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Antibacterial and Antimicrobial Activity of *Lactobacillus plantarum* Isolated from Cow Milk against Bacterial Pathogen Isolated from Burns Infection

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ABSTRACT

Burns infection is one of the most common consequences and the leading causes of death. The burn infections are mostly contaminated with bacterial species resistant to several types of antibiotics. This study comes to evaluate a safe and alternative antimicrobial in order to control the multi-drugs resistant (MDR) isolates. In this study, the minimum inhibitory concentrations (MIC) and minimum biofilm inhibitory concentrations (MBIC) of cell free supernatants prepared from Lactobacillus plantarumisolates (CFS-LP) were determined against the most bacterial contaminants isolated from burn infections. Forty two of cow's milk samples were collected from different areas in Baghdad, Iraq. In addition, 187 swabs of burns infection were collected from patients admitted to the Burns Specialized Hospital/ Medical city in Baghdad. We reported that Pseudomonas aeruginosa (34.22%) and Staphylococcus aureus (27.27%) were the most predominant MDR isolates. L. plantarum spp. 2 was more effective than the other Lactobacilli isolates. The MIC90 of CFS-LP2 ranged from 25%-50%, preventing more than 90% of the bacterial contaminants growth. In addition, the MBIC50 of CFS-LP1, 2 and 3was in rang (6.25%-25%), inhibiting more than 50% of biofilm formed by burns infection isolates. The authors concluded that CFS of L. plantarumisolated from cow's milk is effective and safe substances could be used to control the pathogenic bacterial caused burns infection and their biofilm formation.

Key words: Burns infection, *Lactobacillus plantarum*, Cell free supernatants, Anti-bacterial, Anti-biofilm

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INTRODUCTION

Skin is the largest organ in the body, accounting for about 15% of the total body weight of an adult. It is made up of Three layers, including (from top to bottom) the epidermis and its appendages, the dermis and the hypodermis. These layers having their own specific functions; including (i) protection from external physical, chemical and biological factors, (ii) preventing the loss of excess water from the body and (iii) thermoregulation in addition to being (iv) a sensory organ. The body is easily susceptible to harm due to the exposure of the skin layers to burning or tearing [1]. Microbial infections are the main cause of increased morbidity and mortality in burned-skin patients.

Skin infections could be viral, fungal and bacterial infections, especially infections caused by multidrug-resistant strains (MDR). The most common Gram-positive bacteria involved in burn infections are Staphylococcus. spp., Enterococcus spp. and beta-hemolytic Streptococcus group A, while Gram-negative bacteria include *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella spp*. *Stenotrophomonas spp*., *Escherichia coli* and *Enterobacter cloacae* [2,3]. The virulence factors of these bacterial species play an important role in their resistance to antibiotics and increase the difficulty to control such infections.

Biofilms formation onto a variety of biotic (tissues) and abiotic (devices) surfaces is an important virulence factor of pathogenic bacteria [4,5]. Bacteria form biofilm to protect themselves from the harmful environmental circumstances such osmotic stress, metal toxicity, and antibiotic exposure [6].

Pathogens that develop biofilms are linked to persistent infections in up to 80% of cases [7]. About 90% of

the biofilm mass is made up of proteins, DNA, and extracellular polysaccharides (EPS). In addition to cell stability and mediating surface adhesion, EPS also serves as a scaffold for the attachment of cells, enzymes, and antibiotics [8].

Alternative approaches to controlling MDR pathogens have been evaluated in several studies including; the use of honey, some plants such as aloe vera and oatmeal, bacteriocins, essential oils (EOs), antibodies and nanotechnology (engineered nanostructures) has recently employed in the therapeutic burns applications [9-11]. Furthermore, therapeutic microorganisms are two newer methods being investigated (probiotics and bacteriophages)[9, 10, 11].Our study is focusing on the probiotic potential of lactic acid bacteria and their antimicrobial activity against burns infection-associated pathogens.

Lactic acid bacteria (LAB) are group of bacteria that have beneficial uses. They are Gram positive, non-spore forms, catalase negative, anaerobic or aerotolarate, fastidious bacteria. LAB produces lactic acid as a principal metabolic end product of carbohydrate fermentation [12]. LAB are divided into several genera and have a large number of species, including; Lactobacillus species in addition to Bifidobacterium genera. Also, some LAB from other bacterial species were reported to show a probiotic potential, such as *Propionibacterium acidilactici, Lactococcuslactis, Leuconostocmesenteroides, Bacillus subtilis, Enterococcus fascism, Streptococcus thermophiles,* and *Escherichia coli* [13]. These bacteria are a key component of fermentation starters, particularly for dairy products, and some of them are also found naturally in the gastrointestinal microflora. One of the most important LAB genera is Lactobacillus [14].

Probiotics are living microorganism that provides a health benefit to the host when given in appropriate amounts [15]. The concept of probiotic foods has gained a lot of attention recently by the public due to the increased interest in health consciousness [15]. Probiotics play an important role in the regulation of the host immune system by raising cytokine production and cellular activity in addition to reduceclustering of pathogens [16]. Furthermore, probiotics' therapeutic properties can be attributed to the production of antibacterial agents such as organic acids, short-chain fatty acids, hydrogen peroxide, ethanol and bacteriocins [17]. This study comes to assess the MIC90 and MBIC50 of CFS-LP against the most bacterial contaminants isolated from burn infections.

MATERIALS AND METHODS

Sample collection

Forty two samples of cow's milk was collected from different areas in Baghdad using sterile containers and carried to the laboratory under aseptic conditions and inoculated onto MRS broth. In addition, 187 swabs of burns infection were collected from different sites of patients obtained from Burns Section in Baghdad. The samples were inoculated onto brain-heart infusion agar, MacConkey agar and Mannitol Salt agar and incubated for 24 hours at 37 ºC aerobically.

Identification of bacterial species

The identification of Lactobacillus species and bacterial contaminants of burns infection were performed using two methods; (a) Initial (manual) biochemical test (Catalase Test, Oxidase Test, Triple Sugar Iron (TSI), Citrate Utilization Test, Indole Test, Arginine Hydrolysis Test, Esculine Hydrolysis Test, (HiMedia, Mumbai, India) and (b) Automatic method (VITEK 2 compact system) (Biomerieux, Craponne, France).

Determination of the Minimum Inhibitory Concentration (MIC)

The clinical and laboratory standards institute defined MIC90 as the lowest concentration of antimicrobial agent that cause inhibit 90% or more of microbial growth after 24 hours incubation [18]. A broth micro-dilution experiment was performed as described by [19] with slight modifications. Briefly, a 24-hour growth of the pathogenic bacteria of burns infection was diluted with an appropriate proportion of BHI to obtain a concentration of 108CFU/ml. In a non-tissue-culture 96-well microplate, the CFS of Lactobacillus spp. was serially two-folds diluted with BHI broth into96-well micro plate, the final volume of 100 µl for each well. A 100 µl of diluted bacterial suspension (108 CFU/ml) was added separately to the wells of a microplate containing pre-determined concentrations of CFS. The microplate was then incubated aerobically at 37°C for 24-36 hours. The kinetic reading was statistically evaluated using plate reader (Bio-Rad Laboratories, USA) and the MIC of each CFS-LP was based on the definition of CLSI (2020).

Biofilm inhibition assay

According to [19], biofilm formation inhibition analysis was followed. Bacteria isolated from burn infections were cultivated aerobically for 24 hours at 37°C into BHI supplemented with 1% glucose (BHIG). CFS-LP species was two-folds serially diluted with a final volume of 100 µl in a tissue culture 96-well microplate. The overnight grown bacteria was diluted to 108 CFU/mL in fresh BHIG broth, and 100µlof the diluted suspension was added into each well of a 96-well micro plate, the plates was incubated at 37°C for 24 hours under aerobic conditions. After incubation, the non-attached bacteria were carefully withdrawn and transferred to a new 96 microplate for turbidity measurement using microplate reader. The biofilm cells at the bottom of the micro plate were rinsed once or twice with phosphate buffer saline (PBS). Then, the plate was heat-fixed for 60 minutes at 60°C. After biofilm fixation, each well was treated with 125µlof crystal violet (CV) (0.1%) and incubated at room temperature for 20-25 minutes. The plate was then rinsed three to four times with PBS and 150 µl of 95 % ethanol was added to each well to dissolve the CV-stained biofilm. The plate was incubated at 4°C for 30 minutes. After that, the absorbance was measured using

a microplate reader at 630 nm in a new 96-well micro-titer plate. The biofilm mass in each treated wells was compared to the positive control (non-treated wells) to identify to MBIC50.

RESULTS

Minimum Inhibitory Concentration (MIC-90)

The MIC90 which was determined using the broth micro dilution method was calculated by selecting the lowest concentration that inhibits 90 % of microbial growth. A series of different concentrations were prepared for Lactobacilli CFS (50%-6.25%). At these concentrations, bacterial growth was significantly inhibited ($p<0.05$). No MIC90 values were determined for the CFS-LP1, against the four isolated pathogens, even when the highest concentration (50%) was used compared to the control (bacterial growth without treatment) (Figure 1). However, when using 50% of CFS-LP1, the growth inhibition percentages were23.78%, 20.54%, 19.66% and 17.55% for *A. baumannii, P. aeruginosa, K. pneumoniae,* and *S. aureus,* respectively. While a slight growth inhibition was reported when the lower 50% CFS-LP1 was applied and as following;25% CFS produced 17.47%, 9.57%, 11.06% and 16.06%, respectively, the 12.5% CFS caused 15.32%, 5.85%, 9.60% and 13.85%, respectively, while the 6.25% CFS led to 13.99%, 5.22%, 9.26% and 11.23%, respectively. We identify a significant differences in bacterial growth inhibition when CFS-LP1 was used in all concentration (6.25, 12.5, 25 and 50)% when compared with the positive control, p-value (<0.05) (Table 1).

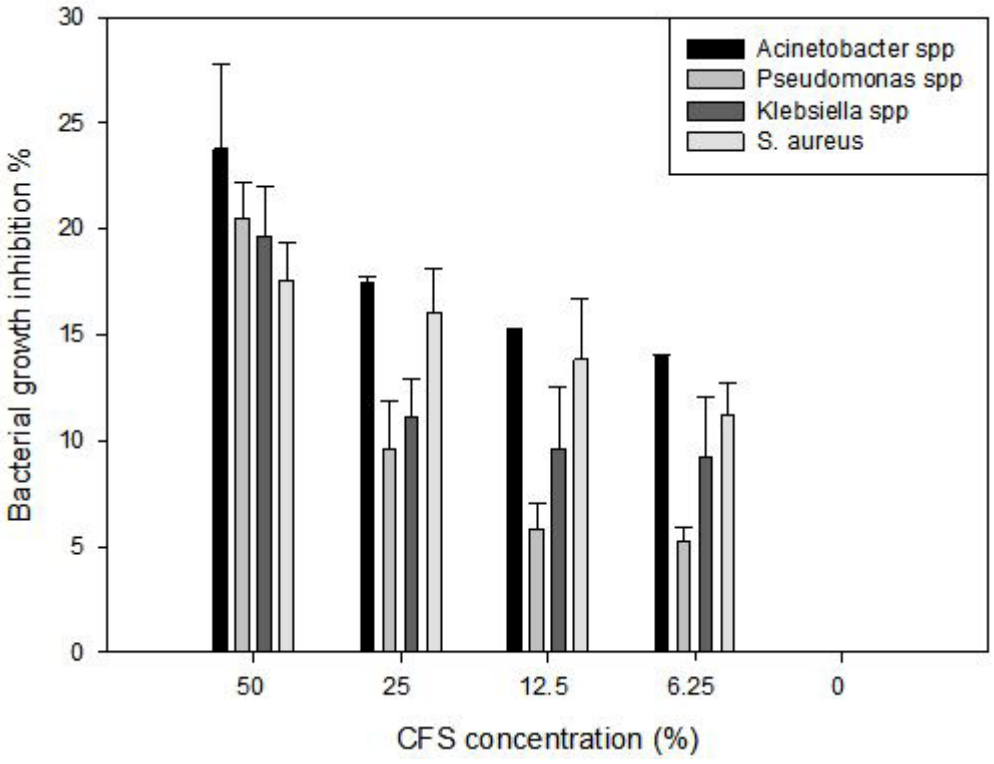

Figure 1: Antimicrobial activity of CFS-LP1 against isolated pathogens.

Table 1: Antibacterial activity of CFS-LP1 against pathogenic bacterial isolates.

Types of Bactria	50 %Mean ± Std. Error	
A. baumannii	23.78 ± 2.82	P. Value>0.05
P. aeruginosa	20.54 ± 1.14	
K. pneumoniae	19.66 ± 1.68	
S. aureus	17.55 ± 1.29	
25 %Mean ± Std. Error		
A. baumannii	17.47 ± 0.20	*A. baumannii vs. P. aeruginosa* 0.011
P. aeruginosa	9.57 ± 1.59	*A. baumannii vs. K. pneumoniae* 0.023
K. pneumoniae	11.06 ± 1.29	*P. aeruginosa vs. S. aureus* 0.022
S. aureus	16.06 ± 1.44	*K. pneumoniae vs. S. aureus* 0.048
12.5 %Mean ± Std. Error		
A. baumannii	15.32 ± 0.00	*A. baumannii vs. P. aeruginosa* 0.011
P. aeruginosa	5.85 ± 0.81	*A. baumannii vs. K. pneumoniae* 0.054
K. pneumoniae	9.60 ± 2.07	*P. aeruginosa vs. S. aureus* 0.020
S. aureus	13.85 ± 2.00	

	6.25 %Mean ± Std. Error	
A. baumannii	13.99 ± 0.06	*A. baumannii vs. P. aeruginosa* 0.005
P. aeruginosa	5.22 ± 0.48	*A. baumannii vs. K. pneumoniae* 0.042
K. pneumoniae	9.26 ± 1.96	*P. aeruginosa vs. S. aureus* 0.020
S. aureus	11.23 ± 1.02	

The MIC90 of the CFS-LP2 against *P. aeruginosa* was 50%, which inhibited 97.17% (Figure 2). In regards to other bacterial species, when 50% of CFS-LP2was used against *A. baumannii, K. pneumoniae,* and *S. aureus* produced 82.46%, 85.06% and 70.96, respectively. A 25% of CFS-LP2 produced; 8.18%, 10.23%, 49.70% and 16.43%, respectively, the 12.5% CFS caused 2.97%, 8.66%, 44.76% and 9.07%, respectively, while 6.25% led to 2.35%, 4.96%, 44.13% and 1.46%, respectively. The results showed significant different in bacterial growth inhibition when CFS-LP2 was used in all concentration (6.25, 12.5, 25 and 50) % when compared with the control. P-value (<0.05) and p-value (<0.001) as explained in Table 2.

We identified that the MIC90 of CFS-LP3 was 50% which inhibited 99.92%, 99.87%, 99.85% and 99.55% of *A. baumannii, P. aeruginosa, K. pneumoniae*, and *S. aureus*, respectively (Figure 3). The other concentration of CFS-LP3; 25% of CFS produced; 17.60%, 24.18%, 79.18% and 16.08%, respectively, the 12.5% CFS caused 8.60%, 6.31%, 4.78% and 11.78%, respectively. While, 6.25% led to 3.64%, 1.21%, 0.81% and 8.01%, respectively. The results showed different significant in bacterial growth inhibition when CFS-LP3 was used at (6.25, 12.5, 25, 50) % when compared with the control p-value (<0.05), and p-value (<0.001) as explained in (Table 3).

Minimum Biofilm Inhibitory Concentrations (MBIC50)

The minimal biofilm inhibitory concentration (MBIC50) is the lowest concentration of an antimicrobial that inhibits 50% or more of biofilm formation when compared to a control not treated with antimicrobial [20]. MBICs were determined using the broth-micro-dilution method. The anti-biofilm activity of the CFS of Lactobacillus species was examined against the four pathogenic strains.

The MBIC50 of CFS-LP1 was 12.5% inhibited 67.14% of *A. baumannii* biofilm (Figure 4). When the other CFS-LP1 concentration (50, 25 and 6.25) % inhibited 97.71%, 94.85% and 15.42%, respectively of biofilm formation by *A. baumannii*. Significant differences were observed in biofilm formation inhibition when CFS-LP1were used at all concentrations compare to the control, p-value (<0.05), and p-value (<0.001) as in Table 4.

The MBIC50 of CFS-LP2 was 12.5% inhibited71.36 % of *A. baumannii* biofilm (Figure 5).When the other concentrations (50, 25 and 6.25) % of CFS-LP2 was evaluated against *A. baumannii*; 97.20%, 94.72% and 2.17% of biofilm were inhibited, respectively. Significant differences were observed in biofilm formation inhibition when CFS-LP2were used at all concentrations compared to the control, p-value (<0.05) and p-value (<0.001) as in Table 5.

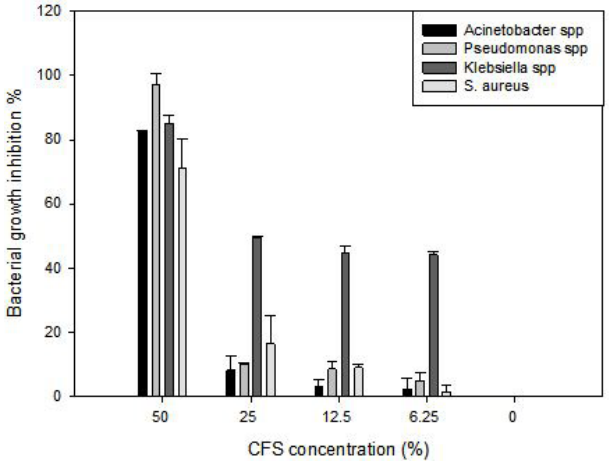

Figure 2: Antimicrobial activity of CFS-LP2 against isolated pathogens.

Table 2: Antibacterial activity of CFS-LP2 against pathogenic bacterial isolates.

Types of Bactria		50 %Mean ± Std. Error
A. baumannii	82.46 ± 0.33	*A. baumannii vs. P. aeruginosa* 0.043
P. aeruginosa	97.17 ± 2.32	*P. aeruginosa vs. S. aureus* 0.006
K. pneumoniae	85.06 ± 1.85	*K. pneumoniae vs. S. aureus* 0.049
S. aureus	70.96 ± 6.45	
25% Mean ± Std. Error		
A. baumannii	8.18 ± 3.30	*A. baumannii vs. K. pneumoniae* 0.001
P. aeruginosa	10.23 ± 0.25	*P. aeruginosa vs. K. pneumoniae* 0.001
K. pneumoniae	49.70 ± 0.29	*K. pneumoniae vs. S. aureus* 0.003
S. aureus	16.43 ± 6.14	
12.5 % Mean ± Std. Error		
A. baumannii	2.97 ± 1.65	*A. Baumannii vs. P. aeruginosa* 0.051
		A. baumannii vs. Klebsiella sp. <0.000
P. aeruginosa	8.66 ± 1.75	*A. baumannii vs. S. aureus* 0.041
K. pneumoniae	44.76 ± 1.44	*P. aeruginosa vs. K. pneumoniae* <0.000
S. aureus	9.07 ± 0.70	*K. pneumoniae vs. S. aureus* <0.000
6.25 % Mean ± Std. Error		
A. baumannii	2.35 ± 2.27	*A. baumannii vs. K. Pneumoniae* <0.000
P. aeruginosa	4.96 ± 1.94	*P. aeruginosa vs. K. pneumoniae* <0.000
K. pneumoniae	44.13 ± 0.81	*K. pneumoniae vs. S. aureus* <0.000
S. aureus	1.46 ± 1.46	

The MBIC50 of CFS-LP3 was 25% which inhibited 74.19% of *A. baumannii* biofilm (Figure 6). When the other concentrations of CFS-LP3 (50, 12.5 and 6.25) were applied against *A. baumannii*, 98.26%, 35.14% and 29.17%, respectively of biofilm were inhabited. Significant differences were observed in biofilm formation inhibition when CFS-LP3 used at all concentrations compared to the control, p-value (<0.001) as in Table 6.

The MBIC50 of CFS-LP1 against *P. aeruginosa* was 6.25% inhibited 62.92 %of biofilm formation (Figure

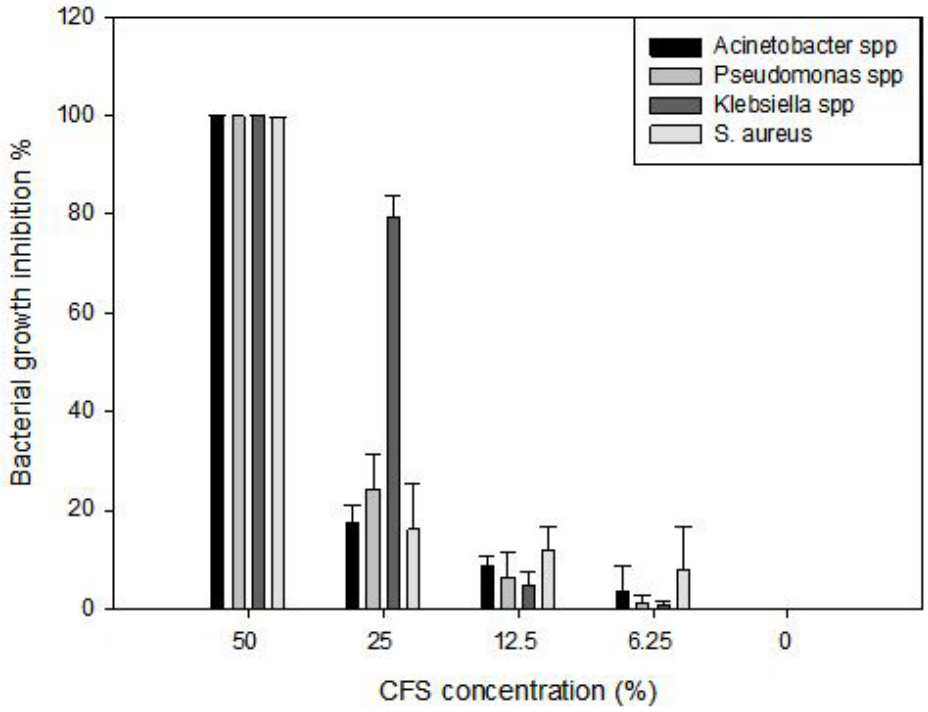

Figure 3: Antimicrobial activity of CFS-LP3 against isolated pathogens.

Table 3: Antibacterial activity of CFS-LP3against pathogenic bacterial isolates.

Types of Bactria	50% Mean ± Std. Error	
A. baumannii	99.92 ± 0.07	P. Value >0.05
P. aeruginosa	99.87 ± 0.00	
K. pneumoniae	99.85 ± 0.14	
S. aureus	99.55 ± 0.04	
25 %Mean ± Std. Error		
A. baumannii	17.60 ± 2.32	*A. baumannii vs. K. pneumoniae* 0.001
P. aeruginosa	24.18 ± 5.12	*P. aeruginosa vs. K. pneumoniae* 0.001
K. pneumoniae	79.18 ± 3.22	*K. pneumoniae vs. S. aureus* 0.001
S. aureus	16.08 ± 6.58	
12.5 %Mean ± Std. Error		
A. baumannii	8.60 ± 1.46	P. Value >0.05
P. aeruginosa	6.31 ± 3.56	
K. pneumoniae	4.78 ± 1.95	
S. aureus	11.78 ± 3.56	
6.25 %Mean ± Std. Error		
A. baumannii	3.64 ± 3.50	P. Value >0.05
P. aeruginosa	1.21 ± 1.03	
K. pneumoniae	0.81 ± 0.60	
S. aureus	8.01 ± 6.03	

7). While the other concentrations (50, 25 and 12.5) % of CFS-LP1when evaluated against *P. aeruginosa*; 99.97%, 76.49% and 63.91%, respectively of biofilm were inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP1were used at all concentrations compared to the control, p-value (<0.001) as in Table 7.

The MBIC50 of CFS-LP2 against *P. aeruginosa* was 6.25% inhibited 72.89% of biofilm formation (Figure 8). When the other concentrations (50, 25 and 12.5) % of CFS-LP2 were evaluated against *P. aeruginosa*; 99.65%, 99.82% and 84.34%, respectively of biofilm inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP2were used at all concentrations compared to the control, p-value (<0.001) as in Table 8. The MBIC50 of CFS-LP3 was 12.5% against *P. aeruginosa*, inhibited 64.58% of its biofilm formation (Figure 9).When the other concentrations (50, 25 and 6.25) % of CFS-LP3 were evaluated against *P. aeruginosa*; 99.88%, 86.04% and 42.37%, respectively of biofilm were inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP3were used at all concentrations compared to the control, p-value (<0.001) as in Table 9.

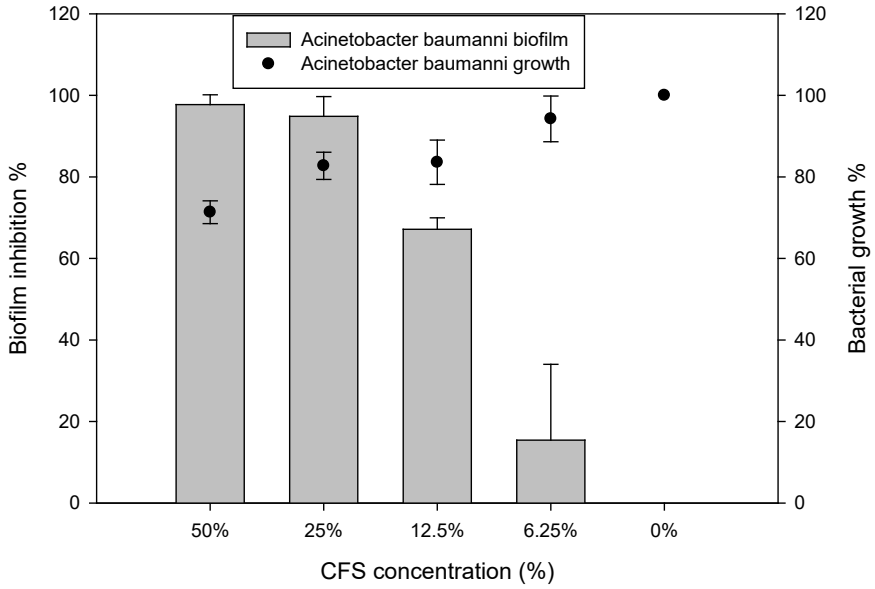

Figure 4: Effect of CFS of CFS-LP1 on *A. baumannii* biofilm.

Table 4: Anti-biofilm activity of CFS-LP1 against *A. baumannii*.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 0.0138
6.25	15.42 ± 13.14	0 vs. 12.5 0.001
12.5	67.14 ± 2.00	0 vs. 25 <0.000
25	94.85 ± 3.42	0 vs. 50 <0.000
50	97.71 ± 1.71	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.208
6.25	94.22 ± 3.95	0 vs. 12.5 0.009
12.5	83.58 ± 3.84	0 vs. 25 0.008
25	82.71 ± 2.35	0 vs. 50 0.001
50	71.33 ± 1.98	

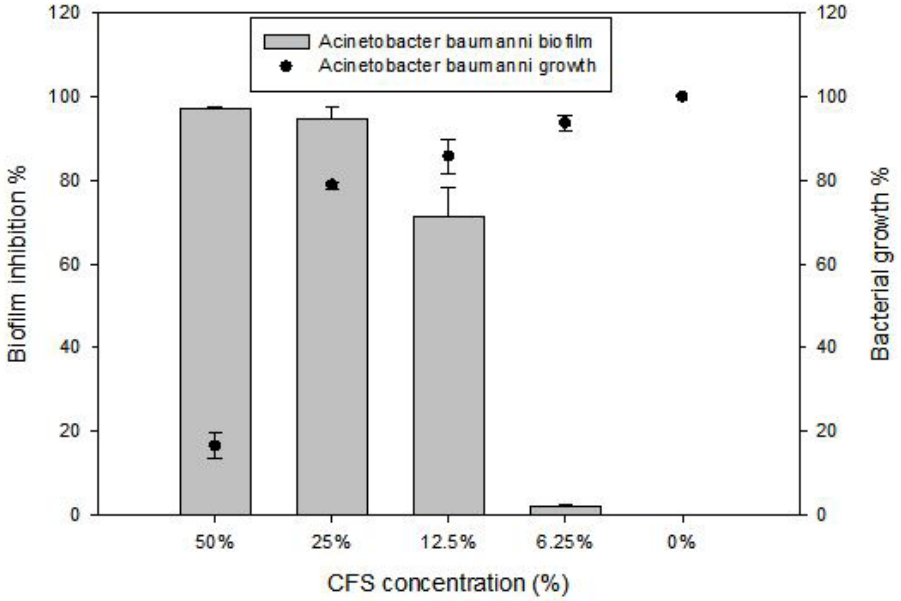

Figure 5: Effect of CFS of CFS-LP2 on *A. baumannii* biofilm.

Table 5: Anti-biofilm activity of CFS-LP2 against *A. baumannii*.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 0.551
6.25	2.17 ± 0.31	0 vs. 12.5 <0.000
12.5	71.36 ± 4.90	0 vs. 25 <0.000
25	94.72 ± 2.17	0 vs. 50 <0.000
50	97.20 ± 0.31	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.050
6.25	93.75 ± 1.25	0 vs. 12.5 0.002
12.5	85.69 ± 2.91	0 vs. 25 <0.000
25	78.88 ± 0.55	0 vs. 50 <0.000
50	16.52 ± 2.08	

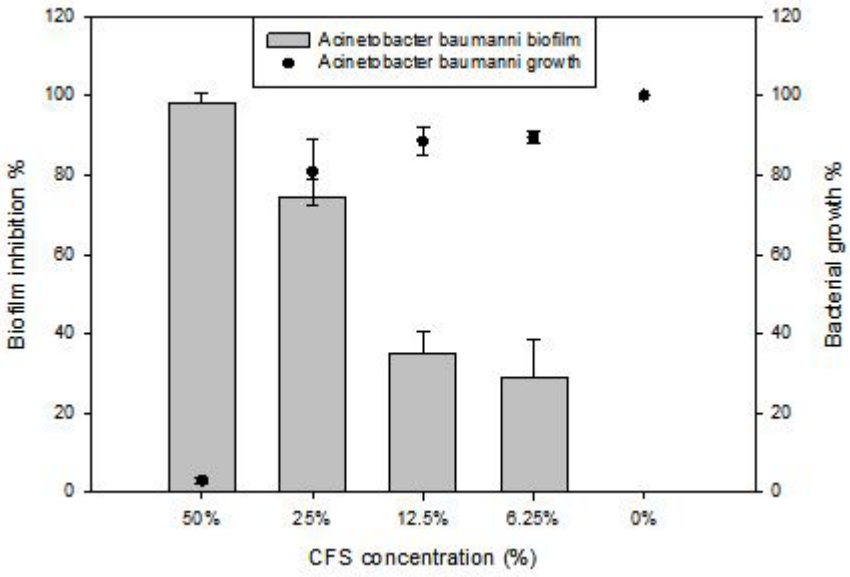

Figure 6: Effect of CFS of CFS-LP3 on *A. baumannii* biofilm.

Table 6: Anti-biofilm activity of CFS-LP3 CFS against *A. baumannii*.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 0.003
6.25	29.17 ± 6.61	0 vs. 12.5 0.001
12.5	35.14 ± 3.90	0 vs. 25 <0.000
25	74.19 ± 3.36	0 vs. 50 <0.000
50	98.26 ± 1.73	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.051
6.25	89.41 ± 1.11	0 vs. 12.5 0.039
12.5	88.45 ± 2.43	0 vs. 25 0.006
25	80.72 ± 5.95	0 vs. 50 <0.000
50	2.93 ± 0.59	

The MBIC50 of CFS-LP1 was 12.5% against K. pneumoniaeinhibited63.04%of its biofilm formation (Figure 10). When the other concentrations (50, 25and 6.25) % of CFS-LP1were evaluated against K. pneumoniae; 90.21%, 71.19% and 47.82%, respectively of biofilm were inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP1were used at all concentrations compared to the control, p-value (<0.001) as in Table 10. The MBIC50 of CFS-LP2 was 12.5% against K. pneumoniae, inhibited 53.12% of bacterial biofilm formation (Figure 11). When the other concentrations (50, 25 and 6.25) % of CFS-LP2 were evaluated against K. pneumoniae; 94.67%, 66.86% and 38.24%, respectively of biofilm were inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP2were used at all concentrations compared to the control, p-value (<0.001) as in Table 11.

The MBIC50 of CFS-LP3 was 6.25% which inhibited 53.01%of K. pneumoniae biofilm (Figure 12). When the other concentration (50, 25 and 12.5) % of CFS-LP3 were evaluated against K. pneumoniae; 96.65%, 97.65% and 81.77%, respectively of biofilm were inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP3were used at all concentrations compared to the control, p-value (<0.001) as in Table 12. The MBIC50 of CFS-LP1 against S. aureus was 25%, inhibited 67.91%of bacterial biofilm (Figure 13). When the other concentrations (50, 12.5

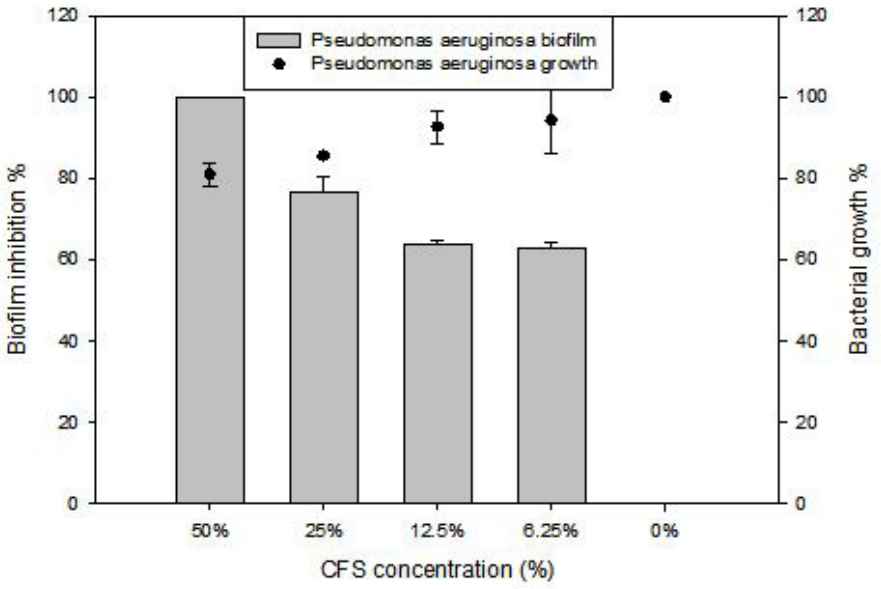

Figure 7: Effect of CFS-LP1 on *P. aeruginosa* biofilm.

Table 7: Anti-biofilm activity of CFS-LP1 against *P. aeruginosa*.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 <0.000
6.25	62.92 ± 0.92	0 vs. 12.5 <0.000
12.5	63.91 ± 0.68	0 vs. 25 <0.000
25	76.49 ± 2.81	0 vs. 50 <0.000
50	99.97 ± 0.02	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.232
6.25	94.22 ± 5.77	0 vs. 12.5 0.147
12.5	92.71 ± 2.82	0 vs. 25 0.019
25	85.52 ± 0.20	0 vs. 50 0.007
50	81.03 ± 1.90	

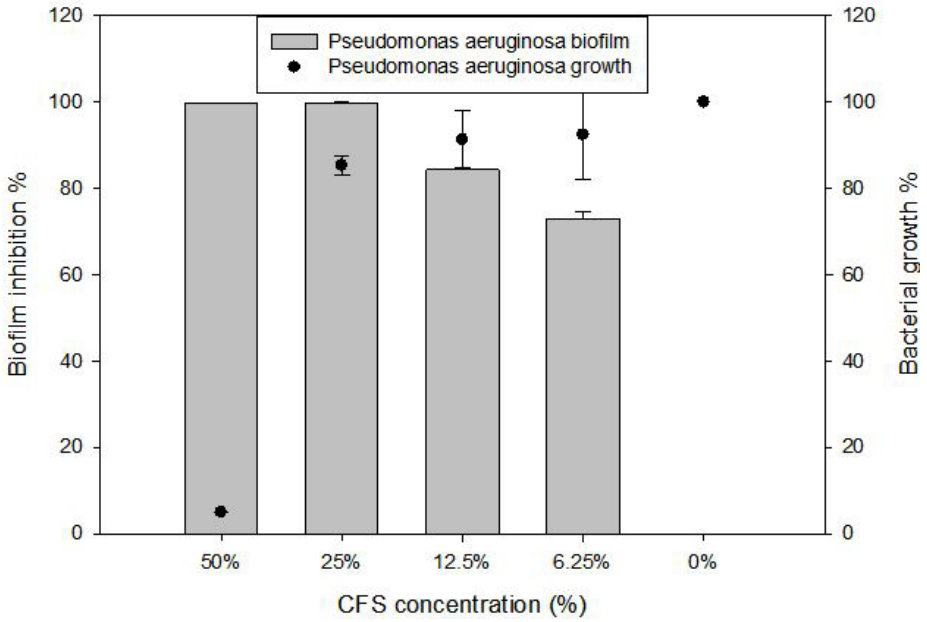

Figure 8: Effect of CFS-LP2 on *P. aeruginosa* biofilm.

Table 8: Anti-biofilm activity of CFS-LP2 against *P. aeruginosa*.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 <0.000
6.25	72.89 ± 1.31	0 vs. 12.5 <0.000
12.5	84.34 ± 0.41	0 vs. 25 <0.000
25	99.82 ± 0.10	0 vs. 50 <0.000
50	99.65 ± 0.06	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.232
6.25	92.41 ± 7.32	0 vs. 12.5 0.181
12.5	91.31 ± 4.64	0 vs. 25 0.261
25	85.34 ± 1.62	0 vs. 50<0.000
50	4.95 ± 0.06	

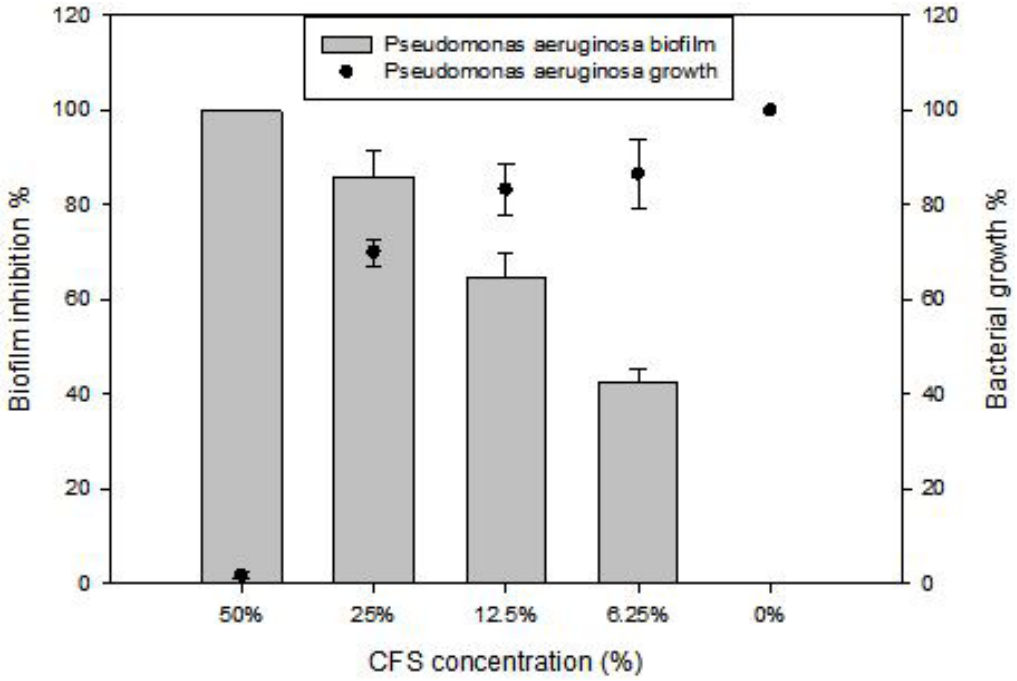

Figure 9: Effect of CFS-LP3 on *P. aeruginosa*biofilm.

Table 9: Anti-biofilm activity of CFS-LP3 against *P. aeruginosa*.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 <0.000
6.25	42.37 ± 2.07	0 vs. 12.5 <0.000
12.5	64.58 ± 3.73	0 vs. 25 <0.000
25	86.04 ± 3.87	0 vs. 50 <0.000
50	99.88 ± 0.03	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.023
6.25	86.58 ± 5.03	0 vs. 12.5 0.010
12.5	83.25 ± 3.69	0 vs. 25 0.001
25	70.03 ± 2.00	0 vs. 50<0.000
50	1.80 ± 0.48	

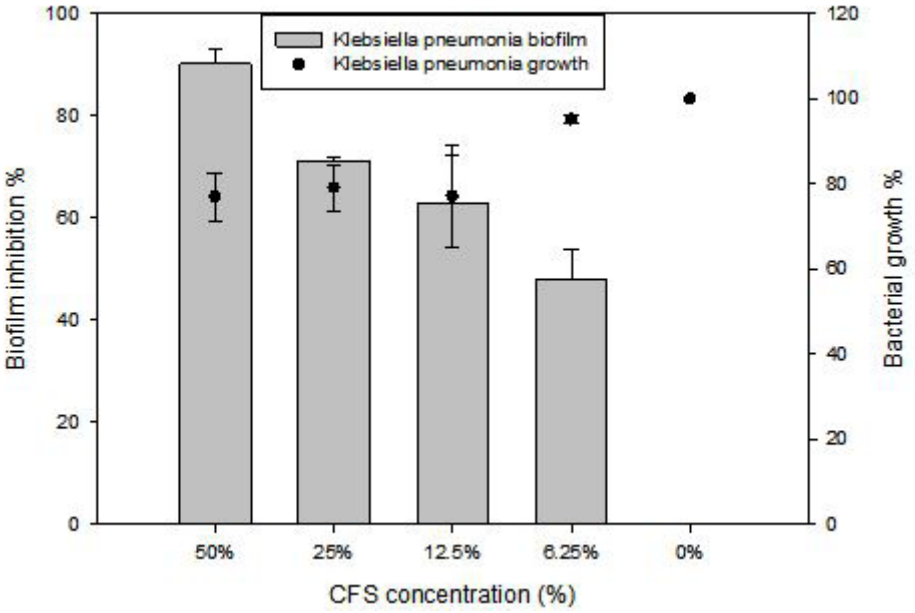

Figure 10: Effect of CFS-LP1on K. pneumoniae biofilm.

Table 10: Anti-biofilm activity of CFS-LP1 against K. pneumonia.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 <0.000
6.25	47.82 ± 4.34	0 vs. 12.5 <0.000
12.5	63.04 ± 6.52	0 vs. 25 <0.000
25	71.19 ± 0.54	0 vs. 50 <0.000
50	90.21 ± 2.17	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.483
6.25	92.14 ± 0.67	0 vs. 12.5 0.038
12.5	77.12 ± 8.44	0 vs. 25 0.022
25	79.07 ± 3.95	0 vs. 50 0.016
50	77.01 ± 3.92	

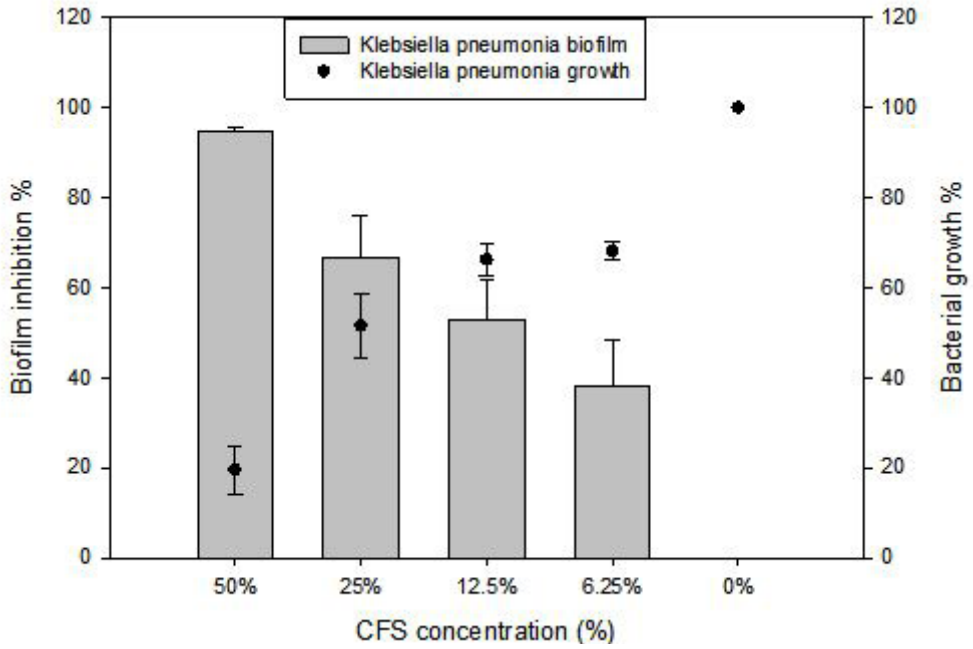

Figure 11: Effect of CFS-LP2 on K. pneumoniae biofilm.

Table 11: Anti-biofilm activity of CFS-LP2 against K. pneumonia.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 0.003
6.25	38.24 ± 7.31	0 vs. 12.5 <0.001
12.5	53.12 ± 6.04	0 vs. 25 <0.000
25	66.86 ± 6.50	0 vs. 50 <0.000
50	94.67 ± 0.59	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.001
6.25	68.21 ± 1.46	0 vs. 12.5 0.001
12.5	66.22 ± 2.45	0 vs. 25 <0.000
25	51.51 ± 4.92	0 vs. 50 <0.000
50	19.57 ± 3.75	

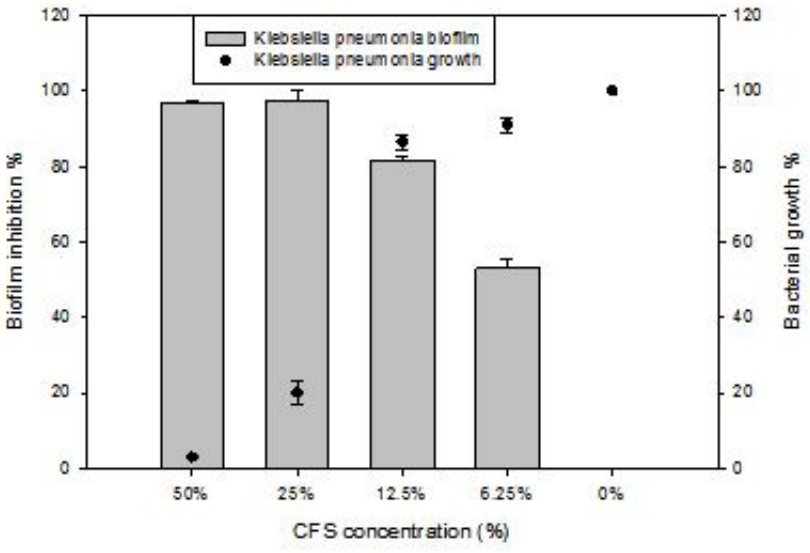

Figure 12: Effect of CFS-LP3 on K. pneumoniae biofilm.

Table 12: Anti-biofilm activity of CFS-LP3against K. pneumonia.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 0.003
6.25	53.01 ± 1.83	0 vs. 12.5 <0.001
12.5	81.77 ± 0.50	0 vs. 25 <0.000
25	97.65 ± 1.76	0 vs. 50 <0.000
50	96.65 ± 0.66	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 <0.000
6.25	90.92 ± 1.46	0 vs. 12.5 <0.000
12.5	86.50 ± 1.27	0 vs. 25 <0.000
25	20.02 ± 2.28	0 vs. 50 0.572
50	2.96 ± 0.20	

and 6.25) % of CFS-LP1 were evaluated against S. aureus; 72.66%, 44.84% and 6.61%, respectively of biofilm were inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP1were used at all concentrations compared to the control, p-value (<0.05), and p-value (<0.001) as in Table 13.

The MBIC50 of CFS-LP2 against S. aureus was 25%, inhibited 58.59%of its biofilm formation (Figure 14). When the other concentrations (50, 12.5 and 6.25) % of CFS-LP2 were evaluated against S. aureus; 95.50%, 45.50% and 9.37%, respectively of biofilm were inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP2were used at all concentrations compared to the control, p-value<0.05, and p-value (<0.001) as in Table 14. The MBIC50 of CFS-

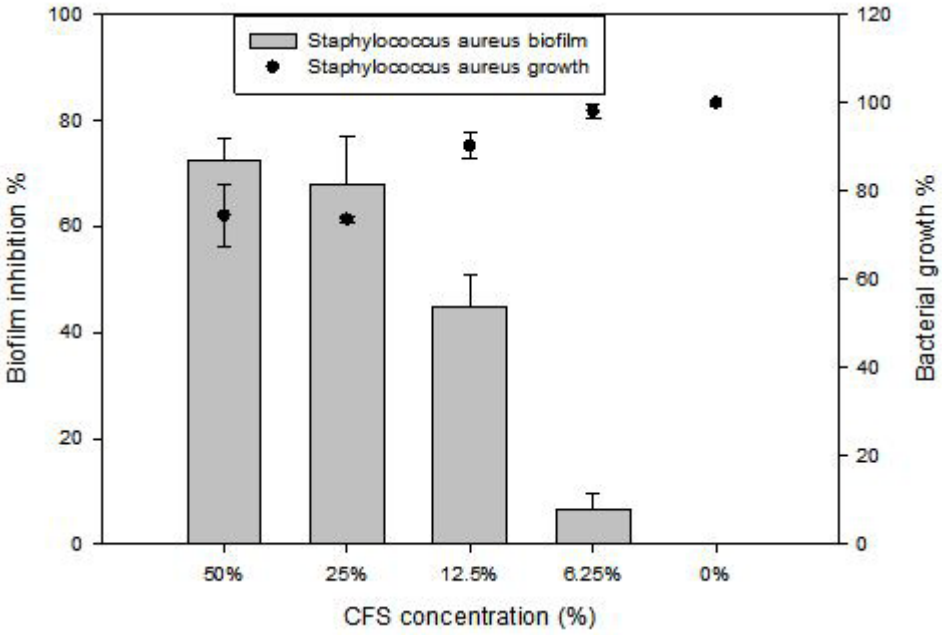

Figure 13: Effect of CFS-LP1 on S. aureus biofilm.

Table 13: Anti-biofilm activity of CFS-LP1 against S. aureus.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 0.272
6.25	6.61 ± 2.12	0 vs. 12.5 <0.000
12.5	44.84 ± 4.37	0 vs. 25 <0.000
25	67.91 ± 6.35	0 vs. 50 <0.000
50	72.66 ± 2.76	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.597
6.25	98.06 ± 1.21	0 vs. 12.5 0.037
12.5	90.30 ± 2.06	0 vs. 25 0.001
25	73.60 ± 0.57	0 vs. 50 0.001
50	74.49 ± 4.84	

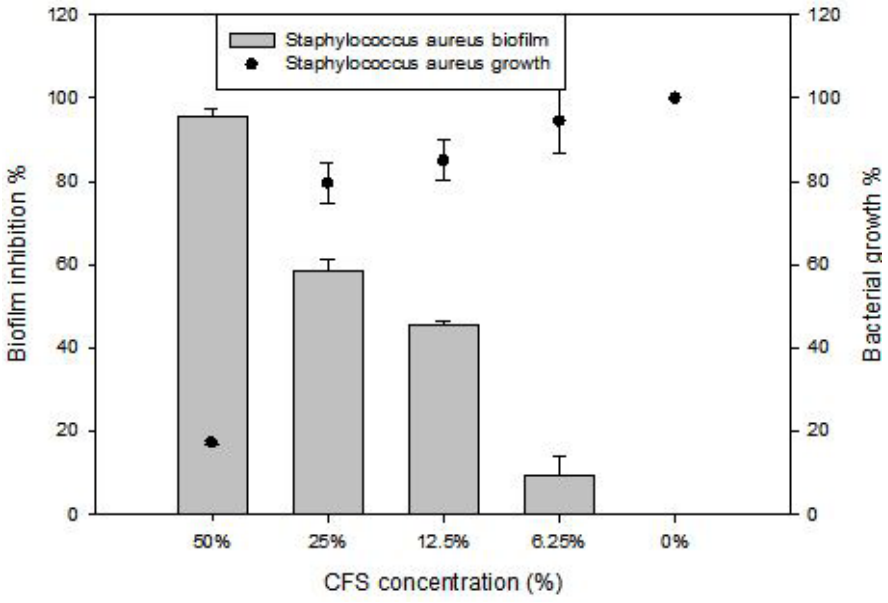

Figure 14: Effect of CFS-LP2 on *S. aureus* biofilm.

Table 14: Anti-biofilm activity CFS-LP2 against S. aureus.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 0.014
6.25	9.37 ± 3.12	0 vs. 12.5 <0.000
12.5	45.50 ± 0.58	0 vs. 25 <0.000
25	58.59 ± 1.95	0 vs. 50 <0.000
50	95.50 ± 1.63	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.289
6.25	94.46 ± 5.53	0 vs. 12.5 0.024
12.5	85.00 ± 3.41	0 vs. 25 0.007
25	79.55 ± 3.47	0 vs. 50<0.000
50	17.23 ± 0.35	

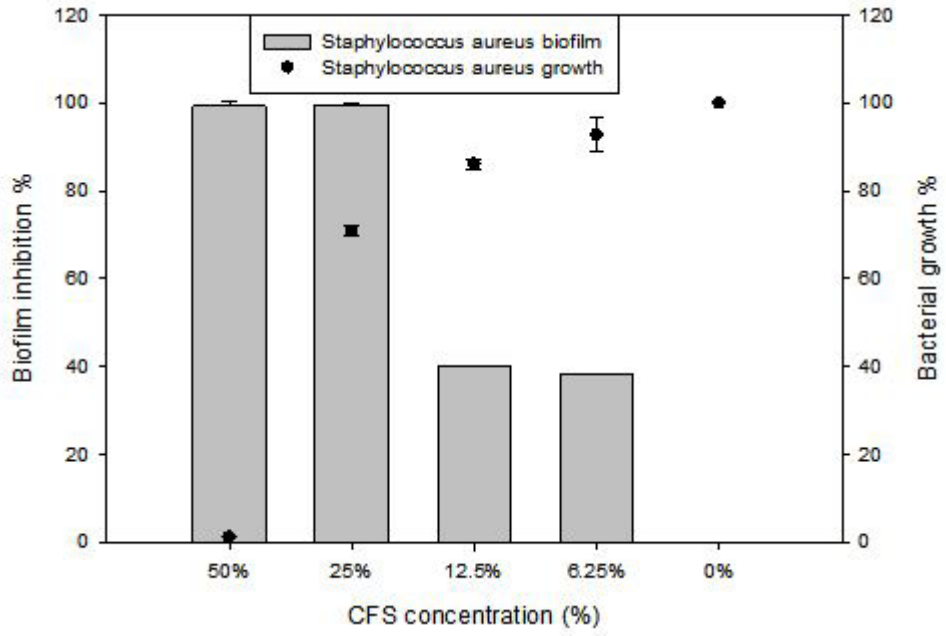

Figure 15: Effect of CFS-LP3 on S. aureus biofilm.

Table 15: Anti-biofilm activity of CFS-LP3 against *S. aureus*.

Biofilm inhibition % CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	0.00 ± 0.00	0 vs. 6.25 <0.000
6.25	38.16 ± 0.11	0 vs. 12.5 <0.000
12.5	40.13 ± 0.00	0 vs. 25 <0.000
25	99.65 ± 0.11	0 vs. 50 <0.000
50	99.30 ± 0.69	
Bacteria growth% CFS		
Concentration (%)	Mean ± SE	ANOVA P-value
0	100.00 ± 0.00	0 vs. 6.25 0.012
6.25	92.73 ± 2.77	0 vs. 12.5.001
12.5	86.11 ± 0.79	0 vs. 25 <0.000
25	70.94 ± 0.74	0 vs. 50<0.000
50	1.23 ± 0.08	

LP3 was 25%, inhibited 99.65%of *S.* aureus biofilm (Figure 15). When the other concentrations (50, 12.5 and 6.25) % of CFS-LP3 were evaluated against S. aureus; 99.30%, 40.13% and 38.16%, respectively of biofilm were inhibited. Significant differences were observed in biofilm formation inhibition when CFS-LP3were used at all concentrations compared to the control, p-value (<0.001) as in Table 15.

DISCUSSION

Burned skin is a type of skin injury that occurs often and frequently. Death after exposure to a burn is considered one of the most common consequences, especially after the burn site was exposed to bacteria, especially MDR. The multi-drug resistance among bacterial pathogens has complicated the management of surgical burn infections [21]. Our study comes to evaluate an alternative antimicrobial substances, CFS, prepared from Lactobacillus species isolated from cow's milk samples to control the pathogenic bacteria causing burns infection.

The MIC90 was determined using the broth micro dilution method after preparing a series of different concentrations of Lactobacilli CFS (50-6.25) %. The antimicrobial activity of Lactobacilli CFS were various against the isolated pathogen, even among the same species of Lactobacilli. The MIC90 of CFS-LP2 & 3 was determined against *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*. While, no MIC90 were identified for CFS-LP1. The proposed antimicrobial mechanisms of Lactobacillus. Spp. including; (i) the secretion of antimicrobial molecules such as ethanol, fatty acid, hydrogen peroxide, and bacteriocins, (ii) production of inhibitory substances, (iii) Nutrient competition, (iv) immune stimulation, (v) the ability to lower pH through the production of lactic acid, acetic acid, formic acid, and other acids, and (vi) competition for binding sites [22,23]. Several studies have shown that CFS has antibacterial action against a variety of bacterial pathogens [24], observed a strong effect of five CFS of Lactobacilli isolates against the majority of carbapenemase-producing Enterobacteriaceae (CPE) isolates. Another study done by [25] reported antibacterial activities and biofilm removal potential of L. acidophilus LA5 and L. casei 431 CFS against S. aureus ATCC 25923. In contrast to our study, [26] found that CFS of Lactobacillus isolated from curd and human milk has no antibacterial activity against S. aureus, L. monocytogenes, E. coli, or K. pneumonia. This result indicating that Lactobacillus strains are various in their antagonistic activity against indicator pathogens.

The MBIC50 is defined as the concentration of an antimicrobial that inhibits 50% of biofilm formation compared to the control (untreated biofilm) [27]. In our study, we found the MBIC50 of all CFS of Lactobacillus spp. which was used against four pathogenic bacteria was in rang (25-6.25) % of CFS. The CFS produced by Lactobacillus spp. had the best inhibitory effect on biofilm formation, according to the findings of the current study. Probiotics, particularly Lactobacilli spp., have been discovered to prevent or disperse pathogenic biofilm formation by assaulting the bacterial membrane, causing it to become rough and wrinkled, which may eventually lead to biofilm inhibition [25]. Lactobacilli have the ability to interfere with pathogenic bacteria by competing for nutrition, co-aggregating, and producing antimicrobials such as lactic acid, hydrogen peroxide, bacteriocin, and organic acids in addition to the possibility of immunomodulatory effects [28]. Several studies have shown that CFS of Lactobacillus spp. have anti-biofilm activity and compete with pathogens [29], showed the anti-biofilm ability of CFS-LP was investigated against multidrug-resistant *P. aeruginosa*, *S. aureus*, and *E. coli*. The authors found that the CFS not only reduced pathogenic bacteria biofilm development but also disrupted preformed biofilms [30], showed that L. rhamnosus and L. paracasei isolates with satisfactory probiotic potential and higher biofilm inhibition and antibacterial activity were found [31], showed that the *L. plantarum* 12 exopolysaccharide were found to reduce polysaccharide production in *S. flexneri's* extracellular polymeric matrix and limit biofilm formation [32], reported that the exopolysaccharide generated by L. acidophilus A4 inhibited the formation of E. coli O157:H7 biofilms.

CONCLUSION

The majority of hospital-acquired bacterial contaminants in burned patients are *P. aeruginosa*, *S. aureus*, *A. baumannii* and *K. pneumonia*. These contaminate bacterial isolates have a variety of virulence factors; the most importantly is biofilm formation, which enhances bacterial colonization in burn site and increase their antibiotic resistance. *Lactobacillus plantarum* was the most LAB species isolated from the cow milk. Probiotics such as, Lactic acid bacteria, especially Lactobacillus spp. isolated from cow's milk possess a mixed of natural antimicrobial molecules making them attractive candidates for preventing the pathogenic biofilm formation. Furthermore, the antimicrobial activity of CFS of Lactobacillus strains could be different, even among the same species, however, they still a good alternative therapy to controlling biofilm-associated multidrug-resistant bacteria.

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