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Molecular Diagnosis of *Lactobacillus* Microbiota in Some Healthy Iraqi Women

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Abstract: Molecular diagnosis of *Lactobacillus* species resident in vaginal ecosystem in some healthy women of Baghdad city was investigated using 16S-23SInternal Transcribed Spacer region (ITS-1) in multiplex PCR reactions with two stages. The first stage used for grouping of Lactobacilli, followed by second stage multiplex reactions. Different PCR products derived from 35 vaginal swaps were obtained, 10 of these products were chosen randomly and subjected for sequencing. Sequences alignment and *in silico* diagnosis using BLAST against nr databases, showed that *Lactobacillus crispatus* comprised the majority (40%),followed by *Lb. gasseri* (30%), the rest *Lb. jensenii*, *Lb. acidophilus* were 10% for each. It was noticed that *Lb. crispatus* and *Lb. gasseri* showed a little variation which is expected due to inter individual and environment variation.

Key words: Vaginal Lactobacilli, Multiplex PCR, Iraqi Women, Molecular Diagnosis , *Lb. crispatus, Lb. gasseri*

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Introduction

It is well known that the vaginal microbiota of healthy women of reproductive age is dominated by *Lactobacillus* species (1) and can effect human vaginal environment in different ways (2), in addition bacterial biota can have a profound impact on the health of women and their neonates (3). Studies showed that human vagina hosts numerous bacterial species that are either uncultivated or not easily identified using cultivation methods (3), so the healthy females vaginal flora were characterized by different approaches and had been found that *Lactobacillus acidophilus* is the most dominated, and followed by *Lactobacillus crispatus, Lb. gasseri, Lb. jensenii*as recently shown using sophisticated analysis methods (1,4).

Changes in vaginal microbiota have been associated with different adverse outcomes, so it is important to identify what kind of *Lactobacillus* species are present in the vaginal ecosystem and which species are most likely to have the potential protective effects (2,3). It is widely recognized that identification of Lactobacilli to species or strain levels on the basis of physiological and biochemical criteria is very ambiguous and complicated ,therefore ,culture – independent approaches have consistently documented the diagnosis of fastidious bacteria in a variety of ecological niches . The new molecular techniques for microbial community analysis that do not require isolation of the microorganisms are very promising (3, 5), especially for accurate analysis of major vaginal commensals (1). Choosing the target for amplification is

quite important, many studies carried out the amplification and sequencing of some housekeeping genes such as 16SrRNA (6,7). Diversity studies based on the 16S rRNA genes are with considerable limitations (3), since The precise results using 16SrRNA sequencing needs the whole gene sequencing which is big due to presence of variable regions (8). Therefore it has been suggested to use 16S-23S region (Internal Transcribed Spacer ITS-1), this region accumulates mutation and are more variable ,and is small region in Lactobacilli , it has been used to identify closely related species (2,5,9). An accurate identification to species level is fundamental for each of estimation of microbiota members to introduce strains for probiotic use (9,10). The aim of this work was to identify the *Lactobacillus* species by non-cultured methods using PCR of DNA in a single multiplex reaction for healthy swaps of Iraqi women.

Materials and Methods

Thirty five healthy women were enrolled for this study ,they had no medicines for at least 30 days and all of them at reproductive age (13-50 years), Vaginal swaps were collected from these women in Health Centers at North-East of Baghdad city. DNA extraction and purification were done as previously mentioned by using genaid Kit (11).

PCR reactions were performed by multiplex PCR in two stages using the primers based on those suggested previously (2,12), as shown in (Table 1).

Table 1: Primers used in this study

First stage		**Primer sequence**		**Amplicon Size**
Group I	Ldel-7	ACAGATGGATGGAGAGCAGA	*L. delbrueckii*	450 bp
	Lac-2	CCTCTTCGCTCGCCGCTACT		
Group II	LU-1P	ATTGTAGAGCGACCGAGAAG	Go to PCR 1	300 bp
	Lac-2	CCTCTTCGCTCGCCGCTACT		
Group III	LU-5	CTAGCGGGTGCGACTTTGTT	Go to PCR 2	400 bp
	Lac-2	CCTCTTCGCTCGCCGCTACT		
Group IV	LU-3P	AAACCGAGAACACCGCGTT	Go to PCR 3	350 bp
	Lac-2	CCTCTTCGCTCGCCGCTACT		

Second stage

	Primer	5'-3' sequence	Strain	Amplicon Size
PCR 1	Laci-1	TGCAAAGTGGTAGCGTAAGC	*L acidophilus*	210bp
	23-10C	CCTTTCCCTCACGGTACTG		
	Ljen-3	AAGAAGGCACTGAGTACGGA	*L. jensenii*	700bp
	23-10C	CCTTTCCCTCACGGTACTG		
PCR 2	Lcri-3	AGGATATGGAGAGCAGGAAT	*L. cripatus*	522bp
	Lcri-2	CAACTATCTCTTACACTGCC		
	Lgas-3	AGCGACCGAGAAGAGAGAGA	*L. gasseri*	360bp
	Lgas-2	TGCTATCGCTTCAAGTGCTT		
PCR 3	Lpar-4	GGCCAGCTATGTATTCACTGA	*L. paracasei*	312bp
	LU-5	CTAGCGGGTGCGACTTTGTT		
	RhaII	GCGATGCGAATTTCTATTATT	*L. rhamnosus*	113bp
	LU-5	CTAGCGGGTGCGACTTTGTT		
PCR 4	Lfer-3	ACTAACTTGACTGATCTACGA	*L. fermentum*	192bp
	Lfer-4	TTCACTGCTCAAGTAATCATC		
	Lpla-3	ATTCATAGTCTAGTTGGAGGT	*L. plantarum*	248bp
	Lpla-2	CCTGAACTGAGAGAATTTGA		
	Lreu-1	CAGACAATCTTTGATTGTTTAG	*L. reuteri*	303bp
	Lreu-4	GCTTGTTGGTTTGGGCTCTTC		
	Lsal-1	AATCGCTAAACTCATAACCT	*L. salivarius*	411bp
	Lsal-2	CACTCTCTTTGGCTAATCTT		

Ref (12,13)

The PCR program used is shown in Table 2

Table 2 : PCR program used in this study

First step	Temperature	Time	Number of cycle
Pre- denaturation	95ºC	5min	**1**
Denaturation	95ºC	30sec	**35**
Annealing	55ºC	2 min	
Extension	72ºC	2 min	
Final extension	72ºC	5min	**1**
Second step	**Temperature**	**Time**	**Number of cycle**
Pre-denaturation	95ºC	5min	**1**
Denaturation	94ºC	1 min	**30**
Annealing	56ºC	1 min	
Extension	72ºC	1 min	
Final extension	72ºC	5min	**1**

Ten DNA samples were chosen randomly for sequencing (Macrogene,USA)

BLASTNhttp://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) was used for searching and identification of the sequences ,using database and organisms *Lactobacillus* (Taxid 1578) with expected value 0.05 and the rest parameters were default of program.Criteria for *in silico* identification were dependented on percent of identity (%ID), E-values (14), Query coverage , Max identity score combined with Total identity score. The ranges and candidate Accession numbers for strains were recovered from BLAST report. Sequences were aligned using Clustal W program and NJ phylogenetic tree were performed using (Mega 6.0) (15,16).

Results and Discussion

First round of PCR reaction led to dominated Lactobacilli in to different groups, which subjected to second round of PCR reactions (11,12,13), as results are shown in figure 1.

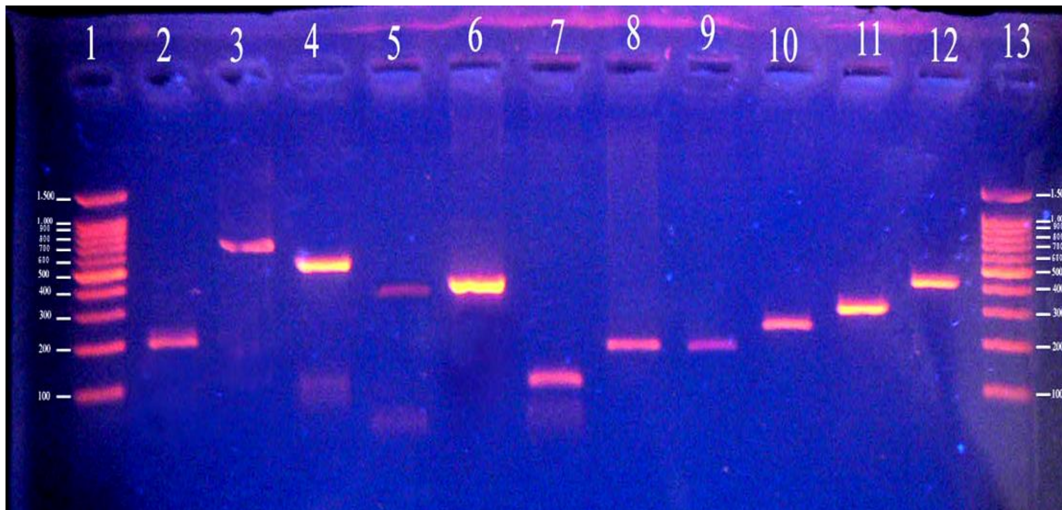

Figure 1 : PCR electrophoresis ; 1,13: DNA ladder (100 bp) , Lane 2 : *Lb. acidophilus* (210 bp) , Lane 3 *Lb. jensenii* (700 bp) , Lane 4 : *Lb. crispatus* (522 bp) , Lane 5 : *Lb. gasseri* (360 bp) , Lane 6 : *Lb . delbrueckii* (450 bp), Lane 7: *Lb. rhamnosus* (113 bp), Lane 8: *Lb.fermentum* (192 bp), Lane 9 : *Lb. plantarum* (248 bp), Lane 10: *Lb. reuteri* (303 bp), Lane 11 : *Lb. paracasei* (312 bp) , Lane 12 : *Lb. salivarius* (411 bp).

Random selected DNA products (10 samples) were subjected for sequencing. The sequences were processed and checked using BLAST program. Identification of bacteria were deduced according to different criteria (13) and the results are shown in (Table 3and figure 2).

Table 3: Blast report

Strain ID	E value	% ID	Max Score & Total Score	Query cover	Range & Accession #
Lb. paracasei	5e-135	99	490, 490	98	88-362 **AB035487.1**
Lb.jensenii	0	98	994 ,994	89	79–644 **AB035486.1**
Lb.acidophilus	1e-40	91	178 ,178	20	391910- 392044 **AB092642.1**
Lb. gasseri 1	2e-154	96	555 ,555	95	292 – 629 **DQ445775.1**
Lb. gasseri 2	2e-130	92	475 ,475	94	377207– 377549 **DQ445775.1**
Lb. gasseri 3	1e-161	99	579 ,579	96	377186 -377508 **DQ445775.1**
Lb.crispatus 1	6e-155	99	556, 556	94	325 – 635 **EU161625.1**
Lb.crispatus 2	3e-157	99	564 ,564	94	324- 635 **EU161625.1**
Lb.crispatus 3	3e-152	98	547 ,547	93	324 – 635 **EU161625.1**
Lb.crispatus 4	2e-154	99	555 ,555	91	333 – 635 **EU161625.1**

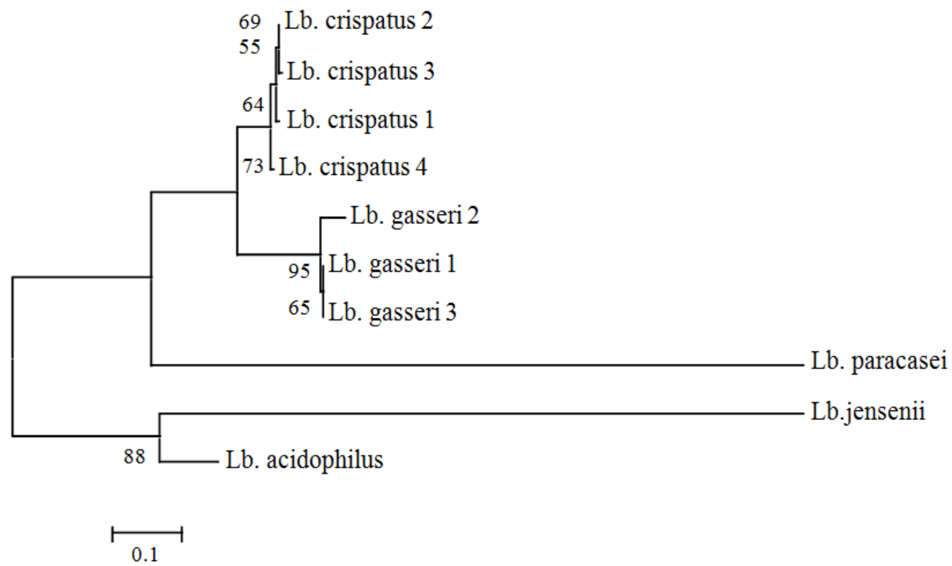

Figure 2 :Relationship of the isolates using NJ method

As shown *Lb. crispatus* represented highest percent (40%), followed by *Lb. Gasseri* (30%). The phylogenetic relationship is shown in (Figure 2). It is obivous that *Lb. crispatus* grouped togather as well *Lb.gasseri* strains,this confirmed in (Figure 3 A and 3B).

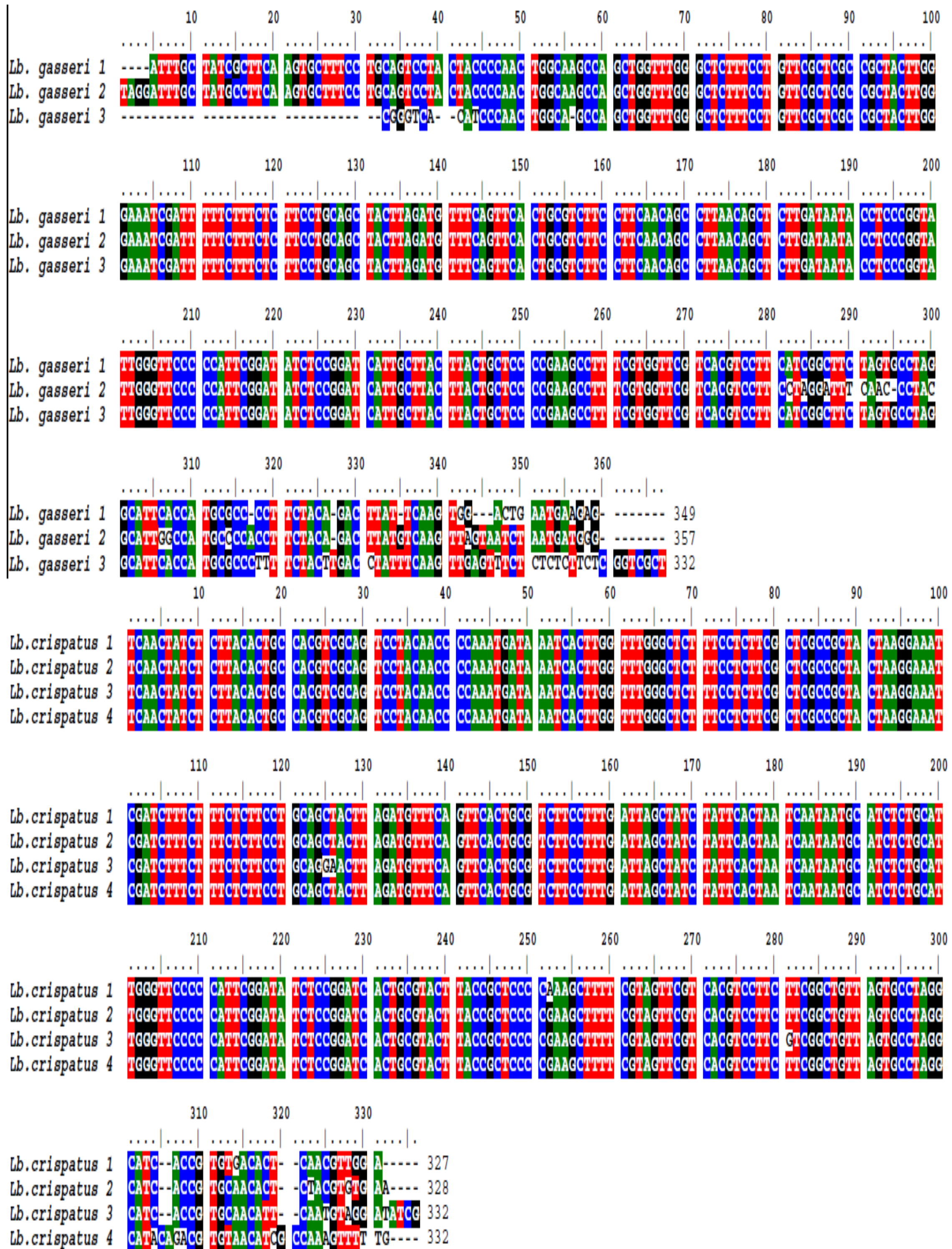

Figure 3A: Sequence alignment of *Lb.gasseri* and *Lb.crispatus*ITS regions

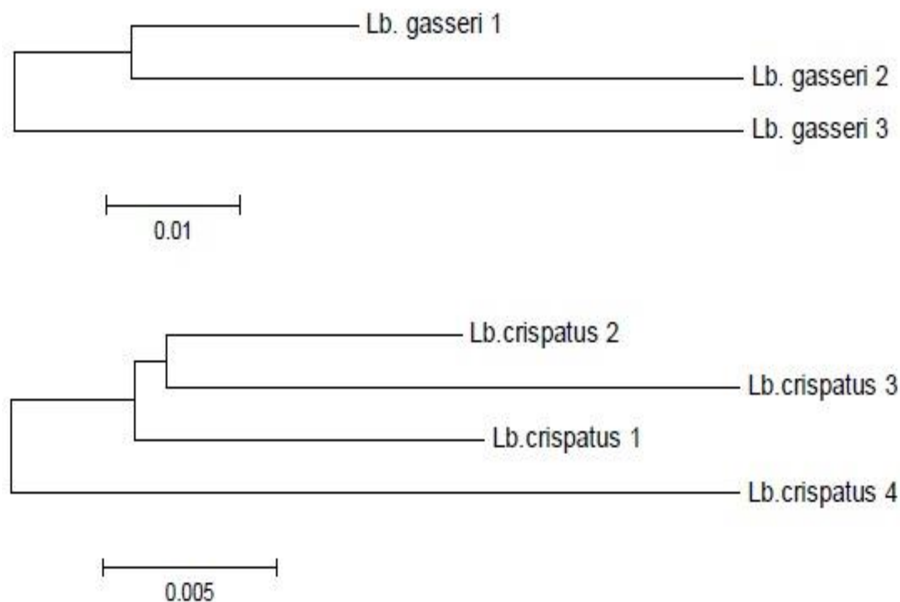

Figure 3B: Phylogeny of *Lb.gasseri* strains and *Lb.crispatus* strains

Lactic acid bacteria are commonly used for animal and human consumption due to their probiotic properties .One of the major genera used is *Lactobacillus* ,a highly diverse genus comprised of several closely related species , *Lactobacillus* genus is extremely diverse with about 190 known species (9,17) . Probiotic ability is often strain dependent and it is impossible to distinguish strains by basic morphological methods. 16SrRNA presents the most common target region for phylogenetic analysis at the species level and sequence data of this region can be used for taxonomic purposes (18), in addition rrn sequences are important tools for inter and intraspecific identification (19) . Intergenic spacer regions (ITS) of 16S-23S rRNA are commonly used to identify lactic acid bacteria especially *Lactobacillus* species (18,20) and it has been suggested that ITS has a promising nature for evolutionary studies and is recommended for microdiversity analyses (9) , as this region are under minimal selective pressure during evolution and therefore should vary more extensively than sequences within genes that have functional roles (21,22).

On the other hand, chosing the PCR primers from 16S-23S rRNA was not always successful at least for some target species, since the ITS in Lactobacilli is not long enough and not variable enough to choose specific primers for multiplex PCR which led some researchers to use more than one step PCRing process (12,13,as shown in this study) , and this could led to miss some species .The limitations can be exacerbated by other reasons such as the quality and exhausitivity of databases, and if the database is not complete (no enough strains of different species or different origins),then the results will not be a good as they could be (6) .The results of this study are in agreement

with the results from other parts of the world in that *Lb.crispatus* is the major *Lactobacillus* in healthy women depending on molecular diagnosis as in India (23), and China (24).It has been demonstrated that the presence of *Lb.crispatus* and *Lb. jensenii* is recognized as important marker of vaginal health ,and this species is consistently negatively associated with bacterial vaginosis by either microbiological or clinical criteria(10,25,26).However, the sequence data variation were detected within different strains (as shown in Figure 3A and 3B), although the sequence similarity was very high (21). It was found that groups of bacterial clusters were associated according to ethic groups and considerable inter-and intraindividual differences have been documented (27,28,29) .

Concerning *Lb.iners* which would not be expected in this study since no primers specific were used, in addition, it has been found that the dominance of *Lb. iners* might indicate an abnormal flora or the tendency towards bacterial vaginosis and during the pregnancy (5,10) , *Lb.iners* is unable to grow on MRS medium and was placed into *Lb. acidophilus* complex group according to 16S rRNA gene sequencing so it has been left in this study (10,29) .

The differences shown in Figure 1and Table 1 (see Materials and Methods) would be expected , as 16S-23S ITS may vary in size with different operons and type of tRNA included in it , as the bacteria get many operons of rrn (8) . It has been shown that the number of rrn operons in lactic acid bacteria vary from 2 in *Lb. brives* (30) , to 6 in *Lactococcus lactis* and *Lb.delbraeckii* (31,32,33). In addition the sequence of different rrn operons existing in a given genome was found to vary up to 5% (34,35,36). This means that heterogenicity was found between the different rRNA encoded in the same genome (30)and the spacer region were those which exhibited the highest degree of variation (37).

Finally, the vaginal microbiome is shown to have a higher stability than other bodily habitats , namely the oral regions ,skin and distal gut (38), and its flora members has a presumptive difference in physiological roles in the vaginal environment ,and up to date, many efforts have been made to develop administrable probiotic *Lactobacillus* species to reduce the recurrence of infections and promote healthy vaginal flora (1,4). In Iraq the folk medicine practice the use of yoghurt to get rid of vaginosis as one of this applications.

In Conclusion ,the probiotic properties is strain dependent and could work in the right place not in the vagina only, but could antagonist colon cancer in rats either *in vitro* or *in vivo* (39) , and some *Lb.acidophilus* strains exert antagonistic effect against different human cancer cells by induction a apoptosis (40) .

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