

# ISOLATION, MOLECULAR CHARACTERIZATION AND ANTIBACTERIAL PROFILING OF METABOLITES PRODUCED BY *BACILLUS SIAMENSIS* AND *STAPHYLOCOCCUS EQUORUM* ISOLATED FROM TRADITIONALLY FERMENTED COW MILK

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## ABSTRACT

The increasing emergence of antibiotic-resistant pathogens has intensified the search for alternative and natural antimicrobial agents. This study investigated the isolation, molecular characterisation, and antibacterial profiling of metabolite-producing lactic acid-related bacteria from traditionally fermented cow milk (nono). Fermented milk samples were collected from the Zango (Tudun Wada) and Rafinguza areas of Kaduna State, Nigeria. Bacterial isolates were obtained using de Man, Rogosa, and Sharpe (MRS) agar plates. The bacterial isolates were characterised based on their morphological, biochemical, and physiological properties, including gram reaction, catalase activity, carbohydrate fermentation, salt tolerance, and temperature tolerance. The microbes were extracted using ammonium sulfate precipitation and evaluated for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* using the agar well diffusion assay. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. Molecular identification of selected potent isolates was carried out through PCR amplification and sequencing of the 16S rRNA gene, followed by NCBI Blast sequences. Gas chromatography–mass spectrometry (GC–MS) was used to characterise the bioactive compounds in the mixed culture. The lactic acid bacterial counts ranged from  $25.5 \times 10^6$  to  $73.0 \times 10^6$  CFU/mL, confirming that milk is a rich source of Lactic acids related bacteria. All isolates were gram-positive and catalase-negative, with rod- and coccus-shaped morphologies, and demonstrated varying degrees of tolerance to NaCl concentrations (4–6.5%) and temperatures (10–45°C). The identified components exhibited concentration-dependent antibacterial activity against all test pathogens, with stronger inhibition observed against *Staphylococcus aureus* and *Escherichia coli* than against *Pseudomonas aeruginosa*. The MIC and MBC assays confirmed the bacteriostatic and bactericidal effects of the bacterial isolates. Molecular analysis identified the selected potent isolates was performed through genomic DNA extraction, PCR amplification of the 16S rRNA gene, agarose gel electrophoresis, purification, sequencing and BLAST analysis against NCBI Genbank database. The isolates were identified as *Bacillus siamensis* and *Staphylococcus equorum*, sequences and with a Query Cover (%) of 99, an E-value of 0.0, and Percentage Similarity (%) of 99.08 and 96.03, respectively, through NCBI Blast sequences confirming their taxonomic relationships. GC–MS profiling revealed the presence of several bioactive compounds, including fatty acids and terpenoid derivatives, associated with antimicrobial activity. Traditionally fermented cow milk harbours LAB isolates with

significant antibacterial potential, which can serve as natural antimicrobial agents for food preservation and therapeutic applications.

**Keywords:** *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* Fermented milk, *Staphylococcus equorum*, *Bacillus siamensis*.

## INTRODUCTION

### Background of the Study

The growing concerns over antibiotic resistance have led researchers to explore natural antimicrobial agents as potential alternatives to synthetic ones. Among these, bacteriocins, which are synthesised antimicrobial peptides produced by lactic acid-related bacteria, have gained significant attention because of their ability to inhibit pathogenic and spoilage bacteria (Cotter *et al.*, 2021). Bacteriocins are particularly attractive because of their safety, specificity, and potential applications in food preservation and therapy (Chikindas *et al.*, 2021).

Fermented dairy products, including cheese and yoghurt, provide an ideal environment for the growth of lactic acid-related bacteria, such as *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus siamensis*, and *Staphylococcus equorum*, which are known to produce potent bacteriocins (Kumari *et al.*, 2022). These naturally occurring antimicrobials exhibit broad-spectrum activity against foodborne pathogens, such as *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* spp. (Gopal *et al.*, 2020).

The increasing global challenge of antimicrobial resistance (AMR) has created an urgent need for alternative and sustainable antimicrobial agents. Conventional antibiotics are becoming less effective due to the rapid emergence of resistant strains of pathogenic microorganisms, posing serious threats to public health, food safety, and clinical treatment outcomes (Chikindas *et al.*, 2021). Consequently, attention has shifted toward naturally occurring antimicrobial compounds, particularly those produced by beneficial microorganisms, such as lactic acid bacteria (O'Connor *et al.*, 2021).

Lactic acid bacteria are a group of gram-positive, non-spore-forming, catalase-negative microorganisms that are widely distributed in nature, especially in fermented foods (Wang *et al.*, 2021). They are known for their ability to ferment carbohydrates, primarily producing lactic acid as the major metabolic end product. In addition to lactic acid, these bacteria produce a wide range of antimicrobial metabolites, including organic acids (lactic and acetic acids), hydrogen peroxide, diacetyl, fatty acids, and other bioactive

compounds. These metabolites contribute significantly to food preservation, safety, and therapeutic applications (Todorov and Dicks, 2020). Traditionally fermented cow milk, locally known as cow milk in Nigeria, serves as a rich ecological niche for lactic acid bacteria. The natural fermentation process involves spontaneous microbial activity, leading to the proliferation of diverse lactic acid species, such as *Staphylococcus equorum*, *Lactococcus lactis*, and *Lactobacillus fermentum*. These microorganisms not only enhance the sensory qualities of fermented milk but also produce antimicrobial metabolites that inhibit the growth of spoilage and pathogenic microbes (Mills *et al.*, 2021).

The isolation and characterisation of lactic acid-related bacteria from traditionally fermented cow milk provides an opportunity to explore these beneficial metabolites. Molecular characterisation techniques, particularly 16S rRNA gene sequencing, have enhanced the accurate identification and classification of lactic acid bacteria. This approach allows for a deeper understanding of microbial diversity and the selection of potent strains with strong antimicrobial properties (Kim *et al.*, 2021). Furthermore, antibacterial profiling of lactic acid-related bacterial metabolites against common pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* is essential for evaluating their potential applications. These pathogens are associated with foodborne illnesses and increasing antibiotic resistance, making them suitable indicators for antimicrobial research (Field *et al.*, 2022). Despite the recognised importance of lactic acids in food fermentation, there is limited information on the diversity, molecular characteristics, and antimicrobial metabolite profiles of lactic acid-related bacteria isolated from traditionally fermented cow milk in many regions. Therefore, this study focused on the isolation, molecular characterisation, and antibacterial profiling of metabolites produced by lactic acid-related bacteria from fermented cow milk, with the aim of identifying potent strains and bioactive compounds for potential applications in food preservation and medicine (Field *et al.*, 2022).

## MATERIALS AND METHODS

### Study Area

The study area include Zango (Tudun Wada) and Rafin Guza in Kaduna State, Nigeria. Kaduna State is situated in the northwestern region of Nigeria, between latitudes 10°35' North and longitudes 7°25' East. The state shares boundaries with Igabi Local Government Area to the north and west, Kaduna South Local Government Area to the southwest, and Chikun Local Government Area to the east. The region is characterized by relatively flat topography, with elevations ranging between 600 and 650 m above mean sea level. The geological composition predominantly consists of metamorphic rocks from the Nigerian basement complex, including biotite gneisses and older granite. Zango, located in the Tudun Wada area, houses one of the major abattoirs in Kaduna Metropolis. Covering an area exceeding 27,000 square meters, this facility meets the standard requirements for abattoirs regarding design, space, and functionality. Rafin Guza is one of the 12 subdivisions or electoral wards within the Kaduna North Local Government Area. The area is part of the urban expanse of Kaduna City and contributes to the diverse cultural and economic activities of the metropolis (Ahmad *et al.*, 2023).

### Samples Collection

Fermented cow milk samples, commonly known as "nono", were collected from two locations: Zango (Tudun Wada) and Rafinguza. These places are integral to the local dairy trade, involving the rearing of different animals, and each serves as a hub for the distribution and consumption of traditional fermented dairy products. The milk samples were collected in sterile, corked plastic bottles and packed in an iced container. All purchased samples were taken to the Department of Biotechnology Laboratory at the Nigerian Defence Academy, where all analyses were conducted. The indicator microorganisms used to determine antibacterial activity were obtained from the same institution..



Figure 1: Map showing the sample collection sites (Source: OSGOF Modify by RS/GIS Lab. Department of Geography, NDA, 2025).

## Isolation of Lactic acid-Producing Bacteria from fermented Cow milk.

### Serial Dilution and Plating

The fermented cow milk sample was initially serially diluted in a sterile saline solution, with dilution factors ranging from  $10^{-1}$  to  $10^{-6}$ . Each dilution was thoroughly mixed to ensure homogeneity, and a portion of each was then plated onto de Man, Rogosa, and Sharpe (MRS) agar plates. MRS agar is commonly used to cultivate lactic acid bacteria because of its selective components that promote the growth of lactobacilli, *Staphylococcus equorum*, enterococci, and other related microorganisms (Gänzle *et al.*, 2022). Following plating, the agar plates were incubated under anaerobic conditions at 37°C for 24–48 h. Anaerobic incubation is essential for promoting the growth of obligate anaerobes and microaerophilic organisms, which might otherwise be outcompeted by aerobic species (Mohammed *et al.*, 2023).

### Bacterial counts

The incubation period allowed for colony development, after which the number of colonies was counted to determine the microbial load in the original fermented milk sample. This method of quantifying microbial populations is commonly referred to as plate counting, and it provides valuable insights into the microbial diversity and population dynamics in fermented foods (Zhang *et al.*, 2023).

### Biochemical Characterization

#### Gram Staining

Gram staining was performed to differentiate bacterial isolates based on their cell wall composition. A fresh bacterial colony from the MRS agar plate was aseptically transferred onto a clean glass slide, mixed with a drop of sterile water, and heat-fixed by passing the slide over a flame. The smear was then sequentially treated with crystal violet stain for 60 s, followed by iodine solution (mordant) for 60 s, decolourised using 95% ethanol for 10–15 s, and counterstained with safranin for 30 s. After each step, the slides were gently rinsed with distilled water. The prepared slide was examined under an oil immersion microscope at 1000× magnification to observe bacterial morphology and the Gram reaction. Gram-positive bacteria, including lactic acid-related bacteria, appeared purple, whereas Gram-negative bacteria retained the safranin stain and appeared pink (Singh *et al.*, 2020). Gram staining is a crucial step in bacterial identification that provides preliminary insights into the taxonomy of microbial isolates (Jørgensen *et al.*, 2021).

#### Catalase Test

A catalase test was conducted to determine the presence of catalase, which breaks down hydrogen peroxide ( $H_2O_2$ ) into water and oxygen. A small amount of bacterial culture was transferred onto a clean glass slide, and a drop of 3% hydrogen peroxide was added. The reaction was observed by the immediate formation of bubbles, which indicated a positive catalase reaction. Lactic acid-related bacteria, including *Lactobacillus*, *Staphylococcus equorum*, *Lactococcus*, and *Streptococcus* spp., are typically catalase-negative, whereas catalase-positive bacteria, such as *Staphylococcus* spp., produce visible oxygen bubbles (Gopal *et al.*, 2021). The catalase test is an important biochemical assay for distinguishing lactic acid-related bacteria from other bacterial

groups and confirming their aerotolerance characteristics (Kim *et al.*, 2021).

#### Citrate Utilization test

Lactic acid-related bacterial isolates were streaked onto Simmons' citrate agar and incubated at 37°C for 48 h. A colour change from green to blue indicates positive utilization while absence of blue colouration showed negative which is commonly observed in *Leuconostoc* and *Lactococcus* spp (Hammes & Hertel, 2021).

#### Arginine Hydrolysis

Lactic acid-related bacterial isolates were inoculated into arginine-containing broth and incubated at 37°C for 24–48 h. The hydrolysis of arginine of ammonia ( $NH_3$ ) was detected using Nessler's reagent, which produced a yellow to brown colour in positive isolates (Gänzle & Follador, 2022).

#### Gas Production from Glucose

Lactic acid-related bacterial isolates were inoculated into MRS broth with an inverted Durham tube and incubated at 37°C for 48 h. Gas production ( $CO_2$ ) was observed as gas bubbles in the Durham tube indicating a positive heterofermentative reaction, while absence of gas indicated a homofermentative pathway (Chen *et al.*, 2022).

#### Bile Salt Tolerance Test

Lactic acid-related bacterial isolates were inoculated into MRS broth supplemented with 0.3% (w/v) bile salts and incubated at 37°C for 24 h. Growth was assessed by measuring the OD600. Isolates showing  $\geq 50\%$  survival were considered bile-tolerant (Cleveland *et al.*, 2020).

#### Growth at Different Temperatures and Salt Concentrations

Lactic acid-related bacterial isolates were inoculated into MRS broth and incubated at 10, 37, and 45°C for 48 h. Similarly, the ability to tolerate salt was assessed by growing the isolates in MRS broth containing 4% and 6.5% NaCl at 37°C for 48 h. Growth was monitored by measuring the optical density at 600 nm (OD600). LAB typically grow at 37°C and tolerate up to 4% NaCl, while some strains exhibit limited growth at 10°C or 6.5% NaCl (Holzapfel & Wood, 2021).

#### Extraction of Crude Antimicrobial Metabolites

To obtain the cell-free supernatant, bacterial cultures were grown in De Man, Rogosa, and Sharpe (MRS) broth at 37°C for 24 h. This incubation period allowed the bacteria to reach the stationary phase of growth. Following incubation, the cultures were centrifuged at 10,000 rpm for 10 min to separate the bacterial cells from the culture medium. The supernatant, which contained the bacteriocins and other soluble components, was carefully decanted to ensure complete removal of the cells. Subsequently, the pH of the collected supernatant was adjusted to 6.5 using 1N sodium hydroxide (NaOH) to neutralise any acidic components and maintain optimal stability for subsequent purification steps (Javed *et al.*, 2021).

#### Concentration of Crude metabolites by Ammonium Sulphate Precipitation

The resulting supernatant containing the crude extract was collected for further processing. Solid ammonium sulfate was

gradually added to the cell-free supernatant to achieve a specific saturation level, commonly between 40% and 80%. For instance, an 80% saturation was found to enhance antimicrobial activity by 10.31-fold, with a 48% yield (Zhang *et al.*, 2018). The mixture was gently stirred to ensure complete dissolution of ammonium sulfate. The solution was then incubated overnight at 4°C for further analysis (Jorgensen *et al.*, 2021).

### Antibacterial Activity of Crude Antimicrobial Metabolites

#### Test for Microorganisms

The test pathogens, including *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, were obtained from the Department of Biotechnology NDA Lab and maintained on nutrient agar slants at 4°C (Chen *et al.*, 2022).

#### Antibacterial Activity Assay

Pathogenic bacteria were inoculated onto the surface of solidified Mueller-Hinton agar plates. Sterile cork borers were used to create wells in the agar plates. Each well was then filled with 50 µL of the purified metabolites produced by lactic acid-related bacteria. The plates were incubated at 37°C for 24 h. Following incubation, the zones of inhibition around the wells were measured to assess the antibacterial activity of the metabolites produced by lactic acid-related bacteria (Elayajara *et al.*, 2021).

#### Determination of Minimum Inhibitory and Minimum Bactericidal Concentration

To determine the MIC and MBC of the bacterial isolates, a two-fold broth microdilution assay was performed. Serial dilutions of the bacterial isolates were prepared in a suitable broth medium. Each dilution was inoculated with the target pathogenic bacteria and incubated under the appropriate conditions. The MIC and MBC were defined as the lowest concentrations of the related bacteria that inhibited visible bacterial growth after the incubation period, and the MBC was determined as the lowest concentration that killed bacteria completely (Das *et al.*, 2022).

#### Molecular characterization DNA Extraction

Genomic DNA was extracted from the bacterial isolates using the protocol by the Department of Biotechnology Lab, NDA. The cultured cells were centrifuged for 5 minutes at 300xg, and the supernatant was carefully discarded without disturbing the pellet. The pellet was resuspended in 200 µL of 1x PBS. Next, 20 µL of Proteinase K was added, followed by 10 µL of RNase mix, and the mixture was incubated for 2 min at room temperature. Subsequently, 200 µL of GB Buffer was added and mixed well. The samples were then incubated at 60°C for 10 min. Subsequently, 400 µL of absolute ethanol was added and mixed well using pipetting. The solution was transferred to a binding column containing a silica membrane. The tube was closed and centrifuged at 8000 rpm for 1 min to bind the DNA to the column. The flow-through was then discarded. Then, 500 µL of WB2 Buffer was added and centrifuged. The flow-through was discarded, and the column was centrifuged at 13,000 rpm. The binding column tube was transferred to a new 1.5 mL tube for elution, and EA Buffer (50–200 µL) was added. The mixture was allowed to sit for approximately 1 min at room temperature (15–25°C) and centrifuged at 8000 rpm for 1 min to elute the DNA.

#### PCR Amplification

The 16S rRNA gene was amplified using universal bacterial primers 27F (5-GGACTACAGGGTATCTAAT-3) and 1492R (5-AGAGTTTGATCCTGG-3) (Zhang *et al.*, 2016), which target the conserved regions of the bacterial gene. Each PCR reaction contained 1 µL of template DNA, 10 µL of PCR buffer, 0.5 µL of Taq polymerase, 1 µL of each primer (10 µM), 1 µL of dNTP mix, and 36.5 µL of nuclease-free water to a final volume of 50 µL. The PCR conditions included initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min (Elayajara *et al.*, 2021).

#### Gel Electrophoresis

The amplified products were visualised on a 1.5% agarose gel stained with ethidium bromide (Sigma-Aldrich). Electrophoresis was conducted at 100 V for 30 minutes, and the bands were observed under UV light. A 100 bp DNA ladder was used as a molecular marker to confirm the expected amplicon size of the 16S region (Ahmad *et al.*, 2021).

#### Purification of PCR Products

PCR products were purified using a commercial EasyTaq PCR SuperMix kit following the manufacturer's instructions. The purified DNA was eluted in 30 µL of nuclease-free water and quantified using a NanoDrop spectrophotometer to ensure sufficient quality and quantity for sequencing (Chen *et al.*, 2022). The amplified materials were sequenced along with the pertinent sequencing fragments. The bacterial isolates were subsequently validated and categorized at the species level using GenBank NCBI's BLAST search (National Center for Biotechnology Information). Identification of the isolates was based on percentage sequence similarity, query coverage and E- value obtained from BLAST analysis. This analysis confirmed the most active isolates as *Bacillus siamensis* and *Staphylococcus equorum*.

#### Gas Chromatography Mass Spectrometry of Antimicrobial Metabolites

Metabolite extraction and chromatographic separation were performed to identify the bioactive metabolites in the extract. The active components of the antimicrobial metabolites were isolated and characterised via GC–MS analysis using a Shimadzu GC–MS-QP2010 PLUS (Japan). Lyophilised samples were dissolved in GC-grade methanol, filtered through a syringe filter, and injected into the GC-MS instrument following the manufacturer's protocols (SHIMADZU GC-MS) (Koilybayeva *et al.*, 2023). Helium was used as the carrier gas, and the metabolites were identified by matching retention times and mass spectra obtained via GC–MS software and fragmentation analysis to determine compound structures and mass spectrum values (m/z) (Koilybayeva *et al.*, 2023).

#### Statistical Analysis

The results are presented as the mean ± standard deviation of three determinations, which were subjected to one-way analysis of variance (ANOVA) to compare the means of three or more groups to determine if at least one group was significantly different from the other groups, with a significance level of  $P < 0.05$ .

## RESULTS

### Bacterial count obtained from milk extract at two different locations in Kaduna Metropolitan area

The results presented in Table 1 show that the bacterial counts in milk obtained from two different locations in the Kaduna Metropolitan Area varied notably. The highest LAB count was observed in the sample from Tundun Wada (Zango) Black Cow B1 at  $73.0 \pm 2.0 \times 10^6$  CFU/mL, followed by Rafingusa White Cow W2 at  $65.0 \pm 0.0 \times 10^6$  CFU/mL. Tundun Wada (Zango) Black Cow B3 had a moderate count of  $45.0 \pm 2.0 \times 10^6$  CFU/mL, while the lowest Bacterial counts was recorded in the sample from Rafingusa White Cow W4 with  $25.5 \pm 0.5 \times 10^6$  CFU/mL. This variation suggests that the differences in environmental conditions, such as ambient temperature, hygiene practices during milking and fermentation, water quality, and handling methods, may account for the observed disparities (Iranmanesh *et al.*, 2025).

**Table 1:** Bacterial count obtained from Nono extract at two different locations in Kaduna Metropolitan area

SAMPLES ID	X10 <sup>6</sup> CFU/mL
ZBC1	73.0±2.0
RWC2	65.0±0.0
ZBC3	45.0±0.2
RWC4	25.5±0.5

**Key:** ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafingusa white cow

### Morphological Characteristics of the Lactic acid related bacteria isolated from milk

Morphological characterisation of lactic acid-related bacteria isolated from milk, a traditionally fermented milk product, revealed that all isolates were gram-positive, which is a hallmark characteristic of lactic acids. Gram-positive staining indicates a thick peptidoglycan cell wall, consistent with their taxonomic classification and known resistance to environmental stress in fermented food systems. The isolates displayed two primary shapes: rod-shaped 0.5-1.2 µm wide and 2-10 µm long. Coccus is 0.5-1.5 µm long in diameter in table 2. Most of the isolates, including ZBC1, RWC2, ZBC3, and RWC4, exhibited rod

morphology, suggesting their identification as members of lactic acid-related bacteria. In contrast, RWC2 was the only isolate with a cocci shape, supporting its tentative identification as lactic acid-related bacteria which are also commonly found in dairy fermentations and contribute to flavour and preservation. The morphological and biochemical characteristics of lactic acid related bacteria isolated from cow milk in the present study are in strong agreement with earlier reports by (Axelson, 2020; Javed *et al.*, 2020) on traditionally fermented milk products.

**Table 2:** Morphological Characteristics of the Lactic acid related bacteria isolated from Milk

Samples ID	Gram reaction	Shape	Color	Elevation	Margin
ZBC1	+ve	Rod	White	Raised	Entire
RWC2	+ve	Coccus	Cream	Convex	Undulate
ZBC3	+ve	Rod	White	Flat	Entire
RWC4	+ve	Rod	Cream	Convex	Entire

**Key:** ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafingusa white cow

### Biochemical Characteristics of the Lactic acid related bacterial isolates from Milk

The biochemical characteristics of the bacterial isolates obtained from milk are presented in Table 3. All isolates (ZBC1, RWC2, ZBC3, and RWC4) were gram-positive rods. Each isolate tested negative for catalase activity, confirming their classification as lactic acid bacteria. The citrate utilisation test showed that isolates ZBC1 and ZBC3 were citrate-positive, whereas RWC2 and RWC4 were citrate-negative. Variation was also observed in the bile solubility test, with RWC2 and ZBC3 showing positive reactions, whereas ZBC1 and RWC4 were bile-negative. All isolates demonstrated arginine hydrolysis, indicating their ability to metabolize arginine. Gas production was observed only in isolate RWC2, whereas ZBC1, ZBC3, and RWC4 were non-gas producers. This is in agreement with the study by Javed *et al.* (2020), who reported similar outcomes on traditionally fermented milk products.

**Table 3:** Biochemical characteristics of the bacterial isolates from the Milk

Sample ID	Catalase Test	Citrate	MSA	TSIA	Bile solubility	Arginine hydrolysis	Gas Production	Protobal Organism
ZBC1	-	+	+	-	-ve	+ve	-ve	<i>Streptococcus spp</i>
RWC2	-	-	-	+	+ve	+ve	+ve	<i>Lactococcus spp</i>
ZBC3	-	+	-	-	+ve	+ve	-ve	<i>L.bacillus/ Fermentum</i>
RWC4	-	-	-	+	-ve	+ve	-ve	<i>L.bacillus/ Plantarum</i>

**Key:** ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafingusa white cow

### Stress Tolerance Test of the Bacteria Isolates

The observed stress tolerance suggests that the isolates, particularly ZBC1 and RWC4, are well adapted to the harsh and variable conditions of fermented cow milk. These traits are important because high salt tolerance enhances survival in fermented milk products (Ahmad *et al.*, 2023), and temperature

flexibility ensures persistence during ambient fermentation. These characteristics also support their potential as probiotic metabolite producers, as previously reported (Tamang *et al.*, 2020). The results in table 4 showed that ZBC1 was the most salt-adapted isolate, whereas RCW3 was comparatively less tolerant. This result is consistent with that reported by Tamang *et al.* (2020).

**Table 4: Stress Tolerance Test of the bacteria isolates**

Samples ID	Salt Tolerance Test (%)		Temperature Tolerance Test (°C)		
	4%NaCl (O.D <sub>600</sub> )	6.5%NaCl (O.D <sub>600</sub> )	10°C (O.D <sub>600</sub> )	37°C (O.D <sub>600</sub> )	45°C (O.D <sub>600</sub> )
ZBC1	0.556±0.003 <sup>c</sup>	0.402±0.003 <sup>d</sup>	0.428±0.004 <sup>c</sup>	0.706±0.007 <sup>c</sup>	0.550±0.002 <sup>b</sup>
RWC2	0.380±0.008 <sup>b</sup>	0.279±0.092 <sup>b</sup>	0.223±0.001 <sup>a</sup>	0.659±0.008 <sup>c</sup>	0.437±0.006 <sup>a</sup>
ZBC3	0.380±0.06 <sup>b</sup>	0.219±0.008 <sup>b</sup>	0.244±0.006 <sup>a</sup>	0.543±0.002 <sup>b</sup>	0.451±0.008 <sup>b</sup>
RWC4	0.565±0.003 <sup>c</sup>	0.342±0.009 <sup>c</sup>	0.325±0.013 <sup>b</sup>	0.462±0.002 <sup>b</sup>	0.520±0.004 <sup>b</sup>
Control	0.163±0.001 <sup>a</sup>	0.156±0.010 <sup>a</sup>	0.221±0.00 <sup>a</sup>	0.283±0.002 <sup>a</sup>	0.569±0.001 <sup>c</sup>

Values are given as mean ± standard deviation (SD). In each column, values with different superscripts are statistically different (p< 0.05)  
 Key: ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafingusa white cow

**Antibacterial Bioassay**

The antibacterial activity results indicate that the metabolites produced by bacterial isolates from cows possess significant inhibitory effects against both Gram-positive and Gram-negative pathogens in tables 5, 6 & 7 . The inhibitory activity decreased progressively at lower metabolite concentrations, demonstrating a concentration-dependent antimicrobial action. Among the isolates,

ZBC1 and ZBC3 extracts exhibited the most consistent antibacterial activity across all pathogens and concentrations. The ZBC1 extract exhibited strong activity at high concentrations but weaker stability at low concentrations, whereas RWC2 and RWC4 were slightly weaker but more stable. Meanwhile, ZBC3 and RWC2 extracts demonstrated exceptional activity against *E. coli*. This finding is consistent with that of Todorov *et al.* (2020).

**Table 5: Antibacterial Activity (zone of inhibition, mm) of Bacteria isolates against P. aeruginosa at different extract concentration (%)**

Sample ID	100(%)	75(%)	50(%)	25(%)
ZBC1	12.2±0.2 <sup>a</sup>	9.85±0.2 <sup>a</sup>	8.7±0.3 <sup>b</sup>	7.2±0.2 <sup>a</sup>
RWC2	11.0±0.0 <sup>a</sup>	9.2±0.2 <sup>a</sup>	7.4±0.2 <sup>a</sup>	0.0±0.0
ZBC3	12.95±0.2 <sup>a</sup>	10.2±0.2 <sup>a</sup>	8.9±0.2 <sup>b</sup>	0.0±0.0
RWC4	11.1±0.4 <sup>a</sup>	8.9±0.1 <sup>a</sup>	0.0±0.0	0.0±0.0
Control (Cipro)	24.7±0.2 <sup>b</sup>	20.6±0.25 <sup>b</sup>	16.05±0.25 <sup>c</sup>	14.9±0.2 <sup>b</sup>

Values are given as mean ± standard deviation (SD). In each column, values with different superscripts are statistically different (p< 0.05).  
 Key: ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafinuosa white cow.

**Table 6: Antibacterial Activity (zone of inhibition, mm) of Bacteria isolates against S. aureus at different extract concentration (%)**

Sample ID	100(%)	75(%)	50(%)	25(%)
ZBC1	13.2±0.2 <sup>b</sup>	10.1±0.25 <sup>b</sup>	8.15±0.15 <sup>b</sup>	7.2±0.2 <sup>a</sup>
RWC2	13.7±0.3 <sup>c</sup>	10.7±0.6 <sup>b</sup>	8.0±0.0 <sup>b</sup>	0.0±0.0
ZBC3	13.05±0.05 <sup>b</sup>	10.1±0.05 <sup>b</sup>	8.1±0.1 <sup>b</sup>	7.05±0.05 <sup>a</sup>
RWC4	10.25±0.25 <sup>a</sup>	8.05±0.05 <sup>a</sup>	7.35±0.15 <sup>a</sup>	0.0±0.0
Control (Cipro)	22.4±0.35 <sup>d</sup>	17.8±0.05 <sup>c</sup>	15.2±0.1 <sup>c</sup>	12.1±0.1 <sup>b</sup>

Values are given as mean ± standard deviation (SD). In each column, values with different superscripts are statistically different (p< 0.05).  
 Key: ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafingusa white cow

**Table 7: Antibacterial Activity (zone of inhibition, mm) of Bacteria isolates against E.coli at different extract concentration (%)**

Sample ID	100(%)	75(%)	50(%)	25(%)
ZBC1	13.1±0.05 <sup>b</sup>	10.0±0.0 <sup>b</sup>	8.35±0.05 <sup>a</sup>	7.4±0.3 <sup>a</sup>
RWC2	10.8±0.2 <sup>a</sup>	10.3±0.1 <sup>b</sup>	8.15±0.15 <sup>a</sup>	7.2±0.2 <sup>a</sup>
ZBC3	10.9±0.25 <sup>a</sup>	8.3±0.2 <sup>a</sup>	0.0±0.0	0.0±0.0
RWC4	14.4±0.1 <sup>b</sup>	11.9±0.2 <sup>b</sup>	10.1±0.05 <sup>b</sup>	7.7±0.3 <sup>a</sup>
Control (Cipro)	25.2±0.05 <sup>c</sup>	22.8±0.1 <sup>c</sup>	16.6±0.4 <sup>c</sup>	13.1±0.2 <sup>c</sup>

Values are given as mean ± standard deviation (SD). In each column, values with different superscripts are statistically different (p< 0.05).  
 Key: ZBC1=Zango black cow, RWC2= Rafinguosa white cow, ZBC3= Zango black cow and RWC4= Rafinguosa white cow

**Result for the Minimum Inhibitory and Minimum Bacteriocidal Concentration**

The MIC and MBC findings indicate that metabolites produced by the bacterial isolates from cow milk possess appreciable antibacterial activity against both Gram-positive and Gram-negative pathogens (Jørgensen *et al.*, 2021). In tables 8, 9 & 10, lower MIC and MBC values indicate stronger antibacterial potency

because smaller concentrations are sufficient to inhibit or eliminate pathogens. The study suggests that RWC2 and RWC4 cow extracts exhibited the most consistent bacteriocidal activity, whereas ZBC1 and ZBC3 cow extracts displayed strong inhibitory activity. This is in agreement with the study by Jones *et al.* (2022), who reported similar outcomes.

**Table 8:** MIC & MBC ( µg /mL) of bacterial isolates against *P. aeruginosa*

Samples ID	MIC (w/v)	MBC (w/v)
ZBC1	25(%)	75(%)
RWC2	50(%)	50(%)
ZBC3	75 (%)	25(%)
RWC4	75 (%)	100(%)
Control (Ciprofloxacin)	25(%)	25(%)

**Key:** ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafingusa white cow

**Table 9:** MIC & MBC ( µg /mL) of bacterial isolates against *S. aureus*

Samples ID	MIC (w/v)	MBC (w/v)
ZBC1	25(%)	50 (%)
RWC2	50(%)	50(%)
ZBC3	25(%)	50(%)
RWC4	75 (%)	100(%)
Control (Ciprofloxacin)	25(%)	25(%)

**Key:** ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafingusa white cow

**Table 10:** MIC & MBC ( µg /mL) of bacterial isolates against *E. coli*

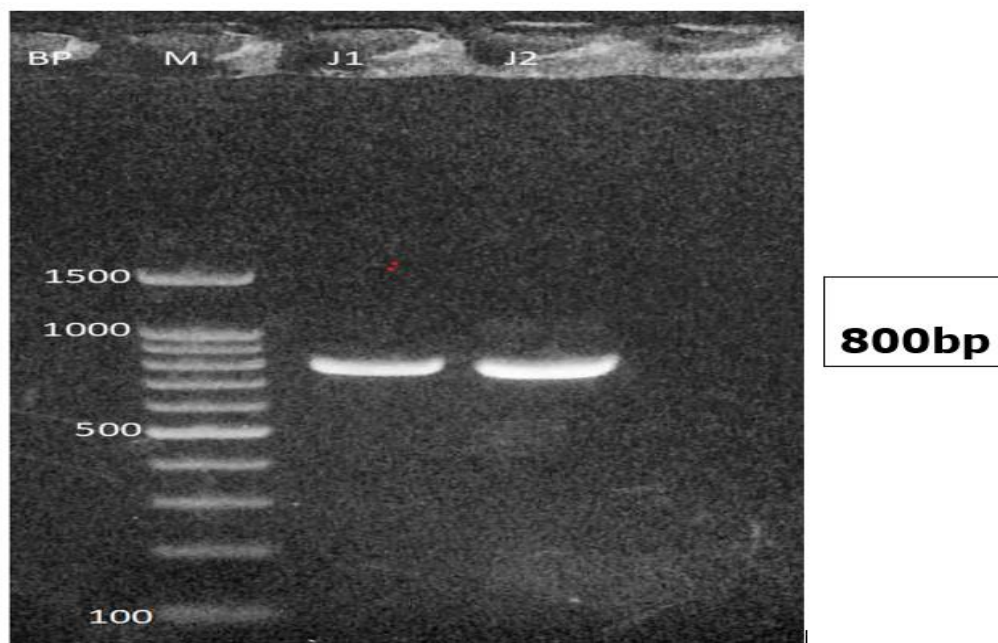
Samples ID	MIC (w/v)	MBC (w/v)
ZBC1	25(%)	75(%)
RWC2	50(%)	50(%)
ZBC3	75(%)	75(%)
RWC4	25(%)	50(%)
Control (Cipro)	25(%)	25(%)

**Key:** ZBC1=Zango black cow, RWC2= Rafingusa white cow, ZBC3= Zango black cow and RWC4= Rafingusa white cow

**Molecular Characterization and Sequencing**

The two bacterial isolates were molecularly characterised to confirm their identities. The 16S rRNA gene bands, with a predictable size of approximately 800 bp, were successfully achieved among the two isolates, as shown in Fig. 2. The electrophoretic profile validated the quality of the PCR products and supported 16S rRNA genomic hybridisation in the molecular identification of bacterial isolates (Sure *et al.*, 2020). Nucleotide sequence BLAST analysis confirmed the two identified isolates:

ZBC1 as *Bacillus siamensis* and RWC2 as *Staphylococcus equorum*, with the same Query Cover (99%) and E-Values (0.0) but different Percentage Similarity (99.08% and 96.03%) and Accession numbers (PZ534545 and PZ534652), as shown in Table 11. The clear separation of RWC2 and ZBC1 into distinct clusters supports the conclusion that the isolates belong to different lactic acid-related bacterial species. The strong bootstrap support observed across key nodes further reinforces the reliability of species-level assignments (Altinoz *et al.*, 2020).



**Figure 2:** Agarose gel electrophoresis of Bacterial isolates.  
 Key: J1=ZBC1 cow, J2= RWC2 cow, M= Ladder

**Table 11:** Nucleotide Sequence Blast

Sample ID	Scientific Name	Query Cover (%)	E- value	Percentage Similarity (%)	Accession Number
ZBC1	<i>Bacillus Sisamensis</i>	99	0.0	99.08	PZ534545
RWC2	<i>Staphylococcus equorum</i>	99	0.0	96.03	PZ534652

### GC-MS Characterization

The GC-MS analysis of lactic acid-related bacteria-derived antimicrobial preparations ZBC1 and RWC2 in this study aligns conceptually with earlier GC-MS-based investigations of bacterial metabolites. This is particularly true for the usefulness of GC-MS as a rapid and sensitive technique for profiling complex bioactive

compounds in microbial extracts (Wang *et al.*, 2022). GC-MS revealed that the bacterial isolates ZBC1 and RWC2 contained multiple fatty acids and lipid-associated compounds, including hexadecanoic acid (palmitic acid), oleic acid, and octadecanoic acid, as well as terpenoids, alcohols, aromatic compounds, and nitrogen-containing molecules (Wang *et al.*, 2022).

**Table 12:** The Major compounds identified from the GC-MS analysis of ZBC1 CFS.

Peak No.	Compound name (best library hit)	Retention time (min)	Molecular formula	Molecular weight (g/mol)	Bio-activity
1	1-Octadecyne	15.30	C <sub>18</sub> H <sub>34</sub>	250	Antimicrobial
2	Hexadecanoic acid (Palmitic acid)	17.93	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256	Anti inflammatory
3	Oleic acid (cis-9-Octadecenoic acid)	20.78	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	282	Anti inflammatory
4	Octadecanoic acid (Stearic acid)	21.00	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	284	Anti inflammatory
5	9,12-Octadecadien-1-ol (Z,Z)	21.22	C <sub>18</sub> H <sub>34</sub> O	266	Antimicrobial
6	1,11,13-Octadecatriene	21.70	C <sub>18</sub> H <sub>32</sub>	248	Antimicrobial
7	Oleic acid (repeat identification)	22.00	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	282	Antimicrobial
8	Caryophyllene oxide	25.58	C <sub>15</sub> H <sub>24</sub> O	220	

**Table 13:** The Major compounds identified from the GC-MS analysis of RWC2 CFS

Peak No.	Compound name (best library hit)	Retention time (min)	Molecular formula	Molecular weight (g/mol)	Bio-activity
1	Benzenepropanoyl bromide (Hydrocinnamoyl bromide)	6.09	C <sub>9</sub> H <sub>9</sub> BrO	212	
2	Ethene, 1,2-bis(methylthio)-	6.63	C <sub>4</sub> H <sub>8</sub> S <sub>2</sub>	120	Antimicrobial
3	(2-Methoxyethyl)benzene (Phenylethyl methyl ether)	6.78	C <sub>9</sub> H <sub>12</sub> O	136	Antimicrobial
4	1-Allyl-1H-imidazole	7.20	C <sub>6</sub> H <sub>8</sub> N <sub>2</sub>	108	Antimicrobial
5	N-Methyl-N-phenylacetamide (N-methylacetanilide)	15.34	C <sub>9</sub> H <sub>11</sub> NO	149	Anti inflammatory
6	5-Amino-1,4-dihydroquinoxaline-2,3-dione	15.53	C <sub>8</sub> H <sub>7</sub> N <sub>3</sub> O <sub>2</sub>	177	Anti inflammatory
7	Isothujol (Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-)	15.82	C <sub>10</sub> H <sub>18</sub> O	154	Antimicrobial
8	n-Hexadecanoic acid (Palmitic acid)	21.08	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256	Anti inflammatory
9	L-(+)-Ascorbic acid 2,6-dihexadecanoate	21.83	C <sub>38</sub> H <sub>68</sub> O <sub>8</sub>	652	Antimicrobial

## DISCUSSION

Figure 1: Map showing the sample collection sites (Source: OSGOF Modify by RS/GIS Lab. Department of Geography, NDA, 2025). The lactic acid-related bacterial counts obtained from nono extracts collected from two locations within the Kaduna Metropolitan area revealed marked variations between samples and locations showed in table 1. Overall, the LAB populations ranged from  $25.5 \pm 0.5 \times 10^6$  CFU/mL to  $73.0 \pm 2.0 \times 10^6$  CFU/mL, indicating that nono is a rich source of lactic acid bacteria, consistent with its nature as a traditionally fermented milk product. Samples obtained from Rafingosa (RW2 and RW4) exhibited notably higher bacterial counts ( $73.0 \pm 2.0$  and  $65.0 \pm 0.0 \times 10^6$  CFU/mL, respectively) than those from Zango (ZB1 and ZB3), which recorded lower counts of  $45.0 \pm 2.0$  and  $25.5 \pm 0.5 \times 10^6$  CFU/mL, respectively. These findings are comparable to those of Akabanda *et al.* (2021) and Mohammed and Ijah (2013), who also reported that differences in environmental conditions, such as ambient temperature, hygiene practices during milking and fermentation, water quality, and handling methods, may account for the observed disparities.

In tables 2 & 3 morphological and biochemical characteristics of lactic acid-related bacteria isolated from cow milk in the present study strongly agree with earlier reports on traditionally fermented milk products. All isolates were gram-positive and catalase-negative, which are defining features of lactic acid-related bacteria and are consistent with their fermentative metabolism and adaptation to acidic dairy environments. Similar observations have been reported for bacteria isolated from cow milk and other fermented milk products, where isolates were described as gram-positive bacilli, non-spore-forming, non-motile, and catalase-negative, which are typical traits of the genus *Lactobacillus* (Jothi *et al.*, 2012; Axelsson 2020; Arokiyamar & Sivakumar, 2021; Usman *et al.*, 2022). Axelsson, 2020; Arokiyamar & Sivakumar, 2021; Usman *et al.*, 2022).

Comparable findings have been reported across different geographical regions and for fermented milk products. Surya *et al.*

(2020) isolated diverse *Lactobacillus* species from dadih, a fermented buffalo milk in Indonesia, while Mobarez *et al.* (2020) reported *Lactobacillus* strains in sheep and cow milk. In Nigeria, Adesokan *et al.* (2011) identified *L. brevis*, *L. casei*, *L. fermentum*, and *L. plantarum* in traditionally fermented cow milk in Ibadan. Earlier studies by Jothi *et al.* (2012) also documented the isolation of LAB from cow milk, which were successfully used to develop starter cultures. In table 2&3 Similarly, Gran *et al.* (2021) reported diverse LAB populations in amasi, a naturally fermented milk product from Zimbabwe. The ability of all isolates to hydrolyse arginine reflects a common adaptive mechanism that enhances survival under acidic fermentation conditions Mobarez *et al.* (2020).

The results in table 4 demonstrated that bacteria isolated from cow milk possessed notable tolerance to salt and temperature stress, with isolates ZBC1 and RWC4 showing the most robust performance across all tested conditions. These findings are in agreement with those of previous studies that have reported high stability of lactic acid-related bacterial isolates under diverse environmental stresses, reinforcing their suitability as natural preservatives in food systems (Iranmanesh *et al.*, 2012).

The present study demonstrated in tables 5, 6 & 7 that lactic acid-related bacteria possess appreciable antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, with inhibitory effects that were strongly concentration-dependent. Among the tested bacterial isolates, ZBC1 and ZBC3 displayed broader-spectrum antibacterial activity, maintaining inhibitory effects against multiple organisms across several concentrations, whereas RWC4 showed comparatively stronger activity against *E. coli* which agree with previous reports showing that related Lactic acid bacteria species produce bioactive metabolites capable of inhibiting gram negative bacteria through mechanisms such as membrane disruption and acidification Elayaraja *et al.* (2021). These isolate-dependent differences are in agreement with the observations of Drider *et al.* (2022) and Pal *et al.* (2019), who reported that lactic acids with similar structural features may nonetheless differ markedly in their antimicrobial spectra owing to variations in peptide structure, stability, and

interaction with specific target receptors on bacterial cells. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays in table 8, 9 & 10 further substantiated the antibacterial efficacy of the lactic acid-related bacteria and revealed notable differences among the bacterial isolates. The lower MIC and MBC values observed for ZBC1 and ZBC3 against *S. aureus* and *E. coli* indicate higher potency, whereas the higher MIC and MBC values recorded for RWC4 against *P. aeruginosa* suggest reduced effectiveness against this intrinsically resistant pathogen. These findings align with the concentration-dependent inhibition observed in diffusion assays and further emphasise the strain-specific nature of bacteriocin activity (Dridier *et al.*, 2022).

In table 10, the findings of this study strongly align with previous reports demonstrating that lactic acid-related bacteria exhibit strain-specific and concentration-dependent antibacterial activity against both Gram-positive and Gram-negative bacteria. Although their activity was lower than that of ciprofloxacin, the ability of certain bacterial isolates, particularly ZBC1 and ZBC3, to inhibit multiple pathogens highlights their potential application as natural biopreservatives and as complementary antimicrobial agents. Further purification and molecular characterisation of these bacteriocins are recommended to enhance their efficacy and better understand the mechanisms underlying their antibacterial activity (Altinoz *et al.*, 2020; Jothi *et al.*, 2021).

The electrophoretic profile obtained validated the quality of the PCR products and supported 16s rRNA genomic hybridisation in the molecular identification of bacterial isolates. Nucleotide Sequence Blast analysis confirms the two identified isolates: ZBC1 is *Bacillus siamensis* and RWC2 is *Staphylococcus equorum*, with the same Query Cover (99%) and E-Values (0.0) but different Percentage Similarity (99.08% & 96.03%). The Accession numbers are (PZ534545) and (PZ534652), as shown in Table 11. The clustering of ZWC1 with *Bacillus siamensis* and RWC2 with *Staphylococcus equorum* confirmed that these organisms are true members of the lactic acid bacteria group, consistent with their expected role in fermented food systems (Altinoz *et al.*, 2020). In Table 12 & 13 Consistent with earlier reports (Wang *et al.*, 2022), this study supports the idea that bacterial antimicrobial activity is rarely attributable to a single compound. Instead, it arises from the synergistic action of multiple metabolites, including fatty acids, volatile organic compounds, terpenoids and heterocyclic molecules. Previous studies have emphasised that the presence of shared volatile compounds among bacterial strains may indicate conserved metabolic pathways essential for survival and competitive fitness (Surya *et al.*, 2020; Wang *et al.*, 2022; Koilybayeva *et al.*, 2023). In the present study, the coexistence of membrane-active fatty acids and secondary metabolites in ZBC1 and RWC2 suggests coordinated antimicrobial strategies that enhance overall bioactivity Surya *et al.*, (2020).

The GC–MS findings of this study in table 12 & 13 are consistent with those of previous reports, demonstrating that bacterial antimicrobial preparations contain diverse bioactive metabolites, with fatty acids serving as key contributors to antibacterial activity (Surya *et al.*, 2020). While differences exist in the dominance of specific compound classes, the overall agreement supports the concept that bacterial metabolites detected by GC–MS play a crucial role in antimicrobial efficacy. These findings further

strengthen the potential application of lactic acid related bacteria as natural antimicrobial agents and justify further purification and mechanistic studies to elucidate the individual and synergistic roles of their constituent compounds (Surya *et al.*, 2020)

## Conclusions

This study achieved its objectives by successfully isolating and molecularly characterizing lactic acid related bacteria that is *Bacillus siamensis* and *Staphylococcus equorum* from traditionally fermented cow milk and evaluating the antibacterial potential of their metabolites against selected pathogens and Identify bioactive compounds present in the metabolites using analytical techniques such as GC–MS and their bio-activity.

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## Declaration of the Competing Interest

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

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