Lactobacillus Acidophilus as Antibiofilm Formed by Staphylococcus Aureus Invitro

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Abstract

Background: The lactobacilli are well known to have a positive effect on human health. These bacilli, which are formed the largest part of the microbiology natural, that characterized by its ability to inhibit the growth of bacteria through the production of various antimicrobial materials such as bacteriocins and biosurfactant, thus preventing the formation of biofilm by lactobacilli, and Staphylococcus aureus for examples of bacteria that have the ability to produce a biofilm**.**

Objective: To determine biofilm production ability by *Staphylococcus aureus* isolates and evaluate effectiveness of *Lactobacillus acidophilus* to elimination of planktonic *Staphylococcus aureus* and their biofilm producers, in vitro.

Methods: This study was carried out for the period December 2012 to April 2013 in Ramadi Teaching Hospital in Ramadi City. Fourty isolates of *Staphylococcus aureus* were isolated from blood, urine, surgical wounds, and intravascular catheters. All specimens were identified using biochemical tests, and they were tested for biofilm production by using Microtiter-plate method. Also, used to study the ability of *Lactobacillus acidophilus* supernatant to inhibit biofilm produced by *Staphylococcus aureus*, and used *L. acidophilus* supernatant to inhibit planktonic *S. aureus* (S2,S7,S11,S12,and S19) which are highest biofilm produced , in vitro.

Results: Fourty *Staphylococcus aureus* were biofilm produced and distributed in to 20 (50%), 15 (37.5%), and 5(12.5%), our result showed that inhibitory effect of *Lactobacillus acidophilus* supernatant on planktonic *S. aureus* (S2, S7, S11, S12, and S19). Significant differences ($P<0.01$) were found between pre and post treatment of *Staphylococcus aureus* biofilm with *Lactobacillus acidophilus* supernatant.

Conclusion: *Staphylococcus aureus* has high ability to produce biofilm, and the Lactobacillus supernatant eradicated planktonic *Staphylococcus aureus* and their biofilms remarkably in vitro.

Key words: *Lactobacillus acidophilus*, *Staphylococcus aureus*, biofilm, drug resistance.

Received: 11 May 2014

Accepted: 26 October 2014

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Introduction

Probiotic bacteria, such as *lactobacilli*, are well known to have a positive effect on the maintenance of human health [1, 2]. These bacteria, which constitute an important part of natural microbiota, are recognized as potential interfering bacteria by producing various antimicrobial substances, bacteriocins, and adhesion inhibitors, such as biosurfactants [2]. Thus, the prevention of biofilm formation by such natural lactobacilli-derived agents is one possible approach, which seems to be a very attractive idea of novel therapy currently tested [3]. Biofilm are involved in the pathogenesis of various infections, and staphylococci are an example of bacteria which are very potent biofilm-producers [4,5]

Biofilms, a surface-associated bacterial community, are complex and ordered bacterial societies that are capable of growing in connection with different biological or inert surface [6]. The major clinical consequence of different disease-causing bacteria correlates with the problems of therapeutic killing of attached cells [7]. Biofilms are commonly associated with many health problems, such as endocarditis, otitis media, periodontitis, prostatitis, and urinary tract infections [8, 9]. Several bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Haemophilus influenza*, and *Pseudomonas aeruginosa*, can form biofilms in the body tissues, leading to different infections [9, 10, 11]. It has been estimated that biofilms account for two-thirds of the bacterial infections that physicians encounter, particularly in immunocompromised patients [12, 13].

Drug resistance in microorganisms is a predictable and perhaps inescapable response to the use of antimicrobial agent. It can arise from the selection of resistant strains among naturally susceptible species or from the ingress of new strains of naturally resistant species. The extent of use of particular agents in a given environment dictates the rate at which resistance arises among microbial populations [14]. Some organisms rapidly acquire resistance e.g. coliforms and *Staphylococcus aureus*, while others rarely do so e.g. *Streptococcus pyogenes* [15]. The emergence of drug resistant bacteria is a major problem in antibiotic therapy.

Staphylococcus aureus is amajor human pathogen that can cause a variety of infections, ranging from minor skin abscesses to more serious, potentially life-threatening infections, sepsis, and invasive endocarditis [16]. Unfortunately, treatment of these severe infections has become increasingly difficult because of the emergence of antibiotic-resistant strains of *S.aureus* [17].

Hence the present study aimed to determine biofilm production ability by *Staphylococcus aureus* isolates and evaluate effectiveness of *Lactobacillus acidophilus* to elimination of planktonic *Staphylococcus aureus* and their biofilm producers, in vitro.

Materials and Methods

Specimens collection and bacterial isolates:

This study was carried out for the period December 2012 to April 2013 in Ramadi Teaching Hospital in Ramadi City. A total of Fourty isolates of *Staphylococcus aureus* were collected from blood, urine, surgical wounds, and intravascular catheters. In the laboratory within aseptic conditions, the collected specimens were streaked directly on Mannitol salt agar plates (HiMedia, India,Ph 7.2) and incubating at 37 °C for 24hr.Further identification tests included the morphological characteristics and biochemical tests were carried out depending on Holt *et al* [18].

***Lactobacilli* isolation and identification:**

L. acidophilus was isolated from a vinegar sample, by spreading 1ml of vinegar on to Man-Rogosa-Sharpe (MRS) agar (HiMedia, India,Ph 5.5) plates which were subsequently incubated at 37°C in anaerobic jar for 24-48hr. After incubation period smooth convex whitish to creamy colonies were isolated and sub-cultured on MRS agar medium incubated for 24-48hr. Identification of *L. acidophilus* was performed by phenotypic criteria. It was initially tested for colony morphology, gram reaction, catalase activity, motility test, and gas production from glucose. It was further characterized by its carbohydrate fermentation [19, 20].

Quantitative determination of biofilm production:

Biofilm production was carried out as described previously [21, 22]. Briefly, overnight grown bacteria in Trypticase Soy Broth (TSB) and incubated for 24hrs. Then, diluted and adjusted to 0.5 McFarland turbidity standard to reach 10^5CFU/ml. An aliquot of 200 µL of diluted bacterial suspension with 0.25% glucose (BDH, England), was added to each well of 96-well flat bottomed polystyrene microtiter plates (Div.Becton,Dickinson&Co.Oxnard California,USA) and incubated for 18-24hrs at 37°C . Media with suspended bacteria were then removed; the plates washed carefully 3-4 times with phosphate buffered saline (PFS.Ph,7.2),air dried and stained with 0.1% crystal violet was added to each well shaking the plates to help the colorant to get the bottom of the well . After 15 minutes at room temperature, each well was washed with 200 µl sterile phosphate buffer slain (PBS). This process was repeated three times. The crystal violet bound to the biofilm was extracted later with 200 µl of ethyl alcohol, and then absorbance was determined at 540 nm in an ELISA reader (Stat-Fax 3200, USA). Controls were performed with crystal violate binding to the wells exposed only to the culture medium without bacteria.

All isolates were tested in triplicate.The data obtained were used to classify the strains as high producers (OD_{540} higher than 0.500), good producers (OD_{540} betweeen 0.500 and 0.100) or poor prouducers (OD_{540} lower than 0.100) [23].

Inhibitory activity of *Lactobacillus* supernatant on planktonic *S. aureus:*

Overnight *L. acidophilus* cultures contained 1.5×10^8 colony forming unit/ml at 37°C for 24 hr. These cultures were centrifuged at 6000 rpm/min for 10 min at 4°C. The resulting supernatants were filtered through a 0.2-µm membrane filter to remove the remaining bacteria and debris. All supernatants were cultured on Man-Rogosa-Sharpe (MRS) agar in order to confirm the absence of lactobacilli cells. Aliquots of supernatants were neutralized with NaOH to pH7 were prepared as well [24]. Thereafter, double fold serial dilutions were made from these supernatants and stored at 4°C until usage.Well diffusion method described by Ikeagwu *et al*. [25]. For the inhibition of blanktonic assay, the highest biofilm producing isolates of *S. aureus* (S2, S7, S11,S12 and S19) were selected to be assayed.

Inhibitory activity of *Lactobacillus* supernatant on biofilm *S. aureus:*

Determination of bactericidal activity of *L. acidophilus* cultures against biofilm was performed by using modified microtiter plate method (MMTP) as described previously by [26, 27]. This assy was made by 10 µl (1:;20) of the acid supernatant was added to prewashed biofilm of standardized bacterial suspension adjusted to 10^5 CFU/ml in microtiter plate for each study isolates.Then ,incubated for 18 hours. at 37°C . The contents of each well was aspirated, and wells were washed three times with 250 µl of sterial distilled water. Plate was shken well so that non adherent bacteria were removed. The bacteria attached to the wells were then

fixed and washed. Stained with crystal violet and re- solubilized by the same previous way in quantitative determination of biofilm .The optical density (OD) of each well was measured at the same previous wavelength by ELISA reader.

Statistical Analysis

All data of our designed study were analyzed using the variance (ANOVA) and the T test. Differences were considered significant when P<0.01.

Results

Fourty bacterial isolates of staphylococcus aureus were isolated and identified from infected cases. Out of 40 isolates, 13(32.5%) were isolated from surgical wounds, 11(27.5%) were isolated from intravascular catheters, 9(22.5%) were isolated from urine and 7(17.5%) were isolated from blood. Figure (1).

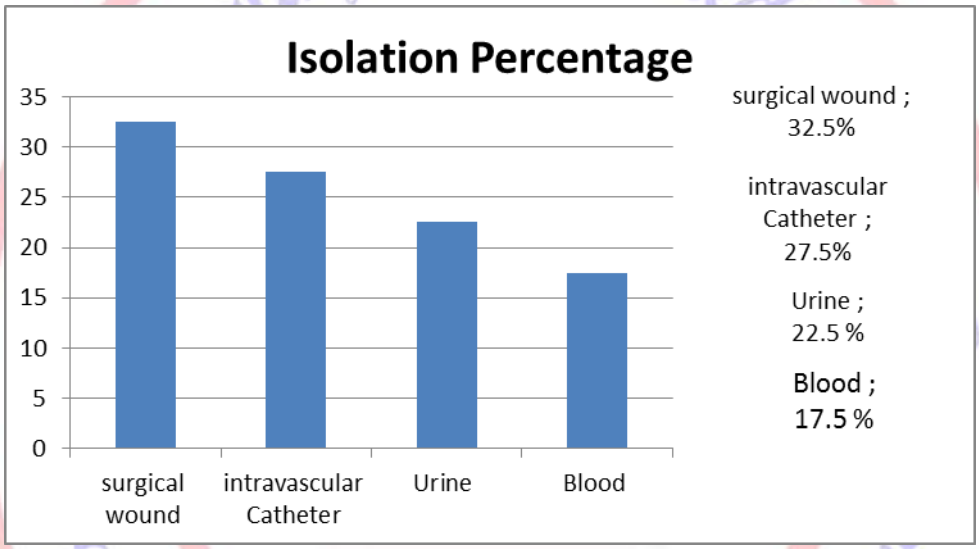

Figure (1): percentages of isolation from different clinical cases.

***Staphylococcus aureus* biofilm formation:**

All of *S. aureus* isolates assayed for the production of biofilm, and the results obtained are presented in table (1), with Mean and Std. Error for optical densities (ODs) at 450nm were: (0.532) and (0.048) with significant difference P-value <0.01; Fourty biofilm producer isolates were distributed in to 20 (50%),15(37.5%), and 5(12.5%) as: High producer (H), Good producer (G), and Poor producer (P) respectively, as shown in figure (2).

Table (1): Optical densitys reading for *Staphylococcus aureus* biofilm at 540 nm with their means and standard deviations for all *S.aureus* isolates.

Isolates code	ODs: 450nm	Isolates code	ODs: 450nm
S1	0.236 G	S21	0.256 G
S2	1.487 H	S22	0.334 G
S3	0.697 H	S23	0.421 G
S4	0.0978 P	S24	0.275 G
S5	0.578 H	S25	0.812 H
S6	0.746 H	S26	0.702 H
S7	0.985 H	S27	0.0675 P
S8	0.635 H	S28	0.346 G
S9	0.597 H	S29	0.522 H
S10	0.0861 P	S30	0.414 G
S11	1.125 H	S31	0.621 H
S12	0.974 H	S32	0.463 G
S13	0.0955 P	S33	0.645 H
S14	0.392 G	S34	0.712 H
S15	0.0792 P	S35	0.345 G
S16	0.388 G	S36	0.574 H
S17	0.472 G	S37	0.519 H
S18	0.476 G	S38	0.681 H
S19	0.983 H	S39	0.811 H
S20	0.395 G	S40	0.298 G
Mean		0.532	
Std.Error		0.048	

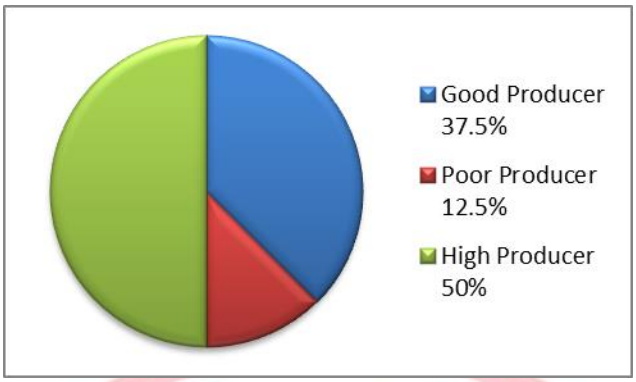

Figure (2): The distribution of S. aureus isolates according to biofilm production.

Inhibitory activity of *Lactobacillus* supernatant on planktonic *S. aureus:*

For the inhibition activity of *Lactobacillus* supernatant on planktonic assay, the highest biofilm producing isolates of *S. aureus* (S2, S7, S11,S12,and S19) were selected to be assayed. Results showed that acid supernatant developed an inhibitory effect observed by formation of inhibition zones around the acidic supernatant-containing wells (figure 3).

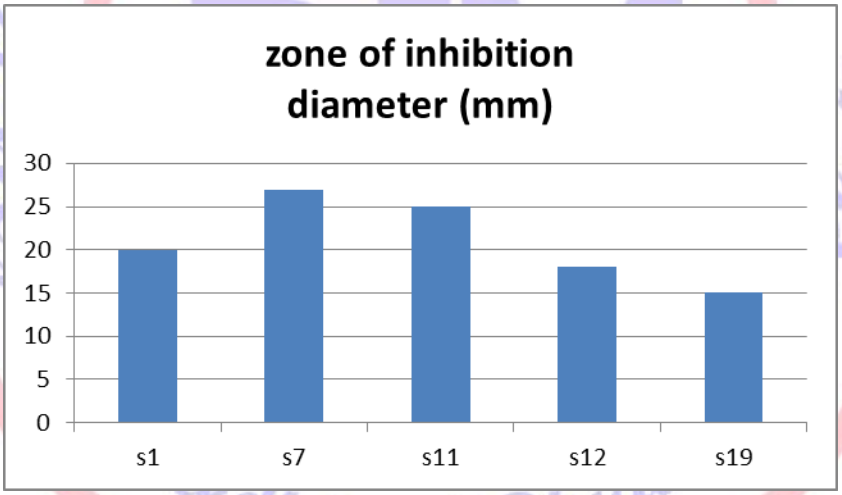

Figure (3): Inhibitory activity of Lactobacillus on plankton S.aureus isolates.

Inhibitory activity of *Lactobacillus* supernatant on biofilm of *S aureus:*

The concerned part of the study included the inhibition of (High and Good)producer biofilm of *S. aureus* with supernatants of *Lactobacillus* .Table (2) demonstrated the readings of optical densities at 450nm with their means(0.598-0.162) and standard Error(0.046-0.028) for study isolates before and after treated with the *Lactobacillus* supernatant. There was high statistically significant difference before and after using *Lactobacillus* supernatant (P-value= 0.0001<0.01).

Table (2): Optical dencity of *Staphylococcus aureus* biofilm after treatment with acidic supernatant of *Lactobacillus acidophilus*.

Isolates code	ODs: 450nm	ODs:450nm After treatmentwith supernatant of *Lactobacillus acidophilus*	Isolates code	ODs: 450nm	ODs:450nm After treatmentwith supernatant of *Lactobacillus acidophilus*
S1	0.236^{G}	o.o35	S23	0.421^{G}	0.068
S2	1.487^{H}	0.532	S24	0.275^{G}	0.024
S3	0.697^{H}	0.121	S25	0.812^{H}	0.420
S5	0.578^{H}	0.095	S26	0.702^{H}	0.395
S6	0.746^{H}	0.134	S28	0.346^{G}	0.026
S7	0.985^{H}	0.437	S29	0.522^{H}	0.084
S8	0.635^{H}	0.101	S30	0.414 ^{G}	0.076
S9	0.597^{H}	0.098	S31	0.621^{H}	0.105
S11	1.125^{H}	0.512	S32	0.463^{G}	0.048
S12	0.974^{H}	0.435	S33	0.645^{H}	0.151
S14	0.392^{G}	0.065	S34	0.712^{H}	0.172
S16	0.388 ^{G}	0.033	S35	0.345^{G}	0.067
S17	0.472^{G}	0.055	S36	0.574 ^{H}	0.072
S18	0.476^{G}	0.057	S37	0.519 ^{H}	0.061
S19	0.983^{H}	0.432	S38	0.681 ^{H}	0.134
S20	0.395^{G}	0.025	S39	0.811 ^{H}	0.489
S21	0.256^{G}	0.030	S40	0.298 ^{G}	0.032
S22	0.334^{G}	0.031			
Mean				0.598	0.162
Std.Error				0.046	0.028

Discussion

In recent years, *Staphylococcus aureus* has been recognized historically as a virulent and important human pathogen. Its capacity to produce human disease has not diminished with the introduction of antibiotics [28] .The failure of antibiotic treatment in the eradication of susceptible organisms has recently induced microbiologists to hypothesize the presence of bacteria ordered in communities , attached to surface, identified as "biofilm" [29].

In this study it has been investigated that S.aureus was able to form biofilm as an alternative method to escape antibiotic treatment and host defenses leading to recurrent infections [30]. So biofilm-associated infections are difficult to eradicate by routine antibiotic doses in compare with planktonic form of bacteria, they need thousands times of doses used for non-biofilm infection [29].

As regard to surgical wound, intravascular catheters, urine and blood biofilm production; 40 (100%) study isolates of *S. aureus*. The differences in biofilm thickness resulted from different reasons such as differences in isolates capacity to form biofilm and greatly influenced by

glucose, other environmental and growth conditions [31, 32]. Furthermore, perhaps the primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (quorum sensing signaling molecules) that produced from each isolate play an essential as well as important role. In other local study done by Makya (2008), biofilm was evaluated by using test tube method, which found that pathogens isolated from urine specimen of catheterized patients, include *Strptococci, Staphylococci,* yeasts, *Proteus* spp., *Klebsiella* spp, *Pseudomonas* spp. and *Candida* spp., formed biofilm but in various thicknesses [33].

Classification of bacteria as high, good and poor biofilm producers regulated by diverse factors, including the good growth, but still poorly understood [34].One possible explanation for the different response of bacteria to environmental conditons could be the results of mutations in genes that control biofilm formation [35].

In the field of studding the Inhibitory activity of *Lactobacillus* supernatant on planktonic *S. aureus*, results showed that acid supernatant developed an inhibitory effect observed by formation of inhibition zones around the acidic supernatant-containing wells.

Kenreigh and Wagner (2006). Pointed out to lactic acid bacteria produces specific natural antibiotics that inhibit and eliminate pathogenic bacterium. For example, *L. acidophilus* produces acidophil in, hydrogen peroxide, bacterial peptides; these are all anti-septic to pathogenic bacterium. Affection mechanism of bacteriocins explained by affection on cellular membranes instability and changing its aspiration by formation of complex or ionic canals by binding itself receiving particles such as lipids or proteins, lead to dispersion and lose ability to formation of protons propelling force [36,37].

Riaz *et al.* (2010) suggested that bacteriocin produced by *L. acidophilus* can be used for the control of infection caused by cephalosporin resistant *E. coli*. Westbroek *et al*. (2010) mentioned that abundant of researches involved the remarkable ability of *Lactobacilli* in inhibiting pathogens growth through its bactericidal activity (such as production of bacteriocins and the hydrogen peroxide and by producing lactic acid as a byproduct of metabolism) and allow the body's immune system to overcome the infection without the use of antimicrobials [38,39].

According to the effect of *Lactobacillus* as anti-biofilm; our results explained that the *Lactobacillus* presented biofilm eradication ability remarkably, in vitro. The main constituents of *L. acidophilus* represented by acids specialy acetic acid ,so *L. acidophilus* used as anti-biofilm in our designed study, due to antibacterial effect of acetic acid that treats infection caused by bacteria or fungus [40,41]. Maldonado *et al*. (2007) studied the inhibitory effect of *Lactobacillus* acid supernatant on both the growth and the formation of biofilm. Because the strain used produces high levels of acetic acid, and hydrogen peroxide, it was able to inhibit bacterial growth. The neutralized supernatant inhibited the biofilm formation in a lower degree than the other fractions evaluated. One of the possible explanations could be the release of different metabolites to the culture media, as for example, biosurfactants, or other substances [42].

More recently, deconvolution microscopy technique was employed to investigate the role of *L. rhamnosus* GR-1 (non H2O2 producer) and *L. reuteri* RC-14 (low H2O2 producer) in inhibiting *Gardnerella vaginalis* biofilm, Saunders *et al.* (2010) showed that pH and hydrogen peroxide alone cannot be deemed responsible for displacement and loss of viability. The authors also stated that it is possible that biosurfactants known to be

produced by *L. reuteri* RC-14 and *L. rhamnosus* GR-1 may have played a role in displacement, while production of antieffective bacteriocins and signaling molecules may have affected viability and pathogen growth [43].

In conclution *Lactobacillus* supernatant eradicated planktonic cell of *Staphylococcus aureus* and their biofilm producer remarkably in vitro, and highly suggested this supernatant as a potent antimicrobial agent against *S. aureus* biofilms. Further studies should be carried out to isolate and purify the active antimicrobial compounds in the *Lactobacillus* acid supernatant responsible for the inhibition of biofilm production in order to decrease the level of the effective dose.

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