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RESEARCH ARTICLE

ANTIBACTERIAL AND ANTIADHESIVE PROPERTIES OF A BIOSURFACTANT ISOLATED FROM *LACTOBACILLUS RHAMNOSUS* AGAINST SOME BACTERIA CAUSING UTI IN IRAQI WOMEN

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ABSTRACT

In this study, production of biosurfactant by *Lactobacillus rhamnosus isolated from* vagina of Iraqi healthy women was studied. The optimal condition of biosurfactant production including aeration, growing at different temperature for different incubation periods of times were studied. Anaerobic condition for 24 hours at 30°Cwas fitting to biosurfactant production. Additionally, biosurfactant was extracted by chloroform: methanol (2:1) and partially purified by acid precipitation, surface activity of crude and partial purified biosurfactant were studied, antibacterial, antiadhesion and antibiofilm activities were evaluated against some Urinary tract infectioncausative bacteria, including *Escherichia coli*, *Klebsiella pneumonia*, *Burkholderia cepacia* and *Staphylococcus aureus* isolated from urine samples of Iraqi women suffering from UTI. Crude biosurfactant showed surface activity higher than partial purified biodurfactant.Both crude and partial purified biosurfactant showed inhibitory effect against UTI causative bacteria at concentration (32) mg\ml against *S.aureus*, *K.pneumonia* and *B.cepacia*, and (64) mg\ml against *E.coli*, and showed inhibitory effect on adherence and biofilm formation of these bacteria, *K.pneumonia*was more sensitive to biosurfactant follow by *S.aureus*, while *B.cepacia* was more resist.

INTRODUCTION

Biosurfactants are amphiphilic compounds produced by micro-organisms with pronounced surface and emulsifying activities. These molecules exhibit a distinct tendency to accumulate at the interface between fluid phases that show different degrees of polarity and hydrogen bonding, such as oil and water or air and water, reducing the surface and interfacial tension (Gudina *et al.,* 2010a). Biosurfactants are organic compounds belonging to various classes including glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipopolysaccharides (Thavasi *et al.,* 2007). They have become recently an important product of biotechnology for industerial, pharmaceutical and biomedical application like anti cancer, anti microbial, antiadhesiveness, anti HIV, microbubbles stabilization, sperm immobilizing and immunomodulatory and various therapeutic activities (Kalyani *et al*., 2011). Biosurfactants may interact with the interfaces and affect the adhesion and detachment of bacteria, they have the potential to be used as anti-adhesive biological coatings for medical insertional materials, also they can be used as a preventive strategy to delay the onset of pathogenic biofilm growth on catheters and other medical insertional materials, thus reducing hospital infections and use of synthetic drugs and chemicals (Rodrigues *et al.,* 2006a; Gudina *et al.,* 2010a). They may also be incorporated into probiotic preparations to combat urogenital tract infections and pulmonary immunotherapy (Rodrigues *et al.,* 2006a). Biosurfactants produced by lactobacilli have been shown to reduce adhesion of pathogenic micro-organisms to glass (Velraeds *et al.,* 1996), silicone rubber (Busscher *et al.,* 1997), surgical implants and voice prostheses (Gudina *et al.,* 2010a). Lactobacilli have been implicated for many years as contributing to the prevention of intestinal and urinary tract infections. Several mechanisms have been proposed to be involved including competitive exclusion and displacement of uropathogens, production of hydrogen peroxide, lactic acid and growth inhibitors, and the release of biosurfactants (surface-active components) which can inhibit adhesion of uropathogenic bacteria (Heinemman *et al.,* 2000). The objective of this study was to determine the antibacterial and antiadhesive properties of a biosurfactant produced by *Lactobacillus rhamnosus* against some bacteria causing urinary tract infection in Iraqi women.

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MATERIALS AND METHODS

Microorganisims

Isolate of *Lactobacillus rhamnosus was isolated from* vagina of Iraqi healthy women, then identified through out cultural, microscopical and biochemical test according to (Kandler and Weiss, 1986; Hammes and Vogal,1995; Carr *et al*., 2002). Four isolates were used to test the antibacterial and antiadhesive properties of the biosurfactant produced by *Lactobacillus rhamnosus*, including *Escherichia coli, Klebsiella pneumonia*,

Burkholderia cepacia and *Staphylococcus aureus*. These isolates were isolated from urine samples of Iraqi women suffering from Urinary tract infection, then identified through out cultural, microscopical, biochemical test according to the criteria established by (Forbes *et al.*, 2002) and Vitek 2 system. Morover, these isolates were tested for susceptibility to antibiotics and for ability to biofilm formation.

Detection of biosurfactant production by *L.rhamnosus*

Blood haemolysis test

Briefly, *L. rhamnosus* isolate was streaked onto blood agar plates and incubated for 48 h at 37 ◦C. The plates were visually inspected for zones of clearing around colonies. This clear zone indicates the presence of biosurfactant (Rodrigues *et al.*, 2006b; Anadaraj and Thivakaran, 2010).

Surface activity test

L.rhamnosus isolate was cultivated in flasks containing MRS broth at 25,30,37,40°C in different aeration conditions (aerobic with shaking speed of120 rpm and anaerobic in CO_2incubator) for 24,48,72 hours. At each times and temperatures and aeration condition, the cultures broth were centrifuged at6,000×g for 20 minat 4°C and the supernatant filter sterilized. Surface activity was measured by the oil spreading assay (Fracchia *et al*., 2010) by using 20 µL of Motor Oil previously deposited onto the surface of 20 mL of distilled water in a Petri dish (90 mm in diameter) to form a thin membrane. Twenty microlitres of bacterial supernatant was gently put onto the centre of the oil membrane. Diameters of clearly formed oil displaced circle were measured.

Emulsification activity (E24)

The emulsifying capacity was evaluated by an emulsification index (E24). The E24 of culture sample was determined by adding 2 ml of Motor Oil and 2 ml of the bacterial supernatant obtained after the centrifugation of sample culture were taken in a test tube, vortexed at high speed for 5 min and allowed to stand for 24h, the emulsion activity was investigated after 24 h. The percentage of emulsification index calculated by using the following equation (Das *et al.*, 2009). The results were compared with PBS as negative control.

$$E24 = \frac{Height\ of\ emulsion\ layer(cm)}{Height\ of\ the\ total\ mixture(cm)} \times 100$$

Biosurfactant production and extraction:

For crude biosurfactant production by *L.rhamnosus*, 1200 ml of culture broth were inoculated with 12 ml of an overnight subculture and incubated for 24 h at 30◦C in CO_2 incubator. Briefly, culture broth was centrifuged at 6000 rpm for 20 min at 4◦C and the cell free culture broth was extracted twice with chloroform and methanol (2:1, v/v). Solvents in the extracts were removed by evaporation and the residue was obtained as a result the "Biosurfactant", and the biosurfactant concentration was expressed as g/ L (dry weight) (Thavasi *et al*., 2011).

Biomass estimation

The cell pellet which obtained after centrifugation in biosurfactant extraction step was washed, resuspended in pre-sterilized distilled water and centrifuged again . The cell pellet was then desiccated in an electric oven at 105◦C until a constant weight was achieved (Raza *et al*., 2006).

Partial purification of biosurfactant

The crude biosurfactant extracted from *L.rhamnosus* was subjected to acidic precipitation. Briefly, the biosurfactant was resuspended in PBS (pH 7.0) and subsequently the pH was adjusted to 2.0 by adding 1MHCl. The acidified sample was kept at 4 ◦C for 2 h and the precipitate was collected by centrifugation (10 000×g, 15min, 4 ◦C) and washed twice with acidic water (pH 2.0). Afterwards the precipitate was dissolved in distilled water by adjusting the pH to 7.0 with 1M NaOH, dialyzed against demineralized water at 4 ◦C in a Cellu-Sep©membrane (molecular weight cut-off 6000–8000 Da) (Van Hoogmoed *et al*., 2000; Gudina *et al*., 2010b). Then the partial purified biosurfactant was expressed as g/ L (dry weight).

Surface activityof crude and partial purified biosurfactant

Surface activity of crude and partial purified biosurfactantat a concentration of 40 mg/ml was measured by the oil spreading assay.

Antibacterial activity

Antbacterial activity of crude and partial purified biosurfactant were determined on the basis of minimum inhibitory concentration (MIC) values, defined as the lowest concentration of biosurfactants at which no visible growth could be observed after incubation for the required time. MIC was determined for *E.coli*, *K. pneumonia*, *B.cepacia* and *S.aureus* by Broth dilution method as described by Morello *et al.* (2003). Briefly, a stock solution of crude and partial purified biosurfactant from *L. rhamnosus* in Muller Hinton broth were diluted to concentrations ranging 4 to 64 mg/ml.

Antibiofilm activity

The Inhibitory effect of the crude and partial purified biosurfactant isolated from *L. rhamnosu s*against biofilm formation of *E. coli*, *K. pneumonia*, *B.cepacia* and *S.aureu s*isolates were quantified by co-incubation experiments using tube method described by Christensen *et al*. (1982) and Mathur *et al*. (2006). Each of UTI causative bacterialsuspensions in brain heartinfusion broth with 2% sucrose were incubated together (1:1 v/v) with sub MIC of the crudeand partial purified biosurfactant (separately) .Control tubes contained brain heart infusion broth with 2% sucrose and bacterial suspensions. All tubes were incubated at 37°C for 24h.The tubes were decanted and washed with PBS (pH 7.2) and dried, dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with distilled water. Tubes were than dried in inverted position and observed for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as (-) absent, (+) weak, (++) moderate or (+++) strong. Experiments were performed in triplicate.

Anti-adhesive activity

The antiadhesive activity of the crudeand partial purified biosurfactant isolated from *L.rhamnosus* against *E. coli* ,*K. pneumonia*, *B.cepacia*and *S.aureus*isolates were quantified by co-incubation experiments according to the procedure described by Ali (2012). Each of UTI causative bacterial suspensionsin brain hear tinfusion broth with 2% sucrose (100 µl) were added to 96-well flat-bottomed plastic tissue culture platestogether with (100 µl) sub MIC of the crude and partial purified biosurfactant (separately). Control wells contained 180 µl of brain heart infusion broth with 2% sucrose and 20µl of bacterial suspensions, The covered microtiter plate was sealed with Parafilm during incubation at 37°C for 24h. Unattached bacterial cells were removed by washing the wells three times with PBS (pH 7.2). After drying at room temperature for 15 min, 200 µl of crystal violet (1%) was added to the wells for 20 min. The stained attached bacterial cells were rinsed three times with PBS (pH 7.2), allowed to dry at room temperature for 15 min, and extracted twice with 200 µl of 95% ethanol, and the absorbance of each well was measured at 630nm using ELISA Reader. The inhibition of adhesion percentages of the crude and partial purified biosurfactant for each pathogenic bacteria were calculated as equation described by Gudina *et al.* (2010a).

$$\% \text{ Inhibition of adhesion} = [1- (\frac{A}{Ao})] \times 100$$

A represents the absorbance of the well with a biosurfactant and A0 the absorbance of the control well. The microtitre-plate antiadhesion assay estimates the percentage of bacterial adhesion reduction in relation to the control wells, which were set at 0% to indicate the absence of biosurfactant and therefore of its anti-adhesion properties. In contrast, negative percentage results indicate the percentage increase in microbial adhesion at a given biosurfactant in relation to the control. The microtitre-plate anti-adhesion assay allows the estimation of the crude and partial purified biosurfactant that are effective in decreasing adhesion of the bacterial isolates studied.

RESULTS AND DISCUSSION

The tested *L.rhamnosus* showed zones of clearing in the blood agar with a diameter 25 mm, this result indicated the *L.rhamnosus* was able to produce biosurfactants and the isolate exhibiting the higher production. The blood agar method was included in this study since it is widely used to screen for biosurfactant production, and in some cases, it is the sole method used. However, despite *Lactobacillus* species are not known to produce hemolysin, demonstrated that some species agglutinate blood cells (Rodrigues *et al.*, 2006b). Anadaraj and Thivakaran, (2010) showed that the culture producing beta haemolysis was able to produce biosurfactants. Ghribi *et al*. (2012) showed that the size of the clear zone developed is in proportion to the amount of the produced biosurfactant. The isolate *L.rhamnosus* showed the highest surface activity with oil displacement diameter (10 mm) at24h of growth under anaerobic condition, (8 mm) both at 48 h of growth under aerobic and anaerobic condition, with the lower values at 72 h (Figure 1). The highest surface activity was observed at (25,30)°C with oil displacement diameter (11mm), and the surface activity at (37,40)°C with oil displacement diameter (10mm) (Figure 2). Rodrigues *et al*. (2006b) observed that biosurfactant production by lactobacilli occurs mainly in the first 4 hours of culture. However, biosurfactant production continues during all 72 h of fermentation but at a very slow production. This slow production rate can be a consequence of product inhibition and pH reduction. The pH reduction results of simultaneous production of lactic acid that changes drastically the media conditions and can be responsible for the inhibition of biosurfactant production. Thavasi *et al*. (2011) that higher biosurfactant concentration after the offset of growth may be because of the release of cell-bound biosurfactant at the early stationary phase, which leads to an increase in extracellular biosurfactant concentration in the culture medium. The highest biosurfactant production by *B. subtilis* was evaluated after 24 h (Ghribi *et al*., 2012). *Lactobacillus* are produced biosurfactants at different temperatures, *Lactobacillus spp.* at (28◦C) (Fracchia *et al*., 2010), *L.pentosus* at (31◦C), *L.paracasei* at (37◦C), (Gudina *et al*., 2010b) and (34◦C) for *L.delbrueckii* (Thavasi *et al*., 2011).

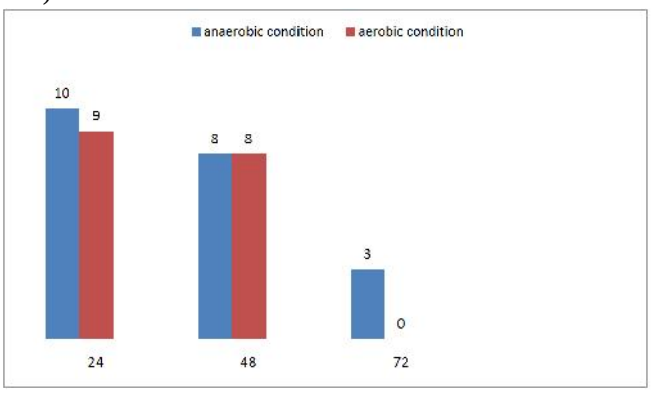

Figure 1. Oil displacement diameter of *L.rhamnosus* at different incubation time and aeration conditions

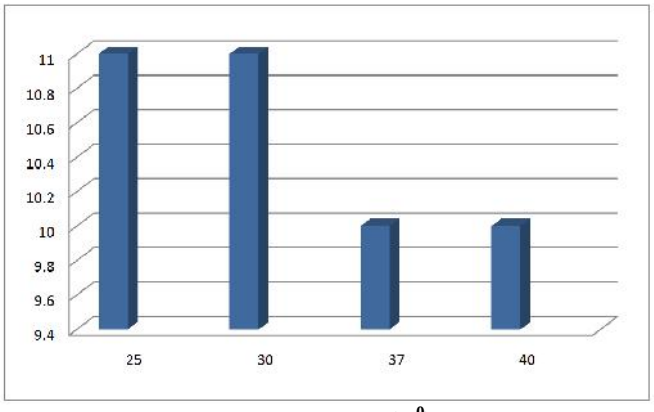

Figure 2. Oil displacement diameter of *L.rhamnosus* at different temperatures

Emulsification activity (E24) of *L.rhamnosus* was measured with motor oil and bacterial supernatant. *L. rhamnosus* showed good emulsifying ability, emulsion formed with E24 50EA%, result is presented in the Figure 3.Emulsification index has been reported to be proportional to the surfactant concentration (Das *et al*., 2009). For another studies *L.rhamnosus* and *L. fermentum* formed good emulsifying ability (Brozozowski *et al*., 2011), while Techaoei *et al*. (2007) showed that the E24 emulsification index of bacteria isolated from soil range from 7.8-63.3%. and Jaysree *et al*. (2011) observed that the biosurfactants produced by *B. subtilis* had an emulsification capacity (E24) of 20% and 15%, and that by *B. cereus* was 30% and 20% for diesel and engine oil respectively.

A **B**

Figure 4: The emulsion form of *L.rhamnosus* (A)Phosphate buffer , (B) *L. rhamnosus* supernatant

Biosurfactant production yield from L.rhamnosus was achieved 48.75g/L of medium (crude biosurfactant), 0.2 g/L (partial purified biosurfactant) and biomass concentration of 1.5 g/L of medium. Lactobacilli produce lower amounts of biosurfactants when compared with other microorganisms, such as Bacillus subtilis or Pseudomonas aeruginosa. The yields of both of these microorganisms are relatively high (approximately 2.5 g/ L of medium), but the amounts released per liter of culture medium by Lactobacillus species were smaller than the amount of these microorganisms (approximately 100 mg/L) (Van Hoogmoed *et al.*, 2000). Gudina *et al.* (2010b) showed that lactobacilli are produced biosurfactant in lower amounts (20–100 mg/IL).

The acid-precipitated fraction obtained from the crude biosurfactant, as well as the crude biosurfactant, were assayed for surface activity at 40 mg/ml concentration. The crude biosurfactant was found to be more surface active than partial purified biosurfactant (the acid precipitated fraction). The crude biosurfactant contains the greater part of the surface active compounds leads to the large diameter (30 mm) of lightened zone formed as a result of contact of oil-water interface surfaces (Figure 5) compared with (7 mm) of partial purified biosurfactant. In that sense, the large diameter of oil displacement by crude biosurfactant was similar to that obtained with the crude biosurfactants isolated from *Lactobacillus rhamnosus* in the study of Brozozowski *et al*. (2011). One explanation of the hight surface activity of crude biosurfactant in present study is the *L.rhamnosus* synthesize biosurfactants which are mixtures of several compounds (Brozozowski *et al*., 2011), and in this study one fraction only purified (acid-precipitated fraction). Youssef *et al*. (2005); Bozozowski *et al*. (2011) showed that the crude biosurfactants contain a protein and amino acids which increased the surface activity. The crude biosurfactant synthesized by *L.rhamnosus* contain a protein and free amino acids precisely (Howard *et al*., 2000). The low surface activity of the acid-precipitated fraction in this study is similar to the result observed by Youssef *et al*. (2005), they showed low surface activity measured by oil displacement method of acid-precipitated biosurfactant purified from *Bacillus mojavens* and explain this result to the fatty acids which causing the decrease of surface activity.

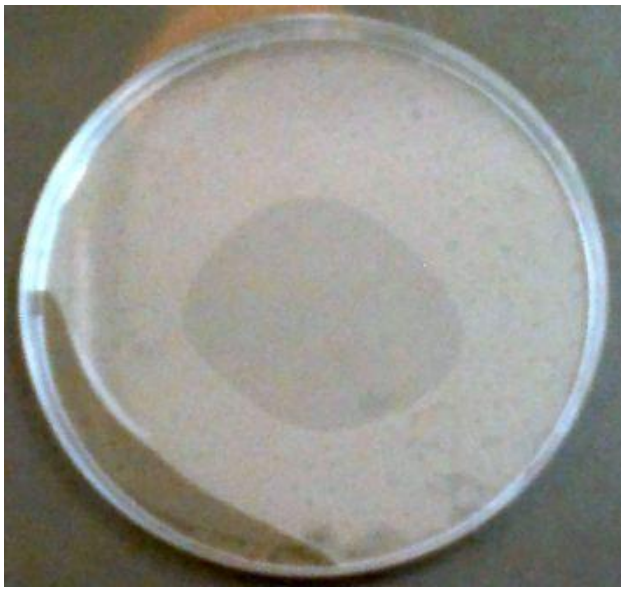

Figure 5. The spreading of crude biosurfactant isolated from *L.rhamnosus* on oil surface layer

The antibacterial activity of the crude and partial purified biosurfactant (acid-precipitated fraction) isolated from *L.rhamnosus* was determined by measuring the growth obtained for some bacteria causing UTI. From those results, the MIC for each bacteria was determined, the minimum concentration (MIC) of the crude and partial purified biosurfactant was found to be 32mg/ml against *K. pneumonia*, *B.cepacia* and *S.aureus* isolates ,and 64 mg/ml against *E. coli.* It is worthy to note that the crude and partial purified biosurfactant produced by *L.rhamnosus* had a good activity against bacteria causing UTI. Several biosurfactants that exhibit antimicrobial activity have been previously described. However, there are few reports about the antimicrobial activity of biosurfactants isolated from lactobacilli; only biosurfactants obtained from *L.paracasei* showed antimicrobial activity against Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis and Streptococcus agalactiae, the minimum inhibitory concentration (MIC) were achieved for

biosurfactant concentrations between 25 and 50 mg/ml (Gudina *et al.*, 2010a). The biosurfactants produced by Streptococcus thermophilus and L. lactis showed significant antimicrobial activity against several bacterial and yeast strains isolated from explanted voice prostheses (Rodrigues *et al.*, 2004, Rodrigues *et al*., 2006c), and the antimicrobial activity of the crude biosurfactant isolated from S. thermophilus and L.lactis observed against S. aureus and S. epidermidis was which completely inhibited the growth of those bacteria with concentrations 100 mg/ ml (Rodrigues *et al.*, 2004). Salman *et al.* (2013) observed that the crude biosurfactant isolated from S. thermophilus showed inhibitory effect against *Klebsiella* spp. and *Pseudomonas aeruginosa*. Another studies, biosurfactant isolated from *B.subtilis, B.licheniformis and Pseudomonas aeruginosa* showed inhibition activity against gram positive and gram negative bacteria (Gomaa *et al*., 2012, Ghribi *et al*., 2012, Lotfabad *et al*., 2013). The mechanism of antimicrobial action of biosurfactant regards to the fact that biosurfactants may disturb membrane structure through interaction with phospholipids as well as membrane proteins (Lotfabad *et al.*, 2013). Another explanation of the antimicrobial effect of biosurfactants is the adhering property of biosurfactants to cell surfaces caused deterioration in the integrity of cell membrane and also breakdown in the nutrition cyle. Also the biosurfactant prevent the protein synthesis by inhibition of the peptidyltransferase in binding mainly the 23S rRNA in the 50S subunit of the bacterial ribosome (Gomaa *et al*., 2012).

The inhibition activity of crude and partial purified biosurfactant was evaluated on biofilm formation of UTI causative bacteria using tube method. Both crude and partial purified biosurfactant showed inhibitory effect on biofilm formation of all bacteria tested (Table 1).

Table 1. Inhibitory effect of crude and partial purified biosurfactant on biofilm formation

Pathogenic bacteria	Biofilm formation		
	Control	Crude biosurfactant	Partial purified biosurfactant
S.aureus	+++	+	+
E.coli	+++	+	++
K.pneumoniae	+++	+	+
B.cepacia	+++	+	++

(+) weak,(++) moderate , (+++) strong

On the other hand, the antiadhesive activity of crude and partial purified biosurfactant was evaluated against UTI causative bacteria. The crude biosurfactant showed antiadhesive activity against all bacteria except *B. cepacia*, the highest antiadhesive percentage was observed for *K. pneumonia* (34%). On the contrary, low activity was obtained for E. coli (6%) (Table2), while partial purified biosurfactant showed antiadhesive activity against *K. pneumonia* and *S. aureus* with antiadhesive percentage (24, 11)% respectively (Table 3). In that sense, the antiadhesive activity of the crude biosurfactant isolated from *L. rhamnosus* against *K. pneumonia* and E. coli was similar to that obtained with the crude biosurfactants isolated from *Lactobacillus fermentum* and *Lactobacillus rhamnosus* which inhibited the adhesion of *Escherichia coli, Klebsiella pneumoniae* (Brozozowski *et al*., 2011). Gudina *et al.* (2010b) showed that the highest anti-adhesive percentages were obtained for *S. aureus, S. epidermidis*and *S. agalactiae* for a biosurfactant isolated from *L.paracasei*, and a low activity was observed for P. aeruginosa and E. coli. Biosurfactants synthesized by *Lactobacillus* had inhibition activity on biofilm formation for *Ecoli, S.*aureus, *Salmonella arizonae* and *Listeria monocytogenus* (Fracchia *et al*., 2010). Ali, (2012) was demonstrated that the biosurfactant isolated from *L.acidophilus* inhibit biofilm formation of *Proteus mirabilis.*

Table 2. Antiadhesive activity of crude biosurfactant isolated from *Lactobacillus rhamnosus*

Pathogenic bacteria	(O.D)		antiadhesive percentages (%)
	Crude biosurfactant	Control	
S.aureus	0.042	0.046	9
E.coli	0.037	0.039	6
K.pneumoniae	0.037	0.056	34
B.cepacia	0.040	0.037	-8

Negative controls were set at 0% to indicate the absence of biosurfactant. Positive percentages indicate the reductions in bacterial adhesion when compared to the control, and negative percentages indicate increased bacterial adhesion. One mechanism that could explain this global inhibition of pathogenic adherence by biosurfactants, the Biosurfactants are amphipathic molecules that have a variety of purposes, including adsorption to surfaces (Spurbeck and Arvidson, 2010). Rodrigues *et al*. (2006c) showed that the main goal of biosurfactant is to modify the physicochemical properties of the surface in order to reduce the force of attraction between microorganisms and the surface of the biomaterial.

Table 3. Antiadhesive activity of Partial Purified isolated from *Lactobacillus rhamnosus*

Pathogenic bacteria	(O.D)		antiadhesive percentages (%)
	Partial purified biosurfactant	Control	
S.aureus	0.041	0.046	11
E.coli	0.043	0.039	-10
K.pneumoniae	0.043	0.056	24
B.cepacia	0.048	0.037	-29

Negative controls were set at 0% to indicate the absence of biosurfactant. Positive percentages indicate the reductions in bacterial adhesion when compared to the control, and negative percentages indicate increased bacterial adhesion

Conclusion

In conclusion, we showed that the crude and partial purified biosurfactant isolated from *Lactobacillus rhamnosus* had antibacterial, antibiofilm and antiadhesive properties against some bacteria causing UTI including *K. pneumonia*, *B.cepacia, E. coli* and *S.aureus*

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