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## Estimating the inhibitory effect of *Lactobacillus* isolated from vagina against some pathogens of genital infections in group of women.

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### Abstract

Thirty- five vaginal swab samples were obtained from women vagina. All samples were subjected to conventional morphological and cultural characteristics, isolates distributed between *Lactobacillus acidophilus* (23) and *Lactobacillus fermentum* (12). Antibacterial activities were done by well diffusion and blank disk method. The *Lactobacillus* was used as a probiotic treatment against bacterial isolates from vagina. The isolated bacteria had strong activity against indicator strains. The results showed that *Lactobacillus* which was isolated from vagina by well diffusion method was effective against pathogenic isolates more than the *Lactobacillus* isolated by blank disc method, the high inhibitory effect of *Lactobacillus* isolates by well give an inhibition zone reached to (18)mm, while the *Lactobacillus* by disc was lower with inhibition zone (12-15) mm. Also the results revealed that *Lactobacillus* spp. whole cell of *Lactobacillus* was more effective against pathogenic isolates more than used supernatant. The supernatant did not show any activity when was treated with NaOH and adjusted to pH 7. This indicates that the organic acid produced by the *Lactobacillus* isolates was may be actually responsible for the inhibition of the indicator bacteria. The result show that the *Lactobacillus* strains could be considered as a potential antimicrobial probiotic strains against some human vaginal pathogens and should be further studied for their human health benefits.

**Keywords:** -*Lactobacillus*, Probiotic, Vaginal flora.

## تقييم التأثير التثبيطي للعصيات اللبنية المعزولة من المهبل ضد عدد من المسببات المرضية لالتهابات التناسلية لدى مجموعته من النساء.

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### الخلاصه

تم الحصول على خمس وثلاثون عينة من مهبل النساء التي شخصيت حسب الصفات المظهرية، الزرع والكيميائية التقليديه. توزعت تلك العينات بين *Lactobacillus acidophilus* (23) و *Lactobacillus fermentum* (12). اظهرت الفعاليه التضاديه ليكتريا *Lactobacillus* بواسطه الاقراص والحفر والطريقه الاخرى استخدام الخلايا الحيه كامله. حيث استخدمت *Lactobacillus* كمعالج ضد البكتريا المعزوله من المهبل، واطهرت العزلات فعاليه تضاديه قويه.

ضد البكتريا المستخدمه بالدراسه. اظهرت النتائج بان استخدام الراشح بطريقه الحفر اكثر فعاليه ضد العزلات الممرضه من المستخدم بطريقه الاقراص. حيث وصل اعلى تاثير تثبيطي بواسطه الحفر الى قطر تثبيط (18) ملم، بينما كان قطر التثبيط بواسطه الاقراص يتراوح بين (12 - 15). كذلك تشير النتائج الى ان استخدام

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*Lactobacillus* كخلايا حيه كامله اكثر فعاليه من استخدام الراشح فقط. تشيرالنتائج ايضا ان معادله الاس الهيدروجيني للراشح الى (7) بواسطه NaOH لايعطي اي فعاليه تثبيطه مما يدل على ان الفعاليه التثبيطيه لبكتريا *Lactobacillus* في هذه الدراسه تعتمدعلى الخاصيه الحامضيه فيه التثبيط البكتريا المستخدمه بالدراسه. كما وتشير الدراسه الى ان *Lactobacillus* يمكن اعتبارها معالج بكتيري ضد بعض المسببات البكتريه المهبلية ، وينبغي ان تدرس لفوائدهاالصحيه البشريه.

## Introduction

The vaginal flora was first reported by Albert Döderlein, as early as 1892. Döderlein found that the microflora was homogenously colonized with Gram-positive rods, which were designated the name "Döderlein's bacilli". Over the years, these bacilli have been identified as *Lactobacillus* spp. *Lactobacilli*, the predominant micro-organisms of the vaginal microbiot. [1]

Probiotics for animals are defined as live microorganism that are able to decrease the number of intestinal infections, increase production and improve food hygiene by contributing to a better gastrointestinal environment [2]. The FAO/WHO have stipulated several criteria for probiotic evaluation. One of the most important parameters by which potentially new probiotic strains must be characterized is the production of antimicrobial substances under *in vitro* conditions [3]. *Lactobacilli* are facultatively anaerobic, catalase negative, non-spore forming, rod-shaped lactic acid bacteria. Several strains of the genus *Lactobacillus* are used as probiotics [4].

*Lactobacillus* play a major role in the maintenance of a healthy genital tract by preventing the colonization of pathogenic bacteria. In healthy women, the vaginal microflora is dominated by *Lactobacillus* species, at a level of  $10^7$ - $10^8$  CFU g<sup>-1</sup> of fluid, which exert a significant influence on the microflora of the ecosystem [5]. It has been observed that indigenous *lactobacilli* prevent the overgrowth and invasion of pathogenic bacteria by a combination of competitive exclusion, competition for nutrients, and release of antimicrobial substances such as hydrogen peroxide, organic acids, bacteriocins, and biosurfactants [6]. In consequence, a depletion of vaginal *lactobacilli* has been directly associated with an increase in the incidence of genital and urinary infections [7]. The *Lactobacilli* have been shown to produce biosurfactants and collagen binding proteins that inhibit pathogen adhesion and displace the pathogens [8].

The study was planned to identify the most common of pathogenic bacteria in the vagina in Iraqi women and estimate the antagonistic effects of *Lactobacillus* that isolated from vaginal tract on the growth of these bacterial isolates including (*Staphylococcus aureus*, *Escherichia coli*, and *Proteus mirabilis*).

## Materials and methods:

### Bacteria and cultural conditions

Three pathogenic bacteria were used in the study: (*S. aureus*, *E. coli*, and *P. mirabilis*). These strains were isolated from genital tract and were identified by using conventional method and vitek 2.

Vaginal samples were collected from vaginal wall of women and were inoculated on de Man, Rogosa and Sharpe agar medium (MRS) and incubated overnight anaerobically (anaerobic jar and gas pack) at 37 °C for 48h. growth was streaked on MRS agar plates several time. The isolates were identified to genus level by: gram staining, oxidase, catalase, growth at various temperatures (10, 15, 45), no growth on nutrient agar, growth at various PH (4, 9) and Finally the carbohydrate fermentation. The *Lactobacillus* isolates were maintained in MRS broth with 20% glycerol at – 18 °C as stock culture.

### Inhibition assay

#### Well diffusion technique

Bacteria were screened for their antibacterial activity by agar –well diffusion technique, the isolates were grown in MRS broth anaerobically at 37 °C for 48hours, cell free solution were prepared by, centrifugation of grown cultures (6000 rpm for 15 min. at 4 °C), followed by filtration using 0.20µm pore size filter, and obtained supernatants. Brain heart infusion broth medium (BHI) was seeded with overnight culture of *E. coli*, *P. mirabilis* and *S. aureus* at final concentration  $10^6$  cell/ ml, poured into sterile petri dishes and allowed to solidify at room temperature, 6mm diameters well that has been cut in Mueller Hinton agar plates and spotted on with the pathogenic bacteria, the wells filled with 6mm diameters well that has been cut in Mueller Hinton agar plates and spotted on with the pathogenic bacteria, the wells filled with 50µl of sterile supernatant separately and allowed to diffuse into agar for 6 h at 4 °C. After 18-24 hours of incubation, the diameters of the zones of growth inhibition were

measured. The screening of the antibacterial substances was performed by using the agar spot test and the well diffusion method described by [9] was used the growth inhibition showed a clear zone around the tested colonies.

#### Disk technique

Bacteria were screened for antibacterial activity by disk technique. The isolates were grown in MRS broth anaerobically at 37 °C for 48 hours, free cell were prepared by centrifugation of grown cultures (6000 rpm for 15 min. at 4 °C), followed by filtration with 0.20 µm pore size filter, and obtained supernatants. Overnight culture of *E.coli*, *P.mirabilis* and *S.aureus* at final concentration (10)<sup>6</sup> cell/ml, that culture preub on BHI was poured into sterile petri dishes and allowed to solidify at room temperature, in Mueller Hinton agar plates and spotted on with the pathogenic bacteria, A cork borer (5mm diameter) was used to withdraw disks of filter paper and put in sterile Lactobacillus supernatant and forceps was used to Place the disks on the surface of the agar, each antimicrobial disk one at a time [10].

### Results and Discussion

#### On solid media (disc method)

Results revealed that propagation of Lactobacillus isolates on MRS agar under anaerobic condition was an efficient method for the production of their inhibitory metabolites against tested pathogens. In this approach Al-Kafaji [9] found that using MRS agar medium in studying the ability of Lactobacillus isolates to produce inhibiting materials under anaerobic condition, in the chosen procedure that gives reasonable result.

The results of antagonistic effects of the *Lactobacillus* strains isolates against three pathogenic strains are characterized by the disc assay against an indicator strain, but the degrees of antagonism varied among the Lactobacillus strains. Our finding revealed that *Lactobacillus fermentum* isolated from healthy women was effective against pathogenic isolates and had the best effect which clarified by the zone of inhibition growth for *E.coli*, which ranged between (12 - 15) mm, while the less effect was observed when *Lactobacillus fermentum* isolates from pregnant women. Our findings agree with previous study [11] who found that supplementing lambs infected with *E.coli* O157:H7 with a mixture of probiotics including *L. acidophilus* in the diet can reduce total number of *E. coli* O157:H7 shed in the feces. Shah, in 2000 selected Lactic acid bacteria as a competitive exclusion product that would inhibit *E.coli* O157:H7 in the intestinal tract of live cattle. Results agree with [12] they found that *L. acidophilus* and *L.plantarum* had inhibitory properties against *E.coli*, *S. aureus*, *S. agalactiae*, *S. uberis*, *S. Enteritidis* and *B. pumilus*. Also results agree with Lema [13] who found that supplementation of cattle with *L. acidophilus* reduce the prevalence and magnitude of fecal *E. coli* O157.

*Lactobacillus acidophilus*, *L. bulgaricus*, *L. plantarum*, *L. lactis* and *L. rhamnosus* isolated from milk samples of buffalo, cow and goat showed antagonistic activity against *E. coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *P. vulgaris* and *Salmonella typhi* by disc diffusion method and the inhibition produced varied between 15 to 24 mm [14]. *Lactobacillus spp.*, isolated from chicken intestine demonstrated inhibitory activity ranged from 12.5 to 18 mm against *S. enteritidis*, *S. pullorum*, *S. typhimurium*, *S. blockley* and three serotypes of *E. coli* and it was suggested that some organic compounds may be responsible for antagonistic activity [15]. The previous study showed that *L. fermentum* showed high inhibitor activities against *E.coli* by inhibition zone between 10 – 15 while *L.acidophilus* was lower antagonistic activities against *staphylococcus* and *proteus* but it was higher effective against *E.coli*. This differs in Lactobacillus effect result to type or strain used among same species because it is one of the factors affecting the influence of effectiveness throughout the growth period, The anti, Antibacterial and quantity of product in the media [16]. These results are shown in table -1. In Statistical analyses showed the highest inhibition effect ( $P < 0.05$ ) was *L. fermentum* isolated from healthy women, with 13.67 (Table -2).

The antibacterial effect of the isolates was demonstrated against both Gram positive and negative bacteria. This observation contradicts the report of [17] which indicates that lactobacilli showed stronger antibacterial effect against Gram positive than Gram negative bacteria.

**Table 1-** The inhibitory effect of *Lactobacillus spp.* isolated from vagina on pathogenic microorganisms using the well diffusion technique which was measured by millimeter In (MRS solid).

Code of <i>Lactobacillus</i>	<i>E.coli</i>	<i>Staphylococcus aureus</i>	<i>Proteus</i>
	Diameter/m.m		
Lb.1	16	17	17
Lb.2	13	13	15
Lb.3	14	14	15
Lb.4	14	14	16

**Table 2-** Mean  $\pm$  SE for *Lactobacillus spp.* isolated from vagina on solid MRS media by disc method

Bacteria	Mean $\pm$ SE			LSD value
	<i>Staphylococcus</i>	<i>Protus</i>	<i>E. coli</i>	
Lb1	9.67 $\pm$ 0.33	10.00 $\pm$ 0.57	10.33 $\pm$ 0.33	1.489 NS
Lb2	9.00 $\pm$ 1.00	9.33 $\pm$ 0.67	11.67 $\pm$ 0.33	2.491 *
Lb3	13.67 $\pm$ 0.67	12.00 $\pm$ 1.15	13.00 $\pm$ 1.00	3.329 NS
Lb4	8.67 $\pm$ 1.85	8.33 $\pm$ 0.33	10.33 $\pm$ 0.33	3.825 NS
LSD value	3.646 *	2.430 *	1.882 *	----

\* (P $\leq$ 0.05).

### In liquid Media (Wells method)

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. High inhibitory effect was obtained when using supernatant of *Lactobacillus* [10].

This influence has ranged between areas of inhibition diameters less than 10 mm and areas of inhibition diameters increases about 13mm. 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 previous study [18] who mented that MRS broth stimulated inhibitory effect against Gram positive (*S. aureus*) and Gram negative bacteria (*E.coli*, *Proteus spp.*) Similar results were also obtained by another researcher [19] who found that best inhibitory effect was gained when liquid media (MRS broth) was used to estimate the effect of *Lactobacillus* on pathogenic bacteria.

The antagonistic activity in liquid media is favoured by rapidly diffusing anti-microbial compounds, including organic acids and co-aggregation of different indigenous bacteria with pathogens [20; 21].

All tested strains showed great sensitivity toward *L.acidophilus* by testing using agar well diffusion method. Results match with the findings of previous invi [22] who reported that *Lactobacillus spp.* isolated from the genital tract have probiotic activities which contribute to health restoration and maintenance. Also the present results agree with Abd El-Moez [23] who showed high activity of in vitro use of *L.acidophilus* as probiotic against *E.coli*, *Bacillus C.diversus*, *E.feacalis* and *Y.enterocolitica* followed by *L.casei*. [24]who proved that lactic acid bacteria display numerous antimicrobial activities and the antimicrobial production by probiotic LAB plays a role during in vivo interactions occurring in gastrointestinal tract hence contributing to gut health.

The Statistical Analyses for *S. aureus* and *E.coli* showed significantly different in inhibition between Lb.1 with( Lb.2,Lb.3and Lb.4 ) in the level of ( $P < 0.05$ ), while for *Proteus* significantly different in inhibition between Lb.1 with Lb.4 and Lb.2 with Lb.4 in the level ( $P < 0.05$ ) ( table -3 ).

This result match with previous study it showed [25] that *L. acidophilus* strains had inhibitory activity towards *S. typhi*, *S. aureus*, *E. coli*, *P. vulgaris* and *Y. enterocolitica*. Our results disagree withold study [26], which reported that none of the *Lactobacillus spp.* was able to inhibit the growth of *S. enteritidis*, *S. typhimurium*, *E.coli* and *S. aureus*. Probiotics have shown to protect against variety of pathogens as *E. coli* [27] and Salmonella as well as Campylobacter [28]. Casey *et al.*, [29] characterized lactobacillus for its antimicrobial activity against *Clostridium difficile* enteropathogenic *E. coli* (EPEC) verocytotoxigenic *E. coli* (VTEC) and *C. jejuni*. They added that lactobacilli displayed variations in their antimicrobial activity with few strains showing inhibitory activity against all pathogens.

When the supernatant was treated with NaOH and the pH was adjusted to 6.5, revealed no inhibition. This indicates that the organic acid produced by the *Lactobacillus* isolates may be actually responsible

for the inhibition of the indicator bacteria.[14 ] reported that organic acids produced by the *Lactobacillus* isolates might be responsible for their inhibitory action. Reid and Bruce [30] have claimed that some strains from the mentioned species; *Lact. rhamnosus* GR-1 and *Lact. fermentum* RC-14 are useful for preventing and treating urogenital infections in women. This confirms once more the specificity of action of a particular probiotic strain.

**Table (3).** Mean  $\pm$  SE for *Lactobacillus spp.*isolated from vagina on solid MRS media by well method.

Bacteria	Mean $\pm$ SE			LSD value
	<i>Staphylococcus</i>	<i>Protus</i>	<i>E. coli</i>	
Lb1	16.67 $\pm$ 0.33	16.67 $\pm$ 0.88	12.67 $\pm$ 0.33	1.997 *
Lb2	13.67 $\pm$ 0.67	15.67 $\pm$ 0.33	9.67 $\pm$ 0.33	1.631 *
Lb3	14.33 $\pm$ 0.33	15.00 $\pm$ 1.52	9.67 $\pm$ 0.88	3.586 *
Lb4	14.67 $\pm$ 0.66	12.00 $\pm$ 0.58	10.00 $\pm$ 1.0	2.663 NS
LSD value	1.718 *	3.074 *	2.306 *	----

\* (P $\leq$ 0.05).

\* Significantly different in the level of (P<0.05) in the antagonistic activity among bacteria species.

### Conclusion

Lactic acid bacteria and its supernatants possessed an inhibitory ability against a number of pathogenic bacterial species used in the study. It was no inhibitory effect of the lactic acid bacteria supernatant when it was neutralized by NaOH and pH of supernatant reaching [7], which indicate that the increase in the pH resulted in inhibition of metabolic activity temporary or terminally, and finally refer to that activity is based on the decrease in the pH level. Lactic acid bacteria grown on MRS liquid medium showed an inhibitory activity more higher than those which are grown on MRS solid medium.

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