

## Evaluation of profertility effect of probiotic *Lactobacillus plantarum* 2621 in a murine model

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**Background & objectives:** Urogenital infections of bacterial origin have a high incidence among the female population at reproductive age, affecting the fertility. Strains of *Escherichia coli* can colonize the vagina and replace natural microflora. *Lactobacillus* the predominant vaginal microorganism in healthy women, maintains the acidic vaginal pH which inhibits pathogenic microorganisms. Studies on *Lactobacillus* have shown that these can inhibit *E. coli* growth and vaginal colonization. An alternative therapeutic approach to antimicrobial therapy is to re-establish *Lactobacillus* in this microbiome through probiotic administration to resurge fertility. Therefore, the aim of the present study was to determine the capability of *L. plantarum* 2621 strain with probiotic properties, to prevent the vaginal colonization of *E. coli* causing agglutination of sperms and to evaluate its profertility effect in a murine model.

**Methods:** Screened mice were divided into five groups *i.e.* control group, *E. coli* group, *Lactobacillus* group, prophylactic and therapeutic groups. The control group was infused with 20  $\mu$ l PBS, *E. coli* group was administered with  $10^6$ cfu/20  $\mu$ l *E. coli*, and probiotic group was administered with *Lactobacillus* ( $10^8$ cfu/20  $\mu$ l) for 10 consecutive days. In prophylactic group, the vagina was colonized with 10 consecutive doses of *Lactobacillus* ( $10^8$ cfu/20  $\mu$ l). After 24 h, it was followed by 10 day intravaginal infection with *E. coli* ( $10^6$ cfu/20  $\mu$ l) whereas for the therapeutic group vagina was colonized with ( $10^6$ cfu/20  $\mu$ l) *E. coli* for 10 consecutive days, followed by 10 day intravaginal administration with *Lactobacillus* after 24 h.

**Results:** Upon mating and completion of gestation period, control, probiotic and the therapeutic groups had litters in contrast to the prophylactic group and the group administered with *E. coli*.

**Interpretation & conclusions:** Results indicated that *Lactobacillus* intermitted colonization of pathogenic strains that resulted in reinforcement of natural microflora and resurge fertility.

**Key words** *Escherichia coli* - infertility - intravaginal administration - *Lactobacillus* - spermagglutination - vaginal lavages

The prevalence of male factor infertility has been reported to be 30 per cent while female factor infertility is found to be 40-55 per cent<sup>1,2</sup>. Infections may lead to male infertility with a prevalence of 15 per cent<sup>3</sup> while

in females it accounts for 30-40 per cent<sup>4</sup>. Bacterial interactions with spermatozoa lead to morphological defects as well as changes in functional parameters of spermatozoa *in vitro*<sup>5</sup>. These may in turn, lead to

a decrease in the fertilization potential of the sperm. *Escherichia coli* is one of the dominant bacteria isolated from the semen samples of males with infertility. *E. coli* inhibits spermatozoa motility *in vitro* and readily adheres to and agglutinates sperm<sup>6</sup>. Antibiotics have been extensively used as effective therapy for the treatment of bacterial infections but due to increasing drug resistance of urogenital pathogens, development of alternative therapeutics is vital. *Lactobacillus* has been shown as an alternative to antibiotics in bacterial infections which on intravaginal administration colonized by eradicating the pathogens<sup>7</sup>.

Lactobacilli are predominant microorganisms balancing the normal vaginal milieu. Lactobacilli comprise 70 per cent of the total microorganisms isolated (*i.e.* at the level of  $10^7$ - $10^8$  cfu/g) from the vaginal fluid influencing the vaginal ecosystem<sup>8,9</sup>. Recolonizing the vagina with live lactobacilli may offer a choice of treatment. However, the use of lactobacilli in improving compromised fertility induced by bacteria has not been evaluated. Carefully designed trials using well characterized probiotic strains and treatment regimens are required to evaluate the effect of probiotics on fertility outcome. *L. plantarum* 2621 has been shown to display *in vitro* properties relevant to colonization as assessed by adherence to vaginal epithelial cells<sup>10</sup>. The present study was undertaken to determine the capability of *L. plantarum* 2621 to overcome vaginal colonization and infertility induced by spermagglutinating *E. coli*, in a mouse model.

### Material & Methods

This study was conducted in the Microbiology department of Panjab University, Chandigarh, India. The strain of *E. coli* used in the present study was isolated earlier in our laboratory from the semen sample of a patient with complains of infertility. A standard strain of *L. plantarum* MTCC 2621 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. These strains were maintained on Luria agar (LA) and de Mann Rogosa Sharpe (MRS) agar (5% CO<sub>2</sub>). Strains were stored as glycerol stocks at -60°C.

**Animals:** Sexually mature, 5-6 wk old male (25±2 g) and 4-5 wk old female (22±2 g) BALB/c mice were used in the present study. 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.

**Extraction of mouse spermatozoa:** Male mice (6-7 wk old) were used for isolation of the spermatozoa. Mice were sacrificed by cervical dislocation and sperms from the cauda epididymis and vas deferens were collected by teasing in different media kept at 37°C to maintain sperm motility. Motility of mouse spermatozoa was maintained in phosphate buffer saline (PBS, pH 7.2). Isolated sperms were utilized to screen mouse vagina harbouring spermagglutinating strains.

**Sperm bacteria interaction:** Sperm bacteria interaction was studied *in vitro* by incubating equal volumes (50 µl) of sperm sample and (50 µl) cell culture to evaluate spermagglutinating property at 37°C for 30 min and 1h. The adherence of immotile spermatozoa to each other or of motile spermatozoa to mucus strands, non-sperm cells or debris was considered to be non-specific aggregation. Agglutination specifically refers to motile spermatozoa sticking to each other, head-to-head, tail-to-tail or in a mixed way.

**Screening of animals:** The animals were first screened for micro-organisms that naturally inhabit the BALB/c mouse vagina. The microbiota of the mice induced in estrous cycle by whitten 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 24 h and MRS agar in 5 per cent CO<sub>2</sub> for 48 h. Strains growing on BHI plates were further checked for spermagglutinating/immobilizing properties. Mice harbouring *Lactobacillus* (growth on MRS agar) / *E. coli* (growth on EMB agar with green sheen) or any other bacteria (growth on BHI) with spermagglutinating/immobilizing property were excluded from the study. Screened mice were divided into five groups, *i.e.* control group, *E. coli* group, *Lactobacillus* group, prophylactic and therapeutic group with three mice per group and the experiments were done twice amounting to total number of animals used in this study 30. In the first group as control six mice were inoculated intravaginally with PBS, the second group was used to induce the vaginal infection with spermagglutinating *E. coli*, the third group was used to study colonization by *L. plantarum* 2621 and the remaining two groups were used to evaluate the preventive and therapeutic effects.

**Preparation of inoculum:** *E. coli* was cultivated in Luria broth at 37°C for 24 h. Cell culture was centrifuged at

7267 g for 20 min and washed twice with PBS (50 mM, pH 7.2). Cells were suspended in the same buffer and adjusted to a concentration of  $10^6$  colony forming units (cfu)/20  $\mu$ l. Similarly, *L. plantarum* 2621 was grown in MRS broth and was adjusted to a concentration of  $10^8$  cfu/20  $\mu$ l.

*Effect of spermagglutinating strain of E. coli on fertility outcome:* To demonstrate the colonization and induction of infertility, a group of female mice were administered intravaginally with  $10^6$  cfu of *E. coli* per mouse in 20  $\mu$ l PBS for 10 consecutive days at 24 h interval. As control, mice were inoculated intravaginally with 20  $\mu$ l of PBS for 10 consecutive days. Vaginal lavages were taken every third day upto 35 days, so as to monitor vaginal colonization. The re-isolated bacteria from the vaginal swabs were further confirmed as *E. coli* by culture characteristics and biochemical tests (Hi-Motility biochemical identification kit, Hi-Media Laboratories, Mumbai, India) and were also checked for spermagglutinating activity *in vitro*.

On day 12, these 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 the females were separated and mating was confirmed by observing the presence of vaginal plug in all the female mice.

*Effect of probiotic L. plantarum 2621 on fertility outcome:* The mice of *Lactobacillus* group were administered by intravaginal inoculation with 20  $\mu$ l of the suspension of *Lactobacillus* in PBS adjusted to  $10^8$  cfu/20  $\mu$ l. Vaginal lavages were taken every third day upto 35 days and cultured on MRS agar (5% CO<sub>2</sub> for 48 h) so as to monitor vaginal colonization. On day 12, these 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 the females were separated and mating was confirmed by observing the presence of vaginal plug in all the female mice.

*Prophylactic effect of L. plantarum 2621 against spermagglutinating E. coli induced infertility:* The preventive effect of *Lactobacillus* on the infertility induced by spermagglutinating organisms was studied by administering *Lactobacillus* ( $10^8$  cfu/20  $\mu$ l) for 10 consecutive days into the vagina of mice, 24 h later followed by a challenge of spermagglutinating *E. coli* ( $10^6$  cfu/20  $\mu$ l) for 10 consecutive days intravaginally. Lavages were taken at day 3 interval upto 35 days to monitor the vaginal colonization and viability of *Lactobacillus* and spermagglutinating *E. coli* checked

on MRS (5% CO<sub>2</sub> for 48 h), LA and EMB agar. Mating was allowed 24 h after the last inoculation as described before.

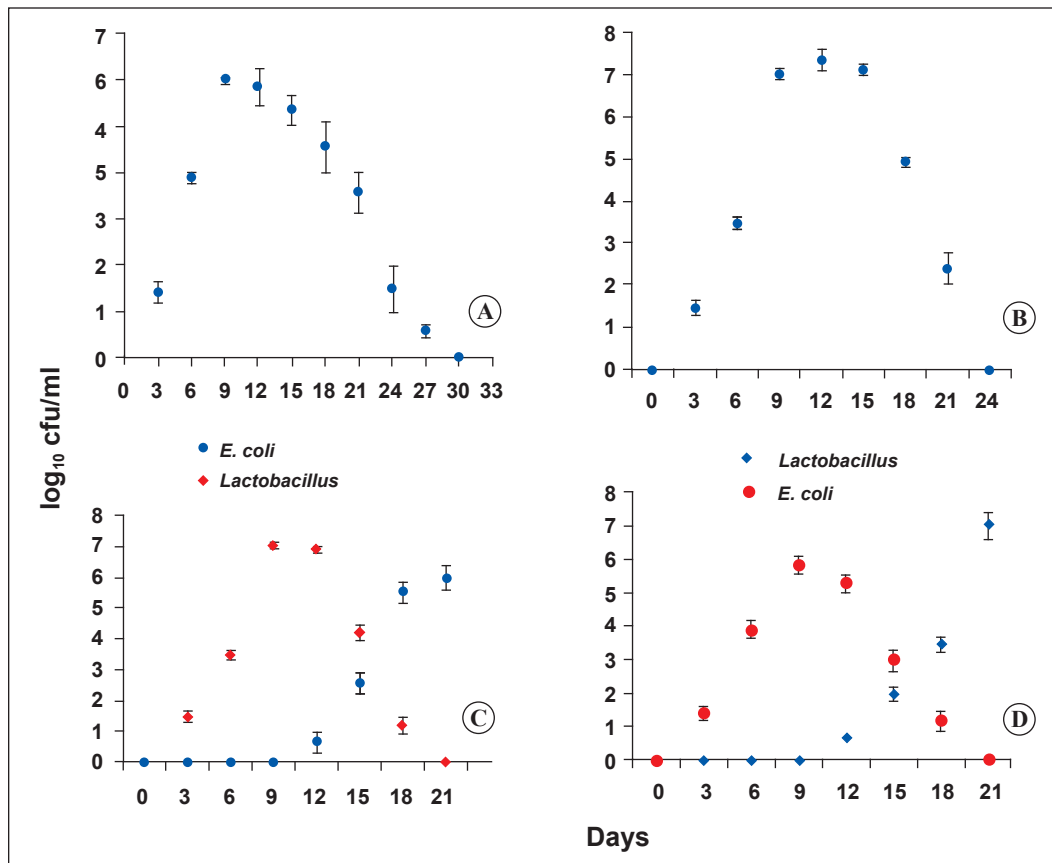
*Therapeutic effect of L. plantarum 2621 against spermagglutinating E. coli induced infertility:* Screened mice were administered intravaginally with the spermagglutinating *E. coli* ( $10^6$  cfu/20  $\mu$ l) for 10 consecutive days to establish an infection, 24 h later followed by infusion with *Lactobacillus* ( $10^8$  cfu/20  $\mu$ l) for 10 consecutive days to ameliorate the impairment caused by *E. coli*. Lavages were taken on every 3<sup>rd</sup> day upto 35 days to assess the establishment of infection and evaluation of colonization of *Lactobacillus* and viability of the organisms on MRS (5% CO<sub>2</sub> for 48 h), LA and EMB agar. Mating was allowed 24 h after the last inoculation.

*Statistical analysis:* All bacterial counts were expressed as mean  $\pm$  SD. An analysis of variance was used for differences in numbers of viable microorganisms from various groups.

## Results

Microbiological analysis of normal flora in mice from vaginal samples was performed by light microscopy and culture characteristics. Gram staining of sample slides showed that higher percentage (48%) of mice harboured Gram +ve cocci followed by Gram -ve rods (23%), very small percentage (8.3%) of mice were found to harbour *Lactobacillus*. These observations correlated with the white colonies on BHI, transparent colonies on Luria agar plates under aerobic conditions. There was growth on very few MRS agar plates. This indicated absence of lactobacilli strains in a large percentage of mice.

*Effect of spermagglutinating strain of E. coli on fertility outcome:* Lavages taken at regular intervals demonstrated persistence of *E. coli* in the mouse vagina at high counts of 4.5 log<sub>10</sub> cfu/ml for more than 17 days (7 days after last inoculation), thereafter started decreasing gradually by day 24 to 1.5 log<sub>10</sub> cfu/ml and was cleared by day 31 and the vaginal cultures were thereafter negative for the respective organism (Figure A). Upon mating on day 12 with the proven male breeder mice in 2:1 ratio in the presence of organisms all the mice failed to conceive and no pregnancy related changes were observed. These results were in contrast to the control mice inoculated with PBS which showed consistent pregnancy related changes



**Figure A.** Vaginal establishment of *E. coli* in BALB/c mice. Results are shown as mean  $\pm$  SD (n=3 observations). **Figure B.** Vaginal colonization of *L. plantarum* 2621 in female mice. Results are shown as mean  $\pm$  SD (n=3 observations). **Figure C.** Prophylactic effect of *L. plantarum* 2621 against *E. coli* in BALB/c mice. Results are shown as mean  $\pm$  SD (n=3 observations). **Figure D.** Therapeutic effect of *L. plantarum* 2621 on *E. coli*. Results are shown as mean  $\pm$  SD (n=3 observations). Significant differences found between the untreated control (**Figure A**) and the treated group ( $P < 0.05$ ).

such as weight gain, strings of pearl by palpation by the day 14, abdominal distension and the delivery of pups at the end of gestation period by day 21.

**Effect of probiotic *L. plantarum* 2621 on fertility outcome:** Lavages taken at regular intervals verified the colonization of *Lactobacillus* in the vaginal samples. *Lactobacillus* was observed to persist in the vagina in high counts of 7.12 log<sub>10</sub> cfu/ml for 15 days, thereafter started decreasing to the count of 2.4 log<sub>10</sub> cfu/ml on day 21 and was cleared by day 24 and the vaginal cultures were negative thereafter for respective organism (Figure B). Mice allowed to mate on day 12 with the proven breeder male mice in 2:1 ratio in the presence of *Lactobacillus* in the vagina were all fertile. These results were analogous to the control group inoculated with PBS and showed pregnancy related changes.

**Prophylactic effect of *L. plantarum* 2621 against spermagglutinating *E. coli*:** It was observed *Lactobacillus* colonized in high counts of 7.12 log<sub>10</sub> cfu/ml on day 10, however, its number decreased progressively after the challenge with each dose of *E. coli*. The count decreased to 4.1 log<sub>10</sub> cfu/ml on day 15 and was cleared by day 21 and cultures were thereafter negative for *Lactobacillus*. With increase in number of doses of *E. coli* challenge the colonization of vagina with *E. coli* tends to increase to 5.52 log<sub>10</sub> cfu/ml on day 18 and by the end of 10 inoculations *E. coli* replaces *Lactobacillus* completely (Figure C). These mice after mating on day 22 in the absence of *Lactobacillus* and presence of *E. coli* failed to conceive, no pregnancy related changes were observed.

**Therapeutic effect of *L. plantarum* 2621 against spermagglutinating *E. coli*:** To evaluate the therapeutic

effect, vaginal lavages collected on regular time intervals were cultured on EMB, Luria agar and MRS (5% CO<sub>2</sub>, 48 h) agar at 37°C. High counts of *E. coli* (5.8 log<sub>10</sub> cfu/ml) were found to colonize the vagina by the last inoculation on day 10. Thereafter its number decreased to 3.01 log<sub>10</sub> cfu/ml on day 15 once the *Lactobacillus* started establishing after the day four of *Lactobacillus* inoculation and *E. coli* was cleared by day 21. The cultures were found negative for *E. coli* as *Lactobacillus* completely removed the *E. coli* (Figure D). A significant difference between the untreated control (Figure A) and the treated group (Figure D) was found ( $P < 0.05$ ). These mice when allowed to mate in the absence of *E. coli* and presence of *Lactobacillus* were fertile.

### Discussion

This study was carried out to evaluate the effect of lactobacilli on infertility due to the presence of spermagglutinating *E. coli*. Many studies have related the absence of lactobacilli, with genital infections such as bacterial vaginosis<sup>11,12</sup>. *Lactobacillus* is the prominent organism present in the healthy human vagina that comprises 70 per cent of the total organisms isolated<sup>13,14</sup>. Lactobacilli play an important role in gastrointestinal tract, urinary tract and in the vagina<sup>8,15</sup>. Substances such as H<sub>2</sub>O<sub>2</sub>, lactic acid and bacteriocin are produced by lactobacilli to protect the human vagina<sup>13,16,17</sup>. To ascertain the important factors in pathogenesis of infertility and the effect of lactobacilli on fertility, mouse model was used for intravaginal inoculations that can simulate ascending genital tract infections in female<sup>18,19</sup>. *L. plantarum* 2621 was able to colonize the mouse vagina efficiently. This showed association between the colonization observed *in vivo* with our *in vitro* studies of *Lactobacillus* attachment to the vaginal epithelium<sup>10</sup>. Other investigators<sup>20,21</sup> have used a large number of doses to ensure the colonization of *Lactobacillus*. Mice inoculated with 10<sup>8</sup> cfu/20 µl of *Lactobacillus* for 10 consecutive days upon mating on day 12 were all fertile delivering litters on completion of gestation period. Same was not true with the spermagglutinating *E. coli*, as all mice administered with 10<sup>6</sup> cfu/20 µl of *E. coli* were rendered infertile. These results were in parallel to the persistence of this strain in high numbers for more than 18 days and were similar to our earlier findings<sup>22</sup>. Bacterial interactions with spermatozoa lead to morphological defects as well as changes in functional parameters of spermatozoa *in vitro*. These may in turn lead to

a decrease in the fertilization potential of sperm. Villegas *et al*<sup>23</sup> demonstrated that a single incubation with *Enterococcus fecalis*, *E. coli* and *Staphylococcus aureus* induced apoptosis in human sperm.

The prophylactic and therapeutic studies were carried out to study the effect of lactobacilli on spermagglutinating *E. coli* strain *in vivo*. In the prophylactic group 10 inoculations of spermagglutinating *E. coli* could successfully eliminate the *Lactobacillus* on day 21. Moreover, upon mating in the presence of *E. coli* and absence of lactobacilli female mice were rendered infertile. The infertility could be due to the high dose of spermagglutinating organisms causing infection that rendered the mice infertile. However, *Lactobacillus* administration in the mouse vagina after *E. coli* establishment resulted in complete elimination of the pathogen resulting in fertility and exhibiting a significant antimicrobial effect in post-infection treatment. Results obtained were parallel to the results of group administered with *Lactobacillus*. These findings were similar to studies by Pascual and his group<sup>7</sup> reaffirming antimicrobial property *in vitro* and *in vivo*.

From the above results it could be concluded that the presence of probiotic strain *L. plantarum* 2621 in vagina had no effects on fertility whereas spermagglutinating organism induced infertility in mice. The therapeutic study indicated that establishment of spermagglutinating organism and its displacement by probiotic strain restored the fertility.

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