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ORIGINAL PAPER

Beneficial lactobacilli: effects on the vaginal tract in a murine experimental model

Priscilla Romina De Gregorio · María Silvina Juárez Tomás · Viviana Santos · María Elena Fatima Nader-Macías

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Abstract Vaginal probiotics containing lactic acid bacteria with activity towards pathogenic microorganisms that cause urogenital tract infections have been proposed as a valid strategy for their prophylaxis and therapy. A murine experimental model was set up to evaluate the colonization capability of beneficial human lactobacilli and their effects on the mouse vaginal mucosa and innate immune cells. Five *Lactobacillus* strains were intravaginally inoculated into previously estrogenized BALB/c mice. The significance of the effects observed in the vaginal tract was determined by analysis of variance using the general linear model. The numbers of viable vaginal lactobacilli were significantly higher at proestrous–estrous than those at the metaestrous–diestrous phase and decreased markedly on the days after inoculation. Lactobacilli inoculation did not cause cytological or histological modifications of the murine vaginal tract. Moreover, the intravaginal administration of *Lactobacillus salivarius* CRL (Centro de Referencia para Lactobacilos culture collection) 1328 and *Lactobacillus gasseri* CRL 1263 did not affect the amounts of granulocytes and macrophages present in vaginal washings. In conclusion, the results demonstrate that vaginal lactobacilli did not produce adverse effects on the murine vaginal tract. Therefore, they could be proposed as safe probiotic candidates to promote a balanced microbiota in the urogenital tract.

Keywords Urogenital probiotic candidates · Mouse experimental model · *Lactobacillus* vaginal colonization · Vaginal innate immune cells

Introduction

The human vagina is an ecosystem in which the cultivable microbiota, constituted mainly by lactic acid bacteria (LAB), is in dynamic equilibrium with the vaginal environment (Ravel et al. 2011). A decrease in the protective LAB is the consequence of an imbalance of the female urogenital ecosystem that makes it more susceptible to urogenital tract infections (UGTI) (Srinivasan et al. 2010; Ravel et al. 2011). One of the novel alternatives proposed for the prevention and/or treatment of UGTI is the application of probiotic products containing LAB (Barrons and Tassone 2008).

Numerous studies, performed mainly in vitro, have demonstrated that vaginal LAB can afford protection against pathogenic microorganisms through several

P. R. De Gregorio · M. S. Juárez Tomás · M. E. F. Nader-Macías (✉)
Centro de Referencia para Lactobacilos (CERELA)-CONICET, Chacabuco 145, 4000 San Miguel de Tucumán, Tucumán, Argentina
e-mail: fnader@cerela.org.ar

V. Santos
Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, San Miguel de Tucumán, Tucumán, Argentina

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mechanisms that include biofilm formation on the vaginal mucosa (by previous adhesion and colonization), production of antimicrobial substances (organic acids, hydrogen peroxide and bacteriocins), competition for nutrients and stimulation of the immune system (Ocaña et al. 1999a; Juárez Tomás et al. 2003; Martín et al. 2008).

International organizations such as the World Health Organization and the Food and Drug Administration of the US have established guidelines to consider a product or microorganism as a probiotic (Reid et al. 2003). Some of the requirements are based on the use of animal models to study the effects of potential probiotic microorganisms on the host, e.g. colonization, proliferation and activity of epithelial and immune cells, and maintenance of mucosal integrity (Yao et al. 2007; Saksena et al. 2011). Moreover, although LAB are food-grade microorganisms and generally regarded as safe, it must be demonstrated that a given LAB strain produces no adverse effects on the host by using an animal model prior to the performance of clinical trials.

In previous studies by our research group a murine experimental model was set up to determine the effects of mouse vaginal LAB and pathogenic microorganisms on the urinary tract (Silva de Ruiz et al. 2001; Silva de Ruiz et al. 2003). Our long term goal is the design of probiotic products containing LAB to restore human urogenital microbiota in order to prevent UGTI in women. Therefore, human vaginal lactobacilli were isolated and selected for their beneficial properties such as inhibition of the growth and/or adhesion of urogenital pathogens that are etiological agents of aerobic vaginitis, bacterial vaginosis, vulvovaginal candidiasis, urinary tract infections and some sexually transmitted diseases (Ocaña et al. 1999a; Ocaña et al. 1999b; Juárez Tomás et al. 2003; Juárez Tomás et al. 2005; Juárez Tomás et al. 2011). Evaluation of the robustness of *Lactobacillus* during biomass production was also carried out (Juárez Tomás et al. 2003).

The aim of this work was to study mouse vaginal colonization of beneficial *Lactobacillus* strains and their effects on the vaginal mucosa and innate immune cells.

Materials and methods

Microorganisms and culture conditions

L. gasseri CRL (Centro de Referencia para Lactobacillos Culture Collection) 1263, *L. gasseri* CRL 1509, *Lactobacillus reuteri* CRL 1324, *Lactobacillus salivarius* CRL 1328 and *Lactobacillus rhamnosus* CRL 1332 were originally isolated from human vagina in Tucumán, Argentina (Ocaña et al. 1999a). The strains were previously selected for their beneficial properties: inhibition of urogenital pathogens by organic acids produced during the growth of all strains (except *L. rhamnosus* CRL 1332) (Juárez Tomás et al. 2011), release of hydrogen peroxide by all strains (except *L. salivarius* CRL 1328) or salivaricin CRL 1328 (a bacteriocin) by *L. salivarius* CRL 1328 (Ocaña et al. 1999b; Vera Pingitore et al. 2009), and auto-aggregation ability of *L. gasseri* CRL 1509 (Juárez Tomás et al. 2005).

Before experimental use, each strain was propagated three times in LAPTg broth (% (w/v): 1.5 meat peptone, 1 tryptone, 1 glucose, 1 yeast extract and 0.1 Tween 80; pH 6.5; individual components obtained from Britania Laboratories, Argentina) (Raibaud et al. 1973), as previously described (Juárez Tomás et al. 2011).

Animals

2-month-old female BALB/c mice, weighing 25–30 g, from the inbred colony of CERELA (Centro de Referencia para Lactobacilos) were used (40–50 mice per experiment). Animals were housed in plastic cages and fed ad libitum with a conventional balanced diet, keeping their environmental conditions constant. 2 days before intravaginal inoculation, all mice were boosted intramuscularly with a single dose of 0.5 mg estradiol hemisuccinate (Eutocol, Craveri S.A.I·C. Laboratory, Argentina) to induce the estrogen dominated phase of the estrous cycle.

Vaginal inoculation procedure

Two experimental designs were carried out. In the first experimental design, the estrogenized animals were separated into seven experimental groups. Five of them were intravaginally inoculated with each of the beneficial *Lactobacillus* strain (*L. gasseri* CRL 1263, *L. gasseri* CRL 1509, *L. reuteri* CRL 1324, *L. salivarius* CRL 1328 and *L. rhamnosus* CRL 1332) twice a day (with 10 h in between) for 2 days. The *Lactobacillus* inoculum was prepared from the cell pellet of the third subculture resuspended in 50 µl of agarized peptone (% (w/v): 1 meat peptone, 1.5 agar;

Britania Laboratories, Argentina). The viable bacteria were quantified by the plate dilution method in LAPTg agar and inoculated into mice at doses of 10^7–10^8 colony forming units (CFU). Two control groups were used: hormone control mice (inoculated only with saline) and agarized peptone control mice (inoculated only with agarized peptone). Figure 1 shows the sequence of vaginal inoculation and the sampling days.

In the second experimental design, only four experimental groups were assayed: hormone control mice, agarized peptone control mice, *L. gasseri* CRL 1263- and *L. salivarius* CRL 1328-treated mice. The *Lactobacillus* strains were intravaginally inoculated into mice twice a day (with 10 h in between) for 2 days (a total of four doses, schedule 1) or 4 days (a total of seven doses, schedule 2) (Fig. 1).

The experiments were independently repeated three times using at least three animals at each sampling time. Animals were randomly assigned to the different treatment groups. The Institutional Committee of Laboratory Animals Care and use of CERELA approved the experimental protocol CRL-BIOT-LMP-2010/1A used in this work.

Sampling and analytical procedures

Every sampling day, vaginal washings were obtained under sterile conditions, using automatic pipettes with tips loaded with 50 µl of 2 % (w/v) foetal bovine serum (FBS, from NATOCOR, Argentina)-PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, pH 7.2). Seven vaginal washes with fresh FBS–PBS were pooled from each mouse to be used for the different protocols. Subsequently, mice were killed by cervical dislocation and dissected to remove the vaginal tissue aseptically.

Cytological and histological studies

A 20 µl aliquot of vaginal washes was spread onto glass slides, stained with Papanicolau (PAP) technique (reagents from Biopur, Argentina) and observed with light microscopy at 400× (Silva de Ruiz et al. 2001). The phases of the estrous cycle (proestrous (PE), estrous (E), metaestrous (ME) and diestrous (DE)) were evaluated from PAP-stained vaginal smears on day zero, and from PAP-stained vaginal smears and histological slides (described later) on the subsequent

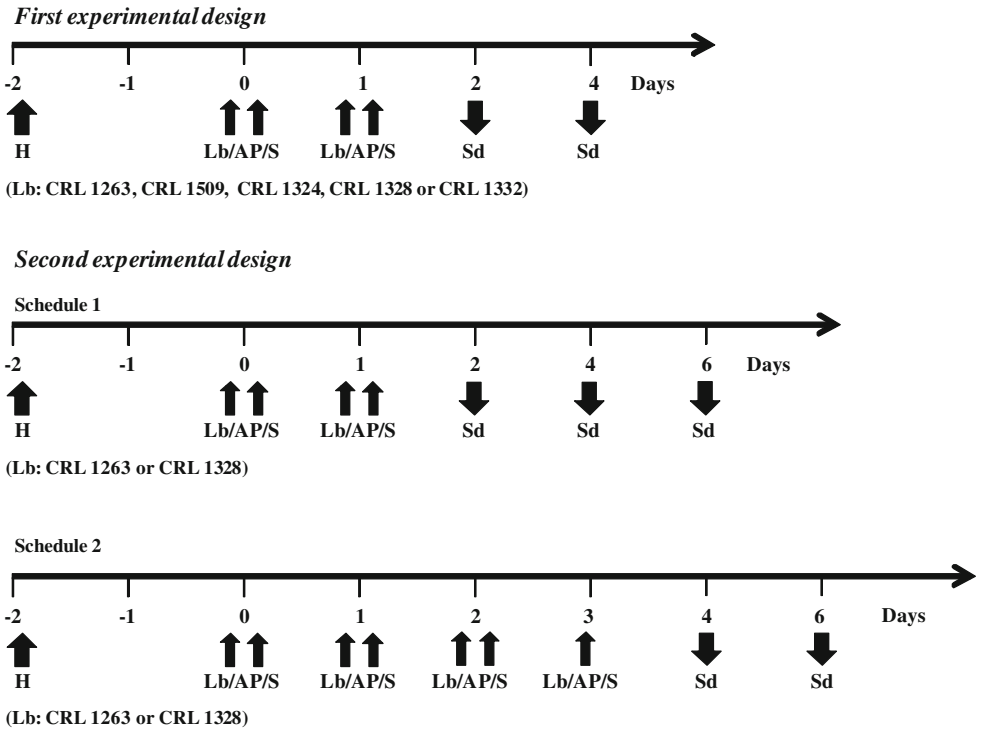


Fig. 1 Sequence of inoculation and sampling days used in the first and second experimental designs. H: Hormone administration (0.5 mg estradiol hemisuccinate). Lb/AP/S: Each *arrow* indicates one inoculation of lactobacilli (*Lb*), agarized peptone (*AP*) or saline (*S*) to *Lactobacillus*-treated mice, agarized peptone control mice or hormone control mice, respectively. *Sd* sampling days

sampling days (Silva de Ruiz et al. 2001). Animals in each experimental group were classified as either mice at PE–E or mice at ME–DE, according to the hormonal similarities between the different phases of the estrous cycle (Bezirtzoglou et al. 2008).

The vaginal tissue was fixed in 4 % (v/v) formaldehyde at 4 °C and embedded in paraffin according to standard histological methods (Silva de Ruiz et al. 2003). Sections were cut at 4 μm, stained with hematoxylin–eosin (reagents from Biopur, Argentina) and examined by light microscopy at 100× and 400×. Pictures were taken with an Axio Scope A1 Carl Zeiss microscopy. The images were processed using AxioVision Release 4.8 software.

Colonization of vaginal tract by different Lactobacillus strains

Aliquots of vaginal washings were Gram-stained (Gram stain kit from Britania Laboratories, Argentina) and serial 10-fold dilutions were plated. To obtain vaginal homogenates (only second experimental design), the vaginas were opened longitudinally, transferred to 1 ml peptone water (0.1 % (w/v) meat peptone) and homogenized with a sterile Teflon pestle. Appropriate dilutions of vaginal washings or homogenates were plated on De Man-Rogosa-Sharpe (MRS) agar pH 5.5 (Merck, Germany) (De Man et al. 1960). MRS agar was supplemented with 15 μg/ml vancomycin (Sigma Chemical Co, USA) to selectively quantify *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332, and with 10 μg/ml nitrofurantoin (Sigma Chemical Co, USA), 1 μg/ml tetracycline (Sigma Chemical Co, USA) and 10 μg/ml vancomycin for *L. gasseri* CRL 1263, *L. gasseri* CRL 1509 and *L. salivarius* CRL 1328, respectively (Juárez Tomás et al. 2005; Ocaña et al. 2006). MRS plates were incubated under aerobic conditions at 37 °C for 48–72 h.

Flow cytometry for the determination of innate immune cells

Viable leukocytes in vaginal washings were quantified with trypan blue stain (Sigma Chemical Co, USA) and microscopic count. For flow cytometric analyses, cell suspensions were washed twice with 2 % (v/v) FBS–PBS and incubated with phycoerythrin (PE)-labeled anti-mouse Gr-1 (granulocyte marker) (BD Pharmingen™, USA), allophycocyanin (APC)-labeled anti-mouse CD45 (leukocyte marker) (BD Pharmingen™, USA) and biotinylated anti-mouse F4/80 (macrophage marker) (Invitrogen, Argentina) antibodies for 30 min at 4 °C. After incubation, the cells were washed with FBS–PBS. Samples treated with biotinylated antibodies were incubated with Streptovidin-PerCP (BD Pharmingen™, USA) for 15 min at 4 °C.

Data were acquired on a BD FACScalibur cytometer (BD, USA) and analyzed using FSC Express V3 software. The leukocyte gate was selected from the plots of forward scatter (FSC) versus side scatter (SSC) according to cell size (FSC), complexity (SSC) and CD45 expression. Afterwards, the expression of Gr-1 and F4/80 was evaluated from the $CD45^+$ leukocyte gate.

Statistical analysis

An analysis of the variance (ANOVA) using the general linear model was applied to determine the main and interaction effects of factors (experimental group and day post-inoculation) on the number of viable lactobacilli and innate immune cells recovered from vaginal samples. Mice at PE–E and at ME–DE of the different experimental groups were evaluated separately. Significant differences between mean values were determined by Tukey's test, using the MINITAB software (version 15 for Windows). A P value <0.05 was considered as statistically significant.

Results

Cytological and histological studies of vaginal tract

The administration of estrogen to BALB/c mice induced a clear estrogenic phase (mainly PE) in 89 % of the mice on day zero of all the experiments. On days 2, 4 (of both experimental designs) and 6 (only of the second experimental design) after lactobacilli inoculation, 70, 50 and 45 % (respectively) of the mice were at PE–E (mainly estrous phase). In these cases, the PAP-stained vaginal smears revealed a vaginal discharge of eosinophilic cornified surface cells with pyknotic nucleus or flakes and keratinized

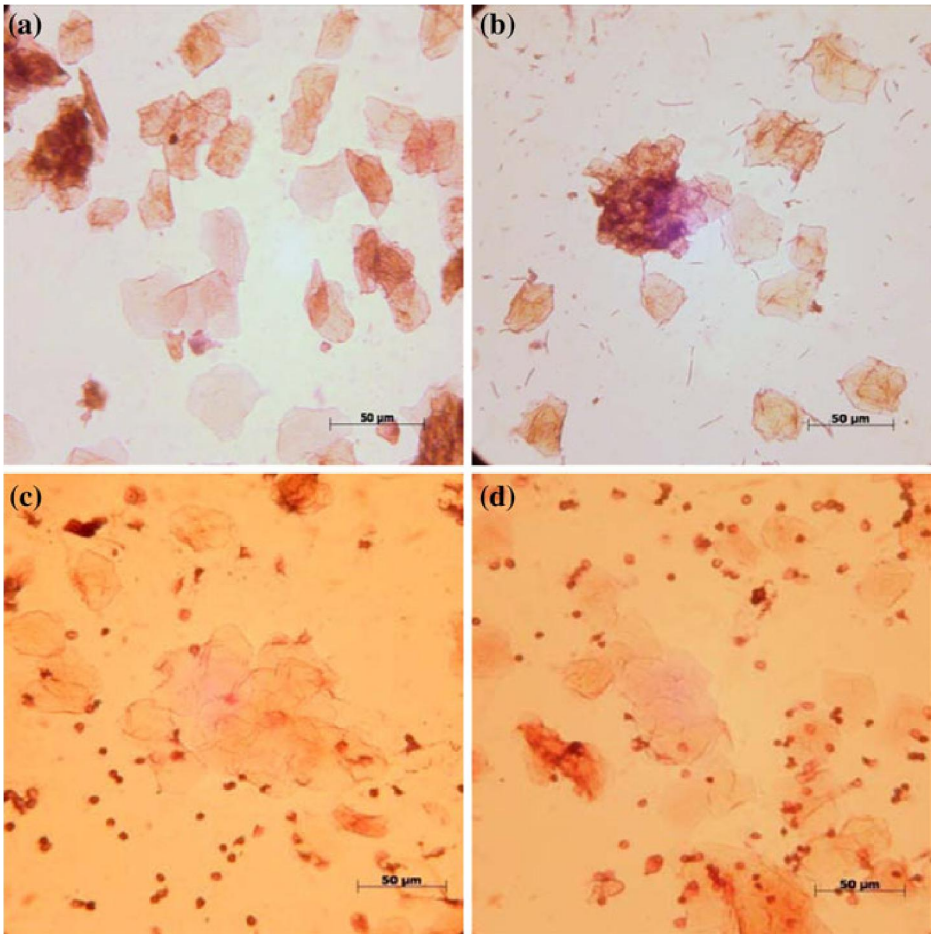


Fig. 2 Photomicrographs of PAP-stained vaginal smears from BALB/c mice on the second day post-inoculation. **a** Hormone control mice at estrous. **b** *L. gasseri* CRL 1263-treated mice at estrous. **c** Hormone control mice at ME. **d** *L. gasseri* CRL 1263-treated mice at ME. Similar results were observed in mice inoculated with the different *Lactobacillus* strains up to 2 days post-inoculation. On the following days, the cytological patterns were similar, but *Lactobacillus* cells were not detected. Results are representative of at least three independent experiments. Magnification: ×400

cell groups in both control and *Lactobacillus*-treated mice (Fig. 2a–b).

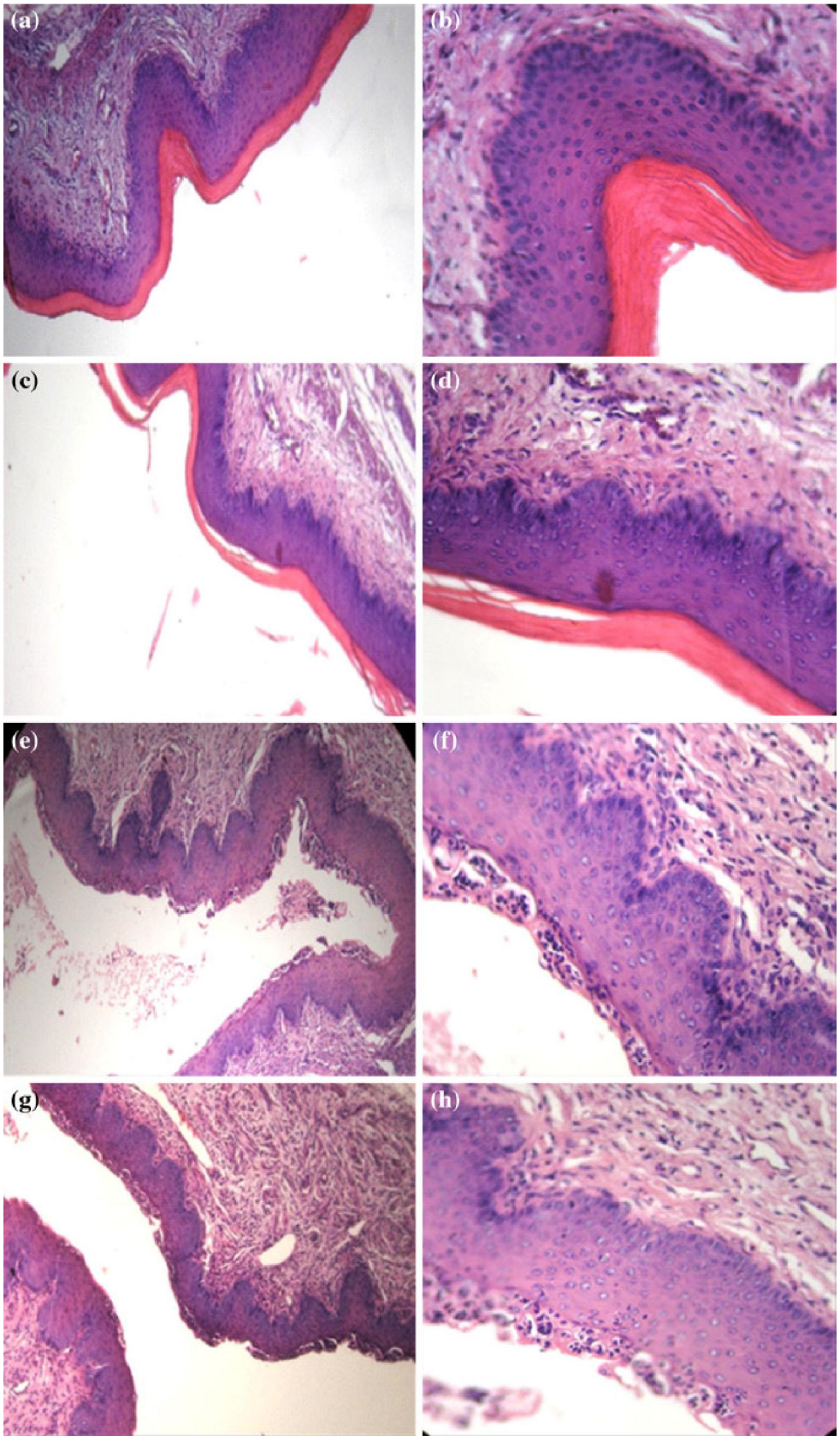
On the other hand, vaginal smears of mice at ME–DE showed the presence of leukocytes, a few eosinophilic surface cells with pyknotic nucleus and many intermediate and parabasal basophilic cells in both control and *Lactobacillus*-treated mice (Fig. 2c–d).

In mice at the same phase of estrous cycle, the histological studies of vaginas evidenced that the characteristics of the epithelium and lamina propria were similar in *Lactobacillus*-treated and control groups at all the days post-inoculation. Figure 3 shows the photomicrographs of vaginal slices obtained from *L. gasseri* CRL 1263-treated and hormone control mice at estrous and ME. Similar numbers of layers composing the stratified squamous epithelium (basal, intermediate and surface cells) were observed in both *L. gasseri* CRL 1263-treated and control groups. In mice at estrous, characteristic keratinization of the vaginal surface was observed (Fig. 3a–d). On the other hand, at ME, typical leukocyte infiltration was present in the surface epithelial cell layers (Fig. 3e–h). These patterns are similar to those obtained from animals inoculated with different *Lactobacillus* strains and agarized peptone control (data not shown).

Colonization of vaginal tract by different *Lactobacillus* strains (first experimental design)

Gram staining of vaginal washings from control mice showed the presence of Gram-positive cocci and Gram-negative bacilli. In *Lactobacillus*-treated mice, the presence of Gram-positive bacilli, with the characteristic shapes of inoculated lactobacilli, was observed up to 2 days post-inoculation. Each of the *Lactobacillus* strains inoculated was selectively enumerated in the culture medium added with antibiotics.

In this experimental design, significant differences were observed between the numbers of viable lactobacilli in vaginal washings of mice at PE–E (higher recovery of lactobacilli) and those at ME–DE at 2 and 4 days after intravaginal inoculation of *L. gasseri* CRL


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

◀ **Fig. 3** Photomicrographs of vaginal slices stained with Hematoxylin–Eosin technique (for histological studies) from BALB/c mice on the second day post-inoculation. **a**, **b** Hormone control mice at estrous at ×100 and ×400, respectively. **c**, **d** *L. gasseri* CRL 1263-treated mice at estrous at ×100 and ×400, respectively. **e**, **f** Hormone control mice at ME at ×100 and ×400, respectively. **g**, **h** *L. gasseri* CRL 1263-treated mice at ME at ×100 and ×400, respectively. Similar results were observed in agarized peptone control mice and in mice inoculated with the different *Lactobacillus* strains and on different days post-inoculation. Results are representative of at least three independent experiments

1263, *L. gasseri* CRL 1509, *L. reuteri* CRL 1324, *L. salivarius* CRL 1328 and *L. rhamnosus* CRL 1332 (Fig. 4).

In mice at PE–E, the number of viable lactobacilli in vaginal washings was around 10^4–10^5 CFU/ml at 2 days post-inoculation, the five strains being able to persist in the vaginal tract up to the 4th day (Fig. 4a). ANOVA results indicated that the *Lactobacillus* viable cells in vaginal washes was affected only on

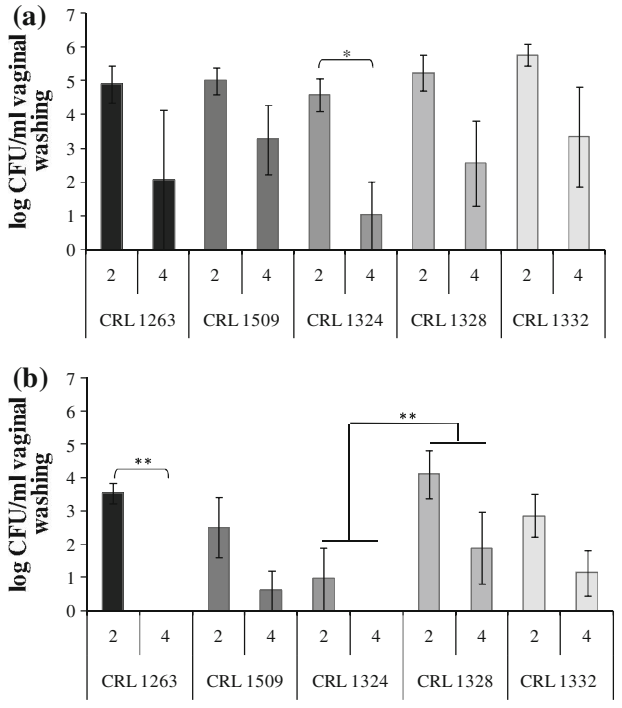


Fig. 4 Viable counts of lactobacilli (*L. gasseri* CRL 1263, *L. gasseri* CRL 1509, *L. reuteri* CRL 1324, *L. salivarius* CRL 1328 and *L. rhamnosus* CRL 1332) from vaginal washings of mice at 2 and 4 days post-inoculation. Mice at PE–E (**a**) or at ME–DE (**b**). The results were obtained from the first experimental design. The data are plotted as the average values of viable numbers (log CFU/ml ± standard error). Statistically significant differences between the log CFU/ml of *Lactobacillus* strains at different days post-inoculation are indicated by *($P < 0.05$) and **($P < 0.01$)

the sampling days ($P < 0.01$). In the specific case of *L. reuteri* CRL 1324, there was a significant decrease ($P < 0.05$) in the number of viable cells between 2 and 4 days after inoculation (Fig. 4a).

In mice at ME–DE, the number of viable lactobacilli in vaginal washings was around 10^3–10^4 CFU/ml at 2 days post-inoculation, *L. gasseri* CRL 1509, *L. salivarius* CRL 1328 and *L. rhamnosus* CRL 1332 being able to persist in the vaginal tract up to the 4th day (Fig. 4b). Similar to results observed in mice at PE–E, there were significant differences in the number of *Lactobacillus* viable cells on the different sampling days (for *L. gasseri* CRL 1263; $P < 0.01$). In addition, the average levels of *L. reuteri* CRL 1324 recovery between days 2 and 4 were significantly lower than those of *L. salivarius* CRL 1328 ($P < 0.01$).

Effects on vaginal tract of selected *Lactobacillus* strains (second experimental design)

Vaginal colonization of lactobacilli inoculated at different doses

Following schedule 1 of the second experimental design, the number of viable cells of *L. gasseri* CRL 1263 and *L. salivarius* CRL 1328 in the vaginal washings and homogenates was affected only on the sampling days (Fig. 5). For both strains, the viable numbers on day 2 were significantly higher than those on day 4 and 6 in mice at the different phases of the estrous cycle. Only *L. salivarius* CRL 1328 was recovered up to the 6th day from vaginal washes and homogenates (10^{1-2} CFU/ml vaginal washing, mainly in mice at PE–E).

The administration of both lactobacilli at higher doses (schedule 2) did not produce a higher colonization of the vaginal tract compared with the results obtained with lower doses (schedule 1) (data not shown).

Effects of selected lactobacilli on innate immune cells

Since the different doses of *Lactobacillus* inoculation did not affect vaginal colonization, the effects of lactobacilli on the innate immune cells were evaluated following only schedule 1 of the second experimental design. In *L. gasseri* CRL 1263- or *L. salivarius* CRL 1328-treated groups, there were no significant differences in the number of vaginal leukocytes on all

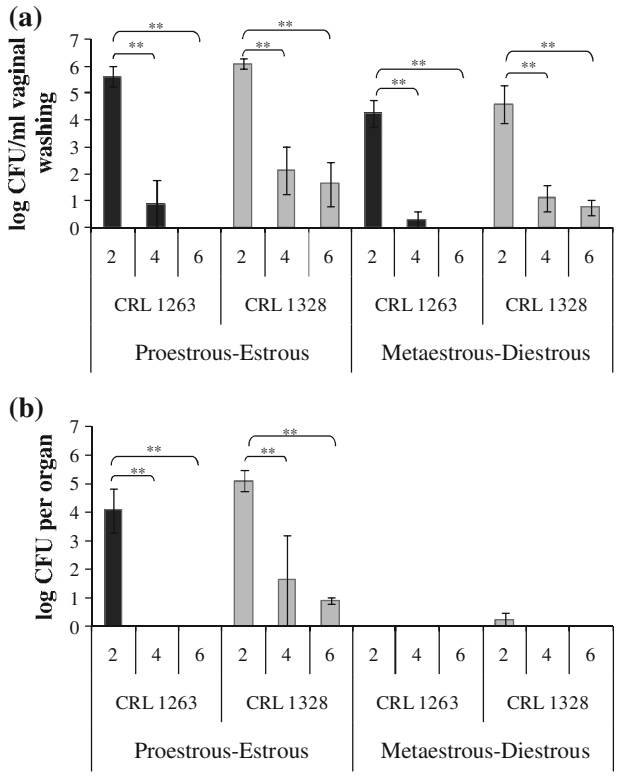


Fig. 5 Viable counts of *L. gasseri* CRL 1263 and *L. salivarius* CRL 1328 in vaginal tract of mice at 2, 4 and 6 days post-inoculation. Vaginal washings (**a**) and homogenates (**b**) from mice at PE–E or at ME–DE phases. The results were obtained from the second experimental design (schedule 1). The data are plotted as the average values of viable numbers (log CFU/ml ± standard error) of lactobacilli. Statistically significant differences between the viable cells of each *Lactobacillus* strain on different days post-inoculation are indicated by * ($P < 0.05$) and ** ($P < 0.01$)

sampling days compared with the control groups ($P > 0.05$) (Table 1). The absolute numbers of viable leukocytes from mice at PE–E were in general lower (10^5–10^6/ml vaginal washing) than those from mice at ME–DE (10^6–10^7/ml vaginal washing).

Significantly higher numbers of $CD45^+$–$Gr\text{-}1^+$ granulocytes (95–99 % of $CD45^+$ leukocyte gate, which include $CD45^{high}$–$Gr\text{-}1^{high}$ and $CD45^{low}$–$Gr\text{-}1^{low}$ cells) than $CD45^+$–$F4/80^+$ macrophages (1–3 % of $CD45^+$ leukocyte gate) were detected in vaginal washes of mice at PE–E and ME–DE phases (Table 2) in *Lactobacillus*-treated and control groups on all sampling days.

The expression level of CD45 leukocyte and Gr-1 granulocyte markers on $CD45^+$–$Gr\text{-}1^+$ cells depended mainly on the phase of the estrous cycle. Therefore, 91.51 ± 1.18 % expressed $CD45^{high}$–$Gr\text{-}1^{high}$ (high expression level of CD45 and Gr-1 markers) and 5.60 ± 0.99 % $CD45^{low}$–$Gr\text{-}1^{low}$ (low expression level of CD45 and Gr-1 markers) in mice at PE–E. However, 68.11 ± 5.03 % expressed $CD45^{high}$–$Gr\text{-}1^{high}$ and 28.24 ± 4.47 % $CD45^{low}$–$Gr\text{-}1^{low}$ in animals at ME-DE (Table 2 and Fig. 6). There were no significant differences in the percentages of $CD45^{high}$–$Gr\text{-}1^{high}$, $CD45^{low}$–$Gr\text{-}1^{low}$ and $CD45^+$–$F4/80^+$ cells between *Lactobacillus*-treated and control groups at different sampling times (Table 2).

Discussion

The use of probiotic products to restore the human vaginal microbiota is a natural and novel strategy to prevent or treat UGTI. Several commercial probiotic formulations have made this claim but most of them have not been previously and properly evaluated in experimental animal models or clinical trials (Nader-Macías et al. 2008). The mouse animal model was employed successfully in numerous studies to assess the colonization of pathogenic microorganisms in the urogenital tract (Meysick and Garber 1992; Harriott et al. 2010; Packiam et al. 2010), the role of virulence factors (Ricci et al. 2001), the stimulation of mucosal immunity (Packiam et al. 2010; Yadav et al. 2005), and the efficacy and toxicity of local microbicides before clinical studies (Catalone et al. 2004). A few animal studies were conducted to evaluate the functionality and safety of *Lactobacillus* strains that can potentially be included in probiotic products for vaginal application (Pascual et al. 2010; Muench et al. 2009).

In this work, the effects of the intravaginal administration of different beneficial *Lactobacillus* strains to mice were evaluated by microbiological, cytological, histological and flow cytometric approaches. Culture media added with antibiotics were appropriate to selectively enumerate the lactobacilli inoculated; however later studies on antibiotic resistance of selected strains are required to evaluate other safety aspects. *L. gasseri* CRL 1263, *L. gasseri* CRL 1509, *L. reuteri* CRL 1324, *L. salivarius* CRL 1328 and *L. rhamnosus* CRL 1332 persisted in the murine vaginal tract up to the 4th day post-inoculation. The numbers of lactobacilli were significantly higher in mice at PE–E than in those at ME–DE. Pascual et al. (2010) reported that vaginal *L. fermentum* L23 was

Table 1 Quantification of leukocytes from vaginal washings of control and *L. gasseri* CRL 1263- and *L. salivarius* CRL 1328-treated mice

Experimental group	Day post-inoculation	Mice at PE–E Total of viable leukocytes ($\times 10^5$)/ml vaginal washing[a]	Mice at ME–DE Total of viable leukocytes ($\times 10^6$)/ml vaginal washing[a]
Hormone control mice	2	7 ± 2	4 ± 0.2
	4	14 ± 4	8 ± 3
	6	19 ± 2	6 ± 6
Agarized peptone control mice	2	5 ± 2	18 ± 11
	4	6 ± 3	6 ± 3
	6	3 ± 2	2 ± 1
L. salivarius CRL[b] 1328-treated mice	2	2 ± 0.5	21 ± 3
	4	11 ± 5	2 ± 0.6
	6	9 ± 4	1 ± 0.6
L. gasseri CRL[b] 1263-treated mice	2	8 ± 4	1 ± 0.5
	4	14 ± 1	9 ± 2
	6	11 ± 1	10 ± 10

[a] The data represent the absolute average values of viable leukocytes ± standard error from vaginal washings of at least three mice

[b] *CRL* Centro de Referencia para Lactobacilos culture collection

Table 2 Percentage of innate immune cells from vaginal washings of control and *L. gasseri* CRL 1263- and *L. salivarius* CRL 1328-treated mice determined by flow cytometry

Experimental group	Day post-inoculation	Mice at PE–E			Mice at ME–DE		
		Innate immune cells[a] (%)			Innate immune cells[a] (%)		
		$CD45^{high}$–$Gr\text{-}1^{high}$ [b]	$CD45^{low}$–$Gr\text{-}1^{low}$ [c]	$CD45^{+}$–$F4/80^{+}$[d]	$CD45^{high}$–$Gr\text{-}1^{high}$ [b]	$CD45^{low}$–$Gr\text{-}1^{low}$ [c]	$CD45^{+}$–$F4/80^{+}$ [d]
Hormone control mice	2	95.36 ± 0.74	3.16 ± 1.10	0.15 ± 0.07	48.33 ± 22.07	44.49 ± 19.63	0.07 ± 0.04
	4	86.40 ± 5.06	12.35 ± 4.93	1.91 ± 1.40	80.89 ± 14.16	31.59 ± 8.80	0.21 ± 0.03
	6	80.56 ± 17.05	16.62 ± 15.57	0.46 ± 0.10	70.35 ± 15.87	25.64 ± 14.06	4.88 ± 1.32
Agarized peptone control mice	2	93.76 ± 0.84	2.90 ± 0.61	1.64 ± 0.34	95 ± 0.41	3.44 ± 0.33	0.05 ± 0.00
	4	91.03 ± 3.82	4.52 ± 1.61	3.68 ± 0.34	73.96 ± 5.60	24.51 ± 5.10	1.31 ± 0.30
	6	86.92 ± 5.70	6.41 ± 3.14	2.6 ± 1.95	66.07 ± 10.28	29.37 ± 9.97	2.42 ± 1.08
L. salivarius CRL[e] 1328-treated mice	2	95.62 ± 1.86	2.11 ± 0.88	3.4 ± 3.25	93.96 ± 3.18	5.075 ± 2.77	0.04 ± 0.03
	4	88.84 ± 3.27	10.29 ± 3.16	0.06 ± 0.01	65.63 ± 12.69	33.96 ± 12.46	0.07 ± 0.00
	6	90.56 ± 5.57	3.94 ± 0.49	6.60 ± 1.27	77.20 ± 4.72	20.03 ± 5.45	1.10 ± 0.31
L. gasseri CRL[e] 1263-treated mice	2	95.17 ± 0.27	2.29 ± 0.36	0.25 ± 0.08	75.67 ± 2.21	18.83 ± 1.59	0.06 ± 0.00
	4	95.26 ± 1.20	3.70 ± 1.44	1.58 ± 0.02	58.41 ± 2.84	32.29 ± 2.99	0.58 ± 0.08
	6	94.80 ± 1.41	3.34 ± 0.64	1.46 ± 1.34	78.08 ± 3.35	20.55 ± 3.61	0.18 ± 0.05

[a] The data represent the average values of percentages of innate immune cells ± standard error from vaginal washings of at least three mice. The absolute average values of total leukocytes of each experimental group and day post-inoculation are showed in Table 1

[b] $CD45^{high}$–$Gr\text{-}1^{high}$: granulocytes with high expression level of CD45 and Gr-1 markers (of $CD45^{+}$ leukocyte gate)

[c] $CD45^{low}$–$Gr\text{-}1^{low}$: granulocytes with low expression level of CD45 leukocyte and Gr-1 granulocyte markers (of $CD45^{+}$ leukocyte gate)

[d] $CD45^{+}$-$F4/80^{+}$: macrophages (of $CD45^{+}$ leukocyte gate)

[e] *CRL* Centro de Referencia para Lactobacilos culture collection

able to colonize the vaginal tract of mice for at least 4 days after a single local inoculation. However, previous hormonal treatment of the animals was not performed in those trials.

Several murine models employing estrogenized animals were validated to support the persistence of pathogenic microorganisms (e.g. *Trichomonas vaginalis, Neisseria gonorrhoeae* and *Candida albicans*)

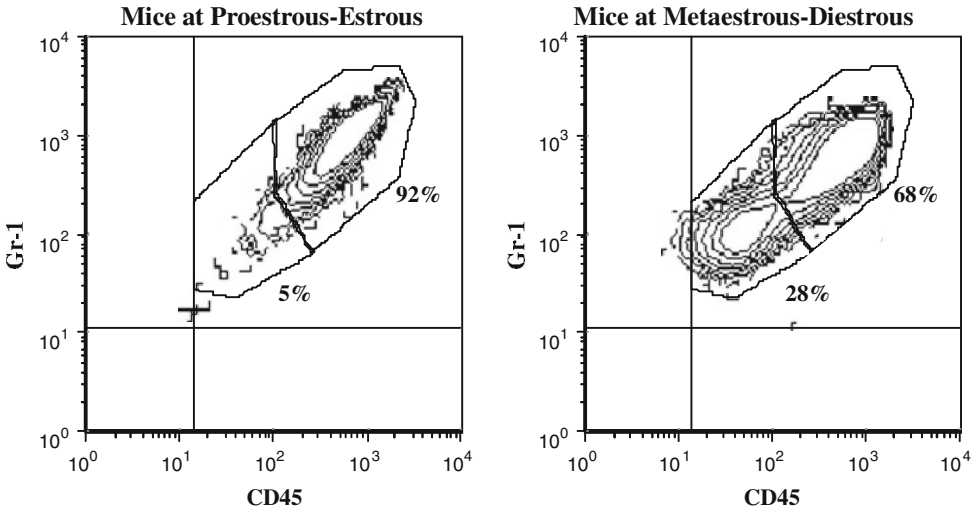


Fig. 6 Flow cytometry of innate immune cells of mouse vaginal washings. Contour plots of $CD45^{low}$–$Gr\text{-}1^{low}$ and $CD45^{high}$–$Gr\text{-}1^{high}$ granulocytic cells in vaginal washings from hormone control mice at PE–E and at ME–DE. Results are representative of at least three independent experiments. Similar results were observed in agarized peptone control mice and *Lactobacillus*-treated mice on different days post-inoculation

in the reproductive tract (Meysick and Garber 1992; Harriott et al. 2010; Packiam et al. 2010; Tarry et al. 2005). In these mice, microbial colonization could be stimulated by the higher amounts of available nutrients such as amino acids, carbohydrates and proteins or by the estrogen-induced changes in the vaginal epithelium, which could promote the growth or adhesion of microorganisms (Larsen 1993).

On the other hand, Muench et al. (2009) reported that estradiol treatment of BALB/c mice was required to promote long-term colonization by human *Lactobacillus* strains intravaginally inoculated, during interaction studies between lactobacilli and *N. gonorrhoeae*. The above authors observed that murine vaginal pH was slightly lower in estradiol-treated mice than untreated control group. Together with the higher availability of nutrients, the low pH could favor the colonization of acidophilic lactobacilli. Also, the murine model with estrogenized animals was used by our research group to achieve an adequate vaginal colonization of lactobacilli and thus to assess their effects in the genital tract.

In healthy non-pregnant women of reproductive age, high estrogen levels promote the proliferation and increase in vaginal epithelium, glycogen deposition and transudation (Larsen 1993). Glycogen is a carbon source metabolized to organic acids, mainly by protective lactobacilli (Boskey et al. 2001). The acidic environment stimulates the growth of lactobacilli and inhibits several pathogenic microorganisms. Considering the urogenital probiotics are intended for human use, the results obtained from the animal model employed in this work could be extrapolated to diverse groups of users, such as non-pregnant women of reproductive age, women who use hormonal contraceptives containing estrogens and post-menopausal women receiving hormone replacement therapy (Heinemann and Reid 2005; Bradshaw et al. 2006). Thus, the administration of lactobacilli together with hormonal treatments could exert a preventive effect against UGTI.

Probiotics must be safe, and therefore have no harmful effects on the host such as negative consequences on mucosa and natural immune protection. In the first experimental design performed in this work, none of the five *Lactobacillus* strains studied caused undesirable effects on the murine vaginal tissue, because the cytological and histological patterns observed in the different groups assayed were typical of the different phases of the estrous cycle.

L. gasseri CRL 1263 ($H_2O_2^+$) and *L. salivarius* CRL 1328 (bacteriocin$^+$) were selected for further studies because of their relevant beneficial and technological characteristics. The two strains selected did not show inhibitory activity between them, i.e. they are compatible and could be combined in vaginal products (Juárez Tomás et al. 2011). *L. gasseri* CRL 1263 and *L. salivarius* CRL 1328 did not affect the normal amount and composition of the immune cell populations (i.e., total leucocytes; predominance of granulocytes over macrophages) found in the vaginal washings. Several components of the innate or specific immune system (neutrophils, macrophages, Langerhans and dendritic cells, lymphocytes, IgG and secretory IgA antibodies) are present in the female genital tract (Wira et al. 2010). These components

may exert an immune surveillance by participating in antigen presentation and in protection against pathogens.

Milligan et al. (2002) demonstrated that approximately 93–97 % of the leukocytes present in vaginal washings of control mice were neutrophils and only 1–2 % were macrophages. However, in mice intravaginally inoculated with a candidate detergent-based microbicide (Conceptrol), the absolute numbers of neutrophils and macrophages progressively increased for 8 h after vaginal application, resulting in an inflammatory response.

In this work, a marked increase in the total number of leukocytes and $CD45^{low}$–$Gr\text{-}1^{low}$ granulocytes and a lower number of $CD45^{high}$–$Gr\text{-}1^{high}$ granulocytes were observed in mice at ME–DE compared with those at PE–E in all the groups under evaluation. An increase in granulocytes in the vaginal tract normally occurs during the ME phase, probably due to a murine neutrophil-specific chemokine (Sasaki et al. 2009). On the other hand, Fleming et al. (1993) demonstrated that the expression of Gr-1 increases with the maturation of granulocytes. Therefore, $CD45^{low}$–$Gr\text{-}1^{low}$ cells could correspond to a population of immature granulocytes, and $CD45^{high}$–$Gr\text{-}1^{high}$ cells to mature granulocytes.

The results obtained demonstrated that different *Lactobacillus* strains transiently colonized the vaginal tract of BALB/c mice. They did not produce adverse effects on the vaginal mucosa, which suggests their safe application as probiotic candidates. Besides, neither *L. gasseri* CRL 1263 nor *L. salivarius* CRL 1328 affected the immune cell populations assayed. Further studies are needed to evaluate the mechanisms of microorganism-host interaction in preventive or therapeutic assays against urogenital pathogens using the murine model proposed in this work.

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