# Estimating the inhibitory effect of *Lactobacillus* isolated from different sources on some pathogens of urogenital infections in women group

# تقييم التأثير التثبيطي للعصيات اللبنية المعزولة من مصادر مختلفة ضد عدد من المسببات المرضية للالتهابات البولية التناسلية لدى مجموعة من النساء

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**Abstract**

**This study was aimed to identify of pathogenic organism isolated from urogintal tract and estimate the effect of *Lactobacillus* which isolated from different sources on the growth of these pathogens including (*Candida albicans, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Gardnerella viginals, Klebsiella oxytoca, Proteus mirabilis*). The results showed that *Lactobacillus* which was isolated from vagina on solid medium was effective against pathogenic isolates more than the *Lactobacillus* species isolated from yoghurt, cow milk, human milk with inhibition zone (12-16) mm, while the highs inhibitory effect of *Lactobacillus* isolated from vagina in liquid media with inhibition zone reached to(18)mm. Adversely, lowest inhibitory effect was shown with supernatant of *Lactobacillus spp.* isolated from human milk with inhibition zone reached to (11)mm comparison with *Lactobacillus spp.* isolated from yoghurt and cow milk with inhibition zone (13-15) mm. Also the result revealed that *Lactobacillus spp.* isolated from vagina, human milk, cow milk and yoghurt by overlay method had no effect on pathogenic bacteria but high effect was shown only with the vaginal *Lactobacillus* isolates on *C. albicans*.**

**المستخلص**

**هدفت الدراسة إلى تعيين المسببات المرضية للالتهابات البولية التناسلية وعزل العصيات اللبنية من مصادرمختلفة و تقييم التأثير التثبيطي لهذه العصيات ضد نمو المسببات المرضية المعزولة من القناة البولية التناسلية والتي ضمت *Staphaphlococcus aureus, Eschrichia coli,Pseudomonas aeruginosa, Gardnerella vaginils ,Klebsilla oxytoca ,Proteus mirabilis,Candida albicanis.* , أظهرت النتائج أن العصيات اللبنية المعزولة من المهبل في الوسط الصلب أعطت فعالية تثبيطية جيدة تجاه ممرضات القناة البولية التناسلية أكثر من للعصيات المعزولة من حليب الأم وحليب البقر واللبن وبقطر تثبيطي (12-16) ملم بينما تم الحصول على أعلى قطر تثبيطي للعصيات المعزولة من المهبل في الوسط السائل وبقطر تثبيطي يصل إلى (18) ملم وعلى العكس ظهر تأثير تثبيطي اقل لطافي العصيات المعزولة من حليب الأم وبقطر تثبيطي يصل**

**إلى 11 ملم مقارنة مع العصيات المعزولة من اللبن وحليب البقر وبقطر تثبيطي ( 13-15) ملم كما أظهرت النتائج في طريقة overlay method أن العصيات اللبنية المعزولة من المهبل وحليب الأم وحليب البقر واللبن ليس لها تأثير على البكتريا المرضية لكن هناك تأثير كبير في حالة استخدام العصيات اللبنية المهبلية ضد *Candida albicanis* .**

## Introduction

Although antimicrobial therapy is generally effective in eradicating urogintal infections, there is still a high incidence of recurrence [1]. Patient's quality of life is affected and many women become frustrated by the cycle of repeated antimicrobial treatment whose effectiveness is diminishing due to increasing development of microbial resistance [2]. So, need for alternative treatment encouraged the researchers for seeking to reach the best outcome [3]. Use of *Lactobacillus* in such cases appears to be a promising answer as a current challenge experienced due to excessive and misuse of antibiotics. *Lactobacillus* is used in urogenital tract infections to re-establish the vaginal flora and prevent future episodes, bacterial vaginitis, vaginal Candidiasis, recurrent UTI, bladder cancer and complications of antibiotic therapy [4]. Because of their relative safety, ease of use, and excellent tolerability, the use of *Lactobacillus* should be considered, not just as adjunctive replacement after antibiotic administration, but as possible first-line therapy when clinically indicated.

This study was designed to isolate and identify of pathogens from urogintal infection, selecting *Lactobacillus* from human and food sources and estimating the effect of these organisms on the growth of pathogens recovered from urogenital women patients.

## Material and methods

### Isolation and identification of pathogens:

#### From vaginal swabs

Two vaginal swabs from 250 patients were transported to the laboratory by inoculating the swab into a sterile tube containing 3.0 ml of saturate transport medium (Sabourauds dextrose broth or Brain heart infusion broth/ Difco- USA). One of the swabs was directly inoculated onto each of Sabourauds dextrose agar (Difco- USA), Blood agar (Oxoid- England) and MacConkey agar (Difco- USA) for microbiological investigation. The other was used for direct examination by wet mounted film and Gram stained for detection of yeasts and bacteria. Inoculated culture plates were incubated at 37°C for 24-72 hrs. Colonies were identified by morphological and biochemical tests and motility test was also preformed. Stock culture was made by inoculating single colony of the isolates into a slant of Sabourauds dextrose ager (for yeast) and Nutrient agar (for bacteria).

#### From urine sample [5].

Urine samples(20-45 ml) were collected in sterile glass containers of 50 ml volume by using mid stream specimens method , mid stream urine samples were taken after cleaning the genital area with soap and water, The sample was divided into two parts , one used for microscopic examination , and the other for culturing.

**Microscopical examination of urine sample [5].**

By using portable centrifuge (Buch- Germany), 10 ml of urine was centrifuged at 3000 rpm for 5min, then the supernatant was discarded and one drop of the retained sediment in the centrifuge tube was placed on glass slide, and covered by cover slip, then examined under the power 40 x.

**Culturing the urine sample [6].**

Quantization of organism in urine was done by using full loop inoculation. A volume of 0.001ml of human urine was spreaded on enriched media (blood agar) and selective media (MacConky agar). The samples were incubated at 37°C for 24hr. After that the number of bacterial colonies on the agar was calculated and the number of organism in I ml of urine was measured.

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**Identification of yeasts**

Yeasts isolates were identified through the following steps as described by [7].

**1.** Gram stain [8].

Small portion of yeast colony was transferred by sterile loop, smeared and fixed on microscopic slide for staining by Gram stain to examine cells shape, grouping and reaction.

**2. Production of chlamydospores [9].**

Corn meal agar medium was inoculated with single colony of the isolated yeast. Inoculation was done by making 3-6 parallel cuts of 1 cm in length on surface of the media, the streaks were covered by a sterile cover slip the inoculated plates were incubated at 28°C up to 2 days. Examination of plates for the presence of chlamydospores was done under microscope.

**3. Production of germ tube [10].**

A small portion of the isolated colony was emulsified in one ml of sterile human serum, then incubated for 2-3 hrs at 37°C, one drop of the suspension was placed on clean slide with drop of lactophenol cotton blue then examined microscopically for the production of germ tube.

**4.** Biochemical tests including :

**A. Sugar fermentation test [11].**

A set of sugars consists of glucose, lactose, maltose and sucrose, which were used for identification and differentiation between *Candida* species. The test was done by inoculating tubes containing fermentation media and 2% sugar with part of the colony, shaking gently then incubated at 28-30°C for three days .The positive result was recorded by changing the color of bromocrysol puraple indicater to yellow and production of $CO_2$ gas bubbles in Durham tube.

**B. Carbohydrate assimilation test [11].**

The test depends on the ability of different species of yeasts to grow in various sugar solutions (glucose, lactose, trehalos, raffinose and sucrose). Carbohydrate assimilation medium was poured in Petri dishes and inoculated with *Candida spp* ,then six wells were made by cork borer in the inoculated plates, each well were filled with 2% sugar and incubated plates at 30° for 2-4 days.

**Identification of bacteria**

The isolated bacteria were identified according to [12,13,14,15,16] by using Gram stain and biochemical tests Including: - oxidase, indole, catalase, urea hydrolysis, geletinase, kiliglar iron agar, coagulase, phenylalnine deaminase, motility and Whiff test, Carbohydrates fermentation test (glucose, sucrose, maltose, mannitol ,rafenose ,lactose, trehalose, rhaminose, arabinose, fructose, galactose, sorbitol, salicin, cellobose, ribose, melibiose,mannose and xilose) growing at 45°C, production of ammonia from arginin, acid and cured production from litmus milk.

**Isolation of *Lactobacillus***

**From vagina**

*Lactobacillus* isolates were isolated from vaginal swabs, samples of the80 women attended obstetric and gynecology clinics of Fatima AL-Zahra hospital in Baghdad. The samples were inoculated in 10 ml MRS broth (Oxoid- England) then cultured onto MRS agar (Oxoid- England) of pH 5.2 and incubated at 37°C for 48 hour, followed by subculturing onto MRS agar of pH 4.3 at 37°C in a candle jar for 48 hour. *Lactobacilli* were identified on the basis of growth on selective MRS agar, colony morphology, Gram staining, catalase activity, beside motility test and other biochemical testes Further identification of the species of the *Lactobacilli* was performed by carbohydrate fermentation test , growth at 15°C and 45°C in MRS broth according to [12 , 14].

**Isolation of *Lactobacillus* from human milk.**

Seven samples (2-5ml) of human milk were taken from breasts healthy women and put into 10 ml MRS broth. The *Lactobacilli* were identified according to [12, 14].

**From cow milk and yoghurt**

*Lactobacillus* isolates were obtained from six samples of cow milk and yoghurt: one sample of yoghurt was home made from cow milk, two samples from retail markets, one sample was dried cow milk and two samples was fresh cow milk. The isolation was performed by the routine microbiological procedure mention above according to [12 ,14].

**Determining inhibitory effect of *Lactobacillus spp*. on pathogenic organisms**

**On sold medium (MRS Agar)**

Culture of *Lactobacillus* previously grown in MRS broth was streaked on MRS agar plate and then plates were incubated under anaerobic condition at 37°C for 18 hr.

[17]. A cork borer (5mm diameter) was used to withdraw disks of *Lactobacillus* growth which were incubated and put on surface of Sabourauds dextrose agar and Nutrient agar that was inoculated previously with 0.1ml of yeast or bacteria suspensions then incubated at 37°C for 24 hr, the inhibition zones around the disk were estimated in millimeter.

**In liquid medium (MRS broth)**

MRS broth was inoculated with 1% of *Lactobacillus* isolates then incubated at 37°C for 18 hr. [18] After incubation, the culture was centrifuged at 6000 rpm for 15 min, and

filtered through millipor filter unit (0.22um) .According to well diffusion method that mentioned by [19]. Sabourad and Nutrient agar plates were inoculated with 0.1ml of each pathogenic organism (*C. albicans, G. vaginalis,Staph.aureus P. aeruginosa, E.coli,Klebsiella oxytoca* or *Proteus mirabilis*) by a spreader then ,5mm wells were made by the cork borer. Each well was filled with *Lactobacillus* supernatant, then incubated at 37°C for 24 hr. Inhibition zones around the wells were measured by (mm) and compared with that of control which contained MRS broth only.

**Agar overlay method [19].**

*Lactobacillus* was cultured on MRS agar and incubated at 37°C for 18 hr and a thin layer of Nutrient agar (Oxoid- England) was poured over it and keept at 4°C for 2hr, then incubated at 37° C for 48hr after pathogenic organism was streaked on the surface of agar.

**Results and discussion**

From a total of vaginal swabs examined (32.81%) were identify *C.albicans*, followed by (16.14%*) Staph .aureus* then (10.93%) *E.coli*, (9.37%) *P. aeruginosa*, (5.72%) *Klebsiella oxytoca*, (5.20%*) G. virginals* and (3, 12%) *Proteus mirabilis***.**

In the urine samples *E.coli* was the common pathogen with percentage of (26.29%) followed by *Staph.aureus* (24.13%), *C.albicans* (20.68%), such percentage were very high when compared to other microorganisms ( *P. aeruginosa* (9.91%), *G.viginals* (4.31%) , *Klebsiella oxytoca*.(3.01% ) and *Proteus mirabilis* ( 2.58%) this observation come in accordance with [20and 21**]** who found that all above pathogens were highly present in female urogenital tract**.**

**Identification of *Lactobacillus* bacteria**

Suspected colonies appeared, pale, round, convex, soft,mucoid and surrounded by zone as a result of dissolving calcium carbonate . When part of the colonies were examined microscopically cells appeared Gram positive bacilli, mainly grouped in chains containing 3-8 cells, and non–sporformers. Biochemical tests showed positive results to lactose fermentation, growth at 45 °C and 15 °C. Furthermore, curd formed in litmus milk, while gave negative result to catalase ,oxidase ,urease , motility and production of NH3 from arginine. These results come in concord with those obtained by [16, 22].

**Inhibitory effect of *Lactobacillus* on pathogenic organisms**

**On solid medium (MRS agar)**

Result revealed that propagation of *Lactobacillus* isolates on MRS agar under anaerobic condition was an efficient method for producting of their inhibitory metabolites against tested pathogens. In this approachAl-Kafaji [17] found that using MRS agar medium in studying the ability of *Lactobacillus* isolates to produce inhibiting materials under anaerobic condition, if the chosen procedure that gives reasonable result.

Our finding revealed that *Lactobacillus* isolated from vagina was effective against pathogenic isolates and had the best effect which clarified by the zone of inhibition growth for pathogenic isolate, which ranged between (16-12) mm, while the less effect

was observed when *Lactobacillus* isolates from yoghurt, cow milk, human milk were used as shown in table (1) .

**Table (1): Inhibitory effect of *Lactobacillus spp*. isolated from different sources, on pathogenic microorganisms expensed as diameter of inhibition zones (mm) .**

| Code of Lactobacillus | E.coli | Staph.aureu | P.aeruginos | G.vaginali | K.oxytoca | P.mirabilis. | C.albecanis |
|---|---|---|---|---|---|---|---|
| | Diameter /mm | | | | | | |
| Lb.1 | 16 | 16 | 12 | 14 | 13 | 13 | 14 |
| Lb.2 | 14 | 15 | 10 | 12 | 10 | 11 | 12 |
| Lb.3 | 11 | 11 | 9 | 11 | 11 | 10 | 12 |
| Lb.4 | 8 | 9 | 5 | 7 | 5 | 8 | 9 |

**Lb.1=*Lactobacillus* from vaginal swab, Lb.2= *Lactobacillus* from yoghurt, Lb.3= *Lactobacillus* from cow milk , Lb.4= *Lactobacillus* from human milk**

Our finding indicated that *Lactobacillus* isolated from vagina possessed the highest inhibitory effect among other isolates, because it has the same ecosystem of pathogens. Therefore, produced variety of compounds with antimicrobial activity and the end products during *Lactobacillus* fermentation, causing a reduction in pH [23]. These finding come in accordance with those other workers [24, 25].

### In liquid Media (MRS broth)

Inhibitory effect of *Lactobacillus* isolates grown in MRS broth was evaluated also .Well diffusion method was used to determine the inhibition activity of *Lactobacillus* against pathogenic isolates. Highs inhibitory effect was obtained during using supernatant of *Lactobacillus* isolated from vagina, the inhibition zones reached to 18 mm. Adversely, lowest effect appeared with supernatant of *Lactobacillus* isolated from human milk when the inhibition zone reached only11mm .While Inhibitory effect of *Lactobacillus* isolates recovered from yoghurt and cow milk ranged between( 13-15) as shown in table (2).

**Table (2): Inhibitory effect of *Lactobacillus spp* isolated from different sources on pathogenic microorganisms which was measured by millimeter (In MRS broth).**

| Code of *Lactobcillus* | *E.coli* | *Staph.aureus* | *P.aeruginosa* | *G.vaginalis* | *Koxytoca* | *P.mirabilis* | *C.albecanis* |
|---|---|---|---|---|---|---|---|
| | **Diameter/m.m** | | | | | | |
| Lb.1 | 18 | 18 | 17 | 16 | 15 | 14 | 14 |
| Lb.2 | 15 | 15 | 14 | 15 | 14 | 15 | 14 |
| Lb.3 | 13 | 12 | 10 | 12 | 11 | 10 | 12 |
| Lb.4 | 11 | 9 | 9 | 11 | 9 | 8 | 9 |

**Lb.1=*Lactobacillus* from vaginal swab, Lb.2= *Lactobacillus* from yoghurt, Lb.3= *Lactobacillus* from cow milk, Lb.4= *Lactobacillus* from human milk**

Maximum inhibition zone was obtained from vaginal *Lactobacillus* isolates, which were grown in MRS broth, in comparison to those grown on solid medium, it was obvious that MRS broth was a better stimulator for inhibitory product than MRS agar .Such finding was confirmed by Fang *et al* [26] who mention that MRS broth stimulated inhibitory effect against Gram positive (*Staph. aureus*) and Gram negative bacteria ( *E.coli ,Klebsiella spp.,Proteus spp.*) Similar results were also obtained by Kubba [27] who found that best inhibitory effect was gained when liquid media (MRS broth) was used to estimate the effect of *Lactobacillus* on pathogenic bacteria.

**Overlay method**

The target of this method was to test the ability of *Lactobacillus* bacteria for inhibiting growth of pathogenic organism by the effect of substance diffused in agar. Results showed that *Lactobacillus* isolated from vagina, human milk, cow milk and yoghurt had no effect on pathogenic bacteria but there was a slight effect on *Candida*. High effect was exhibited only when the vaginal Lactobacillus isolates were used on *Candida*. This inhibition indicates presence of antimicrobial substances in *Lactobacillus* grown on agar which absence of such effect in this method on most pathogen may be related to high molecular weight substances that can not diffuse through the agar [28]. Similar observation were recorded by other investigators [29 - 30].

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