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RESEARCH ARTICLE

Effect of *Lactobacillus* sp. Crude Bacteriocin (CB) and Cell-Free Supernatant (CFS) Against *E.coli* Growth and Adherence on Vaginal Epithelial Cell Surface.

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Abstract

Lactobacilli are gram positive bacteria, are naturally present as normal flora in the gastrointestinal tract, genital sewer and have several benefits, including: prevents infection of many pathogenic bacteria, e.g. *Escherichia coli* and *Salmonella*, helps fermentation of some foods such as different fermented milks, many industrial applications and have therapeutic properties against various infections and cancers. Cell-Free Supernatant (CFS) and Crude Bacteriocin (CB) have an anti-microbial effect that are produced by broad-spectrum of bacteria as lactobacillus; also have toxic properties to other strains of pathogenic bacteria, and other therapeutic properties. CFS and CB form a natural agents produced by probiotics live microorganisms, when administered in adequate amounts confers a health benefit on the host. So the objective of this study is to determine the effect of CFS and CB as natural agents produced from *Lactobacillus sp*. aginst *E. coli* growth and adherence on vaginal epithelial cell surface. The result showed loss ability to a growth of *E. coli* and form clear zone around culture after treated with serial concentration of CFS and CB, when a mixture of *E. coli* with human vaginal epithelial cells treated with a serial concentration of CFS and CB showed loss ability of bacteria to adherence on epithelial cell surfaces. So our study concluded that these products are very necessary to use as antibiotic alternative agents.

INTRODUCTION

Lactic acid bacteria (LAB) is a large group of Gram positive, rods / cocci, non sporulating, and anaerotolerant bacteria that producing lactic acid as their major end product which is a colorless liquid and highly soluble in water (Ljungh and Wadstrom, 2006; Park *et al.*, 2002). Lactic acid formed naturally in several foods and it is primarily founding in fermented milk products, also in meats, fruits, tomato juice, wine, molasses, blood and muscles of animals (Quinto *et al*., 2014; Khalid, 2011).

LABs have complex nutritional requirements especially amino acid and vitamins, the largest genus of LAB are *Lactobacillus* (Leroi, 2010). *Lactobacillus* is a genus of Gram-positive bacteria, non-motile, non-spore forming, vary in shape, are usually facultative anaerobic or microerophilic, it is typically chemoorganotrophic and ferment carbohydrates to produce lactic acid as a major end product (Quinto *et al*., 2014; Gayathri and Devaraja, 2011). Inhabited of gastrointestinal pathogens can be occurs by variety microorganisms as numerous intestinal bacteria recognized as probiotics which are live microbial feed supplements containing potentially bacteria which have positive impact onto their host health by improving its intestinal microbial balance (Ezema, 2013). Indeed they have many benefits either by implantation or by colonization in their host, and useful as antibiotic associated diarrhea, inflammation, and gastroenteritis, also the some probiotic bacteria act as natural treatment of urinary tract infections

(UTIs) by lowering the medium pH and reduce opportunities for spoilage organisms to grow (Gayathri and Devaraja, 2011).

Lactobacillus is a major part of beneficial bacterial group; also are present in human vagina to prevent UTIs (Aguirre and Collins, 1993), and in the gastrointestinal tract where they make up a small portion of the gut flora (Maestromarino *et al*., 2009). Bacteriocin is antimicrobial polypeptides that are produced by broad-spectrum of bacteria. Its produced by Gram-positive bacteria particulary by several strains of bacteria that belong LAB e.g. *Lactococcus, Lactobacillus, Streptococcus,* and *Pediococcus sp*. (Sun *et al*., 2014;De Vuyst and Leroy, 2007) that isolated from different habitat such as: infant feces, fermented meat products, milk, cheese, fermented cucumber and smoked salmon (El-Shouny *et al*., 2012).For these reasons, this study was objected to determine the effective of *Lactobacillus* products as a probiotic as cell free supernatant (CFS) and crude bacteriocins (CB) to the growth and adherence of some pathogenic bacteria such as *E. coli* on human vaginal epithelial cell surface. So the objectives of our study are concluded that these products (CFS and CB) are very necessary to use it as antibiotic alternative agents.

Material and methods:

- Isolation of *Lactobacillus* spp.:

The Cucumber was fermented in D.W with 3% of NaCl (Lab. rassayan, India), at 37^{o}C for 3 days, then implanted 1% of fermented cucumber in MRS broth (Hi-Media, India), and incubated at the anaerobic condition at 37^{o}C for 48 hrs. Serial dilution was prepared and from the final tube was taken 0.1ml to inoculate MRS agar, incubated at the anaerobic condition at 37^{o}C for 48 hrs, then prepare smear slide of pick up colony and staining, finally examined under a microscope.

- Preparation of CFS solution:

Set tubes of MRS broth with isolated bacteria was inoculated, then incubated them at anaerobic condition at 37^{o}C for 48 hrs., tubes centrifuged at 4000 rpm for 20 min, (CFS_1) are collected and discard the pellet, then distributed into three labeled vials, let CFS_1 vial as it's and incubated CFS_2 and CFS_3 vials in oven for(4 – 48) hrs. to obtain once and twice concentration of CFS_2 and CFS_3 respectively.

- Preparation of CB solution:

To prepare CB solution, previous procedure of CFS solution preparation was used, it's treated first with 1M of NaOH (Lab. rassayan, India), then readjusted to pH 6.0 to obtain the CB1 solution, and sterile by (0.2µ) Millipore filter. The CB_1 was distributed into three labeled vials, let CB_1 vial as it's and incubated CB_2 and CB_3 vials in oven for (4 – 48) hrs. to obtain once and twice concentration of CB_2 and CB_3 respectively.

-Determine the antibacterial effects of CFS and CB solutions:

Depending on wells diffusion method Muller Hinton agar medium (MHA, Hi-Media, India) was streaking and inoculated with 0.1ml of *E. coli* (compared with 0.5 McFarland standard to give approximately (1X107CFU/ml), added 0.1ml of CFS, CB, and MRS broth as a control in each well prepared by cock poorer, the plates were incubated at 37^{o}C for 18-24 hrs., the previous step was repeated for twice and triple CFS concentration, and measured in each treated (Bilkova *et al*., 2011).

- Determine the adherence of of *Lactobacillus* sp. on epithelial cell surface:

I. Prepare of epithelial cells suspension:

Urine specimens were collected from non-infected UTI healthy young women at the morning, specimens were centrifuged at 1000 rpm for 10 min, the supernatant was collected in sterile tubes, epithelial cells pellet were washed with sterile PBS, and the pellet was centrifuged at 1000 rpm for 5 min, steps were repeated three times(Inass *et al*., 2010).

II. Determine the adherence of *E. coli* on epithelial cell surface:

0.5ml of *E. coli* suspension (1X10^{7}CFU/ml) was mixed with 0.5ml of epithelial cells suspension in a sterile tube, incubated at 37^{o}C for 60 min with stirring every 10 min, the mixture suspension was washed with PBS, and centrifuged at 1000 rpm for 5min, the steps were repeated four times. A smear of the epithelial pellet was prepared by taken a drop of suspension and mixed with drop PBS that placed on a clear slide, passages slide onto flame rapidly, smear was stained with gram stain and examined under a microscope, 50 epithelial cells were counted and adherent bacterial cells were calculated.

III. Effect of CFS and CB to *E. coli* adherence on epithelial cell surface:

A. Determine of MIC of *E. coli*:

Set tubes of Brain heart Infusion broth (BHIB, Hi-Media, India) were inoculated with *E. coli*, the first, second and third tubes were treated with CB_1, CB_2, and CB_3 respectively, the treated process of CFS with other tubes were conducted,, and incubated at 37^{o}C for 60 min to determine MIC of bacteria compared to control BHIB tube.

B. Determine effect of CB and CFS to *E. coli* adherence on epithelial cell surface:

Labeled tubes of BHIB were inoculated with mixture suspension of *E. coli* and epithelial cells, the first tube was inoculated with CB that given MIC, also with CFS into other tubes, and incubated at 37^{o}C for 60 min to determine adherence of *E. coli*, smear of epithelial pellet was prepared by taken a drop of suspension and mixed with drop of PBS that placed on a clear slide, passages slide onto flame rapidly, smear was stained with gram stain and examined under microscope.

Results and Discussion:

- Isolation of Lactobacillus:

Lactobacillus spp. was isolated from fermented cucumber, based on Gram staining, various biochemical tests and then diagnostic by using VITEK 2 apparatus. Lactic acid bacteria especially, Lactobacillus is common in fermented cucumber products (Leroi, 2010; Fugelsang and Edwards, 2007).

-Determine the antibacterial effect of CFS and CB against growth of *E. coli*:

The antimicrobial activity of *Lactobacillus sp*. isolate was tested against pathogenic *E. coli*; results were summarized in table (1) by using well diffusion assay.

Table (1): The effect of *Lactobacillus* CFS and CB against *E. coli* growth.

Inhibition Zone (mm)		Natural Agents / Conc.
Crude bacteriocin (CB)	Cell-Free Supernatant (CFS)	
5	5	Not Concentrated
10	20	Once Concentrated
25	30	Twice Concentrated

Figures (1, 2, and 3) were illustrated the zones of inhibition of *Lactobacillus* CFS and CB against pathogenic bacteria *(E.coli***)** under present study.

Figure (1): Effect of *Lactobacillus* CFS and CB (not concentrated) against pathogenic bacteria (*E. coli*) growth.

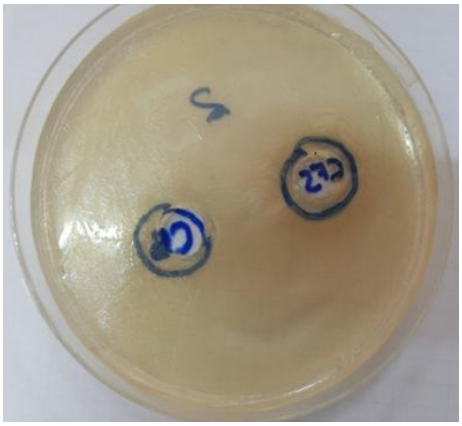

Figure (2): Effect of *Lactobacillus* CFS and CB (once concentrated) against pathogenic bacteria (*E. coli*) growth.

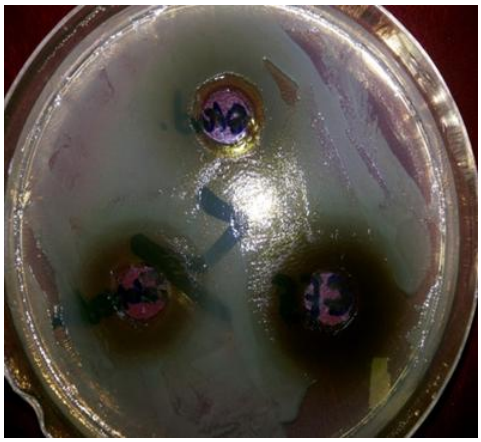

Figure (3): Effect of *Lactobacillus* CFS and CB (twice concentrated) against pathogenic bacteria (*E. coli*) growth.

The results of oure study were indicated the diameters of the inhibition zones; it's ranged between 5 to30 mm and 5 to 25 mm for CFS and CB respectively. This revealed that *E. coli* inhibited by *Lactobacillus* according by both materials CFS and CB which mentioned. A similar study was carried out who's studied the activity of *Lactobacillus*

on *E. coli* (De Vuyst and Leroy, 2007; Cotter *et a*l., 2005; Todorov and Dicks, 2005; Servin, 2004). *Lactobacillus* inhibition activity was varied, that due to a combination of many factors by *Lactobacillus* e.g. production of lactic acid which reduce pH of the medium (like CFS) or other inhibitory substances such as bacteriocin which are responsible for the most antimicrobial activity (like CB), present study agreed with many others studies like (Corr *et al*., 2009; Cotter *et al*., 2005).

- Determine the adherence of *Lactobacillus* sp. on human vaginal epithelial cell surface:

Results from determining the adherence of *E. coli* onto the human vaginal epithelial cells surface was classified into two lines:

Firstly: highly adherence before treatment.

Secondly: poorly adherence after treatment with CFS and CB, which were administrated in figures (4, 5).

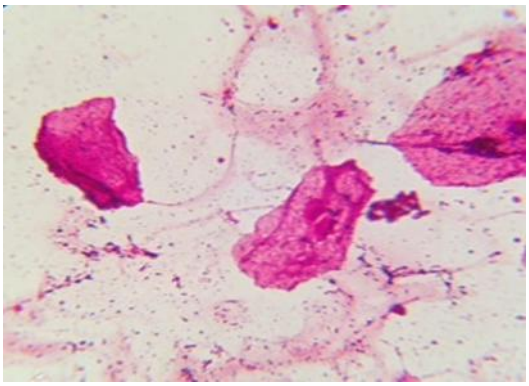

Figure (4): Adherence of pathogenic bacteria (*E. coli*) on humane vaginal epithelial cell surface before *Lactobacillus* CFS and CB treatment.

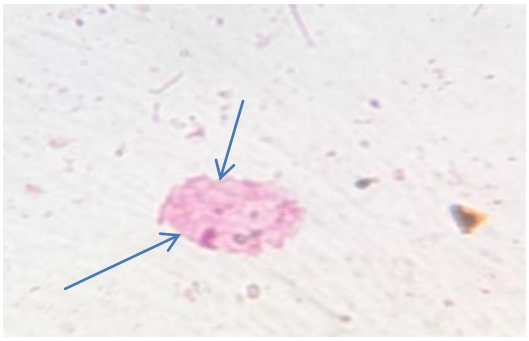

Figure (5): Adherence of pathogenic bacteria (*E. coli*) on humane vaginal epithelial cell surface after *Lactobacillus* CFS and CB treatment.

The mechanisms underlying *Lactobacillus* inhibition of test bacteria (*E. coli*) may be due to presence many fractions containing proteins with a molecular mass below 5.000Da and the finding of in vitro pointed to the peptideic nature of the *Lactobacillus* linked to bacteria inhibition (O'Hara and Shanahan, 2007).However, there are also reports about compounds of proteinaceous nature with antagonistic activity against all bacteria (these proteinaceous inhibitors target of the cell membrane and depolarize it, and also inhibit synthesis of the cell wall, there are one of those peptides were characterized as Bacteriocin called plantracin (Collado *et al*., 2009; Soonro *et al.*, 2002).

Diacetyl and hydrogen peroxide (H_2O_2) have a strong oxidizing effect on membrane lipids and cellular proteins, organic acids such as lactic acid, acetic and propionic acids, the most documented kind of metabolites.

The antagonistic actions of acids are believed to be:
(1) Interference with the maintenance of cell membrane potential.
(2) Inhibition of active transport.
(3) Reduction of intracellular pH.
(4) Inhibition of various metabolites functions.

They have a broad mode of action and inhibit both gram-negative and positive bacteria as well as yeasts and molds (Atassi and Servin, 2010; Corr *et al.*, 2009). Besides the production of inhibitory compounds, *Lactobacillus* have the ability to compete with the pathogens for nutrients during the growth, the combined influence of large numbers of competing *Lactobacillus* and the resulting decrease in pH produce an unfavorable environment for many pathogens such as UT pathogens (Daba and Saidi, 2015;Corr *et al*., 2009).

The possible of protection mechanisms by *Lactobacillus* include: inactivation of pathogens by compete *Lactobacillus* with other bacteria for resources, secrete natural antibacterial chemicals as H_2O_2 which is play role in controlling the micro-environment of the vagina and inhibiting the overgrowth of potentially pathogenic organisms as *Escherichia coli* and prevent bacteria from adhering to the urinary tract. Bacteriocins can able to inhibit the pathogenic Gram-positive bacteria, causes permeate the outer membrane of Gram-negative bacteria, and induce the inactivation of Gram-negative bacteria in conjunction with other enhancing antimicrobial environmental factors, such as low temperature, organic acid, and detergents.

Also, some bacteriocins which provide valuable alternatives to traditional therapeutic antibiotics in the treatment of infectious diseases; because it's lethal against closely related of pathogenic species were identified (Sun *et a*l., 2014; Munoz *et al*., 2011). All reasons above give clear reasons for the high activity that obtained in our results.

Conclusions:

Present study was concluded that the *Lactobacillus* isolate will be helpful in the management of bacterial disease as probiotic especially UTI caused by pathogenic bacteria as *Escherichia coli (E. coli*). The species identification, optimization of *Lactobacillus* growth, there in vivo effect on pathogen in human under pathology status will be a further course of work**.**

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