A Study on the Inhibitory Effect of Vaginal Lactobacilli on Uropathogenic *Escherichia Coli*

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Abstract: Background: The appearance of multiresistant bacteria in recurrent urinary tract infections (UTI) calls for alternative and advanced medical solutions, one promising alternative is the therapeutic use of probiotics. *Lactobacillus* constitutes a major part of the lactic acid bacteria group, has a potent inhibitory effect on some harmful bacteria as uropathogenic *E. coli*, a property that would made it an effective probiotic in prevention and treatment of recurrent UTIs in women. **Aim:** to study the inhibitory effect of vaginal *lactobacillus* strains on the growth of uropathogenic *Escherichia coli* isolated from cases of recurrent UTI. **Methods:** the study included 200 female patients who were put into two groups of 100: Group (1) patients, collected from the urology outpatient, were suffering from recurrent urinary tract infection, from whom the midstream urine samples were collected after taking the patient's consent. Group (2) patients, collected from the gynecology and obstetrics clinic and weren't having any symptoms of urinary tract infection, from whom the vaginal swab samples were taken after taking the patient's consent. **Results:** *Staphylococcus saprophyticus* and *E. coli* were the commonest organisms isolated from the urine samples of UTI patients (53% - 49% respectively). 64.7% of *E. coli*isolates produce complete hemolysis on blood agar, while Presence of mannose resistant haemagglutinating adhesins (MRHA) was detected in 82% of the studied urinary *E. coli.* All the vaginal *lactobacilli* strains and the standard strain (*lactobacillus acidophilus* ATCC L 1295) were inhibit *E. coli* seeded plate. While 3 strains (22.3%) failed to inhibit *E. coli* seeded plate when the supernatant culture was neutralized. It is concluded that vaginal *Lactobacillus* strains isolated from healthy women in the fertility age, are able to inhibit the growth of uropathogens especially *E.coli* by the effect of lactic acid with or without other inhibitory substances.
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1. Introduction:

UTI is one of the most common type of community-acquired, hospital-acquired and recurrent type of infection that occur more commonly in women **(Mulvey, 2002).**

E. coli is one of the commonest organisms in UTI. It possess specific virulence factors that enable it to colonize and produce symptoms of upper or lower UTIs **(Archamoud *et al.*, 2006)**.

The appearance of multiresistant bacteria in recurrent UTI calls for alternative and advanced medical solutions, one promising alternative is the use probiotics to prevent and treat recurrent complicated and uncomplicated UTI **(Reid *et al.*, 2003).**

*Lactobacillus*is a major part of the lactic acid bacteria group, which inhibits the growth of some harmful bacteria as uropathogenic*E. coli* So it is effective as probiotic in prevention and treatment of recurrent UTIs in women **(*Stephanie*, 2010).**

The aim of this work is to study the inhibitory effect of vaginal *lactobacillus*strains on the growth of uropathogenic *Escherichia coli* strains that isolated from cases of recurrent UTI. *Lactobacillus acidophilus* (L 1259) was used as a standard strain tostudy the inhibitory effect of lacticacid and other inhibitory substance produced by this strain and other vaginal strains on the growth of uropathogenic *E.coli* strains.

2. Patients, material and methods:

The study was conducted on 200 patients in urology and gynecology outpatient clinics Beni–Suef university hospital, Faculty of Medicine, Beni - Suef university.

The patients were divided into two groups:

Group (1) 100 patients from the urology outpatient they were suffering from recurrent urinary tract infection, from whom the Midstream urine samples (100 samples) were collected after taking the patient's consent.

Group (2) 100 patients from the gynecology and obstetrics they weren't suffering from any symptoms of urinary tract infection, from whom the Vaginal swab samples (100 samples) were taken after taking the patient's consent.

- Mid stream voided urine samples were collected during active phase of infection under aseptic conditions in sterile containers then transported to the bacteriological laboratory within half an hour.

- Urine samples were studied bacteriologically as follows:
1- Quantitative viable bacterial counts.
2- Culture for isolation and identification of different organisms.
3-Seventeen strains of urinary *E.coli* were isolated and tested for virulence factors namely hemolysin production and mannose resistant hemagglutinationadhesins.

- Culture of the vaginal swabs on MRS medium aims to isolate *Lactobacilli.* Randomly selected 10 strains were identified using API 50 CHL test and were classified into different biotype.

- The inhibitory effect of *lactobacilli* isolates on uropathogenic *E. coli* was tested in parallel with a standard strain *(Lactobacillus acidophilus CRL 1259*) obtained from the CERELA Culture Collection by the following tests:
-The inhibitory effects of lactic acid produced by *lactobacilli* isolates and standard strain on the growth of uropathogenic *E.coli* by the plate-diffusion technique

- Quantitative assay of lactic acid produced by both isolated *lactobacilli* strains and the standard strain using a lactic acid dehydrogenase (LDH)(commercial test kit)
-The minimum inhibitory concentration (MIC) of lactic acid on *E. coli* growth was determined.
- The bactericidal effect of other inhibitory factors was studied after neutralization of lactic acid by sodium carbonate using plate diffusion technique.

3. Results:
1-Significant bacteriuria was found in all urine samples of UTI patients (100%).
2- *Staphylococcus saprophyticus* and *E.coli* were the commonest organisms isolated from the urine samples of UTI patients (53% - 49% respectively).
3- As regard hemolysin production, 11 out of 17 strains of urinary *E.coli* (64.7%) produce complete hemolysis on blood agar.
4- Presence of mannose resistant haemagglutinatingadhesins (MRHA) was detected in 82% of the studied urinary *E. coli*.

Table (1): Virulence factors of urinary *E. coli* isolated strains.

***E. Coli* Isolated strains**	**Total numbe tested**	**Haemolytic activity**		**Haemagglutination activities**					
				HA		**MRHA**		**MSHA**	
		No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
	17	11	64.7%	17	100%	14	82%	3	18%

5- API CH 50 identification of 10 randomly selected *Lactobacilli* isolates were:
- *lactobacillus acidophilus CCUG20531* subtypes1,2 and 3(30%).
-*Lactobacillus delbrueckii subspecies delbrueckii CCUG 34222, Lactobacillus delbrueckii subspecies lactis CCUG 35572* (20%).
-*Lactobacillus fermentum ATCC 14931, Lactobacillus fermentum ATCC 156411* (20%).
- *Lactobacillus paracasei NCFB 151* (10%).
- *Lactobacillus plantarum ATCC 14917* (10%).
- *Lactobacillus curvatus NCFB 166* (10%).
6-Regarding the concentration of lactic acid that was secreted by the different *Lactobacilli* strains: the highest concentration of lactic acid was secreted from the standard strain (*Lactobacillus acidophilus* ATCC L 1295) that was 5.64 mg/ml and the lowest concentration of lactic acid was from *Lactobacillus curvatus*that was 3.89mg/ml.

Table (2): Lactic acid assay of different *lactobacilli*strains (standard & isolated).

***Lactobacillus* strains**	**The concentration of lactic acid mg/ml**
1- Lactobacillus acidophilus standard strain	5.64 mg/ml
2- Lactobacillus acidophilus vaginal strain (I)	5.38 mg/ml
3- Lactobacillus acidophilus vaginal strain (2)	5.28 mg/ml
4- Lactobacillus acidophilus vaginal strain (3)	5.61 mg/ml
5- Lactobacillusfermentum (1)	5.21 mg/ml
6- Lactobacillusfermentum (2)	5.20 mg/ml
7- Lactobacillusdelbrueckii (1)	4.99 mg/ml
8- Lactobacillusdelbrueckii (2)	5.001 mg/ml
9- *Lactobacillusparacasei*	4.57 mg/ml
10- Lactobacillusplantarum	4.22 mg/ml
11- Lactobacilluscurvatus	3.89 mg/ml

7- The minimal concentration of lactic acid that inhibits the growth of uropathogenic*E.coli* was 2.82 mg/ml.
8- Regarding the result of agar well diffusion technique:
- All the vaginal *lactobacilli* strains and the standard strain (*Lactobacillus acidophilus* ATCC L 1295) were shown inhibitory zone diameter >5mm.
- There was a highly significant statistical difference (p value < 0.05) between the mean diameter of the inhibitory zone diameter of standard *lactobacillus* strain and *Lactobacillusplantarum ATCC 14917* (p value = 0.040) *and Lactobacilluscurvatus NCFB 166*($p$value = 0.020). But nosignificant statistical difference (p value > 0.05) between the mean diameter of the inhibitory zone diameter of standard *lactobacillus* strain and the other vaginal *lactobacilli* isolates under study.

Table (3): Statistical difference in the inhibitory zones between the standard and vaginal strains of *lactobacilli* under study.

***Lactobacilli* strains**	**N**	**Mean**	**±Std. deviation**	**±Std. error mean**	***Pvalue**
L.1 *L*	17	14.7059	5.46311	1.32500	
St	17	19.5882	5.18482	1.25750	0.1072
L.2 *L*	17	14.9412	5.87868	1.42579	
St	17	19.5882	5.18482	1.25750	0.220
L.3 *L*	17	14.0000	5.91608	1.43486	
St	17	19.5882	5.18482	1.25750	0.106
L.4 *L*	17	14.1765	4.70685	1.14158	
St	17	19.5882	5.18482	1.25750	0.101
L.5 *L*	17	12.7647	5.80441	1.40778	
St	17	19.5882	5.18482	1.25750	0.098
L.6 *L*	17	9.8824	3.62081	0.87818	
St	17	19.5882	5.18482	1.25750	0.073
L.7 *L*	17	8.2353	2.10741	0.5112	
St	17	19.5882	5.18482	1.25750	0.040
L.8 *L*	17	7.5882	2.31999	0.56268	
St	17	19.5882	5.18482	1.25750	0.020
L.9 *L*	17	15.6765	6.2229	1.50913	
St	17	19.5882	5.18482	1.25750	0.265
L.10 *L*	17	14.2941	6.8164	1.65279	
St	17	19.5882	5.18482	1.25750	0.103

9- Neutralization of *lactobacillus* supernatants showed zones of inhibition > 5 mm in 72.7% of the tested strains. The diameter of the zones of inhibition varies between the different *lactobacilli* strains.
10- 3 strains (22.3%) failed to inhibit *E. coli* seeded plate when the supernatant culture was neutralized.

Table (4): Results of the inhibitory effect of the different *lactobacilli* supernatants on the uropathogenic*E. coli* strain by the plate diffusion technique

Lactobacillus strains	**Diameter of inhibition zone**	
	Non neutralized *lactobacilli* supernatants (control)	**Neutralized *lactobacilli* supernatants**
	Diameter of the zone	**Diameter of the zone**
***Lactobacillus acidophilus* ATTC L1259 (standard strain)**	28 mm	26 mm
***Lactobacillus acidophilus*CCUG5917 subspecies 1 (vaginal strain)**	2o mm	16.5 mm
***Lactobacillus acidophilus*CCUG5917 subspecies 2 (vaginal strain)**	22 mm	17 mm
***Lactobacillus acidophilus*CCUG5917 subspecies 3 (vaginal strain)**	18 mm	16 mm
***Lactobacillus fermentum*ATCC 14931**	15 mm	11 mm
***Lactobacillus fermentum*ATCC 156411**.	20 mm	9 mm
***Lactobacillus delbrueckiisubspecies delbrueckii*CCUG 34222**	20 mm	6 mm
***Lactobacillus delbrueckii subspecies lactis*CCUG 35572**	12 mm	No inhibition zone
***Lactobacilluparacasei*NCFB 151**	13 mm	No inhibition zone

***Lactobacillus curvatus*NCFB 166.**	6 mm	No inhibition zone
***Lactobacillus plantarum*ATCC 14917**	9 mm	8.5 mm
The total positive results	100 %	72.7 %

4.Discussion:

Urinary tract infections are common cause of illness among ambulatory and hospitalized patients, particularly within females. Among the commensally isolated organisms is *E. coli* which is known to cause the greatest proportion of these infections **(Roose*t al.*, 2006).** In addition, *E. coli* has been implicated in a large number of diseases **(Xie*et al.*, 2006).** The most commonly seen UTIs are cystitis and pyelonephritis. More than 50% of the diagnosed cases are caused by *E. coli* (**Lane and Takha, 2011).** Their findings came in agreement with our results where the cases with cystitis represented 55 out of 100 UTI studied cases (55%).

In the present study, hemolysin is produced from 11 of 17 pure isolates of *E. coli* (64.7%) and these isolates were from patients with pyelonephritis and ureteric complications. This result came congruent with that of **Stark,*et al.*, (2003).** They found that 62/95 *E.coli* strains that were isolated from a wide range of urine specimens in four hospitals, were positive for hemolysin production when grown on sheep blood agar.

Moreover, **Hooton, 2010** reported that, agglutination of red blood cells of various species by some serovars was due to the presence of certain fimbriae, while, others gave negative haemagglutination due to absence of fimbriae. MRHA is considered a simple economic and specific for detection of fimbriae (pili) of *E. coli*.

In the present study, it was found that all the isolated uropathogenic*E.coli* strains express haemagglutination. Moreover, 14 out of 17 (82%) had MRHA.

In the present study, API 50 CHL test applied to the API web database system was used for identification of *lactobacilli* species by their biochemical profiles. The majority of vaginal lactobacilli isolates were identified as *L. acidophilus* strains (30%) followed by *L. fermentum* (20%) and *L. delbrueckii*(20%). **Eliae,*et al.* (2011)**, reported that the majority of vaginal *lactobacilli* isolates were identified as *L. acidophilus* (32.6%), which was not detected by Multiplex PCR identification. Other species (*L. reuteri, L. rhamnosus*and *L. salivarius*) are rarely found in the vagina of healthy women, suggesting that they have fewer competitive characteristics compared to the most frequent species.

As regarding the MIC of *lactobacilli* supernatants lactic acid on uropathogenic*E.coli*, showed that the minimal concentration of lactic acid that inhibits uropathogenic*E.coli* growth was 2.82 mg/ml.

In a study conducted by **Domitille, *et al.* (2005)**, they used a commercial kit to determine the concentration of lactic acid in *lactobacilli* culture supernatants. The inhibitory effect of increasing concentrations of lactic acid (concentrations ranging from 4-5.5 mg/ml) was a dose-dependent killing activity.

Concerning the antibacterial activity of *lactobacilli*, the results of the present study paralleled what was reported by **Domitille, *et al.* (2005)**, The highest concentration of lactic acid in overnight broth of *lactobacillus acidophilus* vaginal strain and the standard strain which was (5.32 and 5.64 mg/ml) respectively, and the killing activity was dose dependant as evident in MIC test. Moreover, the killing activity was inhibited when the concentration of lactic acid was less than 2.82 mg/ml.

This results also came in agreement with a study conducted by **Francesco, (2011)**, who reported that 27 strains of *Lactobacillus* examined possess an antimicrobial effect against six species of uropathogenic*E. coli* isolated from recurrent UTI patients with the mean of MIC of the lactic acid intested *Lactobacillus* supernatants was 2.64 ±1.04 mg/ml.

Kiňová, *et al.* (2001) studied the antagonistic activity of *L. acidophilus* against several potential pathogens. They reported that the highest inhibitory activity was detected against *E. coli*, with inhibition zone of (20.75 ± 4.90 mm SD). This result is compatible with the present study as the mean diameter of the inhibition zone of *L. acidophilus* ATCC L1259 standard strain was (19.5 ± 5.18 mm SD).

There was statistical significant difference between the mean inhibitory zones of standard *lactobacillus* strain and *lactobacillus plantarum ATCC 14917* (*p*value = 0.040) *and lactobacillus curvatus NCFB 166* (*p* value = 0.020). On the other hand, there was no significant difference (*p*value > 0.05) between the mean diameter of the inhibitory zone of standard *lactobacillus* strain and the other vaginal *lactobacilli* isolates under study.

Our findings came in agreement with that of **Chan *et al.*(2010)**, who reported that the phenotypic characteristics revealed that different strains of *L.acidophilusand L. delbrueckii*from human vagina was found to be most active in inhibiting *E. coli* and *P. aeruginosa* followed by *L.fermentum,*

*L.paracaseii*respectively with the zones of inhibition from 6 to 25 mm.

The mean of the inhibitory zones diameter of *L. plantarum* was 8.2 mm and for *L. curvatus* was 7.6 mm. These differences were statistically significant ($p< 0.05$) in comparison with the mean diameter of the standard strain inhibitory zone.

In the present study, the inhibitory effect of *lactobacilli* was found to be not only due to lactic acid production but also due to other *lactobacilli* inhibitory substance other than lactic acid such as biosurfactants, bacteriocins, and hydrogen peroxide. This finding was studied by testing the inhibitory effect of the *lactobacillus* supernatants after neutralization by sodium carbonate on uropathogenic*E.c*oli. The recorded inhibitory effect, represented by a zone of inhibition in agar well diffusion with a diameter > 5 mm, was detected in 72.7% of the studied strains. These strains were *lactobacillus acidophilus CCUG20531(1),(2),(3), Lactobacillus delbrueckii subspecies delbrueckii CCUG 34222, Lactobacillusfermentum ATCC 14931, Lactobacillusfermentum ATCC 156411* and*Lactobacillusplantarum ATCC 14917* as well as the standard strain*Lactobacillus acidophilus ATCC L 1259* .

These findings came in agreement with that of **Klaenhammer, (2000)**, who reported that the antibacterial properties of *Lactobacillus* strains isolated from the vagina of healthy women on uropathogenic*E. coli* is not only due to lactic acid. But he observed that the *Lactobacillus acidophilus* strains have bactericidal activity against *E. coli* due to the presence of antibacterial molecules (such as H_2O_2) and different types of bacteriocins that showed an inhibitory activity against Gram-negative pathogens.

It was observed in this study that 3 strains of *Lactobacilli* (22.3%) (*Lactobacillus delbrueckii subspecies lactis*CCUG 35572, *Lactobacilluparacasei*NCFB 151 and *Lactobacilluscurvatus*NCFB 166) could inhibit the growth of *E. coli* by lactic acid production (before neutralization) and failed to inhibit *E. coli* seeded plate when the supernatant culture was neutralized probably due to lack of other inhibitory substance.

McGreoty,*et al.*, (2009), showed that the inhibitory effect produced by *Lactobacillus paracasei* species against the growth of uropathogens was investigated using two *E. coli* indicator strains Hu 734 and ATCC 25922. By using an agar diffusion technique and the result showed that the inhibition was present under acidic conditions and disappear by buffering the acidity. So this data indicated that the inhibitory effect was not due to bacteriocin or hydrogen peroxide that may sectreted by certain strains of *lactobacilli.* Although the *in vivo* inhibition can be via competitive inhibition of uropathogens from attaching to uroepithelial cells and from causing urinary tract infection.

In a study conducted by **Mastromarino, (2010),** he demonstrated that an unexpected ability of the chosen *L.paracaseii*strains to colonize the vagina via oral administration and intestinal establishment. This result pointed out that probiotics administration of *L.paracaseii*strains were effective comparing with strains that more frequently isolated from the vagina (more frequently occurring species) such as *lactobacillus acidophilus*.

This result matched with the present study as *L.paracaseii* had inhibitory effect on the tested uropathogenic*E. coli* strains with mean of the inhibition zones 9.9 ±3.6 mm with no significant difference in comparison with the mean of the standard strain inhibitory zones (*p* value 0.073).

A study conducted by **Gregor Reid. (2011),** showed that the proof of urogenital colonization and protection from infection was obtained from a clinical trial in which 55 postmenopausal women were given one suppository of *lactobacilli probiotic* weekly for one year. The patients were followed up after 2 weeks and then monthly. Their data were included to examine infection rates, and the side effects. The UTI infection rate decreased from 6.0 to 1.6 (73% decrease) for those given *lactobacilli*. The viable *lactobacillus* counts recovered from vaginal swabs increased with therapy, especially for months 7–12 for *lactobacilli*-treated patients, during which time lower UTI rates were seen.

Conclusion:

It can be concluded that vaginal *Lactobacillus* strains isolated from healthy women in the fertility age , are able to inhibit the growth of uropathogens especially *E.coli* by the effect of lactic acid with or without other inhibitory substances . These results might push the investigators for using *L. acidophilus* L 1259 either alone or combined with other strains of vaginal *lactobacilli*, as a prophylactic or therapeutic probiotic product for UTI.

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