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RESEARCH ARTICLE

Study the Antibacterial and Antibiofilm Activity of Purified Exopolysaccharides (EPSs) from *Lactobacillus acidophilus* Against Bacteria Isolated from Burn Wounds

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Abstract

The current study was investigated the antibacterial and antibiofilm activity potential of purified Exopolysaccharides (EPSs) from *lactobacillus acidophilus* against bacteria isolated from patients with Burn wounds that showed some resistance to antibiotics, the results of isolation and identification were showed the predominant bacteria was *S. aureus* 90% followed by P.aeruginosa 80%, *B.subtilis* 60%, *S.hominis* 50%, *E coli* 40%, *P.mirabilis* 40%, E.feacalis 40%. Furthermore some isolates were sensitive to ciprofloxacin, *P.aeruginosa* 37.5%, *S. hominis* 100%, *B. subtilis* 16.6%, *E.feacalis* 50%, *P.mirabilis* 100%, However the other isolates showed sensitivity to Tetracycline such as S.aureus 44.4 %, *E.*coli 100%, *B. subtilis* 100 %. While Azithromycin and Novobiocin, Ampicillin, Cefotaxime, Erythromycin, Imipenem, Doxycycline were showed no effect toward isolates .Meanwhile Chloromphenicol, Amoxicillin /clavulanic acid, Gentamicin showed intermediate effects toward isolates. In addition EPSs showed a positive antibacterial activity against the isolates with different concentrations and the highest value recorded in 500 mg/ml concentration in each isolates but in 200mg/ml concentration in some isolates showed no inhibition zones. Finally anti biofilm activity of EPSs, the study noticed EPSs showed more effective against biofilm formation withing 24 hr more than performed biofilm of isolates (after 24 hr). Furthermore MIC of antibiotic showed more effective than MIC of EPSs in biofilm assay, but both MIC of antibiotic and EPSs were decreased the strength of biofim of isolates from strong biofilm and moderate biofilm formation to weak biofilm formation. The study was revealed depended on obtained results, purified EPSs from *Lactobacillus acidophilus* had antibacterial and antibiofilm activities against some bacteria isolated from burn wounds showed some resistance to antibiotics.

Keywords: *Extraction of Exopolysaccharides from lactobacillus acidophilus, Antibacteria, Antibiofilm activity of EPS, Burn wounds.*

Introduction

Burns is an injury to the skin that damaged or destroyed the skin cells and tissues. Generally caused when skin contacted with flames, chemicals, electricity, or radiation [1].With several species of potentially pathogenic micro-organisms, including *Pseudomonas aeruginosa* and *Staphylococcus sp*are colonized in burn wounds are a major focus for infection. The common aerobic microorganisms were isolated from burn wounds included *S. aureus, S,pyogenes, E. coli, Klebsiella Spp., Proteus spp, P. aeruginosa,* and the anaerobic organisms like B.fragilis, Peptostreptococcus spp, Propionibacterium Spp., Fusobacterium Spp. and fungi like Aspergillus *niger, Candida Spp and Zygomycetes.*

Several studies performed multiple-drug resistant organisms (MDROs), *Pseudomonas aeruginosa* [2, 3]. *Staphylococcus aureus* and and other isolates were in burn patients. These pathogens are often resistant to many of the antimicrobial treatments we use today [3], other studies revealed Lactobacillus (Lb.) used for treatment many cases such as burn wounds as probiotic bacteria and ability to produce lactic acid [4] also exopolysaccharides (EPSs) synthesized by Lactobacillus (Lb.) has commercially

physiological as well as therapeutic activities that was reported Recent studies, concerned the microbial exopolysaccharides (EPSs) because their health benefits. Such as antitumor effect, immunostimulatory potential activity, the ability to decrease blood cholesterol level [4, 5]. In addition antibacterial activity and antibiofilm activity against pathogens [6]. This study was aimed to investigate the antibacterial and anti-biofilm activities of purified EPSs from *lactobacillus acidophilus* toward some resistant bacteria isolated from patient with burn wounds.

Material and Methods

Samples Collection

A total 10 burn wound swabs were collected from burn wounds from Al kindy teaching hospital, the burn wound swabs were transported by the amies medium to laboratories of department of Medical laboratories Techniques, Al Rafidain University College.

Isolation and Identification of Bacteria

Burn wound swabs were inoculated in nutrient broth and incubated for 24 hr and subcultured to different media, the isolates were identified by morphological and cultural characteristics of the colonies were studied, including color, shape, size, margin, elevation, texture, diameter, and others [7].And biochemical tests, finally confirmed by Vitek 2.

Antimicrobial Susceptibility Test

The purpose of this test was to select the antibiotics that more effective against isolates. Anyway the chosen antibiotics were ciprofloxacin, Tetracycline, Ampicillin, Chloromphenicol Amoxicillin /clavulanic acid, Gentamicin, Novobiocin, Azithromycin, Cefotaxime, Erythromycin, Imipenem, Doxycycline, its standard inhibition diameters was used as it recommended by [8]. The procedure of this test was performed according to [9] by the Kirby-Bauer standardized method.

Determination Minimum Inhibitory concentration of Antimicrobial

After determination of the most effective antimicrobial agents by the Kirby-Bauer standardized method for each bacterial, MIC of the most effective antimicrobial agent for each bacterial isolate(Ciprofloxacin and Tetracycline) were measured by using the broth macrodilution method recommended by [8]. the inoculum was prepared adjusted the to 0.5 McFarland standard., then 1ml was added to tubes containing 1 ml of antimicrobial agent (Tetacycline and ciprofloxacin) in the dilution series.

The inoculated tubes were incubated 37 °C for 24 hr the amounts of growth in tubes containing the antibiotics were compared the growth-control tubes (no antimicrobial agent) as control. Inhibited growth of the organism in the tubes as detected by the unaided eye.

Purification and Extraction EPSs from Lactobacillus Acidophilus

L. Acidophilus was obtained from laboratories of department of microbiology / College of Veterinary Medicine / Al-Muthanna University. Iraq. The process of extraction of EPSs, was *L. acidophilus* cultured in MRS broth for 24 hr, after centrifugation (8,000 ×g for 20 min at 4ºC) of culture, the supernatant was collected then added with a final concentration of 14% trichloroacetic acid to denature the proteins content.

The supernatant was then added to cold absolute ethanol (two-fold volume of supernatant) at 4ºC for 24 hours, followed by centrifugation at 8000 ×g at 4ºC for 20 min. The precipitated exopolysaccharide centrifuged for 15 min for at 4°C and the supernatant was removed [10]. Finally, the precipitate was dissolved in deionized water and dialyzed using with dialysis membrane for 24~48 hours. The precipitated exopolysaccharides (EPSs) dried at 60°C to remove ethanol, then the concentration was prepared.

Determination MIC of EPSs

100 µl of bacterial suspension (Adjusted 0.5 McFarland standards) added to Eppendorf tubes then 50 µl of the tested agent were added, the tubes were incubated for 284 hrs. Then the tubes were examined to see if there was any turbidity (turbidity indicates bacterial growth), the tubes that showed signs of turbidity were excluded while the tubes that lack turbidity were identified as the minimum inhibitory concentration [11].

Determination the Antibiotic activity of EPSs

The isolates was prepared from Nutrient broth (18- to 24-hour) then adjusted to 0.5 McFarland standards. Approximately 1.5×108 colony-forming units CFU/ml, and sub cultured on Mueller-Hinton agar contained wells of equal size (6 mm in diameter) were prepared using pasteur pipette under aseptic conditions, well was filled with 0.1 ml of prepared concentrations of EPS [11].

Effect MIC of EPSs on Biofilm Formation

Biofilm formation was measured as described [12] and modified .the chosen concentrations of EPS extract were added to sterile culture medium and 100 µl of each concentration was then added into the well (96-well polystyrene micro titer plate). Overnight cultures of each isolates were diluted to 0.2 in fresh TSB.

Subsequently, 200 *µl* of these suspensions was added into the wells. wells filled with only broth (without bacteria) served as control (blank) to check sterility and non-specific binding of used media then incubated at 37°C for 24 hr.

Effect MIC of EPSs on Preformed Biofilm

100 µl of bacterial cultured in TSB medium were added into the wells (96-well micro titer plate), incubation 37°C for 24 hr to form biofilm, the medium was kindly aspirated, the formed biofilm washed three times with PBS to remove the non-adherent cells. 200 µl of EPS dilution in tryptic soy broth was added. The plate was incubated in 37°C for 24 hr, this assay was repeated in absence of MIC of EPS as a control [12].

Results

The result of isolation the bacteria was showed the predominant bacteria *S. aureus* 90% followed *p. aeruginosa* 80%, *B.subtilis* 60%, *S. hominis* 50%, *E coli* 40%, *P.mirabilis* 40%, *E. feacalis* 40% as in Figure 1.

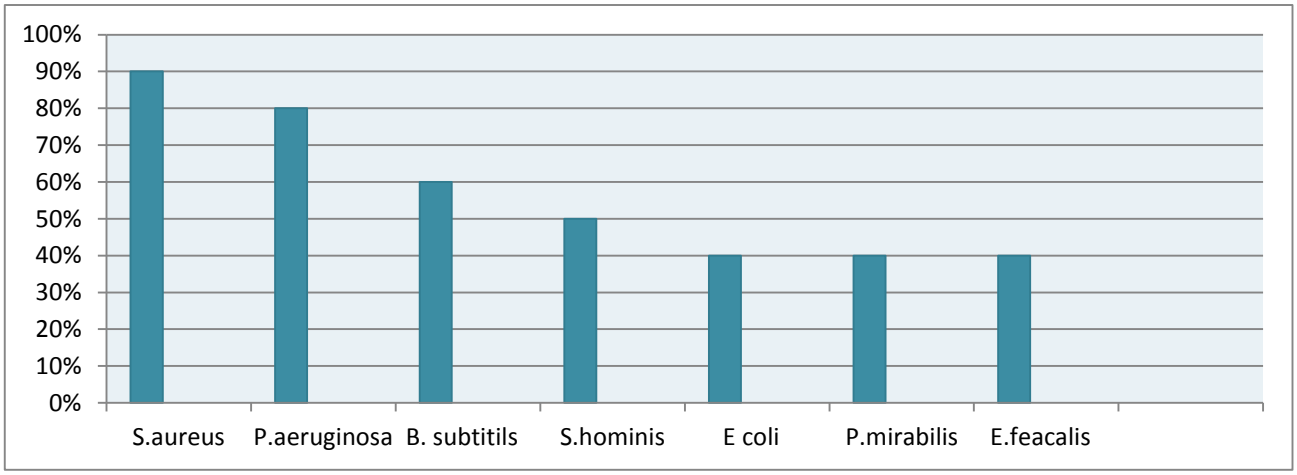

Figure 1: showed the isolates rate in 10 Burn wound swabs

The results of antimicrobial test revealed some isolates were sensitive to ciprofloxacin, *P.aeruginosa* 37.5%, *S. hominis* 100%, *B. subtilis* 16.6%, *E.feacalis* 50%, *P.mirabilis* 100%, However the other isolates showed sensitivity to Tetracycline such as S.aureus 44.4 %, *E.*coli 100%, *B. subtilis* 100 %.

Furthermore , noticed the Azithromycin and Novobiocin, Ampicillin, Cefotaxime, Erythromycin , Imipenem, Doxycycline, were showed no effect against the isolates, however chloromphenicol, amoxicillin /clavulanic acid, Gentamicin showed intermediate effects, depended on those results, the current study were selected ciprofloxacin and tetracycline for determination the minimum inhibitory concentration (MIC) of Ciprofloxacin for *P.aeruginosa. S. hominis, E, feacalis, P.mirbalis*, as well as the minimum inhibitory concentration (MIC) of tetracycline for *S.aureus, E.coli, B. subtilis*. The results of minimum inhibitory concentration of EPS and Tetracycline and Ciprofloxacin for isolates were reported a different MIC for isolates as in Table 1.

Table 1: showed the minimum inhibitory concentration of EPSs

Isolates	MIC of EPSs (µg /ml)
P.aeruginosa	250
S.aureus	215
E.coli	200
S. Hominis	200
B.subtilis	200
P.mirbalis	150
E.feacalis	180

The results of minimum inhibitory concentration Tetracycline for *S.aureus* 6 (*µg* /ml), *E.coli* 5.2(*µg* /ml*)*, *B.subtilis* 4 (*µg* /ml) while MIC of Ciprofloxacin to *P.aeruginosa* was 0.2 (*µg* /ml), *S. Hominis* 0.06 (*µg* /ml), *P.mirbalis* 0.09 (*µg* /ml) , *E.feacalis* 0.08 (*µg* /ml). The antibacterial activity of EPSs results were reported there was a positive effect of EPSs against the isolated used in study and showed inhibition zone and recorded various results and significant differences between the concentrations within same isolated and also with different isolates in addition as *S.aureus*, *P. aeruginosa*, *S.hominis* were showed no inhibition zone at 200 (*m*g/ml) of EPSs as in Table 2.

Table 2: showed the inhibition zone diameter (mm) of Exoploysaccharides against isolates

	Isolates						
Conc (µg /ml)	***S.aureus***	***P. aeruginosa***	***B.subtilis***	***S.hominis***	***E.coli***	***P.mirablis***	***E. feacalis***
200	0.0±0.00	0.0±0.00	1.21±.008	0.0±0.00	1.0 ±0.00	2.14±0.01	1.14±.03
250	1.18±0.09	1.26±0.14	2.13 ±0.01	1.06±0.06	2.07±0.07	4.06±0.06	3.19±0.03
300	5.47±0.37	3.33±0.17	4.0±0.00	2.54±0.22	5.07±0.03	6.2±0.01	5.20±0.06
350	7.27±0.17	5.13±0.03	6.15±0.03	4.59±0.24	7.13±0.06	7.28±0.06	6.0±0.00
400	9.16±0.16	6.26±0.14	9.14±0.01	9.34±0.12	14.73±3.26	11.10±0.10	8.10±0.05
450	12.28±0.06	8.46±0.22	13.29±0.06	12.55±0.19	10.33±0.02	13.20±0.05	10.06±0.06
500	14.17±0.08	12.25±0.12	15.14±0.07	15.0±0.00	13.19±0.10	16.07±0.03	13.11±0.06

The results of biofilm assay were showed various strength of biofilm formation, strong biofilm producers were *P.mirabilis* 0.397±0.07, *P.aeruginosa* 0.365 ±0.12, *E coli* 0.362±0.43 ,moderate biofilm producers were *S.aureus* 0.319±0.02 *S.hominis* 0.337±0.32, *B.subtilis* 0.304 ±0.22, *E.feacalis* 0.3±0.12 .The results of determination the antibiofilm activity showed both MIC of antibiotic and EPSs was decreased the strength of performed biofilm after 24 hr of isolates to weak biofilm formation in all isolates used in study, but noticed the MIC of antibiotics showed more effective than MIC of EPSs with highly significant differences (P≤ 0.05) followed by *P.aeruginosa* (0.121 ±0.34, 0.142 ±0.14), *S.aureus* (0.119 ±0.22, 0.143 ±0.05).*S hominis* (0.113±0.02, 0.12 ±0.09), *E coli* (0.124±0.46, 0.127±0.37), *B.Subtilis* (0.114 ±0.24, 0.142±0.22), *P.mirabilis* (0.139±0.00, 0.153±0.17), *E. feaclis* (0.121±0.09, 0.136±0.07) respectively as in Figure (2). Meanwhile MIC of antibiotics and EPSs were showed more effective against the biofilm formation within 24 hr as following *P.aeruginosa* (0.102±0.82, 0.130±0.06), *S.aureus* (0.111±0.00, 0.125±0.29).*S hominis* (0.122±0.52, 0.121±0.62), *E coli* (0.101±0.67, 0.124±0.48), *B.Subtilis* (0.102 ±0.22, 0.140±0.44), *P.mirabilis* (0.131±0.59, 0.140±0.00), *E.feacalis* (0.096±0.72, 0.123±0.80) in addition EPSs was showed no significant differences (P≤ 0.05) with

MIC of Ciprofloxacin toward the biofilm formation of *S. hominis* (0.122±0.22, 0.121 ±0.22) as in Figure (3).

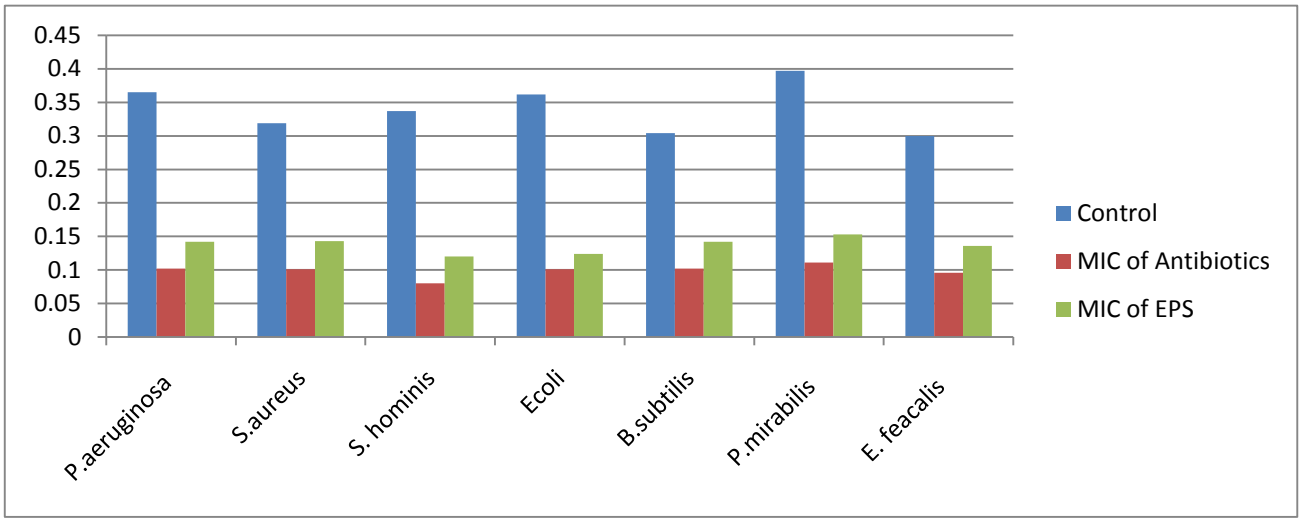

Figure 2: showed the inhibition effect MIC of antibiotic and EPS toward the performed biofilm after 24 hr

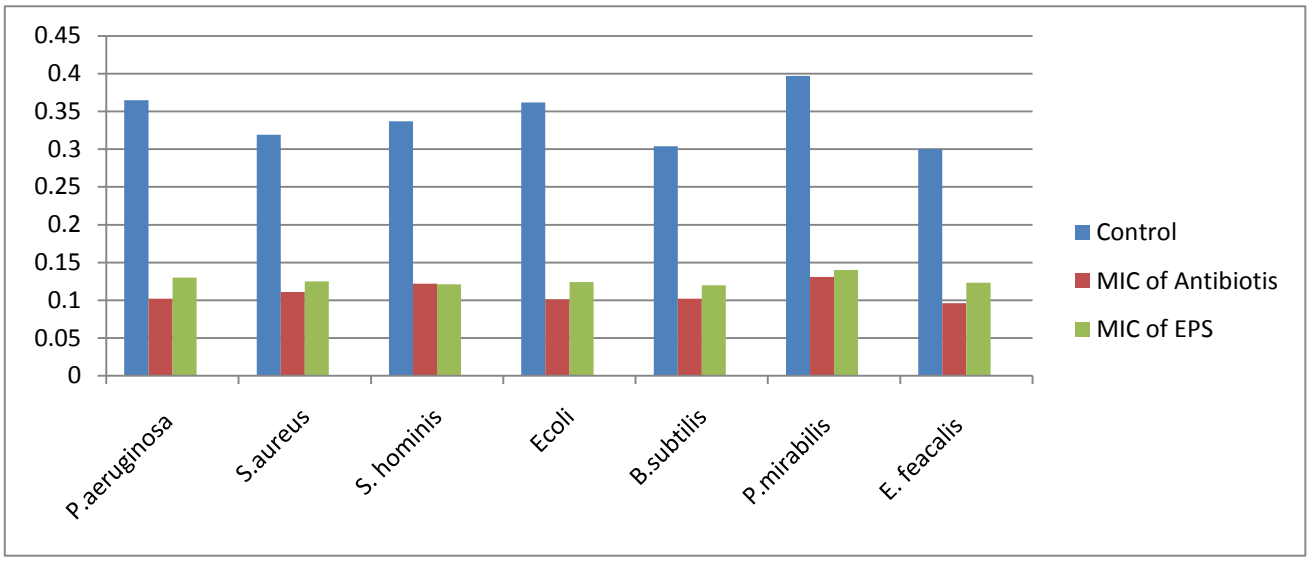

Figure 3: showed the inhibition effect MIC of antibiotic and EPS toward biofilm formation within 24 hr

Discussion

The recent studies [13] used the lactobacillus spp as probitic to treatment burn wounds as previous researches have shown that *L. acidophilus* had antibacterial potentials toward certain pathogenic bacteria, opportunistic that may causes wound infections [14, 15]. The probiotic lactic acid bacteria could interfere with the growth and colonization of pathogens on the wound surface with different mechanisms [16].

Thus, probiotic will be in competitions with pathogenic bacteria that cause infection in wounds also the probiotic bacteria may have some anti-inflammatory and anti-infection effects [13]. Moreover in this current study the researchers were investigating the antibacterial and anti-biofilm activity of purified EPS from *l.acidophilus* against some resistant bacteria isolated from patients with burn wounds, the reason to use the antibiotic resistance bacteria to evaluate capacity of antibacterial, anti-biofilm activity of EPS, considering in many recent studies approved the EPS has an immunmodulatory potential [17] as well as some studies showed EPS has free radical scavenging properties [18].In addition, some studies showed the ability of EPs to act as antioxidant to inhibition of lipid peroxidation by radicals [19].

Moreover EPSs showed ability to inhibition erythrocyte hemolysis by radicals, depended on the mentioned studies, the researchers used EPSs against some resistant isolated bacteria from the burned wound that were showed some to some antibiotic commonly used, attributed to there were benefits in vivo experiments more than side effect of

EPSs even in concentrations higher than antibiotics concentration as well as the biological importance of EPSs as mentioned earlier that leads to prevent the side effects of antibiotic when used constantly against the antibiotic resistance isolates [20], the second reason was to avoid using living probiotics that may lead invasion the wounds consequently cause systemic in patients with immunosuppressive status [21].

Finally to determine what was the main substances produced by probiotics played the main role of benefit in wounds burn, this work was concentrated on exopolysaccharides (EPSs) produced by *L. acidophilus*. Isolation and identification results were showed the highest ratio recorded in *S. aureus* 90% followed *P.aeruginosa* 80%, *B.subtilis* 60%, *S.hominis* 50%, *E coli* 40%, *P.mirabilis* 40%, in *E. feacalis* 40% this agreed with [22, 25] who noticed that *S. aureus* and *P. aeruginosa* were the predominant pathogens and the major agents of nosocomial infections.

However, the reasons beyond distribution of pathogens were attributed to factors involved with nosocomial pathogens in patient with long term hospitalization .Generally [26] mentioned that transmission mechanism of microorganisms to the burn wounds happened by transferring from the hands of health care persons and through hydrotherapy treatments.

Finally the immunosuppressive status of the patient and the immediate lack of antibodies allow the pathogens to multiply because of moisture and nutrients in the physical environment; the temperature, gaseous requirements, for growth. Therefore, a single bacterium cell can increase in numbers within a 24 hr period to over 10 billion cells [27].

Azithromycin and Novobiocin, Ampicillin, Cefotaxime, Erythromycin, Imipenem, Doxycycline, were showed no effect against the isolates, however chloromphenicol, amoxicillin /clavulanic acid, Gentamicin showed intermediate effect also the results pf determination MIC of antibiotics were various, some isolates were sensitive to ciprofloxacin, *P. aeruginosa* 37.5%, *S. hominis* 100%, *B. subtilis* 16.6%, *E. feacalis* 50%, *P. mirabilis* 100%, However the other isolates showed sensitivity to Tetracycline such as *S. aureus* 44.4%, *E.*coli 100%, *B. subtilis* 100%. This attributed to horizontal gene transfer (HGT) can allow antibiotic resistance genes to be transferred between different species of bacteria [28].Resistance can also occur through mutation [29], and overuse, of antibiotics are overprescribed worldwide and.

The results of antibacterial activity of EPSs revealed was an inhibition in growth both gram positive and gram negative bacteria in different concentrations these results agreed with [30], who reported the of EPS from *Pleurotus tuber-regium* showed activity toward E *feacalis*, *E. coli*, *S. aureus* and *Salmonella*, also the results were agreed with [31], who revealed the aqueous EPS extract was active against *S.s epidermis* and *S. typhimurium*. In addition the result agreed with [31], who studied the antibacterial properties of the crude exopolysaccharides of Lentinus subnudus against *E. coli* and *P. aeruginosa* in rats, also the results were revealed the diameter of inhibition zone of EPS in different concentration was from 0-13 mm against the isolates this agreed with [31, 32], who revealed in EPS against four isolates against *E. coli*, *Klebsiella spp*., *S. typhi* and *Staphylococcus* sp, also there were agreements with [33] who reported exopolysaccharides isolated from *B. bifidum* and *L.plantarum* were exhibited antimicrobial activities against *C. sakazakii, E.coli, L.monocytogenes, S. aureus, C. albicans, B. cereus, S. typhimurium, and S. sonnei* at concentration 300 µg/mL. many hypothesis about antibacterial activity of EPS were attributed to such as impairing cell division, disrupting the cell wall and cytoplasmic membrane, and decomposing DNA [34, 35], while the second antibacterial activity mechanism of EPS was attributed to presence of phenolic and carboxylic compounds that work as in antibacterial [36].

However, antibiofilm assay results were revealed the EPSs activity against performed biofilm after 24 hr was less than MIC of antibiotics this attributed to the biofilm already produced by isolates, the function of bacterial biofilms is generally to avoid antibiotics, phagocytosis, and other disinfectant components [37], this led to the activity of antibiotics and EPSs was less, while EPSs showed more effective against biofilm formation during 24 hr this due to

the antibacterial activity against free bacterial cells and still no biofilm produced [32]. However these results were in agreement with [38], who revealed a purified EPS exhibited excellent capacity to inhibit the adhesion of *E.coli* O157:H7 to HT-29 cells and against biofilm formation by several pathogenic as well as there were agreements with [39].

Purified EPS from L. *helveticus* MB2-1 were showed activity against biofilm formation by pathogens, with inhibition rates , *P. aeruginosa* PAO, *E. coli* , *S. aureus* ATCC 6538), in addition the same study showed *EPS of L. plantarum* YW32 showed inhibition activity against *S. aureus* AC1*), (Salmonella Typhimurium S50333),* and *(E. coli O157:H7.)*. The inhibition mechanisms of EPS to the biofilm were suggested in many studies , such as inhibiting the attachment of the bacterial cell to surface [40], influencing the release of substances play a role in biofilm formation such, chemical reactions blocking biofilm matrix synthesis, and or distrusting the bacterial signaling molecules interfering with cell-to-cell communication, required for normal biofilm formation [41], or induces modifications in the bacterial cell surfaces [42], Generally during the biofilm formations the bacteria secreted many active compounds were mediated the interaction (adhesion and deadhesion) between microorganisms and interfaces [43] As well as receptor polysaccharides on bacteria surface was recognized by a complementary protein adhesin were reported to mediate the establishment of productive cell-to-surface and cell-to-cell contacts [44]. The EPS may have the potential to interfere with adherence and biofilm formation [45].

Conclusions

The study was revealed depended on the obtained results purified EPSs from *Lactobacillus acidophilus* had positive antibacterial and antibiofilm activities against some bacteria isolated from burn wounds showed some resistance to antibiotics, also the study noticed the EPSs was more effective against biofilm formation within 24 hr that will help in future to develop drugs derived from EPSs used as creams could be apply over burn wounds surface after exposure to caused agent (burn accidents), which may help to prevent growth of bacteria and biofilm formation within 24 hr without be side effects because the biological importance's of EPSs in vivo studies. The researchers will continue this study through extraction another substances from probiotics bacteria to evaluate its effects against the wounds burn.

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