

Antibiofilm Efficacy of Curcumin: FTIR Spectroscopy and Molecular Docking Evidence for Matrix Disruption in Bovine Mastitis Pathogens

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Bovine mastitis, an inflammatory condition of the mammary gland predominantly caused by microbial infection, remains a substantial challenge to global dairy production. Although the broad-spectrum antibacterial activity of curcumin is well-documented, its precise mechanisms of action against the extracellular matrix of mastitis-associated biofilms are poorly understood. This study evaluated the efficacy of curcumin against biofilms formed by three key mastitis pathogens *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Escherichia coli*, and characterized its ability to alter the biochemical architecture of the biofilm matrix. We assessed the antibiofilm efficacy of curcumin using biofilm formation, dispersion, and membrane leakage assays. Fourier-transform infrared (FTIR) spectroscopy was employed to determine the associated biochemical changes in the biofilm matrix, and molecular docking analysis was performed to evaluate the potential binding of curcumin to selected biofilm matrix proteins. Curcumin significantly ($p < 0.05$) inhibited biofilm formation, promoted the dispersion of mature biofilms, and induced biomolecule leakage. FTIR analysis of *S. aureus* and *S. dysgalactiae* biofilms treated with 600 $\mu\text{g/mL}$ curcumin showed spectral alterations in regions associated with lipids (2800–3000 cm^{-1}), proteins (Amide I & II, 1600–1700 cm^{-1} & 1480–1575 cm^{-1}), nucleic acids (1450–1200 cm^{-1}), and carbohydrates (1150–1000 cm^{-1}). For *E. coli*, spectral changes occurred in regions associated with lipids, proteins, and nucleic acids, but not carbohydrates. Molecular docking revealed that curcumin exhibited strong binding affinity for the biofilm-associated aminopeptidases from *S. dysgalactiae* (A0A380JWY5), *E. coli* (P04825), and *S. aureus* (Q6GIP8) with Vina scores of -7.6 kcal/mol, -8.2 kcal/mol, and -7.4 kcal/mol, respectively. These findings demonstrate that the antibiofilm activity of curcumin is mediated through the targeted disruption of the extracellular matrix in mastitis pathogens, offering new insights into its potential therapeutic applications.

Keywords: Antibacterial, antibiofilm, curcumin, biofilm, bovine mastitis

Received: February 2026; Accepted: July 2026

Bovine mastitis is an inflammation of the breast tissue, often associated with infection. It commonly occurs in lactating animals due to milk stasis when the milk is not properly drained from the breast. The symptoms of mastitis include swelling, warmth, redness, fever and chills [1]. In severe cases, an abscess can form, requiring medical intervention. According to Morales-Ubaldo et al. [2], bovine mastitis is one of the most serious illnesses in dairy herds globally as it reduces output and culls, incurring substantial economic losses. The primary pathogens causing bovine mastitis are bacteria, with *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Escherichia coli* being the most common [3, 4]. *S. aureus* can cause both clinical and subclinical mastitis, often leading to persistent infections due to its ability to form biofilms. *S. agalactiae* is usually associated with subclinical infections and can spread between cows and heifers

in dairy farms. *E. coli*, typically an environmental pathogen, can cause acute and severe mastitis.

Biofilm formation is a significant virulence factor in mastitis-causing pathogens, contributing to persistent infections and treatment resistance [5, 6]. Studies have shown that *S. aureus*, *E. coli*, and *S. dysgalactiae* commonly form biofilms, with *S. aureus* demonstrating the highest biofilm-forming capacity [7]. Biofilm-embedded bacteria exhibit increased resistance to antibiotics compared to their planktonic counterparts, with enrofloxacin, ceftriaxone, and doxycycline showing the highest efficacy against biofilm-associated cells [7, 8]. The biofilm formation process involves bacterial invasion, adherence, and persistence, with species-specific adaptive strategies [9]. Research on *E. coli* and *S. uberis* biofilms has also revealed both similarities and differences in

their formation mechanisms [9]. Understanding biofilm dynamics is crucial for developing effective mastitis control strategies and ensuring milk quality and safety [6].

Curcumin is a polyphenolic compound derived from the rhizome of the *Curcuma longa* plant, commonly known as turmeric. Its chemical structure includes two aromatic rings containing o-methoxy phenolic groups connected by a seven-carbon linker that forms an α,β -unsaturated β -diketone moiety [10]. This structure is responsible for its antioxidant, anti-inflammatory, and antibacterial properties. Praditya et al. [11] has stated that curcumin has potential in treating infectious diseases caused by bacteria, viruses, and fungi, as well as non-infectious conditions such as cancer, diabetes, cardiovascular diseases, and neurodegenerative disorders. Curcumin has shown potential as an anti-inflammatory agent against *Staphylococcus aureus*-induced mastitis by suppressing the TLR4-mediated NF- κ B signaling pathway [12]. It attenuates inflammatory cytokine levels, reduces oxidative stress markers, and restores mammary gland histology [13]. This evidence suggests that curcumin could be a promising alternative to antibiotics for treating mastitis, addressing concerns about anti-microbial resistance.

Despite the known antimicrobial properties of curcumin [14–16], there is a gap in understanding its specific effects on the biochemical composition of biofilms formed by mastitis pathogens, which are increasingly resistant to conventional antibiotics. Existing studies have primarily focused on curcumin's broad-spectrum antibacterial activity, but its mechanistic impact on biofilm matrices particularly the alterations in proteins, polysaccharides, and eDNA in extracellular matrix remains poorly elucidated. This lack of detailed knowledge hinders the development of curcumin as a viable alternative or adjunct therapy for biofilm-associated bovine mastitis, necessitating systematic investigations to delineate its biochemical modulatory effects and potential synergies with existing treatments. Therefore, the objective of the present study was to determine antibiofilm efficacy of curcumin compound against *S. aureus*, *S. dysgalactiae*, and *E. coli*, and its effects on biochemical composition of those mastitis pathogens biofilm.

EXPERIMENTAL

Test Compounds

Curcumin (95–99% purity) was purchased from Bangalore Sales Corporation, India. For all assays, curcumin solution was prepared in 5% (v/v) dimethyl sulfoxide (DMSO).

Test Pathogens

Mastitis pathogens *Staphylococcus aureus* ATCC 43300, *Streptococcus dysgalactiae* ATCC 12394,

and *Escherichia coli* ATCC 25922 were obtained from the Veterinary Laboratory Service Unit (VLSU), Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). Each pathogen was cultured overnight in tryptic soy broth (TSB) at 37°C. Purity of the culture was routinely confirmed by Gram staining, examination of colonial morphology, and standard biochemical assays. Bacterial density was standardized for all experiments by adjusting the optical density (OD₆₀₀) to 0.5 using a spectrophotometer.

Minimum Inhibitory Concentration Assay

Microbroth dilution method and resazurin dye were used to determine the minimum inhibitory concentrations (MIC) against the examined bacteria. Overnight-grown culture strains with a 0.5 McFarland turbidity standard containing about 10⁸ CFU/mL were cultivated on 96-well plates. Various concentrations of curcumin (18.75, 37.5, 75, 150, 300, and 600 μ g/mL) were tested. Ciprofloxacin (1 μ g/mL) and 0.5% (v/v) DMSO served as the positive and negative controls, respectively, for all mastitis pathogens. The plates were covered with plastic film and left to incubate for 16 hours at 37 °C. The microplates were then filled with 30 μ L of 0.02% (w/v) resazurin aqueous solution and incubated for two hours at 37 °C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited microbial growth, indicated by the absence of a color change in resazurin from blue to pink.

Biofilm Formation Assay

The biofilm formation assay was performed following the method described by Amran et al. [17]. Briefly, 100 μ L of bacterial suspension was dispensed into microplate wells, followed by the addition of 100 μ L curcumin solution (18.75, 37.5, 75, 150, 300, and 600 μ g/mL). The microplate was incubated at 37°C for 24 h. Ciprofloxacin (1 μ g/mL) and 0.5% (v/v) DMSO served as the positive and negative controls, respectively, for all mastitis pathogens. After incubation, planktonic cells in the stationary phase were removed from the 96-well microplate. The biofilm fractions were washed twice with saline solution to remove non-adherent cells. Following heat fixation (60°C, 15 min), biofilms were stained with 200 μ L of 1% (w/v) crystal violet solution and incubated at room temperature for 15 min. To remove residual stain, plates were rinsed three times with sterile distilled water. Finally, 200 μ L absolute ethanol was added to dissolve the stained biofilm, and absorbance was measured at 600 nm using a microplate reader (BioTek Synergy).

Biofilm Dispersion Assay

A 200 μ L aliquot of bacterial suspension was dispensed into microplate wells and incubated for 24 h at 37°C. After incubation, planktonic cells in the stationary phase were removed from the 96-well microplate.

Biofilms were washed twice with saline solution to remove non-adherent cells. Next, 200 μ L of curcumin solution was added to each well (18.75, 37.5, 75, 150, 300, and 600 μ g/mL). Ciprofloxacin (1 μ g/mL) and 0.5% (v/v) DMSO served as the positive and negative controls, respectively, for all mastitis pathogens. The microplate was incubated for an additional 24 h at 37°C. Following this second incubation, planktonic cells were again removed, and biofilms were washed twice with saline solution. Biofilms were then heat-fixed at 60°C for 15 min and stained with 200 μ L of 1% (w/v) crystal violet solution for 15 min at room temperature. Excess stain was removed by rinsing plates three times with sterile distilled water. Finally, 200 μ L of absolute ethanol was added to dissolve the stained biofilms, and absorbance was measured at 600 nm using a microplate reader (BioTek Synergy).

Biofilm Leakage Assay

The biofilm leakage assay was performed following the method of Sulistyani et al. [18], with modifications to the microbial fractions and analyte types. Biofilms were grown in 6-well plates at 37°C for 24 h. After incubation, the growth medium was discarded, and biofilms were washed twice with saline buffer. Test wells received 2 mL of curcumin suspension (600 μ g/mL) plus 2 mL of TSB, while control wells contained 2 mL of saline buffer plus 2 mL of 0.5% (v/v) DMSO. Following a 24 h incubation at 37°C, the medium was centrifuged at 5,000 $\times$ g for 15 min, and the supernatant was collected for spectrophotometric analysis: nucleic acids (260 nm), carbohydrates (phenol-sulfuric acid assay, 595 nm), and proteins (Bradford assay, 595 nm).

FTIR Spectroscopy

FTIR spectroscopy of biofilms was performed according to the method described by Hamdan et al. [19]. Biofilms were grown on glass coverslips in 6-well plates at 37°C for 24 h. For treatment, 2 mL of bacterial suspension was combined with 2 mL of curcumin solution (600 μ g/mL), while negative controls contained 2 mL of bacterial suspension mixed with 2 mL of 0.5% (v/v) DMSO. After incubation, the coverslips were washed twice with distilled water, and dried at 40°C for 40 min. FTIR spectra were acquired using a Bruker Tensor 27 spectrometer equipped with an HTS-XT microplate adapter (Bruker Optics GmbH). Measurements were performed in transmission mode, scanning from 4000 to 600 cm^{-1} .

Molecular Docking Analysis

Molecular docking was performed using the publicly accessible CB-DOCK2 program (<https://cadd.labshare.cn/cb-dock2/php/index.php>) to evaluate the binding affinity of curcumin for the aminopeptidase protein, a key component of the biofilm matrix in the mastitis

pathogens *Streptococcus dysgalactiae* (A0A380JWY5), *Escherichia coli* (P04825), and *Staphylococcus aureus* (Q6GIP8). The three-dimensional structures of the proteins were retrieved from the AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk>) by their UniProt accession code while the ligand (curcumin) was retrieved PubChem database (<https://pubchem.ncbi.nlm.nih.gov>). Tetracycline and ciprofloxacin retrieved from PubChem were used as positive controls. CB-DOCK2, like most automated docking servers, performed a standard pre-processing pipeline on the submitted structures. For the protein, it removed water molecules and any heteroatoms (like ions or co-factors) that are not part of the standard amino acids by default. It also added polar hydrogens and calculated partial charges to prepare the protein for docking. For the ligand, it protonated the ligand molecule at a physiological pH (around 7.4) and assigned appropriate charges and atom types.

Data Analysis

All assays were performed in triplicate. Mean absorbance values were used to calculate the percentage of: (1) biofilm formation inhibition, (2) biofilm dispersion, and (3) biofilm leakage according to the following formulas:

- (1) % Inhibition = $[(\text{OD}_{\text{negative control}} - \text{OD}_{\text{test}}) / \text{OD}_{\text{negative control}}] \times 100$
- (2) % Dispersion = $[(\text{OD}_{\text{negative control}} - \text{OD}_{\text{test}}) / \text{OD}_{\text{negative control}}] \times 100$
- (3) % Leakage = $[(\text{OD}_{\text{test}} - \text{OD}_{\text{negative control}}) / \text{OD}_{\text{negative control}}] \times 100$

Significant differences between control and test groups ($p < 0.05$) were determined using independent t-tests.

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration

Curcumin demonstrated potent antibacterial activity against all tested mastitis pathogens, exhibiting a consistent MIC value of 18.75 μ g/mL against *S. aureus*, *S. dysgalactiae*, and *E. coli*.

Inhibition of Biofilm Formation by Curcumin

Curcumin inhibited biofilm formation in *S. aureus*, *S. dysgalactiae*, and *E. coli* in a clear, dose-dependent manner across concentrations ranging from 18.75 μ g/mL to 600 μ g/mL (Fig. 1). Among the tested pathogens, curcumin exhibited the highest inhibitory efficacy against *E. coli* across all concentrations, while *S. aureus* displayed the lowest initial susceptibility but still reached over 40% inhibition at the maximum dose of 600 μ g/mL.

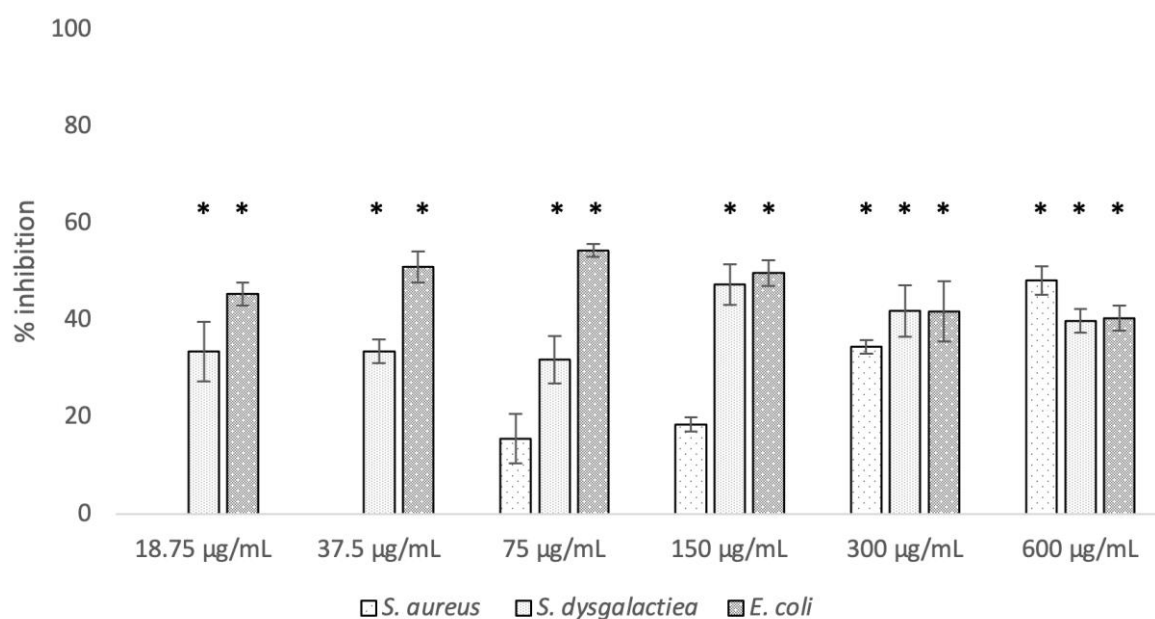


Figure 1. Inhibition of biofilm formation by curcumin at different concentrations.

Biofilm Dispersion by Curcumin

Treatment with curcumin was found to significantly ($p < 0.05$) disperse the preformed biofilms at all concentrations (Fig. 2). *E. coli* biofilm showed the

highest percentage dispersion across all concentrations except 37.5 µg/mL and 75 µg/mL, indicating its preformed biofilm is more susceptible to curcumin. The data obtained from biofilm dispersion assay suggests the damage of the biofilm matrix.

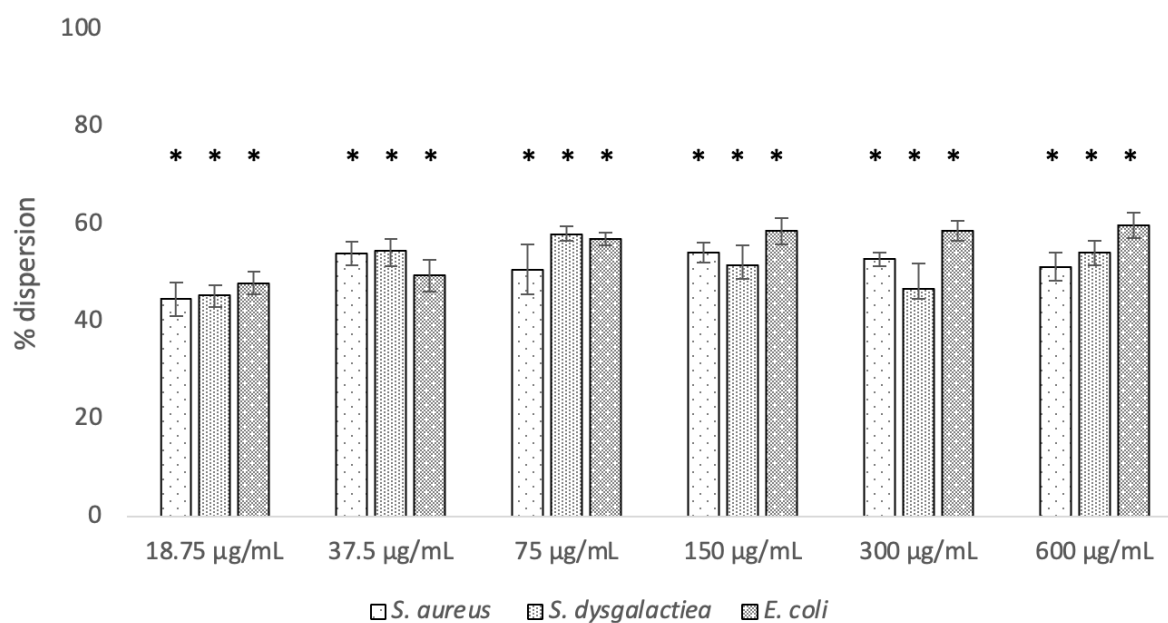


Figure 2. Biofilm dispersion by curcumin at different concentrations.

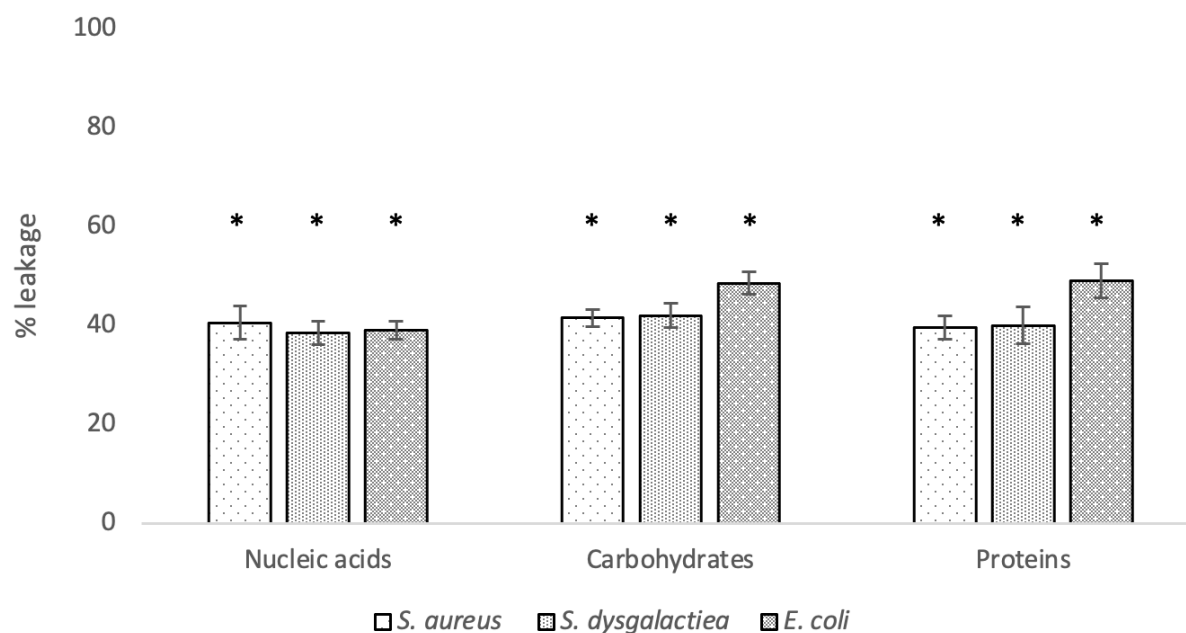


Figure 3. Biofilm leakage following exposure to curcumin at 600 µg/mL.

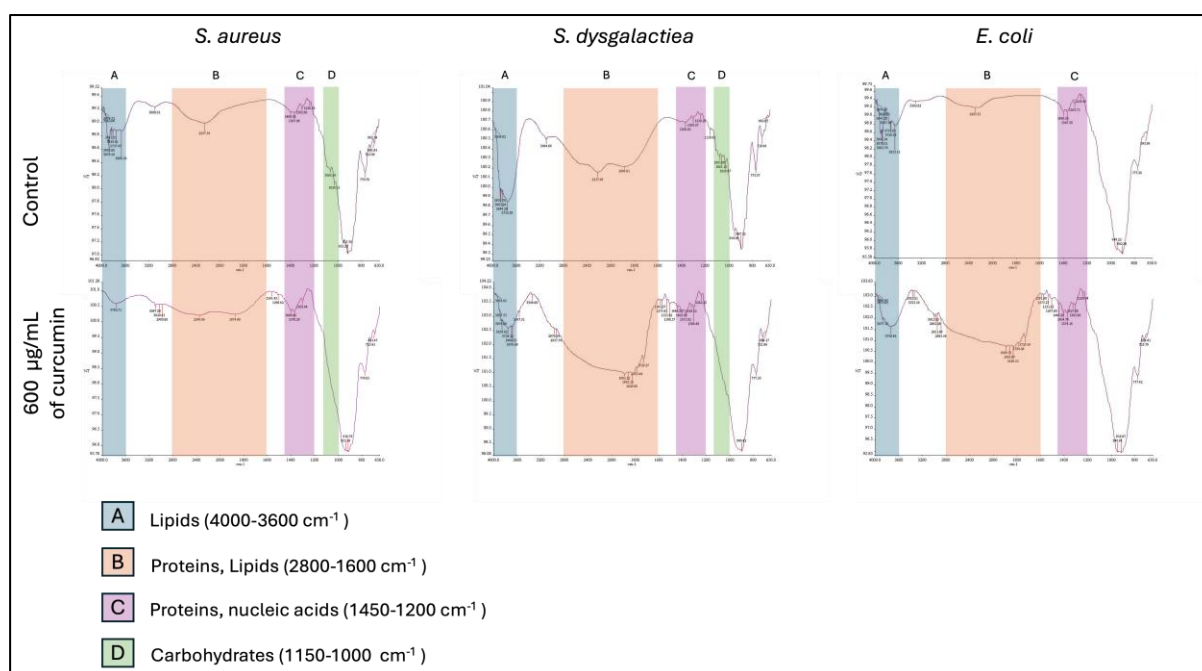


Figure 4. FTIR spectral changes in biofilms following exposure to curcumin at 600 µg/mL.

Biofilm Damage by Curcumin

The leaked cellular components such as nucleic acids, carbohydrates, and proteins were measured to assess biofilm damage (Figure 3). Treatment with 600 µg/mL curcumin significantly ($p < 0.05$) increased the concentration of those cellular components in growth medium. *E. coli* biofilm showed the highest percentage leakage of carbohydrates and proteins.

These results indicate that curcumin disrupt the integrity of the biofilm matrix, leading to the release of intracellular or matrix-embedded components.

Biochemical Changes in the Biofilm Following Treatment with Curcumin

FTIR spectroscopy was also used to determine biochemical changes underlying antibiofilm mechanism

of curcumin. The FTIR spectral changes between the control biofilm and treated biofilm may indicate alterations in the biochemical composition of the biofilms due to the treatment (Fig. 4, Table 1). In the biofilms of *S. aureus* and *S. dysgalactiae* treated with 600 µg/mL of curcumin, FTIR spectral peaks in regions associated with lipids (4000–3600 cm⁻¹), proteins (2800–1600 cm⁻¹), nucleic acids (1450–1200 cm⁻¹), and carbohydrates (1150–1000 cm⁻¹) were altered. In contrast, for *E. coli*, spectral changes occurred only in the regions associated with lipids, proteins, and nucleic acids. The reduction in FTIR spectral peak intensities (4000–3600 cm⁻¹) and enlargement of FTIR spectral peaks (2800–1600 cm⁻¹) observed herein may indicate damage of the biofilm

matrix, cell lysis and active defence response (EPS overproduction) in the biofilms.

Protein-Curcumin Interactions

Aminopeptidases play a key role in maintaining the biofilm matrix and facilitating the metabolic adaptations required for persistent infection, making them strategic targets for antibiofilm agent discovery. Molecular docking results (Table 2, Figure 5) indicate that curcumin bound strongly to aminopeptidases from *S. dysgalactiae* (A0A380JWY5), *E. coli* (P04825) and *S. aureus* (Q6GIP8), with Vina scores of -7.6 kcal/mol, -8.2 kcal/mol, and -7.4 kcal/mol respectively.

Table 1. Assignment of FTIR spectral peaks corresponding to the major biomolecules in mastitis pathogen biofilms.

Spectral region	Biomolecules	Functional groups	References
4000-3600 cm ⁻¹	Lipids	O-H stretching, N-H stretching, C-H stretching	Fadlelmoula et al. (2022)
2800-1600 cm ⁻¹	Proteins, Lipids	C=O stretching (Amide I), CH ₂ symmetric stretching	Kamaruzzaman et al. (2022)
1450-1200 cm ⁻¹	Proteins, Nucleic acids	C-N stretching vibration/N-H bending vibration (Amide III), P=O symmetric stretching	Davis and Mauer (2010)
1150-1000 cm ⁻¹	Carbohydrates	C-O stretching	Kamaruzzaman et al. (2022)

Table 2. Potential binding of curcumin to aminopeptidase in biofilms.

Pathogen	Uniprot ID	Protein	PubChem CID	Ligand	Vina Score [kcal/mol]	Cavity size
<i>S. dysgalactiae</i>	A0ABU0A5J0	Aminopectidase	54675776	Tetracycline	-8.6	749
			2764	Ciprofloxacin	-7.8	374
			969516	Curcumin	-7.6	749
<i>E. coli</i>	P04825	Aminopectidase	54675776	Tetracycline	-8.2	2180
			2764	Ciprofloxacin	-8.2	1946
			969516	Curcumin	-8.2	2180
<i>S. aureus</i>	Q6GIP8	Aminopectidase	54675776	Tetracycline	-7.6	189
			2764	Ciprofloxacin	-7	189
			969516	Curcumin	-7.4	185

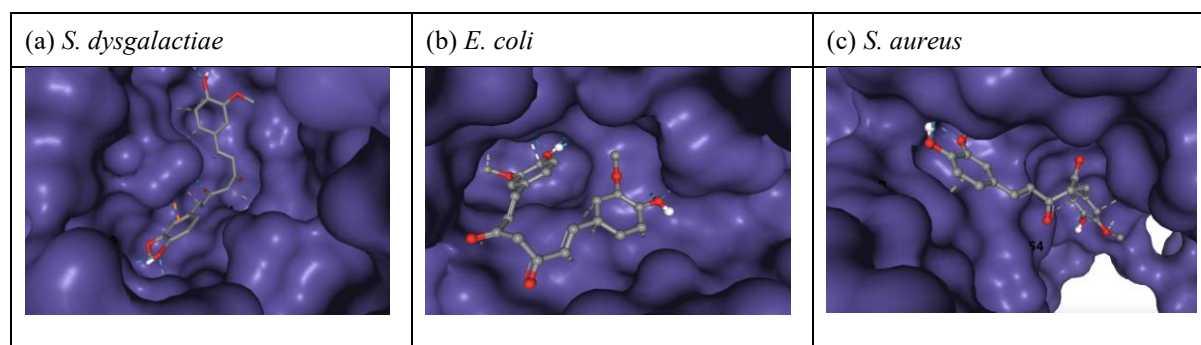


Figure 5. Binding mode of curcumin within the aminopeptidase active site in (a-c). Curcumin is depicted in ball-and-stick form, and the bacterial aminopeptidase is shown as a purple surface. Dashed lines indicate potential interactions, such as hydrogen bonds, between specific atoms of the ligand and amino acid residues of the protein.

A comparison of curcumin's binding affinity with that of antibiotics reveals a context-dependent relationship. For the *S. dysgalactiae* protein, curcumin's binding (-7.6 kcal/mol) was weaker than that of both tetracycline (-8.6 kcal/mol) and ciprofloxacin (-7.8 kcal/mol). In contrast, for the *E. coli* protein, curcumin (-8.2 kcal/mol) exhibited binding strength equal to the antibiotics. For the *S. aureus* protein, curcumin (-7.4 kcal/mol) bound more strongly than ciprofloxacin (-7.0 kcal/mol) but less strongly than tetracycline (-7.6 kcal/mol). Therefore, curcumin does not consistently outperform the antibiotics, as its efficacy is highly dependent on the specific target protein.

DISCUSSION

Curcumin is known to disrupt biofilm formation and reduce bacterial adhesion, thereby enhancing the effectiveness of antimicrobial treatments [20]. This antibiofilm property is particularly valuable in treating mastitis caused by biofilm-forming bacteria. By targeting both planktonic bacteria and biofilms, curcumin can potentially improve treatment outcomes and reduce the recurrence of infections. The present study demonstrates that curcumin effectively inhibited newly formed and preformed biofilms of mastitis pathogens. This result corroborates previous studies demonstrating effectiveness of curcumin in inhibiting biofilm formation and reducing established biofilms of *Streptococcus mutans*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Serratia marcescens* [21-23].

The present study demonstrates that curcumin effectively disrupts preformed biofilms, triggering the leakage of critical biomolecules proteins, nucleic acids, and carbohydrates into the surrounding medium. This leakage confirms the structural disintegration of the biofilm matrix, as evidenced by spectrophotometric quantification of the supernatant following curcumin treatment. The highest concentration of curcumin (600 µg/mL) was selected for the leakage assay to ensure maximal biofilm disruption, allowing clear

detection of released components. Using this concentration helps establish the upper limit of curcumin's antibiofilm efficacy and provides definitive evidence of its ability to compromise membrane integrity and extracellular matrix stability. These findings suggest that curcumin not only disperses biofilms but also induces significant biochemical damage, further supporting its potential as a biofilm-disrupting agent [24-26].

Fourier-transform infrared (FTIR) spectroscopy is a powerful analytical technique used to study the antimicrobial mode of action by analysing the biochemical composition of microbial cells. In FTIR spectroscopy, the laser beam does not deeply penetrate bacterial cells. Instead, it interacts primarily with the surface and near-surface regions of the cell. The present study monitored specific absorption bands corresponding to various biochemical compounds such as proteins, lipids, and nucleic acids in mastitis pathogens biofilm to identify alterations induced by curcumin. This finding allows us to propose antimicrobial mechanism of action and potency of curcumin [27-30].

The extracellular matrix is crucial for the stability and integrity of biofilms, providing a protective environment for microbial cells. Damage to the extracellular matrix disrupts this protective barrier, leading to biofilm dispersal where bacteria disperse from the biofilm matrix into the surrounding environment. The present study demonstrated reduction in spectral peak size and spectral peak broadening following treatment with curcumin. According to Jin et al. [31], FTIR spectral peaks can indeed enlarge after exposure to antimicrobials, with studies demonstrating significant molecular-level changes in bacterial responses. Specifically, long-term low-level exposure drives bacterial cells into changes that cause broader spectral ranges, including alterations in cellular components like fatty acids. In contrast, FTIR spectral peaks also get smaller after exposure to antimicrobials, with consistent evidence across

multiple bacterial species and antimicrobial agents [27-28]. It is understood that FTIR spectral changes in biofilm indicate alterations in bacterial metabolism and extracellular matrix [32]. The FTIR spectroscopic analysis suggests that curcumin exerts its antibiofilm effects through broad and biochemically complex mechanisms, though the response varies meaningfully across bacterial species. This species-specific difference may reflect intrinsic variations in outer membrane architecture or biofilm matrix composition. The overall spectral pattern argues against a single-target action and instead supports a catastrophic, multi-component biochemical assault on mastitis pathogen biofilms.

Lipids are a diverse group of hydrophobic molecules, primarily composed of carbon and hydrogen atoms, with a significantly lower amount of oxygen compared to carbohydrates. Structurally, lipids are characterized by long hydrocarbon chains or rings, making them insoluble in water. In the present study, treatment with curcumin was found to alter FTIR spectral region associated with lipids. Changes in bacterial lipid composition may indicate the sensitivity of bacterial cells to antimicrobial agents [33, 34].

Proteins are complex macromolecules made up of amino acids linked by peptide bonds. They have a hierarchical structure: primary (sequence of amino acids), secondary (alpha helices and beta sheets), tertiary (three-dimensional folding), and quaternary (assembly of multiple polypeptides). The current investigation observed that curcumin administration caused changes in the FTIR spectral area linked to proteins. This result is in line with Hamdan et al. [19] demonstrating that *Etlingera elatior* extracts altered FTIR spectral regions associated with proteins in *S. aureus* biofilm. This FTIR spectral changes suggest altered protein conformation in biofilm [35].

Nucleic acids, which include DNA and RNA, are polymers of nucleotides composed of a sugar, a phosphate group, and a nitrogenous base. DNA (deoxyribonucleic acid) contains the genetic blueprint for the synthesis of proteins and is structured as a double helix. The current study discovered that treatment with curcumin modified the spectral area linked to nucleic acids. This alteration may point out the damage of extracellular nucleic acids. Previous studies have provided insights that antimicrobial agents can inhibit bacteria by damaging nucleic acids [36].

Carbohydrates are composed of carbon, hydrogen, and oxygen, typically in a ratio of 1:2:1. The present study showed that the spectral area linked to carbohydrates changed upon curcumin treatment. This FTIR spectral alteration suggests reduction in the initial adhesion and structural stability of the formed biofilms [37]. Similarly, Johari et al. [33] reported that treatment of *Salmonella typhimurium* biofilm with chloroxylonol-based disinfectants caused alteration in FTIR spectral peaks associated with nucleic acids.

Aminopeptidase was selected as the molecular target for bioinformatic analysis of curcumin's potential antibiofilm activity based on a compelling and convergent body of evidence demonstrating that this enzyme class plays a direct, causal, and evolutionarily conserved role in biofilm formation across multiple bacterial species, including the three mastitis pathogens under investigation. The foundational study by Zhao et al. [38] established that aminopeptidase is not merely an intracellular housekeeping enzyme but functions extracellularly within the biofilm matrix, and critically, that its catalytic activity is essential for the normal development of *Pseudomonas aeruginosa* biofilms. This finding was subsequently extended to *S. aureus* by Singh et al. [39], who demonstrated that the aminopeptidase inhibitor bestatin reduces aminopeptidase activity in a concentration-dependent manner and that this enzymatic inhibition significantly suppresses biofilm formation, establishing a direct pharmacological link between aminopeptidase activity and biofilm persistence. For *E. coli*, the work of Valverde-Pozo et al. [40] provided complementary genetic evidence showing that overexpression of the *pepN* gene, which encodes alanine aminopeptidase, enhances the ability of *E. coli* to form biofilms, confirming that increased aminopeptidase activity directly promotes biofilm development. Collectively, these studies from independent research groups across distinct bacterial species provide strong, multi-layered evidence that aminopeptidase activity is not a passive byproduct of bacterial metabolism but rather an active, required contributor to biofilm formation. This makes aminopeptidase a strategically attractive and mechanistically justified target for bioinformatic screening of potential biofilm inhibitors such as curcumin. Our molecular docking results predicted strong binding of curcumin to aminopeptidases from *S. dysgalactiae* and *E. coli* and a moderate affinity for the *S. aureus* enzyme. The combination of multiple strong hydrogen bonds and a large surface area of hydrophobic sub-pockets of the enzyme explains the high binding affinity, as indicated by a favourable docking score. This binding mode likely sterically hinders the enzyme's access to its natural peptide substrate and/or disrupts the conformational dynamics essential for catalysis. In addition, curcumin does not follow a consistent hierarchy of efficacy whereby it matches antibiotics exactly for the *E. coli* target, outperforms ciprofloxacin yet underperforms tetracycline for *S. aureus*, and ranks below both antibiotics for *S. dysgalactiae*. This variability suggests that curcumin's mechanism is not a simple one-size-fits-all inhibition but rather target-structure-dependent, likely reflecting differences in the shape, charge distribution, and hydrophobicity of the aminopeptidase active sites across bacterial species. Collectively, our *in silico* data suggests that curcumin binds to aminopeptidases with favorable energies which is consistent with potential enzyme inhibition and that aminopeptidases represent candidate molecular targets requiring experimental validation. This computational finding is also supported by a

substantial body of literature which has demonstrated that curcumin possesses potent anti-biofilm and anti-virulence activity against a wide spectrum of pathogens, including *Staphylococci* and *E. coli* [41]. According to Cadel et al. [42], curcumin is identified as one of the most effective inhibitors of aminopeptidase among the tested plant-derived phenolic compounds. It acts as a non-competitive inhibitor with an inhibition constant (K_i) value of $46 \mu\text{mol.L}^{-1}$, indicating a relatively high potency. Thus, curcumin likely inhibits and disperses mastitis pathogen biofilms by binding strongly to aminopeptidases and modify extracellular matrix.

To our knowledge, the present study represents the first effort to propose that curcumin may interfere with the biofilm matrix of mastitis pathogens and disrupts its structural integrity, leading to biofilm dispersal and a reduction in biofilm biomass (Figure 6). Herein, biofilm formation assays demonstrate that

curcumin disrupts the aggregation and stability of bacterial community on surfaces. FTIR spectroscopy reveals substantial changes in the biofilm's chemical structure, indicating that curcumin interacts with and breaks down key components of the extracellular matrix, such as lipids, proteins, polysaccharides, and nucleic acids. Biofilm dispersion assays further support this, showing optimal biofilm dispersion. This combined evidence suggests that curcumin may disrupt biofilm integrity by chemically altering the biofilm matrix, leading to weakened biofilm structure and increased bacterial dispersal from the surface. Our suggestion aligns with Pechaud et al. [43] reporting that when curcumin targets and degrades the matrix components, the cohesive forces that hold the biofilm together weaken and causes the microbial cells to detach from the surface and from each other. This process is critical for biofilm management and eradication, as it addresses the root cause of biofilm resilience and persistence.

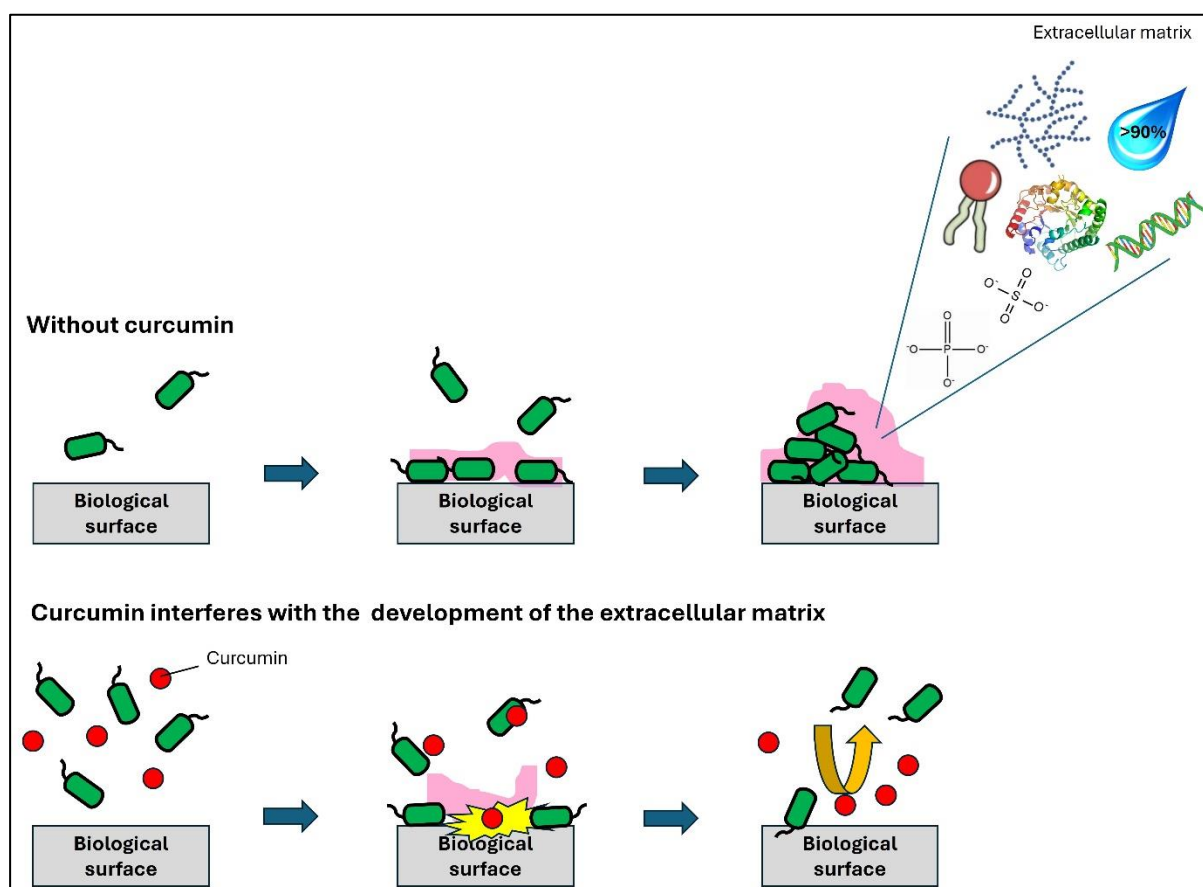


Figure 6. A proposed mechanism of biofilm dispersion by curcumin.

While the integrated *in vitro* and *in silico* data presented are promising, several critical limitations must be acknowledged when interpreting the antibiofilm potential of curcumin against mastitis pathogens. The *in vitro* biofilm assays were conducted under standardized laboratory conditions using synthetic media in static 96-well plates, which do not replicate the complex biophysical and biochemical environment of the bovine mammary gland, where biofilms develop in the presence of milk components such as casein, whey proteins, lipids, and lactoferrin that can alter bacterial physiology, antibiotic susceptibility, and matrix composition. Additionally, the static model lacks the hydrodynamic shear forces present in the teat canal and gland cistern during milking, which influence biofilm architecture, nutrient gradients, and antimicrobial penetration. The concentration of curcumin tested (600 µg/mL) may not be achievable in mammary tissue following systemic administration due to its poor oral bioavailability and rapid metabolism, and even following intramammary infusion, dilution in milk and binding to milk proteins would likely reduce the effective concentration reaching biofilm-embedded bacteria. The limitation of the *in vitro* FTIR spectroscopic analysis is its inherently population-averaged and end-point nature, which cannot capture the temporal dynamics of biofilm disruption or distinguish between viable but stressed cells versus irreversibly damaged or lysed cells. The observed reduction in lipid-associated peaks and enlargement of protein-associated peaks represent composite signals from both living bacteria actively mounting a stress response and dead or dying cells releasing cellular contents, but FTIR alone cannot deconvolute these contributions. The docking calculations assume a rigid protein structure, yet aminopeptidases undergo conformational changes upon substrate binding and during catalysis, meaning the predicted binding poses and scores may not accurately reflect the true energy landscape of enzyme-inhibitor interaction. The Vina scores represent binding affinity to the isolated, purified aminopeptidase enzyme, but in the actual biofilm environment, curcumin must penetrate the extracellular matrix and achieve sufficient local concentration before any meaningful enzyme inhibition can occur. Docking scores also do not account for curcumin's well-documented poor aqueous solubility, chemical instability under physiological conditions, or potential binding to off-target proteins, which would reduce the effective concentration available for aminopeptidase inhibition. Therefore, we emphasize that our findings are preliminary and discovery-oriented, and validation in *ex vivo* milk-based biofilm models and *in vivo* mastitis models is essential before any clinical recommendations can be made.

CONCLUSION

This study provides compelling evidence that curcumin exhibits potent antibiofilm activity against

major mastitis pathogens (*Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Escherichia coli*) through multifaceted mechanisms targeting the extracellular matrix. Our integrated approach combining biofilm formation, dispersion, and leakage assays with FTIR spectroscopy showed that curcumin inhibits biofilm formation and disrupts pre-established biofilms. FTIR spectral analysis detected changes in key biofilm matrix components including lipids, proteins, polysaccharides, and nucleic acids, suggesting that curcumin alters the structural composition of pathogenic biofilms. These observations, taken together with leakage assay data and molecular docking results, point toward a potential mechanism involving interference with extracellular matrix development; however, further experimental validation is required to confirm this proposed mode of action. These findings address a critical knowledge gap by elucidating curcumin's specific effects on biofilm architecture, moving beyond its well-documented broad-spectrum antibacterial properties. Future studies should explore optimal delivery methods and synergistic combinations with conventional antimicrobials to enhance curcumin's practical application in dairy farming. This work establishes a foundation for further investigation into phytochemical-based alternatives to antibiotics for managing biofilm-associated infections in veterinary medicine.

ACKNOWLEDGEMENTS

The authors are highly grateful to the Faculty of Applied Sciences, Universiti Teknologi MARA Shah Alam and Mines Pet Medicate for providing research facilities and a research grant (600-TNCPI/PBT 5/3 PRI (029/2025)), respectively.

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