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Antibiofilm and Antimicrobial Activity of *Lactobacillus fermentum* EMCC1346 Cell Free Supernatant Against *MRSA*

Noha M. Abbas*[1], Omnia K. Riad[2], Salwa S. Afifi[2]

[1] Egyptian Drug Authority (EDA), Al Agouza, Giza, Egypt.
[2] Department of Microbiology and Immunology, Faculty of Pharmacy, Al-Azhar University, Nasr City, Cairo, Egypt.

***** Correspondence: drnohataha@yahoo.com

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Abstract: *Methicillin-resistant Staphylococcus aureus* (MRSA) infection possesses significant risks to humans and animals, raising concerns among health authorities. MRSA has emerged as the leading nosocomial pathogen globally. In the current study, sixty MRSA clinical isolates underwent antibiotic sensitivity testing and biofilm capacity test, to select the strongest biofilm-producing isolates. *Lactobacillus fermentum* EMCC 1346 was obtained from Cairo Microbiological Resources Centre (MIRCEN), Egyptian Microbial Culture Collections Network (EMCCN), to evaluate its efficacy against MRSA isolates. The bactericidal efficacy of *L. fermentum* was evaluated via agar well diffusion and the minimal inhibitory concentration (MIC) using the microdilution method, while the antibiofilm activity was evaluated using 96-well polystyrene microtiter plate method. Results indicated that *L. fermentum* EMCC 1346 was declared possible probiotic features (acid and bile tolerance), and it displayed no hemolytic activity. Moreover, it was susceptible to most common classes of antibiotics which proves its safety. *Lactobacillus fermentum* has antibacterial activity through agar diffusion as the average inhibition zone diameters against MRSA isolates ranged from 9.5±0.5 to 13±0.1 mm, while MIC ranged from 125 µg/mL to 500 µg/mL. Results demonstrated that *L. fermentum* exhibits antibiofilm efficacy in a concentration-dependent pattern, as the cell-free supernatant inhibited biofilm formation at concentration ranging from 31.5 µg/mL to 500 µg/mL and eradicated the preformed biofilm up to 70 %. One-way ANOVA showed a significant ($P<0.05$) variability in inhibition of biofilm formation and eradication. In conclusion, the supernatant *of L. fermentum* presents a promising alternative treatment for MRSA infections.

Keywords: Antibacterial; antibiofilm*; MRSA* biofilm; *Lactobacillus fermentum*; probiotics; cell-free supernatant

1. INTRODUCTION

*Methicillin resistant staphylococcus aureus (*MRSA*)* is one of the important drug-resistant pathogens which can cause skin and soft tissue infections (SSTIs), occur in hospitalized patients or may develop into serious, even life-threatening conditions including endocarditis, osteomyelitis, pneumonia and invasive disease [1].One of the existent virulence factors is the production of biofilms as it represents a major trait in MRSA resistance [2] due to generation of an extracellular polymeric substance (EPS) matrix that serves as a sticky substance which enables microorganism to attach to a living or non-living surface. The process of biofilm formation is an essential part of the prokaryotic life cycle, as it enhances survival chances in harsh environments, allowing for continuity, and the spread necessary to create new ecological system [1]. Further, the biofilm life cycle is divided into three stages: adherence, development and dispersal, as bacteria can evade phagocytosis and participate in the recurrence or chronicity of inflammation, to cause disease [3].Moreover, the biofilm formation and development are more common on rough surfaces due to their increased surface area and favorable physicochemical characteristics [3]. This implies that materials used in

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implanted medical devices or biomaterials can be particularly susceptible to biofilm formation [4]. Thus, the urgent development and innovation of novel antibiofilm agents targeting MRSA is extremely needed. Natural products have been increasingly investigated as significant sources of novel antibiofilm agents, aimed at avoiding the adverse effects associated with common antibiotics on human health and ecological systems. Further, the usage of essential oils derived from plants [5,6], flavonoids [7,8] and phenolic acids [9] have proven to be the most popular natural products with biofilm inhibitory and disrupting activities through various mechanisms, which include the reduction of adhesins, degradation of the biofilm matrices and disruption of bacterial communication [6,7], however, these natural plant derived products should be used with some limitations, as some studies have reported that essential oils can lead to intestinal tissue irritation, resulting in a reduction of the intestinal surface area, which may negatively impact gut health and probiotic colonization [9], moreover, essential oils can interact with various food components, such as fats, proteins, and carbohydrates, which can affect their antimicrobial activity in real food systems [10]. This makes it difficult to achieve the desired antimicrobial effect against pathogens without compromising probiotic viability [9,10]. One notable source of anti-MRSA compounds is the marine bacterium *Bacillus amyloliquefaciens*, which has been shown to produce a range of secondary metabolites with significant antibacterial properties. Among these, compounds such as abyssomicin C and marino pyrroles have been identified for their ability to inhibit MRSA growth. The mechanisms of action include disruption of biofilm formation and interference with quorum sensing, which is crucial for bacterial communication and biofilm stability. Furthermore, electron microscopy and flow-cytometry analyses indicated that these compounds could alter the cell membrane integrity of MRSA, leading to cell lysis [3,9].

Microorganisms that form a biofilm have been found to be the main cause of chronic disease in up to 80% of registered case [12]. Approximately 90% of the biofilm mass consists of: proteins, DNA, and extracellular polysaccharides (EPS) which is responsible of the cellular stability by facilitating surface adhesion, also EPS functions as a scaffold for the attachment of cells and enzymes [13]. Multiple strategies for eradication of multidrug-resistant microorganisms have been issued in various studies, which include the usage of honey, specific plant extracts such as aloe vera, essential oils (EOs), antibodies, and nanotechnology (engineered nanostructures), all of which have experienced as therapeutic applications[14,15]. Moreover, the examination of therapeutic microorganisms represents a novel approach currently under investigation [16]. Our research concentrates on evaluating the antimicrobial efficacy of lactic acid bacteria (LAB) and their antibiofilm efficacy against MRSA infections. LAB are a group of microorganisms recognized for their beneficial applications. LAB produces lactic acid as the primary metabolic product resulting from carbohydrate fermentation [17]. LAB can be divided into multiple species, representing a wide selection of species, including *Lactobacillus* spp. and *Bifidobacterium* spp. These bacteria are vital components of fermentation processes, especially in dairy production, and some species are also naturally occurring within the gut normal flora. *Lactobacillus* spp. is considered as one of the most significant species of LAB [18].

Probiotics have an avital role in modulating the immune system of the host through enhancing cytokine production and cellular activity, while also eradication of pathogen clustering [19]. Furthermore, the therapeutic contribution of probiotics can be ascribed to the synthesis of bactericidal compounds such as organic acids, short-chain fatty acids, hydrogen peroxide, ethanol, and bacteriocins [20]. Moreover, the process of producing secondary metabolites, LAB also produces a variety of compounds that have antimicrobial and anticancer properties. It is thought that bacteria release secondary metabolites into their growth medium, so it is possible that the cell free supernatant could perform multiple biological functions[19]. In addition, multiple in-vivo and in-vitro investigations have shown that LAB possesses the ability to prevent pathogen biofilm formation and disrupts the preformed biofilm. LAB is not prone to trigger or promote the emergence of bacterial resistance for pathogens. Moreover, the European Food Safety Authority (EFSA) declared that the beneficial role of probiotics is that they do not have antibiotic resistance genes which could spread through plasmids or transposons. Among LAB strains, *Lactobacillus* and *Bifidobacterium* have sprung up as the most common probiotics [21]. Moreover, *Lactobacillus* species can also interfere with the adhesion of pathogens to epithelial cells, a critical step in the establishment of infections. Research indicates that certain *Lactobacillus* strains can block the adherence of antibiotic-resistant *S. aureus* and *Pseudomonas aeruginosa* to mammalian cells, thereby preventing colonization and subsequent infection, this anti-adhesive property is particularly valuable in clinical settings, where biofilm formation on medical devices can lead to persistent infections[19]. Thus, the aim of this study is to investigate at the first time the bactericidal and

antibiofilm efficacy of CFS obtained from *L. fermentum* against locally isolated MRSA at different concentrations.

2. METHODS

2.1. Materials

Blood agar base medium (Merck, Germany),Sheep blood 5% (Vacsera ,Egypt), Rabbit plasma (Becton, Dickinson &MD, USA), Mannitol agar (Oxoid, Canada) ,Baird-Parker agar (Oxoid, Canada), Tryptic soy broth (TSB; Oxoid, Canada), MRS agar De Man–Rogosa–Sharpe(Himedia ,USA), Muller Hinton agar (Oxoid, USA), NaCl(El Nasr, Egypt), Oxacillin (sigma Aldrich), all antibiotic discs were purchased from (Oxoid, USA), Bile salt (Himedia, USA), HCl(sigma Aldrich),Acetic acid (sigma Aldrich), phosphate buffer saline (Sigma Aldrich), Crystal violet (sigma Aldrich).

2.2. Methods

2.2.1. Probiotic isolation and growth condition

Lactobacillus fermentum EMCC 1346 was obtained from Egyptian Microbial Culture Collections (Cairo MIRCEN) isolated from vegetable juice and isolated on Lactobacilli MRS agar medium.

2.2.2. Isolation and identification of biofilm-forming MRSA isolates

2.2.2.1. MRSA isolates collection and identification

Sixty *S. aureus* isolates were collected from the Bacteriology Laboratory, Al kasr El Ainy hospital, faculty of medicine, Cairo university. *S. aureus* isolates were from different sources including wounds, abscesses, urine, and blood. The isolates were cultured on Blood Agar medium as blood agar base medium was added to aseptically 6% of sterile defibrinated sheep blood and incubated at 37°C for 24 hours. Following Gram staining, Gram-positive cocci strains were selected for additional biochemical tests, which included catalase, coagulase with rabbit plasma, mannitol agar fermentation, and selection on Baird-Parker agar. The identified isolates were preserved in tryptic soy broth with 25% glycerol at −80°C to be used later, MRSA (ATCC 43300; Manassas, USA) was used as a reference strain.

2.2.2.2. Determination of antibiotic susceptibility pattern

2.2.2.2.1. Determination of methicillin resistance using Oxacillin agar method

The clinical isolates were tested for methicillin resistance using the Oxacillin agar method[23] for screening on Muller Hinton agar MHA that had been altered by adding 4% NaCl and Oxacillin (6 µg/mL), and the plate was dried , then, the plates were incubated in ambient air atmosphere for 18 – 24 h. at 35°C. Evidence of growth suggested that the isolates were oxacillin resistant.

2.2.2.2.2. Determination of antibiotic sensitivity

Antibiotic sensitivity test was carried out by disk agar diffusion method consonance with the National Committee for Clinical Laboratory Standards (NCCLS)' Manual of antimicrobial susceptibility test [24]. Ten antibiotic discs that were selected represent the most clinically relevant antibiotics for MRSA[25] , were belonged to multiple classes of antimicrobials, aminoglycosides: Gentamicin (CN, 10 µg), Aminopenicillins: Ampicillin (Amp,10 µg),Cefoxitin (Fox,30 µg) Cloxacillin (CX,5 µg), Carbapenems: Meropenem (MEM, 10 µg); Macrolides: Erythromycin (E, 15 µg); Lincosamide: Clindamycin (DA, 2 µg), Cephalosporins: Ceftriaxone (CTX, 30 µg); Cefuroxime (CXM,30 µg), and Fluoroquinolones: Norfloxacin (NOR, 10 µg).

2.2.3. Analysis of MRSA isolates for biofilm-forming capacity

The capacity of *MRSA* isolates to form biofilm was quantitatively analyzed using the tissue culture plate method (TCP) [26]. In brief, the bacterial culture was allowed to grow on the ELISA microtiter plate (California, USA), and incubated at 37 ℃ for 24 h. After fixation at 60℃ for 1 h., the plate was rinsed twice with phosphate buffer (pH7) and stained with a solution of crystal violet (0.1%). After 15 min, the plates were washed twice with phosphate buffer and left to dry for 1 h. The crystal violet staining was dissolved using 33% acetic acid, and the optical density (OD) at 630 nm was recorded using a microplate reader (Bio-Rad, Hercules, California, USA). The experiment was done three times, and the OD reading values were taken for each isolate; Interpretation of biofilm formation was performed as per the criteria described by Saidi et al. [27] as shown in

Table 1.

2.2.4. Characterization of L. fermentum

2.2.4.1. Acid and bile tolerance

Acid and bile tolerance were done according to the methods described by El Mansy et al. [28]. Briefly, overnight cultures of the test isolate were inoculated (1% v/v) in MRS broth previously adjusted to pH 2.5% by 1N HCl or treated with a bile salt concentration of 0.3%. After incubating for 3 h at 37 °C, 1 mL of the culture medium was mixed with 9 mL of MRS broth and incubated for 24 h at 37 °C. Resistance was assessed in triplicates in terms of

Table 1. Interpretation of the formed biofilm grade

OD	Result
ODc<ODt<2×ODc	Weak biofilm
2×ODc<ODt<4×ODc	Moderate biofilm
ODt≥4×ODc	Strong biofilm

ODc : optical density of control; ODt: optical density of test sample

optical density measurement at 620 nm. The control comprised MRS broth adjusted to pH 7.0±0.2. The percentage of resistance was calculated according to **Eq. 1**, as ODs is the optical density of the test sample and ODc is the optical density of control. Lactobacillus isolates that showed more than 50% resistance at 0.3% (w/v) bile salt or 2.5%(v/v) acid were considered bile and acid resistant as this is the minimum range for being a probiotic isolate.

Resistance (%) = ODs ⁄ ODc × 100 **(Eq. 1)**

2.2.4.2. Hemolytic activity

Fresh bacterial culture was streaked in triplicates on blood agar plates, containing 5% (w/v) sheep blood, and incubated for 48 h at 37 °C. Blood agar plates were examined for signs of β-hemolysis, α-hemolysis or γ-hemolysis. Experiments were done at least three times on separate days [28].

2.2.4.3. Determination of antibiotic susceptibility

The susceptibility of the strain to different types of antibiotics was evaluated using the agar disc diffusion method[28,29], including Penicillin(10µg), Gentamicin(10µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), Norfloxacin (10 µg), Streptomycin (10 µg), Aztreonam (30 µg), Ofloxacin (5 µg), and Clindamycin (2 µg) were used. The overnight culture was aseptically swabbed on MRS agar plates, and the antibiotic discs were placed on the surface of the agar plates. The inoculated plates were incubated at 37°C for 24 h. and zones of inhibition were measured and recorded in millimeters.

2.2.5. Evaluation of L. fermentum CFS antimicrobial activity

2.2.5.1. Preparation of cell-free supernatant (CFS) of L. fermentum isolate

The CFS derived from *Lactobacillus fermentum* was obtained by growing strain in 30 mL of MRS. Next came centrifugation, at 10000 × g for 20 min. at 4°C using a cooling centrifuge, the culture supernatants were filtered twice through a sterile 0.22 µm nitrocellulose membrane filter. The liquid remaining after the process and the samples, with the culture medium, were frozen and dried using a Lyophilizer LS3000 (Terroni, in São Carlos, Spain). Following lyophilization the samples were carefully. Stored at 20°C. After that, the samples were reconstituted in deionized water before using [30].

2.2.5.2. Antimicrobial activity by well diffusion assay

The antimicrobial efficacy of the CFS derived from *L. fermentum EMCC1346* was determined against MRSA isolates utilizing the agar well diffusion methodology [28]. The MRSA isolates were cultivated in Muller Hinton broth (MHB) at a controlled temperature of 37 °C for a period of 24 h. The culture inoculum was calibrated to a 0.5 McFarland standard (1.5 × $10^{\wedge 8}$ CFU/mL) and employed to inoculate melted MHA at a temperature of 45°C. After the solidification of the medium, wells were created in the agar using a cork borer. The tissue culture plate wells were subsequently filled up with 100 µL of *L. fermentum* CFS and the plates were incubated at aerobic conditions, at 35°C for 24 h. The inhibition zones were then measured to detect their antimicrobial activity.

2.2.5.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The broth microdilution techniques were used to determine the minimum inhibitory concentration and minimum bactericidal concentration of CFS. In summary, 100 µL of Muller Hinton broth (MHB), which had been prepared with an overnight culture of MRSA isolates adjusted to 0.5 McFarland standards, was carefully added to each well of a 96-well plate. Then, the same volume of CFS, previously prepared in two-fold serial dilutions from 1000 to 31.25 µg/mL, was added to appropriate wells to complete the set-up for testing. The bacterial suspensions without CFS served as the positive control for the experiments to have a valid comparison of results. The uncultured broth was blank and allowed for a negative control to ensure the accuracy of the results; subsequently, all experimental plates were incubated at 37°C for 24 h. Finally, after incubation, the final OD

obtained was normalized against the blank control, while the MIC was determined by the absence of turbidity, the result was determined visually. Then, 100 µL was transferred from the cultures to plates filled up with MHA medium. After the transfer, incubation of the plates was done for 24 hours to have the proper estimation of MBC [30,31].

2.2.6. Antibiofilm activity of L. fermentum's CFS

2.2.6.1. Biofilm inhibition activity by L. fermentum's CFS

To assess the capacity of the CFS to prevent biofilm formation and detect the biofilm inhibition percentage, the microtiter plate method was assessed following the protocol previously delineated by Khaleghi et al. [32]. In this essay, the biofilm inhibition characteristics of varying concentrations of CFS (ranging from 100 to 31.25 µg/mL) were evaluated by amalgamating 100 µL of CFS solution with 100 µL of bacterial suspensions and incubated for 24 h. Following incubation, the plates were drained, washed, fixed, and subsequently stained with crystal violet (CV). The stained biofilm was then solubilized in 200 µL of acetic acid 33%, and it was measured at 630 nm. The percentage of inhibition was computed according to the equation provided **Eq.2**. An uncultured medium served as the blank (negative control), while a medium with cultured bacteria, excluding CFS, was designated as the positive control. In this context, C represents the average absorbance of control wells, B denotes the mean reading absorbance of the blank wells, and T signifies the mean absorbance of treatment wells.

Inhibition (%) = ((C - B) - (T - B) / (C - B)) *100

(Eq. 2)

2.2.6.2. Biofilm eradication activity by L. fermentum CFS

The capacity of the cell-free supernatant (CFS) to eradicate the bacterial biofilm formations and eliminate bacteria was evaluated utilizing the microtiter plate method as described by Khaleghi et al., with minor changes [32]. To detect biofilm eradication percentage, an Aliquot of 100 µL of the bacterial suspension from each MRSA isolate (0.5 McFarland) was introduced into the wells and subsequently incubated for a duration of 24 h. Following this incubation period, the wells were drained, after that, they were replenished with 100 µL of CFS at concentrations ranging from ٢000 µg to 31.25 µg/mL. Upon completion of another 24 h. of incubation, the wells were drained, washed, fixed, and stained utilizing crystal violet (CV). Subsequently, the stained wells with biofilm were filled with 200 µL aliquot of 33% acetic acid, and the reading absorbance was measured at a wavelength of 630 nm; the percentage of eradication was computed by **Eq. 2.**

2.2.7. Statistical analysis

The results were expressed as mean ± standard deviation. All experiments were performed three times. Statistical Package for Social Sciences (SPSS) was used. A one-way analysis of variance -ANOVA with Dunnett test for post hoc analysis. *p-value* ≤0.05 is considered significant.

3. RESULTS

3.1. Isolation, phenotypic identification, and determination of antibiotic susceptibility pattern of MRSA isolates

Among 60 isolates of *S. aureus*: 26 (43.3%) were from infected wounds; 19 (31.7%) from blood culture; and 15 (25%) from urine, all isolates were Gram-positive and positive for catalase, coagulase, and fermented mannitol. **Table 2** shows the resistance of the clinical isolates against nine antibiotics. The results declared that all the isolates were resistant to multiple antibiotics such as Ampicillin 91.7%, Cloxacillin73.3%,Cefoxitin 73.3%, Cefuroxime73.3%, Cephalexin65%, Gentamicin 45%, Meropenem 63.3%, Erythromycin53.3%, and Norfloxacin50%, Clindamycin 55% respectively. By the Cefoxitin and Cloxacillin disc-diffusion Resistance test (≤20 mm), 44(73.3%) of clinical isolates were identified as being MRSA phenotypically.

3.2. Analysis of biofilm-forming capacity of MRSA isolates

The biofilm-forming capacity of MRSA isolates was qualitatively analyzed using a quantitative microtiter plate assay. As shown in **(Fig. 1)**, 18 and 16 MRSA isolates were weak and moderate biofilm producers, however, 10 MRSA isolates were strong biofilm producers as they were above 0.5 at optical density (OD) 630 nm according to **Table 1**.

3.3. Evaluation of probiotic characteristics of *L. fermentum*

3.3.1. Acid and bile salt tolerance

In the present study, L. fermentum was challenged at low pH and it showed a survival rate of 63.257 ± 0.56% after 3 h. Concerning bile salt tolerance, L. fermentum showed considerable resistance to exposure to 0.3% bile salts and a survival rate of 60.5 ± 0.83% was recorded.

Table2. Antibiogram of methicillin-resistant *Staphylococcus aureus (MRSA)* and methicillin-sensitive *Staphylococcus aureus (MSSA)*.

Antibiotics (µg)	MSSA		MRSA		Total sensitive N (%)	Total resistant N (%)
	S [n (%)]	R [n (%)]	S [n (%)]	R [n (%)]		
Ampicillin (AMP 10 µg)	5 (31.2)	11 (68.8)	0 (0.0)	44 (100)	5 (8.3)	55 (91.7)
Cefoxitin (FOX 30 µg)	16 (100.0)	0 (0.0)	0 (0.0)	44 (100)	16 (26.7)	44 (73.3)
Cloxacillin (CX 5 µg)	16 (100.0)	0 (0.0)	0 (0.0)	44 (100)	16 (26.7)	44 (73.3)
Cefuroxime (CXM 30 µg)	9 (56.3)	7 (43.7)	7 (15.9)	37 (84.1)	16 (26.7)	44 (73.3)
Cephalexin (CN30 µg)	13 (81.3)	3 (18.3)	8 (18.2)	36 (81.8)	21 (35.0)	39 (65.0)
Gentamycin (GEN 10 µg)	14 (87.5)	2 (12.5)	19 (43.2)	25 (56.8)	33 (55.0)	27(45.0)
Norfloxacin (Nor 5 µg)	15 (93.8)	1 (6.2)	15 (34.1)	29 (65.9)	30 (50.0)	30 (50.0)
Erythromycin (E 15 µg)	15 (93.8)	1 (6.2)	13 (29.5)	31 (70.5)	28 (46.7)	32 (53.3)
Meropenem (MEM 10 µg)	16 (100.0)	0 (0.0)	6 (13.6)	38 (86.4)	22 (36.7)	38 (63.3)
Clindamycin (DA2 µg)	16 (100.0)	0 (0.0)	11 (25)	33 (75)	27 (45)	33 (55)

MRSA: *Methicillin-resistant Staphylococcus aureus*; **MSSA:** Methicillin-sensitive *S. aureus*; **R:** Resistant; **S:** Sensitive; **n:** number of isolates.

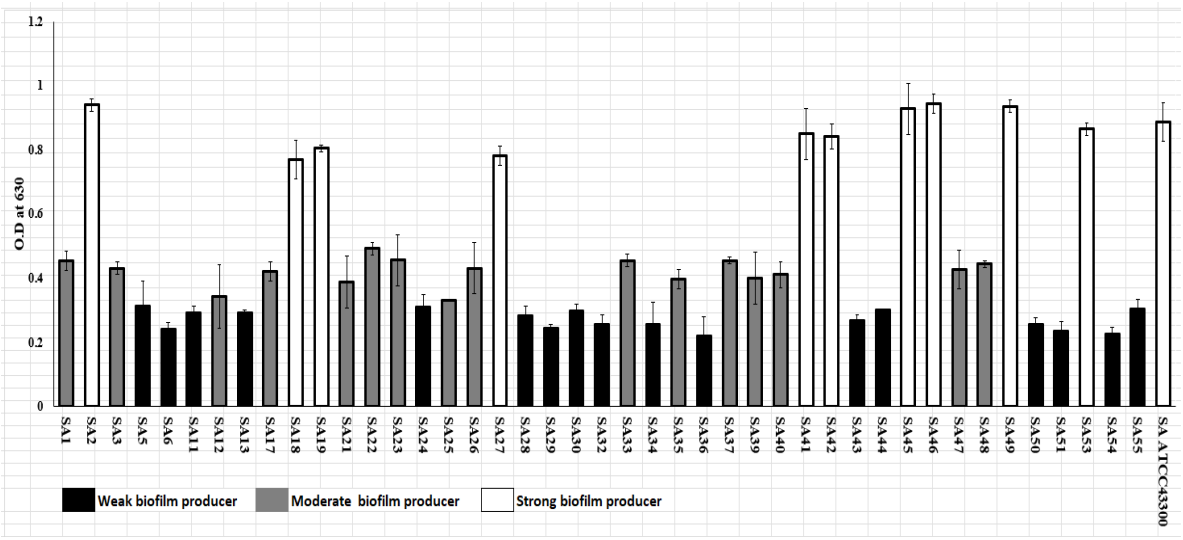

Fig.1. Quantitative analysis of biofilm formation by MRSA isolates; Spectrophotometric analysis for the biofilm formation for MRSA isolates. The plot shows mean optical density (OD) values ± standard deviation.

3.3.2. Blood hemolysis

L. fermentum did not show any blood hemolysis on the blood agar plates.

3.3.3. Antibiotic resistance of L. fermentum

Data in table 3 revealed that L. fermentum had variable susceptibility against different classes of antibiotics, as it was sensitive towards penicillin, imipenem, tetracycline and clindamycin, On the other hand, it was resistant to aztreonam, norfloxacin, gentamicin, ciprofloxacin, and ofloxacin and streptomycin.

Table 3. The antibiotic susceptibility of *L. fermentum*

Class Antibiotic	Antibiotics	Concentration	Sensitivity
Aminoglycoside	Streptomycin	10 µg	Resistant
	Gentamicin	10µg	Resistant
Tetracyclines	Tetracycline	30 µg	Sensitive
B-Lactams	Penicillin	10µg	Sensitive
	Aztreonam	30 µg	Resistant
	Imipenem	10µg	Sensitive
Quinolones	Ciprofloxacin	5 µg	Resistant
	Norfloxacin	10µg	Resistant
	Ofloxacin	5 µg	Resistant
Lincosamide	Clindamycin	2 µg	Sensitive

Table 4: Zone of inhibition, MIC, and MBC of ***L. fermentum*** CFS* against MRSA clinical isolates

	L. fermentum EMCC 1346		
Strain no	zone of inhibition IZD [mm]	MIC µg/mL	MBC µg/mL
SA2	12.5 ± 0.1	125	250
SA18	10.6 ± 0.3	250	500
SA19	11.5 ± 0.3	250	500
SA27	9.5 ± 0.2	500	1000
SA41	11.2 ± 0.3	250	1000
SA42	11.5 ± 0.1	125	250
SA45	13 ± 0.6	125	500
SA46	12.5 ± 0.2	125	250
SA49	10 ± 0.3	250	500
SA53	11.3 ± 0.5	500	500
MRSA ATCC43300	10.2 ± 0.1	125	250

MIC: minimum inhibitory concentration, **MBC:** minimum bactericidal concentration, *Standard Methicillin-Resistant *S. aureus* strain, **CFS** *cell free supernatant.

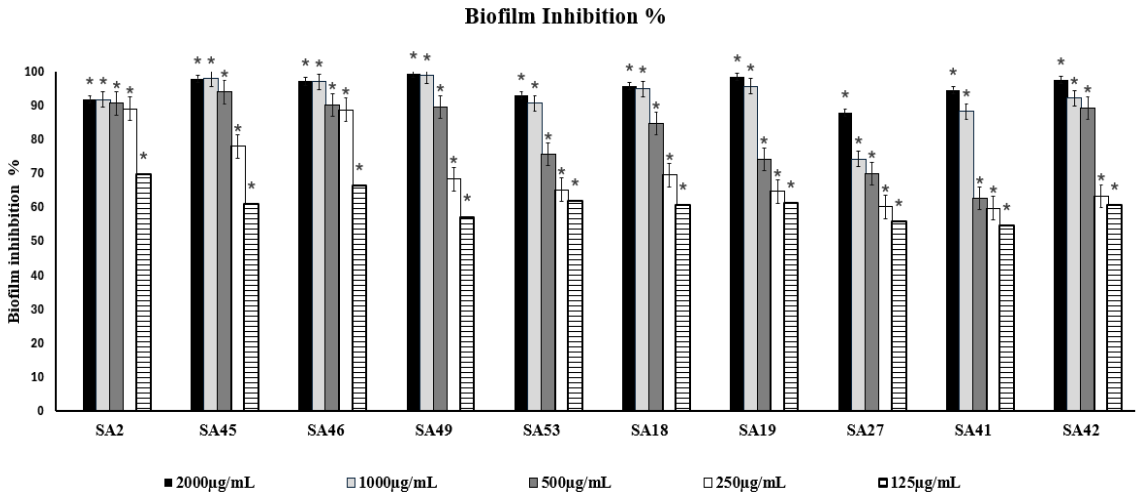

Fig. 2. The percentage of the biofilm formation inhibition of MRSA isolates SA2, SA45, SA46, SA49, SA53, SA18, SA19, SA42, SA41 and SA27. Error bars represent standard deviations (SD). *$P < 0.05$.

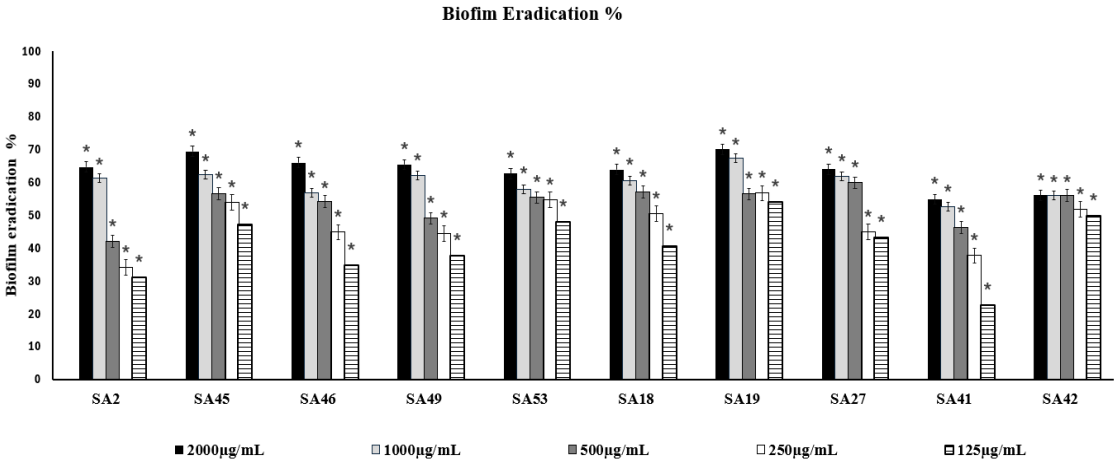

Fig 3. The percentage of the biofilm eradication regard to SA2, SA45, SA46, SA49, SA53, SA18, SA19, SA42, SA41 and SA27. Error bars represent standard deviations (SD). *$P < 0.05$**.**

3.4. Antimicrobial activity of *L. fermentum* cell free supernatant (CFS)

The CFS of *L. fermentum* inhibited the growth of the selected ten MRSA isolates, as shown in in **Table 4.** The most antimicrobial activity was observed in SA45 (13 ± 0.6 mm). It was found that the range of the (MIC) among the strains was between 125 and 500 µg/mL and the (MBC) ranged from 250 to 1000 µg/mL as shown in **Table 3.**

3.5. Antibiofilm activity of *L. fermentum* cell free supernatant (CFS)

3.5.1. Biofilm inhibition activity of L. fermentum CFS

The anti-biofilm efficacy of cell free supernatant of Lactobacillus spp. was determined against the ten MRSA clinical isolates. The results revealed that the effects of CFS on the initial attachment ability of MRSA isolates indicated that CFS at descending concentrations ranged from 500 µg/mL to 31.25 µg/mL exhibited an inhibition from 55% to 98% of biofilm formation significantly ($P < 0.05$). As shown in Fig. 2. The least concentration of CFS that can inhibit the biofilm formation up to 55% was 31.25 µg/mL. A higher concentration of CFS treatment was able for better inhibition of biofilm formation in all isolates in a concentration-dependent manner.

3.5.2. Biofilm eradication activity of L. fermentum CFS

The biofilm disruption activity of cell free supernatant of *Lactobacillus spp*. was evaluated against the ten MRSA clinical isolates. The results revealed that the clearance (%) of the preformed biofilm under the treatment with CFS at descending concentration ranging from 125 µg/mL to 2000 µg/mL exhibited an inhibition from 31% to 70% of biofilm formation significantly ($P < 0.05$). As shown in Fig. 3. The least concentration of CFS that can inhibit the biofilm formation up to 50% was 125 µg/mL. A higher concentration of CFS treatment was able for better inhibition of biofilm formation in all isolates in a concentration-dependent manner.

4. DISCUSSION

The occurrence of antibiotic resistance among bacterial pathogens is one of the specific examples of very difficult to treat infections [33]. The production of biofilm by *Staphylococcus aureus* acts as a first line of defense against the host's immune responses; as the bacteria attaches to the tissues of the host, enhancing their capacity for spreading through living hosts[34]. Furthermore, the protective mechanism of biofilm exhibits an intrinsic resistance to most currently used antibiotics among bacteria embedded within mature biofilms [35]. The susceptibility test that was evaluated in the current study revealed that a substantial proportion of *Staphylococcus aureus* isolates exhibited resistance to the majority of most common utilized antibiotics, as the clinical isolates displayed an increased resistance percentages to Ampicillin (91.7%),Cefoxitin (73.3%), Meropenem (65%), Erythromycin (53%), Cloxacillin (73.3%) and Gentamicin (45%) which is found to be in agreement with other findings from previous studies [36]. Thus, there was an urgent need for finding sustainable alternatives for the management of bacterial resistance issues, particularly against different classes of commonly used antibiotics [37-40]. The target of this study is to discover an innovative therapeutic approach for eradication of MRSA

resistance and preventing biofilm development. A multitude of studies have demonstrated that *lactobacillus* CFS exhibits antibacterial efficacy against a diverse type of bacterial pathogens [41].

In the present investigation, the viability and existence of probiotic bacteria in acidic circumstances is one of the urgent criteria for providing therapeutic functions. The pH of the stomach generally ranges from pH 2.5 to pH 3.5 and this is a barrier against the entry of bacteria into the intestinal tract [28]. In concurrence with our findings, El Mansy et al. [28] reported a good survival rate for *Lactiplantibacillus plantarum* after 90 min in MRS broth at pH 2.5 %. Moreover, the ability of *L.fermentum* to survive in bile salts assists colonization and metabolic activity of bacteria in the host's small intestine [28]. In vivo, bile salts act as biological detergents that emulsify and solubilize lipids, thereby playing an essential role in fat digestion [28]. Probiotic bacteria tolerance to different pH and salt concentrations which can be a common occurrence during food processing is another essential merit of probiotics as it gives the opportunity to the bacteria to start metabolism, which generates acids, that further suppresses the growth of unwanted organisms[28]. This finding resembled a preceding study that revealed *Lactobacillus fermentum* possessed no hemolytic activity[28] , in addition, it was urgent to study the antibiotic resistance of *Lactobacillus fermentum* to avoid severe medical dangers. Probiotic bacteria can possess intrinsic and mobile genetic elements that ascertain resistance to a wide variety of antibiotic classes which could transfer to pathogens. Also, the lack of an antibiotic's target site in a LAB cell may explain the resistance to that antibiotic [28,29] . It was reported that *Lactobacillus fermentum* is generally sensitive to the most common classes of antibiotics such as: β-lactams antibiotics, such as penicillin, meropenem and other broad-spectrum antibiotics like tetracycline and clindamycin [28]. The substantial antibacterial efficacy *of L. fermentum* CFS was detected against the selected clinical isolates of MRSA through the agar well diffusion assay alongside a minimum inhibitory concentration/minimum bactericidal concentration assay. Additionally, the anti-biofilm properties of *L. fermentum* CFS were also evaluated.

Based on the results, the *Lactobacillus* isolate exhibited a significant antibacterial effect as the highest inhibition was (average 13mm) and lowest (average respectively. In other previous studies, the inhibition zone diameter induced by probiotic lactobacilli cell free supernatant was ranged from 17 mm to 20.3 mm against *P. aeruginosa* isolates, and from 9.4mm to 12 mm against MRSA isolates [42,43]. The lowest MIC value at 125 µg/mL was observed, these results are in good agreement with previous studies conducted as the reported MIC values for inhibiting *P. aeruginosa* by *Lactobacillus* spp. strains ranged from 100 to 300 µg/mL, and from 2 to 4 mg/mL for inhibiting MRSA isolates, respectively[44,45] .

Our findings are in a good agreement with Maleo et al., as it was reported that CFS of *Lactobacillus* spp. isolated from fermented food can inhibit MRSA growth as well as its biofilm which isolated from human skin, and they declared that, the derived metabolites from *Lactobacillus* isolate are considered as a proper therapy for many wound infections [45]. Our results indicated that the cell-free supernatant of *L. fermentum* exhibited a marked capability to prevent the formation of MRSA biofilms [34], these findings were in a good agreement with a study conducted previously [46] that reported the bactericidal activities and biofilm eradication activity of *L. acidophilus* and *L. casei* CFS against *S. aureus* ATCC 25923. In contrast to our findings, another study identified that the CFS obtained from Lactobacillus strains isolated from yogurt and fermented milk exhibited no antibacterial activity against S. aureus, L. monocytogenes, E. coli, or K. pneumonia [47]. This observation indicates that Lactobacillus strains exhibit multiple variability in their antagonistic capabilities against multiple pathogens[47].

Our result showed that the CFS of L. fermentum exhibited a significant reduction in biofilm formation up to 98% and the elimination of performed biofilms was over 65%. This phenomenon may be due to the presence of antimicrobial metabolites synthesized by lactic acid bacteria, which include organic acids, fatty acids, proteinaceous substances (bacteriocins), hydrogen peroxide, and various other volatile compounds [48]. Moreover, in previous literature, a probiotic strain P. acidilactici was also reported that it has a significant antibiofilm activity up to 77% against P. aeruginosa [49].

Probiotics, particularly Lactobacilli spp. have been recognized for their capability to inhibit or disrupt bacterial biofilm formation by targeting the bacterial cell wall, which results in alterations such as roughness and wrinkling, potentially leading to the inhibition of biofilm development [46]. Lactobacilli possess the ability to interfere with pathogenic bacteria through mechanisms such as nutrient competition, co-aggregation, and the production of antimicrobial compounds, besides, their effective immunomodulatory impact [50].

Several studies have indicated that the CFS derived from Lactobacillus spp. possesses anti-biofilm activity and engages in competition with pathogens [51], demonstrating the biofilm-hindering efficacy of probiotics against MRSA. The researchers, in a previous study, observed that the CFS not only diminished the biofilm development of pathogenic bacteria but also removed the pre-existing biofilm[52], while another study reported that Lactobacillus spp. isolates exhibited commendable probiotic effectiveness along with enhanced biofilm prevention and bactericidal activity [53]. Additionally, in previous literature, it was revealed that the exopolysaccharides produced by L. plantarum were very powerful in reducing polysaccharide production within the extracellular polymeric matrix of S. flexneri, thereby limiting biofilm formation [54].

Many studies have indicated that prolonged exposure to probiotics at sub-MIC levels can influence the development of antimicrobial resistance. For example, Tiwari et al. noted that exposure to sub-MIC concentrations of certain antimicrobial agents can lead to biofilm formation and increased resistance in bacteria, suggesting that similar mechanisms could occur with probiotics[55]. This phenomenon raises concerns about the potential for Lactobacillus strains to contribute to the emergence of resistant bacterial populations when used over extended periods or at sub-MIC levels.

Additionally, the safety profile of probiotics has been scrutinized in clinical settings. While probiotics are generally considered safe, there have been rare instances where they have caused bacteremia or fungemia, particularly in immunocompromised individuals [56]. This highlights the need for caution in the use of probiotics, especially in vulnerable populations, as the risk of developing antibiotic-resistant infections could be exacerbated by using certain probiotic strains.

Moreover, while Lactobacillus probiotics hold promise for combating antibiotic-resistant pathogens, their use must be approached with caution. Prolonged or sub-MIC exposure may contribute to the occurrence of superbugs through the transfer of antibiotic-resistance genes and the potential for increased resistance development. Therefore, it is crucial to screen probiotic strains for safety and to monitor their impact on the gut microbiome to mitigate the risk of enhancing antibiotic resistance [56].

5. CONCLUSIONS

In conclusion, CFS of *L. fermentum* EMCC1346 demonstrated significant efficacy as a potent antibacterial and antibiofilm agent against MRSA. The CFS not only hinders the proliferation of bacterial cells and induces their mortality, but it also possesses the capacity to hinder biofilm formation, disrupting the pre-existing biofilms, and eliminating bacterial cells embedded in the biofilm matrix. These characteristics showed the prospective application of *Lactobacillus* CFS in the management of severe infectious diseases, as they are an excellent alternative and innovative therapeutic approach for controlling bacterial biofilm-associated MRSA infection. The results of this research should be supported by further different studies that probiotics CFS could be used as a potent drug in preventing MRSA infections alone or in combination with different antibiotics.

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List of Abbreviations: ANOVA: analysis of variances, CFS: cell free supernatant; CV: crystal violet; EFSA: European Food Safety Authority; EMCCN :Egyptian Microbial Culture Collections Network ;EPS: extracellular polymeric substance, Eos: essential oils; LAB: lactic acid bacteria; MBC: minimum bactericidal concentration; MHA: Muller-Hinton agar; MHB: Muller-Hinton broth; MIC: Minimum inhibitory concentration; MIRCEN: Egyptian Microbial Culture Collections; MRSA: Methicillin resistant S. aureus; MRS :De Man–Rogosa–Sharpe medium; OD: Optical density; QS: quorum sensing, TCP: tissue culture plate.

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