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Molecular study of vaginal *Lactobacillus acidophilus* carried class III bacteriocin gene and effect of different conditions on the presence of *Lactobacillus* in some Iraq women

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Abstract

Background: Vaginal *Lactobacillus acidophilus* has ability to bacteriocins production the greatly broader inhibitory spectra that bacteriocins make and different conditions effect on the presence of vaginal *Lactobacillus* .

Objective: Molecular study of vaginal *L. acidophilus* carried class III bacteriocin gen and effect of different conditions on the presence of vaginal *Lactobacillus*.

Patients and Methods: Vaginal swabs of healthy women from hospitals in Baghdad city for isolation of *Lactobacillus acidophilus* to study effect of different conditions on the presence of vaginal *Lactobacillus* were inoculated in MRS broth medium, after 24 h incubation in the presence of 5% CO_2 the specimens were sub-cultured on MRS agar, 11 isolates from 49 isolates identified by PCR technique were *Lactobacillus acidophilus* from 104 *Lactobacillus* isolates from total 214 vaginal swabs.

Results: The first identification presented (n=49)(22.9%) *L. acidophilus* isolates then using conventional polymerase chain reaction (PCR) with specific primers gene which showed that (n=11)(22.4%) isolates were *Lactobacillus acidophilus* carried class III bacteriocin gene with high molecular weight 4000bp, also 11 *L. acidophilus* isolates characterized by their ability to inhibit the growth of *Pseudomonas aeroginosa*, *Escherichia coli* and *Staphylococcus aureus* through the production of bacteriocin.

Conclusion: This study suggested highly significant difference during *Lactobacillus acidophilus* (n=11)(22.4%) isolates from (n=49)(22.9) isolates by PCR technique and highly significant difference during studied parameter effect on the presence of vaginal *Lactobacillus* (Antibiotic intake , hormonal contraceptive,age, intravaginal products for douching, menstruation and breast feeding).

Recommendation: 1- Conventional PCR technique is more accurate for identification.

2- Purification and characterization of *L. acidophilus* bacteriocin 3- Bacteriocin applied on catheter to prevent catheters problems by using a natural safe compound (bacteriocin) from *L. acidophilus* (normal flora) 4- Reduce used antibiotics, hormonal contraceptive, alkaline and acidic products for douching which effect on presence of *L. acidophilus* and on the production of bacteriocin 5- *Lactobacillus acidophilus* bacteriocin may be used an adjuvant or alternative therapy in bacterial vaginitis and/or urinary tract infection in women because they are considered safe, non-pathogenic and normal inhabitants of women urogenital tract.

Key words: *Lactobacillus acidophilus*, *Lactobacillus*, vagina, bacteriocin.

1. INTRODUCTION:

The vagina is an internal organ, is not sterile, such as the heart or pancreas, because it is opening is connected to the anus, it is a place with a very high numbers of intestinal pathogens bacteria for that reason it is necessary for the vagina to have protection system to prevent from the disorderly causing proliferation of microbes and the protection against growth of pathogen based on the presence of great numbers of non-pathogenic bacteria in the vagina, which prevent the presence of unnecessary microbes[1] and these bacteria are generally called Döderlein's bacillus, Döderlein who first discovered their occurrence in the vagina, *Lactobacillus acidophilus* is an obligatory homofermentative, that growth in the presence of 5% Co_2 and this bacterium lacks oxidase therefore unable to undergo oxidative and utilize sugars e.g. (Glucose, lactose, maltose, (galactose and sucrose) as substrates for fermentation which inhabit environments with high sugar[2].

Probiotic *L. acidophilus* must be of human origin, genetically stable and non-pathogenic[3].

Mechanisms of probiotic action, it is possible to get the inhibition of pathogens by production of lactic acid, Hydrogen peroxide (H_2O_2) and bacteriocin competitive elimination of pathogens by blocking adhesion sites, competition for nutrients and modulation of the immune system, included decrease of inflammation[4].

L. acidophilus play an important role in maintaining the balance of the active ecosystem of the urogenital tract and *L. acidophilus* can protect against urogenital tract infections by preventing pathogenic from aggregating and adhering to the epithelial surface with producing antimicrobial substances (hydrogen peroxide, organic acids and bacteriocins),competing for nutrients and stimulating the local immune response[5].

They also give benefits to the host, for example lactose intolerance easing, decrease cholesterol level by absorption of nutrition by the intestinal normal microbiota and dysbiosis ameliorating, inhibition of toxin production, destruction of toxin receptors in the intestine, maintain of normal intestinal pH, high intestinal movement and help to preserve the reliability of the intestine permeability[6] main compositional changes during a woman's life from childhood until puberty, the limited presence of estrogens suggests a low vaginal bacterial content, which occurs during reproductive years or through menopause, as estrogen levels drop, not only the epithelial fragility of vaginal mucosa is affected because the decrease in the thickness of the different cellular layers but also the vaginal flora because the decrease of *L. acidophilus* which depend on the presence of glycogen to survive for that reason the vagina loses amount of its natural defenses [7].

It is also that several factors influence the balance of the vaginal microbiota in one way or another through the long period of a woman's reproductive life. The more important ones are described(Menstrual cycle, coition, use of antibiotics, hormonal contraceptives intake, use of intravaginal products for douching such as alkaline products (Sodium bicarbonate) and acidic products such as (Alum) and breastfeeding.

2. METHODS:

2.1 Isolation and identification: Vaginal swabs were obtained.from 214 women between the ages of (18 – 50) years with healthy vaginal environments without urinary tract infection (UTI) and /or bacterial vaginitis. The samples were diluted to MRS broth, then incubated at 37°C for 24 h in the presence of 5% CO_2 [8].

The identification of *Lactobacillus acidophilus* done by PCR by DNA extraction method and by using specific primers[9] F:5-TGCAAAGTGGTAGCGTAAGC-3 R:5-CCTTTCCCTCACGGTACTG-3 were prepared depending on the manufacturer's instructions (Alpha DNA /Canada)and using DNA Ladder 1Kb.

Table1: PCR amplification program

Steps	Temperature	Time	Number of cycles
Initial Denaturation	94 °C	3min	One cycle
Denaturation	94 °C	30sec	35cycles
Annealing	57 °C	60sec	
Extension	72 °C	30sec	
Finally Extension	72 °C	5min	One cycles

PCR product was analyzed by gel electrophoresis.in.2% agarose containing red safe TM (Nucleic acid staining solution [10].

2.2 Detection of class III bacteriocin gene by (PCR): By using specific primers [11].
F:5- AAGAGTTTGATCCTGGCTCAG -3
R:5-CTACGGCTACCTTGTTACGA from Promega/ USA for detection of bacteriocin gene.

Table 2: PCR amplification program for detection of class III bacteriocin gene

Steps	Temperature	Time	Number of cycles
Initial Denaturation	95°C	3min	One cycle
Denaturation	95°C	30sec	35cycles
Annealing	61°C	40sec	
Extension	72°C	1min	
Finally Extension	72°C	5min	One cycle

The amplified PCR products were checked for the expected size on 1% (w/v) agarose gel and visualized after staining with Red Safe under ultraviolet transillumnater[12].

2.3Bacteriocin production

Agar-well diffusion assay was used by aliquots of 50 µl of the sterile supernatant were placed in 5 mm diameter wells on Muller-Hinton-agar plates against *Pseudomonas aerogenosa Escherichia coli* and *Staphylococcus aureus* [13].

Previously seeded with the respective indicator bacteria, after incubation18h at 37°C, the diameters of the zones of growth inhibition were measured [14] .

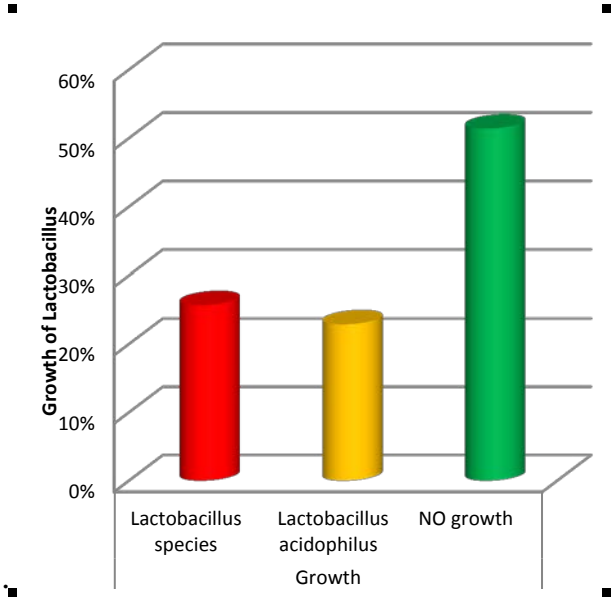
2.4. Statistical Analysis

The usual statistical methods were adjusted in order to assess and analyze the results according to Ying *etal.* In (2015) [15].

3. RESULTS:

Table (3): Bacterial growth of *Lactobacillus* results according to biochemical test

Growth of *Lactobacillus*	Number of isolates	Percentage	Kolmogorov-Smirnov Test (P-value)
Lactobacillus species	55	25.7%	P=0.00 Highly sign. (P<0.01)
Lactobacillus acidophilus	49	22.9%	
No growth	110	51.4%	
Total	214	100%	



Figure(1): Growth of *Lactobacillus* species and *L. acidophilus* in the presence of 5% Co_2 at 37°C .

Table (4): Mean age / Year comparison among growth of *Lactobacillus* results

Growth	N	Mean age /Year	Std. Deviation	Std. Error	Range		ANOVA test (P-value)
					Mini.	Maxi.	
Lactobacillus species	55	30.76	5.196	0.701	20	43	P=0.00 Highly sign. (P<0.01)
L. acidophilus	49	26.94	6.929	0.991	18	43	
NO growth	110	33.28	6.527	0.622	22	50	
Total	214						

Multiple Comparisons LSD test		
Growth		P-value
Lactobacillus species	*L. acidophilus*	P=0.002 Highly sign. (P< 0.01)
	NO growth	P=0.017 Sign. (P< 0.05)
L. acidophilus	NO growth	P=0.00 Highly sign. (P< 0.01)

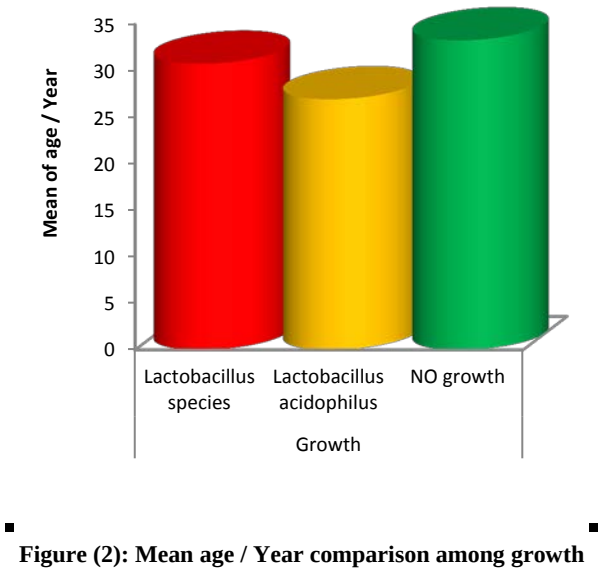
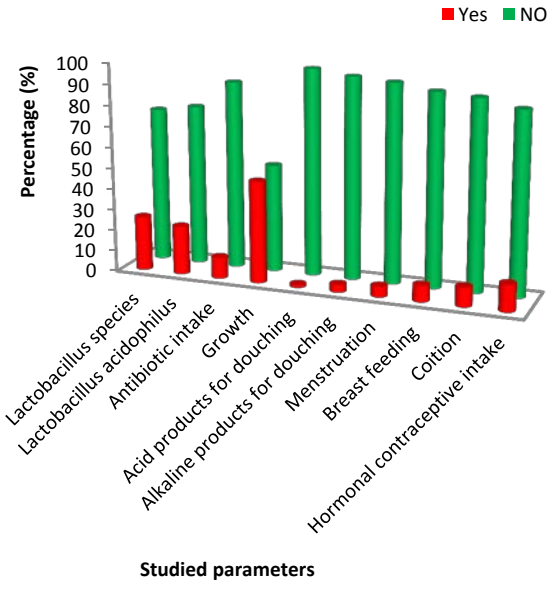


Figure (2): Mean age / Year comparison among growth

Table (5): Distributions of studied parameters according to bacterial growth.

Studied parameters	Yes (N=104)		NO (N=110)		Binomial (Z) Test (P-value)
	N	%	N	%	
Lactobacillus species	56	26.17	158	73.83	P=0.00 Highly sign. (P<0.01)
Lactobacillus acidophilus	50	23.36	164	76.64	P=0.00 Highly sign. (P<0.01)
Antibiotic intake	22	10.28	192	89.72	P=0.00 Highly sign. (P<0.01)
Growth	104	48.60	110	51.40	P=0.733 Non sign. (P>0.05)
Acid products for douching	3	1.40	211	98.60	P=0.00 Highly sign. (P<0.01)
Alkaline products for douching	8	3.74	206	96.26	P=0.00 Highly sign. (P<0.01)
Menstruation	11	5.14	203	94.86	P=0.00 Highly sign. (P<0.01)
Breast feeding	17	7.94	197	92.06	P=0.00 Highly sign. (P<0.01)
Coition before few hours	20	9.35	194	90.65	P=0.00 Highly sign. (P<0.01)
Hormonal contraceptive intake	28	13.08	186	86.92	P=0.00 Highly sign. (P<0.01)



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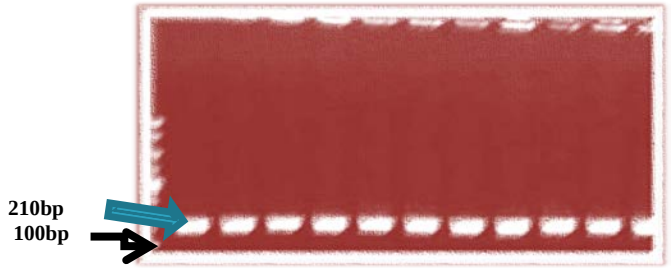


Figure (4) *Lactobacillus acidophilus* identification bands at 210bp by using Ladder 1kb and 2% agarose for electrophoresis.

Table (6): Distributions of *L. acidophilus* isolation according to PCR test results.

Growth			PCR		Total	Binomial (Z) Test (P-value)
			Positive	Negative		
	L. acidophilus	N	11	38	49	P=0.00
		%	22.4%	77.6%	100%	Highly sign. (P<0.01)

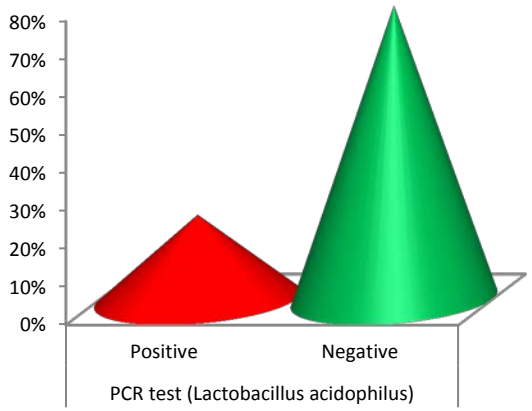


Figure (6): Distributions of *L. acidophilus* isolation according to PCR test results.

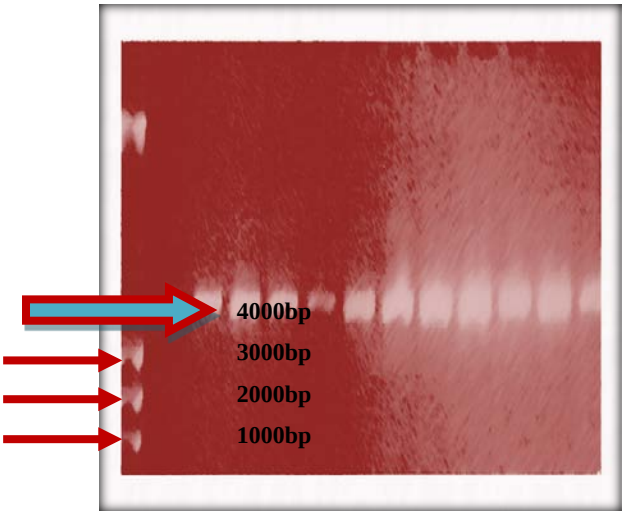


Figure (6) *Lactobacillus acidophilus* class III bacteriocin gene band 4000 bp by using Ladder 10 kb and 1% agarose for electrophoresis.

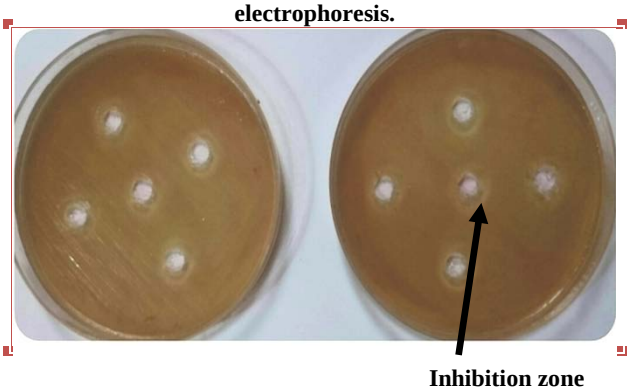
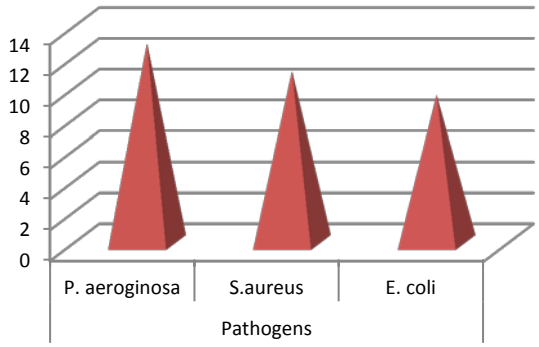


Figure (7) *L. acidophilus* inhibition zone against *P.aeroginosa* on Muller Hinton agar medium.

Table (7) Mean of *L. acidophilus* bacteriocin inhibition effect against pathogens (*P. aeroginosa, S.aureus and E. coli)* by agar diffusion assay.

Pathogens	N	Mean of inhibition zone(mm)	Std. Deviation	Std. Error	Range		ANOVA test (P-value)
					Mini.	Maxi.	
P. aeroginosa	11	12.9091	1.30035	.39207	11	14	P=0.00 Highly sign. (P<0.01)
S.aureus	11	11.0909	1.22103	.36815	9	12	
E. coli	11	9.5455	.52223	.15746	9	10	
Total	11						

Multiple Comparisons LSD test		
Pathogens		**P-value**
P. aeroginosa	*S.aureus*	P=0.00 Highly sign. (P<0.01)
	E. coli	P=0.00 Highly sign. (P<0.01)
S.aureus	*E. coli*	P=0.002 Highly sign. (P<0.01)



Figure(8): Mean of *L. acidophilus* bacteriocin inhibition effect against pathogens (*P. aeroginosa, E. coli* and *S. aureus)* by agar diffusion assay.

4. Discussion

Dependingfon.the.first.identification of bacteria, all 49 *Lactobacillus acidophilus* isolates were amplified with specific primers; 11 isolates (22.4%) produced a product 210 bp as in (figure 4).

The vaginal environment undergoes main compositionalichanges during a woman's life from childhood until puberty , the limited presence of…estrogens...suggests..a.low.vaginal .bacterial content, whichkoccurs during reproductive years or through menopause[7]. It is also that several factors (Menstrual cycle, coition, use of antibiotics, use of intra vaginal products for doughing and breastfeeding influence the balance of the vaginal microbiota.

Presented the results all 11 *Lactobacillus acidophilus* isolates were molecular identified carrying bacteriocin gene by amplified with the bacteriocin gene primers, all 11 isolates produced a product with high molecular weight 4000 bp as in (figure 6) therefore classified to classIII bacteriocin which agree with classification of bacteriocin according to molecular weight by Klan hammer in (1993) and Balciunas *etal.* in (2013)[16][17]

Lactic acid bacteria (LAB) are produced somelsubstances such as organiclacids hydrogen peroxide, carbon dioxide and bacteriocins[13].

Bacteriocins have been reported tokbe inhibitory against several other bacteria, most of bacteriocins produced

by Gram positive bacteria arelfrom lactic acid bacteria [18] .

Some LAB bacteriocins can inhibit the growth of Gram-positivelpathogenic bacteria and also inhibit the growth of some Gram-negativelspecies, therefore, such thesellactic acid bacteria canlbe used as probiotic [19] .

The isolates shown a different capacity to class III bacteriocin production under the same conditions of experimentation, the results represented the cell-free-supernatants exerted varying inhibitory effect on thelindicator pathogens and inhibition was assessed against *Pseudomonas aerogenosa Escherechia coli* and *Staphylococcus aureus* and thislstudy agree with Abo-Amer (2007)[20] and Kyoung-Sik *et al.* (2007)[21].

5. Conclusion:

This study suggested highly significant difference during *Lactobacillus acidophilus* (n=11) (22.4%) isolates from (n=49) (22.9) isolates by PCR technique and highly significant difference during studied parameter effect on the presence of vaginal *Lactobacillus* (Antibiotic intake, hormonal contraceptive, age, intravaginal products for douching, menstruation and breast feeding).

Recommendation:

1- Conventional PCR technique is more accurate for identification.
2- Purification and characterization of *L. acidophilus* bacteriocin.
3- Bacteriocin applied on catheter to prevent catheters problems by using a natural safe compound (bacteriocin) from *L. acidophilus* (normal flora).
4- Reduce used antibiotics, hormonal contraceptive, alkaline and acidic products which effect on presence of *L. acidophilus.* and on the production of bacteriocin
5- s*Lactobacillus acidophilus* bacteriocin may be used an adjuvant or alternative therapy in bacterial vaginitis and/or urinary tract infection in women because they are considered safe, non -pathogenic and normal inhabitants of women urogenital tract.

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