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RESEARCH ARTICLE

Influence of Surfactants Solutions on Staphylococci Biofilm

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Abstract

Staphylococci known to form biofilm on various clinical and biomaterials, so increase resistance to antimicrobial agents through biofilm formation strategy. The intracellular adhesion had been required for biofilm formation by Staphylococci. In this research, the main goal was detecting the biofilm formation capability regarding *Staphylococcus sp.* isolates on 96-well microtiter plates, also studying the impact of surfactants solutions extracted from *Lactobacillus acidophilus* in biofilm formed through *Staphylococcus sp.* Seventy-four Staphylococci isolates had be utilized in this research, the solid biofilm forming capacity had be confirmed through incubating these isolates in microtiter plates for 48 hours and at a temperature of 37 Celsius. Afterward, during the next 24-48 hours, observations have made over removing the established biofilms of Staphylococci isolates with surfactants solution. The results hinted at the elimination of these biofilms in surfactants concentration-dependent way, with an observation that after 48 hours of incubation, the biofilm biomass reduction had been considerably greater.

Keywords: *Lactobacillus; Surfactants; Biofilm; Adhesion; Staphylococci.*

Introduction

Biofilms can be defined as an assemblage of microorganisms, associated with the pathogenesis of different infections, an important example of bacteria that is considered very effective biofilm maker is staphylococci [1,2].For the purpose of forming well-ordered multicellular assemblages, biofilms are developed in multiple phases, at first, bacteria should stick to a tissue of the host or to a surface (basic attachment stage) prior to proliferating to form multicellular assemblages (accumulation stage), throughout the maturation phase, for allowing the deeper biofilm layers to be penetrated by the nutrients, structures of mushroom shape and channels are formed [3]. Lastly, throughout the dispersal phase, the bacteria separate from the biofilm and spread to new spots [4].

An extracellular matrix is what covers the staphylococcal biofilms; composed of host factors (presumably), extracellular DNA (eDNA), polysaccharides and proteins, preventing forming biofilms and disrupting the established ones could occur when the matrix components are dissolved via some compounds. The matrix offers defense from a different damaging aspects like the exposure to antimicrobials and attack by immune cells.

The exact components of the biofilm matrix differ significantly according to *Staphylococcus* strain, dominant physical conditions, existing nutrients, and its physiological status. Even though a lot of overlapping occurs between the development of biofilm in *S. epidermidis and S. aureus*, it must be noticed that biofilm matrix in the above-mentioned species are not equal and differences have regularly been detected between the strains of the same species [5, 7].

Preventing the formation of biofilms via surface active substances (surfactants) originated from probiotic micro-organisms appears to be a very appealing thought, it is hard to treat them efficiently with traditional antimicrobial agents, due to the high-resistance of biofilms [8,9].

There are various natural roles and chemical structures in the surfactants which are produced via fungi and bacteria. They could be released from cells, attached to an integral cell wall components or to their surface [10, 11]. *Lactobacillus acidophilus* has an important repressive impact on the development of pathogenic *Staphylococcus sp* [12].

Lactobacillus spp. is effective surfactants producing micro-organism mainly located in the microflora of the urogenital tracts in females. Surfactants originated from the lactic acid bacteria (LAB) appeared to have high prevention attributes of bacterial infections and biofilm development [13, 15]. *Lactobacillus acidophilus* is known as effective interfering bacteria through forming different antimicrobial agents, with surface active substances among them, that recorded as a sufficient to completely abolish biofilm formation [14,15]. This research investigated whether surfactants derived from *Lactobacillus acidophilus* can be playing as an active agent against biofilm that is created via isolates of *S. epidermidis* and *S. aureus*.

Bacterial Isolates

In this study, the utilized bacterial isolates included 14 *S. epidermidis* and 76 *S. aureus* isolates taken from patients with UTI at the laboratory of Alramadi Teaching Hospital, all 90 patients had documented pyuria (WBCs > 5/hpf). *Staphylococcus sp*. were characterized by API Staph-Ident system [16], 11 isolates of *Lactobacillus acidophilus* were obtained as previously described via Ali O.A. 2012 [13], *Lactobacillus acidophilus* isolates were obtained from urogenital tracts of healthy women via vaginal swabs. Vaginal swabs were cultured on MacConkys agar, blood agar, chocolate agar, and M.R.S. agar, then the plates incubated at 37 Celsius for 24 - 48 hours under 5 percentages of carbon dioxide conditions.

The lactobacilli isolates were characterized by Grams stain, catalase test, production of acids from raffinose and mannitol, production of ammonia from arginine, culturing the isolates in M.R.S. broth anaerobically, culturing the isolates in nutrient agar aerobically, and growing on 45 Celsius.

Surfactant Production and Isolation

For producing crude surfactant via *Lactobacillus acidophilus* in flasks, six-hundred milliliter of MRS broth had be inoculated with six milliliters of a subculture left for the duration of overnight and undergo of shaking incubation for 72 hours at 37 Celsius with 120 rpm. After 72 h, cells have been harvested through centrifugation (10000×g, 5 minutes, 10 Celsius), and washed two times in distilled water, then resuspended in 100 milliliters of phosphate-buffered saline (PBS: 10mM KH_2PO_4/K_2HPO_4 and 150mM NaCl with pH adjusted to 7.2). Bacteria have been placed at room temperature for two hours with stirring lightly for surfactant release. Then, bacteria have been taken away via centrifugation, residual supernatant liquid went through the filtering process via a 0.22µm pore size filter (Millipore, Bedford, United States).

The supernatant has been dialyzed against distilled water at a temperature of 4 Celsius in Cellu-Sep© membrane (molecular weight cut-off 6000–8000 Da, Membrane Filtration Products, Seguin, United States). As soon as it is dried, the surfactant has been stored at a temperature of -20 Celsius for additional researches [17].

Oil Spreading Test Was carried out in a Petri dish containing 20µL of kerosene with 50 ml of distilled water. 10 µL of 10 percentages surfactants dissolved in PBS were added at the surface of oil and the diameters of clear zones were determinate [17].

Emulsification activity (EA) was evaluated using a mixture of 1mL crude surfactants, 3 mL distilled water and 1 mL sunflower oil or kerosene, vigorously shaken for 2 min, allowed to stand for 10 min then the turbidity at 540 nm was measured. For examination the emulsion stability, the absorbance was measured every 10 min over a period of 60 min [17].

Medium for Biofilm Cultivation

Tryptic soy broth (Sigma-Aldrich) is most regularly utilized medium for growing staphylococcal biofilms that usually consist of 0.25 percent of glucose, also it was supplemented with an extra 1 percent of glucose to increases the ability of staphylococci to form biofilm [18].

Staphylococcal Biofilm Formation

Bacteria have been grown for twenty-four hours at a temperature of 37 Celsius in 5 milliliters of Tryptic Soy Broth supplemented with 1 percent of glucose (TSBGlc). Cultures have been diluted 1: 40 in TSBGlc, then 170µl have been added to the wells of 96 well microtiter plate. The formation of biofilm in presence or absence of 30µl surfactants solutions (the range of concentration was 0.5 to 5 milligram/milliliter in PBS, PH=7.2) has been assessed afterward, respectively,

twenty-four hours and forty-eight hours incubation periods at a temperature of 37 Celsius. The adherent biofilm layer, which is created in each microtiter plate well, stained with 150 µL crystal violet that is utilized for Gram staining (2% Hucker crystal violet) for fifteen minutes at room temperature, after that, through the use of a pipette, the stain must be aspirated and by putting the microtiter-plate under tap water, the excess stain rinsed off, the washing process is continued up until there are no stains in the washings. In order of quantitative assays of biofilm formation, the optical density regarding each well that is stained with crystal violet will be evaluated at 570 nm through using microtiter plate reader [18].

Statistical Analysis

The equation below, describes the calculation process of percentages for biofilm prevention:

$$\% \text{ biofilm prevention} = 1 - (Ac/A_0) \times 100$$

Differences in biofilm formation were tested for significance ($P < 0.001$) via applying t-test of students, and the outcomes are displayed as means of biofilm formation percentages ± standard deviation of values taken from triplicate tests.

Results

Emulsification activity of surfactants was measured on sunflower oil and kerosene. It was determined that sunflower oil were efficiently emulsified by all surfactants (OD540nm= 0.539 to 1.062) and the emulsions formed with kerosene were less stable (OD540nm= 0.194 to 0.457), the surfactants that developed best results for Emulsification activity was used in biofilms inhibition.

Quantitation of Staphylococcal biofilm

The capability to produce biofilm by the 76 *S. aureus* and 14 *S. epidermidis* isolates were evaluated, 68 (89.47%) of *S. aureus* and 6 (42.85%) *S. epidermidis* isolates showed the highest capability of biofilm production, these isolates were used for quantitation of biofilm inhibition by surfactants solutions.

Table 1: Influence of surfactants solutions on biofilm formed by *S. aureus* isolates

Surfactants conc. mg/ml	Incubation period	
	24 h	48 h
0.5	19.78 ± 1.38	29.77 ± 0.93
1.0	22.10 ± 0.68	37.91 ± 1.00
1.5	26.23 ±1.56	44.61 ± 1.14
2.0	30.51 ± 1.24	50.75 ± 0.64
2.5	34.77 ± 0.96	56.09 ± 1.12
3.0	40.87 ± 1.28	61.85 ± 1.08
3.5	43.97 ± 0.71	67.05 ± 0.98
4.0	48.83 ± 0.87	74.72 ± 1.23
4.5	50.26 ± 1.12	79.63 ± 1.17
5.0	*56.34 ± 1.56	*88.49 ±1.26

*Significance differences ($P < 0.001$)

Table 2: Influence of surfactants solutions on biofilm formed by *S epidermidis* isolates

Surfactants conc. mg/ml	Incubation period	
	24 h	48 h
0.5	18.60±1.10	17.30±0.82
1.0	24.81±0.77	26.85±0.56
1.5	29.74±0.84	37.42±1.16
2.0	44.54±1.27	44.79±0.73
2.5	50.10±0.98	57.62±1.03
3.0	57.07±0.80	75.20±1.12
3.5	63.87±0.74	*85.90±0.77
4.0	73.15±0.92	84.36±0.93
4.5	81.22±0.61	85.65±0.69
5.0	*85.47±0.46	85.53±0.67

*Significance differences ($P < 0.001$)

Quantitation of Biofilm Inhibition

In (Table 1), we will see the effect of co-incubation surfactant solutions on the biofilm formation of *S. aureus*, results are expressed as the mean of biofilm prevention percentages ± standard deviation of values taken from triplicate tests. Co-incubation of 5mg/ml of crude surfactant significantly reduced the mean of *S. aureus* biofilm formation by 88.49 ($P < 0.001$) after 48h of incubation, while the same concentration has

reduced the mean of *S. aureus* biofilm formation by 56.34 (P< 0.001) after twenty-four hours of incubation at a temperature of 37 Celsius.

Mean of *S. epidermidis* biofilm formation was significantly reduced by 85.47 (P< 0.001) at 5 mg/ml after 24h of incubation at 37 Celsius, while at 48h incubation period, 3.5 mg/ml. surfactant solution reduced the mean of *S. epidermidis* biofilm formation by 85.90 (P< 0.001), and the increasing of surfactants concentration was not significantly affected on biofilm formation (Table 2).

Discussion

Nosocomial infections are usually related to *S. aureus* and *S. epidermidis* happen at a high rate of recurrence. The capability of forming biofilms and mediating intercellular adhesion in these two species is not likely to have developed lately in conjunction with various infections, instead, it might have a previous impact on the survival and development of these organisms. *S. epidermidis* and *S. aureus* are considered the gram-positive bacteria which are most frequently related to the infections of the urinary tract [19]. *S. aureus* isolates that used in this study were shown high capability for producing biofilms in 96 well microtiter plates (89.47%), while *S. epidermidis* isolates produce biofilm by 42.85%.

Cell-surface interaction and cell-cell adhesion of biofilm formation are both mediated by *S. epidermidis* and *S. aureus* [19,20]. Many previous articles studied the role of dairy lactobacilli on biofilm formation of standard Staphylococci strains [15,21,22], so it seems to be a very good idea to detect the roles of surfactants solution extracted from normal microbiota *Lactobacillus acidophilus* in biofilms formation of clinical pathogenic staphylococci.

The surfactants solution obtained from *Lactobacillus acidophilus* was used in previous studies to the prevention of *Proteus mirabilis* biofilm formation in 96 well microtiter plate and for prevention of uropathogenic Citrobacter adhesion [13,14]. *S. aureus* is considered as an effective source of biofilm production and the process of biofilm inhibition via surfactants solutions, the observations showed a significant reduction in biofilm formation ($p < 0.001$) (table 1).

Treatment through (5 milligrams/milliliter) concentration for 48 hours was very useful against the biofilm formation of *S. aureus*, while after 24 hours incubation period it seemed less effective, the length of exposure period played a significant role in biofilm inhibition. There has been a considerable difference in forming biofilms when using (5mg/ml) surfactants concentration ($p < 0.01$), in both incubation periods, and there were no statistically significant differences between the two experiments.

The biofilms of *S. epidermidis* reduced significantly by 85.90 ($p < 0.01$) when treated only with 3.5 mg/ml for 48 hours incubation periods, the higher a concentration was not significantly effective, and it's reduced by 85.47 ($p < 0.01$) when treated with 5 mg/ml surfactants solution for 24 hours. (table 2) The surfactants solution extracted from different lactic acid bacilli was demonstrated that it had an anti-adhesive and anti-biofilm activity against staphylococci [15,21,22].

The mechanism of surfactants interaction seems to be based on the properties and surfactant type of intended bacteria. The general method for explaining surfactant anti-adhesion and anti-biofilm actions will be their direct anti-microbial activity. Surfactants inhibited pathogen biofilm and adhesion with no impact on cell development, therefore, the way used by surfactants to impact Staphylococci-surface interactions appear to be associated with alterations in bacterial cell-wall charge and surface tension.

Both the cell to surface and cell to cell interactions could be impacted by surfactants [20], in primary attachment stage of forming biofilms, with the existence of surfactants solutions, the surface has become less supportive of the bacterial deposition; bacterial cell-surface exposed hydrophobins reduce, impacting their co-aggregation and/or adhesion capability [11], these factors are highly important in the process of overcoming the initial electrostatic repulsion barrier between the substrate and the microorganism cell. Usually, influencing the supportive surface of biofilm hydrophobicity via surfactants treatment could offer another

way for handling the development of biofilms and impacting the adhesion capability of bacterial pathogens [20].

Conclusion

Clinical pathogenic *S. epidermidis* and *S. aureus* were produced high rates of biofilm and surfactants produced by normal microbiota *Lactobacillus acidophilus* significantly reduced the biofilm production rates. Regarding to the outcomes acquired in current research, surfactants launches innovative future possibilities to be used as anti-colonization agent, making it an appropriate and useful supporter to conventional anti-microbials.

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