

# Lactobacillus reuteri can reduce Gardnerella induced bacterial vaginosis in mice and modulate immune markers

Kiran Shahzadi

University of the Punjab

Syed Zeeshan Ahmad

University of the Punjab

Syed Shoaib Ahmad

The University of Lahore

Najma Arshad (✉ [najmaarshad@gmail.com](mailto:najmaarshad@gmail.com))

University of the Punjab <https://orcid.org/0000-0002-0244-6757>

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## Research article

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# Abstract

**Background** Healthy vaginal microbiome is dominated by Lactobacilli, which constitute a strong line of defense against vaginal diseases like Bacterial vaginosis (BV). Bacterial Vaginosis is a polymicrobial disease characterized by gradual replacement of predominant population of Lactobacillus with anaerobic uropathogens such as Gardnerella vaginalis (GV), Prevotella and Mobiluncus spp. Due to antibiotic resistance in these pathogens, Lactobacillus spp. have been given attention in the prophylaxis and prevention of infections such as, urinary tract infections, genital infections and BV. Current study describes role of L. reuteri in reducing BV in a GV induced BV murine model. In addition, immunomodulatory effects of L. reuteri were assessed by analyzing gene expression of inflammatory and anti-inflammatory markers by real time PCR in vaginal tissue.

**Methods** Study was divided into two parts. In phase 1, two groups of mice were intravaginally administered with L. reuteri and GV at a dose of  $1 \times 10^6$  CFU/ml<sup>-1</sup> and  $5 \times 10^5$  CFU/ml<sup>-1</sup> respectively and their colonization was confirmed by re-isolation of these strains in vaginal washes. In phase 2, prophylactic efficacy of L. reuteri in GV induced BV mice model were observed by considering clinical scoring, re-isolation frequency, bacterial load, change in epithelium of vaginal tissue and tissue inflammatory and anti-inflammatory markers.

**Results** A significant reduction in clinical scores and re-isolation frequency was noticed in GV challenged pre- L. reuteri colonized animals (prophylactic group). On 10 th day, L. reuteri restricted the growth of GV and kept its count significantly lower than in GV positive control group. Reduction in epithelial thickness and exfoliation was noticed in histological sections of treated group as compare to that in positive control group. In addition to their antimicrobial potential against GV, a depletion in IL-6, IL-1 $\beta$  and elevation in IFN- $\gamma$  was noticed in L. reuteri treated GV challenged mice.

**Conclusions** Based on aforementioned findings, it is concluded that L. reuteri could be considered a natural candidate for therapies of female genital infections.

## Background

Bacterial vaginosis (BV) is one of the most prevailing bacterial infections in human that results from overgrowth of microorganisms naturally present in the vagina. The clinical features include thick grey discharge, an amine or “fishy” vaginal odor, an elevated pH and/or the presence of superficial squamous cells studded with bacteria (clue cells) in wet mounts (1). Women with BV are at increased risk of severe health problems such as pelvic inflammatory disease, increased susceptibility to sexually transmitted infections and preterm birth (2). The prevalence of BV is about 18-35.3% in women of 16-25 years of age in Pakistan (3, 4). Although the etiology of bacterial vaginosis remained poorly understood, previous studies have declared that this disorder is a consortium of bacteria that live in symbiotic association and cause dramatic changes in vaginal microbiota by decline in level of *Lactobacillus* spp. and an overgrowth of several aerobic and anaerobic organisms (5).

Recent genomic data have advocated that microbial community in BV vary from one individual to another (6). Moreover, potential role of each species to the cellular, biochemical and clinical characteristics of BV remain difficult to track down. *Gardnerella vaginalis* (GV) was the first bacterium incriminated in the pathogenesis of BV and claimed to be related with the disease (7). The isolation of GV from almost 100% cases of BV has been reported by Srinivasan et al. (8). Studies have confirmed the pathogenic potential of *G. vaginalis* by cell adhesion, penetration, biofilm formation and cytolytic toxin production (9, 10, 11). *G. vaginalis* has a symbiotic relationship with anaerobes present in vagina (12). It produces amino acids which are utilized by *P. bivia* that in return secretes ammonia which is utilized by GV. The mechanisms employed in symbiotic relationship support not only the growth of strict anaerobes normally present in low number in vagina but also cause a shift to more alkaline pH which is unsuitable for beneficial microbes e.g., *Lactobacillus* spp. (13).

*Lactobacillus* spp. are probiotics that antagonize interruptions of indigenous microflora (8, 14) and/or produce secondary metabolites to ameliorate harmful effects of bacterial pathogens (15). Probiotic bacteria are also well known to modulate innate and adaptive responses of host (16). Bacterial vaginosis (BV) is treated by clindamycin or metronidazole but recurrence rate is escalating with the passage of time (8, 17). Under this scenario, it would be imperative to check *Lactobacillus* spp. isolated from healthy humans as therapeutic probiotics to restore and maintain a healthy genital tract (18). Previous literature reports antagonistic effects of *L. johnsonii*, *L. rhamnosus*, *L. acidophilus* and *L. fermentum* against GV in mice, but to the best of our knowledge, no *in vivo* data exists on inhibitory activity of *L. reuteri* in GV induced BV model. In addition, most preventive and therapeutic mechanisms are typically species and strain specific (19). Moreover, most the studies reported their beneficial efficiencies through *in vitro* assays. Animal models are obligatory to confirm beneficial effects of potential strains in *in vivo* settings (20, 21, 22, 23). Furthermore, animal studies can help to study human infections more in detail and help to find treatments for such infections. *L. reuteri* strain used in this study was isolated from healthy women in our previous study. The aim of the current study was to check colonization of *Lactobacillus reuteri* in mice and find its role in reduction of Bacterial vaginosis (BV) induced by using its major etiological agent GV in murine model. Lactobacilli are also reported to provide indirect protection through modulation of immune system. The expression of inflammatory and anti-inflammatory cytokines was also compared among groups to probe the role of *L. reuteri* in immunomodulation.

## Methods

### Study Design

Experiments was conducted using completely randomized design with control groups using female mice (*Mus musculus*) as test units. The study was divided in two phases. In phase one, colonization of GV and *L. reuteri* were checked in immunocompromised mice. In phase 2 effectiveness of prophylactic application of *L. reuteri* was studied by challenging animals with GV.

### Study Samples

Clinical signs of the disease, reisolation of *L. reuteri* and GV from vaginal washes, bacterial load and expression of Immune markers in vaginal tissue and histological changes in vaginal sections were taken into consideration.

### **Characterization of microbes used in study**

*L. reuteri* and GV were isolated from healthy women and patients diagnosed with Bacterial vaginosis (BV) respectively in a previous study. In *in vitro* setting *L. reuteri* was confirmed for its probiotic and antagonistic ability through *in vitro* procedures. It displays good adherence ability, self-aggregation and co-aggregation, acid, bile salt, NaCl and lysozyme tolerance as well as antagonistic activity against *G. vaginalis*, *E. faecalis*, *E. coli*, *S. aureus* and *Streptococcus* spp. (Data not shown). Antibiotic profile shows that strain was sensitive to erythromycin (E15) streptomycin (S10), and gentamicin (CN10) and resistant to ampicillin/sulbactam (SAM20), oxacillin (OX5) and Methicillin (MET5).

GV is Gram variable, pleomorphic rod shaped and show small, transparent colonies on GV selective media (Columbia agar containing nalidixic acid (5mg/L), gentamicin sulfate (10mg/L) and human blood (5%). It shows  $\beta$ -hemolysis on human blood agar but not on sheep's blood. GV was negative for oxidase, catalase, nitrate reduction and Voges Proskauer and ferment starch, sucrose, fructose and maltose but not mannitol. In addition, it was resistant to ampicillin/sulbactam (SAM20).

### **Phase 1: (Establishment of GV murine model)**

#### **Animal housing and grouping**

Two-month-old female BALB/c mice (n=15) weighing 20-30g were purchased from stocks of Government College University Lahore. Mice were housed in standard sized cages (12"×18") at 28-33°C temperature and 40% humidity and they were fed conventional balanced diet and water *ad libitum*. After initial weighing, mice were equally distributed into three groups (A-C). Group A was maintained as negative control, group B and C were exposed to GV and *L. reuteri* respectively.

#### **Initial screening of mice**

Vaginal endogenous flora of each mice was collected by flushing vagina with 50  $\mu$ l sterile Phosphate buffer saline (PBS) using micropipettes (Dragon LAB, China). Each vaginal sample obtained was cultured on MRS and GV selective media. Each isolated colony grown on these agar plates was studied for colony morphology, Gram staining, oxidase catalase, nitrate reduction, Voges Proskauer and antibiotic profile to distinguish vaginal flora of mice from probiotic *L. reuteri* and etiological agent GV.

#### **Preparation of mice for Bacterial colonization**

All mice were prepared for bacterial colonization (24). Briefly, mice were injected subcutaneously  $\beta$ -estradiol 17-valerate weekly throughout the experiment. Dexamethasone sodium phosphate injection was also given daily 2 and 4 days prior to exposure to *L. reuteri* and GV respectively and continued up to 6

days post exposure to GV. A dose of  $5 \times 10^5$  CFU ml<sup>-1</sup> of *G. vaginalis* and  $1 \times 10^6$  CFU ml<sup>-1</sup> of *L. reuteri* was intravaginally inoculated in mice of groups B and C respectively. Details of experimental treatments are given in Table 1.

## **Monitoring of infection**

### **(a) Clinical signs**

Animals were monitored for clinical signs of morbidity such as animal irritation, ruffled fur, vaginal itching, discharge and redness which may be associated with GV induced vaginosis.

### **(b) Re-isolation of *reuteri* and GV**

Animals were screened for the presence of *L. reuteri* and GV for 9 days post exposure to GV. Five vaginal washes were collected on alternative days and spread on MRS and GV selective agar plates for re-isolation of inoculated *L. reuteri* and GV respectively. These plates were incubated for 24-48h under anaerobic condition. After incubation, plates were observed for presence of *G. vaginalis* and *L. reuteri*. Colonies on Columbia agar plates were reconfirmed as GV using biochemical procedures (Negative oxidase, catalase, nitrate reduction and Voges Proskauer tests). *L. reuteri* was detected on basis of colony morphology, Gram staining and matching antibiotic susceptibility profile. On 10<sup>th</sup> dpi, mice were euthanized following ULAM guide lines of euthanization by intraperitoneal injection of ketamine (200 mg/kg of body weight). Vaginal tissues were removed to quantify bacterial load of *L. reuteri* and GV (CFU/g).

## **Phase:2 Protective effects of *L. reuteri* against GV**

### **Animals**

Two-month-old female BALB/c mice, weighing 20-30g were procured from stocks of Government College University Lahore. Mice were housed in cages as mentioned in phase 1. A total of 32 mice were randomly divided into four groups I-IV (n = 8). Experimental treatments to each group are summarized in Table 2.

### **Exposure to *L. reuteri***

After confirming the absence of *L. reuteri* and GV in experimental mice, Estrogen and dexamethasone injections were given to all mice as described in phase 1. Exposure to probiotic was given following (25) with slight modification. Freshly cultured *L. reuteri* was re-suspended in agarized peptone (0.1%, 0.5% w/v peptone and agar respectively) at a concentration of  $1 \times 10^6$  CFU ml<sup>-1</sup>. Two-days post  $\beta$ -estradiol administration, group III and IV were intravaginally inoculated with 50  $\mu$ l of *L. reuteri* suspension with an average inter-inoculation period of twelve hours.

### **Exposure to *G. vaginalis***

On 5<sup>th</sup> day of experiment, the mice were exposed to GV in 50 µl inoculum ( $5 \times 10^5$  CFU ml<sup>-1</sup>) as mentioned in Table 2. These doses of *L. reuteri* and GV were selected following (25) with slight modifications and initial application in phase 1.

### **Monitoring of *G. vaginalis* infection**

#### **(a) Recording of clinical signs/symptoms**

Mice were observed for clinical signs of BV including irritation, ruffled fur, vaginal itching, discharge and redness. All symptoms were given equal weightage. Animals were scored from 0-1 depending upon mean number of symptoms present e.g. animal showing two of the five symptoms was scored as 0.4. After scoring, each animal mean score of each group was used for comparison of general health condition of each group.

#### **(b) Re-isolation of *reuteri* and *G. vaginalis***

Vaginal washes were collected and *L. reuteri* and GV were confirmed on the basis of biochemical characteristics and antibiotic profile (as mentioned in phase 1).

### **Sampling of tissue**

On 17<sup>th</sup> day of experiment, mice were euthanized by using over dose of ketamine (200 mg/kg), and vaginal tissues were harvested. A part of vaginal tissue was preserved by placing in liquid nitrogen later on these were stored at -80°C till further use. Similarly, 100 -150 mg of tissue was used for determination of bacterial load and rest of the tissue was fixed in 10% formalin for histological studies.

### **Bacterial load**

Vaginal tissue (bisected longitudinally) was weighed and homogenized in sterile saline and 100 µl of suspension was spread on MRS and GV selective agar plates at 37°C for 24-48h under anaerobic condition. After incubation period, colonies were enumerated and expressed as log<sub>10</sub> CFU/ g of vaginal tissue (26).

### **Histopathological examination**

In brief, paraffin-embedded vaginal sections (5µm) were stained with hematoxylin and eosin (H &E) and observed under light microscope to assess histopathological changes. Images were captured and degree of thickness and exfoliation of epithelium were observed. The pathology of vaginal tissue was recorded following (7).

### **RNA extraction and cDNA preparation**

Total RNA was extracted from stored tissues at -80°C using TRIZOL RNA extraction protocol (27). RNA extracted was quantified using Nanodrop spectrophotometer followed by complementary DNA (cDNA)

synthesis using 500 ng of RNA with Superscript™ IV First-Strand cDNA Synthesis Kit according to manufacturer's protocol. Quantity of cDNA was measured using aforementioned Nanodrop spectrophotometer.

## Real-Time PCR

The PCR reaction mixture having a final volume of 25 µl containing 12.5 µl SYBR green Dye, 1 µl cDNA, 1 µl forward and reverse primer each of final concentration of 10 Pmole and 9.5 DNase - and RNase-free water. Details of the primers along with annealing temperatures are given in Table 3. Real- Time PCR was carried out on a Bio-Rad CFX Real-Time PCR system for 40 cycles using the following conditions: denaturation at 95 °C for 3 min, annealing at 54-59 °C for 10s and elongation at 72°C for 30s. Average quantification cycle (Cq) values from duplicate measurements were used to determine mRNA expressions. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as Normalization control (28). It was normalized with the average Cq value of the control group.

## Normalized expression ratio (NER) calculation

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as normalization control (Eissa et al., 2016). The degree of expression of all markers was evaluated by difference in Ct value ( $\Delta$ Ct value). The Ct values were normalized by both the "Ct value of GAPDH" and "Ct value of control group" to get  $\Delta\Delta$ Ct values. Mean of  $\Delta\Delta$ Ct values were compared between groups II, III and IV.

## Statistical analysis

Data of bacterial load and inflammatory markers was parametric while Clinical symptoms and degree of epithelial exfoliation data was non parametric. Parametric data was analyzed by Independent sample T-test and ANOVA following post-Hoc Tukey's test. Kruskal wallis H-test was applied to non- parametric data. The p value < 0.05 was considered significant. Data was analyzed using software IBM SPSS version 21 Chicago IL, USA.

# Results

## Phase 1: (Establishment of GV murine model)

### Screening for the absence of *L. reuteri* and GV

There was no growth on GV selective agar plates. On MRS agar plates, only acid producing cocci were observed. Furthermore, normal microbial fauna of mice was susceptible to antibiotic ampicillin/sulbactam (SAM 20).

## Clinical symptoms

Mice exposed with GV (group B) showed clinical signs of morbidity including irritation, ruffled fur, turbidity of vaginal discharge, itching and redness beginning at the 3<sup>rd</sup> dpi. *L. reuteri* exposed mice (group C) seemed active except irritation at the beginning that might be due to inoculation procedures. As expected, Mice from negative control (group A) remained healthy throughout the experiment.

### **Re-isolation of *L. reuteri* and *G. vaginalis***

After intravaginal inoculation of *L. reuteri* and *G. vaginalis*, vaginal washes taken from 8<sup>th</sup> to 16<sup>th</sup> days of experiment were detected for presence of these strains. Distinguishing features of *G. vaginalis* including Gram variable, negative for oxidase, catalase, nitrate reduction, Voges Proskauer tests and resistance to ampicillin/sulbactam (SAM20) were observed in bacterial colonies grown on GV selective agar plates. While growth of *L. reuteri* on MRS plates was confirmed by characteristics like Gram positive, rod-shaped and antibiotic sensitivity to erythromycin (E15) streptomycin (S10), and gentamicin (CN10) while resistance to ampicillin/sulbactam (SAM 20), oxacillin (OX5) and Methicillin (MET5). Based on these assays, their re-isolation was confirmed. *L. reuteri* and GV were found to account for 4.8 log CFU/g and 4.39 log CFU/g respectively (Table 4).

### **Phase:2 Protective effects of *L. reuteri* against GV**

#### **Clinical signs/ symptoms of infection**

Mice in groups I and III remained healthy and their average clinical score was 0 and 0.04 respectively throughout sampling period while mice challenged with GV only were less irritated at the beginning of experiment and scored 0.08 at 2<sup>nd</sup> dpi but other clinical symptoms appeared in succeeding days and at end of the experiment and average score recorded was 0.84. Mice in prophylactic group showed clinical signs during initial period, scoring 0.24 that was reduced to 0.16 at the end of study (Fig 1).

### **Re-isolation frequency of *L. reuteri* and *G. vaginalis***

Vaginal washes obtained at sampling intervals were detected for presence of *L. reuteri* and *G. vaginalis* to confirm their colonization. All mice in group III were positive for *L. reuteri* while it was observed in 7 out of 8 animals in group IV (prophylactic group). *G. vaginalis* colonized successfully in all GV exposed group II and was continuously observed in vaginal washes throughout experiment but there was significant reduction (2 out of 8) in prophylactic group (Fig 2).

#### **Bacterial load**

Bacterial load of *L. reuteri* was  $4.86 \pm .16$  and  $4.91 \pm .13$  CFU/g in group III and IV respectively. GV count was  $4.71 \pm .27$  in group II and reduced significantly  $1.5 \log_{10}$  CFU/g in prophylactic group (Table 5).

#### **Histopathological examination**



Vaginal sections were observed for epithelial thickness and exfoliation in response to GV infection as compared to negative control group. Epithelial exfoliation was higher in GV infection group which correlated positively with increased thickness of transitional epithelium (Fig 3). Both these factors were observed normal in I, III and IV groups.

### Inflammatory and anti-inflammatory cytokines expression

In order to investigate the role of innate immune system in anti-BV mechanism of probiotic therapy, the transcript levels of Pro and anti-inflammatory cytokines were determined in all groups. The relative mRNA expression of these cytokines is presented in Fig 4. All results are expressed as normalized expression ratio (NER). In immunocompromised mice, the exposure to *L. reuteri* resulted in 1.18-fold and 1.19-fold decrease expression of IL-6 and IL-1 $\beta$  in comparison to GV exposed (group II). On the other hand, GV exposure resulted in 4.43 and 4.37-fold increase IL-6 and IL-1 $\beta$  expression respectively. Pre-exposure with *L. reuteri* decreased IL-6 and IL-1 $\beta$  gene expressions to the levels of 2.14 and 2.08-fold in GV infected mice (group IV). Interferon- $\gamma$  which is anti-inflammatory cytokine expressed 0.98-fold and 0.25-fold in *L. reuteri* and GV exposed groups respectively but *L. reuteri* increased its expression to 0.30-fold in prophylactic group than GV exposed group (group II). ROR- $\gamma$ t levels were not influenced by *L. reuteri* in prophylactic group.

## Discussion

Bacterial vaginosis (BV) is the most serious disorder in women of childbearing age, contributing to over 60 per cent of all vulvovaginal infections (32). It can cause serious health hazards, including acquisition and transmission of multiple sexually transmitted agents (33), endometritis (34), spontaneous abortion (35) and pre-term birth (36). BV is a growing international concern due to high prevalence rate worldwide. Antimicrobial therapy is generally used to eradicate these infections but their effectiveness is currently diminishing due to adverse sequel and recurrence of infections (37). Role of probiotic fauna in reducing BV are being explored as alternative approach for restoration and maintenance of healthy vaginal flora and the prevention of recurrent diseases. Several *in vitro* studies provide evidence for antimicrobial effects of *Lactobacillus* spp. such as *L. rhamnosus*, *L. acidophilus*, *L. reuteri*, *L. crispatus*, *L. gasseri*, *L. salivarius* against uropathogens *G. vaginalis*, *E. coli*, *S. agalactiae* and *C. albicans* (25) but their confirmation in *in vivo* setting is mandatory. The mechanism used by lactobacilli in promoting local immunity to pathogens are not completely understood (18) in particular the role of *L. reuteri* on cytokine levels in female genital tract is least reported. Current study focused on prophylactic application of *L. reuteri* as a mean of controlling GV induced BV infection in mice. Initially, endogenous flora was explored for the presence of *L. reuteri* and GV by analyzing vaginal spreads obtained from each of the mice. we observed the presence of Gram-positive cocci on MRS plates and there was no growth on GV selective media. GV were absent in vaginal washes in consistent with our results no previous study has reported GV isolation from laboratory mice. Enterobacteriaceae, streptococci, Staphylococci are predominant vaginal flora of mice. After finding negative results for both strains, study was preceded further. Pre-

oestral stage is prerequisite to induce BV in mice. It is well known that estrogen production positively influences colonization of lactobacilli in healthy human vagina (1).

In the present study when Dexamethasone and  $\beta$ estradiol-immunosuppressed mice were intravaginally inoculated with *L. reuteri* and GV. Successful colonization was observed at the dose of  $1 \times 10^6$  CFU ml<sup>-1</sup> and  $5 \times 10^5$  CFU ml<sup>-1</sup> respectively. Both strains colonized successfully evident from their re-isolation up to 10th dpi and log<sub>10</sub> CFU/g count in tissue homogenate. Animal model was declared established based on re-isolation and bacterial load of both strains in phase 1. In order to determine inhibitory effect of *L. reuteri* against GV, mice were prepared and colonized at the same dose used in phase 1. Clinical signs might be used as a tool to assess progress of infection in mice. Turbidity of discharge, redness and itching were sharply observed in mice exposed with GV. The mice were more sensitive to holding or touching due to vaginal irrigation. These signs were improved (normal range) in prophylactic group. Animal irritation and fur texture are reported first time in this study in addition to other clinical signs mentioned in a previous study (38). The culture of GV in vaginal lavage showed that *L. reuteri* has restricted colonization of GV as compared to GV positive group. In general, infected mice are not to control bacterial proliferation, showing increased bacterial load and/or delayed clearance compared to healthy mice.

Bacterial load is a simple and easy method to determine level of infection. Bacterial load was quantified in homogenate of vaginal tissue removed by euthanizing mice. GV count was 4.71 log<sub>10</sub> CFU/g in GV exposed group but reduced significantly 1.5 log units in prophylactic group (group IV). This finding clearly indicated prophylactic influence of *L. reuteri* on GV count. It might be due to beneficial effects of different mechanisms such as antimicrobial compounds (hydrogen peroxide, organic acid, biosurfactants and bacteriocins) (39, 40) host immune response (25) biofilm formation and colonization ability (41) suggested for probiotics. *L. reuteri* compete out to a significant extent that 80% mice were found free of GV strain in prophylactic effect (group IV). Our results are consistent with findings (42) who reported anti-candida activity of *L. reuteri* in *in vivo* model. The prophylactic effect of *L. reuteri* was further confirmed by histological study of hematoxylin and eosin (H &E) stained vaginal sections and measuring expression levels of pro-inflammatory IL-6, IL-1 $\beta$  and ROR  $\gamma$ t and anti-inflammatory IFN- $\gamma$  markers. GV resulted in robust epithelial exfoliation and increased thickness of transitional epithelium in GV exposed animal. These two features were comparable to normal range in prophylactic and *L. reuteri* exposed groups. Epithelial shedding exfoliation and epithelial thickness have long been reported with BV. Exfoliation removes adherent pathogens and appears one of the defense mechanisms against pathogens but excessive exfoliation increases the establishment of BV-associated bacteria and risk of secondary infection. Our results are consistent with well-known study of (7). It is reasonable to suspect that local immunity may contribute local protection against potential pathogens. The epithelial cells layer possesses "*Toll-like receptors*" (TLR) in their surface recognize molecular patterns associated with the pathogens (PAMPs) and trigger sequence of events to release pro-inflammatory cytokines (IL-6, TNF-  $\alpha$  and IL-1 $\beta$ ) and consequently, the activation of the acquired immune system, which is, the activation of lymphocytes T and B and release of anti-inflammatory cytokines (IFN- $\gamma$ ). IL-6 is a multifunctional

cytokine produced by immune cells following infection. IL-6 production induces acute phase proteins such as serum amyloid A, C-reactive protein (CRP) and  $\alpha$ 1-antitrypsin as part of an inflammatory response (43).

IL-1  $\beta$  is highly inflammatory, activates a range of cells including macrophages and T lymphocytes that may thus lead to production of other cytokines. ROR $\gamma$ t belongs to nuclear hormone receptor superfamily and is required for differentiation of the Th17 lineage and also sufficient to direct the expression of the hallmark cytokines of this lineage. Interferon-  $\gamma$  produced by Th1-cells, NK cells, NKT cells is an important cytokine in recognizing and eliminating pathogens. It increases the efficiency of immune system by enhancing its competence to deliver anti-microbial effector functions. In this study, *L. reuteri* significantly reduced the mRNA expressions of pro-inflammatory markers IL-6, IL-1 $\beta$  in prophylactic group in comparison ROR  $\gamma$ t expression was not influenced significantly. This might be due to fact that effects on cytokine production are strain dependent. IFN- $\gamma$  which is anti-inflammatory expressed higher in probiotic group (group III) as compared to GV exposed mice (group II) but *L. reuteri* elevated IFN-  $\gamma$  expression in prophylactic group (group IV). These findings suggest that *L. reuteri* regulate immune system of host to attenuate GV- induced vaginosis in mice.

Studies suggest that the expression levels of pro-inflammatory cytokines, such as IL-6, TNF-  $\alpha$ , ROR $\gamma$ t and IL-1 $\beta$  in patients with BV infection has been reported higher than normal values and such inflammatory cytokines can attract inflammatory cells chemotactically and further worsen the local mucosal injury (31, 44, 45).

## Conclusions

It is concluded that *L. reuteri* can inhibit clinical symptoms of BV by reducing the growth of pathogen GV and can induce immunity response of host which is comparable to the role of antibiotics. These findings suggest clinical applications of metabolites in of *L. reuteri* as pharmabiotics in treatment of Bacterial vaginosis in human beings.

## Limitations Of The Study

Animal models have had limitations in modeling the complex clinical entity of BV. The genetic modification and fluorescent labeling of strains would made these experiments more informative.

## Abbreviations

GV  
Gardnerella vaginalis  
BV  
Bacterial vaginosis  
H &E

hematoxylin and eosin

## **Declarations**

### **Ethics approvals**

The sampling procedures and animal handling were performed following the guideline of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (UK) with prior approval by Ethical Committee for Animal Experimentation, Department of Zoology, University of the Punjab, Lahore, Pakistan.

### **Consent for publication**

N/A

### **Availability of data and materials**

All data analyzed during this study are included in this published article.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Author's contributions**

All authors have read and approved the manuscript. The experiments were conceived and designed by NA and KS. The experiments were performed by KS and SZA. Real time PCR was performed and interpreted by SZA and SSA. Data was analyzed by NA. KS wrote the first draft of manuscript. NA edited and finalized the manuscript.

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### **Author Information**

### **Affiliations**

Department of Zoology, University of the Punjab, Lahore, Punjab, Pakistan (Najma Arshad, Kiran Shahzadi and Syed Zeeshan Ahmad)

## Corresponding author

Correspondence to Professor Dr. Najma Arshad

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## Tables

**Table 1 Experimental schedule and treatments for phase 1**

Time (Day)	Treatment	Group <sup>#</sup>		
		A	B	C
1	Estrogen	+	+	+
1-4	Dexa <sup>a</sup>	+	+	+
3, 4	<i>L. reuteri</i> <sup>b</sup>	-	-	+
5, 6	Infection <sup>c</sup>	-	+	-
7	Estrogen & Dexa	+	+	+
8	Sampling <sup>c</sup> & Dexa	+	+	+
9	Dexa	+	+	+
10	Sampling & Dexa	+	+	+
11	Dexa	+	+	+
12	Sampling & Dexa	+	+	+
14, 16	Sampling <sup>e</sup>	+	+	+

<sup>#</sup> Group A: Negative control, B: GV (positive control), C: *L. reuteri*

<sup>a</sup>Dexamethasone sodium phosphate

<sup>b</sup>*L. reuteri* exposure at the dose of  $1 \times 10^6$  CFU ml<sup>-1</sup>

<sup>c</sup>GV dose was given at  $5 \times 10^5$  CFU ml<sup>-1</sup>

<sup>d</sup>Vaginal washes were collected on each sampling day to confirm presence of *L. reuteri* and GV

<sup>e</sup>Animals were sacrificed on 10<sup>th</sup> dpi and vaginal tissue was removed to determine bacterial load

**Table 2 Experimental schedule and treatments (phase 2)**

Schedule (Day)	Treatment	Group <sup>#</sup>			
		I	II	III	IV
1	Estrogen	+	+	+	+
1-4	Dexa <sup>a</sup>	+	+	+	+
3, 4	<i>L. reuteri</i> <sup>b</sup>	-	-	+	+
5, 6	Infection <sup>c</sup>	-	+	-	+
7	Estrogen & Dexa	+	+	+	+
8	Sampling <sup>c</sup> & Dexa	+	+	+	+
9	Dexa	+	+	+	+
10	Sampling & Dexa	+	+	+	+
11	Dexa	+	+	+	+
12	Sampling <sup>d</sup> & Dexa	+	+	+	+
14, 16	Sampling <sup>e</sup>	+	+	+	+

# Group I: Negative control, II: GV- positive, III: *L. reuteri*, IV, Prophylactic

<sup>a</sup> Dexamethasone sodium phosphate

<sup>b</sup> *L. reuteri* exposure at the dose of  $1 \times 10^6$  CFU ml<sup>-1</sup>    <sup>c</sup> GV dose was given at  $5 \times 10^5$  CFU ml<sup>-1</sup>

<sup>d</sup> Vaginal washes were collected on each sampling day to confirm presence of *L. reuteri* and GV    <sup>e</sup> Animals were sacrificed on 10<sup>th</sup> dpi and vaginal tissue was removed to determine bacterial load

**Table 3 Annealing temperatures of primers of selected target genes and reference gene (GAPDH)**

Gene	Primer sequence	Annealing Temp. (°C)	Reference
GAPDH	F 5' TGCAGTGGCAAAGTGGAGAT 3' R 5' TTTGCCGTGAGTGGAGTCAT 3'	54	Kozera and Rapacz, (2013)
IL-6	F 5' GAGGATACCACTCCCAACAGACC 3' R 5' AAGTGCATCATCGTTGTTTCAT 3'	55	Zarepour et al. (2013)
IL-1β	F 5' CAGGATGAGGACATGAGCACC 3' R 5' CTCTGCAGACTCAAACCTCCAC 3'	55	Zarepour et al. (2013)
RORγt	F 5' ACAGCCACTGCATTCCCAGTTT 3' R 5' TCTCGGAAGGACTTGCAGACAT 3'	55	Celiberto et al. (2018)
IFN-γ	F 5' TGAACGCTACACACTGCATCTTGG 3' R 5' CGACTCCTTTCCGCTTCCTGAG 3'	59	Celiberto et al. (2018)

**Table 4 Clinical signs and successful colonization of *L. reuteri* and GV**

Clinical symptoms	Group A	Group B	Group C
Irritation	-	+	+
Fur condition	-	+	-
Vaginal itching	-	+	-
Vaginal discharge	-	+	-
Vaginal redness	-	+	-
Re-isolation of <i>L. reuteri</i> and GV			
<i>L. reuteri</i>	-	-	5
GV	-	4	-

Table 5 Comparison of bacterial load (CFU/g) of *L. reuteri* and GV in all experimental groups

GROUPS	<i>L. reuteri</i>	GV
I	0	0
II	0	4.71±0.27
III	4.86± 0.16	0
IV	4.91± 0.13	3.07± 0.09

## Figures

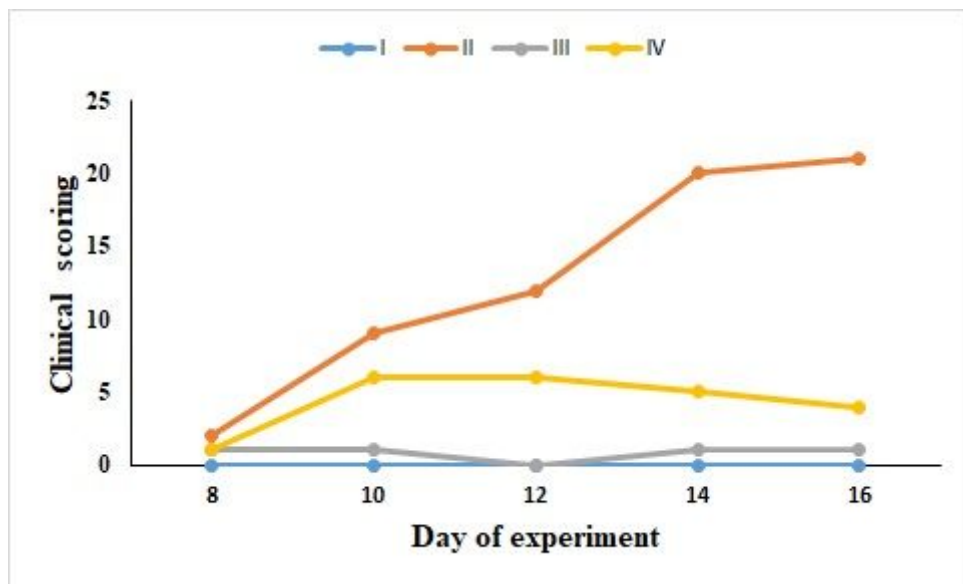
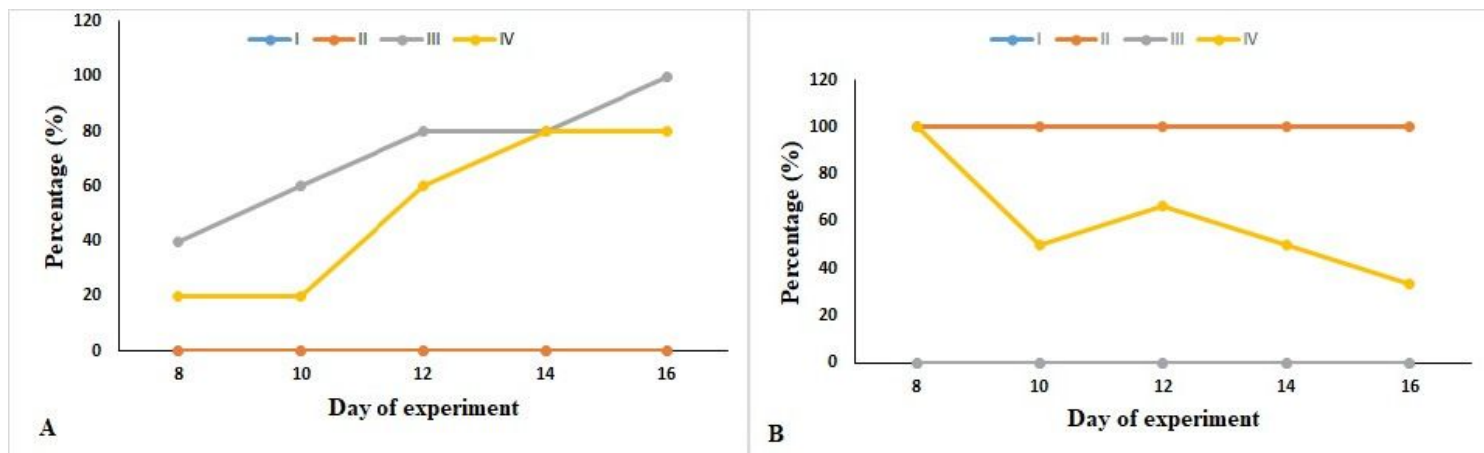


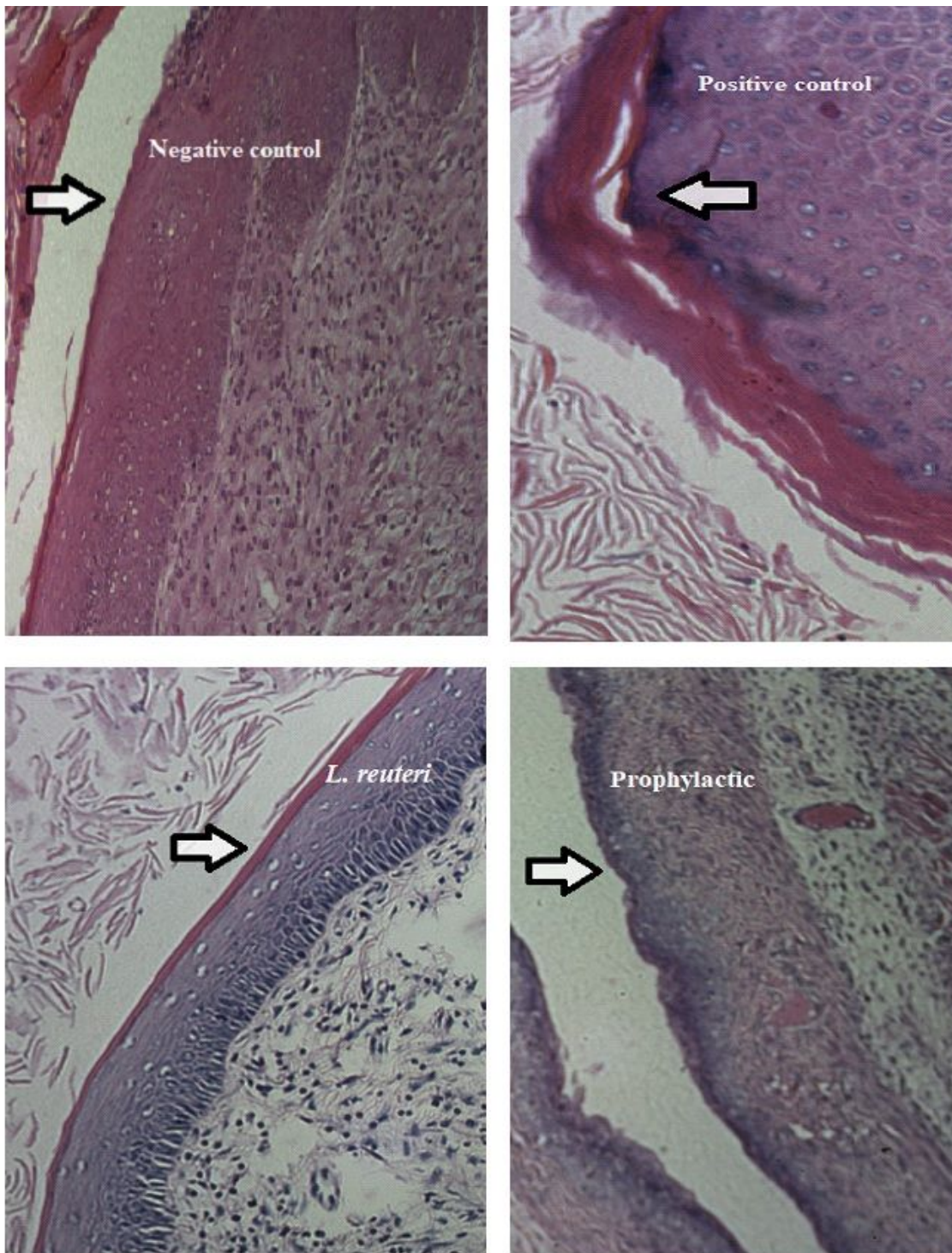
Figure 1

Clinical scoring during the course of experiment



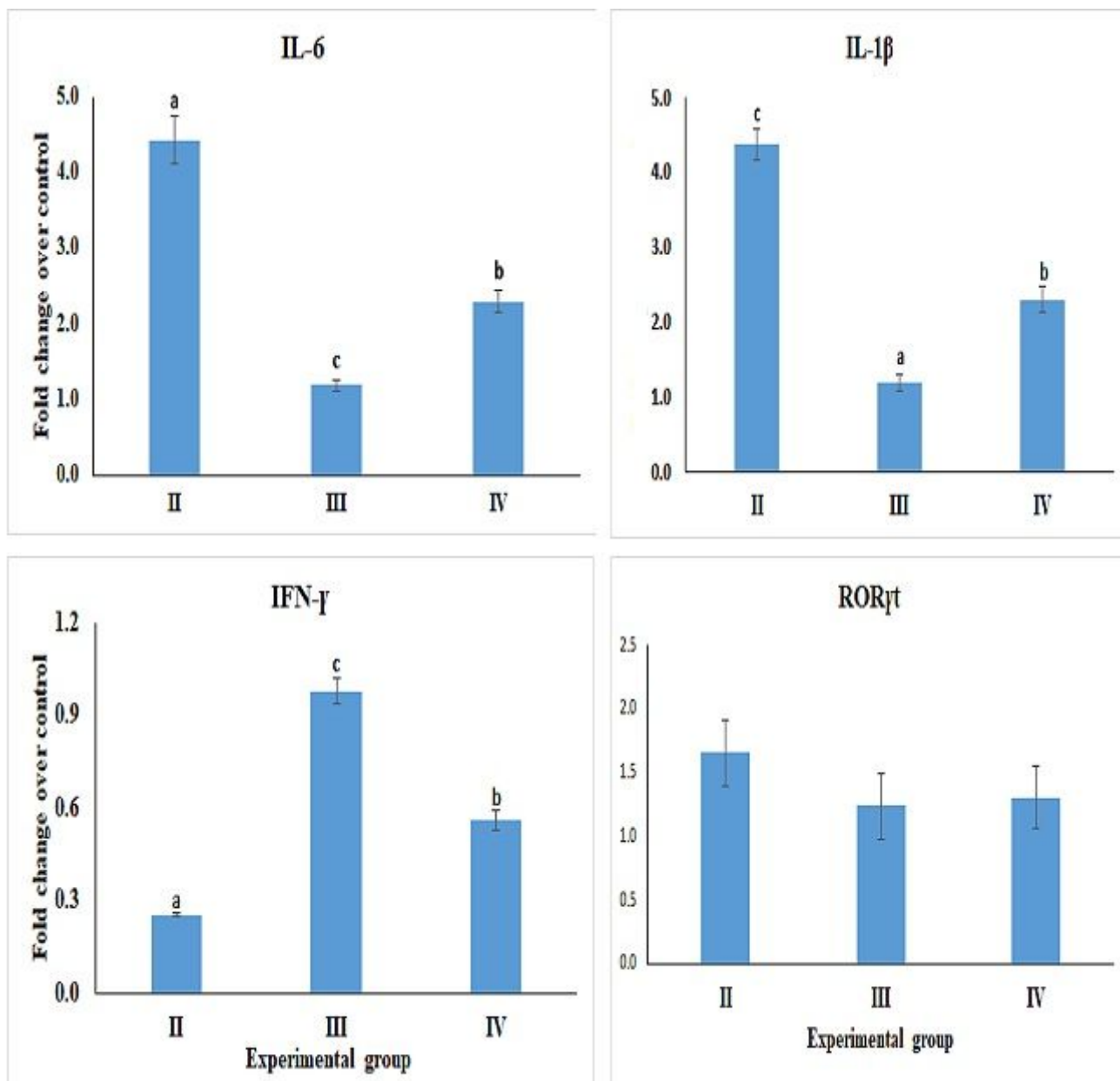
**Figure 2**

Comparison of proportion of animals positive for (a) *L. reuteri* and (b) GV following 9 days post infection



**Figure 3**

Epithelial exfoliation and thickness in H &E stained slides of vaginal tissue. Arrows in the figure show degree of thickness of transitional epithelium.



**Figure 4**

Effect of Probiotic therapy on mRNA levels of pro-inflammatory and anti-inflammatory cytokines in mice BALB/c mice. Different letters indicate statistical difference using One Way ANOVA and Tukey as a post-test ( $p < 0.05$ )

## Supplementary Files

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