

Inhibition of Microbial Biofilm on Different Surfaces Treated with Biosurfactant from *Lactobacillus spp.*

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Abstract

Background: The use and potential commercial application of biosurfactants in the medical field has increased during the past decade. Their antibacterial, antifungal and antiviral activities make them relevant molecules for applications in combating many diseases and as therapeutic agents. In addition, their role as antiadhesive agents against several pathogens indicates their utility as suitable anti-adhesive coating agents for medical insertional materials leading to a reduction in a large number of hospital infections. In the present study the ability of the *Lactobacillus acidophilus* biosurfactant to inhibit the *Enterococcus faecalis* and *Staphylococcus epidermidis* biofilm on Foley catheter and microtiter plate wells were investigated.

Materials and Methods: The surfaces were coated with biosurfactant solution, subsequently the tested bacteria were inoculated to the surfaces. Quantification of biofilm was performed by a spectrophotometric method (measuring the optical density (OD₅₄₀)), which measures the total biofilm biomass, including bacterial cells and extracellular matrix.

Results: The amounts of biofilm were determined after 24 hr of incubation. biosurfactant layers caused a marked inhibition of *Enterococcus faecalis* biofilm formation on microtiter plate well (OD= 0.227) and Foley catheter (OD= 0.112) compare with uncoated surfaces (OD= 0.439, 0.297 respectively), similar antiadhesive activity were obtained on *Staphylococcus epidermidis* biofilm, as the amount of biofilm formation on microtiter plate well (OD=0.118) and Foley catheter (OD=0.099) were reduced markedly when compare with uncoated surface (OD=0.213,0.188 respectively). The biofilm amount formed by *Enterococcus faecalis* and *Staphylococcus epidermidis* were reduced by 48.2% and 44.6 % on microtiter plate wells and 62.2% and 47.3% on Foley catheter respectively, after coating those surfaces by biosurfactants.

Conclusions: Biosurfactants have the potential to be used as a preventive strategy to delay the onset of pathogenic biofilm growth on catheters and other materials, thus may lowering the large number of hospital infections without the use of synthetic drugs and chemicals.

Introduction

The origin of the uropathogens in uncomplicated urinary tract infection and bacterial vaginosis is the fecal flora. For more than 30 years, the key factor in pathogenesis has been regarded as the ability of the pathogens to attach to epithelial cells, thereby allowing them a niche in which to establish, multiply, spread, and avoid host defenses (Reid, 2001).

It has been reported that more than 50 species colonize the healthy vagina (Redondo-Lopez, 1990). Studies have shown that urogenital cells are covered by dense bacterial biofilms whose composition changes constantly but in which lactobacilli predominate, at least until menopause (Reid, 2001). Uropathogenic organisms emerge from the intestine and come into contact with these biofilms on vaginal and urethral cells, yet little is known about what happens thereafter. It is presumed that uropathogens can either bypass the microflora or successfully enter the biofilms, survive, and continue their ascension into the bladder (Wold *et al.*, 1992).

Biofilms have been described in the human host for more than 20 years but their role in health has not been studied as well as their association with disease (Costerton *et al.*, 1981). These biofilms are defined as structured communities of microbial species embedded in a biopolymer matrix on either biotic or abiotic substrata. Both abiotic and biotic surfaces such as mineral, metal, animal or plant surfaces, lung and intestine, and all types of medical implants are subject to bacterial colonization and biofilm formation (Fux *et al.*, 2003). Most importantly, the biofilm is characterized by its resistance to biocides, antibiotic chemotherapy, and clearance by humoral or cellular host defense mechanisms (Kreft, 2005).

Lactobacilli are Gram-positive rods, primarily facultative or strict anaerobes that generally have a fastidious growth requirement. They prefer an acidic environment and help create one by producing lactic and other acids. In general, lactobacilli have not been associated with disease and for more than 100 years have been regarded as nonpathogenic members of the intestinal and urogenital floras (Reid, 2001).

In more recent years, the use of probiotics *per se* and lactobacilli specifically has received greater attention as an alternative, inexpensive, and natural remedy to restore and maintain health (Shalev *et al.*, 1996), namely the ability to adhere to and colonize tissues and the capacity to inhibit the pathogenesis of disease causing organisms (Hamilton-Miller, 1997).

Microbial compounds that exhibit pronounced surface and emulsifying activities are classified as biosurfactants. Biosurfactants comprise a wide range of chemical structures, such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids (Ahimou, *et al.*, 2001). Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases that have different degrees of polarity and hydrogen bonding, such as oil and water or air and water interfaces. Some biosurfactants are suitable alternatives to synthetic

medicines and antimicrobial agents and may be used as safe and effective therapeutic agents (Singh and Cameotra, 2004 and Rodrigues *et al.*, 2006). The aim of this study was to investigate the potential of *Lactobacillus acidophilus* biosurfactant as an antiadhesive coating for different solid surfaces.

Materials and Methods

Bacterial Strains

Two uropathogenic bacteria were used in the study: *Enterococcus faecalis* and *Staphylococcus epidermidis* and these strains were clinical isolates from urinary tract infections and were identified by using conventional methods.

For isolation and identification of *Lactobacillus acidophilus*, vaginal swabs were taken from lateral vaginal wall of healthy women and were inoculated on MRS medium (de Man, Rogosa and Sharpe) and incubated overnight at 37°C in an atmosphere containing 5% CO₂. To identify the strain each colony from MRS agar was subjected to Gram stain test, as well as test for catalase activity, cell shape, motility, growth at various temperatures (15, 45 and 50 °C) and tolerance of different salt levels (MRS broth with 2,4 and 6.5% NaCl). Finally the carbohydrate fermentation characteristics were determined by API 50 CHL system (Biomerieux, France) (Velraeds *et al.*, 1996).

Adhesion assay

1- Microtiter Plate

The quantification assay of biofilm was performed as previously described (Mireles *et al.*, 2001). Typically, 25µl of an overnight culture of tested bacteria (*Staphylococcus epidermidis* and *Enterococcus faecalis*) in Trypticase soy broth (TSB) was used to inoculate at least 20 wells of microtiter plate containing 175 µl of sterile TSB. The covered microtiter dish was sealed with Parafilm during incubation at 35°C. Cultures were removed and the wells were rinsed with distilled water. After drying at room temperature for 15 min, 200 µl of crystal violet (1%) was added to the wells for 20 min. The stained biofilms were rinsed several times with distilled water, allowed to dry at room temperature for 15 min, and extracted with 200 µl of 95% ethanol. The optical densities (OD) were estimated using ELISA reader spectrophotometer (230S Organon Teknika, Austria). At least 10 wells filled with 200 µl of sterile TSB and used as controls.

2- Foley catheters assay

An overnight culture (10µl) of tested bacteria (*Enterococcus faecalis* and *Staphylococcus epidermidis*) in TSB was inoculated into 500 µl of sterile TSB and injected into clear urethral catheters. The catheters were capped at both ends and incubated at 35°C overnight. Cultures were removed and the catheters were rinsed with distilled water. After drying at room temperature for 15 min, 700 µl of crystal violet (1%) was added to the catheters for 20 min. The stained biofilms were rinsed several times with distilled water and allowed to dry at room temperature for 15 min, then 700 µl of 95% ethanol were added for distaining, finally 200 µl of them was transferred to a clean MTP for measuring the optical density (Mireles *et al.*, 2001).

Production of Biosurfactant

For a select number of Lactobacillus strains, 600-ml cultures in MRS broth were grown overnight (for 18 h), then the cells were harvested by centrifugation at 10,000 rpm for 5 min, washed twice in demineralized water, and resuspended in 100 ml of PBS. The lactobacilli were left at room temperature for 2 h with gentle stirring for biosurfactant production. Subsequently, the bacteria were removed by centrifugation, and the remaining supernatant liquid was filtered through a 0.22-µm-pore-size filter (Millipore). About 10-ml of the supernatant was used immediately in the adhesion assay (Velraeds *et al.*, 1996).

Inhibition assay

Both MTP wells and catheter were filled with (200 µl and 500 µl respectively) of freshly produced biosurfactant and left at room temperature for overnight adsorption. Subsequently, the two tested bacteria (*Enterococcus faecalis* and *Staphylococcus epidermidis*) suspension was added. Bacterial inoculums, media and growth conditions and reading the results were as described above for adhesion assay (Velraeds *et al.*, 1996, Mireles *et al.*, 2001).

Measuring the optical density (OD)

The OD of all assays was measured as following:

$$\text{Mean OD (control)} - \text{Mean OD (test)} = \text{OD biofilm}$$

For tested bacteria at least mean of 20 MTP wells were used and for control at least mean of 10 wells were used. Three catheters were used for bacterial biofilm formation and one catheter as control. The optical densities of control wells subtracted from the test wells to estimate only the biofilm binding stain.

Results

Different amount of biofilm (bacterial adhesion) were recorded on different abiotic surfaces by two uropathogenic bacteria. As shown in figure 1, the amount of biofilm formed by *Enterococcus faecalis* on microtiter plate wells as measured by optical density (0.439) were higher than those formed on Foley catheters (0.297), similar results were observed when *Staphylococcus epidermidis* were used as the amount of biofilm formed on microtiter plate wells were higher (0.213) than those formed on Foley catheter (0.188), (Figure 2).

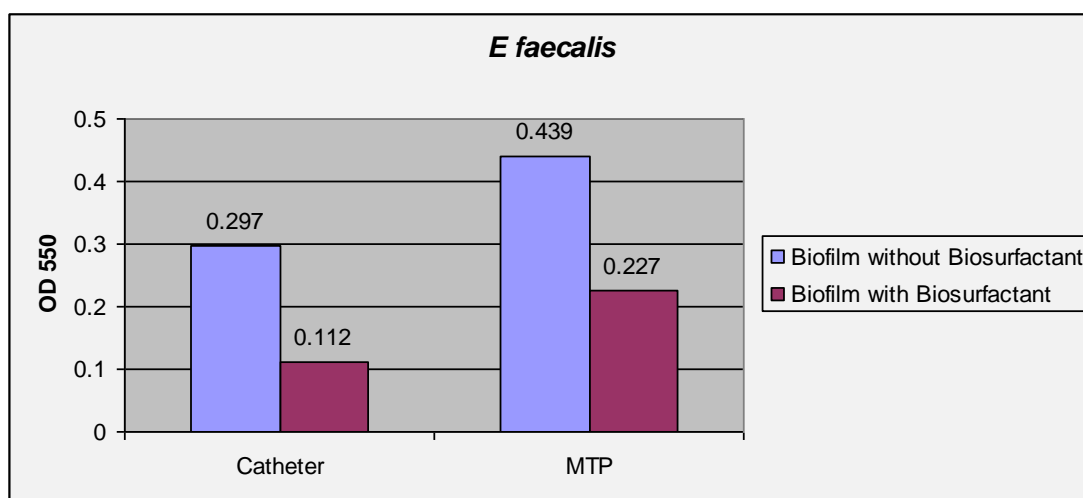


Fig.1: The mean optical densities of stained *Enterococcus faecalis* biofilm on different surfaces with and without biosurfactant

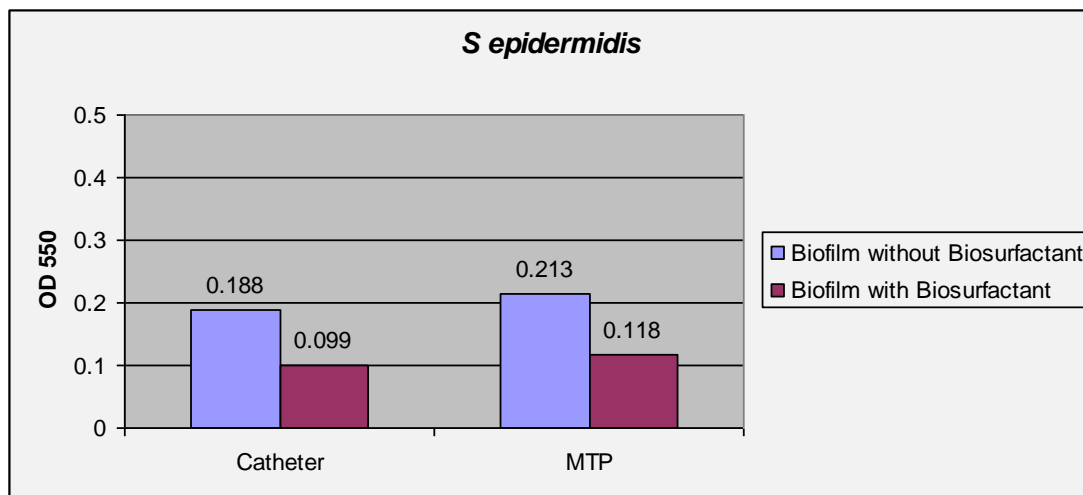


Fig.2: The mean optical densities of stained *Staphylococcus epidermidis* biofilm on different surfaces with and without biosurfactant

The antiadhesive activities of biosurfactant were investigated in the present study, stationary phase *Lactobacillus aciophilus* were used for biosurfactant production.

The amounts of *Enterococcus faecalis* biofilm (adherent bacteria) were markedly reduced when the microtiter plate wells (0.227) and Foley catheter (0.122) pre-coated with biosurfactant compared with the uncoated surfaces (Figure 1, 3 and 4). Similar antiadhesive effect of biosurfactant were recorded on *Staphylococcus epidermidis* adhesions on microtiter plate wells (0.188) and Foley catheters (0.099) compared to uncoated surfaces (figure 2).

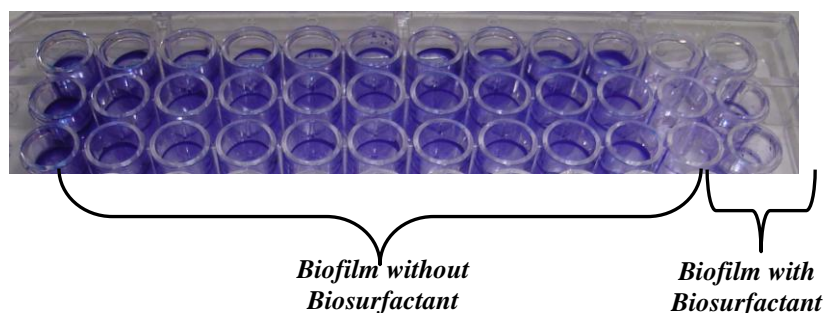


Fig.3: Stained *Enterococcus faecalis* biofilm with and without biosurfactants after 24hr of incubation.



Fig. 4: Stained *Enterococcus faecalis* biofilm on Foley catheter with and without biosurfactants after 24hr of incubation

On the basis of the data, it was concluded that the biosurfactants from *Lactobacillus acidophilus* decreased the amount of biofilm of *Enterococcus faecalis* and *Staphylococcus epidermidis* by 62.2% and 47.3% on catheters and by 48.2% and 44.6% on microtiter plate wells respectively (table1).

Table 1: Reduction rate of biofilm formation after the use of biosurfactant on different surfaces.

Reduction rate	<i>E faecalis</i>*	<i>S epidermidis</i>
Catheter	62.20%	47.30%
MTP	48.20%	44.60%

* ($p < 0.05$; Student's t test).

Discussion

Velraeds and coworkers (1996) reported that fifteen isolates of lactobacillus were found to produce biosurfactant.

Many studies found that the production of biosurfactant per bacterial cell was generally greater and more rapid when the bacteria were harvested in the stationary phase than when they were harvested in the mid-exponential growth phase, the crude substance was analyzed and they were found that crude substance contain proteins and carbohydrates, and the activity was not due to lipoteichoic acid or glycosyldiglycerides or to factors such as acid or bacteriocins, which inhibit bacterial growth (Reid, 2001, Mireles *et al.*, 2001, Velraeds *et al.*, 1997 and Velraeds *et al.*, 1998).

In our study we used a stationary phase *Lactobacillus acidophilus* (18 hr) for biosurfactant production. Pre coating Foley catheters and microtiter plate wells with biosurfactant solution before inoculation with uropathogenic bacteria resulted in a decrease in the amount of biofilm formed by *Enterococcus faecalis* (40.80%, 62.20%) and *Staphylococcus epidermidis* (37.70%, 48.20%), these results have great potential for practical applications.

Velraeds and his group (1998) reported that the adsorbed biosurfactant (surlactin) layers were particularly effective at preventing adhesion to silicone rubber of *Enterococcus faecalis*, *E. coli* and *Staphylococcus epidermidis* strains. (Ried, 2001) also found that the biosurfactant adsorbed to surfaces and inhibited the initial adhesion of *Enterococcus faecalis* by 70%. (Heinemann *et al.* 2000) showed that *Lactobacillus fermentum* RC-14 releases surface-active components that can inhibit adhesion of uropathogenic bacteria, including *Enterococcus faecalis*.

Efforts in the development of strategies to prevent the microbial colonization of silicone rubber voice prostheses have been reported by Rodrigues *et al.* (2004 and 2006) The ability of biosurfactants obtained from the probiotic strains, *Lactococcus lactis* 53 and *Streptococcus thermophilus* A, to inhibit adhesion of four bacterial and two yeast strains isolated from explanted voice prostheses to pre-coated silicone rubber was evaluated. The results obtained by those researchers showed that the biosurfactants were effective in decreasing the initial deposition rates, as well as the number of bacterial cells adhering after 4 h, for all microorganisms tested. Over 90% reductions in the initial deposition rates were achieved for most of the bacterial strains tested.

Mireles and co-workers (2001), reported that Pre-coating vinyl urethral catheters by running the surfactin solution through them before inoculation with media resulted in a decrease in the amount of biofilm

formed by *Salmonella typhimurium*, *Salmonella enterica*, *E. coli* and *Proteus mirabilis*.

Singh *et al* (2004) concluded that biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites; thus, prior adhesion of biosurfactants to solid surfaces might constitute a new and effective means of combating colonization by pathogenic microorganisms.

As a conclusion, biosurfactants produced by *Lactobacillus acidophilus* have the potential to be used as a preventive strategy to delay the onset of pathogenic biofilm growth on catheters and other medical insertional materials, thus lowering the large number of hospital infections without the use of synthetic drugs and chemicals, especially at a time when drug resistance among causal organisms for many life-threatening diseases is on the rise.

Acknowledgment

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تنشيط تكوين الغشاء الحيوي البكتيري على الاسطح المختلفة المعالجة بمادة (biosurfactants) المعزولة من *Lactobacillus* *spp.*

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الخلاصة:

المقدمة: لقد كثرت استخدامات وتطبيقات التجارية لمواد (biosurfactants) في المجال الطبي خلال العقد الماضي. فأنشطتها المضاد للبكتيريا والفطريات و الفيروسات جعلتها من المواد المهمة في مكافحة العديد من الأمراض ، وبالإضافة إلى ذلك، دورها كعامل مانع لالتصاق الجرثومي (antiadhesive) جعلتها من المواد المستعملة لطلاء العديد من المواد الطبية مما يؤدي إلى انخفاض في عدد كبير من الإصابات في المستشفيات. في هذه الدراسة تم التحقق في قدرة biosurfactants المعزولة من *Lactobacillus acidophilus* لمنع تكوين الغشاء الحيوي البكتيري على قسطرة الجهاز البولي (Foley catheter) و الاسطح البلاستيكية (Microtiter plate).

المواد والطرق العمل: تم طلاء السطوح بمحلول ال (biosurfactant) المعزولة من *Lactobacillus acidophilus* و من ثم لقتت بالبكتيريا لتكوين الغشاء الحيوي. تم إجراء القياس الكمي للغشاء الحيوي بواسطة طريقة الطيف الضوئي (قياس كثافة بصرية OD540) والذي يقيس إجمالي الكتلة الحيوية ، بما في ذلك الخلايا البكتيرية و المواد الاصقة خارج الخلية.

النتائج : تم قياس الغشاء الحيوي المتكون بعد مرور 24 ساعة من الحضان ، . تسببت طبقات biosurfactant في إعاقة ملحوظة في تكوين الغشاء الحيوي لبكتيريا *E faecalis* على الاسطح البلاستيكية (OD = 0.227)، وقسطرة الجهاز البولي (OD = 0.112) مقارنة مع الأسطح غير المطلية (OD = 0.439، OD = 0.297 على التوالي)، وقد تم الحصول على نشاط مماثل على الغشاء الحيوي لبكتيريا *S epidermidis* ، حيث ان تشكيل الغشاء الحيوي على الاسطح البلاستيكية (OD = 0.118)، وقسطرة الجهاز البولي (OD = 0.099) وانخفضت بشكل ملحوظ عند مقارنة مع الاسطح غير المطلية (OD = 0.213، OD = 0.188 على التوالي). نسبة اختزال الغشاء الحيوي التي شكلتها *E faecalis* و *S epidermidis* على الاسطح البلاستيكية كانت 48.2% و 44.6% و على قسطرة الجهاز البولي كانت 62.2% و 47.3% على التوالي، بعد طلاء هذه السطوح بمحلول biosurfactants.

الاستنتاجات: Biosurfactants لديها القدرة على أن تستخدم كاستراتيجية وقائية لتأخير ظهور و نمو الغشاء الحيوي المسببة للأمراض في القسطرة وغيرها من المواد، وبالتالي قد يؤدي الى خفض عدد كبير من الإصابات في المستشفى من دون استخدام العقاقير الاصطناعية و المواد الكيميائية.