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Lactobacillus reuteri and *Lactobacillus salivarius*, Two Prevalent Vaginal Species Isolated from Healthy Women in Western Algeria.

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Abstract

Background & objectives: *Lactobacillus* species or their metabolites (such as lactic acid or bacteriocin) are known to protect the vagina from urogenital infections and to maintain vaginal hygiene. The goal of the current study was to identify lactobacilli species for a possible probiotic and postbiotic perspective. In Mostaganem city (Algeria), an effective molecular detective technique for lactobacilli in the vagina of women is seriously lacking. Therefore, our current studies promise to contribute to molecular diagnostic.

Materials and Methods: 37 samples of vaginal lactobacilli from 32 healthy women were isolated. By using polymerase chain reaction (PCR), strains were identified. For identification, genus specific, group specific and species-specific PCR protocols were implemented.

Results: All isolates were confirmed as spp. *Lactobacillus.* All 37 isolates belonged to Group IV of the *Lactobacillus* genus and were further divided into two *L. reuteri* and *L. salivarius* species.

Result interpretation & conclusion: In this study, the prevalent species in vagina were *L. reuteri* (70, 27%), followed by *L. salivarius* (29,73%). The probiotic potential of *L. salivarius* and *L. reuteri* from vaginal origin has not been fully exploited yet but studies to date have shown that these two species may be reliable candidates for use as new probiotics or as 'postbiotic' metabolites.

Keywords: Vaginal lactobacilli; Multiplex PCR; *Lactobacillus reuteri*; *Lactobacillus salivarius*; Probiotics; Postbiotics.

1. Introduction

The presence of beneficial bacteria which product lactic-acid- in the vaginal area, particularly from the *Lactobacillus* genus, is what defines vaginal eubiosis. *Lactobacillus* spp. can produce vaginal eubiosis by killing healthy and pathogenic bacteria by lactic acid, either natively or as probiotics. Other antimicrobial factors can also be released, such as bacteriocins (Aroutcheva et al., 2001; Selle and Klaenhammer, 2013).

Recent studies have shown that lactic acid (O'Hanlon, 2011; Gong et al., 2014) is the key antimicrobial factor produced by lactobacilli. It was proposed by Gil et al. (2010) that *L. salivarius* was the top source of lactic acid. The benefits of *L. reuteri* have also been enumerated by various reports as a probiotic (Indrio et al., 2008; Spinler et al., 2008; Hou et al., 2015). *L. reuteri* in both bacteriocidal and bacteriolytic forms, produced an antimicrobial agent (bacteriocin) which was active against sensitive cells (Kawai et al., 2001). Bacteriocins generation by *L. salivarius* has been also identified (Barrett et al., 2007; Strahinic et al., 2007; Busarcevic et al., 2008).

A clearer understanding of each population's natural vaginal biota seems important in order to suggest improved probiotics. Some studies have analyzed vaginal microbiota and reported numerous findings from different populations (Vasquez et al., 2002; Zhou et al., 2007). Some studies have confirmed that *L. crispatus*, *L. gasseri, L. jensenii*, and *L. iners* were predominated in healthy vaginal microbiota (Ravel et al., 2011), but different findings were documented in other studies (Garg et al., 2009; Damelin et al., 2011).

Within the *L. acidophilus* the closely related species it is really challenging. and often hard to distinguish *acidophilus* complexes by phenotypic techniques (Klein et al.,1998). Furthermore, so many of the distinctions of vaginal *Lactobacillus* observed in numerous researches may be due to the lack of effective detection methods (Redondo-Lopez et al., 1990; Zhong et al., 1998). Accurate molecular biological methods are therefore required to identify the presence or type of vaginal

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Lactobacillus, not just for infection therapy, but also to restore the natural microbiota in a variety of conditions.

Multiplex PCR methods have also been used for the simultaneous identification and discriminating of a large number of bacteria. Multiplex PCR was performed utilizing 16S rRNA or 16S-23S rRNA intergenic spacer region (ISR) primers based on sequences unique to each species encompassing the 16S rRNA or 23S rRNA flanking region (Schleifer et al., 1995).

Probiotics, particularly *Lactobacillus* species, have gained prominence in recent years. There are few studies on *L. salivarius* and *L. reuteri* from the human vagina and their use as a probiotic or its 'postbiotic' metabolites to prevent or treat vaginal disorders, opening the door to this important public health issue.

2. Materials and methods

2.1. Specimen collection

Vaginal isolates were collected by a midwife from 32 volunteer women working in Oued El Kheir public health unit in Mostaganem city (Algeria), who were premenopausal, non-menstruating between the ages of 18 and 45. For these experimental purposes, volunteer consent was obtained. With cotton swabs, vaginal samples were taken aseptically.

2.2. Lactobacillus strains isolation from vaginal swabs

Once vaginal swab samples were collected, they were sent to the bacteriology laboratory of Aïn-Tedeles Public Hospital, in Mostaganem city (Algeria). The swabs were inoculated into 5 ml MRS (de Man, Rogosa, and Sharpe) broth and allowed to enrich for 24 hours at 37 °C. MRS plates were streaked with the enhanced bacterial suspension and incubated at 37 °C for 48 hours under anaerobic conditions (Gordana et al., 2011).

2.3. Phenotypic characterization

All isolates were first examined for colony shape, Gram stain, catalase, and oxidase activity. These studies were carried out in the city of Mostaganem's Microbiology and Plant Biology laboratory (Algeria). All isolates that were gram-positive and catalase-negative, were stored in 30 percent glycerol at -80 °C.

2.4. Molecular Diagnosis

Genotypic identification of the lactobacilli isolates was carried out at Microbiology Laboratory in the Faculty of Veterinary Medicine at Mustafa Kamel University, Antakya city (Turkey).

2.5. Genomic DNA extraction:

Isolates were streaked on MRS agar for nucleic acid isolation. Only one colony from each type of strain resuspended in 500 µl of sterile PBS (phosphate buffer saline pH: 7.2) after an overnight incubation at 37°C in microaerophilic conditions. Bacterial cells were collected by centrifugation at 3000 x g for 10 minutes, then resuspended in 350 µl TE buffer [10 mM tris chloride, 1 mM EDTA (pH8.0)] with 20 mg of lysozyme (Sigma, USA) per ml and incubated at 37°C for 60 minutes. Every 15 minutes, each tube was vortexed. Then 350 µl 10 percent SDS was added, along with 100 g proteinase-K (Vivantis Technologies, Malaysia) per ml, then incubated for 60 minutes at 37°C. According to Sambrook and Russell, (2001) the phenol/chloroform extraction technique was employed for nucleic acid extraction. The precipitate of DNA was diluted in 100 µl of TE buffer [10 mM Tris chloride-1 mM EDTA (pH 8.0)], and kept at -20°C till use.

2.6. PCR analysis

2.6.1. Genus Specific PCR:

Amplification using the genus-specific primer LbLMA-rev (5' CTC AAA ACT AAA CAA AGT TTC3') and the universal primer R16-1 (5' CTT GTA CAC ACC GCC CGT TCA3') was used to identify each isolate at the genus level. Each PCR was carried out in a 25 µl volume of the reaction comprising 2 µl of DNA that has been extracted, 2.5 µl of the Taq buffer (10X, Vivantis, Malaysia), 200 µM of each of the deoxynucleotide triphosphates (dNTP), 20 µM of each of the forward and reverse primers, and 1 U of the Taq DNA polymerase (Vivantis, Malaysia).

The reaction protocol was set at initial denaturation at 94°C for 5 minutes was followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C, and extension at 72°C for 30 seconds and 7 minutes (Dubernet and Desmasures, 2002).

2.6.2. Group Specific Multiplex PCR

After verifying the genus and to establish which group an isolate belonged to, multiplex PCR-G was employed using a primer mixture that contains equimolar amounts of four forward primers, Ldel 7(5'ACAGATGGATGGAGAGCAGA3'), LU-1'(5'ATTGTAGAGCGACCGAGAAG3'), LU 3'(5'AAACCGAGAACACCGCGTT3'), LU-5' (5'CTAGCGGGTGCACTTTGTT3') and 1 common reverse primer Lac-2 (5'CCTCTTCGCTCGCCGCTACT3') as per Song *et al.* (2000).

Each PCR was carried out in a 25 µl reaction volume containing 2 µl of extracted DNA, 2.5 µl of the Taq buffer, 200 µM of each dNTP, 20 µM of each of reverse and forward primers, and 1 U of the Taq DNA polymerase. for group specific PCR, a program of denaturation at 95°C for 1 minute, then 35 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 2 minutes, and extension at 72°C for 30 seconds and 7 minutes thereafter, was employed.

2.6.3. Species Specific Multiplex PCR

After group detection, a species-specific multiplex PCR was used according to Song *et al*. (2000), using the primers shown in **table 1**. Each PCR was carried out in a 25 µl the volume of the reaction containing 2 µl of the DNA that has been extracted, 2.5 µl of the Taq buffer, 200 µM of each dNTP, 20 µM of each of the forward and reverse primers, and 1 U of the Taq DNA polymerase. PCR program for species specific multiplex PCR was employed at denaturation at 95°C for 3 minutes then, 35 cycles of denaturation for 20 second at 95°C, annealing for 60 second at 60°C and extension for 60 sec at 72°C and thereafter 7 minutes for final extension.

Table 1: Species that have been identified by PCR

Program	Primer (5'-3' sequence)	Species	Amplicon (bp)
	Lsal-1	*L. salivarius*	
	AATCGCTAAACTCATAACCT		411
	Lsal-2		
	CACTCTCTTTGGCTAATCTT		
	Lreu-1		
	CAGACAATCTTTGATTGTTTAG	*L. reuteri*	303
	Lreu-4		
PCR- IV	GCTTGTTGGTTTGGGCTCTTC		
	Lpla-3	*L. plantarum*	
	ATTCATAGTCTAGTTGGAGGT		
	Lpla-2		248
	CCTGAACTGAGAGAATTTGA		
	Lfer-3		
	ACTAACTTGACTGATCTACGA	*L. fermentum*	192
	Lfer-4		
	TTCACTGCTCAAGTAATCATC		

2.7. Agarose Gel Electrophoresis and Imaging

Electrophoresis of aliquots of the amplified products in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2) on 2% agarose gels was performed. Ethidium bromide was used to stain the gels, which were then viewed under UV light. As a molecular mass marker, a 100-bp polymer was employed (Vivantis).

3. Results and Discussion

3.1. Molecular identification

The results of genus specific PCR are shown in **fig.1,** which indicates that all vaginal isolates tested gave an amplicon of about 200 bp long. In the results of group specific PCR, all isolates detected belong to group IV with amplicons of 350 bp and in the species-specific PCR detection two different species were identified. The amplification products of group and species-specific PCR were shown in **fig.2**.

In the analyses of species, 26 (70, 27%) of isolates were found to be *L. reuteri* (303 bp) and 11 (29, 73%) *L. salivarius* (411 bp). These 2 species of *Lactobacillus* belong to group IV **(fig.2).**

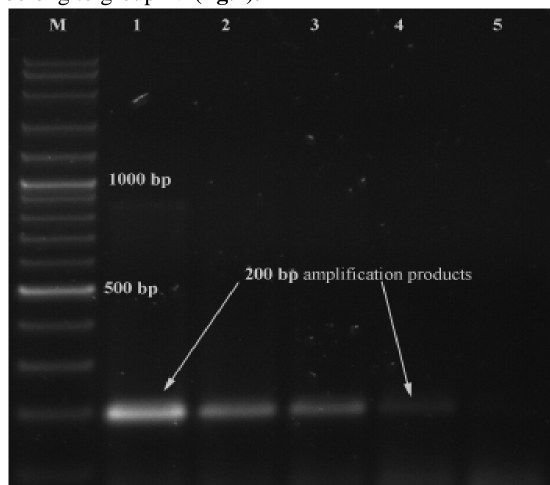

Figure 1. Genus-specific PCR results that are representative of 5 isolates on 2% agarose gel.

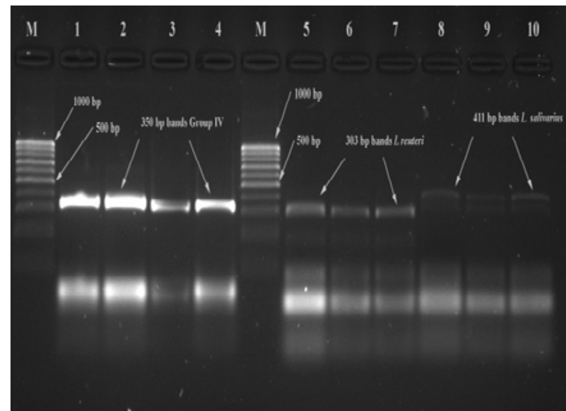

Figure 2 Agarose gel electrophoresis of PCR products from multiplex PCR-G and species-specific multiplex PCR.

It was necessary to first identify the *Lactobacillus* species that colonize women in this region before administering exogenous *Lactobacillus* or their metabolites to preserve vaginal health in west Algerian women.

Vaginal isolates were characterized in our sample base d on cell morphology and physiological and biochemical studies.

Our finding reveals difficulties in separating these isolates on the basis of tests for the fermentation of carbohydrates, which are also supported by Chagnaud et al. (2001) who also reported the reference *L. salivarius* UCC43321 strain as *L. Paracasei* with this technique. Thus, phenotypically characterized strains, even those with a good level of identification, could be misidentified. According to Song et al. (1999), API tests typically identify *L. acidophilus* isolates as belonging to the species *L. crispatus.*

Molecular techniques, such as PCR, can reduce observer-dependent errors and offer more comprehensive and precise findings than biochemical approaches (Yan et al., 2009). On the basis of Gram positivity and catalase negativity, genomic DNA was amplified from 37 vaginal isolates of 32 women cultured on MRS medium and identified as *Lactobacillus*. By genus-specific PCR **(Fig 1),** all of the isolates examined had an amplicon of around 200 bp, indicating that they all belonged to the *Lactobacillus* genus (Garg et al., 2009).

The group-specific PCR utilized a combination of one reverse primer and forward primers to distinguish *Lactobacillus* into classes I, II, III, and IV. A total of 100% of the isolates with a 350 bp amplicon were found to belong to group IV **(Fig 2)**.

In a study conducted by Garg et al. (2009), 80 vaginal *Lactobacillus* isolates using group-specific PCR were examined. They found that 80% of isolates belonged to Group IV, 13.75% to Group II, and 6.25% to women's Group III. There was no isolate belonging to Category I.

All the 37 isolates were analyzed at species level, by species specific primers. These species were identified as *L. reuteri* and *L. salivarius*. These two species which are part of the usual vaginal microbiota were tested for their essential characteristics as possible probiotics (data not shown). Garg et al. (2009) found that *L. salivarius* (16.25%), *L. fermentum* (25%), and *L. reuteri* (32.5%) were the most frequent species in women's vaginal

Lactobacillus species in Delhi, northern India, when they used the same primers, we used.

While the species *L. crispatus* and *L. jensenii* were discovered in Mysore, southern India, and are similar to those identified in American women studied by Pavlova et al (2002), such variations imply that vaginal *Lactobacillus* species can differ across Indian subpopulations. *Lactobacillus crispatus*, *L. gasseri*, and *L.jensenii* are the most widely cultivated vaginal lactobacilli, according to studies from throughout the world (Chaban et al., 2014; Mendes-Soares et al., 2014; Van de Wijgert et al., 2014).

L. acidophilus and *L. fermentum* dominated the vaginal biota of healthy women in the past, *L. brevis*, *L. jensenii*, and *L. casei* are the next species., and other species (Lachlak et al., 1996). It is also worth noting that the quantity of prevalent lactobacilli differs significantly between women from various ethnic groupings. For example, *L. crispatus*, *L. iners, L. gasseri*, and *L. jensenii* were found in 91%, 75%, 67%, and, 43% of healthy Chinese women, respectively (Yan et al., 2009). But the abundance of these lactobacilli in Iranian women was 66.7%, 55%, 29.6% and, 29.6% respectively for the same species (Motevaseli et al., 2013).

We also note major variations in Algeria, with two species, *L. iners* and *L. delbrueckii*, identified in the study conducted by Alioua *et al*. (Alioua et al., 2016) on 15 pregnant women in Annaba, eastern Algeria. Although *L. gasseri* and *L. plantarum* were found in Jijel, northeastern Algeria, they were obtained from 60 healthy women (Bouridane et al., 2016). *L. fermentum* was the dominant species identified by Ouarabi *et al*. (2017), followed by *L. plantarum* in 10 healthy women from Bejaia, another region in eastern Algeria.

It is crucial to understand that the presence of certain health-promoting LAB (Lactic Acid Bacteria) genera in the vaginal canal, such as *L. reuteri*, could be a source for inoculation of newborn babies' guts during vaginal delivery for initial colonization of the neonates' colon with these beneficial bacteria (Al-Balawi and Morsy, 2020).

4. Conclusion

This study shows that there are similarities and variations between the vaginal lactobacilli composition found in Algeria and those in environments that are markedly different.

The vaginal biota of Algerian women is little studied., and particularly in the western region, so our research fills a gap in the literature. The results of this study also indicate that *L. reuteri* and *L. salivarius* are promising candidates for probiotics or postbiotics that are usually isolated from healthy Algerian women.

These findings suggest that further research is needed into the microbial variety of women's vaginal biota in different regions of the world and how it impacts vaginal health. Because of the current study's limitations, metagenomic research to identify the whole vaginal microbiome may be performed in the future. Our results will need to be verified in a larger study utilizing molecular techniques and long-term research designs paired with data on participants' activities in order to further characterize this important defense and to help women suffering from vaginal ecosystem disruptions.

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Data availability statement

This published paper contains all of the data collected or evaluated during this investigation.

Contribution of the Author

BN conceived the presented idea, developed the theory, and wrote the manuscript with support from EY and DR. EM's encouragement to begin this work.

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Declarations

Conflict of interest: The authors state that no commercial or financial ties were present throughout the research. Therefore, a conflict of interests does not exist.

Ethical approval

The individuals who provided us with their samples for this study had given their consent. There were no animals involved in this study.

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