),
162 contingency tables, Chi-square (χ^2) tests, and multiple correspondence multivariate analysis,
163 using XLSTAT software (Lumivero, 2023). Heat maps were constructed to graphically
164 represent multidrug resistance in bacterial strains.

165

166 **3. Results**

167 **3.1. Mastitis prevalence**

168 A total of 239 cows and 959 mammary quarters were sampled. Table 1 shows the results of
169 the CMT for farms by municipality. Statistically significant differences in herd size were
170 determined among production units by municipality ($p < 0.01$), with San Salvador having
171 herds of 31.36, Ixmiquilpan 12.25, and Francisco I. Madero 10.56 cows, respectively. The
172 prevalence of subclinical mastitis in cows and mammary quarters was determined as follows:
173 San Salvador (47.07% and 37.47%), Ixmiquilpan (42.21% and 27.97%), and Francisco I.
174 Madero (24.99% and 17.28%).

Concerning evaluated mammary quarters using the CMT, no significant differences were observed in the reaction grades (T, G1, G2, and G3) among municipalities. The highest occurrence of grade 3 was observed in the municipality of San Salvador ($p > 0.01$). The prevalence of clinical mastitis was below one percent, with no significant differences between municipalities ($p > 0.01$).

3.2. Factors associated with mastitis

The multiple correspondence analysis identified two factors explaining 63.19% of the data variability (Figure 1). The aspects associated with Factor 1 (F1), which accounted for 39.35% of the variance, included the presence of a milking room (MR), milking area conditions (MAC), milker training (MT), teat drying (TD), cow hygiene (CH), teat cleaning (TC), forestripping practice (FP), and milker hand disinfection (MHD).

Along Factor 2 (F2), which explained 23.84% of the variability, the variables most strongly associated with the prevalence of cows positive for mastitis (CPM) in the production units were the use of a pre-dipping solution (PDS) and a post-dipping disinfectant (SEAL).

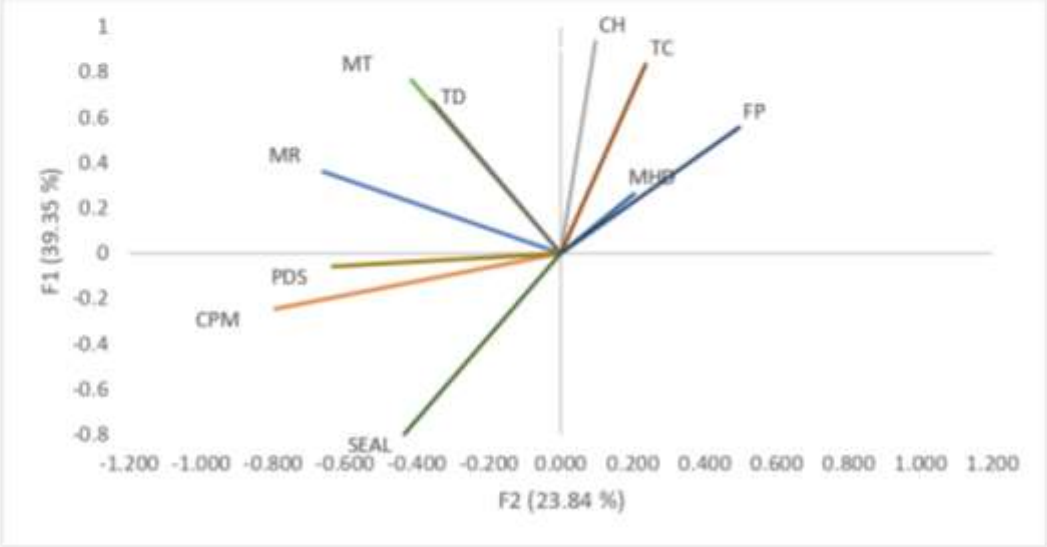

Figure 1. Factors associated with the prevalence of subclinical mastitis in dairy production units in the Mezquital Valley, Hidalgo, Mexico.

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196 **Table 1.** Prevalence of clinical and subclinical mastitis in dairy production units in the Mezquital Valley, Hidalgo, Mexico.

			Prevalence						
			Subclinical mastitis (%)				Subclinical mastitis (%)		Clinical mastitis (%)
Municipality	**DPU**	**Cows**	**T**	**G1**	**G2**	**G3**	**quarters**	**cows**	
Francisco I. Madero	9	10.56±4.2[b]	1.10±2.3[a]	7.42 ± 4.7[a]	3.13±3.0[a]	7.79±11.9[a]	17.28±16.2[a]	24.99±18.92[a]	0.17±0.5[a]
San Salvador	3	31.36±6.8[a]	2.64±2.9[a]	11.22±3.9[a]	9.79±3.6[a]	13.20±13.4[a]	37.47±15.1[a]	47.07±3.0[a]	0.66±1.3 [a]
Ixmiquilpan	4	12.25±2.5[b]	0.41± 0.8[a]	5.72±6.6[a]	6.35±5.6[a]	4.64±0.7[a]	27.97±12.5[a]	42.21±8.5[a]	0.69 ±1.3[a]

197 DPU: Dairy production units, T: trace, G1: grade 1, G2: grade 2, G3: grade 3. μ±s: mean ± standard deviation. [a,b] values sharing the same letter in a column are not
198 statistically different ($p < 0.01$).

199

200

201

202 In the association analysis using Pearson´s correlation (R), five factors with statistical
203 significance were identified as being related to the prevalence of mastitis. Likewise, the Chi-
204 square (X2) test revealed a significant association between mastitis prevalence and the factors
205 cow hygiene, presence of a milking room, teat drying and use of a post-dipping disinfectant
206 (Table 2). Factors related to the milker, the use of a pre-dipping solution, and forestripping
207 showed no significant influence on the prevalence of subclinical mastitis ($p > 0.05$).

Table 2. Factors associated with the prevalence of subclinical mastitis in dairy production units in the Mezquital Valley, Hidalgo, Mexico.

Factor	R	p<0.05	X^2	p<0.05
Cow hygiene	-0.55	0.02*	7.51	0.02*
Presence of a milking room	0.48	0.05*	7.11	0.02*
Milker training	0.16	0.54	1.50	0.47
Milker hand disinfection	-0.32	0.22	2.50	0.28
Teat cleaning	-0.61	0.01*	5.84	0.11
Pre-dipping solution	0.32	0.21	1.78	0.18
Forestripping practice	-0.33	0.2	0.41	0.51
Teat drying	-0.38	0.13	4.37	0.03*
Post-dipping disinfectant	0.53	0.04*	7.71	0.005**

208 p value ≤ 0.05 indicates statistical significance; **p value ≤ 0.01 indicates high statistical significance

209 **3.3. Bacterial identification**

210 A total of 22 bacterial strains associated with subclinical mastitis were isolated: 12 strains in
211 San Salvador, nine strains in Francisco I. Madero and only one strain in Ixmiquilpan.
212 According to the Gram staining classification, 16 Gram positive and six Gram negative
213 strains were identified, corresponding to 72.72% and 27.28 %, respectively (Table 3). In the
214 study area 13 bacterial species were identified, belonging to seven genera: *Staphylococcus*
215 (36.36 %), *Enterococcus* (27.27 %), *Escherichia* (9.09 %), *Pseudomonas* (9.09 %),
216 *Macrococcus* (9.09 %), *Stenotrophomonas* (4.55 %) y *Serratia* (4.55 %) (Fig. 2).

217

218

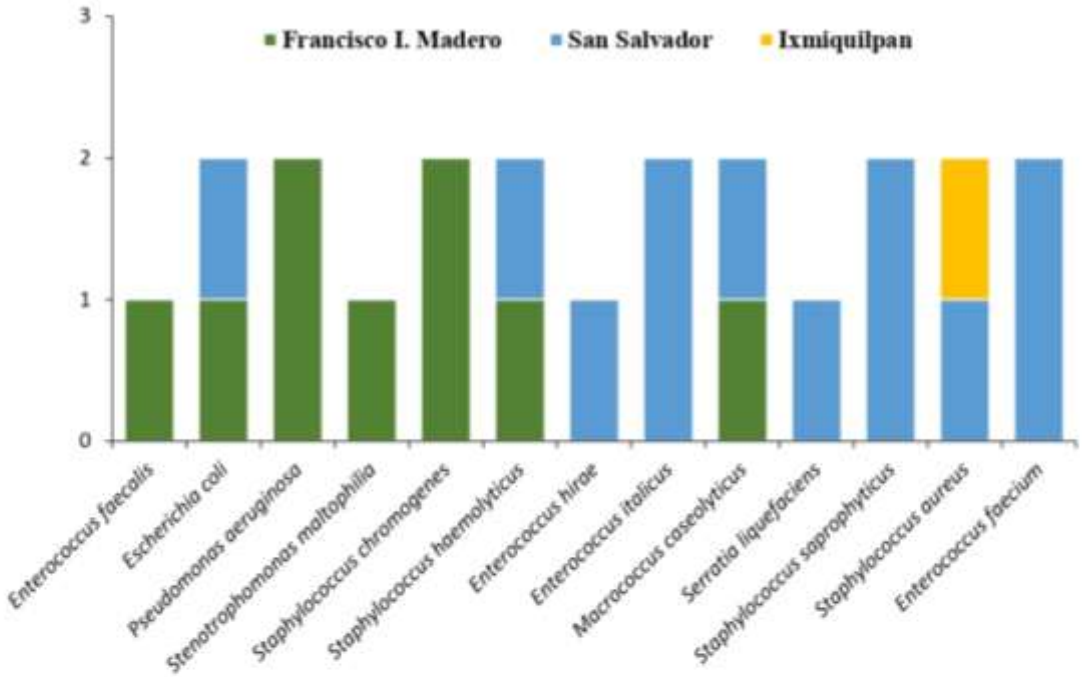
219 **Table 3.** Bacteria identified by MALDI-TOF associated with subclinical mastitis in dairy
220 production units in the Mezquital Valley, Hidalgo, Mexico.

Municipality	Total of strains	Catalase test	Gram staining	Specie
Francisco I. Madero	9	-	+	*Enterococcus faecalis*
		+	-	*Escherichia coli*
		+	+	*Macrococcus caseolyticus*
		+	-	*Pseudomonas aeruginosa*
		+	-	*Pseudomonas aeruginosa*
		+	-	*Stenotrophomonas maltophilia*
		+	+	*Staphylococcus chromogenes*
		+	+	*Staphylococcus chromogenes*
		+	+	*Staphylococcus haemolyticus*
San Salvador	12	-	+	*Enterococcus hirae*
		-	+	*Enterococcus italicus*
		-	+	*Enterococcus italicus*
		+	+	*Macrococcus caseolyticus*
		+	-	*Serratia liquefaciens*
		+	+	*Staphylococcus haemolyticus*
		+	+	*Staphylococcus saprophyticus*
		+	+	*Staphylococcus saprophyticus*
		+	+	*Staphylococcus aureus*
		+	-	*Escherichia coli*
		-	+	*Enterococcus faecium*
		-	+	*Enterococcus faecium*
Ixmiquilpan	1	+	+	*Staphylococcus aureus*

221 +, positive; -, negative

222 In the municipality of San Salvador, most frequent isolated bacteria were *Enterococcus*
223 *faecium*, *Enterococcus italicus* y *Staphylococcus saprophyticus*. In Francisco I. Madero,
224 predominant species were *Pseudomonas aeruginosa* y *Staphylococcus chromogenes*, while
225 in Ixmiquilpan, the only strain of interest was *Staphylococcus aureus*.

226



227

228 **Figure 2.** Frequency of bacteria isolated from subclinical mastitis samples in dairy
229 production units in the Mezquital Valley, Hidalgo, Mexico.

230 **3.4. Resistance profile of bacteria associated with mastitis**

231 The Gram positive isolated strains showed the highest resistance to B-lactam antibiotics,
232 including ampicillin (100%), dicloxacillin (94%), cefotaxime (94%), cephalothin (94%), and
233 penicillin (100%). Resistance to clindamycin (81%), ciprofloxacin (56%), gentamicin (50%)
234 vancomycin and tetracycline (38%) also was observed. Antibacterials with less resistance
235 were sulfamethoxazole/trimethoprim and erythromycin (31%) (Figure 3).

236 Gram negative bacteria showed higher resistance to ampicillin (100%), cephalothin (100%),
237 cefotaxime (83%), carbenicillin (66%). Resistance to amikacin (100%), Resistance to
238 amikacin (100%), chloramphenicol (83%), netilmicin (66%), nitrofurantoin and norfloxacin
239 (50%). The antibacterial with less resistance were ciprofloxacin, gentamicin (33%) and
240 sulfamethoxazole/trimethoprim (17%)

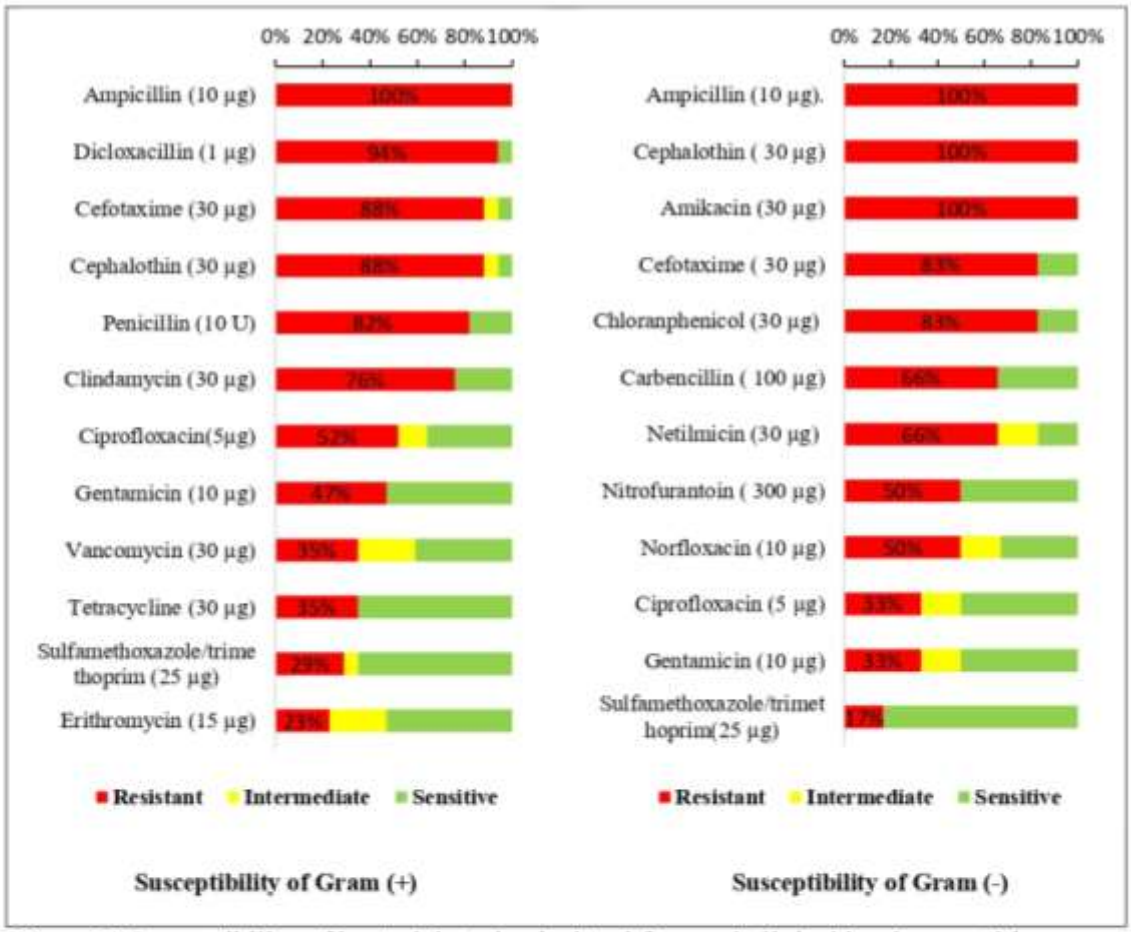


Figure 3. Susceptibility of bacterial strains isolated from subclinical bovine mastitis cases in in dairy production units in the Mezquital Valley, Hidalgo, Mexico.

From all isolated strains, 16 Gram positive strains were classified as multidrug-resistant (MDR). In Francisco I. Madero, the values of the Multiple Antibiotic Resistance Index (MARI) were as follows: *Staphylococcus chromogenes* (MARI = 0.91 y 0.58), *Enterococcus faecalis* (MARI = 0.91) y *Macrococcus caseolyticus* (MARI = 0.75) (Figure 4).

In the municipality of San Salvador, the Gram positive strains were classified was MDR. Strains with the highest values of the Multiple Antibiotic Resistance Index were *Enterococcus faecium* (MARI = 1.0), *Staphylococcus aureus* (MARI= 0.83), *Staphylococcus haemolyticus* (MARI= 0.83) and *Enterococcus hirae* (MARI=0.83) (Table 4). In Ixmiquilpan was isolated a strain belonging to *Staphylococcus aureus* (MARI= 0.41).

It was isolated six Gram negative multidrug-resistant strains (Table 5), values of multiple antibiotic resistance index of *Stenotrophomonas maltophilia* (MARI=0.83), *Pseudomonas*

aeruginosa (MARI=0.83 y 0.75), *Serratia liquefaciens* (MARI=0.41) and *Escherichia coli* (MARI=0.33 y 0.75).

Table 4. Gram-positive strains isolated from cows affected by subclinical mastitis with their resistance profiles and multiple antibiotic resistance indices

Gram positive strains	Municipality	Ampicillin	Cephalotin	Cefotaxim	Dicloxacillin	Penicillin	Clindamycin	Ciprofloxacin	Gentamicin	Sulf/Trim	Erythromycin	Vancomycin	Tetracycline	MARI	Resistance category
Staphylococcus chromogenes	FIM	R	R	R	R	R	R	R	R	R	R	R	S	0.91	MDR
Enterococcus faecalis	FIM	R	R	R	R	R	R	R	R	R	R	I	R	0.91	MDR
Macrococcus caseolyticus	FIM	R	R	R	R	R	R	I	R	R	I	R	R	0.75	MDR
Sthaphylococccus chromogenes	FIM	R	R	R	R	R	R	R	S	S	S	S	S	0.58	MDR
Enterococcus faecium	SSA	R	R	R	R	R	R	R	R	R	R	R	R	1.00	MDR
Staphylococcus aureus	SSA	R	R	R	R	R	R	R	R	R	R	I	S	0.83	MDR
Staphylococcus haemolyticus	SSA	R	R	R	R	R	R	R	R	S	S	R	R	0.83	MDR
Enterococcus hirae	SSA	R	R	R	R	R	R	I	R	S	R	R	R	0.83	MDR
Sthaphylococccus saprophyticus	SSA	R	R	R	R	R	R	S	R	S	S	S	R	0.66	MDR
Enterococcus faecium	SSA	R	R	R	R	R	R	R	S	S	I	I	S	0.58	MDR
Enterococcus italicus	SSA	R	R	R	R	R	R	S	S	S	S	S	S	0.50	MDR
Macrococcus caseolyticus	SSA	R	R	R	R	S	S	R	S	S	I	S	S	0.41	MDR
Staphylococcus haemolyticus	SSA	R	R	R	R	R	S	S	S	S	S	S	S	0.58	MDR
Staphylococcus saprophyticus	SSA	R	R	I	R	R	S	S	S	S	S	I	S	0.33	MDR
Enterococcus italicus	SSA	R	S	R	S	S	R	R	S	S	S	S	S	0.33	MDR
Staphylococcus aureus	IXM	R	R	R	R	R	R	S	S	I	I	R	S	0.41	MDR

R Resistant; I Intermediate; S Sensitive; FIM, Francisco I. Madero; SSA, San Salvador; IXM, Ixmiquilpan; MARI, Multiple Antibiotic Resistance Index; MDR, Multidrug-resistant strains.

Table 5. Gram-negative strains isolated from cows affected by subclinical mastitis with their resistance profiles and multiple antibiotic resistance indices.

Gram negative strains	Municipality	Ampicillin	Cephalothin	Amikacin	Cefotaxim	Chloramphenicol	Carbenicillin	Netilmicin	Nitrofurantoin	Norfloxacin	Ciprofloxacin	Gentamicin	Sulf / Trim	IMARI	Resistance category
Stenotrophomonas maltophilia	FIM	R	R	R	R	R	R	R	R	R	I	I	R	0.83	MDR
Pseudomonas aeruginosa	FIM	R	R	R	R	R	I	R	R	R	R	R	S	0.83	MDR
Pseudomonas aeruginosa	FIM	R	R	R	S	R	S	R	R	R	R	R	S	0.75	MDR
Serratia liquefaciens	FIM	R	R	R	S	R	S	R	S	I	S	S	S	0.41	MDR
Escherichia coli	FIM	R	R	R	R	S	S	S	S	S	S	S	S	0.33	MDR
Escherichia coli	SSA	R	R	R	R	R	R	R	S	R	R	S	S	0.75	MDR

R Resistant; I Intermediate; S Sensitive; FIM, Francisco I. Madero; SSA, San Salvador; IXM, Ixmiquilpan; MARI, Multiple Antibiotic Resistance Index; MDR, Multidrug-resistant strains.

4. Discussion

Bovine mastitis, a multifactorial disease, affects animal health and milk production, representing a threat to global food security. The prevalence is influenced by some factors such as animal, environmental, zootechnics management and milking routine (Pérez et al., 2022).

In the present study, the prevalence of subclinical mastitis was from 24.99% and 47.07% and from 17.28% to 37.47% for mammary quarter, comparable with international studies, which report values ranging from 20% to 65% (Khasapane et al., 2024). In Etiopia, Colombia, Bangladesh and China have recorded prevalences of 59.2%, 50%, 62.7% y 37.7%, respectively (Abebe et al., 2016; Medrano et al., 2021, Fazal et al., 2023; Chen et al., 2023).

In Mexico, prevalence of mastitis in small-scale production units varies according to the region: 20.5% in Guerrero (Olivares et al., 2015), 35.64% in la Ciénega, Jalisco (Aguilar et al., 2014) and 25.65% in the Mezquital Valley, Hidalgo (Vargas et al., 2020). These differences are attributed to climate, management, infrastructure, and training.

Our factorial analysis revealed that the prevalence is related with infrastructure, the milkers training, and procedures during milking. Other factors, such as cow hygiene, milking room, teat drying, and the use of post-dipping disinfectants were determinants, coinciding with those reported by Belage et al. (2019). Our findings confirm that cow hygiene is a key factor in reducing the prevalence of mastitis (Medrano et al., 2021; Rana et al., 2022).

In the evaluated production units, we observed udder contamination and the lack of pre-dipping and post-dipping, which increases the risk of intramammary infections. Differences in prevalence were related to management practices, breed, age, number of lactations, milking practices, infrastructure, and training (Ashraf et al., 2018; Belage et al., 2019; Rana et al., 2022; Chen et al., 2023).

Most frequent bacteria corresponded to Gram positive strains, in this respect different authors explain that Gram positive bacteria are responsible for up to 87% of subclinical mastitis cases (Abebe et al., 2023; Bonifaz et al., 2024), while 40% of clinical cases were associated to Gram negative bacteria (Steele et al., 2020).

293 The Gram positive identified bacteria corresponded to *Staphylococcus*, *Enterococcus* and
294 *Macrococcus* genera, while the Gram-negative included *Escherichia*, *Pseudomonas*,
295 *Stenotrophomonas* and *Serratia* genera. All 13 species isolated have been reports as
296 pathogens associated with bovine mastitis, variating in its prevalence and incidence
297 (Schukken et al., 2012; Kim et al., 2022; Kaczorowski et al., 2022; Ocak y Turkyilmaz, 2023;
298 Morales et al., 2023).

299 *Staphylococcus aureus* was one of the identified species in this study, *S. aureus* is a highly
300 contagious pathogen, which resides within the udder and is transmitted between cows during
301 the milking process (Liu et al., 2022; Rana et al., 2022; Morales et al., 2023). With respect
302 to *saprophyticus*, *S. chromogenes* y *S. haemolyticus,* coagulase negative staphylococci they
303 are present in the lying areas and in the teat skin microbiota and are considered emerging
304 opportunistic pathogens associated with persistent subclinical mastitis and an increased
305 somatic cell count in cows without evident clinical signs of mastitis (De Buck et al., 2021;
306 Freu et al., 2024).

307 *Enterococcus faecalis*, *E. faecium* y *E. hirae* have been isolated from cows with mastitis in
308 South Corea, presence of these species is associated with environmental contamination, since
309 they are part of intestinal microbiota of humans and animals. Additionally, authors reported
310 a high prevalence of *E. faecalis* and *E. faecium*, species that were also identified in the present
311 study (Kim et al., 2022).

312 The Gram-positive bacterium with the lowest frequency in this study was *Macrococcus*
313 *caseolyticus*, a species of under skin microbiota with low pathogenicity. Similar studies in
314 Brazil reported a prevalence of 1.3% for this species (De Oliveira et al., 2022).

315 *Escherichia coli* was identified, this bacterium is reported as the causative agent in up to 50%
316 acute clinical mastitis cases, potentially reducing daily milk production by up 30% (Goulart
317 y Mellata, 2022). This enteric bacterium can invade the mammary gland through fecal
318 contamination of the teat, particularly in immunocompromised cows (Salamon et al., 2020;
319 Zhou et al., 2021). Its infective capacity is associated with high genetic variability and a
320 diverse array of virulence factors (Goulart y Mellata, 2022).

The less frequently identified non-coliform Gram-negative bacteria in the present study were *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* y *Serratia liquefaciens*. *P. aeruginosa*. *P. aeruginosa* is an opportunistic environmental pathogen, whose occurrence in bovine mastitis is sporadic, although it exhibits significant pathogenic potential (Badawy et al., 2023).

Stenotrophomonas maltophilia is part of the ruminal microbiota, previous studies have reported that this bacterium migrates to the mammary gland during acidosis, inducing mastitis (Ocak y Turkyilmaz, 2023; He et al., 2023).

Finally, *Serratia liquefaciens* is an uncommon bacterium in bovine mastitis, this pathogen is present in environment and in digestive tract of multiple animal species (Schukken et al., 2012; Friman et al., 2019).

The antimicrobial susceptibility test indicated that bacteria isolated in the present study exhibited multidrug resistance. This resistance may be associated with the prolonged use of antibacterials in empirical treatments, previous studies as documented in previous studies, which could explain the high resistance rates observed in this investigation (Monte et al., 2018; Rana et al., 2022; Sharifi et al., 2023; Shahzad et al., 2024).

Regarding the isolated strains, two *Staphylococcus aureus* strains were identified, a bacterium widely reported for its multidrug resistance, including penicillin, erythromycin, clindamycin, cefoxitin, penicillin G, sulfamethoxazole-trimethoprim, ciprofloxacin, norfloxacin, polymyxin B, levofloxacin, chloramphenicol, clarithromycin, spectinomycin, amoxicillin, cephradine, cefotaxime, azithromycin, and methicillin, showing a resistance profile similar to that observed in the present study. This resistance has been associated with various genes, including mecA, *blaZ*, *ermC*, *rpoB*, *ant (4')-Ia*, *hla*, *hlb*, *clfA* and *clfB*, which contributes to the evasion of antibacterial treatments. These findings can explain resistance profiles determined in *S. aureus* isolated strains (Liu et al., 2022; Sharifi et al., 2023; Silva et al., 2023; Shahzad et al., 2024).

With respect to *S. chromogenes*, *S. haemolyticus* y *S. saprophyticus*, resistance to ampicillin, amoxicillin/clavulanic acid, cefoxitin, ceftriaxone, ciprofloxacin, cefepime, gentamicin, erythromycin, tetracycline, penicillin, vancomycin, cefepime, erythromycin, penicillin,

350 vancomycin, oxacillin, and spectinomycin, results that are consistent with those observed in
351 the present study. In coagulase negative staphylococci it has been documented the presence
352 of various resistance genes, including *blaZ*, *icaD*, *pvl*, *mecA*, *hlb*, *sec* and *hla*, which promote
353 their persistence and resistance to antibacterial agents (Fazal et al., 2023; Khasapane et al.,
354 2024; Getahun et al., 2024).

355 Concerning *Enterococcus spp.*, multidrug-resistant strains have been reported that act as
356 reservoirs of resistance genes, which can be transmitted to humans through the food chain.
357 *Enterococcus faecalis* and *Enterococcus faecium* are the most prevalent in dairy production
358 units, where genes such as *gelE*, *esp*, and *cylA,* associated with biofilm formation, gelatinase
359 and hemolysin production, as well as *tetK*, *tetM*, *tetO*, and *tetS* (Juliano et al., 2022; Kim et
360 al., 2022) have been identified. These findings could explain the MAR index of 1 observed
361 in the *E. faecalis* strain isolated in this study.

362 For *Macrococcus caseolyticus*, resistance genes to methicillin (*mecB*, *mecD*) and macrolides
363 (*ermB*, *ermC*) have been reported, which can be horizontally transferred to bacteria of the
364 genus *Staphylococcus* and other genera (Schwendener et al., 2020).

365 The strains of *Escherichia coli* isolated in the present study exhibited multidrug resistance to
366 ampicillin, cephalothin, cefotaxime, dicloxacillin, penicillin, clindamycin, ciprofloxacin,
367 sulfamethoxazole-trimethoprim, and erythromycin. Our results are consistent with those
368 reported in other studies, which have documented resistance to streptomycin, sulfisoxazole,
369 and tetracycline, associated with genes such as *blaCTX-M*, *tetB*, *tetA*, *strA*, *strB*, *qnrB*, and
370 *blaTEM* (Saini et al., 2012; Salomon et al., 2020; Rana et al., 2022; Dos Santos et al., 2023).

371 Regarding *Pseudomonas aeruginosa*, multidrug resistance has been reported in dairy
372 production units against sulfamethoxazole, imipenem, cefepime, piperacillin-tazobactam,
373 and gentamicin, associated with resistance genes such as *drfA*, *sul1*, *ermB*, *fosA*, as well as
374 mutations in *gyrA*, *parC*, *oprD*, and *ampC*, which are involved in the regulation of resistance
375 mechanisms through efflux pumps (Aslantas et al., 2022; Badawy et al., 2023). Turkyilmaz
376 and Ocak (2023) also reported associations between antimicrobial resistance and virulence
377 genes in *P. aeruginosa*.

Stenotrophomonas maltophilia has been characterized by resistance to ticarcillin/clavulanate, ceftazidime, ciprofloxacin, and doxycycline, attributed to genes involved in biofilm formation (*smf-1*, *rpfF*), as well as genes encoding efflux pumps and drug-inactivating enzymes for multiple drugs (Gil et al., 2020; Azimi et al., 2020). *Serratia liquefaciens* exhibits resistance to various antibacterials, including cefpodoxime, mediated by chromosomal *AmpC* production and its association with efflux pumps for multiple drugs (Friman et al., 2019).

Finally, 100% of the strains isolated in the present study exhibited a MAR index equal to or greater than 0.33, in this regard, Moffo et al., (2024) reported that a MAR index above than 0.2 is associated with bacteria originating from high-risk sources of antimicrobial contamination or from environments where antimicrobials are frequently used, such as the dairy production units sampled in the present study

5. Conclusion

In small-scale dairy farms in the Mezquital Valley, Hidalgo, Mexico, the prevalence of subclinical mastitis was determined to range between 24.9% and 47.0% per cow, and between 17.2% and 37.4% per mammary quarter, associated with factors related to milking practices, cow hygiene, presence of a milking room, teat drying, and the use of teat sealants. The bacteria isolated from grade 3 subclinical mastitis cases belonged to the genera *Staphylococcus*, *Enterococcus*, *Escherichia*, *Pseudomonas*, *Macrococcus*, *Stenotrophomonas*, and *Serratia*, and their resistance profiles showed multidrug resistance. 72% of the strains exhibited a MAR index equal to or greater than 0.5. The *Enterococcus faecium* strain exhibited resistance to 100% of the antibacterials evaluated. The strains isolated in the present study represent a risk to both animal health and public health therefore, it is recommended to identify the main resistance genes present in the isolates to understand the mechanisms of resistance as well as their potential transfer.

Ethical approval

The authors confirm that all ethical policies of the journal have been adhered to, as indicated in the journal's author guidelines. Fieldwork (mastitis detection and sample collection) was conducted following good animal husbandry practices in dairy production units in Mexico

(Federal Animal Health Law), and all laboratory procedures in the present study were performed according to the protocols of the Academic Area of Veterinary Medicine and Zoothecnics at the Universidad Autónoma del Estado de Hidalgo.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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11 Capítulo 3. Actividad bactericida del aceite esencial de orégano mexicano contra bacterias multirresistentes asociadas con mastitis

Microbial Pathogenesis

Bactericidal activity from Mexican Oregano Essential Oil against multidrug-resistant bacteria associated with mastitis

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Abstract:	TThe aim was to evaluate the antibacterial activity of Lippia graveolens essential oil (AELg) against multidrug-resistant bacteria associated with bovine mastitis and to determine its possible mode of action. The oil was obtained by steam distillation, and its antibacterial activity was determined using the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and the ratio between these concentrations. The chemical composition of AELg was determined by gas chromatography-mass spectrometry (GC-MS). The cytotoxicity of LAE was determined by hemolytic activity and cell membrane damage by the release of proteins and DNA. Molecular docking analysis was performed to determine its interaction with a target molecule. The AELg exhibited antibacterial activity against all bacteria tested, with significant differences ($p \leq 0.01$) in MIC and MBC. The greatest activity was observed against Gram-positive bacteria, especially Staphylococcus aureus, Macrococcus caseolyticus, and Enterococcus faecium, with MIC and MBC values below 100 µg/mL. The AELg showed bactericidal effect against 100% of the strains tested. The major compound present in the AELg was the thymol chemotype (68.9%), and a concentration-dependent hemolytic effect and membrane damage were observed. Molecular docking revealed thymol's affinity for the FabH enzyme of S. aureus and DHPS of E. coli. The results suggest that the AELg could be an alternative against multidrug-resistant bacteria; however, its application requires further safety studies and the determination of appropriate therapeutic doses for the clinical treatment of bovine mastitis.
Opposed Reviewers:	

Bactericidal activity from Mexican Oregano Essential Oil against multidrug-resistant bacteria associated with mastitis

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Abstract

The aim was to evaluate the antibacterial activity of *Lippia graveolens* essential oil (AELg) against multidrug-resistant bacteria associated with bovine mastitis and to determine its possible mode of action. The oil was obtained by steam distillation, and its antibacterial activity was determined using the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and the ratio between these concentrations. The chemical composition of AELg was determined by gas chromatography-mass spectrometry (GC-MS). The cytotoxicity of LAE was determined by hemolytic activity and cell membrane damage by the release of proteins and DNA. Molecular docking analysis was performed to determine its interaction with a target molecule. The AELg exhibited antibacterial activity against all bacteria tested, with significant differences ($p \leq 0.01$) in MIC and MBC. The greatest activity was observed against Gram-positive bacteria, especially *Staphylococcus aureus*, *Macrococcus caseolyticus*, and *Enterococcus faecium*, with MIC and MBC values below 100 µg/mL. The AELg showed bactericidal effect against 100% of the strains tested. The major compound present in the AELg was the thymol chemotype (68.9%), and a concentration-dependent hemolytic effect and membrane damage were observed. Molecular docking revealed thymol's affinity for the FabH enzyme of *S. aureus* and DHPS of *E. coli*. The results suggest that the AELg could be an alternative against multidrug-resistant bacteria; however, its application requires further safety studies and the determination of appropriate therapeutic doses for the clinical treatment of bovine mastitis.

Keywords: molecular docking, *Staphylococcus aureus, Escherichia coli*.

1. Introduction

The milk consumed by the world's population comes mostly from the mammary glands of cows; however, its safety can be compromised by mastitis, an inflammation of the mammary gland caused mainly by intramammary bacterial infections that affect the health and well-being of the animals, reduce milk production and quality, and generate significant economic losses in dairy farms [1, 2]. The presence of bacteria in the farm environment, associated with poor hygiene practices during milking, significantly influences the prevalence and severity of the disease, which can manifest in subclinical, clinical, or chronic forms [3].

Mastitis control programs on farms aim to reduce the risk of infection through hygiene practices, health monitoring, and early diagnosis to enable timely treatment and reduce transmission [4]. Traditionally, treatment is based on the use of antimicrobials administered by intramammary infusion or parenteral injection. Although antimicrobials allow for rapid clinical recovery, bacterial eradication can take between 7 and 28 days, during which time bacteria may remain present in the milk [5].

Prolonged use of antimicrobials has contributed to the development of antimicrobial resistance (AMR) among mastitis-causing bacteria, posing a growing threat to animal and human health [5, 6]. AMR is currently a critical public health problem worldwide due to the horizontal transmission of resistance genes between humans and animals, leading to increased treatment failures and mortality from infections [7].

In response to this problem, research into alternative treatments for mastitis in dairy cows has been conducted in recent years. Plant extracts contain compounds with antimicrobial, anti-inflammatory, and antioxidant activity, making them a promising phytotherapeutic alternative for mammary gland health and ensuring milk safety [8, 9].

Lippia graveolens, commonly known as Mexican oregano, has been extensively studied for its antimicrobial effects. Its extracts contain bioactive compounds such

as flavonoids and terpenoids capable of destabilizing bacterial membranes, causing lysis and cell death [10-13]. Furthermore, these compounds modulate the inflammatory response and protect against oxidative damage, which could contribute to the control of bovine mastitis [14, 15]. Therefore, the aim of this study was to determine the antibacterial activity of *Lippia graveolens* essential oil against multidrug-resistant bacteria associated with bovine mastitis.

2. Materials and methods

2.1. Plant material

The aerial parts of *Lippia graveolens* were collected from hills in the town of Orizabita, municipality of Ixmiquilpan, Hidalgo, Mexico (20°34'59"N 99°12'32"W) at an altitude of 1900 m above sea level. For identification, the herbarium of the National Autonomous University of Mexico (UNAM) was consulted, and the plant was verified as *Lippia graveolens* Kunth (IBUNAM: MEXU:308815). The fresh material was dried at room temperature, protected from sunlight.

2.2. Essential oil extraction

The essential oil was extracted using steam distillation, 250 g of aerial plant material from *Lippia graveolens* and 2.5 L of water were used. The essential oil yield was calculated as the percentage of the weight of oil obtained relative to the weight of the plant material used.

2.3. Antibacterial activity of AELg

Reference strains (ATCC) of *Escherichia coli*^{35218}, *Staphylococcus aureus*^{6538} were used as indicator strains and field strains isolated from bovine mastitis samples, previously identified at the species level and classified as extremely resistant in antimicrobial susceptibility tests, with multiple resistance indices (MRI) greater than 0.75. The Gram-positive field strains and their MRI were *Staphylococcus aureus* (0.83), *Enterococcus faecium* (1.0), *Staphylococcus chromogenes* (0.92), *Enterococcus faecalis* (0.92), *Micrococcus caseolyticus* (0.83), *Enterococcus hirae*

(0.83) and *Staphylococcus haemolyticus* (0.83). The Gram negative field strains and their MRI values used were *Stenotrophomonas maltophilia* (0.83) and *Escherichia coli* (0.75). These strains were obtained from the Microbiology Laboratory collection of the Institute of Agricultural Sciences at the Autonomous University of the State of Hidalgo.

The bacterial strains were reactivated after cryopreservation on Müller-Hinton agar (BD Bioxon, Heidelberg, Germany). One colony of each strain was inoculated into nutrient broth (BD Bioxon) and incubated for 24 h at 37 °C.

The antibacterial activity of AELg was determined using the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) according to CLSI guidelines and as described by Zaragoza-Bastida *et al.* [16]. The bactericidal or bacteriostatic effect was determined by the MBC/MIC ratio; a bacteriostatic effect was considered present when the ratio was greater than 4, and a bactericidal effect when values less than or equal to 4 were obtained [17].

2.3.1. Concentración Mínima Inhibitoria (CMI)

La CMI se determinó por el método de microdilución en placa de 96 pozos donde se evaluaron por triplicado concentraciones seriadas del AELg las concentraciones evaluadas de 20 mg a 18.7 µg/mL. En cada pozo se añadieron 100 µL de cada concentración junto con 10 µL de suspensión bacteriana ajustada al 0.5 del estándar de McFarland (Remel, R20421, Lenexa, USA). Se empleó Kanamicina (AppliChem 4K10421, Alemania) como control positivo a concentraciones de 64 a 0.5 µg/ml y como control negativo se usó caldo nutritivo. Las placas se incubaron a 37 °C durante 24 h, después se adiciono 20 µL de una solución de p-yodonitrotetrazolio al 0,04 % (p/v) (Sigma-Aldrich I8377, USA) a cada pocillo, se incubo la placa por 30 minutos. La CMI se determinó en la concentración a la que la solución cambio a un color rosado [18].

2.3.2. Minimum Bactericidal Concentration (MIC)

To determine the MBC before adding p-iodonitrotetrazolium, 5 µL were extracted from each well and inoculated onto Mueller-Hinton agar Petri dishes (BD Bioxon) properly labeled with the AELg concentration. The plates were incubated for 24 hours at 35 °C. The MBC reading was taken on those plates with concentrations where no bacterial growth was visible [18].

2.4. Chemical composition of AELg

The chemical composition of AELg was determined by gas chromatography-mass spectrometry (GC-MS) analysis, performed at the National Laboratory of Macromolecular Structure of the Center for Chemical Research of the Autonomous University of the State of Morelos (LANEM-CIQ-UAEM) using a 7890B gas chromatograph coupled to an Agilent Technologies 7000D triple quadrupole mass spectrometer, operated in EI mode at 70 eV (CA, USA). Helium was used as the carrier gas (1.3 mL/min). The oils were diluted in hexane (1:5, v/v), and 1 µL of each sample was injected. The constituents were identified based on the retention index (RI) on the column, determined in relation to homologous series of n-alkanes (C7–C30) described in the literature. The relative quantification of the compounds was performed based on the areas under the curve of each peak.

2.5. Indirect Hemolytic Activity of AELg

Indirect hemolytic activity was performed on 8% blood agar plates. Four 6 mm diameter wells were prepared on the plate surface. Five microliters of each concentration to be evaluated (0.86 mg, 0.43 mg, 0.21 mg, 0.10 mg, and 0.05 mg) were added. Treatments were performed in triplicate. 100% Tween 80 (Sigma-Aldrich) and nutrient broth (BD Bioxon) were used as positive and negative controls, respectively. The plates were incubated for 24 h at 37 °C. After the incubation period, the hemolysis halos were measured in mm. [16].

168 2.6. Cell membrane integrity

169 Damage to bacterial cell membrane integrity by AELg was examined by measuring
170 the release of proteins and DNA, indicating leakage through the bacterial membrane.
171 Bacteria cells in the logarithmic growth phase (*E. coli*^{35218}, *S. aureus*^{6538}) were
172 subjected to treatment with the AELg at 75 (1x MIC), 150 (2x MIC) and 300 µg/mL
173 (3x MIC), cell lysis solution (Promega, USA) (positive control), thermal shock
174 (positive control) and saline solution (0.9%) (negative control), the treatments were
175 performed in triplicate.

176 The treatments were incubation period lasted for 30 minutes at a temperature of 37
177 °C. Following the incubation, the bacteria were separated from the supernatant by
178 centrifugation at 10,000 g for 5 min. The concentrations of proteins and DNA in the
179 supernatant was quantified using a NanoDrop Thermo Fisher Scientific 1000
180 (Delaware, USA) with an absorbance of 280 nm y 260 nm respectively.

181

182 2.7. Molecular Docking of Thymol

183

184 2.7.1 Ligand Preparation

185 Conformational analysis was performed to evaluate the structural stability of the
186 main monoterpene in the essential oil of L. graveolens. A set of gas-phase
187 conformers was generated by random search using the semi-empirical AM1 method
188 with Spartan 06 software [19]. To represent the optimized chemical structure of the
189 monoterpene, lower-energy conformers were selected using Density Functional
190 Theory (DFT) and the three-parameter hybrid density functional (B3LYP) model with
191 the extended base set 6-311++G(d, p) [20, 21]. Molecular construction and quantum
192 DFT calculations of the compound's ground state were performed using Gaussian
193 [22] and GaussView 06 [23].

194

195

196

197 2.7.2. Docking Configuration

198

199 The interaction potential of the monoterpene with two bacterial enzymes was
200 evaluated: β-ketoacyl-ACP synthase III (FabH) from Staphylococcus aureus (PDB
201 ID: 3IL7), essential in bacterial fatty acid synthesis [24], and dihydropteroate
202 synthase (DHPS) from Escherichia coli (PDB ID: 1AJ0), essential in bacterial folic
203 acid formation by catalyzing the formation of dihydropteroate [25]. The
204 crystallographic structures of the enzymes were obtained from the Protein Data Bank
205 database. Prior to docking, water molecules and other unwanted ligands were
206 removed.
207 To define the docking, AutoDock 4.2 software and a grid-based procedure were used
208 to prepare the structural inputs and define all binding sites. A rectangular grid of 55
209 x 115 x 80 Å was used for FabH from *S. aureus* (PDB ID: 3IL7) and 60 x 70 x 80 Å
210 for dihydropteroate synthase from *E. coli* (PDB ID: 1AJ0), with points separated by
211 0.375 Å, centered on the active site of each enzyme [26-28]. ,0 x 107 [25, 29].
212

213 2.8. Statistical analysis
214 The normality and homoscedasticity of the data (CMI, CMB) were verified and
215 analyzed using a completely randomized design through analysis of variance
216 (ANOVA), with α=0.05. Differences between means were determined by multiple
217 comparison using Tukey's test. A regression analysis was performed using
218 hemolysis halos and halos and cell membrane integrity of the AELg. The analyses
219 were performed using Minitab version 18.
220

221 **3. Results**

222 3.1 Antibacterial activity of AELg

223 The AELg yield was 2.8%, and regarding antibacterial activity, an inhibitory effect of
224 AELg was determined against multidrug-resistant bacterial strains associated with
225 bovine mastitis, with statistically significant differences between them (p=0.0001)

(Table 1). AELg showed the best activity against Gram-positive bacteria for the *S. aureus*[6538] indicator strain and the *E. faecium*, *S. chromogenes*, *E. faecalis*, *M. caseolyticus*, and *S. haemolyticus* strains, with an MIC of 37.5 µg/mL, and the lowest activity against *S. aureus* and *E. hirae*, with an MIC of 75 µg/mL.

Regarding Gram-negative strains, the best activity was determined against Stenotrophomonas maltophilia with an MIC of 18.7 µg/mL. The MIC for the indicator strain of Escherichia coli35218 and Escherichia coli was 37.5 µg/mL.

The AELg showed bactericidal activity with statistically significant differences between the bacterial strains evaluated (p=0.0001) (Table 1). Regarding Gram positive strains, the AELg exhibited the best activity against the indicator strain of *S. aureus*[6538], *E. faecium*, *M. caseolyticus*, and *S. haemolyticus*, with an MBC of 75 µg/mL. The highest MBC was observed against *S. aureus, S. chromogenes*, *E. faecalis*, and *E. hirae* at a concentration of 150 µg/mL. For Gram-negative strains, the best activity was determined against *S. maltophilia*, with an MBC of 37.5 µg/mL. Bactericidal activity against *E. coli*[35218] and *E. coli* was determined at a concentration of 75 µg/mL.

The AELg exhibits a bactericidal effect on the evaluated strains, as evidenced by an MBC/MIC ratio of less than or equal to 4 (Table 1). The oil had a bactericidal effect similar to the control antimicrobial kanamycin; however, it showed less effect on *S. chromogenes* and *E. faecalis*.

Tabla 1. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration, as well as the MBC/MIC ratio (µg/mL) against multidrug-resistant bacteria associated with bovine mastitis.

	Strains evaluated	MRI	CMI (µg/mL)		CMB (µg/mL)		(CMB/CMI)
			AELg	Kanamicina	AELg	Kanamicina	
Gram +	*Staphylococcus aureus* [6538]	IS	37.5[b]	1.0	75.0[b]	2.0	2.0
	Staphylococcus aureus	0.83	75.0[c]	1.0	150.0[d]	2.0	2.0
	Enterococcus faecium	1	37.5[b]	2.0	75.0[c]	4.0	2.0
	Staphylococcus chromogenes	0.92	37.5[b]	0.5	150.0[d]	1.0	4.0
	Enterococcus faecalis	0.92	37.5[b]	2.0	150.0[d]	4.0	4.0
	Macrococcus caseolyticus	0.83	37.5[b]	1.0	75.0[c]	2.0	2.0
	Enterococcus hirae	0.83	75.0[c]	1.0	150[c]	2.0	2.0
	Staphylococcus haemolyticus	0.83	37.5[b]	8.0	75.0[c]	16.0	2.0
Gram -	*Escherichia coli* [35218]	IS	37.5[b]	0.12	75.0[b]	0.25	2.0
	Escherichia coli	0.75	37.5[b]	0.12	75.0[c]	0.25	2.0
	Stenotrophomonas maltophilia	0.83	18.7[a]	8.0	37.5[a]	16.0	2.0
	Valor de *P*	0.0001					

[a,b,c,d,e,f] Different letters in columns are statistically different ($p \leq 0.01$). IS indicator strain.

Tabla 2. Chemical composition by gas chromatography coupled to mass spectrometry (GC-MS) of *Lippia graveolens* essential oil.

No	Compound	Molecular Formula	Retention Time (min)	Abundance (%)	Compound Type
1	p-Cimeno	$C_{10}H_{14}$	7.39	4.6	Monoterpene
2	m- Cimeno	$C_{10}H_{14}$	7.39	4.6	Monoterpene
3	2-Etil-m-xiyleno	$C_{10}H_{14}$	7.39	4.6	Aromatic hydrocarbon
4	o-Cimeno	$C_{10}H_{14}$	7.39	4.6	Monoterpene
5	2-Etil-p-xyleno	$C_{10}H_{14}$	7.39	4.6	Aromatic hydrocarbon
6	Terpineno 4- acetate	$C_{10}H_{18}O$	10.03	1.1	Monoterpenoid alcohol
7	α-Terpineol	$C_{10}H_{18}O$	10.26	0.7	Monoterpenoid alcohol
8	3-Metoxi-p-cimeno	$C_{11}H_{16}O$	10.61	4.6	Ether
9	Timol	$C_{10}H_{14}O$	11.30	68.9	Monoterpene
10	Caryophyllene	$C_{15}H_{24}$	13.11	1.1	Sesquiterpene
11	Humulen	$C_{15}H_{24}$	13.55	0.3	Sesquiterpene
12	1,4,7, Cicloundecatrieno, 1,5,9,9-tetrametil-, Z,Z,Z-	$C_{15}H_{24}$	13.55	0.3	Sesquiterpene

3.2 Chemical composition of the essential oil of *Lippia graveolens*

In the chromatographic analysis, 12 compounds were determined in the essential oil of *Lippia graveolens* (Table 2). Monoterpenes were the predominant compounds, with thymol being the major compound at 68.9%. Additionally, cymene iosomes (p-, m- and o-cymene) and associated compounds such as 2-ethyl-xylene were identified, which showed a similar retention time (7.39 min) and abundance in the oil (4.6%). Terpinene-4-acetate (1.1%), α-terpineol (0.7%), and a 3-methoxy-p-cymene ether (4.6%) were identified. In a smaller proportion, sesquiterpenes such as caryophyllene (1.1%) and humulene (0.3%) were also present, in addition to a complex cyclic compound (1,4,7-Cyclondecatriene,1,5,9,9-tetramethyl-, Z,Z,Z-) with an abundance of 0.3%.

3.3. Indirect hemolytic activity of AELg

AELg exhibited concentration-dependent hemolytic activity (R^2 = 0.84). At 0.86 mg/mL, a hemolysis halo of 18.7 mm was generated. A reduction in the effect was observed at lower concentrations, with halos of 3.0 mm and 1.87 mm observed for concentrations of 0.21 mg/mL and 0.10 mg/mL, respectively. No hemolytic activity was detected at a concentration of 0.05 mg/mL of AELg (Figure 1).

3.4. Cell membrane integrity and DNA release.

Regarding membrane integrity, a positive correlation (R^2= 0.99) was determined between protein release and the evaluated concentrations of AELg, with no significant differences between the release values for *E. coli* and *S. aureus* ($p \geq 0.05$). At 300 µg/mL, 14.96 and 14.26 mg/mL of protein were quantified (*E. coli* and *S. aures*), as shown in Figure 2. A positive correlation (R^2= 0.99) was determined between DNA release and the evaluated concentrations of AELg, as shown in Figure 2. Higher AELg concentrations resulted in greater DNA release. Statistical differences were found between the indicator strains and the positive and negative controls ($p<0.001$). Greater DNA release was observed in the *S. aureus* strain. At

the highest concentration evaluated (300 µg/mL), an average of 362.71 ng/µL of DNA was quantified for *S. aureus* and 287 ng/µL for *E. coli*.

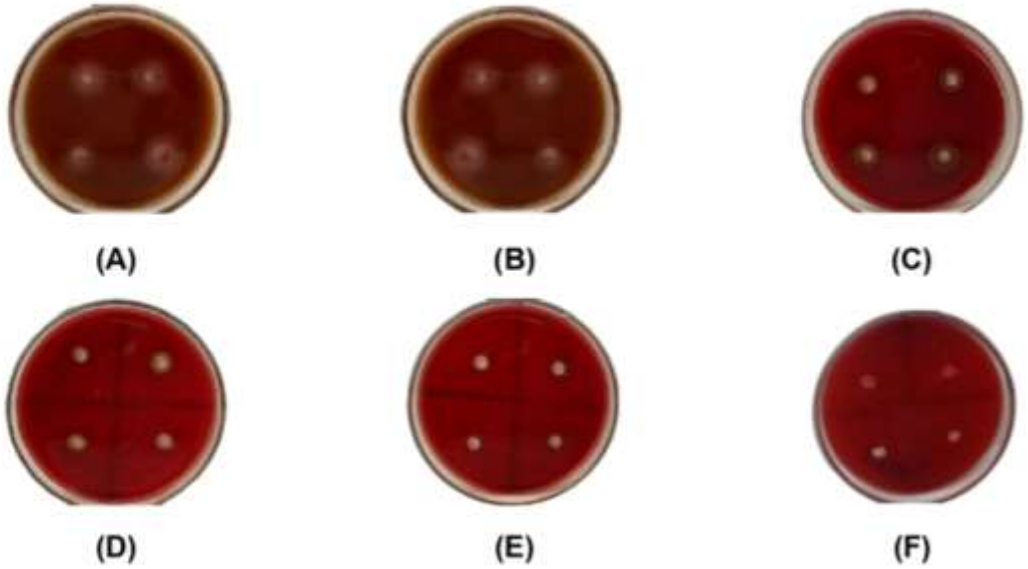


Figure 1. Hemolytic activity of AELg on blood agar. **A:** 0.86 mg/mL-inhibition halo 18.7 mm, **B:** 0.43 mg/mL-inhibition halo 17.5 mm, **C:** 0.21 mg/mL -inhibition halo 3.0 mm, D: 0.10 mg/mL-inhibition halo 1.87 mm, **E:** 0.05 mg/mL-inhibition halo 0 mm and **F:** Physiological saline solution / no hemolysis.

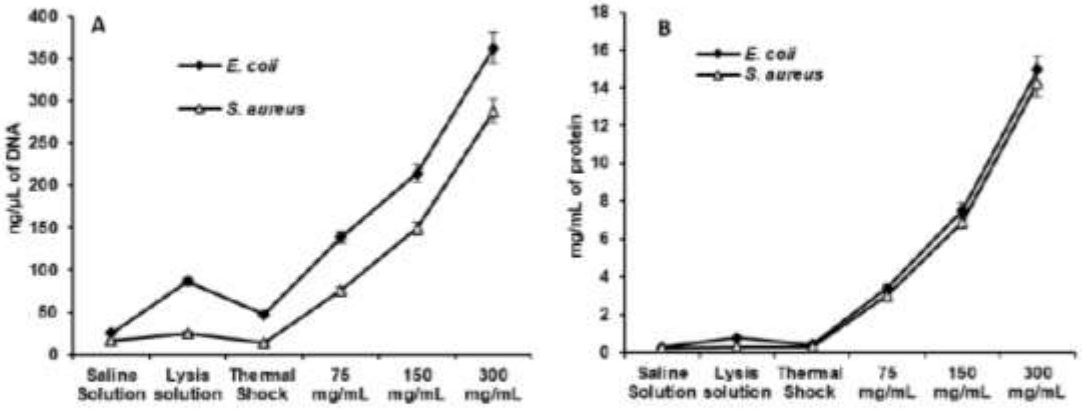


Figure 2. Leakage of DNA (A) and proteins (B) from *E. coli* and *S. aureus* treated with *Lippia graveolens* essential oil.

3.5. Molecular Docking of Thymol

In the docking study using AutoDock 4.2 to determine the interaction mode and binding free energy (ΔG) of thymol with the enzymes β-ketoacyl-ACP synthase III (FabH) from *S. aureus* (PDB ID: 3IL7) and dihydropteroate synthase (DHPS) from *Escherichia coli*, ΔG values of -6.14 kcal/mol were found for the interaction of thymol with DHPS and -5.84 kcal/mol with FabH (Table 3), suggesting a moderate affinity of the compound for both enzymes.

Table 3. Binding energy (ΔG) and types of interactions of thymol with the enzymes FabH (PDB ID: 3IL7) from *S. aureus* and DHPS (PDB ID: 1AJ0) from *E. coli*.

Enzyme	Binding energy ΔG (kcal/mol)	Interacting amino acids	Type of interaction
β-cetoacil – ACP sintasa III (FabH)	-5.84	Cys112	Hydrogen Bond
		Ile145, Ala111, Phe157, Leu142, Ala81, Leu199	π–Alkyl
		Phe87	π–Sigma
Dihidropteroato sintasa (DHPS)	-6.14	Phe190	π–Alkyl
		Thr147	Hydrogen Bond

3.5.1. Enzyme Binding energy

Molecular interactions between thymol and the active site of the bacterial enzyme β-ketoacyl-ACP synthase III (FabH) from *S. aureus* (PDB ID: 3IL7) show thymol at the center of the catalytic complex, stabilized by hydrophobic and Van der Waals interactions with residues Ala81, Ala111, Phe87, Ile145, Leu142 and Phe15 (Figure 2. A.).

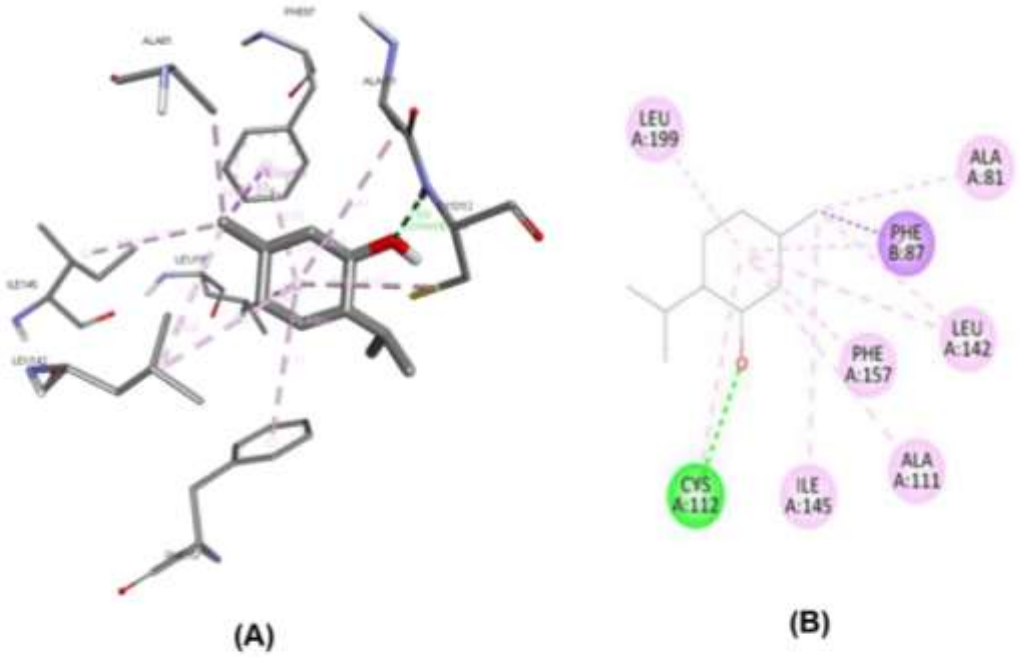
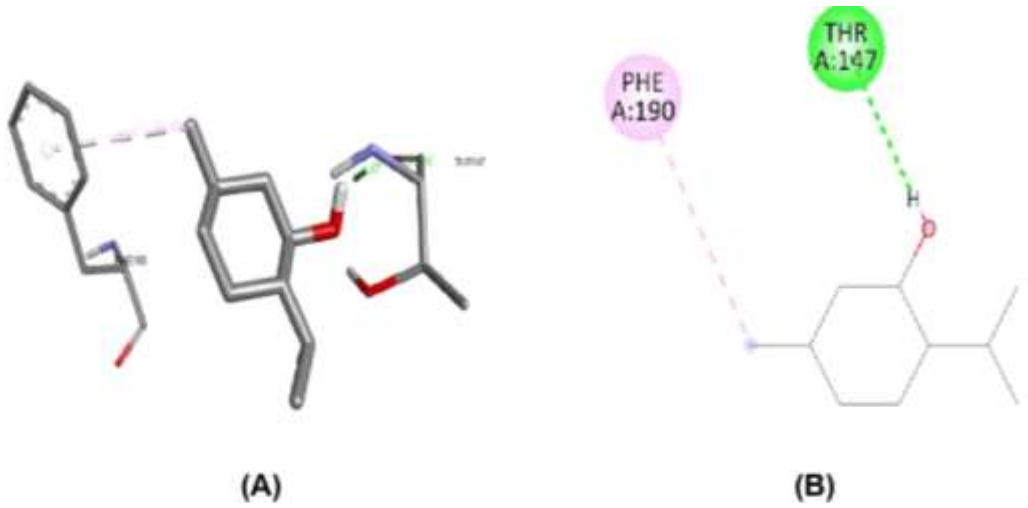


Figure 3. Three-dimensional (A) and two-dimensional (B) interaction of thymol with active site of FabH enzyme (PDB ID: 3IL7) of *S. aureus*.

The purple dotted lines represent non-covalent interactions that contribute to ligand binding within the catalytic site. Among the specific interactions of thymol with residues in the active site, a hydrogen bond with Cys112, a key amino acid in FabH catalysis, stands out (Figure 2.B).

Molecular interactions between thymol and the active site of the *E. coli* dihydropteroate synthase enzyme DHPS (PDB ID:1AJ0) show hydrogen bonding of the thymol hydroxyl group with Thr147 and a hydrophobic interaction of the aromatic ring with Phe190 within the catalytic site (Figure 3.A). These interactions contribute to the stability of the ligand-enzyme complex, which could be associated with thymol interfering with the catalytic function of DHPS, affecting the bacterial metabolism (Figure 3.B).



320 **Figure 4.** Three-dimensional (A) and two-dimensional (B) interaction of thymol with
321 the active site of the DHPS enzyme (PDB ID:1AJ0) of *E. coli*.

322

323 **4. Discusión**

324 The essential oil of *L. graveolens* has bacteriostatic and bactericidal effects on
325 multidrug-resistant bacterial strains isolated from cases of bovine mastitis. The
326 antimicrobial activity of EALg is associated with its higher concentration of
327 monoterpene compounds with antibacterial effects, such as thymol, which acts at
328 the level of bacterial cell membranes [30-33]. The effect of EALg was greater on
329 Gram positive bacteria, such as *S. aureus* and *M. caseolyticus*, due to significantly
330 lower MIC and MBC values compared to Gram negative strains. However, its
331 antibacterial activity is similar to that of kanamycin, so it may represent a therapeutic
332 alternative in the management of intramammary infections in dairy cattle, given the
333 growing problem of antimicrobial resistance [14, 34].

334 Regarding S. maltophilia, an emerging bacterium that is difficult to treat [35], AELg
335 showed antibacterial activity with MICs and MBCs at very low concentrations,
336 confirming the possibility of using essential oils as alternative or adjuvant agents in

337 the treatment of bovine mastitis [36, 37]. However, the MICs found in this study differ
338 from those reported in other research. For *L. graveolens* essential oil, MICs of 0.17
339 mg/mL against *S. aureus* have been reported [38, 39], y Calamaco [40] reported
340 MICs for *L. graveolens* extract of 1.5 mg/mL for *E. coli* and 1.0 mg/mL for *S. aureus*.
341 The differences in antibacterial activity are attributed to the phytochemical
342 composition of the oil and the specific intrinsic genotypic characteristics of each
343 bacterial strain associated with multidrug-resistant bovine mastitis [34, 41].

344 AELg induces progressive destabilization of the cytoplasmic membrane, leading to
345 complete rupture as evidenced in this study. Furthermore, several authors mention
346 that it can affect the transmembrane potential, similar to the effect of surfactants
347 such as polymyxin B. It also affects ATP synthesis by interfering with the respiratory
348 chain and ATP synthase. At the structural level, it causes cytomembrane separation,
349 affecting cellular integrity. These multiple mechanisms of action of AELg in bacteria,
350 centered on plasma membrane dysfunction and metabolic energy collapse, suggest
351 a low potential for the development of bacterial resistance and reinforce its value as
352 a natural antimicrobial [11, 12, 44].

353 Gas chromatography-mass spectrometry (GC-MS) analysis of the AELg revealed a
354 composition dominated by monoterpenes, with thymol being the major component
355 at 68.9%. The predominance of thymol is characteristic of high-quality Mexican
356 oregano (*L. graveolens*); this compound is recognized for its high biological activity
357 as an antimicrobial, antifungal, and antioxidant agent. [10, 13].

358 The presence of p-cymene isomers, a direct biosynthetic precursor of thymol and a
359 synergistic agent in cell membrane dysfunction, enhances the antimicrobial activity
360 of essential oil of *Lippia* spp [32]. The presence of α-terpineol and terpinene 4-
361 acetate has been shown to contribute to the oil's antibacterial properties, while
362 methyl ethers such as 3-methoxy-p-cymene can influence its lipophilicity [45]. In vitro
363 studies on Gram negative enterobacteria from aquaculture have shown that a higher
364 concentration of thymol in *Lippia* spp. essential oil is associated with greater
365 antibacterial activity [32, 43].

366 The hemolytic activity found in this study, which was dependent on AELg
367 concentrations, may be associated with the synergy of the oil's compounds and their
368 direct interaction with the plasma membrane, causing erythrocyte rupture and
369 hemoglobin release [46]. This effect coincides with that reported by Neira [47] who
370 described how L. origanoides thymol chemotype essential oils act as irritants,
371 showing signs of acute toxicity. Similar behavior was found in cytotoxicity studies of
372 peritoneal macrophages and L. mexicana promastigotes, where Lippia spp.
373 essential oils, thymol, and carvacrol were evaluated separately. Thymol had the
374 greatest cytotoxic effect; however, in essential oils rich in thymol, the cytotoxic effect
375 was lower, demonstrating that interactions among the oil compounds reduce the
376 cytotoxic effect of thymol [48]. In our study, the absence of hemolysis at a
377 concentration of 0.05 mg/mL suggests the existence of a minimum threshold for
378 establishing safe limits in therapeutic applications.

379 In molecular docking, thymol showed moderate binding affinities to the enzymes
380 DHPS (-6.14 kcal/mol) and FabH (-5.84 kcal/mol), consistent with its nature as a
381 hydrophobic phenolic monoterpene with a single polar functional group (-OH)
382 capable of forming specific hydrogen bonds [49]. In DHPS, interactions with Phe190
383 and Thr147 suggest that the molecule is primarily stabilized by hydrophobic
384 contacts. In FabH, thymol interacted at the enzyme's catalytic site with Cys112, a
385 nucleophilic residue belonging to the catalytic triad (Cys112, His244, and Asn274),
386 suggesting potential inhibition of fatty acid biosynthesis.

387 Even though the binding energies obtained are lower in magnitude than those
388 reported for functionalized thymol derivatives ($\Delta G \approx -7.3$ and -19.70 kcal/mol, DHPS
389 and FabH, respectively [24, 25], this is explained by the absence of additional groups
390 included by the authors, capable of generating electrostatic or multiple-bridge
391 interactions. Structurally, thymol has a hydrophobic nature, which favors its binding
392 to nonpolar cavities, while its phenolic group participates in one or two directed
393 interactions, which is consistent with the fact that simple phenolic compounds act as
394 non-covalent modulators of bacterial enzymes involved in the biosynthesis of folates
395 and fatty acids.

The results obtained regarding antibacterial activity, hemolytic effects, and molecular docking of the major compound of AELg, reported in this study, support the possibility that thymol acts on multiple bacterial targets through a complex of weak but cooperative interactions [49, 50], therefore it is necessary to carry out complementary evaluations to determine the mechanisms of action at the level of cell membranes of the bacteria associated with bovine mastitis.

5. Conclusion

The essential oil of *L. graveolens* exhibited antibacterial activity against multidrug-resistant bacteria isolated from bovine mastitis, with a bactericidal effect against 100% of the strains evaluated. The AELg has a thymol chemotype abundance of 68.9%, showed hemolytic activity and cytoplasmic membrane damage depending on the concentration. Molecular docking indicates a moderate affinity of thymol for the enzymes DHPS and FabH, primarily through hydrophobic contacts and hydrogen bonds, suggesting an inhibitory effect on essential bacterial metabolic pathways. This suggests that AELg could represent a therapeutic alternative against multidrug-resistant bacteria. However, cytotoxicity studies with cell lines are recommended to ensure its safety before its use *in vivo* models.

CRediT authorship contribution statement

Jorge Vargas-Monter: Writing – original draft, conceptualization, methodology, data curation, formal analysis. Benjamin Valladares-Carranza, Armando Peláez-Acero: formal analysis, validation. Olmedo-Jurez Agustin: writing – review and editing, formal analysis. María Inés Nicolás-Vázquez, Montserrath Abigail León-Flores: methodology, data curation, formal analysis. Adrian Zaragoza-Bastida, Nallely Rivero-Perez: Conceptualization, writing – original draft, Investigation, resources, methodology, writing – review and editing, supervision, formal analysis, validation.

Ethical approval

This research did not involve testing on humans or animals, therefore approval from an ethics committee was not required.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

The data supporting the study findings are available upon request to the corresponding author.

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13 ANEXOS

Congreso Internacional

Ciencias Veterinarias y Producción Animal

26 y 27 de octubre de 2023

"Una salud y estrategias no farmacológicas que promuevan la salud y producción animal sustentable"

ConcerVet

GEN 074/23

Memorias en extenso 2023

PREVALENCIA DE MASTITIS EN PEQUEÑAS UNIDADES DE PRODUCCIÓN DE LECHE DEL VALLE DEL MEZQUITAL EN EL ESTADO DE HIDALGO MÉXICO

PREVALENCE OF MASTITIS IN SMALL FARMS DAIRY IN THE MEZQUITAL VALLEY IN THE STATE OF HIDALGO MEXICO

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RESUMEN

El objetivo fue determinar la prevalencia de mastitis en las vacas en unidades de producción en tres municipios del Valle del Mezquital Hidalgo. Se realizó prueba de mastitis california (CTM) a 174 vacas para determinar la prevalencia de mastitis a nivel de vaca y cuarto mamario. Para la identificación de los factores asociados a mastitis se realizó una guía de verificación para 10 aspectos relacionados con la higiene de la vaca, el equipo de ordeño, el ordeñador y el proceso de la rutina de ordeño. Los factores asociados a la prevalencia de mastitis se determinaron por análisis multivariado de factores de correspondencia múltiple. La mayor prevalencia de mastitis en las vacas fue en el municipio de San Salvador e Ixmiquilpan, con valores de 52.7 % y 42.9 % respectivamente, a nivel de cuartos mamarios se encontró prevalencia del 37.8 % y 35.1 % en los municipios de San Salvador e Ixmiquilpan. Los factores con mayor correlación a la prevalencia de mastitis son el uso del sellador, despunte, presencia de sala de ordeño. En la definición de programas de prevención y control de mastitis se deben considerar estos aspectos inherentes a la rutina de ordeño y al ambiente en el que vive el animal.

Palabras clave: mastitis bovina, ganado lechero, factores de riesgo.

ABSTRACT

The objective was to determine the prevalence of mastitis in cow's small farms dairy in the Mezquital Hidalgo Valley. California mastitis test (CTM) was performed on 174 cows to determine the prevalence of mastitis at the cow and mammary quarter level. To identify the factors associated with mastitis, a verification guide was created for 10 aspects related to the hygiene of the cow, the milking equipment, the milker, and the milking routine process. Descriptive and variance statistics were obtained. The identification of factors associated with the prevalence of mastitis was determined by multivariate analysis of multiple correspondence factors. The highest prevalence of mastitis in cows was in the municipality of San Salvador and Ixmiquilpan, with values of 52.7 % and 42.9 % respectively. At the level of mammary quarters, a prevalence of 37.8 % and 35.1 % was found in San Salvador and Ixmiquilpan. The factors with correlation to the prevalence of mastitis are the use of sealant, trimming, presence of milking zone. When defining mastitis prevention and control programs, these aspects inherent to the milking routine and the environment in which the animal lives must be considered.
Key words: bovine mastitis, dairy cattle, risk factors.

2024-34-VET

Actividad antibacteriana del extracto hidro alcohólico de *Lippia graveolens* sobre *S. aureus* y *E. coli*

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RESUMEN

La mastitis bovina es una efermedad causada principalmente por bacterias que destruyen el tejido secretor en la glandula mamaria, afecta la producción, la calidad e inocuidad de la leche e incrementa los costos de producción, esto debido al uso de antimicrobianos. El uso continuo de dichos ingredientes activos ha inducido el desarrollo de resistencia por parte de las bacterias asociadas a mastitis, tal es el caso de *Staphylococcus aureus* y *Escherichia coli*. Actualmente se ha buscado el desarrollo de alternativas de tratamiento para la enfermedad, destacando el uso de extractos de plantas con actividad antimicrobiana que sean eficaces al inhibir o matar a los microorganismos. El objetivo del presente estudio fue determinar la actividad antibacteriana *in vitro* del extracto hidroalcohólico de *Lippia graveolens* sobre *Staphylococcus aureus* y *Escherichia coli*. Se obtuvo un extracto hidroalcohólico (EHA-Lg) a partir de las partes aéreas de *L. graveolens* (250 g) por medio de la técnica de maceración, en una solución agua:metanol (70:30). Como material biológico se emplearon cepas de referencia (ATCC) de *E. coli*^{35218} y *S. aureus*^{6538}. La actividad antibacteriana del extracto se evaluó a través de la determinación de la Concentración Mínima Inhibitoria (CMI) y Concentración Mínima Bactericida (CMB). Se evaluaron concentraciones del extracto de 200 a 3.12 mg/mL, por triplicado, como control positivo se empleó Kanamicina a concentraciones de 64 a 0.5 µg/mL. el EHA-Lg presentó mejor actividad inhibitoria frente a *S. aureus* (0.39 mg/mL) en comparación con *E. coli* (3.12 mg/mL), en cuanto a la actividad bactericida, dicho extracto presentó actividad a concentraciones de 0.78 y 6.25 mg/mL sobre *S. aureus* y *E. coli*, respectivamente. En estudios previos se ha reportado la actividad antibacteriana de *L. graveolens*, por su parte Hernández *et al*. (2003), determinaron que el extracto hexanico de *L. graveolens* presentó actividad tanto frente a bacterias Gram positivas como Gram negativas, en un estudio similar Calva-Cruz y colaboradores, determinaron que las oleorresinas de dicha especie presentaron actividad bactericida frente a dos cepas bacterianas, incluída *S. aureus* (Calva-Cruz *et al*.,2021), recientemente, se reportó que el uso del aceite

esencial de esta especie vegetal fue capaz de general halos de inhibición de hasta 30 mm de diámetro (Marín-Tinoco *et al.*, 2023). Los estudios antes mencionados confirman la eficacia antibacteriana de *L. graveolens*. El extracto hidroalcohólico de *Lippia graveolens* presentó actividad antibacteriana frente a *S. aureus* y *E. coli* asociados a mastitis bovina, sin embargo, se sugiere realizar futuras investigaciones para determinar la posible vía de administración del tratamiento.

Palabras clave: Actividad antibacteriana, *Lippia graveolens*, mastitis bovina.

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UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA
INSTITUTO DE CIENCIAS AGRÍCOLAS

En el marco del evento conjunto:

XXIV

REUNIÓN INTERNACIONAL
Sobre Producción de Carne y Leche en Climas Cálidos
IV Congreso Internacional
de Ciencias Veterinarias y Producción Animal

SE OTORGA LA PRESENTE

CONSTANCIA A:

J. Vargas-Monter, N. Rivero-Perez, B. Valladares-Carranza, A.L. Morales-Ubaldo, L. Rangel López, C. Rosenfeld-Miranda, B. Benavides-Benavides, D.M. Sifuentes-

Por su participación con la **Ponencia Corta** titulada:

FACTORES ASOCIADOS A MASTITIS BOVINA EN EL VALLE DEL MEZQUITAL HIDALGO

En el marco del evento conjunto llevada a cabo de forma hibrida en el Departamento de Informática y Biblioteca de la Universidad Autónoma de Baja California, Campus Mexicali, Baja California, México, los días 17 y 18 de octubre de 2024.

"Por la realización plena del ser"

DR. ULISES MACÍAS CRUZ
Presidente del Comité Organizador

UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA

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Cuerpo Académico de Fisiología & Genética Animal

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2025-23-VET

Aislamiento de *Stenotrophomonas maltophilia en muestras de vacas con* mastitis en unidades de producción de lechería en el Estado de Hidalgo

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RESUMEN

La mastitis afecta el bienestar de la vaca, reduce la producción de la leche y genera pérdidas económicas en los establos. La presencia de la enfermedad se asocia a la interacción de factores propios de la vaca, el ambiente de corrales, las prácticas de manejo en el ordeño y la presencia de microorganismos patógenos. El objetivo del trabajo fue identificar las bacterias asociadas a mastitis en vacas lecheras. Se colectaron muestras de leche de vacas con mastitis subclínica positivas a prueba de california grado 3 en 16 unidades de producción de lecherías en el estado de Hidalgo. Para el aislamiento bacteriano y purificación de las cepas se realizó por siembra en extensión superficial de placa de agar MacConkey, la identificación preliminar se realizó por pruebas bioquímicas, para identificación de género y especie se utilizó espectrometría de masas (MALDI-TOF MS 1000). Se aislaron 6 bacilos Gram negativos catalasa positiva, por medio del sistema MALDI-TOF se identificó que una de las seis cepas correspondió con *Stenotrophomonas maltophilia*, bacteria poco frecuente en mastitis bovina y con multirresistencia antimicrobiana (Kabui *et al.*, 2024). He y colaboradores 2023, reportaron que dietas altas en concentrados inducen mastitis en las vacas lecheras y que la presencia de *S. maltophilia* aumentaba significativamente tanto en la microbiota del rumen como en la de la leche. Se aisló *S. maltophilia* de muestras con mastitis en unidades de producción de lechería en el Estado de Hidalgo, bacteria con reporte de migración de rumen a leche y con multirresistencia antimicrobiana.

Palabras clave: mastitis bovina, MALDI-TOF, vacas lecheras

1. He, Z., Zhao, C., He, Y. 2023. Enterogenic *Stenotrophomonas maltophilia* migrates to the mammary gland to induce mastitis by activating the calcium-ROS-AMPK-mTOR-autophagy pathway. J Animal Sci Biotechnol 14, 157. https://doi.org/10.1186/s40104-023-00952-y