Potential of Probiotic *Lactobacillus plantarum* 2621 for the Management of Infertility

Praveen Bhandari¹, Praveen Rishi¹ and Vijay Prabha*¹

¹Department of Microbiology, Panjab University, Chandigarh, India.

Authors’ contributions

This work was carried out in collaboration between all authors. Author VP designed the study. Author PB managed the literature searches, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors VP and PR managed the analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2014/12129

Editor(s):
(1) Giuseppe Blaiotta, Department of Food Science, Via Universita, Italy.
(2) Luis Martinez-Sobrindo, University of Rochester, School of Medicine and Dentistry, NY, USA.

Reviewers:
(1) Abbas Farahani, Dept. of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
(2) Anonymous, University of Athens, Greece.
(3) Shuhong Luo, Institute of Antibody Engineering, School of Biotechnology, Southern Medical University, China.
(4) Anonymous, Kurukshetra University, India.

Peer review History: http://www.sciencedomain.org/review-history.php?id=605&id=8&amp;aid=5878

ABSTRACT

**Background:** Infertility outcomes may be associated with the infections that would lead to morphological defects of spermatozoa *in vitro*. The purpose of this work was to investigate the effect of *Lactobacillus plantarum* 2621 (*L. plantarum*) on adherence of sperm agglutinating *Escherichia coli* (*E. coli*) *in vitro* and *in vivo* as well as its effect on fertility outcome.

**Materials and Methods:** Interference of *E. coli* adherence to vaginal epithelial cells (*VECs*) by *L. plantarum* was studied by carrying out different assays such as exclusion, competition and displacement. Further, *in vivo* study was carried out in mouse model to...
evaluate the effect of presence of *L. plantarum* against *E. coli* and its effect on fertility outcome by administering intravaginally at one hour interval between *L. plantarum* (10^6 c.f.u./20 µl) and different concentrations of *E. coli* (10^2, 10^4, and 10^6 c.f.u./20 µl) for ten consecutive days.

**Results:** 116.8 bacteria/VEC adhesion levels were observed for *L. plantarum* 2621 whereas values for *E. coli* were 60.5 bacteria/VEC. *L. plantarum* interfered to different extents with the adherence of *E. coli*. *L. plantarum* 2621 decreased the adhesion by displacement and competition in a significant level (90.3% and 68.5% of inhibition). *L. plantarum* 2621 also excluded the *E. coli* attached to VEC (25.8% of inhibition). Upon mating and completion of gestation period 100% fertility was observed with 10^6 c.f.u./20 µl *L. plantarum* and 10^2 c.f.u./20 µl *E. coli*, whereas 100% females were infertile when administered with 10^6 c.f.u./20 µl of *E. coli* along with 10^6 c.f.u./20 µl *L. plantarum* and only 50% fertility outcome was observed with 10^4 c.f.u./20 µl *E. coli*.

**Conclusion:** Results indicated that *L. plantarum* displaces colonization of *E. coli* and endows competition that resulted in reinforcement of natural microflora and affects fertility outcome depending on the presence and count of *E. coli*.

**Keywords:** Adherence; Infertility; *Lactobacillus plantarum*; *Escherichia coli*; Intravaginal administration.

**1. INTRODUCTION**

Infertility is defined as getting no pregnancy despite one year of unprotected sexual intercourse. The prevalence of male factor infertility is reported as 25-40% while female factor infertility is found to be 40-55% [1]. Infections may lead to male infertility with a prevalence of 6.6% while in females it accounts for 30-40% [2]. Bacterial interaction with spermatozoa leads to morphological defects as well as changes in functional parameters of spermatozoa in vitro [3]. These may in turn lead to decrease in the fertilization potential of sperm. The human vagina is a complex ecosystem composed by a stratified squamous epithelium and its indigenous microbiota. The vaginal ecosystem harbors a microbiota that is being increasingly recognized as protecting it from invading pathogens, including those that cause urinary tract infections and sexually transmitted diseases [4]. Lactobacilli comprise 70% of total microorganisms isolated i.e Lactobacilli are present at the level of 10^7-10^8 cfu g^-1 of the vaginal fluid influencing the vaginal ecosystem [5-7]. Most frequently isolated among them are those belonging to the *Lactobacillus acidophilus* group and *L. fermentum* although others, such as *L. plantarum*, *L. brevis*, *L. jensenii*, *L. casei*, *L. delbrueckii* and *L. salivarius*, are isolated as well [6].

In fact, it has been observed that a disruption of the microbial ecosystem balance and particularly depletion of vaginal Lactobacilli is associated with colonization of the genitourinary tract with other pathogenic microbes that may lead to an increased incidence of bacterial vaginitis and other urinary tract infections in humans [6,8-10]. For these reasons, in the last few years, interest in the use of Lactobacilli to restore and maintain a normal vaginal flora and prevent disease recurrence has increased and also represents an alternative to conventional chemotherapy [11,12].

The adherence of the uropathogens to the epithelial cells is an important factor in vaginal colonization. Lactobacilli are believed to interfere with pathogens by competitive exclusion for receptors present on the surface of the genitourinary epithelium [13,14] and release of antimicrobial compounds, such as lactic acid, hydrogen peroxide, bacteriocin-like
substances [15,16]. With respect to the urogenital tract, there is in vitro evidence that Lactobacilli can inhibit the attachment of pathogens such as Escherichia coli, Gardnerella vaginalis, Candida albicans, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus and Streptococcus agalactiae to urogenital epithelial cells [17-23].

The aim of the present study was to evaluate the capacity of Lactobacillus plantarum 2621 to block the adherence of sperm agglutinating Escherichia coli to vaginal epithelial cells. The ability of bacteria to attach to mucosal epithelial cells seems to have a crucial impact on the establishment of the indigenous bacterial flora. The adherence of bacteria to exfoliated vaginal cells was tested in an in vitro system by microscopic observations, to demonstrate that L. plantarum and sperm agglutinating E. coli readily adhere in vitro to vaginal epithelial cells. Under the experimental conditions used, differences in the ability of bacteria of these two groups to adhere to vaginal cells were observed. Further, in vivo experiments were carried out to study the efficacy of L. plantarum against the sperm agglutinating E. coli colonization and its effect on the fertility outcome when both strains are present simultaneously.

2. MATERIALS AND METHODS

2.1 Microorganisms

The strain of E. coli used in the present study was isolated earlier in our laboratory from the semen sample of the patient undergoing semen analysis at PGIMER, Chandigarh. A standard strain of Lactobacillus plantarum MTCC 2621 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Sector-39, Chandigarh, India. E. coli was maintained on Luria agar (LA) while Lactobacillus plantarum MTCC 2621 was maintained on De Mann Rogosa Sharpe (MRS) agar (5% CO₂). Strains were stored as glycerol stocks at -60°C.

2.2 Vaginal Epithelial Cells

Vaginal epithelial cells (VECs) were obtained from 10 healthy female mice from proestrous cycle. 10 samples were obtained by flushing the vaginal walls with an autopipette and the VECs exfoliated were transferred to RPMI and pooled to carry the assays. Indigenous bacteria were removed by washing cells with RPMI (800g, 10 min) at least three times [17] and the concentration of VECs were adjusted to 10⁵ cells ml⁻¹.

2.3 In Vitro Studies

2.3.1 Influence of probiotic Lactobacillus plantarum on in vitro adhesion of E. coli to vaginal epithelial cells

2.3.1.1 Adhesion assays

The overnight cultures of E. coli and L. plantarum were centrifuged and the supernatants were discarded. The pellets were washed twice with sterile saline solution and resuspended in Rose well Park Memorial Institute (RPMI) medium to obtain 10⁶ and 10⁸ c.f.u ml⁻¹ respectively.
Three types of assays were performed to study the ability of *L. plantarum* to block the adherence of *E. coli* to VEC: blockage by exclusion, by competition or by displacement using standard methods [18,19].

For the exclusion test, suspensions of *L. plantarum* (10⁸ c.f.u ml⁻¹) and VEC (10⁵ cells ml⁻¹) were mixed (1:1) (100µl each) and incubated at 37°C for 60min, *E. coli* (10⁶ c.f.u ml⁻¹) (100 µl) was then added and the mixture was incubated for another 60min.

For the competition assay, VEC, *L. plantarum* and *E. coli* were incubated together (37°C, 60 min) (same count and same volume was used as in exclusion assay).

For the displacement test, VEC and *E. coli* were mixed and incubated together (37°C, 60 min); *L. plantarum* was added later and incubation was continued for further 60 min (same count and same volume was used as in exclusion assay).

After the incubation period, each mixture was washed by centrifugation at 800g for 10 min to remove nonadhering bacteria. Cells with adhered bacteria were transferred to microscope slides, fixed with methanol and Gram stained. Bacterial adhesion to VECs was assessed by microscopy (×1000) by counting the number of micro-organisms attached to 10 consecutive cells. *L. plantarum* was distinguished from *E. coli* by size and Gram staining (lactic acid bacteria: Gram-positive bacilli; *E. coli*: Gram-negative rods). The results of the three conditions (i.e. exclusion, competition and displacement) were expressed as the average number of *E. coli* per VEC and compared with adhesion without *L. plantarum* (control value). The control values were taken as 100% of adhesion and the blocking effect was determined by comparing the mean of the attachment of the *E. coli* with and without *L. plantarum*.

### 2.4 In Vivo Studies

#### 2.4.1 Animals

Sexually mature, 5–6 week old male (25±2 g) and 4–5 week old female (22±2g) Balb/c mice were used in the present study. Animals were kept in animal room of the Department of Microbiology, Panjab University, Chandigarh from April 2012 to March 2013. Animals were maintained in laboratory conditions (12:12, dark: light cycle), housed in plastic cages and fed with standard pellet diet and water ad libitum. Experimental protocols were approved by Institutional Animal Ethics Committee of the Panjab University, Chandigarh, India vide letter no.330/CAH dated 31/02/12 and were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

#### 2.5 Screening of Animals

First, we screened the animals for micro-organisms that naturally inhabit the Balb/c mouse vagina. The microbiota of the mice induced in estrous cycle by whitten’s effect was studied from vaginal samples taken with sterile cotton swabs moistened with physiological saline. Swabs were cultured at 37°C on Brain Heart Infusion (BHI) agar, Eosin Methylene Blue (EMB) agar for 24h and De Mann Rogosa Sharpe (MRS) agar in 5% CO₂ for 48h. Strains growing on BHI plates were further checked for spermagglutinating/immobilizing properties using mice sperm samples. Mice harbouring *Lactobacillus* (growth on MRS agar)/*E. coli*
(growth on EMB agar with green sheen) or any other bacteria (growth on BHI) with sperm agglutinating/immobilizing property were excluded from the study.

2.6 Preparation of Inoculums

E. coli was cultivated in Luria Broth at 37°C for 24h. Cell culture was centrifuged at 10,000 rpm for 20 min and washed twice with Phosphate Buffer Saline (PBS) (50mM, pH 7.2). Cells were suspended in the same buffer and adjusted to a concentration of $10^2$, $10^3$, $10^6$ c.f.u/20 µl. Similarly L. plantarum 2621 was grown in MRS broth and was adjusted to a concentration of $10^8$ c.f.u/20µl.

2.7 Effect of Administration of Lactobacillus plantarum and E. coli on Fertility outcome

Screened mice were divided into 3 groups consisting of 4 females per group. Group 1 was intravaginally administered with L. plantarum ($10^8$ c.f.u/20 µl) and at one h interval with E. coli ($10^2$ c.f.u/20 µl), similarly, group 2 with L. plantarum ($10^8$ c.f.u/20µl) and E. coli ($10^4$ c.f.u/20µl), Group 3 with L. plantarum ($10^8$ c.f.u/20µl) and E. coli ($10^6$ c.f.u/20µl) for 10 consecutive days. As control, groups of mice (4 females each) were inoculated intravaginally with $10^2$, $10^4$, $10^6$ c.f.u/20µl of E. coli for 10 days. Vaginal lavages were taken every 3rd day so as to monitor vaginal colonization. The vaginal isolates were further confirmed as L. plantarum and E. coli by culture characteristics. E. coli isolates were also checked for sperm agglutinating activity in vitro. On day 12, female mice were allowed to mate with breeder male mice in the ratio 2:1, to check the effect on fertility outcome. Upon mating, next day females were separated and mating was confirmed by observing the presence of vaginal plug in all the female mice. After mating, mice were checked for fertility changes i.e. weight gain, abdominal distension and string of pearls by palpation. All the groups, were allowed to complete their gestation period. The whole experiment was repeated twice (amounting to the total number of 56 mice) for consistency of the results.

3. RESULTS

3.1 Influence of Probiotic Lactobacillus plantarum on in vitro Adhesion of E. coli to Vaginal Epithelial Cells

Both L. plantarum 2621 and E. coli used in the present study were able to adhere, at different degrees, to VECs. Adhesion level observed for L. plantarum 2621 was with the value: 116.8 bacteria/VEC. For E. coli adhesion to VEC, mean value was 60.5 bacteria/VEC. L. plantarum interfered to different extents with the adherence of E. coli and decreased the adhesion by displacement and competition in a significant level (90.3% and 68.5% of inhibition) (Fig. 1). L. plantarum 2621 also excluded the E. coli attached to VEC (25.8% of inhibition) (Fig. 2).
3.2 Effect of Administration of Lactobacillus plantarum and E. coli on fertility outcome

3.2.1 Evaluation of colonization of L. plantarum and E. coli

From the results (Figs. 3,4,5) it was observed that log3.5 c.f.u ml⁻¹, 2.8 log₁₀ c.f.u ml⁻¹, 3.3 log₁₀ c.f.u ml⁻¹ of E. coli were recovered from the vaginal lavages taken on day 9 from mice instilled with L. plantarum (10⁵ c.f.u/20 µl) and one hour later with different concentration (10², 10⁴,10⁶ c.f.u/20 µl) of E. coli for ten consecutive days respectively. Whereas counts of L. plantarum recovered from female mice were 7.1 log₁₀ c.f.u ml⁻¹, 6.2 log₁₀ c.f.u ml⁻¹, 4.7 log₁₀ c.f.u ml⁻¹.

When the female mice were inoculated intravaginally with different doses of E. coli (10², 10⁴, 10⁶ c.f.u/20µl) after one hour inoculation with L. plantarum (10⁵ c.f.u/20 µl) for 10 consecutive days, it was observed that L. plantarum could efficiently colonize mice vagina alongwith 10²,10⁴ and 10⁶ c.f.u/20 µl of E. coli. Upon mating on day 12 with proven male breeder mice 100% female mice were infertile in group 3 administered with 10⁵ c.f.u/20µl E. coli alongwith Lactobacillus (10⁵ c.f.u/20µl). Similarly 100% infertility was observed in groups administered intravaginally with 10², 10⁴, 10⁶ c.f.u/20µl of spermagglutinating E. coli for 10 consecutive days [20].

![Fig. 1. (A) Control vaginal epithelial cell, (B) adherence of L. plantarum to vaginal epithelial cell, (C) adherence of E. coli. The effect of L. plantarum on the attachment of E. coli to VEC under the conditions of (D) exclusion (E) competition and (F) displacement](image)


Fig. 2. Percentage blockage adherence of *E. coli* to vaginal epithelial cells by exclusion assay, competition assay and displacement assay.

Fig. 3. Vaginal colonization with *L. plantarum* (10^8 c.f.u/20 µl) and *E. coli* (10^2 c.f.u/20 µl).
Fig. 4. Vaginal colonization with *L. plantarum* (10⁸ c.f.u/20 µl) and *E. coli* (10⁴ c.f.u/20 µl)

50% fertility outcome was observed in group 2 administered with 10⁴ c.f.u/20 µl *E. coli* along with *L. plantarum* (10⁸ c.f.u/20 µl), however, 100% females were fertile in the group 1 inoculated with 10² c.f.u/20 µl *E. coli* along with *L. plantarum* (10⁸ c.f.u/20 µl) (Table 1) and the control group administered with PBS.

Fig. 5. Vaginal colonization with *L. plantarum* (10⁸ c.f.u/20 µl) and *E. coli* (10⁶ c.f.u/20 µl)
Table 1. Effect of repeated intravaginal inoculations of sperm agglutinating strain of E. coli and L. plantarum on fertility outcome in mice

<table>
<thead>
<tr>
<th>Dose instilled as 10 consecutive inoculations (cfu/20 µl)</th>
<th>No. of treated mice</th>
<th>Fertility outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 10^2</td>
<td>Lactobacillus plantarum 10^0</td>
<td>4</td>
</tr>
<tr>
<td>10^4</td>
<td>10^8</td>
<td>4</td>
</tr>
<tr>
<td>10^6</td>
<td>10^8</td>
<td>4</td>
</tr>
</tbody>
</table>

*Experiment was repeated twice and identical results were obtained

4. DISCUSSION

Present study was carried out to evaluate the effect of presence of Lactobacillus plantarum 2621 in relation to the E. coli strain leading to sperm impairment and agglutination, previously isolated in our laboratory from males undergoing semen sample analysis complaining of infertility. Many studies have related the absence of Lactobacilli, with genital infections such as bacterial vaginosis [9,21]. Lactobacillus is the prominent organism present in the healthy human vagina that comprises 70% of the total organisms isolated [6,22]. Lactobacilli play an important role in gastrointestinal tract, urinary tract and in the vagina [5,23]. Vagina is a delicate ecosystem being maintained by the presence of Lactobacilli [24]. Substances such as H2O2, Lactic acid and bacteriocin are being produced by Lactobacilli to protect the human vagina [25,22,26]. An important step in colonization to different mucosal surfaces such as respiratory, gastrointestinal, and urogenital tracts is adhesion to epithelial cells. In the present study, we assessed the ability of Lactobacillus plantarum 2621 to inhibit the attachment of sperm agglutinating E. coli to mouse vaginal epithelial cells (VEC). The adherence to VEC could be due to the composition of the cell wall of Gram-negative and Gram-positive bacteria, their content of specific adhesion factors. It is supposed that the adhesion of Lactobacilli exclude the colonization of pathogenic bacteria by masking potential binding sites in the vaginal mucosa, [27,28] by the formation of film over it. However, Reid [29] showed that Lactobacilli have the capacity to compete for the same receptors and displace previously attached pathogens. Therefore, we investigated the blockage of sperm agglutinating E. coli adherence by L. plantarum under three possible mechanisms: exclusion, competition and displacement. In the exclusion test, L. plantarum was allowed to adhere to VEC first and sperm agglutinating E. coli was added later. Results showed that the L. plantarum adhered to the surface of VECs were able to exclude sperm agglutinating E. coli to some extent. Competition between sperm agglutinating E. coli and L. plantarum for adhesion on the surface of VECs were observed and adherence was inhibited in significant level by L. plantarum. Higher degree of displacement was observed when sperm agglutinating E. coli was added first to VECs, then L. plantarum. The ability to displace sperm agglutinating E. coli from VEC indicates higher affinity of L. plantarum for the receptors.

These results are in accordance to earlier studies carried out by Boris et al. [25], wherein they reported that vaginal L. acidophilus blocks the adherence of G. vaginalis and C. albicans by displacement and competition but not by exclusion. In concordance, Mastro-marino et al. [30] also showed that vaginal Lactobacilli exerted a greater interference in adhesion of pathogens to VECs by displacement rather than exclusion. However, the use of exfoliated cells in bacterial attachment studies donot mimic in vivo conditions, therefore, to ascertain the important factors in pathogenesis of infertility and the effect of L. plantarum on infertility, mouse model was used for intravaginal inoculations that can simulate ascending
genital tract infections in female [31,32]. E. coli previously isolated in our laboratory leading to sperm impairment and agglutination was found to cause infertility in mouse model when administered intravaginally [20]. As L. plantarum displaces the E. coli in vitro from the VEC, therefore, an in vivo experiment was performed to study the amelioration of the infertility induced by sperm agglutinating E. coli by L. plantarum. Results showed that fertility was affected to different extent when female mice were administered intravaginally with L. plantarum and different doses of E. coli.

5. CONCLUSION

In conclusion, this data suggest the use of L. plantarum in the protection of vaginal epithelium by interfering the adherence and encourage further in vivo studies, such as clinical trials designed to test the capacity to prevent and manage genital tract infections in females leading to infertility.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


© 2014 Bhandari et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=605&id=8&aid=5878