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ORIGINAL ARTICLE

Preventive effect of *Lactobacillus reuteri* CRL1324 on Group B *Streptococcus* vaginal colonization in an experimental mouse model

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Keywords
Group B *Streptococcus*, lactobacilli, mouse experimental model, preventive effect, urogenital tract, vaginal probiotic.

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Abstract

Aims: To assess the preventive effect of different intravaginal (i.va.) doses of *Lactobacillus reuteri* CRL1324 against vaginal colonization by Group B *Streptococcus* (GBS) in a murine experimental model.

Methods and Results: The major virulence factors of four vaginal GBS clinical isolates were determined to select the most virulent strain and set up a murine model of streptococcal vaginal colonization. Later, the effect of four and seven doses of 10^8 viable cells of *Lact. reuteri* CRL1324 i.va. administered, prior to the GBS challenge was studied. Seven doses of lactobacilli were able to significantly reduce the number of viable GBS cells, while four doses showed no preventive effect. Both doses reduced the leucocyte influx induced by GBS. Seven doses caused a slight increase in the *Lact. reuteri* CRL1324 vaginal colonization compared with four doses and reduced murine vaginal pH compared to control mice.

Conclusions: *Lactobacillus reuteri* CRL1324 evidenced a preventive effect on GBS vaginal colonization in an experimental mouse model.

Significance and Impacts of the Study: Maternal GBS colonization is one of the most important risk factors for developing disease in newborns. *Lactobacillus reuteri* CRL1324 could be considered as a new biological agent to reduce infections caused by this micro-organism.

Introduction

The vaginal microbiome of healthy women consists of a diversity of anaerobic and aerobic micro-organisms, with the dominant role of lactobacilli (Ravel *et al.* 2011; Stumpf *et al.* 2013). Lactobacilli play a significant role in the maintenance of women's vaginal health by their ability to colonize the mucous membranes and their competition with other micro-organisms for adherence to the vaginal epithelium, their production of antimicrobial compounds (hydrogen peroxide, organic acids, bacteriocin-like substances), and/or their effect on the modulation of the immune response against the causative agents of infectious diseases (Martín *et al.* 2008; Nader-Macías *et al.* 2010). Therefore, the disruption of the normal vaginal microbiota, mainly by the reduction in the number or activity of lactobacilli, frequently causes the income of opportunistic pathogens and results in the development of vaginosis, vaginitis or urinary tract infections as well as the emergence of other pathological conditions (Verstraelen 2008; Ruiz *et al.* 2009).

Streptococcus agalactiae (Group B Streptococcus in cursive, GBS) is an opportunistic pathogen that predominantly is present as a commensal colonizer in the vaginal tract of around 25% healthy adults (Hensler *et al.* 2005; Lindahl *et al.* 2005; Maisey *et al.* 2008). However, newborns and pregnant women are at high risk of developing invasive GBS infections (Skoff *et al.* 2009). The main source of neonatal infection is maternal genital tract colonization. GBS is transmitted vertically during labour and delivery, and colonization occurs in up to 50–70% of neonates born from colonized mothers (Baker and Edwards 2001). Maternal streptococcal colonization is also associated with an increased risk of urinary tract

infection and pregnancy complications, such as endometritis, chorioamnionitis, preterm birth and intrauterine death (Winn 2007). Also, other syndromes as vaginitis, painful fine superficial fissures and minimal erythema of vulvar skin in nonpregnant adolescent and young adult populations have also been attributed to GBS (Clark and Atendido 2005; Mirowski *et al.* 2012; Savini *et al.* 2013).

Different virulence factors have been identified in GBS, including the exopolysaccharide capsule and a surface-associated toxin, β-haemolysin/cytolysin (β-h/c). Moreover, the expression of the β-h/c has been associated with the production of a carotenoid pigment with antioxidant properties (Spellerberg *et al.* 2000; Pritzlaff *et al.* 2001). These virulence factors could act together preventing macrophage-based host immune clearance (Liu *et al.* 2004; Sendi *et al.* 2009). Usually, isolates displaying hyperpigmentation are typically hyperhaemolytic. Hence, in invasive GBS diseases, a significant pathogenic role has been attributed to the expressed pigment (Liu *et al.* 2004). However, recent studies have shown that hyperpigmented isolates were also described as colonizers of the urogenital tract, but were not associated with severe invasive disease. Also, hyperpigmented isolates showed a decrease in the capsule thickness (Lupo *et al.* 2014).

The absence of an effective vaccine against GBS and the emerging antibiotic resistance (Kasahara *et al.* 2010; Nagano *et al.* 2012; Oviedo *et al.* 2013) exert a strong pressure to develop novel treatments or preventive strategies to control GBS vaginal colonization and transmission to susceptible newborns. The use of probiotic products containing beneficial vaginal lactobacilli (BVL) could represent a novel and interesting alternative strategy for the biological control of GBS.

Previously, autochthonous lactobacilli from the human vaginal tract were isolated and selected on the basis of their beneficial properties (Ocaña *et al.* 1999; Juárez Tomás *et al.* 2005, 2011). In addition, the antagonistic effect of vaginal lactobacilli on GBS strains by the production of organic acids has been demonstrated through *in vitro* studies (Juárez Tomás *et al.* 2011; De Gregorio *et al.* 2014). Also, the different *in vitro* and *in vivo* behaviour of two different BVL (*Lactobacillus gasseri* CRL1509 and *Lactobacillus salivarius* CRL1328) has been evidenced: the strains were able to inhibit GBS in associative cultures, but when these strains were administered to mice, the inhibitory effect was not evidenced, even though BVL were isolated up to the 9th day post-BVL inoculation (De Gregorio *et al.* 2014).

Taking into account that the BVL effects are generally considered as strain- and dose-dependent (ISAPP 2013) the aim of this study was to evaluate the preventive effect of different doses of *Lactobacillus reuteri* CRL1324 against vaginal colonization by a virulent GBS strain in a murine experimental model.

Material and methods

Bacterial strains and growth conditions

Lactobacillus reuteri CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 was originally isolated from human vagina in Tucumán, Argentina (Ocaña *et al.* 1999) and selected on the basis of its beneficial properties such as hydrogen peroxide production, biofilm formation and *in vitro* inhibition of GBS by organic acids (Juárez Tomás *et al.* 2011; Leccese Terraf *et al.* 2012; De Gregorio *et al.* 2014).

Streptococcus agalactiae strains (GBS NH16, NH17, NH18 and GB96, all identified as IB serotype) were previously isolated from women urogenital infections and phenotypically identified at the Nuevo Hospital 'El Milagro' (Salta, Argentina) and at the Instituto de Microbiología 'Luis Verna' of the Universidad Nacional de Tucumán.

Micro-organisms were stored in milk-yeast extract (% (w/v): 13 nonfat milk, 0·5 yeast extract and 1 glucose) at −20°C. Before experimental use, *Lact. reuteri* CRL1324 was grown in De Man-Rogosa-Sharpe (MRS) broth (Merck, Darmstadt, Germany) (De Man *et al.* 1960) at 37°C for 24 h and subcultured twice in the same medium at 37°C for 12 h. GBS strains were cultured overnight on Columbia agar base (Britania, Buenos Aires, Argentina) with 5% sheep blood (blood agar plates) and then subcultured in Bacto™ Todd Hewitt broth (THB) (Becton Dickinson, Le Pont de Claix, France) for 9 h at 37°C up to 0·8 optical density at 600 nm ($OD_{600\ nm}$) (Spectronic 20; Bausch and Lomb, Rochester, NY).

Screening of virulence factors in GBS vaginal clinical isolates

Haemolysin extraction and quantification of haemolytic activity

GBS haemolysin was extracted as described by Liu *et al.* (2004). The haemolytic titer was assessed by a microtiter dilution method, as described previously (Nizet *et al.* 1996). The haemolytic titer was defined as the reciprocal of the highest dilution that produces 50% haemoglobin release compared with SDS control. Afterwards, the results were related to the lytic activity of SDS (100%) and expressed as haemolytic capacity.

Additionally, the β-haemolysis of the GBS strains was evidenced by inoculating 5 μl of a bacterial suspension ($OD_{600\ nm}$ = 0·8) on blood agar plates, which were incubated overnight at 37°C.

Carotenoid pigment detection

Carotenoid pigment production was evidenced by inoculation of 5 μl bacterial suspension ($OD_{600\ nm}$ = 0·8) on

Todd-Hewitt agar plates, incubated overnight under anaerobic conditions to favour orange pigment production (Nizet *et al.* 1996).

Capsule detection
Capsule detection in GBS strains was studied as previously described by Mozzi *et al.* (2000). The capsule was microscopically evidenced (Axio Scope A1 Carl Zeiss microscope; design and quality by Carl Zeiss Germany; made in China by Carl Zeiss) as a clear area around the bacterium.

Animals

Two-month-old female BALB/c mice, weighing 25–30 g, from the inbred colony of CERELA (Centro de Referencia para Lactobacilos) were used. Animals were housed in plastic cages and fed *ad libitum* with a conventional balanced diet, keeping their environmental conditions constant. In order to induce a pseudo-estrous condition and promote bacterial colonization, all the mice received a weekly subcutaneous injection of 0·02 mg β-Estradiol 17-valerate (Sigma-Life Sciences, Bern, Switzerland) dissolved in 100 μl of sesame oil (Sigma-Life Sciences, Federal District, Mexico) throughout the experiment. The experimental protocol was independently repeated three times using at least three animals for each experimental group at each sampling time. The Institutional Laboratory Animal Care and Use Committee of CERELA approved the experimental CRL-BIOT-LMP-2011/2A protocol used in this work.

Preventive model of *Lactobacillus reuteri* CRL1324 against GBS vaginal colonization

Three experimental protocols were applied to evaluate the preventive effect of the intravaginal (i.va.) *Lactobacillus* inoculations. In the first protocol, the animals were randomly assigned to three experimental groups: (i) *Lact. reuteri* CRL1324 (CRL1324)-GBS NH17 (NH17)-treated mice (i.va. inoculated with 10^8 colony forming units (CFU) of CRL1324 twice a day, with 10 h in between, for 2 days (a total of 4 doses) and later i.va. challenged with a single dose of 1×10^5 CFU of NH17), (ii) NH17-challenged mice (i.va. inoculated four times with saline and later i.va. challenged with a single dose of 1×10^5 CFU of NH17) and (iii) CRL1324-treated mice (i.va. inoculated four times with CRL1324 and later inoculated with saline) (Fig. 1).

In the second experimental protocol, the animals were randomly assigned to three experimental groups: (i) CRL1324-NH17-treated mice (i.va. inoculated seven times with 10^8 CFU of CRL1324 and later i.va. challenged with 1×10^5 CFU of NH17), (ii) NH17-challenged mice (i.va. inoculated seven times with saline and later i.va. challenged with 1×10^5 CFU of NH17) and (iii) CRL1 324-treated mice (i.va. inoculated seven times with CRL1324 and later with saline) (Fig. 1).

The third experimental protocol included the same experimental groups described in the second protocol, but CRL1324-NH17-treated mice and NH17-challenged mice were challenged with a 1×10^7 CFU of NH17.

For some analytical determinations (murine vaginal pH and microbiota), two control groups were included: hormone control (HC) mice inoculated only with saline and agarized peptone control (APC) mice inoculated only with agarized peptone (% (w/v) 1 meat peptone, 1·5 agar; Britania Laboratories, Buenos Aires, Argentina).

Inoculum preparation
The *Lactobacillus* inoculum was prepared from the cell pellet of the third subculture (grown in MRS broth for 12 h at 37°C as described above) resuspended in 50 μl of melted agarized peptone, as previously published (De Gregorio *et al.* 2012).

The GBS inocula were prepared with bacterial pellets from the second subculture (in THB for 9 h at 37°C) washed and resuspended in saline. Figure 1 shows the experimental groups, the sequence of vaginal inoculation and the sampling days of the different experimental protocols. *Lactobacillus reuteri* CRL1324 inoculation and pathogen challenge were indicated by two different time lines.

Sampling and analytical procedures

Every sampling day, vaginal washes (v.w.) were obtained under sterile conditions, using automatic pipettes with tips loaded with 50 μl of saline. Seven v.w. with saline were pooled from each mouse to be later used for the different determinations. Mice were later killed by cervical dislocation and dissected to aseptically remove the vaginal tissue.

Microbiological studies
Viable bacteria numbers in v.w. were determined by the serial dilution method by plating in selective media: MRS agar pH 5·5 supplemented with 15 μg ml^{-1} vancomycin (Sigma Chemical Co, St Louis, USA) to selectively quantify *Lact. reuteri* CRL1324 (De Gregorio *et al.* 2012) and chromIDStrepto B agar to quantify GBS NH17 (Poisson *et al.* 2011). Also, MRS agar pH 6·4, Mc Conkey agar (Britania, Buenos Aires, Argentina), Bile Esculin agar (Britania, Buenos Aires, Argentina) and Manitol Salt Agar (MSA; Britania, Buenos Aires, Argentina) were used to quantify the cultivable lactic acid bacteria (LAB), staphylococci, enterococci and enterobacteria, respectively, from the murine vaginal microbiota. The effect of lactobacilli inoculation on the yeast presence was not evaluated because previous studies performed in our laboratory showed that bacteria but no

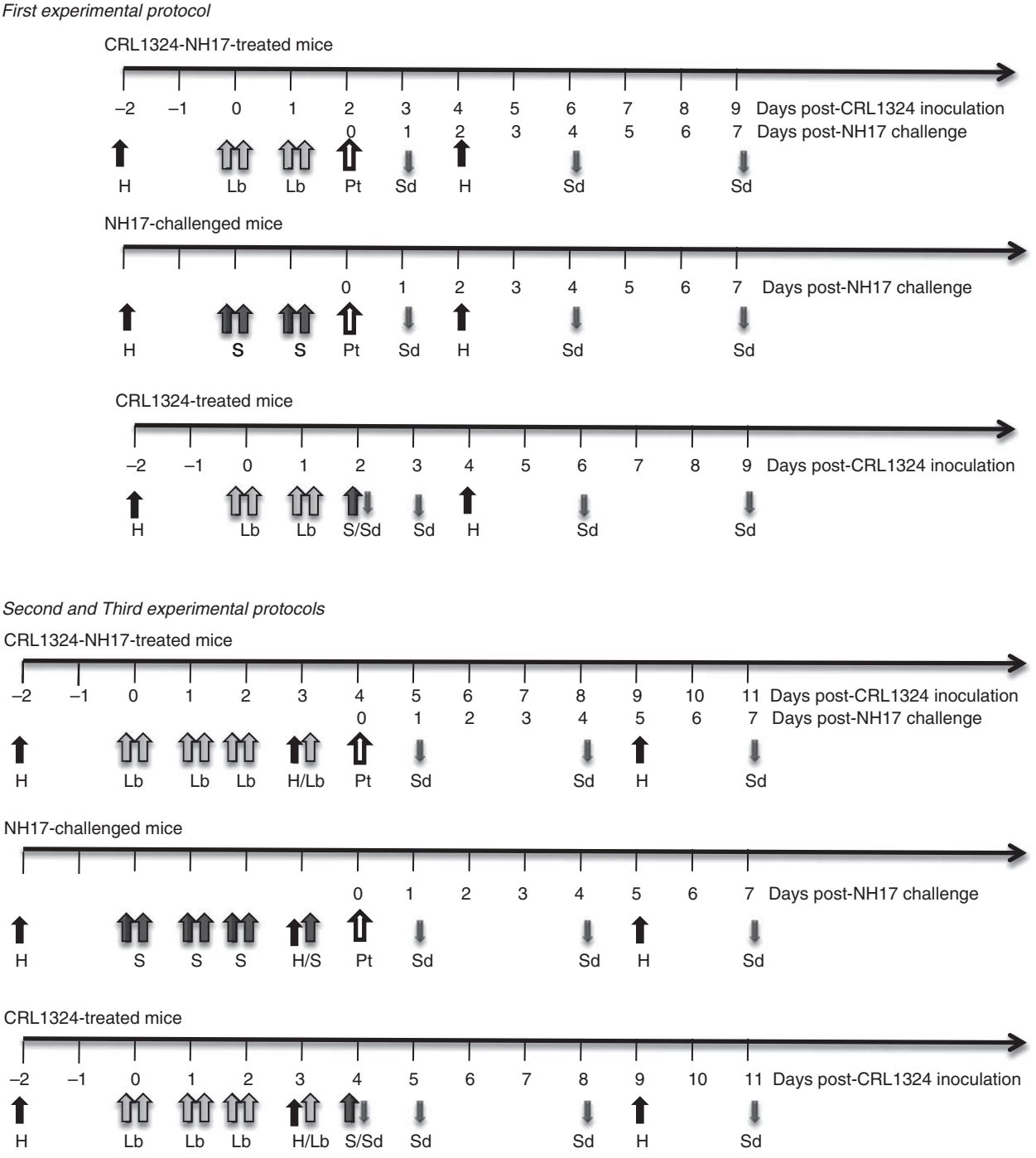

Figure 1 Sequence of inoculation and sampling days used in the experimental protocols. H: Hormone administration (⬆) (0·02 mg β-Estradiol 17-valerate). Each arrow indicates one inoculation of lactobacilli (⇧) in 50 μl containing 10^8 CFU (Lb: *Lactobacillus reuteri* CRL (Centro de Referencia para Lactobacilos Culture Collection)1324 (CRL1324)), pathogen (⇧) in 20 μl containing 1×10^5 CFU (first and second protocols) or 1×10^7 CFU (third protocol) (Pt: Group B *Streptococccus* NH17 (NH17)) or saline (, ⇧S), applied in the following experimental groups: CRL1324-NH17-treated mice, NH17-challenged mice and CRL1324-treated mice. Sd (⇩): Sampling days.

yeast were isolated from the vaginal autochthone microbiota of BALB/c mice (De Gregorio P.R., Juárez Tomás M.S., Nader-Macías M.E.F. data unpublished).

Additionally, vaginal homogenates (v.h.) of CRL1324-treated mice with 4 and 7 *Lactobacillus* doses were obtained to quantify the number of *Lact. reuteri*

CRL1324 viable cells as described above. The v.h. were extracted from the longitudinally opened vaginas, transferred to 1 ml peptone water (0·1% (w/v) meat peptone) and homogenized with a sterile Teflon pestle.

The plates were incubated under aerobic conditions at 37°C for 24 h (for quantification of GBS NH17 and vaginal microbiota) or 48 h (for *Lact. reuteri* CRL1324), and CFU numbers were determined.

Cytological and histological studies

For cytological studies, a 20 μl aliquot of v.w. was spread onto glass slides and stained with the May Grünwald-Giemsa technique. Histological studies of vaginal tissue were performed according to standard methods with Hematoxylin–Eosin stain (Biopur, Rosario, Argentina) (Silva de Ruiz *et al.* 2003). The vaginal smears and the histological slides were evaluated by light microscopy (at 400×) to determine the structural and cell patterns in an Axio Scope A1 Carl Zeiss microscope. The images were processed using Axio-Vision Release 4.8 software.

pH determinations

Vaginal pH of CRL1324-treated mice with 4 and 7 *Lactobacillus* doses, HC and APC mice was determined 1 h prior to the first inoculation of *Lact. reuteri* CRL1324 and on the days corresponding to GBS NH17 challenge (day 0) and on days 1, 4 and 7 post-NH17 challenge. A Sartorius PT-15 combination pH microelectrode (Sartorius AG, Göttingen, Germany) was carefully applied to minimize the disruption of the mucosa during insertion.

Statistical analysis

Analysis of variance (ANOVA) using a general linear model was applied to determine the main and interaction effects of factors (experimental group and day postinoculation) assayed in each experimental protocol on the number of viable microorganisms and vaginal pH. Significant differences (P value < 0·05) between mean values were calculated by Tukey's test. All the statistical analyses were performed using Minitab 16 Statistical Software.

Results

Evaluation of virulence factors in GBS strains

When evaluating the expression of β-h/c toxin in the GBS strains through cultures on blood agar plates, three different patterns were observed. GBS NH17 showed a high haemolytic (HH) phenotype, while GBS NH16 and

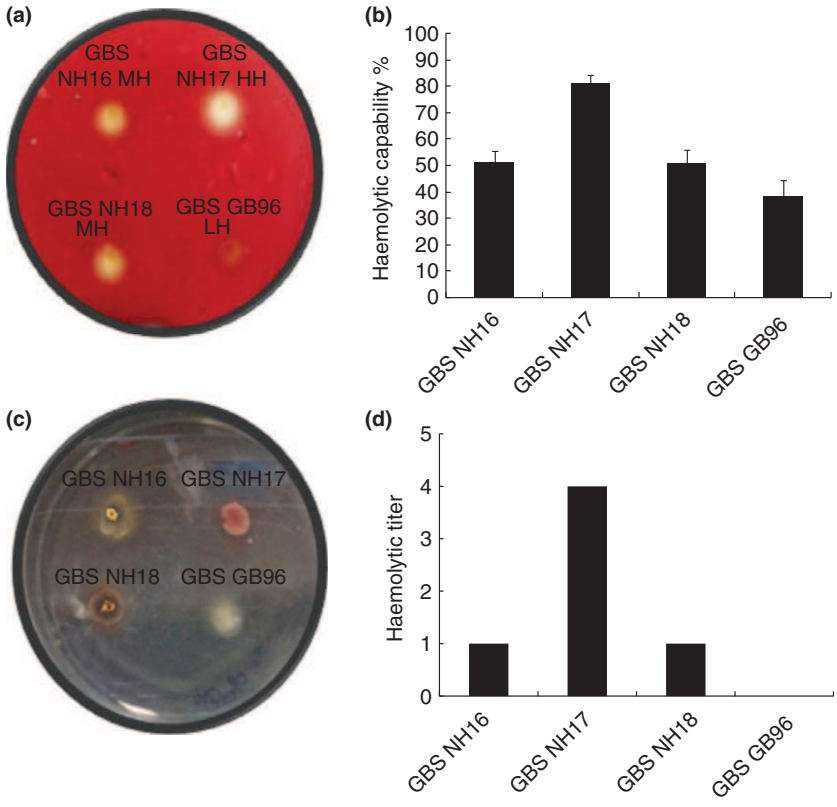

Figure 2 Screening of virulence factors in Group B *Streptococcus* (GBS) vaginal clinical isolates. (a) Haemolytic area on blood agar plate after GBS overnight growth: high haemolytic (HH), moderate haemolytic (MH) and low haemolytic (LH) phenotypes. (b) Haemolysin extract activity plotted as haemolytic capacity related to sodium dodecyl sulphate (SDS, haemolytic capacity = 100%). The data are plotted as the mean values of haemolytic capacity ± standard error. (c) Production of orange carotenoid pigment in GBS strains after overnight growth under anaerobic conditions on Todd-Hewitt agar. (d) Haemolytic titer obtained in a microdilution assay. Haemolytic titer was defined as the reciprocal of the highest dilution that produced 50% haemoglobin release compared with SDS control.

NH18 presented a moderate haemolytic (MH) effect and GBS GB96 a low haemolytic (LH) phenotype (Fig. 2a). The differences in haemolysis were corroborated by the haemolytic activity, because GBS NH17 showed the highest haemolytic titer and capability (4 and 81·34% respectively) compared with the other strains (GBS NH16 and NH18: haemolytic titer = 1 and haemolytic capability approx. 50%; GBS GB96: haemolytic titer = 0 and haemolytic capability = 38·24%) (Fig. 2b,d).

A close link between the expression of β-h/c toxin and the production of orange carotenoid pigment was evidenced. The HH phenotype showed a strong orange pigmentation, while MH and LH displayed a moderate and weak orange pigmentation respectively (Fig. 2c).

By light microscopy, the four strains evidenced the presence of capsule. However, no differences were observed between capsules produced by the GBS strains (data not shown).

Preventive effect of *Lactobacillus reuteri* CRL1324 against GBS NH17 challenge

GBS NH17 was the strain that evidenced the highest haemolytic activity and pigment production. Therefore, this strain was selected to evaluate the preventive effect of *Lact. reuteri* CRL1324 in a murine model of streptococcal vaginal colonization.

When GBS NH17 was i.va. inoculated to mice in low (1×10^5 CFU) and high (1×10^7 CFU) concentrations, it was recovered from v.w. up to the last sampling day (7th day post-NH17 challenge, Fig. 3a,c,e).

In the experimental protocol performed with 4 doses of *Lact. reuteri* CRL1324 i.va. administered prior to the i.va. GBS NH17-low inoculum challenge, no significant differences in the number of GBS viable cells between CRL1324-NH17-treated mice and NH17-challenged mice were observed (Fig. 3a). However, when mice were inoculated with seven doses of *Lact. reuteri* CRL1324 prior to the challenge with a low inoculum of GBS NH17 (second experimental protocol), a lower number of GBS viable cells was recovered in the v.w. of CRL1324-NH17-treated mice compared to NH17-challenged mice. For a same post-NH17 challenge day, this lower number was not significantly different between the experimental groups, probably because of the high dispersion of the data (Fig. 3c).

The protective effect of 7 doses of *Lact. reuteri* CRL1324 on GBS was also evidenced when mice were challenged with a high inoculum of GBS NH17. In this trial, a significant reduction in the number of GBS viable cells was evidenced since day 4 post-NH17 challenge in CRL1324-NH17-treated mice when compared to NH17-challenged mice (Fig. 3e).

The viable *Lact. reuteri* cells in v.w. of CRL1324-treated mice and CRL1324-NH17-treated mice (4 or 7 doses) were similar, indicating that the i.va. challenge with a low or high inoculum of GBS did not affect the number of viable lactobacilli recovered in v.w. In the three experimental protocols applied, a significant decrease in the number of viable lactobacilli was observed throughout the sampling period (Fig. 3b,d,f).

The murine vaginal cytology and histology studies showed a leucocyte influx in the vaginal lumen on day 1 post-NH17 challenge in mice challenged with GBS NH17. This leucocyte influx decreased on day 4 and disappeared on day 7 postchallenge. However, the inoculation of 4 and 7 doses of *Lact. reuteri* prior to GBS challenge reduced the vaginal leucocytes influx observed in NH17-challenged mice. A slightly higher reduction was evident in mice treated with 7 doses compared with 4 doses. No such influx was observed on the different sampling days in CRL1324-treated mice (Fig. 4).

Effect of different *Lactobacillus reuteri* CRL1324 doses on vaginal tract colonization and murine vaginal pH

In order to evaluate if higher number of doses of *Lact. reuteri* CRL1324 favour the colonization of lactobacilli in the vaginal tract, and if they can modify the vaginal milieu (probably by production of antimicrobial substances such as organic acids), a comparison between the number of *Lact. reuteri* viable cells from v.w and v.h of mice treated with 4 and 7 doses and the vaginal pH of CRL1324-treated and control mice was performed.

A higher number of i.va. doses of *Lact. reuteri* slightly increased its colonization in v.w. and v.h. on the days corresponding to GBS NH17 challenge (day 0) and on day 1 post-NH17 challenge (Fig 5a).

When evaluating the effect of lactobacilli doses on the murine vaginal pH, a significant reduction was observed at day 0 (NH17 challenge day) in mice treated with 7 doses of *Lact. reuteri* (pH = 5·98 $\pm$ 0·26) compared with control animals (pH = 6·91 $\pm$ 0·10 for HC, pH = 6·97 $\pm$ 0·09 for APC). The decrease in pH was not significant compared with mice treated with four lactobacilli doses (pH 6·38 $\pm$ 0·15). Vaginal pH (7 doses of *Lact. reuteri*) returned to the value of control mice on the following sampling days (Fig. 5b).

Effect of *Lactobacillus reuteri* CRL1324 on murine vaginal microbiota

The effect of the i.va. administration of *Lact. reuteri* CRL1324 (7 doses) on different mice cultivable vaginal microbial populations (LAB, enterococci, staphylococci

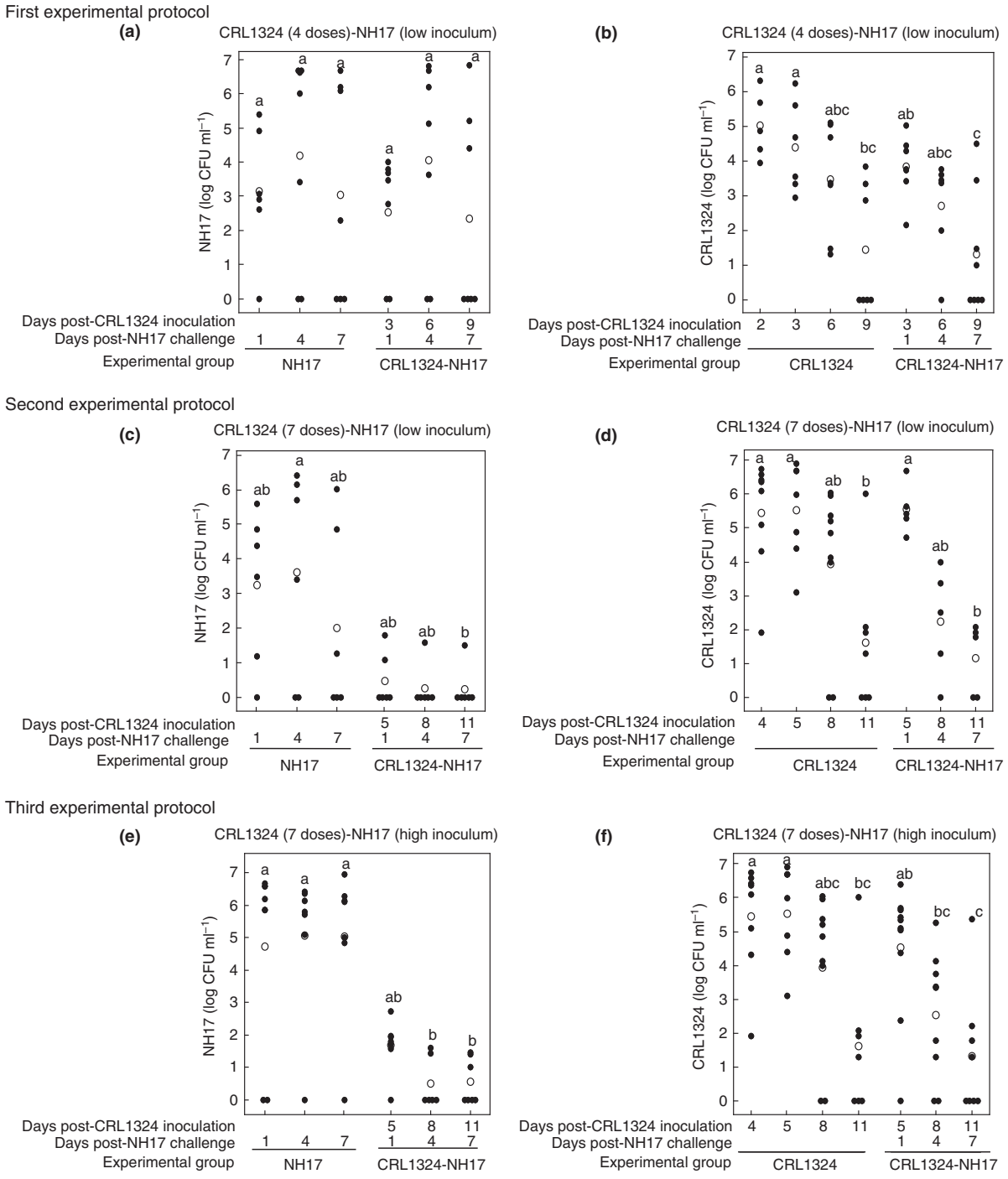

Figure 3 Effects of different doses of *Lactobacillus reuteri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 intravaginally (i.va.) administered against Group B *Streptococcus* NH17 (NH17) i.va. challenged in a murine experimental model. (a, c and e) NH17 viable cells in vaginal washings (v.w.) of CRL1324-NH17 and NH17 mice groups in the different experimental protocols. (b, d and f) CRL1324 viable cells in v.w. of CRL1324 and CRL1324-NH17 mice in the different experimental protocols. CRL1324-NH17 mice were inoculated with 4 doses (panels a-b) or 7 doses of CRL1324 (panels c-f) and later challenged with low (1×10^5 CFU) (panels a-d) or high inoculum (1×10^7 CFU) of NH17 (panels e-f). NH17 mice were challenged with low (panels a and c) or with high NH17 inoculum (panel e). CRL1324 mice were inoculated with 4 doses (panel b) or 7 doses of CRL1324 (panels d and f). Data are plotted as individual (solid circle) or average values (open circle) of the NH17 or CRL1324 viable cell numbers. In each experimental protocol, different letters indicate statistically significant differences ($P < 0{\cdot}05$) between the mean values of NH17 log CFU ml^{-1} or CRL1324 log CFU ml^{-1} between experimental groups on the different days postinoculation.

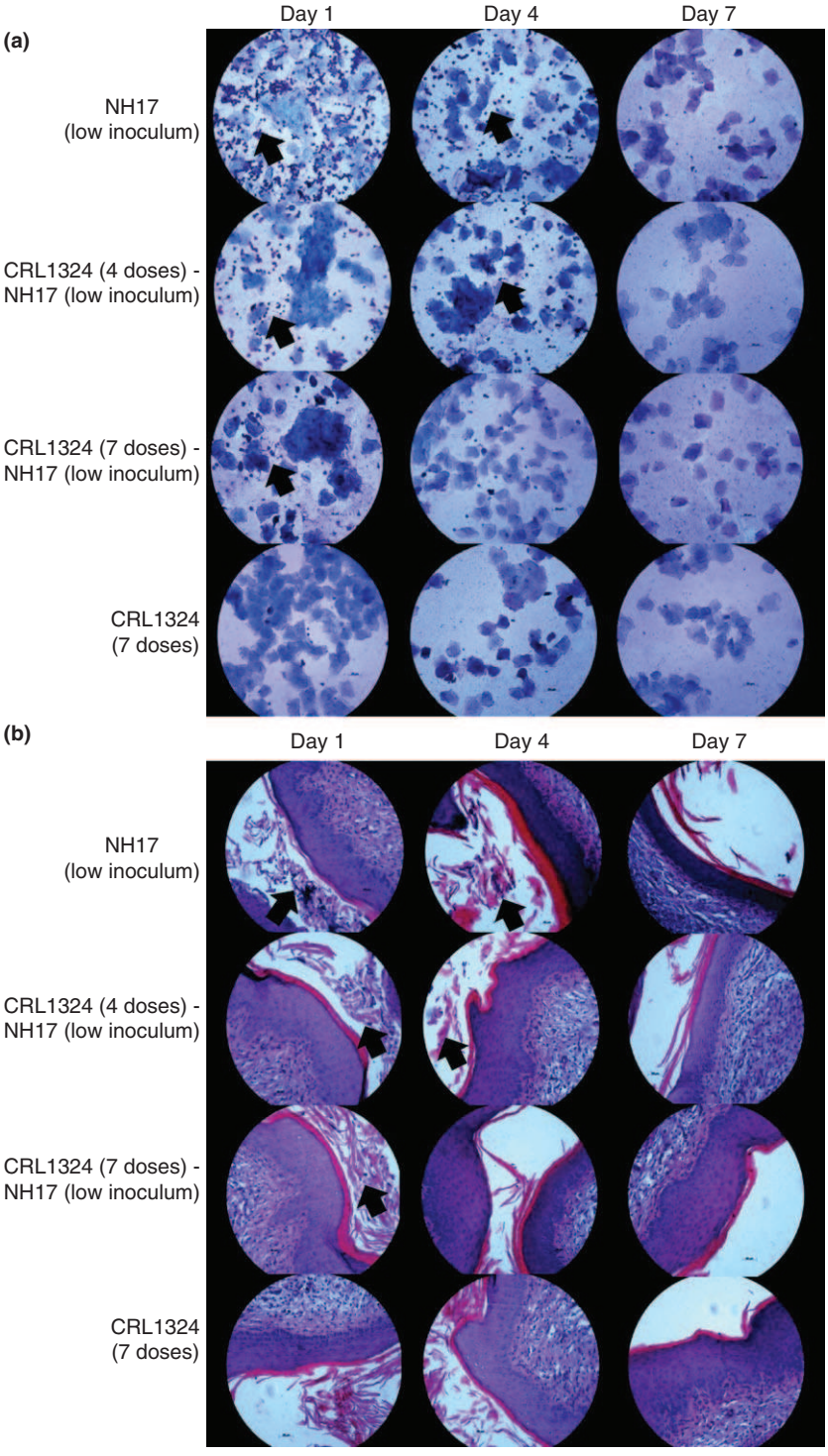

Figure 4 Photomicrographs of (a) May Grünwald-Giemsa-stained vaginal smears and (b) Hematoxylin–Eosin-stained vaginal slides from BALB/c mice (different experimental groups of the three experimental protocols assayed) on different days post-Group B *Streptococcus* NH17 (NH17) challenge. NH17 (low inoculum): mice intravaginally (i.va.) challenged with 1×10^5 CFU of NH17. *Lactobacillus reuteri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 (CRL1324) (4 doses)-NH17 (low inoculum): mice i.va. inoculated with 4 doses of CRL1324 and later challenged with 1×10^5 CFU of NH17. CRL1324 (7 doses)-NH17 (low inoculum): mice i.va. inoculated with 7 doses of CRL1324 and later challenged with 1×10^5 CFU of NH17. CRL1324: mice i.va. inoculated with 7 doses of CRL1324 (the same results were observed in mice inoculated with 4 doses of CRL1324). Leucocyte influx in the vaginal wash and lumen of NH17 challenged mice is indicated with black arrows. Results are representative of at least three independent experiments. Magnification: 400×.

and enterobacteria) was evaluated and compared with HC mice. A significant increase ($P < 0{\cdot}05$) in the number of viable LAB was observed in CRL1324-treated mice at day 4 post-CRL1324 inoculation compared with control animals. CRL1324 inoculation did not affect enterococci. However, a decrease that was not statistically significant ($P > 0{\cdot}05$) in the number of viable enterobacteria and staphylococci was observed on all sampling days in CRL1324-treated mice when compared with HC mice (Fig. S1).

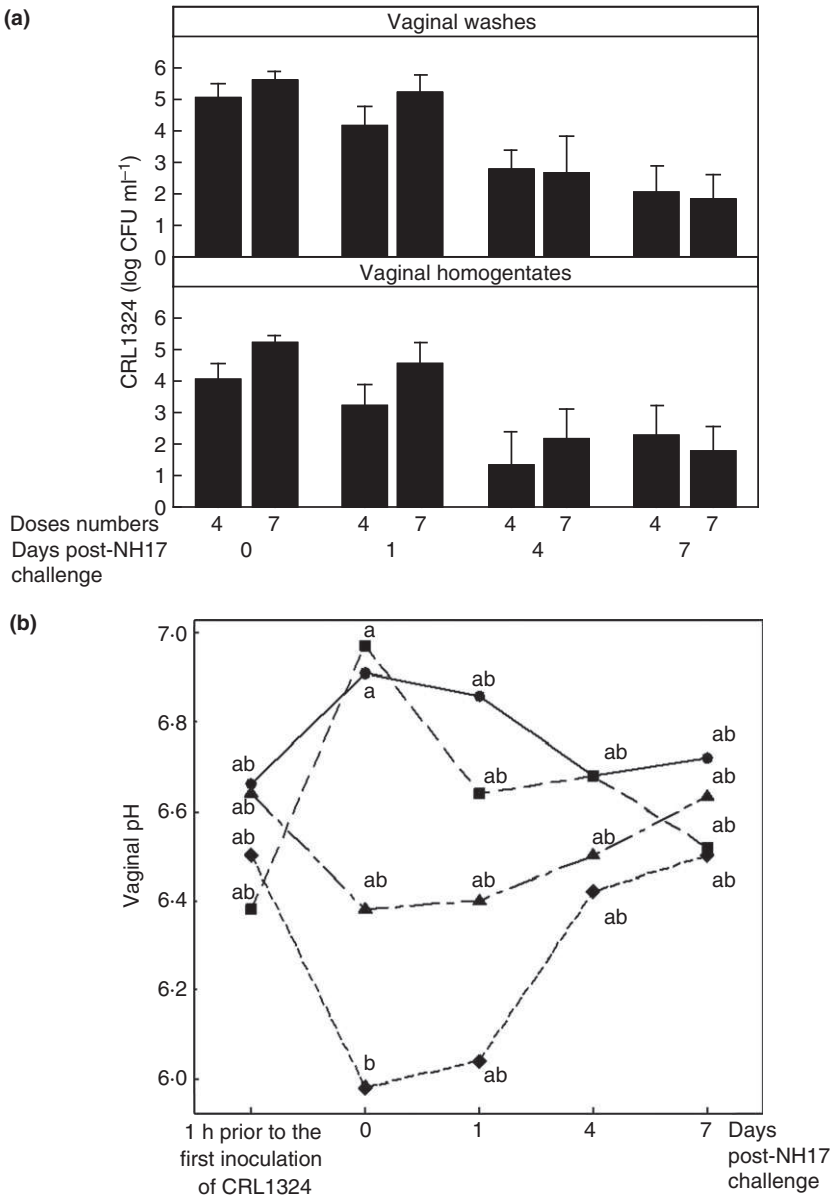

Figure 5 Effect of *Lactobacillus reuteri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 (CRL1324) doses on the murine vaginal colonization and pH. (a) Viable cells of CRL1324 from murine vaginal washings (v.w.) and vaginal homogenates (v.h) of mice treated with 4 or 7 doses of CRL1324, on the days corresponding to Group B *Streptococcus* NH17 (NH17) challenge (day 0) and on days 1, 4 and 7 post-NH17 challenge. The data are plotted as the mean values of viable cells numbers (log CFU ml^{-1} ± standard error). (b) Vaginal pH of CRL1324 (4 (▲) or 7 (♦) doses)-treated mice and control mice (HC (●) and APC (■)) determined 1 h prior to the first inoculation of CRL1324 and on the days corresponding to NH17 challenge (day 0) and on days 1, 4 and 7 post-NH17 challenge. The data are plotted as the mean values of vaginal pH. Statistically significant differences between the mean values of pH between experimental groups on the different days postinoculation are indicated by different letters ($P < 0{\cdot}05$).

Discussion

On account of the increasing antibiotics resistance of GBS strains (Kasahara *et al.* 2010; Nagano *et al.* 2012; Oviedo *et al.* 2013) and considering that maternal GBS colonization continues to be one of the most important risk factors for developing disease in newborns, urgent development of new prevention strategies to replace or improve antibiotic therapy is needed. In the present work, a murine experimental model of GBS vaginal colonization was conducted to evaluate a BVL strain, *Lact. reuteri* CRL1324, as a potential candidate for controlling GBS vaginal colonization.

GBS phenotypic variants with markedly different expression of virulence factors can arise during the process of colonization or during infection with this pathogen. Selective pressures exerted by the host may cause differential expression of certain GBS surface components, providing the pathogen with a survival benefit (Sendi *et al.* 2009). The GBS capsule, which contributes to the immune resistance by reducing the opsonophagocytic clearance through inhibition of the complement fixation and activation on the bacterial surface (Sendi *et al.* 2009), and the *β*-h/c toxin associated with inflammatory activation (Doran *et al.* 2003; Patras *et al.* 2013) and lysis of eukaryotic cells (Sendi *et al.* 2009) have been identified as virulence factors that

can modify their expression in different situations. Although the most of the vaginal GBS isolates are haemolytic, no haemolytic GBS strains (5–8% of all isolates) can be isolated from the vagina of colonized women (Nickmans *et al.* 2012).

In the present work, the phenotypic expression of β-h/c toxin, carotenoid pigment and capsule were evaluated in four vaginal GBS clinical isolates in order to select the most virulent strain to set up an adequate murine model of streptococcal vaginal colonization. Differences in the β-h/c toxin expression and consequently in the carotenoid pigment production were evidenced between the different GBS strains under study. Three haemolytic phenotypes (high, moderate and low) closely associated with the production of orange carotenoid pigment were observed. However, when evaluating capsule production, there were no differences. Taking into account that the β-h/c toxin is related to virulence in animal models (Hensler *et al.* 2005; Patras *et al.* 2013), GBS NH17, the strain showing a higher haemolytic activity and pigment production, was used to evaluate the preventive effect of *Lact. reuteri* CRL1324 in a murine experimental model.

Several research groups have evidenced through *in vitro* studies that lactobacilli strains inhibit the growth of GBS by production of metabolites as organic acids and bacteriocin-like substances (Bodaszewska *et al.* 2010; Juárez Tomás *et al.* 2011; Ruiz *et al.* 2012; De Gregorio *et al.* 2014). Some other publications have demonstrated the *in vivo* protective effect of different lactobacilli strains on several urogenital microorganisms by using a murine experimental model (Pascual *et al.* 2010; Joo *et al.* 2011, 2012; Lazarenko *et al.* 2012). However, the preventive effect of lactobacilli on GBS in animal experimental models was not evidenced up today.

This is the first report where an inhibitory effect on GBS murine vaginal colonization was demonstrated after i.va. inoculation of a BVL (*Lact. reuteri* CRL1324). The preventive effect depended on the number of times that *Lact. reuteri* CRL1324 was inoculated, because when seven doses were used (instead of 4 doses) prior to the GBS challenge, a significant reduction in the vaginal GBS viable cell numbers was evidenced. In this way, most of the clinical trials with vaginal probiotics report promising results by using probiotic preparations containing high doses of lactobacilli (around 10^9 CFU). These results suggest that, together with the specific properties of the strain, the number of viable exogenously applied lactobacilli could have a relevant role in the effectiveness of the product (Mastromarino *et al.* 2009). Also, recent data from prolonged repetitive trials of *Lactobacillus*-containing probiotics appear to be more promising than short courses (Ya *et al.* 2010; Bradshaw *et al.* 2012). Some other studies performed at the intestinal tract showed that the beneficial effects of lactobacilli were dose- and strain-dependent (Perdigón *et al.* 2002; Li *et al.* 2012; Zhu *et al.* 2014).

In this work, the lactobacilli inoculum assayed was selected according to the number of lactobacilli isolated from the genital tract of healthy fertile women ($6{\cdot}59 \pm 2{\cdot}02$ log CFU per vaginal samples) (Rönnqvist *et al.* 2006). On the other hand, the *Strep. agalactiae* concentration used in the first and second protocol was selected to evaluate the inhibitory ability of lactobacilli against the highest number of the pathogen (5·0 log CFU per vaginal samples) reported in women with samples considered positive (Rönnqvist *et al.* 2006). This concentration was increased in the third protocol (10^7 CFU) based on the high variability in GBS numbers recovered from vaginal washing of mice inoculated with 10^5 CFU of the pathogen.

Several reports indicate that high numbers of lactobacilli can contribute to the low vaginal pH, which seems to have a negative influence on Group B streptococci (Rönnqvist *et al.* 2006). Therefore, the effect of *Lactobacillus* colonization on the murine vaginal pH was determined. A slight but not statistically significant increase in *Lact. reuteri* CRL1324 colonization was evidenced in 7 dose-treated mice compared to 4 dose-treated mice on the days representing the GBS NH17 challenge, and on day 1 postchallenge. On the same days, mice inoculated with 7 doses showed significantly lower vaginal pH than control mice, but not than 4 dose-treated mice. Despite the reduced pH evidenced in CRL1324-treated mice (7 doses), a murine vaginal acidity similar to that of normal human vagina (4·0–4·5) (Larsen 1993) was not observed. Muench *et al.* (2009) reported that the murine vaginal pH values were not directly related to the numbers of lactobacilli in the vaginal tract.

Some urogenital pathogenic bacteria, such as GBS, can subvert the immune responses mounted by the host, creating problems with pathogen elimination (Kline *et al.* 2011). Recently, Patras *et al.* (2013) showed that the CovR/S regulatory system of GBS is needed to limit the expression of virulence factors in a GBS vaginal colonization of a murine model, reducing the host innate immune response and promoting the pathogen colonization. In this work, when evaluating the vaginal cytological and histological patterns of mice challenged with GBS NH17, a leucocyte influx in the vaginal lumen was evidenced on the first day post-NH17 challenge. However, this leucocyte influx showed to decrease on day 4 and disappeared on day 7.

Up to date, there is strong evidence related to the role of probiotics, mainly LAB, in the maintenance of the health or in the prevention of disease by modulation of the host's immune response (Karlsson *et al.* 2012). Sev-

eral studies have demonstrated that lactobacilli exert an anti-inflammatory effect in a vaginal infection models (Joo *et al.* 2011, 2012; Wagner and Johnson 2012; Rizzo *et al.* 2013), and can activate the immune system, improving the host's response against urogenital pathogens (Lazarenko *et al.* 2012; Wagner and Johnson 2012). In the present work, the i.va. treatment with *Lact. reuteri* CRL1324 prior to the i.va. challenge with GBS reduced the leucocyte influx to the vaginal lumen induced by the pathogenic microorganism. A higher vaginal leucocyte reduction was observed in mice treated with 7 doses of *Lact. reuteri* CRL1324 than with 4 doses. As some authors have shown, LAB can modulate the host's immune response in a way related to the dose administered (Perdigón *et al.* 2002; Li *et al.* 2012; Zhu *et al.* 2014), then we could suggest that the higher number of i.va. *Lact. reuteri* CRL1324 doses could have some type of effect on the modulation of the immune system. Further research is required to determine the effect of *Lact. reuteri* CRL1324 on different immunological components in order to determine if the decrease in the GBS viable cells produced after the i.va. inoculations of *Lact. reuteri* CRL1324 could be supported by a modulation of the immune system.

Lactobacilli are involved in the maintenance of the equilibrium of the human vaginal microbiota by preventing the overgrowth of pathogenic and opportunistic organisms (Rönnqvist *et al.* 2006; Coman *et al.* 2014; Verdenelli *et al.* 2014). The continuous vaginal and oral application of certain *Lactobacillus* strains has been shown to modify a microbiota indicative of bacterial vaginosis to a normal microbiota dominated by lactobacilli (Reid *et al.* 2003). In the present work, the i.va. administration of 7 doses of *Lact. reuteri* CRL1324 to normal mice induced a slight decrease in enterobacteria and staphylococci and an increase in LAB. The CFU of viable LAB include *Lact. reuteri* CRL1324. In a similar way, Babenko *et al.* (2013) demonstrated that i.va. administration of *Lactobacillus casei* IMV B-7280 had an effective anti-staphylococcal effect and reduced the number of coliform bacteria and fungi in the murine vagina.

From the results obtained in the present work and considering some of the beneficial characteristics of *Lact. reuteri* CRL1324, as the capability to form biofilm (Leccese Terraf *et al.* 2012), co-aggregate with GBS NH17 and inhibit this pathogen by production of organic acids (De Gregorio *et al.* 2014), several mechanisms involved in the *in vivo* preventive effect of *Lact. reuteri* CRL 1324 on GBS could be suggested. *Lactobacillus reuteri* CRL 1324 could form a biofilm on the vaginal epithelium resulting in a competition by nutrients and by binding sites with the pathogen. Moreover, *Lact. reuteri* CRL 1324 could co-aggregate with GBS causing a prolonged exposition to antagonistic compounds, which would reduce the pathogen viability. These modes of action could be complemented with a possible immunomodulatory effect exerted by the BVL. Further studies should be carried out to demonstrate the mechanisms involved in GBS inhibition by lactobacilli.

In conclusion, i.va. administered *Lact. reuteri* CRL1324 decreases the murine vaginal colonization of GBS and therefore could be considered as a new biological agent to reduce infections caused by this microorganism.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effect of intravaginal (i.va.) inoculation of *Lact. reuteri* Centro de Referencia para Lactobacilos Culture Collection (CRL) 1324 (CRL1324) on the mice vaginal microbiota.