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Impact of *Lactobacillus Plantarum* on Reproductive Potential of Male Mice Challenged with Sperm Agglutinating *Escherichia Coli*

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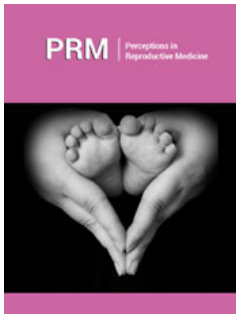

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Abstract

Nowadays, substantial attention is being directed towards using probiotics for treating many diseases. However, the probable role of probiotics in alleviating infertility problems did not receive much attention. Keeping this in mind, the present study was carried out to determine the capability of *L. plantarum* 2621 to ameliorate the uropathogenic colonization and detrimental effects induced by sperm agglutinating Escherichia coli in a male murine model. For this, male Balb/c mice were divided into 3 groups viz. group I (PBS); group II ($10^4/10^8$ of *E. coli*); group III ($10^4/10^8$ of *L. plantarum* 2621) and tissue somatic indices, bacterial load, seminal parameters (sperm count, motility and viability), tissue histology and MDA levels were evaluated to assess the impact of different doses of individual bacteria. The administration of *E. coli* ($10^4/10^8$cfu) alone affected all the parameters negatively irrespective of dose, while in case of *L. plantarum* ($10^4/10^8$cfu), results obtained were comparable to PBS. After this, the combined effect of E. coli and *L. plantarum* 2621 was evaluated and experiments were further carried out to check the ability of *L. plantarum* (10^8cfu) to ameliorate the negative impact induced by *E. coli* ($10^4/10^8$cfu). For this, male mice were divided in to 2 groups viz. group I (PBS) and group II. Group II was further subdivided in to 2 subgroups viz. subgroup I (10^4cfu of *E. coli* + 10^8 of *L. plantarum* 2621) and subgroup II (10^8cfu of *E. coli* + 10^8 of *L. plantarum* 2621). Group I showed no significant change in any of the parameters, while in case of Subgroup I of group II, complete amelioration of the negative impact was observed in all the parameters as compared to subgroup II of group II where the negative impact of *E. coli* was observed.

Introduction

Probiotics are food supplements in the form of live microorganisms when consumed, impart health benefits to the host. Bacterial genera most commonly used in the probiotic preparation are Lactobacillus, Bifidobacterium, Enterococcus, Bacillus and yeasts. Based on many experimental studies and scientific observations, it has been proposed that probiotics can control both digestive and non-digestive diseases [1]. In several cases, urogenital infections are responsible for infertility in both males and females. In females, these infections occur due to the absence of Lactobacillus, which is the main component of the normal flora of the vagina. The probiotic Lactobacilli produce an anti-inflammatory response against pathogenic microorganisms. Hence, it can be hypothesized that inflammation induced infertility can be treated by the use of *Lactobacillus* [2]. The composition and organization of commensal bacterial communities in seminal fluids ascertain the potential causes of male infertility. The consequential inflammation compromises spermatogenesis and sperm cell function due to infection in the male reproductive tract. Sperm abnormalities like aberrant motility, deficient mitochondrial function and DNA integrity loss, are linked with the microorganisms such as Escherichia coli, *Enterococcus faecalis, Neisseria gonorrhoeae, Chlamydia trachomatis, Ureaplasmaurealyticum*, *Candida*. *albicans, Trichomonas vaginalis and Mycoplasma hominis*, which are found in the male urogenital tract. However, the most abundant bacteria in the seminal fluid of normal males include *Staphylococcus*, *Anaerococcus, Corynebacterium, Lactobacillus, Prevotella, Streptococcus, Finegoldia* and others [3]. It can be speculated from

these observations that *Lactobacillus* is not only a major component of the microflora of the female vagina but is also related to the normal semen fluid in males such that the seminal parameters are affected by its presence.

Dardmeh et al. [4] have studied the effect of probiotic *L. rhamnosus* on sperm motility and kinematics and found an increase in the fraction of motile sperm compared to immotile sperms when probiotics are administered. Therefore, in the present study, sperm agglutinating Escherichia coli previously isolated in our laboratory and the standard strain of *Lactobacillus plantarum* (2621) were used to colonize in the mouse in order to study their effect on reproductive potential individually. Further, a study was undertaken to determine the capability of *L. plantarum* to overcome the uropathogenic colonization and reduced reproductive potential induced by sperm agglutinating *E. coli* in the male murine model.

Materials and Methods

Experimental animals

In the present study, sexually mature, 5-6 weeks old Balb/c males weighing 25±5g were used, which were obtained from Central Animal House, Panjab University. Standard laboratory conditions were maintained for keeping animals, with a photoperiod of 12h of light & 12h of darkness. A standard pellet diet consisting of 20-21% crude protein, 4% fat, 5.0-7.5% crude fibre, 8-9% ash, 1.0-1.5% calcium, 0.6-0.8% phosphorus and 50% nitrogen free extract (M/s Ashirwad Industries Pvt. Ltd.) and water ad libitum were given to all mice. All the experimental protocols were reviewed and approved by the Institutional Animals Ethics Committee of the Panjab University, Chandigarh (Approval No: PU/45/99/CPCSEA/IAEC/2018/221) and were performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Microorganisms

A clinical isolate of Escherichia coli (isolated in our laboratory from semen of males undergoing analysis at PGIMER, Chandigarh) causing 100% sperm agglutination in vitro and a standard strain of *Lactobacillus plantarum* (MTCC 2621), were used in the present study. The standard strain was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Sector 39, Chandigarh, India.

Preparation of inoculum

The sperm agglutinating *E. coli* and *L. plantarum* were grown in Luria-Bertani broth (LB) and De Man, Rogosa and Sharpe broth (MRSB), respectively, under shaking conditions for 24h at 37 °C. The culture broths were centrifuged after incubation at 10,000rpm for 10min. The pellet so obtained was washed twice with Phosphate Buffer Saline (PBS) (50mM, pH 7.2). The final concentration of 10^4 and 10^8CFU/20µl of each organism was achieved by suspending the pellet in PBS buffer.

Impact of intravas deferens inoculation of *E. coli*/*L. plantarum* on reproductive potential of male mice

In order to assess the role of *E. coli*/*L. plantarum* on the reproductive potential of male mice, Balb/c mice were divided into three groups (I, II, III):

1. Group I (n = 3): PBS
2. Group II was further subdivided into 2 subgroups:

a) Subgroup E1 (n = 3): 10^4cfu/20µl of *E. coli*

b) Subgroup E2 (n = 3): 10^8cfu/20µl of *E. coli*

3. Group III was also further subdivided into 2 subgroups:

a) Subgroup L1 (n = 3): 10^4cfu/20µl of L. plantarum

b) Subgroup L2 (n = 3): 10^8cfu/20µl of L. plantarum

Mice were anesthetized (ketamine (75mg/kg) and xylazine (121mg/kg)) and the right vas, testis and epididymis were exteriorized aseptically via a vertical incision in the scrotum. By using 27-gauge needle, 20µl of inoculum (single dose) was instilled into the lumen of right vas deferens, towards the direction of epididymis. 3-0 silk suture was used to close incision and animals were housed individually in propylene cages to avoid transmission of organisms. No mortality due to surgical procedure was observed and the animals revived quickly.

Tissue somatic indices TSI (%)

To evaluate any effect on TSI, mice from each group were sacrificed on day 7and TSI (percent organ weight in relation to body weight) was calculated according to equation [5].

Bacterial load

The viable bacterial load was calculated after weighing the organs which were collected under sterile conditions. The organs were immersed in separate Eppendorf's in 500µl PBS (50mM, pH 7.2). These organs were homogenized manually to form a dense mixture. 100µl of this mixture was spread on LA and MRSA plates and incubated at 37 °C for 24h. The number of colonies were counted after 24h and log CFU/g of tissue was calculated.

Confirmation of reisolated microorganisms

The obtained bacterial isolates were streaked on Eosin Methylene Blue (EMB) agar plates. The Group I administered with PBS was culture negative whereas Group II administered with different doses (10^4/10^8) of *E. coli* confirmed the presence of *E. coli* by giving green metallic sheen in all the Subgroups (E1and E2). The obtained bacterial isolates were streaked on MRS agar plates. The Group I administered with PBS was culture negative whereas in Group III administered with different doses of (10^4/10^8) *L. plantarum* confirmed its presence by showing growth when streaked on MRS agar plates.

Analysis of Seminal Parameters

Total sperm count: After inoculation, on day 7 mice were sacrificed by cervical dislocation and with the help of dissecting kit they were dissected and abdomen was cut open. The vas deferens was pulled out and placed in freshly prepared normal saline

(250µl) in glass plate. The spermatozoa were enabled to swim into the freshly prepared normal saline by gentle agitation and teasing of vas deferens. Clean glass slide was prepared with 10µl of sample with the help of micropipette and covered with cover slip (22mm x 22mm). While placing the cover slip, trapping and formation of air bubbles was avoided. For stabilization, preparation was left undisturbed for approximately 1min. It was then viewed under light microscope (Olympus India Pvt. Ltd.) at 400X magnification. Eight fields were scanned and mean number of spermatozoa in the fields was multiplied by 106.

Sperm motility and viability: A fixed volume of 10µl of the sample obtained was delivered on a clean glass slide with a micropipette, covered by a cover slip (22mm x 22mm) and examined various fields under light microscope at 400X magnification. On the basis of motility, spermatozoa were classified as either motile or non-motile. The relative percentage of motile and immotile sperms was determined after assessing different microscopic fields [6]. For viability, above procedure was repeated with spermatozoa along with eosin dye to differentiate between live and dead spermatozoa. Percentage of viable sperm was evaluated.

Tissue histology: Reproductive organs (vas deferens, cauda and testis) of mice from all groups were examined for any histopathological changes by using standard procedure, after organs fixed with 10% formaldehyde for 24hr. the paraffin embedded tissues were sectioned and stained with hematoxylin and eosin. Any significant changes in the reproductive organs were observed under 100 X and 400 X magnifications.

DA estimation: 2ml of TBA-TCA reagent (15% TCA and 0.375% TBA) was added to 100-500µl of tissue homogenates and mixed. The reaction mixture was kept for 20min in boiling water bath. Protein precipitates settled at the bottom after centrifugation at 3500g for 10min and pink color supernatant was collected. Absorbance was read at 532nm. The MDA levels were calculated in nanomoles per gram of tissue by using extinction coefficient of 1.56 x $105mol^{-1}$ (OD X Total volume/ EC X sample volume).

Combined effect of intra vas deferens inoculation of *E. coli* and *L. plantarum* on reproductive potential of male mice: To determine the role of *E. coli* and *L. plantarum* on the reproductive potential of male mice when administered in combination via intra vas deferens route, male Balb/c mice were divided into two Test Groups. Test Group I was administered with PBS while Test Group II was further divided in to two subgroups (I and II):

a) Subgroup I (n = 3): 10^4 CFU of *E. coli*+ 10^8 CFU of *L. plantarum*/20µl

b) Subgroup II: (n = 3): 10^8 CFU of *E. coli*+ 10^8 CFU of L. plantarum/20µl

After inoculation 3 mice from each group were sacrificed on day 7 and TSI (%), bacterial load, seminal parameters, histopathological changes and MDA levels were evaluated as described in section 2.4.

Result

Impact of intra vas deferens inoculation of *E. coli*/*L. plantarum* on reproductive potential of male mice

Tissue somatic indices TSI (%): In case of mice administered with PBS (Group I), the TSI (%) levels of reproductive organs of right side viz. vas deferens, cauda and testis were estimated to be 0.025±0.001, 0.051±0.0025 and 0.51±0.02 and the corresponding values in left side were 0.06±0.002, 0.039±0.001 and 0.522±0.001, respectively.

In Subgroup E1 (10^4) of Group II, the TSI (%) of reproductive organs on right side were 0.023±0.002, 0.03±0.0017 and 0.417±0.0015 whereas on left, the corresponding values were 0.021±0.0025, 0.041±0.002 and 0.506±0.002, respectively. In Subgroup E2 (10^8), the TSI (%) of reproductive organs on right side were 0.031±0.0026, 0.031±0.003 and 0.349±0.002 whereas on left, the corresponding values were 0.023±0.003, 0.039±0.002 and 0.484±0.002, respectively. From the above results, it was observed that Subgroup E2 showed significant changes in TSI (%) in right side of reproductive organs whereas in left side no significant changes were observed in E1 as compared to Group I (PBS).

In Subgroup L1 (10^4) of Group III, the TSI (%) of reproductive organs on right side were 0.023±0.002, 0.051±0.003 and 0.505±0.002 whereas on left, the corresponding values were 0.061±0.002, 0.038±0.002 and 0.516±0.002, respectively. In Subgroup L2 (10^8), the TSI (%) of reproductive organs on right side were 0.0256±0.001, 0.0513±0.0015 and 0.499±0.002 whereas on left side, the corresponding values were 0.061±0.0025, 0.037±0.0015 and 0.511±0.001, respectively. From the above results, it was observed that no significant changes in TSI (%) in right side and left side were observed in both the subgroups of Group III (*L. plantarum*) as compared to Group I (PBS).

Bacterial load: On day 7, when the homogenates of reproductive organs viz. vas deferens, cauda and testis were plated on LA plate, the results showed that the homogenates of reproductive organs of Group I (PBS) demonstrated no viable count. On the other hand, when the various reproductive organs of mice challenged with *E. coli* (Group II) and *L. plantarum* (Group III) were plated on LA plates on day 7 bacterial load were observed in all the reproductive organs (Table 1) (Figure 1 & 2). The bacterial count was present in both sides of reproductive organs in the subgroups of Group and II and III indicating that bacteria could evade to left side, however, with higher bacterial load in right side as compared to left side.

Table 1: Enumeration of bacterial load (in terms of log10cfu/g of tissue) from vas deferens, cauda and testis of mice challenged intravasally with *E. coli*/*L. plantarum* on day 7.

Groups	log_{10} cfu/g of Reproductive Organs					
	Vas deferens		Cauda		Testis	
	Left	Right	Left	Right	Left	Right

Test Group I (PBS)		-	-	-	-	-	-
Test Group II	Subgroup E1	4.05±0.14	5.69±1.01	-	3.98±0.07	-	-
(*E. coli*)	Subgroup E2	7.58±1.04	10±0.7	7.29±0.04	8.11±0.035	7.02±0.16	7.69±0.06
Test Group III	Subgroup L1	Subgroup L1	4.24±0.07	5.13±0.4	-	4.17±0.02	-
(*L. plantarum*)	Subgroup L2	8.71±0.16	10.3±0.209	8.23±0.05	8.31±0.25	7.62±0.1	8.1±0.04

- No viable count, [Values represent Mean±SD]

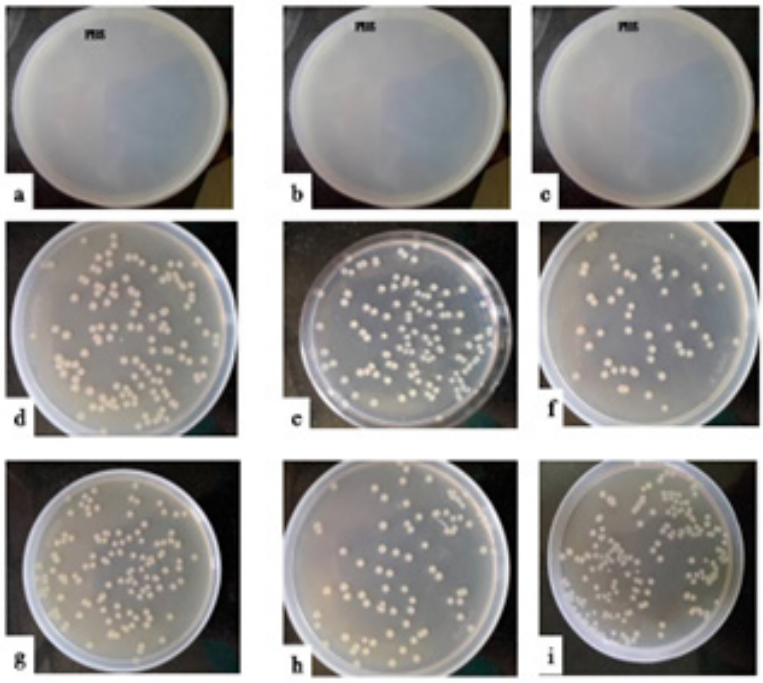

Figure 1: Representative photographs of bacterial load from the homogenates of mice administered with i) PBS (a-c) {a) Left/Right vas deferens, b) Left/Right cauda, c) Left/Right testis}; ii) $10^4/10^8$cfu/20µl of *E. coli* (d-i) {d) Left vas deferens, e) Left cauda, f) Left testis, g) Right vas deferens, h) Right cauda, i) Right testis} on day 7.

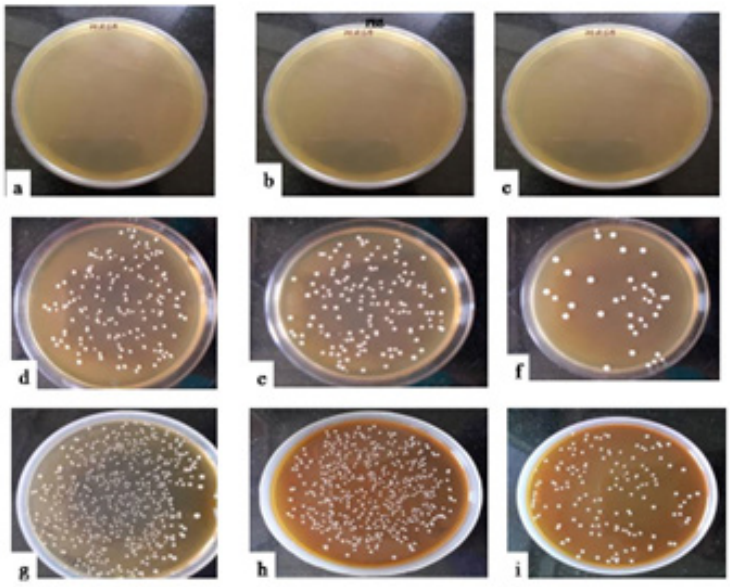

Figure 2: Representative photographs of bacterial load from the homogenates of mice administered with i) PBS (a-c) {a) Left/Right vas deferens, b) Left/Right cauda, c) Left/Right testis}; ii) $10^4/10^8$cfu/20µl of *L. plantarum* (d-i) {d) Left vas deferens, e) Left cauda, f) Left testis, g) Right vas deferens, h) Right cauda, i) Right testis} on day 7.

Reisolation of administered microorganisms: The obtained bacterial isolates were streaked on Eosin Methylene Blue (EMB) and *Lactobacilluson* MRS agar plates. *E. coli* was confirmed by the presence of green metallic sheen on EMB agar and *L. plantarum* was confirmed by its growth on MRS agar (Figure 3 & 4).

Figure 3: Representative photographs of *E. coli* of Group II showing growth on EMB plates on day 7.

Figure 4: Representative photographs of *L. plantarum* (Group III) showing white colonies on MRS agar plates on day 7.

Analysis of seminal parameters: On day 7, all animals from each Group were sacrificed and dissected for evaluation of seminal parameters viz. sperm count, motility and viability. The results showed that Group I, administered with PBS and Group III administered with *L. plantarum*, showed normal seminal parameters (sperm count, motility and viability) in both the sides in comparison to Group II (*E. coli*) where an alteration in seminal parameters was observed (Table 2).

Table 2: Seminal parameters of male mice inoculated with a single dose $10^4/10^8$cfu of *E. coli*/ *L. plantarum* on day 7.

Parameters	Test Group I (PBS)		Test Group II (*E. coli*)				Test Group III (*L. plantarum*)			
			Subgroup E1 (10^4)		Subgroup E2 (10^8)		Subgroup L1 (10^4)		Subgroup L2 (10^8)	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Sperm count (x 10^6/ml)	24.7±0.26	23.56±0.37	14.3±0.61	5.93±0.15	11.7±0.3	-	22.1±0.76	21.5±0.5	23.7±0.3	22.5±0.9
Motility %	56.53±0.61	55.06±0.75	27.76±0.25	4.01±0.06	17.66±0.4	-	47.16±0.47	44.1±0.55	47.4±0.45	44.06±0.4
Viability%	68.1±0.4	66.7±0.35	32.4±0.41	6.76±0.305	24.23±0.25	-	57.13±0.3	56±0.56	54.63±0.35	51.33±0.72

[Values represent Mean±SD]

Histopathological Examination: The reproductive organs viz. testis, cauda and vas deferens obtained from mice were examined for histopathological changes on day 7. The Group I instilled with PBS and Subgroup L1 (10^4), and L2 (10^8) of Group III (*L. plantarum*) revealed normal tissue histology in all the reproductive organs (Figure 5). However, in Subgroup E1 (10^4) of Group II, the right testis showed mild hypo spermatogenesis and cauda demonstrated reduced number of mature spermatozoa. The left set of all the reproductive organs showed normal histology in both the Subgroups. In case of Subgroup E2 (10^8), the right set of organs viz. testis showed severe hypo spermatogenesis, cauda revealed lack of mature spermatozoa and vas deferens showed mild inflammation. The left side showed normal histology in all the organs (Figure 6).

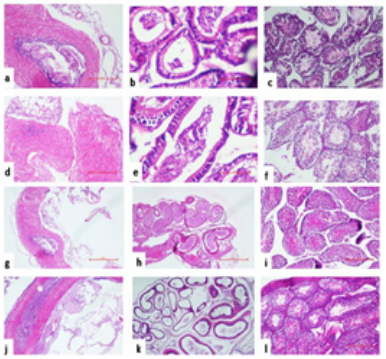

Figure 5: Representative photomicrograph of histopathological examination of various reproductive organs viz. vas deferens, cauda and testis of mice administered in right vas deferens with single dose of i) PBS (Group I) (a-f) {Left side (a,b,c), Right side (d,e,f)}; ii) $10^4/10^8$cfu/20µl of *L. plantarum* (g-l) {Left side (g,h,i), Right side (j,k,l)}on day 7.

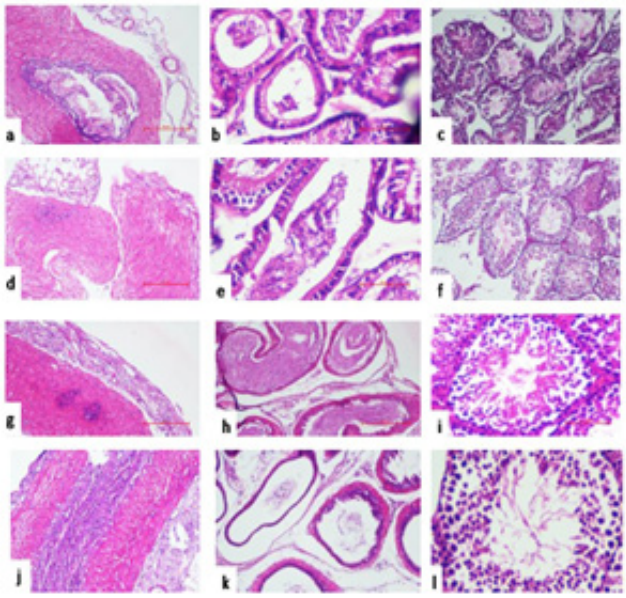

Figure 6: Representative photomicrograph of histopathological examination of various reproductive organs viz. vas deferens, cauda and testis of mice administered in right vas deferens with single dose of i) PBS (Group I) (a-f) {Left side (a,b,c), Right side (d,e,f)}; ii) $10^4/10^8$cfu/20µl of *E. coli* (g-l) {Left side (g,h,i), Right side (j,k,l)}on day 7.

MDA estimation: To study the tissue damage caused by microorganisms, the levels of chief reactive aldehyde i.e., malondialdehyde (MDA) was estimated in reproductive organs, which are formed due to peroxidation of bio-membranes. Hence, reproductive organs viz. vas deferens, cauda and testis were studied for MDA (µmol/g of tissue) production. When the effect of different doses of *E. coli* (Group II) on MDA levels of all the reproductive organs was assessed, highly significant increase in MDA values were observed as compared to Group I (PBS) however, in case of Group III (*L. plantarum*) no significant changes in MDA values were observed as compared to Group I (Table 3).

Table 3: Effect of intravasal inoculation of different doses of *E. coli*/*L. plantarum* on MDA levels in reproductive organs on day 7.

Groups		MDA (µmole/g of Tissue) of Reproductive Organs					
		Vas deferens		Cauda		Testis	
		Left	Right	Left	Right	Left	Right
Test Group I (PBS)		94.16±0.65	94.69±0.74	90.87±0.12	93±0.17	86.79±0.26	87.13±0.2
Test Group II	Subgroup E1	99.4±0.15	101.19±2.49	94.17±0.077	97.18±0.32	95.36±0.43	96.4±0.49
(*E. coli*)	Subgroup E3	435.39±0.17	440.27±0.8	426.95±0.11	430.84±0.42	417.24±0.4	422.2±0.41
Test Group III	Subgroup L1	94.45±0.53	96.77±0.45	91.65±0.16	94.64±0.07	87.07±0.13	88.8±0.19
(*L. plantarum*)	Subgroup L2	109.04±0.522	119.9±0.177	107.8±0.14	114.06±0.202	103.92±0.17	107.49±0.19

[Values represent Mean±SD]

Combined effect of intra vas deferens inoculation of *E. coli* and *L. plantarum* on reproductive potential of male mice

Tissue somatic indices (TSI %): TSI (%) of the reproductive organs excised from groups of mice was determined on day 7. In Test Group I (PBS), the TSI (%) levels of reproductive organs of right side viz. vas deferens, cauda and testis were estimated to be 0.023±0.0025, 0.051±0.003 and 0.507±0.007 and the corresponding values in left side were 0.06±0.0026, 0.039±0.002 and 0.522±0.0015, respectively.

In Subgroup I of Test Group II, the TSI (%) of reproductive organs on right side were 0.025±0.001, 0.048±0.002 and 0.506±0.002 whereas on left side, the corresponding values were 0.061±0.0025, 0.038±0.002 and 0.521±0.002, respectively.

In subgroup II of Test Group II, the TSI (%) of reproductive organs on right side were 0.035±0.002, 0.033±0.002 and 0.38±0.002 whereas on left, the corresponding values were 0.033±0.002, 0.027±0.003 and 0.319±0.001, respectively.

Determination of bacterial load: The homogenates of all reproductive organs viz. vas deferens, cauda and testis of mice inoculated with *E. coli* ($10^4/10^8$cfu) and L. plantarum(10^8cfu), revealed log cfu of both *E. coli* and *L. plantarum* in all the 2 Subgroups of Test Group II whereas Group I (PBS) demonstrated no viable count (Table 4).

Table 4: Enumeration of bacterial load (in terms of log10cfu/g of tissue) from vas deferens, cauda and testis of mice challenged with $10^4/10^8$ of *E. coli* with different doses (10^8) of *L. plantarum*/20µl intravasally on day 7.

Groups			Log cfu of Reproductive Organs					
			Vas deferens		Cauda		Testis	
			Left	Right	Left	Right	Left	Right
Test Group I (PBS)			-	-	-	-	-	-
Test Group II	Subgroup I	E	4.11±0.03	4.52±0.08	-	3.79±0.34	-	-
		L	6.59±0.062	8.26±0.035	5.93±0.08	7.16±0.4	5.37±0.04	6.66±0.09
	Subgroup II	E	7.02±0.11	9.37±0.38	6.85±0.06	7.94±0.06	5.93±0.1	6.71±0.075
		L	6.4±0.03	8.87±0.801	6.24±0.07	7.1±0.02	4.58±0.11	5.9±0.16

- No viable count, [Values represent Mean ± SD]

Analysis of seminal parameters: In order to evaluate the changes in seminal parameters viz. sperm count, motility and viability, all the mice were sacrificed on day 7 of Test Group I receiving PBS and Test Group II receiving single dose ($10^4/10^8$) of *E. coli* in combination with 108cfu of *L. plantarum*/20µl. Significant changes in seminal parameters of right side of Test Group II were observed as compared to left side. While Group I (PBS) showed normal seminal parameters (Table 5).

Table 5: Seminal parameters of male mice inoculated with PBS in Group I and of Test Group I, inoculated with the combination of $10^4//10^8$ of *E. coli* with 10^8cfu of *L. plantarum*/20µl on day 7.

Parameters	Group I (PBS)		Test Group II			
			Subgroup I		Subgroup II	
	Left	Right	Left	Right	Left	Right
Sperm count (x 10^6/ml)	23.7±0.9	22.9±0.36	22.06±0.4	21.6±1.4	12.76±0.32	3.9±0.36
Motility %	55.8±0.2	54.46±0.77	45.76±0.32	43.4±0.6	27.03±0.25	21.26±1.4
Viability %	66.5±0.51	65.4±0.4	54.8±0.2	52.1±0.26	29.18±0.36	25.18±0.28

[Values represent Mean±SD]

Histopathological Examination: On day 7, the reproductive organs viz. vas deferens, cauda and testis obtained from mice inoculated with PBS (Test Group I) and of Test Group II inoculated with *E. coli* ($10^4/10^8$) and *L. plantarum* (10^8) in combinations were examined for histopathological changes. In Subgroup I the right and left set of all the reproductive organs showed normal histology (Figure 5). While in case of Subgroup II, the right set of organs viz. testis showed hypo spermatogenesis and vas deferens showed mild inflammation and the left side of organs revealed normal histology (Figure 6).

MDA estimation: Tissue damage caused by microorganisms can be studied by estimating the levels of MDA in reproductive organs viz. vas deferens, cauda and testis in terms of µmol/g of tissue. The MDA values of subgroup I of Test Group II showed insignificant changes in MDA levels as compared to Group I (PBS). However, the MDA values of subgroup II showed significant increase in MDA levels (Table 6).

Table 6: Effect of intravasal inoculation of PBS (Test Group I) and Test Group II with $10^4/10^8$cfu of *E. coli* and 10^8cfu/20µl of *L. plantarum* on the levels of MDA on day 7.

Groups		MDA (µmol/g of tissue) of Reproductive Organs					
		Vas deferens		Cauda		Testis	
		Left	Right	Left	Right	Left	Right
Test Group I (PBS)		94.92±0.27	94.41±0.46	90.99±0.113	94.33±1.36	86.397±0.16	87.03±0.14
Test Group II	Subgroup I	94.01±0.17	94.95±0.11	92.18±0.3	95.05±0.57	87.79±0.11	89.12±0.28
	Subgroup II	255.7±0.82	265.94±0.075	252.25±0.39	256.34±0.08	247.44±0.06	254.6±0.39

[Values represent Mean±SD]

Discussion

The exploitation of probiotics in the grassland of human reproduction is partial and mainly focuses on the female aspect. In this reverence, there are studies in which probiotics have been used as a therapy against bacterial vaginosis, submitting positive results in clinical trials [7]. Conversely, there are fewer accounts with respect to the effect of probiotics on male fertility in humans. Therefore, this experimental study was planned to investigate the

ameliorative effect of *L. plantarum* over *E. coli* induced infertility in male mice. Although, many microorganisms have been greatly recognized to impede sperm parameters and thereby, reducing the fertilizing potential of males. But *E. coli* shows maximum potential among these microorganisms. Francesco et al. [6] had reported that *E. coli* is the second most common isolate from the infertile male. Thus, the clinical isolate of *E. coli* capable of causing 100% sperm agglutination was used in the present study. The standard strain of *Lactobacillus plantarum* MTCC 2621 was procured from the Institute of Microbial Technology, Sector 39, Chandigarh.

To study the relevance of the experiment, mice were administered intravasally with a single dose of 20µl of PBS and $10^4/10^8$ of *E. coli* and *L. plantarum* as a single dose or in different combinations. Mice were sacrificed on day 7 and their impact on male reproductive potential was determined in terms of tissue somatic indices (%), bacterial load, seminal parameters, histopathological analysis and evaluation of reactive oxygen species in terms of MDA levels. The TSI (%) of reproductive organs (vas deferens, cauda and testis) was carried out to investigate their functional status ensuing the various experimental conditions. The results revealed alterations in TSI (%) values of reproductive organs, which lead to the morphological and functional abnormalities of these organs and this, could be attributed to urogenital tract infection by *E. coli*. However, no significant alterations in TSI (%) values of the various reproductive organs were observed in the groups of mice inoculated either with PBS or *L. plantarum*. These results are in harmony with the study carried out by Jantos et al. [7] wherein, they reported that intravasal administration of *C. trachomatis* biovar mouse pneumonitis induced enlargement of epididymides and caused significant alterations in relative organs weights. This highlights the fact that during pathological conditions, TSI becomes a predictor variable that has a dependence on body condition. Tissue somatic indices have been used as an indicator of Health Status and infectious diseases may alter Tissue somatic indices along with alterations in histopathological and hematological parameters.

In order to maintain an argument that changes in TSI (%) were only due to the pathogen under study, reisolation of both the microorganisms from reproductive tissues of mice was carried out. Bacterial enumeration studies showed bacterial load in both sets of reproductive organs of both microorganisms. In support of this, Hackett et al. [8] have also established that upon inoculation of *E. coli* into the left vas deferens of rabbits, and the organism could be recovered from both sets of reproductive organs. The colonization of *E. coli* (10^8) led to impairment of spermatogenesis as obvious by the absence of spermatozoa (azoospermia) being prominent in right vas deferens, whereas when 10^4cfu of *E. coli* was administered significant decrease in sperm count in right vas deferens was observed. The left vas deferens also revealed a reduction in spermiogram in both the subgroups. On the other hand, the PBS/*L. plantarum* ($10^4/10^8$) administered groups revealed normal spermatozoa on the day of sacrifice. Diemier et al. [9] reported a substantial decrease in sperm concentration when inoculated intravasally with *E. coli*.

The result of colonization of these microorganisms on the histological architecture of reproductive tissue was also inspected. Hence, in the present study, mice in groups administered with (10^8) *E. coli*, the histopathological examination discovered severe changes in the reproductive organs in contrast to PBS/*L. plantarum* ($10^4/10^8$) receiving mice where no changes in the histological organization of organs were seen. Further, the right-side organs showed severe inflammation whereas; mild inflammation was seen in the organs of left side of *E. coli* (10^8) administered group. Similar results have been documented in earlier study that uropathogenic *E. coli* could result in hypospermatogenesis as an outcome of severe histopathological damage in terms of germ cell loss [10].

Reactive oxygen species are the important signaling molecules that play a major role in the sequence of inflammatory disorders. To evaluate severity of infection, tissue homogenates of both sides of reproductive organs of mice were assessed for lipid peroxidation, which was calculated in terms of levels of MDA. From the previous results, it was clear that (10^8) *E. coli* severely affects the right set of reproductive organs and therefore, the results indicated significantly high levels of MDA in the right set of reproductive organs of groups administered with *E. coli* in contrast to PBS/*L. plantarum*. These major rise in levels of MDA in *E. coli* group indicated increased oxidative stress in reproductive organs, which might have resulted in tissue injury, which further affects the process of spermatogenesis, thereby leading to the commencement of male infertility. In PBS/*L. plantarum* ($10^4/10^8$) administered group of mice, no significant changes in MDA levels were noticed. Masroor et al. [11] have correlated the seminal MDA levels in the patients of infertility wherein, they stated increased MDA levels in oligozoospermic patients in comparison to normozoospermic patients. From these results, it is clear that *E. coli* induced inflammation brings about endothelial dysfunction and tissue injury.

Since *L. plantarum* showed normal results, which were in comparison to PBS, studies were further carried out to determine the dose at which *L. plantarum* can ameliorate the devastating effects of *E. coli*. Therefore, the mice were administered with a single dose of 10^4cfu of *E. coli* in combination with $10^4/10^8$cfu of L. plantarum. When *L. plantarum* (10^8) was given in double the dose of *E. coli* (10^4), complete amelioration of all the parameters was observed such that the results were comparable to PBS group indicating that *L. plantarum* has suppressed the infection swayed by *E. coli*. Eaton et al. [12] obtained similar results, who found that *L. reuteri* suppresses the colonization and signs of disease due to *E. coli* in germ-free animals. He also reported that with an increase in the frequency of administration, protection against *E. coli* is enhanced. Abdel-Aziz et al. [13] found that oral administration of probiotic Saccharomyces cerevisiae enhances the growth performance and significantly mitigated the ochratoxin-induced toxicity by preventing oxidative stress and maintaining the glutathione content and protected against OTA-induced genotoxicity and spermatotoxicity. Yang et al. demonstrated the improved effect of L. acidophilus on the gastric inflammation caused by Helicobacter pylori.

When mice were administered with 10^8 of *L. plantarum* and 10^8 of *E. coli* in combination, the negative impact of *E. coli* dominated over *L. plantarum*. Thus, indicating that the ameliorative effect of *L. plantarum* is dependent on the dose being administered. Dose-dependent results were also observed by Fang et al. [14] in which the effective dose of 10^8 cfu of *L. rhamnosus* 35 was able to cure rotaviral gastroenteritis in children when administered for 3 days.

Conclusion

It can be concluded from the present study that administration of *E. coli* alone can negatively affect the seminal parameters irrespective of dose by colonizing in the reproductive organs of mice, whereas administration of *L. plantarum* does not affect the same. In addition to this, double the dose of *L. plantarum* can ameliorate the negative impacts of *E. coli* when given in combination.

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