Positive effect of probiotic Lactobacillus plantarum in reversing LPS-induced infertility in a mouse model

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Bacterial infections have high incidence among the female population at reproductive age and are widely known to cause infertility due to inflammation. The purpose of the present study was to investigate the effect of the inflammatory agent LPS on fertility outcome and to evaluate the ability of Lactobacillus plantarum in ameliorating the LPS-mediated inflammation-induced infertility. Female BALB/c mice infused intravaginally with a single dose of 20 μl sterile normal saline containing 5, 10 or 20 μg LPS were divided into two groups for evaluation of tissue histology and pregnancy outcome. In the first group, aimed at observing changes in tissue histology, inflammation was observed in vaginal sections of mice instilled with a single dose of 20 μg LPS, which were sacrificed on days 2, 5 and 8. In the second group, aimed at evaluating pregnancy outcome, female mice were administered 20 μg LPS, which rendered them infertile upon mating on days 2, 5 and 8. In another experiment, normal histology of vaginal sections was observed in mice administered a single dose of 20 μg LPS, followed by 10^8 c.f.u. L. plantarum in 20 μl at 24 h intervals, until the mice were sacrificed on days 2, 5 and 8. Following similar treatment, female mice, when mated with proven male breeder mice on days 2, 5 and 8, retained their fertility and delivered pups. These results were further confirmed by the downregulation of pro-inflammatory cytokines and an increase in anti-inflammatory cytokines on treatment with L. plantarum, revealing the role of probiotics in ameliorating inflammation-induced infertility.
METHODS

Micro-organism. A standard strain of *L. plantarum* MTCC 2621 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Sector-39, Chandigarh, India. This strain was maintained on de Mann–Rogosa–Sharpe (MRS) agar (5 % CO₂) and was stored as glycerol stocks at −60 °C. Previously, *L. plantarum* was shown to adhere to the mouse vaginal epithelial cells and could replace the adhered spermagglutinating *E. coli*. Hence, this strain was used for intravaginal administration to check for amelioration of inflammation-induced infertility.

Preparation of inoculum. *L. plantarum* was cultivated in MRS broth in 5 % CO₂ at 37 °C for 24 h. The cell culture was centrifuged at 2767 g for 15 min and washed twice with sterile normal saline. The cells were resuspended in normal saline to a concentration of 10⁶ c.f.u. in 20 μL.

Animals. Sexually mature, 5–6-week-old male (25 ± 2 g) and 4–5-week-old female (20 ± 2 g) BALB/c mice were used. Animals were kept in the animal room of the Department of Microbiology, Panjab University, Chandigarh, India. Animals were maintained in laboratory conditions (12 : 12 h, dark : light schedule), housed in plastic cages and fed with a standard pellet diet and water *ad libitum*. Experimental protocols were approved by the Institutional Animal Ethics Committee of Panjab University, Chandigarh, India (no. 504/CAH, 2 April 2014), and experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Screening of animals. First, we screened the animals for microorganisms that naturally inhabit the BALB/c mouse vagina. The microbiota of the mice, induced in the oestrous cycle by the Whitten effect, was studied from vaginal samples, taken with sterile cotton swabs moistened with physiological saline. Swabs were cultured at 37 °C on MRS agar in 5 % CO₂ for 48 h. Mice harbouring lactobacilli (growth on MRS agar) were excluded from the study. Screened mice were divided into appropriate groups according to the needs of the experiment, with four mice per group, and the experiments were done twice. The day of intravaginal administration was designated day 0.

Evaluation of LPS-induced inflammation and infertility in mice. LPS (from *E. coli* O127 : B8) used in the present study was procured from Sigma Chemicals Co.

Inflammation. To ascertain the dose needed for the induction of an inflammatory state, a preliminary study was performed. BALB/c mice of the same age and weight were randomly assigned to experimental groups: a control group (intravaginally administered 20 μL sterile normal saline) and test groups (three subgroups of four mice each intravaginally administered a single dose of LPS, i.e. 5, 10 or 20 μg LPS per animal in 20 μL sterile saline). A mouse from each group was sacrificed by cervical dislocation on days 2, 5 and 8 post-administration (p.a.) and the reproductive organs (ovaries, uterus and vagina) were collected. Histopathological analysis of different reproductive organs was carried out according to standard procedures. Small portions of recovered ovaries, uterus and vagina from animals of each group were dissected out, freed from fat bodies and fixed in 10 % neutral buffered formalin for 24 h. Tissues were dehydrated in a graded series of ethanol, cleared in xylene, and infiltrated and embedded in paraffin wax at 60 °C. The blocks were sectioned at 4 μm using a microtome (Leica) and mounted on glass slides. The tissue sections were stained with haematoxylin and eosin, observed under a microscope (Nikon) using ×10 and ×40 objectives, and photographed. The inflammatory response was determined by leukocyte infiltration and disruption of the vaginal epithelium.

Fertility outcome. To determine the effect of LPS on pregnancy outcome, groups of sexually mature female BALB/c mice were randomly assigned to two groups, test and control. The test group was further divided into three subgroups, with six mice in each. Mice were induced in the oestrous phase by the Whitten effect. All the subgroups were intravaginally administered a single dose of different concentrations of LPS (5, 10 and 20 μg in 20 μL saline per mouse). The control group animals received 20 μL normal saline intravaginally. Two mice from each group were housed with proven breeder male mice at a ratio 2 : 1 (female : male) and were allowed to mate overnight on days 2, 5 and 8 p.a. Next morning, the males were separated and the females were monitored for the presence of a vaginal plug as confirmation of mating. Consistent weight gain, palpation of foetuses as a ‘string of pearls’ and abdominal distension in females were observed as indicators of pregnancy.

Evaluation of probiotic *L. plantarum* as potential intervention against LPS-induced inflammation and infertility

Effect on inflammation. Based on the results of intravaginal LPS infusion, use of *L. plantarum* as a potential therapeutic intervention was evaluated. Four female BALB/c mice were intravaginally administered with 20 μg LPS, prepared as described previously, followed by administration of *L. plantarum* (10⁶ c.f.u. per 20 μL) at 24 h intervals until the mice were sacrificed and the effect on LPS-induced inflammation was evaluated. Animals administered with a single dose of either LPS (*n* = 4) or saline (*n* = 4) served as controls. Finally, mice from each group were sacrificed on days 2, 5 or 8 p.a. As no inflammation was observed in the ovary and uterus of mice intravaginally administered 20 μL LPS, only the vagina was removed and was processed immediately, as described above, for histopathological analysis. Cross-sections of the vagina obtained from LPS/*L. plantarum*-treated animals were compared with those from the saline and LPS groups.

Effect on infertility. Intravaginal infusion of LPS followed by consecutive administration of probiotic *L. plantarum* led to alleviation of inflammation, so we were interested evaluating whether this amelioration could recuperate the fertility. Twelve randomly assigned female BALB/c mice were infused intravaginally with a single dose of 20 μg LPS, prepared as described above, followed by administration of *L. plantarum* (10⁶ c.f.u. in 20 μL) at 24 h intervals until the mice were mated. Animals administered a single dose of 20 μg LPS (*n* = 12) or 20 μL saline (*n* = 12) served as controls. On days 2, 5 and 8 p.a., two mice from each group were allowed to mate overnight with proven breeder male mice (2 : 1, female : male). The next morning, the males were separated and females were observed for a vaginal plug as confirmation of mating and for pregnancy-related changes.

Cytokine level estimation in mice vaginal homogenates

From the previous results, it was observed that the intravaginal administration of LPS led to inflammation, and hence we studied the level of cytokines. For cytokine level estimation, female BALB/c mice were divided in four groups and each group was intravaginally separately administered LPS, 10⁶ c.f.u. *L. plantarum*, LPS plus *L. plantarum* or PBS. Vaginal homogenates were collected to estimate the cytokine levels at various time intervals (0, 2, 4, 8, 24 and 48 h p.a.).

To obtain vaginal homogenates, two mice from each group were killed by cervical dislocation at above-mentioned time intervals. The vagina was removed aseptically in 0.1 ml PBS and homogenized with a Teflon pestle. The homogenate was centrifuged at 3997 g for 20 min, and clear supernatant was used for cytokine estimation. TNF-α and IL-10 were measured using a commercially available ELISA kit (Mouse TNF-α estimation kit and Mouse IL-10 estimation kit; RayBiotech).

The standard curve for the assays ranged from 0 to 1500 pg mL⁻¹ for both TNF-α and IL-10. Cytokine levels in undiluted vaginal homogenates were measured using a commercially available ELISA kit (Mouse TNF-α estimation kit and Mouse IL-10 estimation kit; RayBiotech).
were calculated based on the respective standard curves. Cytokine quantification was expressed as pg ml\(^{-1}\) in tissue homogenates.

**RESULTS**

**Effect of LPS on the reproductive tract of female mouse**

**LPS-induced inflammation in the mouse vagina.** The histopathological analysis of the mouse vagina injected with a single dose of 20 μg LPS showed evidence of inflammation as indicated by infiltration of leukocytes on days 2 and 5 p.a. (Fig. 1b, c) compared with that of the control (Fig. 1a), while inflammation subsided on day 8 p.a. as no leukocyte infiltration was observed (Fig. 1d). However, no histopathological changes were observed on days 2, 5 and 8 p.a. in the mouse vagina administered intravaginally with 5 or 10 μg LPS. Moreover, inflammation was also absent in the samples of ovary and uterus (Fig. 2) from all groups.

**LPS-induced infertility.** When a single dose of different concentrations of LPS (5, 10 or 20 μg) was instilled in the vagina of female BALB/c mice, it was observed that 20 μg LPS induced infertility upon mating on days 2, 5 and 8 p.a., whereas mice receiving lower doses of LPS, i.e. 5 or 10 μg, underwent conception. This was confirmed by consistent weight gain, abdominal distension and palpation of small strings of pearls both in the control group and mice receiving 5 or 10 μg LPS. At the end of the gestation period, on average six to eight pups were born per mouse. On the basis of these observations, we decided to use 20 μg LPS in 20 μl in experiments to test for the pro-fertility effect of *L. plantarum*.

**Protective effect of probiotic *L. plantarum* in a mouse model of LPS-induced inflammation and infertility**

**Attenuation of LPS-induced inflammation.** In order to examine the therapeutic efficacy of probiotic *Lactobacillus* in the amelioration of vaginal inflammation, histopathological studies were carried out. Probiotic *L. plantarum* had a positive effect in reversing the LPS effect in mice, as shown in Fig. 3. Cross-sections of the vagina from LPS-treated mice showed inflammation (Fig. 3c). However, administration of *L. plantarum* resulted in attenuation of LPS-induced infiltration of leukocytes in mice infused with 20 μg LPS (Fig. 3b) and the sections were comparable to those of control mice (Fig. 3a).

**Amelioration of LPS-induced infertility.** Next, we examined whether or not intravaginal administration of the probiotic *L. plantarum* could protect against inflammation-induced infertility. The results of the effect of probiotic *L. plantarum* on LPS-induced infertility are presented in Table 1. The incidence of infertility was 100 % in the LPS group, in contrast to 100 % fertility in

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**Fig. 1.** Histological examination of the vagina on days 2, 5 and 8 of mice administered with saline/representative control (a; day 5) or a single dose of 20 μg LPS in 20 μl (b–d). Inflammation (infiltration of leukocytes; red arrows) was observed on days 2 (b) and 5 (c), with inflammation subsiding on day 8 (d). Original magnification ×400.
the saline group. Fertility was 100% in the group administered *L. plantarum* after LPS treatment. However, the number of pups delivered varied with the number of days of treatment with *L. plantarum* (Table 1). Thus, treatment with *L. plantarum* attenuated LPS-induced inflammation and infertility.

**Cytokine level estimation.** Vaginal homogenates were collected to estimate the cytokine level. For this, two mice from each group were killed by cervical dislocation at 0, 2, 4, 8, 24 and 48 h after administration of LPS, 10^8 c.f.u. *L. plantarum*, LPS plus *L. plantarum* or PBS. Both TNF-α and IL-10 were measured using commercially available

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**Fig. 2.** Haematoxylin and eosin-stained sections of uterus (a–d) and ovary (e–h). Light micrographs of the uterus (b–d) and ovary (f–h) of mice on days 2, 5 and 8 after administration of a single dose of 20 μg LPS and representative control receiving normal saline (a, e). No histopathological changes were observed in the uterus and ovary of the test groups (b–d and f–h) or in the representative control receiving normal saline (a, e). Original magnification × 100.

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**Fig. 3.** Histological examination of the vagina on day 5 after intravaginal administration of normal saline (a), LPS + *L. plantarum* (b) or LPS (c). The vagina of the saline (a) and LPS + *L. plantarum*-treated (b) groups showed normal histology, while the vagina of the LPS-treated mice showing inflammation (infiltration of leukocytes; black arrow). Original magnification × 400.
ELISA-based kits. In the case of TNF-α, elevation was observed at 24 h (1200 pg ml⁻¹) in homogenates from mice administered LPS, followed by a return to a level comparable to that of the placebo (PBS) group (850 pg ml⁻¹). No elevation in TNF-α was observed in the homogenates of mice of the other two groups, i.e. administered L. plantarum or LPS followed by L. plantarum. For the L. plantarum-treated group, the maximum level of IL-10 was observed from 4 h (540 pg ml⁻¹) to 24 h (580 pg ml⁻¹) p.a., followed by a return comparable to a level comparable to that of the placebo group (456 pg ml⁻¹).

**DISCUSSION**

The relationship between genital tract infections and infertility in females has long been postulated. The vaginal tracts of women harbour a wide variety of microbial species. The occurrence of Gram-negative enterobacteria represents a frequent event. Although most of these micro-organisms colonize the vagina asymptomatically, there have been increased incidences of infertility in recent years. Previous in vivo studies carried out in our laboratory demonstrated that intravaginal administration of spmmergagglutinating E. coli caused no histological changes such as inflammation in the vaginal tissue but could lead to infertility in the mice. Infertility in the case of this organism was hypothesized to be due to the spmmergagglutinating factor, which led to immobilization of the spermatozoa (Kaur & Prabha, 2013). Elimination of spmmergagglutinating E. coli by the probiotic L. plantarum resulted in the reversal of infertility (Bhandari & Prabha, 2015). Thus, we were interested in studying the pro-fertility effect of L. plantarum to see whether it could prevent infertility induced by inflammatory changes caused by micro-organisms. Most of sexually transmitted infections cause infertility by inflammation. Gram-negative bacteria can modulate the inflammatory reactions by releasing structural components such as LPS via cell lysis from the outer membrane into the surrounding environment in secretions during active growth. Dal Bosco et al. (2005) observed endometritis-like inflammation in the cervix after directly inoculating 500 μg LPS. Thus, the present study investigated the anti-inflammatory role of the probiotic L. plantarum in relation to the amelioration of inflammation-induced infertility.

Our results suggested that LPS induces infertility in female BALB/c mice through a mechanism involving inflammation of the vagina. This is consistent with previous findings showing that inflammation of the reproductive tract may lead to infertility, as some of the remarkable changes in the vaginal environment might be a consequence of the accumulation of bacterial products by cell lysis or secretion. These changes may result in a strong reduction in the vitality of spermatozoa and increase the level of naturally occurring apoptosis (Galdiero et al., 1994; Gorga et al., 2001). Inflammation of the reproductive tissue is assessed by the magnitude of infiltration of inflammatory cells such as neutrophils, macrophages and lymphocytes. Many of the biological effects (anti-fertility effects) of LPS are mediated by pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6). The induction of pro-inflammatory cytokines by LPS in the vaginal ecosystem may trigger damage in the DNA of spermatozoa, thereby leading to infertility.

Many studies have related the absence of lactobacilli in genital infections such as bacterial vaginosis (Gupta et al., 1998; Pascual et al., 2008). Lactobacillus is the prominent organism present in the healthy human vagina and comprises about 70% of the total number of organisms isolated (Redondo-Lopez et al., 1990; Lepargneur & Rousseau, 2002). Lactobacilli play an important role in the gastrointestinal tract, urinary tract and vagina (Antonio et al., 1999; Cannon et al., 2005). To protect the human vagina, they produce certain substances such as H₂O₂, lactic acid and bacteriocin (Boris et al., 1998; Velraeds et al., 1998; Lepargneur & Rousseau, 2002). Changes in the vaginal microbiota, particularly the decrease in the lactobacilli population and increases in the number of Gram-negative bacteria, are the signs of inflammation. We have shown previously that L. plantarum can colonize the mouse vagina and eliminate pathogens from the vagina without any adverse effect on fertility outcome (data not shown). Our data also indicated that LPS-mediated inflammation in the mouse vagina had an adverse impact on fertility outcome, so it was speculated that alleviation of

<table>
<thead>
<tr>
<th>Fertility/no. pups</th>
<th>Control (days 2, 5 and 8)</th>
<th>LPS (days 2, 5 and 8)</th>
<th>LPS + L. plantarum (day 2)</th>
<th>LPS + L. plantarum (day 5)</th>
<th>LPS + L. plantarum (day 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy rate</td>
<td>4/4</td>
<td>0/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
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<tr>
<td>No. pups (mean ± SD)</td>
<td>7.25 ± 0.95*</td>
<td>0</td>
<td>2.5 ± 0.58†</td>
<td>6.75 ± 0.95*</td>
<td>7 ± 0.81*</td>
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All groups had n=4 mice.

*P<0.001 compared with the LPS group.

†P<0.01 compared with the LPS group.
inflammation might help in the restoration of fertility. Therefore, we further evaluated the effect of the presence of lactobacilli on the amelioration of inflammation-induced infertility. In vivo studies carried out with BALB/c mice revealed that infusion of LPS in the vagina of female mice could compromise fertility and that application of L. plantarum resulted in a reversal of infertility. Lactobacillus spp. may mediate an anti-inflammatory reaction and ameliorate inflammation by downregulation of pro-inflammatory and/or stimulation of anti-inflammatory cytokine production (Fedorak & Madsen, 2004; Di Giacinto et al., 2005). Furthermore, confirmation came from normal pregnancy-related changes in the two experimental groups, one receiving Lactobacillus + LPS and the other saline. This reversal supports the hypothesis that Lactobacillus spp. could be exploited as a therapeutic intervention against inflammation-induced infertility.

From these results, it can be concluded that probiotic Lactobacillus spp. lead to a potential anti-inflammatory response as they attenuate LPS-induced inflammation. Therefore, probiotics may be useful against sexually transmitted organisms such as N. gonorrhoeae and C. trachomatis, which can cause inflammation-induced infertility. These findings also provide an insight into the use of probiotics as prospective agents that could reduce the impact of infection and inflammation without dependence on antibiotic therapies, which contribute to antibiotic resistance.

REFERENCES


