

Original Article

Chemical profiling and antibacterial activity of fermented papaya (*Carica papaya*) and lemon (*Citrus limon*) peel solutions: In vitro, in vivo, and in silico studies

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Abstract

Antimicrobial resistance has driven the search for natural antibacterial agents, including fermentation-derived metabolites. The aim of this study was to evaluate the antibacterial activity and chemical profile of a fermented papaya and lemon peel solution against *Staphylococcus aureus* and *Pseudomonas aeruginosa* using an integrated in vitro, in vivo, and in silico approach. Antibacterial activity was assessed using disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays, followed by validation in a *Drosophila melanogaster* infection model. Chemical profiling was performed using liquid chromatography-high-resolution mass spectrometry (LC-HRMS), followed by molecular docking and molecular dynamics simulation. In the disc diffusion assay, the fermented solution showed greater activity against *S. aureus* than against *P. aeruginosa*, with the largest inhibition zones observed at a 15% concentration (12.26 ± 0.30 mm and 8.49 ± 0.68 mm, respectively). In infected *D. melanogaster* larvae, 5% and 10% fermented solution significantly improved survival compared with infection controls and reduced bacterial recovery to below the detection limit of 1×10^4 colony-forming unit (CFU)/mL under the tested conditions. Gene expression analysis showed increased expression of defensin and catalase, suggesting modulation of innate immune and antioxidant responses during *S. aureus* infection. LC-HRMS identified 18 compounds, and molecular docking indicated that luteolin 7-O-rutinoside had the strongest predicted binding affinity toward DNA gyrase and transpeptidase penicillin-binding protein 2a, with docking scores of -9.8 and -10.4 kcal/mol, respectively. These findings suggest that the fermented papaya and lemon peel solution exhibits antibacterial activity against *S. aureus* and may act through complementary antibacterial and host-response mechanisms. Further studies using isolated compounds, defined detection limits, mammalian models, and clinical bacterial isolates are needed to validate its therapeutic relevance.

Keywords: Fermented solution, antibacterial, *Drosophila melanogaster*, DNA gyrase, transpeptidase penicillin-binding protein 2a

Introduction

Antimicrobial resistance (AMR) has become a major public health concern worldwide. Recent global estimates indicate that bacterial AMR was associated with approximately 4.71 million



deaths in 2021, including approximately 1.14 million deaths directly attributable to resistant infections, and this burden is projected to increase substantially by 2050 if effective interventions are not implemented [1]. *Pseudomonas aeruginosa*, a Gram-negative bacterium, and *Staphylococcus aureus*, a Gram-positive bacterium, are significant contributors to AMR-related mortality [2]. Methicillin-resistant *S. aureus* is one of the primary causes of healthcare-associated infections globally. In 2019, methicillin-resistant *S. aureus* was identified as one of the most consequential pathogen-antibiotic resistance combinations associated with AMR-related deaths, accounting for approximately 13,800 fatalities in Europe and an estimated 121,000 deaths worldwide [3]. The 2024 World Health Organization Bacterial Priority Pathogen List further emphasizes the clinical importance of drug-resistant pathogens, including carbapenem-resistant *P. aeruginosa* and methicillin-resistant *S. aureus*, thereby reinforcing the need for continued research into new antibacterial strategies [4,5]. Since resistance in *S. aureus* is closely associated with reduced susceptibility to β -lactam antibiotics and other clinically important antibacterial classes, the investigation of natural products with potential antibacterial and host-supportive effects remains relevant.

The growing prevalence of antibiotic-resistant bacteria has driven greater interest in investigating natural sources as alternative antibacterial agents. Fruit peels and other agro-industrial by-products have attracted increasing attention as potential sources of bioactive metabolites because they contain phenolic compounds, flavonoids, organic acids, enzymes, and other secondary metabolites with reported antibacterial and antioxidant activities [6,7]. A fermented solution derived from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels, obtained through a fermentation process, represents one such potential candidate. Papaya and lemon peels were selected as a defined mixed-substrate system because they possess distinct and potentially complementary chemical profiles. Papaya peel has been associated with phenolic [8], flavonoid [9], and enzyme-related constituents [10,11], whereas lemon peel contains organic acids, citrus-derived flavonoids, phenolic compounds [12], pectin [13], and volatile terpenoids [14]. This selection was not intended to imply that papaya and lemon peels are superior to other agro-industrial by-products, but rather to evaluate an undercharacterized fermented matrix derived from two compositionally distinct fruit-peel substrates.

During fermentation, microbial hydrolysis and biotransformation may modify complex plant-derived constituents and alter the resulting metabolite profile [15]. However, fermentation does not necessarily or consistently enhance antibacterial or antioxidant activity, because the direction and magnitude of these changes depend on substrate composition, the microorganisms involved, temperature, pH, oxygen availability, and fermentation duration. Fermented solutions prepared from papaya, pineapple, and lemon peels have been reported to contain phytochemicals, including flavonoids, alkaloids, quinones, and saponins; organic acids, such as acetic acid; and hydrolytic enzymes, including proteases, lipases, and amylases [16-18]. These constituents may contribute to biological activity, although their presence alone does not establish an antibacterial effect. Flavonoids and related phenolic compounds have been reported to exert antibacterial effects through several possible mechanisms, including disruption of bacterial membranes, interference with protein and nucleic acid synthesis, inhibition of efflux-pump activity, and suppression of biofilm formation [19].

Previous studies have demonstrated the antimicrobial and bioactive potential of fermented solutions prepared from various fruit peels, including tomato, pineapple, orange, and mango [17,20-22]. These findings highlight the therapeutic potential of fermented fruit peel extracts in combating bacterial infections. To date, the antibacterial potential of fermented papaya and lemon peel solution remains insufficiently characterized, as existing studies have predominantly been limited to in vitro assessments. Therefore, further investigations employing in vivo and in silico approaches are warranted. The combined application of in vitro, in vivo, and in silico approaches provides complementary information for evaluating candidate antibacterial preparations [23,24]. In vitro assays enable direct assessment of antibacterial activity [25], whereas in vivo infection models permit evaluation of infection-associated survival, recoverable bacterial burden, and selected host-response parameters within a whole organism [26-36]. Moreover, in silico approaches can be used to predict potential interactions between selected

metabolites and bacterial targets and to examine the predicted behavior of the resulting complexes [37]

This study integrated antibacterial efficacy testing, evaluation of infection-associated host responses, metabolite profiling, and computational prediction of molecular target interactions within a single experimental framework. The aim of this study was to comprehensively evaluate the antibacterial activity of fermented solution from papaya (*C. papaya*) and lemon (*C. limon*) peels (FSPL), characterize its metabolite profile, assess its effects in an infection model, and predict potential interactions between its constituent metabolites and bacterial targets.

Methods

Preparation of fermented solution from papaya and lemon peels

Fresh papaya and lemon peels were collected in April 2025 from Pa'rappunganta Village, Polongbangkeng Utara District, Takalar Regency, South Sulawesi, Indonesia (5°21'35.32"S, 119°29'5.68"E). The collected peels were washed thoroughly and cut into small pieces. Approximately 300 g of the mixed peels was transferred into a fermentation container and combined with 100 g of molasses and 1 L of distilled water at an initial pH of 7.0. The containers were tightly closed and maintained at room temperature (approximately 25°C) for three months [17,18]. Fermentation was performed spontaneously using the indigenous microbiota naturally associated with the papaya and lemon peels, without the addition of a defined starter culture. The pH and aroma were assessed before and after fermentation. Before fermentation, the mixture had no characteristic acidic aroma and a pH of 7.0. After fermentation, the solution developed an acidic aromatic odor, and the final pH ranged from 3.2 to 3.5. Upon completion of fermentation, the solution was filtered to remove solid debris. The resulting FSPL filtrate was then freeze-dried for subsequent applications. A schematic of the preparation procedure is presented in **Figure 1**.

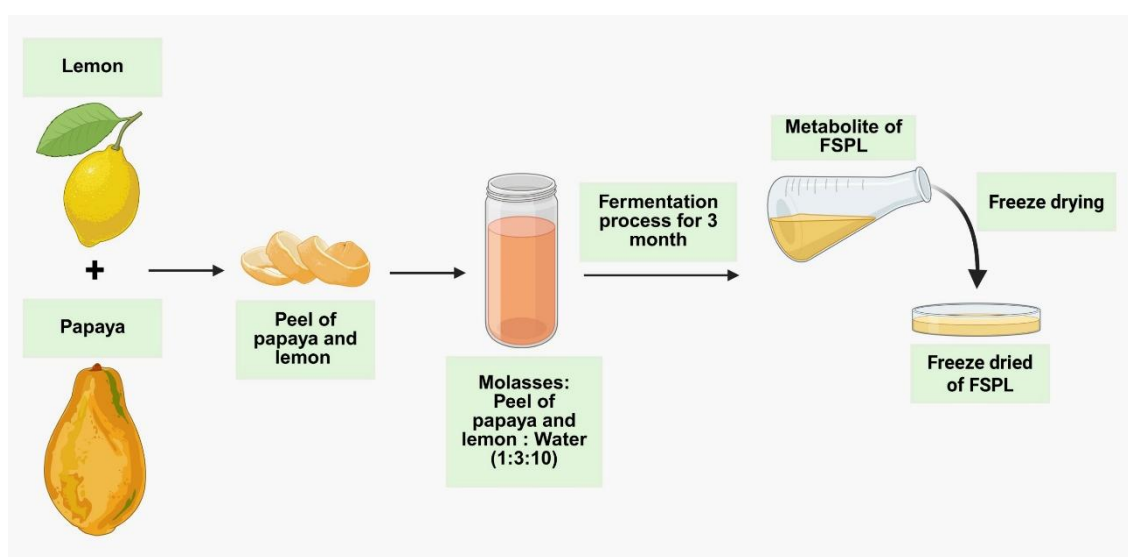


Figure 1. Preparation of fermented solution from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL).

Bacterial strains and media

The bacterial strains *S. aureus* WDCM 00032 Vitroids and *P. aeruginosa* WDCM 00025 Vitroids were used in this study. These strains were selected as representative Gram-positive and Gram-negative bacteria for preliminary antibacterial screening. The identity and purity of both strains were verified based on the Certificates of Analysis (CoA) documented at the Microbiology Laboratory, Faculty of Pharmacy, Universitas Hasanuddin, Makassar, Indonesia. Bacterial suspensions were prepared according to the procedures recommended by the Clinical and Laboratory Standards Institute (CLSI). Each strain was cultured in nutrient broth and incubated at 37°C for 24 h under 95% relative humidity. The media used in the study were Mueller-Hinton

broth, Mueller-Hinton agar, and mannitol salt agar. All media were purchased from Merck Millipore, Darmstadt, Germany.

***Drosophila* stocks**

The *Drosophila melanogaster* Oregon R(R) strain was obtained from the Kyoto *Drosophila* Stock Center, Kyoto Institute of Technology, Japan. The flies were reared at approximately 25°C and maintained on a conventional cornmeal-based medium. Ten adult flies, consisting of five males and five females, were placed in each vial for stock propagation and allowed to mate and produce offspring.

In vitro antibacterial assay

The antibacterial activity of FSPL against *S. aureus* and *P. aeruginosa* was assessed using the Kirby-Bauer disc diffusion technique [38] in accordance with CLSI guidelines [39]. Briefly, each bacterial isolate was cultured in nutrient broth and incubated at 37°C for 18–24 h. After incubation, the bacterial suspensions were standardized to approximately 0.5 McFarland ($1-2 \times 10^8$ CFU/mL) and inoculated onto nutrient agar plates using sterile cotton swabs. Sterile 6 mm filter paper discs containing 20 µL of 1%, 5%, 10%, and 15% (w/v) FSPL solution were then placed on the agar surface. FSPL was dissolved in distilled water. A standard amoxicillin disc was used as the positive control. The diameters of the clear inhibition zones surrounding the discs were measured after incubation at 37°C for 18–24 h. The commercial amoxicillin disc contained a fixed amount of antibiotic (10 µg/disc), whereas FSPL was applied as weight/volume concentrations (% w/v). Therefore, inhibition zones produced by FSPL should not be interpreted as directly equivalent to those produced by the fixed-dose amoxicillin disc, which was included only as a reference positive control. Antibacterial activity was interpreted according to the classification of Davis and Stout, in which inhibition zone diameters of <5 mm, 5–10 mm, 10–20 mm, and >20 mm indicate weak, moderate, strong, and very strong antibacterial activity, respectively [40].

Minimum inhibitory concentration (MIC) assay

The MIC assay was performed according to CLSI guidelines [41]. Each well contained 100 µL of Mueller–Hinton broth, 60 µL of bacterial suspension, and 20 µL of FSPL at final concentrations of 1%, 5%, 10%, or 15%. The bacterial inoculum was adjusted to a turbidity equivalent to 0.5 McFarland ($1-2 \times 10^8$ CFU/mL), before inoculation. Following incubation at 37°C for 18–24 h, 10 µL of 0.1% 2,3,5-triphenyltetrazolium chloride (TTC) solution was added to each well as a viability indicator for assessing bacterial growth. The plates were then incubated for an additional 30 min, after which bacterial growth was evaluated visually based on the presence or absence of TTC color development.

Minimum bactericidal concentration (MBC) assay

The MBC assay was carried out according to the CLSI guidelines [41]. Following determination of the MIC, aliquots (10–100 µL) were collected from the well corresponding to the MIC and from all wells containing higher FSPL concentrations that showed no visible bacterial growth. Each aliquot was streaked onto Mueller-Hinton agar plates and incubated at 37°C for 18–24 h. After incubation, the plates were examined for bacterial colony formation, and the MBC was defined as the lowest FSPL concentration that prevented visible bacterial growth on the agar plates. Based on the in vitro antibacterial findings, the bacterial species exhibiting the highest susceptibility to FSPL was selected for subsequent in vivo and in silico assays.

Developmental toxicity assay

The concentrated FSPL was first diluted in distilled water to prepare stock solutions. The stock solutions were thoroughly mixed with standard cornmeal-based *Drosophila* fly food to obtain final FSPL concentrations of 2.5%, 5%, and 10% in the fly food. The development of *D. melanogaster* from the larval stage through pupation to adulthood was monitored to evaluate the developmental toxicity of FSPL following exposure to diets supplemented with 2.5%, 5%, or 10% FSPL. Developmental progression was monitored daily. The assay was performed using uninfected *Drosophila* in all groups, with the FSPL-treated groups compared with the control group. Five independent biological replicates were included for each condition, with each

replicate comprising ten larvae maintained in a separate vial. This assay was designed as an initial developmental toxicity screening.

Survival assay

The survival assay was conducted to determine the influence of bacterial challenge and FSPL administration on larval survival. The *D. melanogaster* infection model was established using the banana-crush feeding method [42]. Based on the MIC and MBC findings from the in vitro susceptibility assay, *S. aureus* was selected for subsequent in vivo experiments because it exhibited greater susceptibility to FSPL; in contrast, FSPL showed limited antibacterial activity against *P. aeruginosa*. Briefly, a standardized *S. aureus* suspension at a concentration of 5×10^8 CFU/mL was added to the banana crush and used as the infection source. Successful infection was indicated by a significant reduction in larval survival in the infection group compared with the non-infected control group. For each treatment group, five independent biological replicates were used, with each replicate consisting of ten larvae. The number of surviving larvae was recorded for each treatment condition, including infected and non-infected groups with or without FSPL administration. Survival percentages were calculated and compared with those of the corresponding control groups. Different stages of the experiment, including infection preparation, treatment administration, and survival assessment, were conducted by different personnel to minimize procedural bias.

Colony-forming unit (CFU) assay

The CFU assay was conducted to evaluate the antibacterial activity of the treatment by quantifying viable *S. aureus* cells. Samples were plated on mannitol salt agar, a selective medium that supports the growth of *S. aureus*. Briefly, ten larvae were collected per sample, suspended in 1 mL of phosphate-buffered saline, and homogenized using a sterile micropestle. The homogenates were serially diluted and plated onto mannitol salt agar using the pour plate method. Plates were incubated at 37°C for 24 h under aerobic conditions, and visible bacterial colonies were quantified through manual enumeration. The detection limit of the CFU assay was defined as the lowest bacterial concentration at which at least 10 CFU/mL could be detected, based on serial dilutions ranging from 10^{-1} to 10^{-3} and plating 1 mL of each dilution per plate. CFU values below the limit of detection were reported as "<LOD" to indicate that the bacterial count was below the assay detection limit, rather than implying the complete absence of bacteria. For statistical analysis, bacterial counts below the limit of detection (<LOD) were substituted with one-half of the LOD (LOD/2) to enable log transformation ($\log_{10}$ CFU/mL).

Reactive oxygen species (ROS) assay

The ROS assay was performed to assess oxidative stress in *D. melanogaster* by measuring intracellular ROS levels following *S. aureus* infection. ROS levels were evaluated using a nitro blue tetrazolium (NBT)-based assay with spectrophotometric analysis [43]. Briefly, *D. melanogaster* larvae from the untreated control, infection control, and FSPL-treated infected groups were collected and homogenized in phosphate-buffered saline. The homogenates were reacted with NBT solution, and absorbance was measured at 595 nm using a spectrophotometer (Shimadzu UV-VIS 1800, Kyoto, Japan). Increased absorbance was interpreted as greater NBT reduction, indicating higher ROS-associated activity.

Gene expression analysis

The expression of selected immune- and antioxidant-related genes was evaluated to further characterize the host immune and oxidative responses following *S. aureus* infection and FSPL treatment by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The immune-related gene *def* and the endogenous antioxidant genes *sod1*, *sod2*, and *cat* were selected to assess changes in host immune signaling and antioxidant defense, respectively (Table 1). Total RNA was isolated from *D. melanogaster* larvae using the Monarch® Total RNA Miniprep Kit according to the manufacturer's instructions (New England Biolabs, MA, USA). Gene expression levels were subsequently evaluated using one-step RT-qPCR with the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, MA, USA). Each reaction was prepared in a final volume of 10 µL and analyzed using the Rotor-Gene Q system (Qiagen, Germany). Reverse transcription was conducted at 50°C for 10 min, followed by 40 amplification cycles comprising denaturation

at 95°C for 15 s, annealing and extension at 60°C for 30 s, and final extension at 72°C for 30 s. Melt curve analysis was performed to verify the specificity of amplification across a temperature range of 60°C to 95°C. Relative gene expression was calculated using the comparative cycle threshold method.

Table 1. List of primers used in RT-qPCR

Genes	Gene function	Forward primer (5'–3')	Reverse primer (5'–3')
<i>def</i>	Antimicrobial peptide	GTTCTTCGTTCTCGTGG	CTTTGAACCCCTTGGC
<i>sod1</i>	Endogenous antioxidant	AGGTCAACATCACCGACTCC	GTTGACTTGCTCAGCTCGTG
<i>sod2</i>	Endogenous antioxidant	TGGCCACATCAACCACAC	TTCCACTGCGACTCGATG
<i>cat</i>	Endogenous antioxidant	TTCCTGGATGAGATGTCGCACT	TTCTGGGTGTGAATGAAGCTGG
<i>rp49</i>	Reference gene	CGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG

Untargeted liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis

Untargeted metabolite profiling of FSPL was carried out using LC-HRMS on a Thermo Scientific Dionex Ultimate 3000 RSLC Nano system linked to a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA). Chromatographic separation was performed using an Acclaim™ Vanquish C18 column (150×2.1 mm), with mobile phase A consisting of water and 0.1% formic acid and mobile phase B consisting of methanol and 0.1% formic acid. The flow rate was set at 40 µL/min, and the injection volume was 5 µL. The 25-min gradient consisted of 5% B from 0–2 min, 60% B from 2–15 min, and 95% B from 15–22 min, followed by 95% B from 22–25 min, and re-equilibration to 5% B from 25.1–30 min. Metabolomic analysis was performed using an untargeted LC-HRMS acquisition mode with full MS and data-dependent MS/MS acquisition. All raw data were processed using Thermo Scientific Xcalibur software [44–46]. Metabolite annotation was based on accurate mass-to-charge ratio matching against the mzCloud, ChemSpider, and in-house mass list databases. As this was an untargeted qualitative profiling study, authentic reference standards, MS/MS fragmentation confirmation, and quantitative calibration curves were not employed.

Molecular docking

Molecular docking was performed to evaluate potential interactions between FSPL-derived metabolites and two essential *S. aureus* targets: DNA gyrase (PDB ID: 2XCT) and transpeptidase penicillin-binding protein 2a (PBP2a; PDB ID: 1MWU). The three-dimensional structures of both proteins were retrieved from the RCSB Protein Data Bank (<https://www.rcsb.org/>) and prepared using AutoDock Vina integrated into UCSF Chimera [47]. Protein preparation involved removing crystallographic water molecules, eliminating non-essential ligands and cofactors, adding polar hydrogen atoms, and assigning Gasteiger partial charges. Ligand structures were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) in SDF format and converted into PDB files using UCSF Chimera. Ligands were subsequently prepared by adding hydrogen atoms and assigning Gasteiger charges [48].

Prior to docking the test compounds, the docking protocol was validated by re-docking the co-crystallized native ligand into the binding pocket of each target protein. The reliability of the docking protocol was evaluated by calculating the root mean square deviation (RMSD) between the crystallographic ligand pose and the re-docked pose, with an RMSD value below 2.0 Å considered indicative of an acceptable docking protocol. The docking grid was manually defined to encompass the active site of each target protein based on the position of the co-crystallized ligand and previously reported binding site information. For DNA gyrase, the grid box center was set at X=−3.64, Y=38.83, and Z=70.69, with grid box dimensions of 60.23×52.26×61.34 Å. For PBP2a, the grid box center was defined at X=25.92, Y=29.89, and Z=87.89, with grid box dimensions of 24.49×26.43×25.05 Å. Docking parameters were configured within the UCSF Chimera interface prior to executing AutoDock Vina. Docking simulations were performed using the AutoDock Vina algorithm implemented in UCSF Chimera, and the predicted binding poses were ranked according to their binding affinity (kcal/mol). The pose with the lowest binding energy was selected for subsequent interaction analysis. Protein–ligand interactions, including hydrogen bonds, hydrophobic contacts, and ligand orientation within the binding pocket, were analyzed and visualized using Discovery Studio Visualizer [49,50].

Molecular dynamics simulation

Molecular dynamics simulations were conducted using YASARA [51] to evaluate the interaction stability of luteolin 7-O-rutinoside and the native co-crystallized ligands CPF and 7EP with their respective protein targets, DNA gyrase and transpeptidase PBP2a, respectively. The simulations employed the AMBER14 force field under periodic boundary conditions at 310 K and physiological pH 7.4. The systems were solvated using the Transferable Intermolecular Potential 3-Point (TIP3P) water model [52], and appropriate counterions were added to maintain charge neutrality. Each system was simulated for 100 ns with a 0.25 fs timestep. Throughout the simulation, structural stability parameters including RMSD, radius of gyration (Rg), root mean square fluctuation (RMSF), and solvent-accessible surface area (SASA) were recorded at 25 ps intervals to assess conformational dynamics and ligand–protein stability [53,54].

Data processing and statistical analysis

Data on antibacterial effects, developmental toxicity, survival rates, CFU assay, and gene expression were analyzed using one-way analysis of variance (ANOVA) to evaluate differences among the treatment groups. Survival probabilities were estimated using the Kaplan-Meier method, and differences in survival distributions among experimental groups were assessed using the Mantel-Cox (log-rank) test. Prior to performing one-way ANOVA, the assumptions of normality and homogeneity of variance were evaluated using the Shapiro-Wilk test and Levene's test, respectively. Data that met these assumptions were analyzed using one-way ANOVA, whereas data that did not meet these assumptions were subjected to the non-parametric Kruskal-Wallis test. When ANOVA indicated significant differences, Dunnett's multiple-comparison post-hoc test was applied to compare each treatment group with the corresponding control group. Gene expression data, presented as cycle threshold values, were first processed and normalized using Rotor-Gene Q software (Qiagen, Germany). Further statistical analysis of gene expression data and other datasets was performed using GraphPad Prism 9 (GraphPad Software, CA, USA). Statistical significance was set at $p < 0.05$.

Results

In vitro antimicrobial activity of papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL)

FSPL exhibited antibacterial activity against *S. aureus* and *P. aeruginosa*, with activity increasing with increasing FSPL concentration (**Table 2**). For *S. aureus*, the inhibition zone increased from 6.50 ± 0 mm at 1% FSPL to 12.26 ± 0.30 mm at 15%. Similarly, for *P. aeruginosa*, the inhibition zone increased from 6.20 ± 0.01 mm to 8.49 ± 0.68 mm across the same concentration range. At 15% FSPL, the inhibition zone was larger against *S. aureus* than against *P. aeruginosa*, indicating greater susceptibility of the former.

Table 2. Antibacterial activity of fermented solution from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL)

Bacterial group	Bacterial strain	Treatment	Concentration (%)	Inhibition zone diameter (mm)
Gram-positive bacteria	<i>Staphylococcus aureus</i>	Negative control (purified water)	-	0
		Positive control (amoxicillin)	-	11.84 ± 0.23
		FSPL	1	6.5 ± 0
			5	6.74 ± 0.01
			10	8.74 ± 0.11
			15	12.26 ± 0.30
Gram-negative bacteria	<i>Pseudomonas aeruginosa</i>	Negative control (purified water)	-	0
		Positive control (amoxicillin)	-	10.51 ± 0.30
		FSPL	1	6.2 ± 0.01
			5	6.52 ± 0.02
			10	7.39 ± 0.51
			15	8.49 ± 0.68

In the broth microdilution assay, 1% FSPL showed bacterial growth for both organisms, whereas concentrations $\geq 5\%$ resulted in a colorless TTC reaction, corresponding to inhibition of bacterial growth (**Table 3**). Thus, the MIC was 5% for both *S. aureus* and *P. aeruginosa*.

Table 3. Minimum inhibitory concentration (MIC) based on the color change following 2,3,5-triphenyltetrazolium chloride (TTC) solution addition

Bacterial group	Bacterial strain	Concentration (%)	Color after TTC addition	Interpretation
Gram-positive bacteria	<i>Staphylococcus aureus</i>	1	Pink	Bacterial growth observed
		5	Colorless	Bacterial growth inhibited
		10	Colorless	Bacterial growth inhibited
		15	Colorless	Bacterial growth inhibited
Gram-negative bacteria	<i>Pseudomonas aeruginosa</i>	1	Pink	Bacterial growth observed
		5	Colorless	Bacterial growth inhibited
		10	Colorless	Bacterial growth inhibited
		15	Colorless	Bacterial growth inhibited

The MBC results further demonstrated differential susceptibility between the two organisms (**Table 4**). For *S. aureus*, bacterial colonies were not detected at 10% and 15% FSPL, corresponding to an MBC of 10%. In contrast, *P. aeruginosa* showed residual colony growth at 10% and no detectable colonies at 15%, corresponding to an MBC of 15%. Collectively, the inhibition zone, MIC, and MBC results indicate greater susceptibility of *S. aureus* to FSPL than *P. aeruginosa*.

Table 4. Minimum bactericidal concentration (MBC) outcomes for papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL) on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Types of bacteria	Number of colonies			
	1% FSPL solution	5% FSPL solution	10% FSPL solution	15%FSPL solution
<i>Staphylococcus aureus</i>	7	3	0	0
	8	2	0	0
	9	4	0	0
<i>Pseudomonas aeruginosa</i>	27	19	6	0
	20	15	5	1
	25	17	7	1

Developmental toxicity of FSPL in *Drosophila melanogaster*

Exposure to FSPL did not significantly affect the developmental survival of *D. melanogaster* under the tested conditions (**Figure 2**). Larval-to-pupal survival (**Figure 2A**) and pupal-to-adult survival (**Figure 2B**) were comparable between the FSPL-treated and control groups, with no statistically significant differences observed at either developmental stage. These results indicate that FSPL did not induce detectable developmental toxicity under the experimental conditions tested.

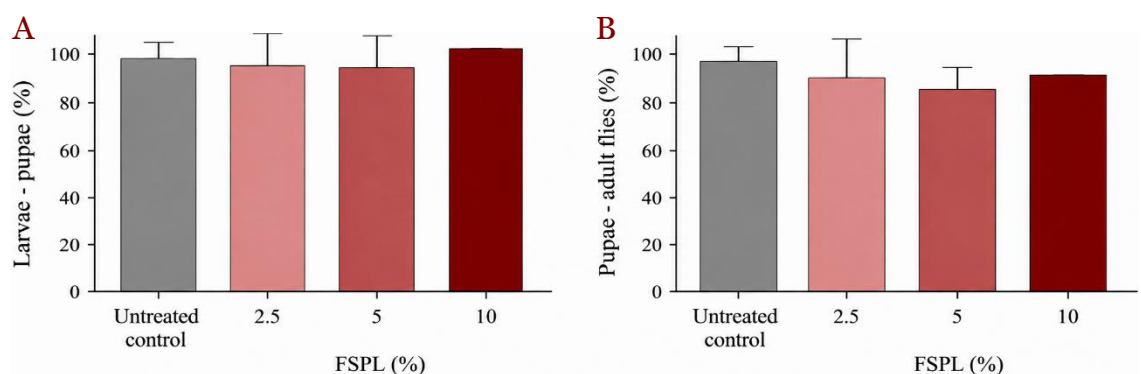


Figure 2. Developmental toxicity assessment of fermented solution from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL) in *Drosophila melanogaster*. Survival rates of (A) larvae-to-pupae and (B) pupae-to-adult stages.

Effect of FSPL on *Drosophila melanogaster* survival following *Staphylococcus aureus* infection

Subsequent in vivo and in silico investigations were focused exclusively on *S. aureus* because this species exhibited greater susceptibility to FSPL during the initial in vitro antibacterial screening. In the *D. melanogaster* infection model, *S. aureus* significantly reduced larval-to-pupal survival compared with the non-infected control group (**Figure 3A**). FSPL supplementation significantly improved larval-to-pupal survival at 2.5%, 5%, and 10%, with the highest survival observed at 10% FSPL.

A similar pattern was observed for pupal-to-adult survival, with FSPL treatment increasing adult emergence compared with the infected group, although the difference at 2.5% was not statistically significant (**Figure 3B**). Kaplan–Meier analysis further demonstrated significantly improved survival among FSPL-treated infected larvae compared with the infected control group, with the greatest improvement observed at 10% FSPL (**Figure 3C**). These findings demonstrate that FSPL supplementation improved developmental and overall survival following *S. aureus* infection under the tested conditions.

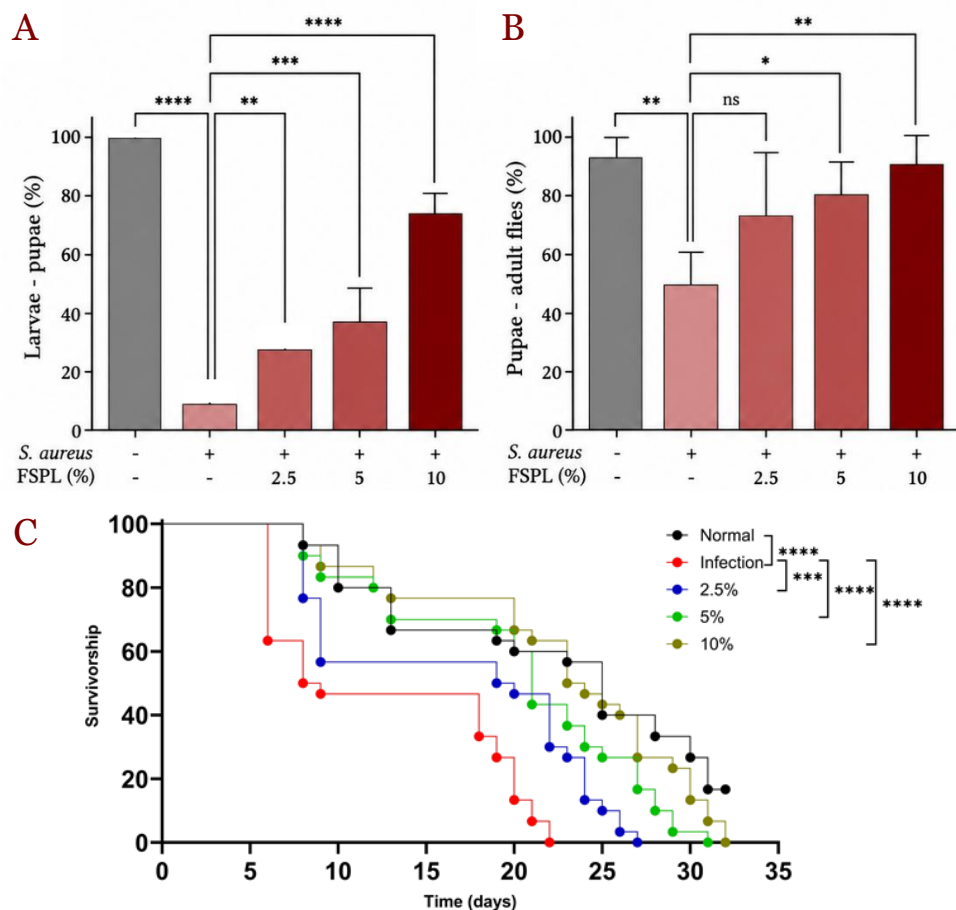


Figure 3. Effect of fermented solution from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL) on survival outcomes in the *Drosophila melanogaster* infection model. (A) Larval-to-pupal survival; (B) pupal-to-adult survival; and (C) Kaplan-Meier survival curves of *S. aureus*-infected larvae treated with different concentrations of FSPL. Asterisks indicate significant differences relative to the infection group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) and “ns” denotes no significant difference.

Dose-dependent inhibitory effect of FSPL on *Staphylococcus aureus* growth

The bacterial burden in infected *D. melanogaster* larvae was quantified to further evaluate the antibacterial activity of FSPL by CFU enumeration (**Table 5**). Bacterial recovery remained detectable following treatment with 2.5% FSPL, although the recoverable bacterial burden was lower than that of the infection control group ($13.33 \pm 5.77 \times 10^2$ CFU/mL versus $17.33 \pm 2.08 \times 10^2$

CFU/mL). At 5% and 10% FSPL, no detectable bacterial colonies were recovered, with bacterial counts reported as below the assay limit of detection (<LOD). These findings indicate a concentration-dependent reduction in recoverable *S. aureus* following FSPL treatment, with concentrations of 5% and 10% producing bacterial burdens below the detection limit under the tested conditions.

Table 5. Assessment of *Staphylococcus aureus* infection in *Drosophila melanogaster* larvae using a colony-forming unit (CFU) assay

Treatment group	CFU/mL	Log ₁₀ CFU/mL
Infection	17.33±2.08×10 ²	2.24±0.05
FSPL 2.5%	13.33±5.77×10 ²	1.10±0.17
FSPL 5%	<LOD (limit of detection)	0.699
FSPL 10%	<LOD (limit of detection)	0.699

FSPL: fermented solution from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL)

Modulation of *def* expression during *S. aureus* infection

The expression of the immune-related gene *def* was evaluated to further characterize the host response to FSPL treatment during *S. aureus* infection by RT-qPCR. *S. aureus* infection significantly increased *def* expression compared with the non-infected control group, indicating activation of an infection-associated immune response (**Figure 4**). FSPL treatment significantly reduced *def* expression compared with the infection control group at all tested concentrations, although expression remained higher than that in the non-infected control group. These findings indicate that FSPL modulated the *def*-associated immune response during *S. aureus* infection.

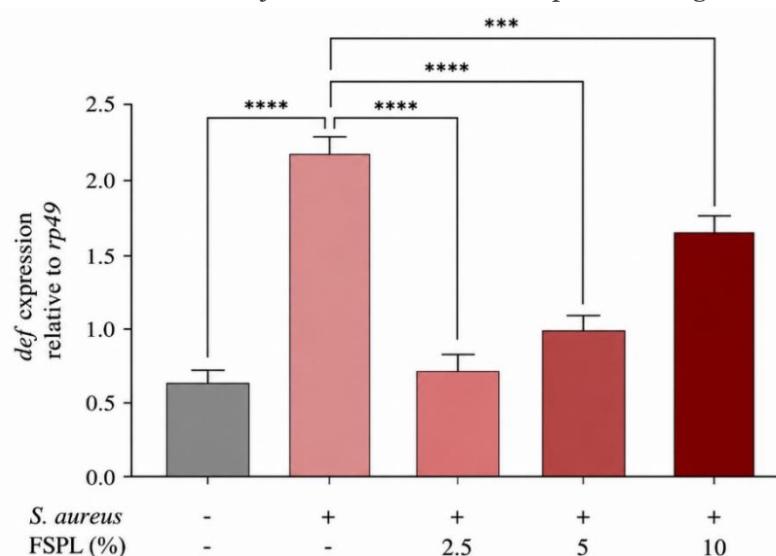


Figure 4. Effect of fermented solution from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL) on the expression of immune-related gene in the *Drosophila melanogaster* infection model. Asterisks indicate significant differences relative to the infection group (*) $p < 0.001$; **** $p < 0.0001$.**

Modulation of oxidative responses during *Staphylococcus aureus* infection

ROS levels and the expression of antioxidant-related genes were subsequently evaluated to characterize the oxidative response to *S. aureus* infection and FSPL treatment (**Figure 5**). *S. aureus* infection significantly increased intracellular ROS levels compared with the non-infected control group (**Figure 5A**). FSPL treatment reduced ROS levels relative to the infection control, indicating attenuation of infection-associated oxidative stress. In contrast, *S. aureus* infection significantly decreased the expression of *sod1*, *sod2*, and *cat* compared with the non-infected control group (**Figure 5B–D**). FSPL treatment did not significantly alter *sod1* or *sod2* expression compared with the infection control, whereas 10% FSPL significantly increased *cat* expression. These findings suggest that FSPL modulated the oxidative response during *S. aureus* infection, characterized by reduced ROS levels and a selective increase in *cat* expression rather than a generalized restoration of antioxidant gene expression.

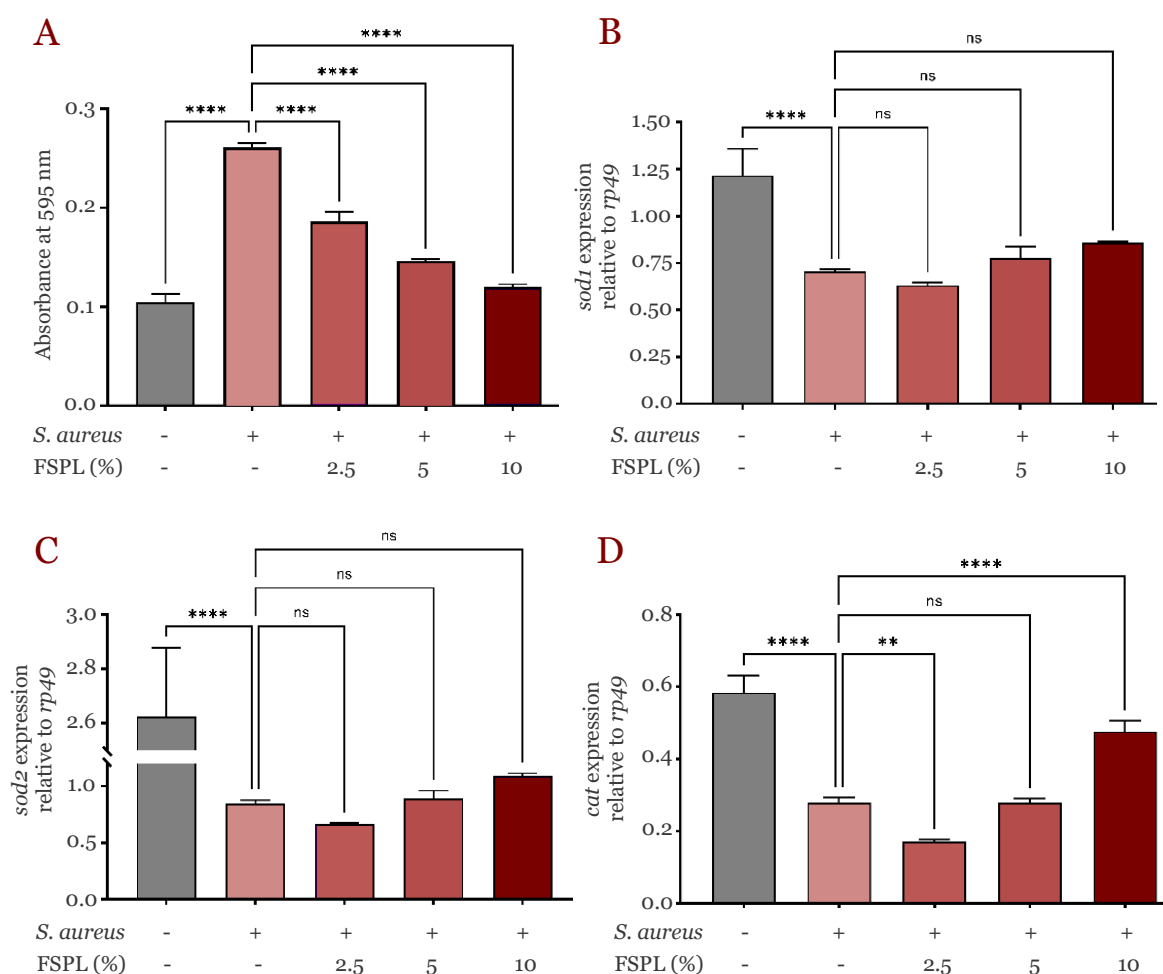


Figure 5. Effect of fermented solution from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL) on oxidative responses in the *Drosophila melanogaster* infection model. (A) Intracellular reactive oxygen species (ROS) levels. Relative expression of (B) *sod1*, (C) *sod2*, and (D) *cat*. Asterisks indicate significant differences relative to the infection group (** $p < 0.01$; **** $p < 0.0001$), and “ns” indicates no significant difference.

Untargeted LC-HRMS profiling of FSPL

LC-HRMS analysis tentatively annotated 18 metabolites in the FSPL based on accurate mass matching against the mzCloud and ChemSpider databases (Table 6). The corresponding total ion chromatogram (TIC) is presented in Figure 6. The annotated metabolites comprised several classes of phenolic and flavonoid-related compounds, including eriodictyol, hesperetin, neohesperidin, and luteolin 7-O-rutinoside. Among these, hesperetin showed the highest peak area (30,591,752.17), followed by eriodictyol (29,524,241.03), 3-[3-(β -D-glucopyranosyloxy)-2-methoxyphenyl]propanoic acid (29,053,773.17), neohesperidin (22,879,559.35), and luteolin 7-O-rutinoside (22,028,254.39). These compounds were subsequently considered for molecular docking based on their relative abundance in the LC-HRMS profile and previously reported biological activities [55-58].

Molecular docking analysis

The re-docked poses showed good agreement with the corresponding crystallographic ligand poses, with RMSD values of 1.053 Å for DNA gyrase and 1.642 Å for PBP2a (Figure 7). Both values were below the predefined 2.0 Å threshold, supporting the suitability of the docking protocol for subsequent analysis. The molecular docking results of the 18 tentatively annotated FSPL metabolites showed variable predicted binding affinities toward both target proteins (Table 7). Several metabolites exhibited lower predicted binding energies than the respective native ligands.

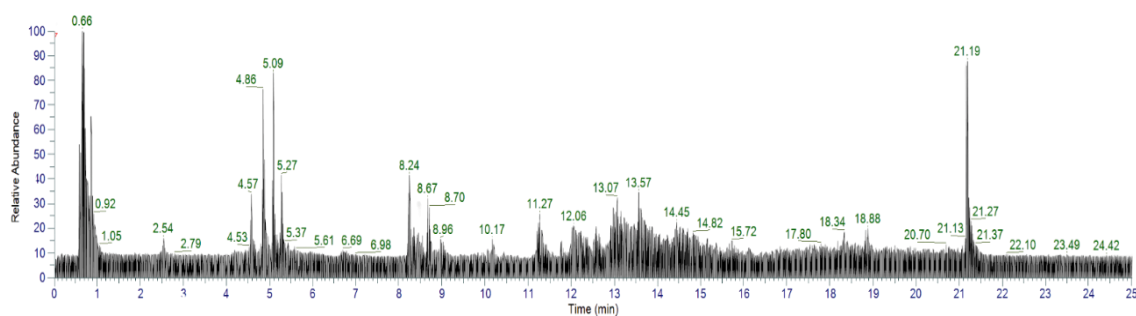


Figure 6. Total ion chromatograms (TICs) of fermented solution from papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL) obtained from untargeted liquid chromatography-high-resolution mass spectrometry (LC-HRMS).

Luteolin 7-O-rutinoside showed the lowest predicted binding energy among the tested FSPL metabolites, with ΔG values of -9.8 kcal/mol against DNA gyrase and -10.4 kcal/mol for PBP2a, compared with -7.7 kcal/mol and -7.3 kcal/mol for the corresponding native ligands, respectively. Interaction analysis further showed that luteolin 7-O-rutinoside formed multiple hydrogen-bond interactions within the binding pockets of both target proteins. For DNA gyrase, interactions involved Lys581, Ser1028, Arg1033, and Arg1122, whereas interactions with PBP2a involved Ser403, Thr444, Asn464, His583, and Ser598 (**Table 7**). The corresponding two-dimensional and three-dimensional interaction profiles are presented in **Figure 8**.

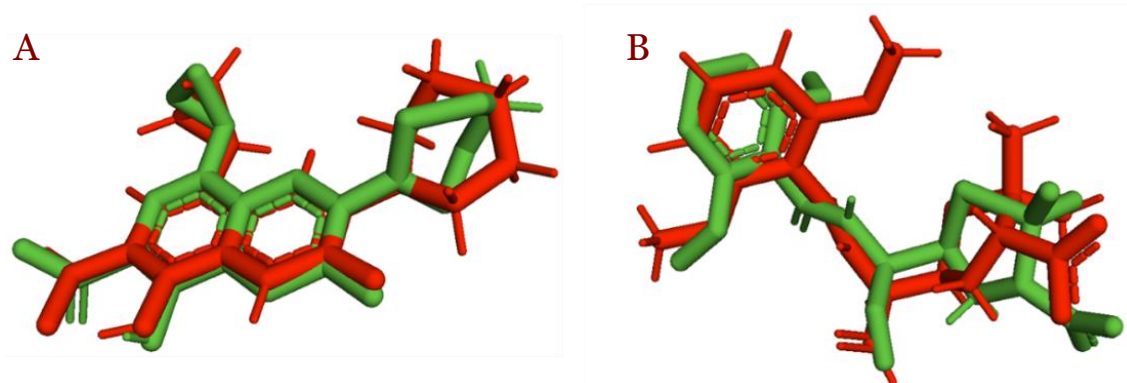


Figure 7. Validation of the molecular docking protocol by re-docking of the co-crystallized native ligands. Superimposition of the crystallographic native ligand (red) and re-docked ligand (green) in the binding sites of (A) DNA gyrase and (B) transpeptidase PBP2a.

Molecular dynamics simulations

The molecular dynamics simulation profiles of the luteolin 7-O-rutinoside and native-ligand complexes with DNA gyrase and transpeptidase PBP2a over 100 ns are presented in **Figure 9**. For the DNA gyrase system, the luteolin 7-O-rutinoside complex exhibited a slightly lower average radius of gyration (Rg) of 38.18 Å, compared with 38.46 Å for the native ligand (CPF) complex, indicating comparable overall compactness of the protein-ligand complexes. The average RMSD values were also lower for the luteolin 7-O-rutinoside complex than for the native ligand complex (3.0 Å vs 3.8 Å, respectively), while the corresponding average SASA values were $55,950$ Å² and $56,340$ Å², respectively.

For PBP2a, both the native ligand (7EP) and luteolin 7-O-rutinoside complexes exhibited an average Rg of 37.42 Å. The average RMSD values were 4.0 Å for luteolin 7-O-rutinoside and 4.3 Å for the native ligand, whereas the average SASA values were $30,030$ Å² and $30,296$ Å², respectively. RMSF analysis revealed localized residue fluctuations in both protein systems (**Figure 9C** and **9G**). In the DNA gyrase complex, the highest RMSF was observed at residue 1009, with values of 4.1 Å for the luteolin 7-O-rutinoside complex and 3.9 Å for the native ligand complex.

Table 6. Untargeted LC-HRMS profiling of papaya (*Carica papaya*) and lemon (*Citrus limon*) peels (FSPL)

Compound	Formula	m/z	Molecular weight (g/mol)	Retention time (min)	Area (max)
3,4,5-Trimethoxyhydrocinnamic acid	C ₁₂ H ₁₆ O ₅	241.1071	240.0999	2.308	6042668.64
3-[3-(beta-D-Glucopyranosyloxy)-2methoxyphenyl]propanoic acid	C ₁₆ H ₂₂ O ₉	357.9185	358.1258	3.417	29053773.17
Dihydroferulic acid 4-O-glucuronide	C ₁₆ H ₂₀ O ₁₀	372.0980	372.1054	3.378	4601524.78
5,3',4',5'-Tetrahydroxy-6,7dimethoxyflavone	C ₁₇ H ₁₄ O ₈	345.0611	346.0684	6.814	2242272.76
5,7,3',6'-Tetrahydroxy-6,8,2'trimethoxyflavone	C ₁₈ H ₁₆ O ₉	376.0718	376.0790	6.815	1691820.74
5-hydroxy-2-(4-hydroxyphenyl)-7[(3,4,5-trihydroxy-6-methyloxan-2yl)oxy]-3-[(3,4,5-trihydroxyoxan-2yl)oxy]-4H-chromen-4-one	C ₂₆ H ₂₈ O ₁₄	563.1412	564.1482	3.341	5187092.92
Sphaerobioside	C ₂₇ H ₃₀ O ₁₄	579.1710	578.1638	4.338	1594466.61
Astilbin	C ₂₁ H ₂₂ O ₁₁	450.0984	450.1162	3.832	2888659.24
Cerarvensin	C ₂₀ H ₁₈ O ₉	402.0874	402.0947	7.184	13258139.96
Eriodictyol	C ₁₅ H ₁₂ O ₆	287.0562	288.0635	5.619	29524241.03
Hesperetin	C ₁₆ H ₁₄ O ₆	301.0720	302.0792	6.811	30591752.17
Mesquitol-4beta-ol 3,8-dimethyl ether	C ₁₇ H ₁₈ O ₇	334.1020	334.1053	5.974	8675475.18
Naringenin 7-O-beta-D-glucoside 6"acetate	C ₂₃ H ₂₄ O ₁₁	476.1245	476.1319	4.197	2039932.32
Neohesperidin	C ₂₈ H ₃₄ O ₁₅	609.1827	610.1898	4.655	22879559.35
Rubone	C ₂₀ H ₂₂ O ₇	374.1289	374.1361	4.197	2110685.21
Luteolin 7-O-rutinoside	C ₂₇ H ₃₂ O ₁₅	595.1675	596.1746	3.819	22028254.39
4-Hydroxycoumarin	C ₉ H ₆ O ₃	161.8245	162.0318	3.695	14200941.95
4-Hydroxyderricin	C ₂₁ H ₂₂ O ₄	338.3590	338.1517	13.168	4226425.57

Table 7. Predicted binding affinities and hydrogen-bond interactions of tentatively annotated FSPL metabolites with DNA gyrase and transpeptidase PBP2a

Compound	DNA gyrase		Transpeptidase PBP2a	
	ΔG (kcal/mol)	H-Bond	ΔG (kcal/mol)	H-Bond
3,4,5-Trimethoxyhydrocinnamic acid	-5.7	Arg1047	-6.1	Lys406, Ser462
3-[3-(beta-D-Glucopyranosyloxy)-2methoxyphenyl]propanoic acid	-7.7	-	-7.7	Ser403, Thr600
Dihydroferulic acid 4-O-glucuronide	-7.7	Tyr1150	-7.7	Ser403, Lys430, Thr444, Ser461, Ser462, Asn464, His583, Thr600
5,3',4',5'-Tetrahydroxy-6,7dimethoxyflavone	-7.8	Arg1122	-8.5	Ser403, Ser462, Asn464, Gly640
5,7,3',6'-Tetrahydroxy-6,8,2'trimethoxyflavone	-7.7	Ser1098, Met1113, Gly1115, Asp1116, Val1268	-7.5	Ser403, Thr444, Asn464, Thr600, Glu602, Met641
5-hydroxy-2-(4-hydroxyphenyl)-7[(3,4,5-trihydroxy-6-methyloxan-2yl)oxy]-3-[(3,4,5-trihydroxyoxan-2yl)oxy]-4H-chromen-4-one	-9.6	Leu457, Gly459, Arg1122	-9.9	Thr444, Tyr446, Ser462
Sphaerobioside	-9.5	-	-9.9	Asn464, Ser598, Thr600, Gly640, Ser643
Astilbin	-8.7	Asp508, Gly582, His1081, Arg1122	-8.6	Ser403, Asn464
Cerarvensin	-8.9	Glu435, Asp512, Met1075	-9.0	Ser403, Thr444, Asn464, Tyr519
Eriodictyol	-7.9	Ser445, Ala588, Asp1096, Gly1108, Thr1296	-8.1	Ser403, Tyr519
Hesperetin	-8.1	Gly584, His1081	-8.1	Ser403, Ser462, Ala642, Ser643

Compound	DNA gyrase		Transpeptidase PBP2a	
	ΔG (kcal/mol)	H-Bond	ΔG (kcal/mol)	H-Bond
Mesquitol-4beta-ol 3,8-dimethyl ether	-7.3	Ser438	-7.7	Ser462
Naringenin 7-O-beta-D-glucoside 6"acetate	-9.2	Glu435, Asp437, Ser438, Arg1122	-9.2	Ser403, Lys406, Glu447, Ser462, Asn464, Ser643
Neohesperidin	-9.5	Asp508, Gly545, Gly584, Arg1122	-9.7	Ser403, Ser462, Asn464, Gly640, Ala642, Ser643
Rubone	-7.7	Asp437, Ser438	-7.6	Asn464, Ser598
Luteolin 7-O-rutinoside	-9.8	Lys581, Ser1028, Arg1033, Arg1122	-10.4	Ser403, Thr444, Asn464, His583, Ser598
4-Hydroxycoumarin	-6.2	Met1113, Val1268	-6.3	Ser403, Thr600
4-Hydroxyderricin	-8.4	Asp437	-7.6	Tyr446, Ser462, Thr600
Native Ligand	-7.7	Arg1122, Tyr1150	-7.3	Ser403, Lys406, Ser462, Ser598, Thr600

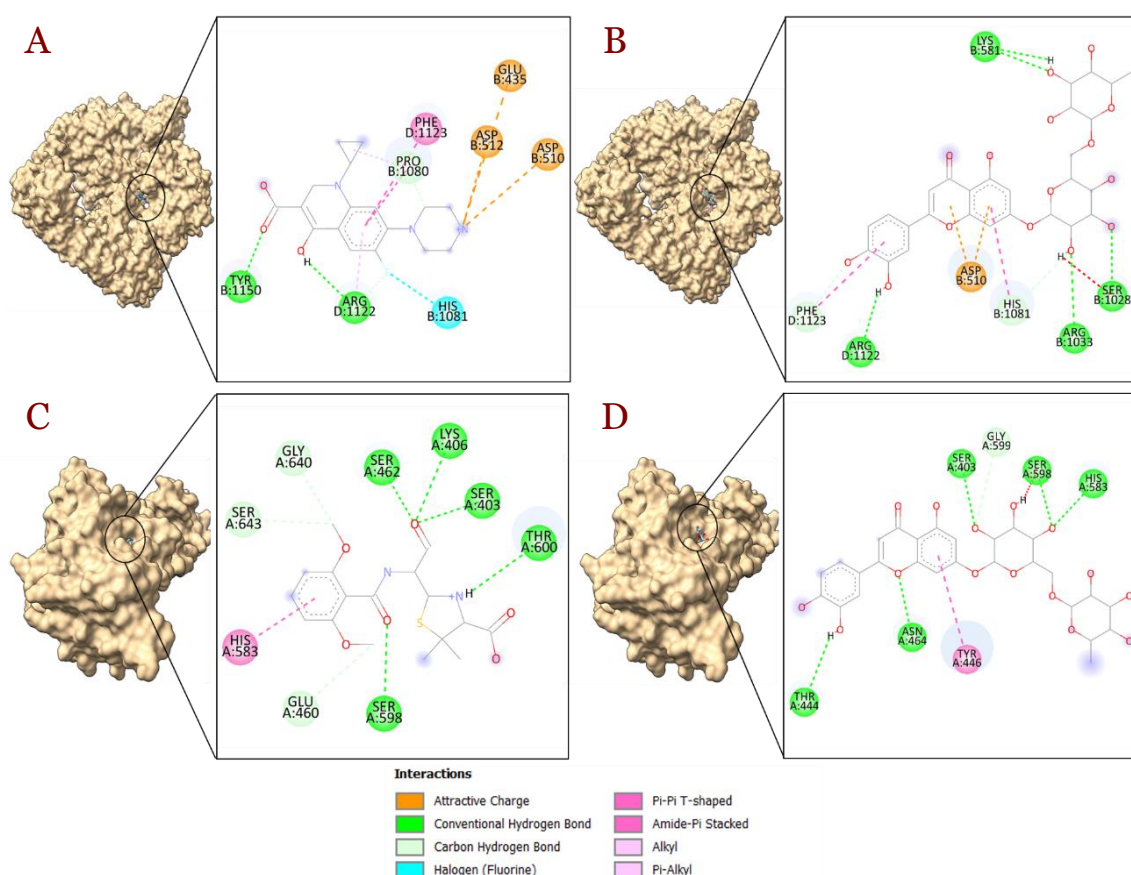


Figure 8. Molecular interactions of native ligands and luteolin 7-O-rutinoside with *S. aureus* target proteins. Three-dimensional (3D) and two-dimensional (2D) representations of the binding interactions of: (A) Native ligand of DNA gyrase (CPF); (B) Luteolin 7-O-rutinoside with DNA gyrase; (C) Native ligand of transpeptidase PBP2a (7EP); and (D) luteolin 7-O-rutinoside with transpeptidase PBP2a.

In the PBP2a system, the highest RMSF values were 7.1 Å at residue 90 for the luteolin 7-O-rutinoside complex and 6.2 Å at residue 204 for the native ligand complex. Taken together, the Rg, RMSD, and SASA profiles showed broadly comparable conformational characteristics between the luteolin 7-O-rutinoside and native-ligand complexes throughout the simulation period, with no marked difference in the global structural parameters assessed.

Discussion

The increasing prevalence of antimicrobial resistance has intensified the need to identify novel antibacterial agents from natural sources. Fermentation represents a promising approach for generating or transforming bioactive metabolites from plant-based materials. In the present study, FSPL exhibited antibacterial activity against both *S. aureus* and *P. aeruginosa*, as demonstrated by the concentration-dependent increase in inhibition zone diameters in the disc diffusion assay. At 15% FSPL, the inhibition zone was larger against *S. aureus* than *P. aeruginosa*, indicating greater susceptibility of the former. This differential susceptibility may reflect differences in bacterial envelope architecture. The outer membrane of Gram-negative bacteria can act as an additional permeability barrier to certain antimicrobial compounds, whereas Gram-positive bacteria lack this outer membrane and may therefore be more susceptible to some plant-derived constituents [59].

The antibacterial activity of FSPL was further supported by the MIC and MBC assays. In the MIC assay, bacterial growth was inhibited at concentrations of 5% and above, whereas the pink coloration observed at 1% indicated residual metabolic activity. The MBC assay further demonstrated that 10% FSPL was sufficient to prevent detectable bacterial growth of *S. aureus*, whereas 15% FSPL was required for *P. aeruginosa*.

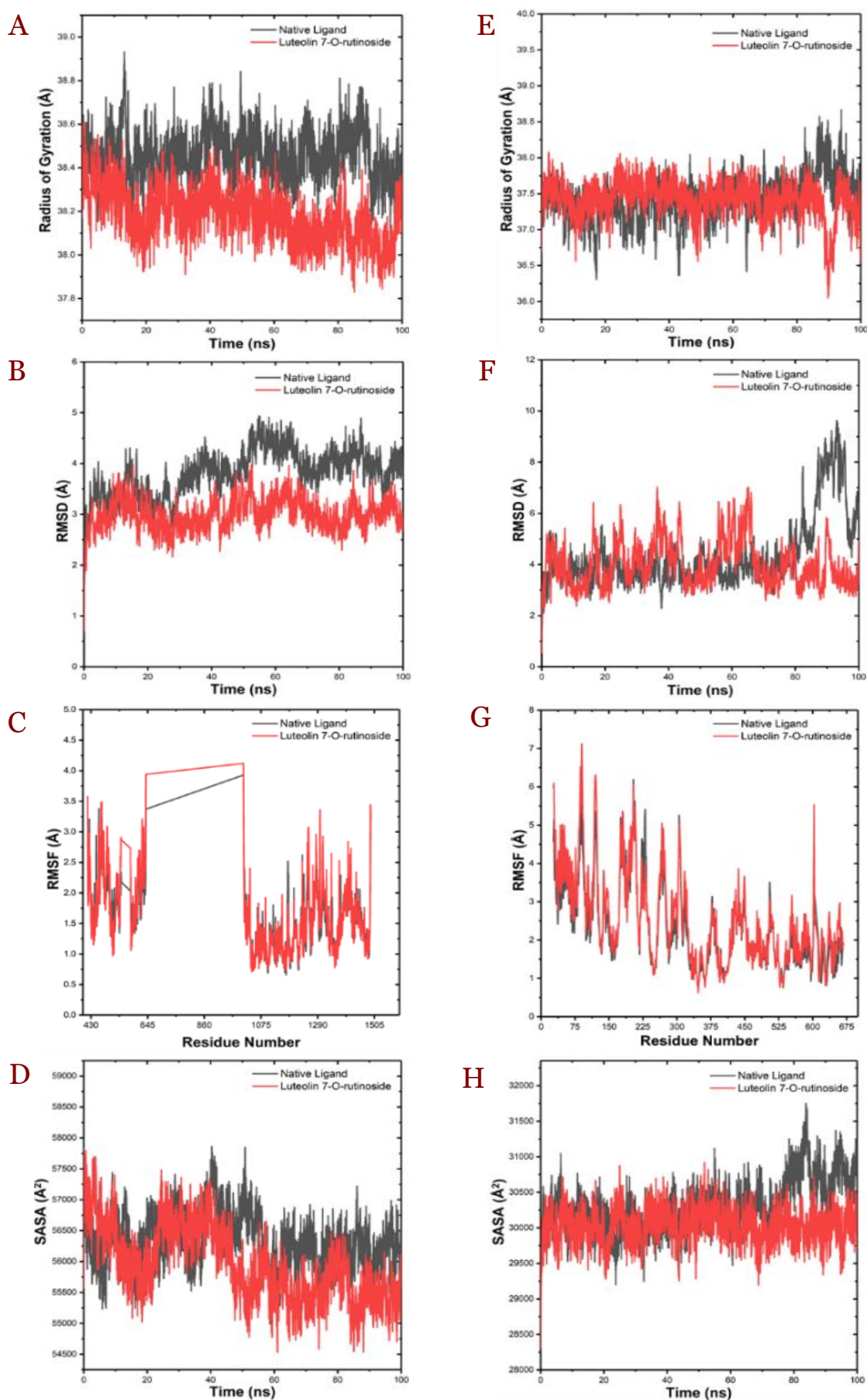


Figure 9. Molecular dynamics simulation profiles of luteolin 7-O-rutinoside and native-ligand complexes with DNA gyrase (A–D) and transpeptidase PBP2a (E–H) over 100 ns. (A, E) Radius of gyration (Rg); (B, F) root mean square deviation (RMSD); (C, G) root mean square fluctuation (RMSF); and (D, H) solvent-accessible surface area (SASA).

These findings indicate that FSPL exhibits a concentration-dependent antibacterial effect, with concentrations required for bactericidal activity differing between the two bacterial species. The greater susceptibility of *S. aureus* across the antibacterial assays is consistent with the possibility that the outer membrane of *P. aeruginosa* limits the access of some FSPL-derived constituents to bacterial targets.

Based on its greater susceptibility to FSPL in the in vitro antibacterial assays, *S. aureus* was selected for subsequent in vivo investigations. FSPL concentrations of up to 10% did not significantly affect larval-to-pupal or pupal-to-adult survival in uninfected *D. melanogaster*, indicating no detectable developmental toxicity under the experimental conditions. This finding supported the use of these concentrations in subsequent infection models and helped distinguish potential treatment effects from nonspecific developmental toxicity. Following *S. aureus* infection, FSPL supplementation significantly improved host survival. Consistent with this finding, CFU analysis showed a reduction in recoverable bacterial burden following FSPL treatment, with bacterial counts below the assay detection limit at 5% and 10% FSPL. Together, these findings provide complementary evidence that FSPL treatment was associated with improved host survival and reduced recoverable *S. aureus* burden in the *D. melanogaster* infection model.

FSPL treatment was also associated with changes in the host immune response. Infection increased *def* expression relative to the non-infected control, whereas FSPL treatment reduced *def* expression compared with the infection control. Because *def* encodes Defensin, an antimicrobial peptide involved in the *Drosophila* innate immune response to bacterial infection [60]. This pattern may reflect reduced immune stimulation following FSPL-mediated suppression of bacterial burden. However, the reduction in *def* expression should not be interpreted as direct evidence of immunosuppression, particularly because it occurred concurrently with reduced bacterial recovery. The observed changes are therefore more consistent with a reduced infection-associated immune stimulus following FSPL treatment.

The potential contribution of oxidative responses was further examined by assessing intracellular ROS levels and the expression of antioxidant-related genes. *S. aureus* infection increased ROS levels compared with the non-infected control, whereas FSPL treatment reduced ROS levels relative to the infection control. In parallel, infection was associated with reduced expression of *sod1*, *sod2*, and *cat* [61]. FSPL did not significantly alter *sod1* or *sod2* expression but significantly increased *cat* expression at 10%. These findings suggest that FSPL modulated infection-associated oxidative responses, although the effect was not uniform across the antioxidant genes examined [62,63]. The reduction in ROS levels together with the selective increase in *cat* expression at the highest FSPL concentration may indicate a contribution of antioxidant responses to the observed protective effect. However, because ROS levels and gene expression were measured as separate outcomes and no direct mechanistic relationship was established, these findings should be interpreted as evidence of an association rather than confirmation of a specific antioxidant mechanism.

Building on the in vitro and in vivo findings, computational analyses were performed to explore potential molecular interactions between FSPL metabolites and two *S. aureus* targets, DNA gyrase and transpeptidase PBP2a. Untargeted LC-HRMS analysis tentatively annotated 18 metabolites based primarily on accurate mass matching. These compounds included flavonoids, phenolic acids, and glycosylated derivatives, providing a preliminary chemical basis for further investigation of the antibacterial activity of FSPL. Luteolin 7-O-rutinoside was subsequently selected for computational analysis based on its relatively high abundance in the LC-HRMS profile and its predicted interactions with the selected bacterial targets. Because the metabolite annotations were not confirmed using authentic standards or comprehensive MS/MS analysis, these compounds should be regarded as tentatively annotated rather than definitively identified.

The molecular docking protocol was first validated by re-docking the co-crystallized ligands into the active sites of DNA gyrase and PBP2a, yielding RMSD values of 1.053 Å and 1.642 Å, respectively. Both values were below the commonly accepted threshold of 2.0 Å [64,65], supporting the ability of the docking protocol to reproduce the experimentally observed ligand-binding poses. Among the evaluated metabolites, luteolin 7-O-rutinoside exhibited the most favorable predicted binding affinities toward DNA gyrase (−9.8 kcal/mol) and PBP2a (−10.4

kcal/mol), with docking scores lower than those of the respective co-crystallized ligands. Furthermore, the compound also formed hydrogen bond interactions with residues within the active sites of both proteins, including Arg1122 and Tyr1150 in DNA gyrase and Ser403 and Lys406 in PBP2a, suggesting that it occupies functionally relevant regions of these targets. Although these findings provide a plausible molecular explanation for the observed antibacterial activity, molecular docking alone cannot establish direct target inhibition or a specific antibacterial mechanism. Instead, the predicted protein-ligand interactions should be regarded as hypothesis-generating and require experimental validation, such as enzyme inhibition assays, target-based biochemical studies, or bacterial genetic approaches.

To further examine the dynamic behavior of the predicted complexes, 100 ns molecular dynamics simulations were performed. The DNA gyrase–luteolin 7-O-rutinoside complex showed average RMSD, Rg, and SASA values of 3.0 Å, 38.18 Å, and 55,950 Å², respectively, compared with 3.8 Å, 38.46 Å, and 56,340 Å² for the DNA gyrase–native ligand complex. For PBP2a, the corresponding values were 4.0 Å, 37.42 Å, and 30,030 Å² for luteolin 7-O-rutinoside and 4.3 Å, 37.42 Å, and 30,296 Å² for the native ligand (7EP). RMSF analysis showed localized residue fluctuations in both protein systems, with the highest fluctuations occurring at residue 1009 for DNA gyrase and at residues 90 and 204 for the PBP2a complexes. The luteolin 7-O-rutinoside complexes exhibited broadly comparable conformational profiles to their corresponding native-ligand complexes throughout the 100 ns simulations. These findings indicate that luteolin 7-O-rutinoside maintained a comparable dynamic profile within the binding environments of both target proteins under the simulated conditions; however, the simulations alone do not establish binding stability or biological activity.

Taken together, the experimental and computational findings provide complementary evidence supporting FSPL as a potential source of antibacterial activity against *S. aureus*. The in vitro assays demonstrated direct antibacterial activity, while the *Drosophila* model showed improved survival and reduced recoverable bacterial burden following FSPL treatment. The associated changes in *def* expression, ROS levels, and *cat* expression suggest that host immune and oxidative responses may also be involved in the response to treatment. The computational analyses further identified luteolin 7-O-rutinoside as a plausible candidate metabolite that may contribute to the antibacterial activity of FSPL through interactions with DNA gyrase and PBP2a. Nevertheless, these findings do not establish luteolin 7-O-rutinoside as the sole active constituent. Other tentatively annotated metabolites and potential interactions among multiple constituents may contribute to the overall biological activity of the fermented preparation [66]. Previous studies reporting antibacterial activity of luteolin and related glycosylated derivatives against Gram-positive bacteria, including *S. aureus*, provide additional support for this possibility [67].

Several limitations should be considered when interpreting the results. First, FSPL was produced through spontaneous fermentation without the use of a defined starter culture, which may introduce batch-to-batch variability in microbial composition and metabolite profiles. Standardization of the fermentation process and systematic assessment of batch-to-batch consistency will therefore be essential to improve experimental reproducibility. Second, metabolite annotations obtained from untargeted LC-HRMS were primarily based on accurate mass matching and thus remain putative. As these annotations were not confirmed using authentic reference standards or comprehensive MS/MS fragmentation analyses, the metabolomic data should be interpreted as a preliminary chemical characterization rather than definitive metabolite identification. Future studies employing targeted metabolomic approaches with tandem mass spectrometry and authentic reference standards are warranted to validate the proposed metabolite identities and provide more robust evidence regarding the chemical constituents potentially responsible for the biological activities of FSPL.

Conclusion

This study provides preliminary evidence that the FSPL possesses antibacterial activity, with greater inhibitory effects against *S. aureus* than *P. aeruginosa* under the experimental conditions tested. In the *D. melanogaster* infection model, FSPL improved host survival, reduced recoverable *S. aureus* burden, and modulated selected immune- and antioxidant-related

markers, including *def* and *cat*. Untargeted LC-HRMS analysis tentatively annotated multiple metabolites in FSPL, whereas subsequent computational analyses identified luteolin 7-*O*-rutoside as a promising candidate for further investigation based on its predicted interactions with DNA gyrase and PBP2a. However, these in silico findings are hypothesis-generating and should not be interpreted as evidence of direct target inhibition or confirmation of the antibacterial mechanism. Collectively, the findings support further investigation of FSPL as a fermented preparation with potential antibacterial properties. Future studies should validate the annotated metabolites using authentic reference standards and tandem mass spectrometry, evaluate the antibacterial activities of individual compounds and their potential synergistic interactions, and assess the efficacy of FSPL in clinically relevant antimicrobial-resistant isolates and more physiologically relevant infection models.

Ethics approval

Ethical approval was not required because the study involved *D. melanogaster*, an invertebrate species that is not covered by the applicable institutional animal ethics review requirements.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

This study used artificial intelligence (AI) tools and methodologies in the following capacities: manuscript writing support. ChatGPT was employed in language refinement (improving grammar, sentence structure, and readability of the manuscript). Technical writing assistance (providing suggestions for structuring complex technical descriptions more effectively). We confirm that all AI-assisted processes were critically reviewed by the authors to ensure the integrity and reliability of the results. The final decisions and interpretations presented in this article were solely made by the authors.

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