International Scholars Journals

African Journal of Parasitology Research ISSN 2756-3391 Vol. 7 (12), pp. 001-005, December, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Isolation and identification of *Lactobacillus* species from the vagina and their antimicrobial properties

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Accepted 07 November, 2020

The microbial resident in the vagina is a heterogeneous flora containing bacteria. This microbial flora plays an important role in regular vagina function and support host defense from attack by pathogens. In this study *Lactobacilli* spp. isolated from healthy women and their antimicrobial activity was evaluated. One hundred and three healthy women referred to women clinics in Ahvaz-Iran were subjected for this study. A vaginal swab of each one inoculated in MRS broth media. After 24 h incubation the specimens were subcultured on MRS agar media. The Gram positive bacilli where isolated for more identification by using polymerase chain reaction (PCR) with genus and species-specific primers. The antimicrobial activity of confirmed lactobacilli where tested against *Candida albicans* and *Staphylococcus aureus*. Fifty-one samples out of 103 were positive for *Lactobacillus* genus primers. Twenty-four isolates were *Lactobacillus acidophilus* and two species were identified as *Lactobacillus plantarum* and 2 as *Lactobacillus casei*. Twenty-three other isolates were just positive by genus primer. Twenty strains of *L. acidophilus* and one strain of *L. plantarum*, showed antimicrobial activity against *S. aureus* and *C. albicans.* The objectives of this study showed that less than 50% of healthy ladies in Ahvaz city- Iran can be supported from vaginal pathogens by lactobacilli probiotics but others are in risk of attack by harmful microbes.

Key word: *Lactobacillus,* vagina pathogens, antimicrobial.

INTRODUCTION

Recently, an increasing interest has developed in microbiota that promotes a woman's health. In particular, *Lactobacillus* species, that are commonly present in the human vagina, have received considerable attention due to their protective and probiotic properties (Andreu, 2004). Lactobacilli produce acids, hydrogen peroxide (H_2O_2), bacteriocins and biosurfactants and thus confer protection of the host (Reid and Bruce, 2003). The vagina of healthy women is a typical balanced ecosystem in which lactobacilli consist of more than 95% of flora. The predominant lactobacilli can be isolated from vagina with a recovery rate of 50 to 80% and a microbial load of about 8×10^7 CFU/ml. The lactobacilli-dominant vaginal flora have been proved to be of utmost importance for preventing various urogenital infections (UI), including recurrent urinary tract infections (UTI) and bacterial vaginosis (BV), a common disease with an infection rate of 27.86% in Chinese women, characterized by decreased lactobacilli and overgrowth of other micro-organism (Xu et al., 2008). Lactobacilli are acidotolerant, and the most frequently described mechanism for the control of growth of other populations by lactobacilli has been through the production of lactic acid from glycogen in vagina of healthy women. Among the properties of most vaginal strains of lactobacilli is their ability to

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Table 1. Primers that used for this study (Massi et al., 2004).

Name		Primer	bp
Lactobacillus genus	Forward	5-GGGTTCCCCATTCGGA-3	560-640
	Reverse	5-GAATCGCTAGTAAATTCG-3	
L. acidophilus	Forward	3-CCTTTCTAAGGAAGCGAAGGAT-5	199
	Reverse	3-AATTCTCTTCTCGGTCGCTCTA-5	
L. casei	Forward	5-AAGCACCCTAACGGGTGCGACT-3	118
	Reverse	5-GCGATGCGAATTTCTTTTTC-3	
L. plantarum	Forward	5-TCGGGATTACCAAACATCAC-3	319
	Reverse	5-CCGTTTATGCGGAACACCTA-3	

to release H_2O_2 in appreciable amounts *in vitro*. The hydrogen peroxide formed by $H_2O_2^-$ generating bacteria can be auto inhibitory or be toxic to adjacent bacteria, fungi, viruses (Klebanoff et al., 1991), particularly in the presence of peroxidase and halide. Bacteriocins are defined as proteinaceous, bacterial substances synthesized by bacteria, which usually have a Review Boris and Barbés narrow spectrum of activity, inhibiting strains of the same or closely related species. A bacteriocin-like substance is a term applied to antagonistic substances, which are incompletely defined or do not fit the typical criteria of bacteriocins. They tend to have a broader spectrum of activity than bacteriocins. A great number of these substances have been reported to be produced by lactobacilli, inhibiting a wide range of both Gram-positive and - negative bacteria as well as fungi. McGroarty and Reid described one such substance which showed activity *in vitro* against uropathogenic *Escherichia. coli* and *Enterococcus* species (McGroarty and Reid, 1998), but the role of these substances in vivo remains to be elucidated. Other mechanisms proposed for their microbial antagonism are competition for nutrients (McFarland, 2000; Reid and Burton, 2002), adhesion inhibition of pathogens to surfaces (Reid and Burton, 2002; Vesterlund et al., 2006), and stimulation of the immune system (Gill et al., 2001; Adel- Patient et al., 2005) then when the vaginal lactobacilli are diminished or absent, other microorganisms may grow excessively, causing disorders including bacterial vaginosis (BV), yeast vaginitis and sexually transmitted diseases (STD) (Burton et al., 2003). The main goal was to define their spectrum of antagonistic activity and to select the uropathogens-inhibiting strains with putative ability to protect the vagina. We attempted to isolate lactobacilli strains from healthy woman vaginal ecosystem that commonly referred to clinics for recheck. These strains were identified and initially tested for their probiotic properties. The inhibitory effect of these strains on both Gram -positive and Gram-negative pathogenic bacteria and fungi was further investigated.

MATERIALS AND METHODS

Isolation and identification of lactobacilli from vaginal specimens

Vaginal specimens were obtained from 103 women between the ages of 25 and 50 years with healthy vaginal ecosystems that referred to women clinics in Ahvaz- Iran. Lateral vaginal walls were swabbed with sterile cotton-tipped applicators. Lactobacilli were isolated by inoculating on de Man-Rogosa Sharpe agar (MRS agar, Difco, Detroit, USA) with 0.3% bile oxgall (Sigma, Lousiana, USA) and 0.2% bromocresol purple (Merck, Darmstadt, Germany), incubated anaerobically at 37°C for 48 h using an anaerobic jar containing anaerobic pak. Identification of *Lactobacillus* species was performed by phenotypic criteria. All isolates were initially tested for colony morphology, Gram reaction, catalase activity, motility test, and gas production from glucose (Voravuthikuncha et al., 2006). Polymerase chain reaction (PCR) analyses are then performed on the extracted DNA from Stock cultures were stored at -70°C in skim milk. 1-2 loop of the confirmed bacteria with phenotypically analysis grown on MRS agar, were resolved in TE (Trace EDTA) buffer and boiled at 100°C for 15 min. Extracted DNA was selected for PCR analyses by using genus and species-specific primers (Table 1).

The PCR conditions were initial denaturation of 94°Cfor 5 min followed by 30 cycle of denaturation of 94°C for 30 s, annealing of 52°C for 30 s for cas-ITS genus specific primers; 45°Cfor 45 s for *Lactobacillus plantarum*; 40°C for 40s for *Lactobacillus casei* and final extention at 72°C for 10 min using a termocycler (eppendorf). The PCR products were analyzed on 1% agaros gel. The confirmed species by PCR were tested for antimicrobial properties.

Determination of antimicrobial products

Antimicrobial compound was isolated using ethyl acetate solvent from *L. acidophilus* and *L. plantarum*. After 5 days incubation, the MRS broth media containing bacteria was mixed with ethyl acetate and agitated with a magnetic stirrer for two days. Then the media was allowed to settle for 30 min. Following settlement, the solution

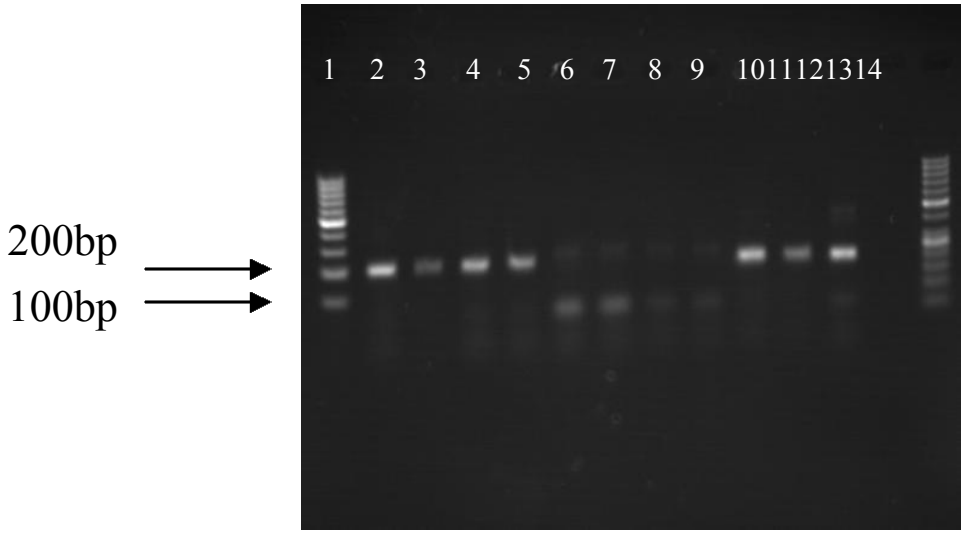

Figure 1. Agarose gel of PCR products amplified by species-specific primers of *L. plantarum* and *L. acidophilus*; 1:marker100bp- 2,3,4,5,10,11,12 *L .acidophilus*; 14: marker 50 bp.

was separated into two phases, which the supernatant was comprised of the extracted antimicrobial compound (Settanni et al., 2005). The color of ethyl acetate was turned yellow after agitation. The supernatant then was dried at 45°C. The yield of dried anti-microbial compound was 20 mg. The activity of antimicrobial agent was tested against pathogen microorganisms after adjustment of pH at 7 using NaOH 5 N. The minimal inhibitory concentration (MIC) of this antimicrobial substance determined using modified E. test (Amin et al., 2009). The target bacteria were *Staphylococcus aureus* (ATCC1189) and *Candida albicans* (ATCC 5027). The tests were performed 3 times.

RESULTS

Fifty- one samples out of 103 were positive for *lactobacillus* genus primers. Twenty - four isolates were *L. acidophilus* and 2 species were identified as *L. plantarum* and 2 spices as *L. casei*. Twenty three other isolates were just positive by genus primer (Figure 1). The antimicrobial compounds showed potent inhibitory activity against all tested microbes. The MICs of anti-microbial compound extracted from *L. acidophilus* were 4 and 1 µg/ml for *S. aureus* and *C. albicans* respectively (Figures 2 and 3), while *L. plantarum* and *L. casei* did not show any antimicrobial effect.

DISCUSSION

It has been well-documented that lactobacilli can act competitively to exclude pathogens, inhibiting their colonization and subsequently preventing infection. We made an attempt to select a good probiotic strain that could be used as therapeutic medicine. A wide range of pathogenic bacteria responsible for urinary tract infections were used in this study. The importance of vaginal *Lactobacillus* as a barrier to infections is of considerable interest. The experiments that demonstrate their protective role in a woman's health would benefit from the development of simple methods, allowing their detection and identification. In the present study, different *in vitro* methods were applied to characterize the antagonistic properties of *Lactobacillus*.

The results of this study showed that 49.5% of healthy women in Ahvaz- Iran have possibility of colonizing the *Lactobacillus* in their vagina. According to benefits of these bacteria for the protection of vagina, the quantity of this probiotic should be increased in the vagina of ladies. 70% of women that were positive for colonizing lactobacilli take place in range between 20 to 40 years old. 42 out of 103 under study women had vaginal infection at lease once in their life and 82% out of them were negative for *Lactobacillus*. To investigate the indigenous lactobacilli from the vagina, Xu et al. (2008) studied on one hundred and three lactobacilli strains were isolated from 60 samples of vaginal secretion from healthy pregnant women. Among them, 78 strains could produce hydrogen peroxide, in which 68, 80, 80 and 88% had antagonistic effects against *Candida albicans, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa,* respectively.

The lactobacillus strains were then further studied by Voravuthikuncha et al. (2006) for their probiotic properties. All of these strains were able to produce H_2O_2. All strains grew well at pH 5, however, only strains L 01A, L 01B, L 19A, and L 22 demonstrated growth at pH. The results of their ability to utilize protein, starch,

Figure 2. E test representing MIC of antimicrobial compound obtained from *L. acidophilus* against *S. aureus*.

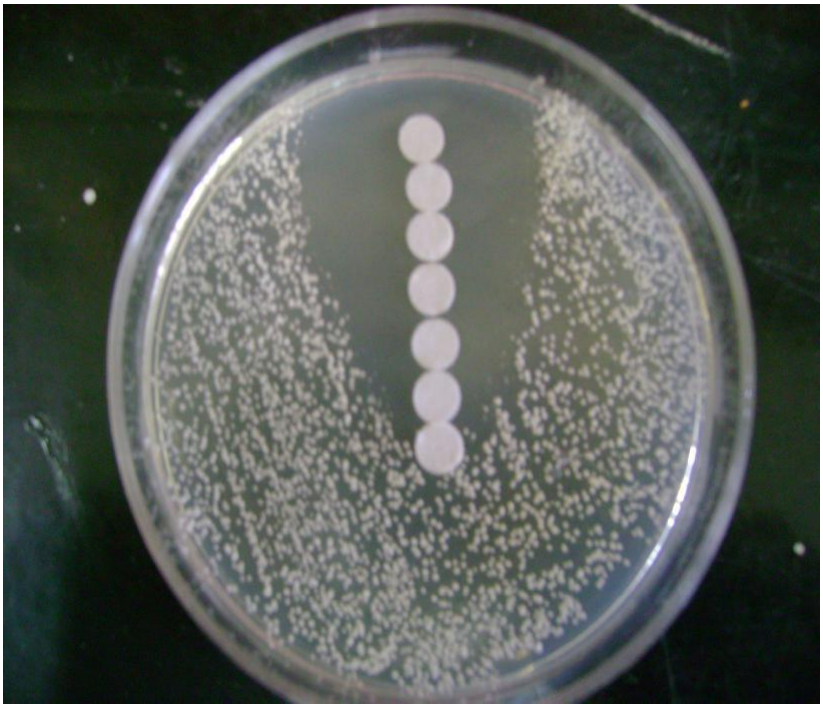

Figure 3. E test representing MIC of antimicrobial compound obtained from *L. acidophilus* against *C. albicans.*

and lipid are presented in. None of these strains were able to utilize starch and lipid. Six strains (L12A, L 12B, L 19A, L 19B, L 20 and L 22) were shown to hydrolyse protein.

Conclusion

The objectives of this study showed that less than 50% of healthy ladies in Ahvaz city- Iran can be supported from vaginal pathogens by lactobacilli probiotics but others are in risk of attack by harmful microbes. The food containing probiotis may be colonized by the *Lactobacillus* and *Bifidobacterium* species in the vagina through oral- fecal-vaginal track.

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