Plant Archives Vol. 19, Supplement 2, 2019 pp. 2025-2029 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EVALUATION OF ANTIBIOFILMS FORMATION OF *E. COLI* ISOLATED FROM RECURRENT URINARY TRACT INFECTIONS IN WOMENBY THERAPEUTIC BACTERIA

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Abstract

Recurrent urinary tract infections (rUTIs) is a difficult situation among women of all ages. The repeated and indiscriminate use of antibiotics has led to the development of increased resistance to antibiotics, ensuring the need to explore new therapeutic agents for prevention and reduction of rUTI. reviews suggested that probiotics are safe and effective in preventing rUTIs, so this study was aimed to detect sensitivity of *Escherichia coli* which isolated from women whose suffered from rUTIs and evaluated the Antibiofilms activity of live cell-free culture from probiotic isolate of *Lb . plantarum* and *Lb. rhamnosus GG*. Sixty seven urine samples were collected from women with rUTI in Female and Children Educational Hospital in Samawa city- Iraq under described and supervision of a specialist doctor. Samples were surveyed and examined for the presence of the *Escherichia coli*, biochemical tests were then carried out, including sugar fermentation, indole test, citrate, catalase, methyl red, Voges Proskauer, urease production. 25 isolates belonging to *E. coli,* all isolates appeared multi resistance to antibiotics, 22 isolates were formed biofilms, live cells- free culture of *Lb. plantarum* and *Lb. rhamnosus GG* shown Antibiofilms activity. live cell-free culture of *Lb. plantarum* was the best in inhibiting the formation of biofilm for *E. coli* isolates.

Keywords**:** *Probiotics, Lb. plantarum, rUTI, cells-free culture, biofilms*

Introduction

Biofilm is a community of microorganisms attached to substrate surface and submerged into extracellular slimy matrix (Donlan, 2002). Genetic diversity of organisms that form the biofilm and variety of environmental conditions where it emerges proves that biofilm is an ancient ubiquitous life form of microorganisms (Trachoo, 2003). The formation of biofilms contributes to the high resistance of bacteria to antibiotics making the treatment of biofilm infections more difficult (Overman, 2000). In addition, bacteria in biofilm were demonstrated to show elevated resistance to the host immune system clearance (Costerton *et al*., 2003). Factors which explain the high resistance of biofilms include decreased diffusion of antibiotics through the biofilm matrix, and biofilms can act as physical diffusion barriers to prevent antibiotics from reaching their targets (Davey and Tool, 2000). Biofilm maturation occur to the growth of the biofilm that occurs after initial attachment to the surface. Mostly due to the interactions of bacteria - bacteria, this process creates a heterogeneous physiochemical environment in which bacteria show distinct physiological traits distinct from their plankton counterparts (Beloin, *et al.,* 2008). Urinary tract infection refers to the presence of microorganisms in urinary tracts, although it may be difficult to distinguish between pollution and colonization or infection (Verrier, 2000). Urinary tract infection is a common infection in society and affects all age groups as well as both male and female infections (Orrett, 2001). And are more likely to occur in females than in males of all ages, except in early childhood (less than three months), where they are more likely to occur in males (usually 5-3% in females and 1% In the male) (England, 2002) Most urinary tract infections are caused by Gram-negative bacteria such as *E. coli, P. vulgaris, P .mirablis,* and *Klebsiella spp*, also *Pseudomonas aeruginosa* and *Acintobacter*. Urinary tract infections are also caused by the Gram positive bacteria, such as *enterococcus,* and *Streptococcus agalacticae,*(Tangho and Mcaninch, 2004). The acquisition of microbes has many mechanisms, including non-permeability of the outer membrane, as well as the production of enzymes and the occurrence of resistance mutations and thus the spread of resistance. Therefore, the need for safe antimicrobial agents for use from natural sources is an urgent need, therefore, it was necessary to find safe antimicrobial agents from natural sources to achieve targets that were unable to achieve antibiotics. Moreover, the development of antibiotic resistance in bacteria is responsible for many of the clinical problems in the treatment of bacterial infections today (Tricia *et al*., 2006) Probiotics have the potential for an alternative strategy for prevention and treatment of urinary tract infection (Borchert *et al.,* 2008). This study was aimed to evaluate the effect of local isolate of *Lactobacillus plantarum* and cell-free culture of *Lactobacillus rhamnosus* GG bacteria in inhibiting the ability of *E. coli* bacteria isolated from women recurrent urinary tract infections to form the biofilms.

Material and Methods

Samples collection

The samples were collected according to the method reported by Rajeshwari *et al* (2010) of various age groups from women with recurrent Urinary Tract Infection. They reviewed Female and Children Educational Hospital in Samawa city-Iraq. They were previously diagnosed by specialists and recorded patient information in a special form. For this purpose, sterile bottles of 50 mL were used. The mid-stream urine sample is taken in the middle of the

urination process and not at the beginning without cutting off the urination using the 50 ml sterile glass bottles. The method of sampling for all patients was clarified with the emphasis on the need not to contact any part of the body to prevent contamination with natural flora in the external area, samples were mixed using the Vortex for 1 minute and were directly streaking on the blood agar (Oxoid) and Differential medium MacConkey (Oxoid) and incubated at 37 °C for 24 hr. The isolates that produce a fish-like odor are selected on the blood agar, whereas on the MacConkey, the pale color of the colonies is adopted as non-lactose fermented, the bright pink selected isolates were transported to a new MacConkey . then lactose fermented isolates were selected and spreading on the Eosin methylene blue (Oxoid) and incubated at 37 °C for 48 hr, The colonies with green metallic luster were selected, phenotypic microscopic characteristics and Traditional biochemical tests were then carried out, including sugar fermentation, indole test, citrate, , catalase, methyl red, Voges Proskauer, urease production (Harold, 2002).

Antibiotic Susceptibility

The sensitivity of *E. coli* isolates to antibiotics was conducted according to the method of (Bauer *et al.,* 1996) with some modified by using antibiotics tablets which included (ampicillin 10µg, cephalexin 30µg, tetracycline 30µg, chloramphenicol 30µg, erythromycin 15µg, gentamicin 10µg, cefotaxime 15µg and amoxicillin 25µg) (Oxoid, England), *E. coli* isolates were spreaded on Mueller- Hinton agar (Oxoid, England) by streaking, antibiotic discs were placed on the agar surface and incubated for 16 -20 hrs. at 37ºC.

Therapeutic Bacteria

local isolation of *Lactobacillus plantarum* (Agriculture college - University of Baghdad) and *Lactobacillus rhamnosus GG* (the North Hollywood American Biological Production Company) in the form of freeze-drying capsule were used.

Preparation of Culture Filtrate cells

Lb. rhamnosus GG and *Lb . plantarum* growing on skim milk (reconstituted 12%, sterilized at 121 °C for 5 min), incubated at 37 °C until skim milk coagulation (repeated three times for activation), then live cells culture was harvested using centrifuge (Huttich, Germany) at 10000 rpm for 20 minutes. The upper part was then carefully removed and filtered using a 0.45 micron filter (Millipore corp, Spain) (Sisto *et al.*, 2002).

Biofilm formation:

Biofilm formation was performed on microtiter plate according to Salo *et al.* (2009) A suspension of *E. coli* isolates is equivalent to the McFarland No.0.5 turbidity standard, 200µL of cell suspension in blood-heart-infusion (BHI) broth was diluted to 1:100 were added to individual wells of sterile polystyrene, 96-well, flat-bottomed, with Negative control wells contained sterile blood-heart-infusion, (to test the efficacy of therapeutic bacteria inhibition to form biofilms, 10% (v/v) of live cells culture of each therapeutic bacteria was added) The plates were incubated at 37°C for 24 hours. after incubation, microtiter plate wells were washed twice with 200 µL of 0.85 % NaCl to remove all non-adherent bacteria. by drying and incubating for 1hr.at 60°C, the attached isolates were fixed and stained with 200 µL of 0.1 % (v/v) crystal violet solution at room temperature for 15min. The amount of crystal violet was extracted by the ethanol 95% (200µl to each well for 10 min.) in each well was directly quantified spectrophotometrically by measuring the OD630 using amicroplate reader.

The results were calculated according to following equation:

Capacity of biofilm formation = Absorption of the sample test - absorption for control.

Results

The results shown that from the total samples 78 samples, 29 isolated Non-sorbitol fermenting were small, circular, colorless or pale compared to the sorbitol fermented colonies which were pink, Twenty-five isolates emerged, fermenting lactose sugar as they culturing in MacConkey agar. Their colonies had a metallic green luster on the EMB medium. A number of conventional microscopic and biochemical tests were used to confirm the diagnosis of *E. coli*. The biochemical tests showed that the isolates were not fermented Cellobiose , not grow with the presence of Potassium cyanide , positive for the tests of the indole, methyl red and catalase, and negative for the tests of Voges Proskauer, Urease and Oxidase (Ejrnaes, 2011).

Antibiotic susceptibility of *E. coli* isolates:

Results in Table (1) showed that *E. coli* isolates have multi resistant to the antibiotics which used, 80% of the isolates showed resistance to Chloramphenicol, 72% to Cephalexin and Cefotaxime, 68% to Erythromycin, 64% to Tetracycline and Cefotaxime, and 60% to Amoxicillin.

Table 1 : Antibiotic susceptibility of *E. coli* isolates from rUTI:

		Resistant	Sensitive	
Antibiotics	**Number of Isolates**	**Percentage of Isolates**	**Number of Isolates**	**Percentage of Isolates**
Ampicillin	15	60%	10	40%
Cephalexin	18	72%	7	28%
Tetracycline	16	64%	9	36%
Chloramphenicol	20	80%	5	20%
Erythromycin	17	68%	8	32%
Gentamicin	16	64%	9	36%
Cefotaxime	18	72%	7	28%
Amoxicillin	15	60%	10	40%

Detection of biofilm formation:

The ability of *E. coli* isolates to biofilm formation tested by Micro-titer plate (MTP) method. This method is a quantitative analysis to detect biofilm formation where it gives numerical value for absorption at 630 nm by ELISA reader to determine the amount of membranes formed through adhesion of bacteria to surfaces of microtiter plate, where absorbance represents the thickness of the membranes which formed on surface by *E. coli* isolates. The results shown that 22/25 (92%) of isolates produce biofilm, but with varying degrees compared to negative control. The isolates are capable of forming the biofilm ranged in intensity among high, medium and weak adherent according to the application of the equation.

Table 2 : Ability of *E. coli* that isolated from rUTI to biofilm formation along and with cells free culture of probiotics bacteria:

Capacity of biofilm formation (biofilm formation values)			
Isolates	**Before CFC Addition**	**After *CFC* of *Lb. plantarum* Addition**	**After *CFC* of *Lb. rhamnosus GG* Addition**
E. coli 1	0.48	0.03	0.11
E. coli 2	0.67	0.07	0.09
E. coli 3	0.41	0.04	0.15
E. coli 4	0.56	0.06	0.17
E. coli 5	0.60	0.04	0.10
E. coli 6	0.44	0.07	0.14
E. coli 7	0.64	0.05	0.07
E. coli 8	0.58	0.06	0.09
E. coli 9	0.37	0.02	0.12
E. coli 10	0.32	0.03	0.16
E. coli 11	0.55	0.05	0.12
E. coli 12	0.42	0.05	0.11
E. coli 13	0.37	0.06	0.09
E. coli 14	0.62	0.03	0.17
E. coli 15	0.44	0.03	0.16
E. coli 16	0.50	0.07	0.07
E. coli 17	-	-	-
E. coli 18	0.33	0.03	0.08
E. coli 19	0.62	0.06	0.16
E. coli 20	0.34	0.05	0.12
E. coli 21	-	-	-
E. coli 22	0.58	0.02	0.11
E. coli 23	-	-	-
E. coli 24	0.46	0.04	0.10
E. coli 25	0.38	0.06	0.14

Discussion

Recurrent Urinary tract infection is one of the health problems facing the communities, spreading in female in different ages to form a phenomenon that has been studied by researches to find suitable treatments for them. Untreated UTIs can lead to complications, such as pelvic and kidney inflammation, low birth weight babies, premature birth and sometimes fetal death (Matuszkiewicz-Rowinska *et al.*, 2015). Most of the bacteria that cause recurrent urinary tract infections are *Proteus* and *E. coli*. The latter are widely spread in nature and are part of the normal flora but represent one of the most important opportunistic pathogens and cause 80% of urinary tract infections. (Jacobsen *et al.,* 2008) Most bacteria that cause urinary tract infections (Especially *E. coli*) are characterized by the emergence of strains with multiple resistance to antibiotics due to the increased and random use of these antibiotics, this make bacteria have different antibiotic resistance mechanisms such as changing the permeability barrier and changing the target location or production of β- lactamase enzymes(Singh *et al.,* 2010), Also, biofilms, one of the virulence factors to antibiotics resistance, studies have shown that a range of anaerobic bacteria associated with urinary tract infections include bactericidal gram negative bacillus, such as *Bacteroides fragilis, Prevotella*, *Clostridium* sp, *Porphyromonas* sp, as well as gram-positive coccus and *actinomycete* and these species are usually integrated with *E. coli* to form a complex synergistic resistance to antibiotics (Byron *et al.,* 2015). Bacteria are make biofilms to protect against external influences, so biofilm is described as microscopic cities inhabited by bacterial colonies to be colonies of one or more species of bacteria (Donlan, 2002) Research confirms the return of 20% of infections treated with antibiotics in women and the higher percentage in pregnant women (Al-Badr and Al-Shaikh 2013) As a result of the possession of bacteria causing urinary tract infections, such as penicillins and cephalosporins, these antibodies are sensitive to beta-lactamases produced by these bacterial species, which are encoded either by plasmids or through bacterial chromosomes or by jumping genes (Tsang, 2017). The reason for the increase in the bacterial resistance of our study isolates may be due to the high frequency of the random use of antibiotics, which allowed the increase of bacterial resistance agents to these different antibiotics , as well as the

probability of acquiring isolates of the genetic factors carrying multiple resistance traits by conjugation, transformation, Transduction (Munita and Cesar 2016). It is therefore necessary to seek safe treatments for these infections, which are mainly used for antibiotics, despite the side effects of antibiotics (Matuszkiewicz- Rowinska *et al.*, 2015). Studies have shown that some strains of therapeutic bacteria have the potential to inhibit the growth of pathogenic *E. coli* in vitro and in vivo (Tsai *et al*., 2005). Carr *et al*. (2002) pointed to the ability of probiotics to inhibit the growth of a wide range of pathogens, including *Shigella dysenteria*, *Shigella flexneri*, *S. typhi Pseu. aeruginosa* and *E. coli*. Lactobacillus promotes the immune system, prevents colon cancer and reduces cholesterol levels by converting it to Coprostanol and then removing it, as well as helping digest and absorb nutrients and diarrheal resistance (Lay-Gaik and Min-Tze 2010). Probiotics have the ability to remove toxins that result in increased efficiency of estrogen metabolism and help to break up some of the carcinogenic compounds and prevent their production such as Nitrosamines. It has the potential to inhibit a wide range of various pathogens such as *Salmonella, Shigella, Clostridium, Bacillus cereus, Campylobacter jejuni, Staphylococcus aureus* (Lin *et al*., 2008). Probiotics have confirmed ability to prevent and treat some infections and appeared safe especially UTI (Heidari *et al.,* 2017). Therapeutic bacteria have direct and indirect anti-pathogenic mechanisms include. (I) Reduce pH by Production of acid and Production of antimicrobial agents (bacteriocins) that inhibited pathogens (II) adhere to cell receptors and block pathogen adhesion (III) stimulate the immune system to fight the pathogen. (Petrova *et al.,* 2013). Studies have indicated the use of probiotics as a treatment has gained attention in the management of various infectious diseases, which showed moderate effectiveness when used as an adjuvant to antibiotics (Hosseini *et al.,* 2017).

Conclusions

Antibiotic resistance is a widespread problem and one of the most urgent public health problems in the world. Antibiotic-resistant bacteria have become more resistant, and the use of excessive antibiotics can lead to other problems. Antibiotics kill good bacteria in natural flora that keeps the body healthy. Probiotics are a wide range of treatments for many diseases. The study found that the use of metabolic products for probiotics may be a safe treatment to inhibit the formation of *E.coli* biofilms of recurrent urinary tract infections in women. These products can be inserted into topical ointments for treatment rUTIs.

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