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RESEARCH ARTICLE

Anti-*Candida* activity of beneficial vaginal lactobacilli in *in vitro* assays and in a murine experimental model.

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One sentence summary: Lactobacilli predominant in human vagina and protect from vulvovaginal candidiasis. The manuscript reports different methods to determine lactobacilli inhibitory effect at the laboratory, and protection against Candida in murine vagina.

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ABSTRACT

Vulvovaginal candidiasis (VVC) is one of the most frequent infections affecting women worldwide. Healthy vaginal microbiota is dominated by lactobacilli, which form a strong defense line against pathogens. In this work, *in vitro* antimicrobial properties of thirty vaginal *Lactobacillus* strains were evaluated against eleven *Candida* vaginal clinical isolates, employing three different methods. Also, the effect of intravaginal (i.va.) administrations (preventive, therapeutic and preventive-therapeutic) of *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332 strains against the i.va. challenge with *Candida albicans* (C.a.) was evaluated in a murine experimental model. From the results of agar overlay and liquid medium assays the selected lactobacilli strains have shown to inhibit the growth of at least one *Candida* strain. The inhibition was mainly due to the effect of organic acids. Anti-*Candida* activity was not evidenced in the agar plate diffusion method. In the experimental murine model, only preventive-therapeutic administration of both lactobacilli was able to significantly reduce viable C.a. numbers recovered in vaginal washes and the leukocyte influx induced by the fungi. In conclusion, lactobacilli exhibited *in vitro* and *in vivo* antimicrobial effects on *Candida*, suggesting that they could be promising candidates for protection against VVC. Lactobacilli predominant in human vagina and protect from VVC. The manuscript reports different methods to determine lactobacilli inhibitory effect at the laboratory, and protection against Candida in murine vagina.

Keywords: *Candida* spp.; beneficial lactobacilli; murine model; vulvovaginal candidiasis; *in vitro* and *in vivo* studies; anti-*Candida* activity

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a high-incidence disease affecting the life quality of women worldwide. Around 75% of all women of reproductive age suffer from VVC at least once during their lifetime, with approximately 5% to 8% experiencing up to four or more episodes per year (Peters *et al.* 2014; Cassone 2015). The most frequent etiologic agent is the opportunistic pathogen *Candida albicans*, the fungal species with highest prevalence in the human microbiota (Workowski and Berman 2010; da Silva Dantas *et al.* 2016). However, other varieties of *Candida* species such as *C. glabrata*, *C. krusei* and *C. tropicalis* are associated with VVC (Mahmoudi Rad *et al.* 2011). It has been suggested that the overgrowth of *Candida* spp. is facilitated by the disruption of the microbial vaginal balance. Factors increasing the risk for the development of VVC are antibiotic therapies, pregnancy status, genetic polymorphisms and susceptibility, use of contraceptives and/or spermicides, sexual intercourse, immunosuppression and diabetes (Babula *et al.* 2005; Sobel 2007; Sangaré *et al.*

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2017). The therapy usually applied against VVC consisted in the use of antifungal azoles. However, these drugs are fungistatic for *C. albicans*, and prolonged exposition can generate high pressure, thus promoting the appearance of azole-resistant *Candida* (Coste *et al.* 2007). In this way, recurrent VVC is a serious clinical condition due to the lack of successful therapies. Long-term fluconazole treatment can promote longer asymptomatic periods between recurrences, but does not provide a definitive cure. This fact leads to a low quality of life together with high expenses associated with medical visits and therapies (Marchaim *et al.* 2012; Foxman *et al.* 2013; Sobel 2015). Therefore, novel antifungal drugs or successful alternative therapies for VVC are urgently required.

Lactobacilli are the predominant microorganisms in the vaginal microbiome of healthy women, preventing the overgrowth of opportunist microorganisms and pathogens (Nader-Macías and Juárez Tomás 2015; Mendling 2016). Recent studies on the human microbiome showed that different species of *Lactobacillus* including *Lactobacillus iners*, *L. crispatus*, *L. gasseri*, *L. jensenii*, and to a lesser extent, *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. delbrueckii*, *L. salivarius*, *L. reuteri*, *L. casei*, *L. vaginalis* and *L. rhamnosus* (Ravel *et al.* 2011; Douillard and de Vos 2014) are host-specific, dominant and exclusive in the human vagina (Human Microbiome Project Consortium 2012; Integrative HMP (iHMP) Research Network Consortium 2014). However, the species present depend on ethnic background, genetic polymorphism, environmental and behavioral factors (Yildirim *et al.* 2014; Mendling 2016). The universality and host-specificity of vaginal *Lactobacillus* species are the support for novel therapeutic opportunities for the treatment of alterations in the vaginal microbiome (Reid 2017).

Lactobacilli can exert their function through different mechanisms, including a) biofilm formation on the vaginal mucosa, a phenomenon affected both by the production of biosurfactants and by the capability of these bacteria for self- or coaggregation; b) adhesion to epithelial cells, mucus and/or extracellular matrix components; c) production of antimicrobial substances (organic acids, hydrogen peroxide, bacteriocins); d) enzyme release (e.g. arginine deaminase) competing for nutrients or e) modulation of the immune system (Leccese Terraf *et al.* 2012; De Gregorio et al., 2014, 2015; Leccese Terraf *et al.* 2014; Nader-Macías and Juárez Tomás 2015; De Gregorio, Juárez Tomás and Nader-Macías 2016; Reid 2016; Leccese Terraf *et al.* 2017). *In vitro* and *in vivo* studies are required to demonstrate the beneficial effect of different *Lactobacillus* strains because the effect is strain-specific (De Gregorio *et al.* 2012; De Gregorio *et al.* 2014). Thus, the aims of this study were to: a) evaluate the *in vitro* antimicrobial properties of 30 different vaginal *Lactobacillus* strains, previously selected for their beneficial properties, against 11 vaginal clinical isolates of *Candida* spp. through different methodologies, and b) determine the effect of intravaginal (i.va.) administration (preventive, therapeutic and preventive-therapeutic protocols) of *Lactobacillus* strains against the i.va. challenge with *C. albicans* in a murine experimental model.

METHODS

Microorganisms and culture conditions

Thirty *Lactobacillus* strains from the Centro de Referencia para Lactobacilos Culture Collection (CRL, Tucumán, Argentina), originally isolated from healthy (without infection) and unhealthy (with infection) human vagina (Ocaña *et al.* 1999), and selected for their beneficial properties (Ocaña, Pesce de Ruiz Holgado and Nader-Macías 1999; Vera Pingitore *et al.* 2009; Juárez Tomás *et al.* 2011; Leccese Terraf *et al.* 2012; De Gregorio et al., 2014, 2015; Leccese Terraf *et al.* 2014; De Gregorio, Juárez Tomás and Nader-Macías 2016; Leccese Terraf *et al.* 2017) were evaluated in this work. The *Lactobacillus* strains used and the health status of the vaginas from which the strains were originally isolated are summarized in Table 1. All the strains were grown in microaerophilic conditions in De Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, France) (De Man, Rogosa and Sharpe 1960) at 37°C for 24 h and subcultured twice in the same medium at 37°C for 12 h before use.

Eleven vaginal clinical isolates of *Candida* sp. were kindly provided by Dr. Virginia Ocaña from the Nuevo Hospital 'El Milagro' (Salta, Argentina) (codified as F11 and F18), Dr. Cristina Gaudioso de Allori from the Acción Social Universidad Nacional de Tucumán (ASUNT, Tucumán, Argentina) (codified as C1 and C2) and Biochemist Cecilia Vallejo from the Universidad Nacional de Tucumán (codified as HE2, HE3, HE4, HE5, HE6, HE7 and HE10). *Candida* strains were grown aerobically for 16 h at 37°C in Sabouraud dextrose (SD) medium [% (w/v): 40 glucose and 10 peptone, pH 5.6; Britania Laboratories, Argentina].

All microorganisms were stored in milk-yeast extract [% (w/v): 13 nonfat milk, 0.5 yeast extract and 1 glucose; Britania Laboratories, Argentina] with 20% glycerol (Cicarelli Laboratories, Argentina) at −20°C.

Identification of *Candida* sp. vaginal clinical isolates

For the identification of *Candida* sp. vaginal clinical isolates at the species level, saline suspensions (previously grown in SD broth) were cultured in CHROMagar *Candida*® (CHROMagar Company, France) plates for 24 to 48 h at 35°C. The use of a chromogenic medium contributes to the presumptive identification of *C. albicans*, *C. tropicalis* and *C. krusei* (Odds and Bernaerts 1994) and allows working with re-isolated colonies. Then, the strains grown in the chromogenic medium were subcultured in SD agar for 24 h at 37°C, identification at the species level being performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This technique allows the identification of the pathogens by generating a protein spectrum or 'fingerprint' that is unique for a given species (Buchan and Ledeboer 2013; Clark *et al.* 2013). For the protein extraction, a colony of each isolate was added in duplicate to a 96-well metallic plate (Bruker Daltonics, Germany) and allowed to dry at room temperature. Then, 1 μL of formic acid (100%) (Sigma-Aldrich, Argentina) and 1 μL of HCCA matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% trifluoroacetic acid; Bruker Daltonics, Germany) were added. Readings were performed in a Microflex LT mass spectrometer using the Flex Control software (version 3.0, Bruker Daltonics, Germany). The spectrometer was calibrated using a protein extract of *Escherichia coli* (Bruker Bacterial Test Standard). The spectra obtained were compared with two databases simultaneously and the results were presented as a score (Clark *et al.* 2013). The following identification criteria were used according to the manufacturer's recommendations: a log score value $\geq$2 indicated correct identification at the species level, a value between 1.7 and 1.9 indicated identification at the genus level and a value $<$1.7 indicated lack of identification. The correct identification of each isolate at the species-level was accepted if at least one of the obtained duplicates scored $>$2.

Table 1. Antimicrobial activity of vaginal *Lactobacillus* strains against clinical isolates of *Candida* spp.

Lactobacillus strains					Inhibition zone (mm)[e]										
Metabolic group[a]	Vaginal status[b]	H_2O_2[c]	pH[d]	Species	*C. albicans*[f]							*C. glabrata*[g]			*C. tropicalis*[h]
					C1	C2	HE3	HE4	HE5	HE6	HE7	F11	F18	HE10	HE2
OHo	HV	2	4.09	*L. gasseri* CRL1252	—	—	—	—	—	—	—	—	—	—	—
	UV-Vno	2	4.06	*L. gasseri* CRL1255	—	—	—	—	—	—	—	—	—	—	—
	UV- Vni	2	4.04	*L. gasseri* CRL1256	—	—	—	—	—	—	—	—	—	—	—
	HV	1	4.59	*L. gasseri* CRL1261	1.33 ± 0.33	+ (wh)	—	—	—	+ (wh)	3.33 ± 1.67 pi	—	—	—	+ (wh)
	HV	2	4.71	*L. gasseri* CRL1263	1.66 ± 0.66	+ (wh)	—	—	—	—	—	—	—	—	—
	HV	2	4.06	*L. gasseri* CRL1264	—	—	—	—	—	—	—	—	—	—	—
	HV	2	4.13	*L. gasseri* CRL1265	—	—	—	—	—	—	—	—	—	—	—
	HV	2	4.07	*L. gasseri* CRL1268	—	—	—	—	—	—	—	—	—	—	—
	UV-Vno	2	4.06	*L. gasseri* CRL1270	—	—	—	—	—	—	—	—	—	—	—
	HV	1	4.33	*L. gasseri* CRL1290	—	—	—	—	—	—	—	—	—	—	—
	UV- Vno	2	4.11	*L. gasseri* CRL1307	—	—	—	—	—	—	—	—	—	—	—
	HV	2	4.12	*L. gasseri* CRL1311	—	—	—	—	—	—	—	—	—	—	—
	HV	2	4.06	*L. gasseri* CRL1314	—	—	—	—	—	—	—	—	—	—	—
	HV	1	4.06	*L. gasseri* CRL1320	8.66 ± 0.66	9.33 ± 0.67	7.00 ± 1.00	9.00 ± 2.65	2.66 ± 1.33 pi	3.66 ± 1.52	6.50 ± 3.08	+ (wh)	+ (wh)	+ (wh)	+ (wh)
	HV	2	4.05	*L. gasseri* CRL1322	5.66 ± 1.20	3.33 ± 1.77 pi	1.66 ± 0.20	7.33 ± 0.67	—	4.33 ± 1.19	+ (wh)	+ (wh)	—	+ (wh)	—
	UV- M-Vno	2	4.10	*L. gasseri* CRL1509	—	—	—	—	—	—	—	—	—	—	—
	UV- M	2	4.13	*L. jensenii* CRL1313	—	—	—	—	—	—	—	—	—	—	—
	HV	2	4.14	*L. jensenii* CRL1317	—	—	—	—	—	—	—	—	—	—	—
	UV- M	1	4.29	*L. jensenii* CRL1333	—	—	—	—	—	—	—	—	—	—	—
	HV	1	4.28	*L. jensenii* CRL1349	—	—	—	—	—	—	—	—	—	—	—
	HV	1	4.12	*L. johnsonii* CRL1292	—	—	—	—	—	—	—	—	—	—	—
	HV	1	4.06	*L. salivarius* CRL1296	5.56 ± 3.21	4.33 ± 1.61	4.00 ± 1.08	8.00 ± 1.53	2.66 ± 1.33	4.33 ± 2.01	4.00 ± 2.08	—	—	—	+ (wh)
	UV- M-Vno	0	4.04	*L. salivarius* CRL1328	4.00 ± 2.31	6.66 ± 1.67	2.66 ± 1.45 pi	6.00 ± 0.58 pi	+ (wh)	3.00 ± 1.53 pi	7.00 ± 0.50	—	—	—	+ (wh)
FHe	HV	1	4.08	*L. paracasei* CRL1512	5.00 ± 2.64	3.33 ± 1.77 pi	3.66 ± 1.86 pi	3.33 ± 1.67	—	2.00 ± 0.48	7.66 ± 2.19 pi	—	+ (wh)	—	—
	HV	2	4.20	*L. rhamnosus* CRL1332	8.00 ± 1.73	13.66 ± 3.29	9.00 ± 1.63	10.66 ± 1.20	9.66 ± 4.49	10.00 ± 0.00	11.00 ± 1.00	+ (wh)	+ (wh)	+ (wh)	+ (wh)
	HV	1	4.08	*L. rhamnosus* CRL1511	6.33 ± 0.88	3.00 ± 1.53 pi	+ (wh)	5.33 ± 0.33	—	4.00 ± 2.01	4.00 ± 2.00	—	+ (wh)	+ (wh)	—
OHe	UV-Vni	2	4.70	*L. fermentum* CRL1287	—	—	—	—	—	—	—	—	—	—	—
	HV	2	4.70	*L. reuteri* CRL1324	2.00 ± 1.01 pi	2.66 ± 1.33 pi	2.00 ± 1.00 pi	3.00 ± 1.53 pi	—	—	+ (wh)	—	—	—	+ (wh)
	HV	2	4.69	*L. reuteri* CRL1327	3.33 ± 1.66 pi	2.66 + 1.45 pi	2.33 ± 1.20 pi	3.33 ± 1.67 pi	—	—	+ (wh)	—	—	—	+ (wh)
	HV	2	4.27	*L. mucosae* CRL1508	8.66 ± 2.40	10.33 ± 1.86	10.66 ± 2.73	9.66 ± 3.18	6.33 ± 3.01	4.00 ± 1.50	11.00 ± 1.53	+ (wh)	+ (wh)	+ (wh)	—

Value represents the mean ± standard error of three experiments performed on different days.

[a]Metabolic group of *Lactobacillus* strains OHo: obligate homofermentative group. FHe: facultative heterofermentative group. OHe: obligate heterofermentative group.

[b]Health status of the vagina from which lactobacilli were isolated. HV: healthy vagina. UV: unhealthy vagina-Vno: vaginosis; Vni: vaginitis; M: mycosis.

[c]H_2O_2 production classified as strong (score 2), moderate (score 1) and negative (score 0) according to the intensity of blue color of the colonies when applying chromogenic method in MRS agar-tetramethylbenzidine (TMB) (MRS-TMB plates).

[d]Final pH of *Lactobacillus* cultures after 12 h of incubation in MRS broth at 37°C.

[e]Inhibitory halos in agar plates (expressed in mm) of vaginal *Lactobacillus* strains against *Candida* spp. + indicate inhibitory activity; when the fungal growth was inhibited only in the area above *Lactobacillus* growth without a definite inhibition halo; pi indicate partial inhibition;—absence of inhibitory activity.

[f,g,h]*Candida* species identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Qualitative determination of H_2O_2 production by lactobacilli

Hydrogen peroxide production by *Lactobacillus* strains was determined by the chromogenic method in MRS agar medium plates supplemented with tetramethyl-bencydine and horseradish peroxidase, as described by Juárez Tomás *et al.* (2004). Briefly, the plates were inoculated with the lactobacilli and incubated at 37°C for 48 h under microaerophilic conditions. H_2O_2-producing strains evidenced blue colonies after exposure to air for 30 min. H_2O_2 production was classified as strong (score 2), moderate (score 1) or negative (score 0) taking into account the intensity of the blue color of the colonies.

In vitro anti-*Candida* activity of lactobacilli

Antimicrobial activity of Lactobacillus strains

The antimicrobial effect of vaginal lactobacilli against *Candida* spp. was evaluated with the agar overlay technique with minor modifications (do Carmo *et al.* 2016). Briefly, MRS agar plates were inoculated with 10 μL of *Lactobacillus* suspensions (10^8 UFC mL^{-1}) and incubated at 37°C for 24 h under microaerophilic conditions. After incubation, 10^6 CFU *Candida* in 10 mL of melted SD agar were added over the MRS containing the grown lactobacilli. The plates were again incubated aerobically at 37°C for 24 h to allow *Candida* growth. Inhibition zones over or around *Lactobacillus* colonies indicated antimicrobial activity. The diameters of the inhibition halo were calculated by subtracting the colony diameter from the total diameter. Three independent experiments were performed.

Antimicrobial activity of cell free supernatant from Lactobacillus strains

Cell free supernatant (CFS) was obtained by centrifugation of the third *Lactobacillus* sub-culture (grown in MRS broth for 12 h at 37°C as described above in the microorganisms and culture conditions section) at 6000 g for 10 min and sterilized through a 0.22 μm filter (Millipore, USA). CFS pH was determined by digital pH meter (Sartorius AG, Germany). The effect of CFS on *Candida* growth was evaluated in both solid and liquid medium assays.

For the solid medium assay, CFS (25 μl) was added to 4 mm holes performed in the SD agar plates (1% agar) containing the *Candida* strains (~10^6 CFU). The plates were incubated for 4 h at room temperature and then for 24 h at 37°C. The inhibition of *Candida* growth by CFS was evidenced by an inhibitory area around the well. Two independent experiments were performed.

The CFS effect in liquid medium was performed in a 96-well microplate assay according to Wang *et al.* (2017) with modifications. 100 μl containing approximately 2×10^5 CFU *Candida* in SD medium were combined with 100 μl CFS in each well of sterile microplates. As a control, MRS broth was used instead of CFS. Moreover, MRS broth adjusted to pH 4.0 with HCl was included to evaluate the effect of low pH on *Candida* growth. After aerobic incubation at 37°C for 24 h, fungus growth was determined by optical density (OD) at 630 nm using a Microplate Spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, CA, USA). Later, the growth inhibition rate was calculated as:

$$\text{Growth inhibition rate (\%)} = [(OD_{control} - OD_{CFS}) / OD_{control}] \times 100$$

Each CFS was assayed in three independent experiments, each with three replicates.

On the other hand, the inhibition assay in liquid medium against *C. albicans* C2 was carried out again employing CFS neutralized with 2 N NaOH, and neutralized-treated with 1000 U mL^{-1} catalase (Sigma, USA) CFS in order to indicate the chemical nature of inhibitory substances (organic acid and/or H_2O_2).

In vivo anti-*Candida* activity of lactobacilli

An experimental murine model was used to evaluate the preventive, therapeutic and preventive-therapeutic effect of the intravaginal (i.va.) inoculation of *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332 on the i.va. challenge with *C. albicans* C2. These strains were selected considering several characteristics explained below.

Animals

Two-month-old female BALB/c mice from the inbred colony of CERELA (Centro de Referencia para Lactobacilos) were used. Animals were housed and fed as previously described (De Gregorio *et al.* 2015). In order to induce a pseudo-estrous condition and promote microbial colonization, the mice received a weekly subcutaneous injection of 0.02 mg b-Estradiol 17-valerate (Sigma-Life Sciences, Switzerland) dissolved in 100 μl of sesame oil (Sigma-Life Sciences, Mexico) throughout the experiment. The experiments were independently repeated three times (with at least three animals) for each experimental group and sampling time. The Institutional Laboratory Animal Care and Use Committee of CERELA approved the experimental CRL-BIOT-LMP-2011/1A protocol applied in this work.

Mice were randomly assigned to seven experimental groups:

(1) one **Control** group: *C. albicans* C2 (C.a.)-challenged mice [i.va. inoculated with saline twice a day for 4 days (a total of 7 times), later i.va. challenged with 20 μL containing 1×10^6 CFU C.a., and again i.va. inoculated seven times with saline (one per day for 7 days)];
(2) two **Preventive** groups: *Lactobacillus* (Lb) (7 doses) + C.a. treated-mice (i.va. inoculated seven times with 10^8 CFU *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332, later i.va. challenged with 1×10^6 CFU C.a., and i.va. inoculated seven times with saline);
(3) two **Therapeutic** groups**:** C.a. + Lb (7 doses)-treated mice (i.va. inoculated seven times with saline, later i.va. challenged with 1×10^6 CFU C.a., and i.va. inoculated seven times with 10^8 CFU *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332) and
(4) two **Preventive-Therapeutic** groups: Lb (7 doses) + C.a. + Lb (7 doses)-treated mice (i.va. inoculated seven times with 10^8 CFU *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332, later i.va. challenged with 1×10^6 CFU C.a., and i.va. inoculated seven more times with 10^8 CFU *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332).

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 (% (w/v): 1 meat peptone, 1.5 agar; Britania Laboratories, Argentina), as previously published (De Gregorio *et al.* 2015). *C. albicans* C2 inoculum were suspensions of bacterial pellets from cultures (in SD broth for 16 h at 37°C, agitated at 70 rpm) washed and resuspended in saline.

The experimental groups, the i.va. inoculation sequence and the sampling days of the experiments are schematized in Fig. 1.

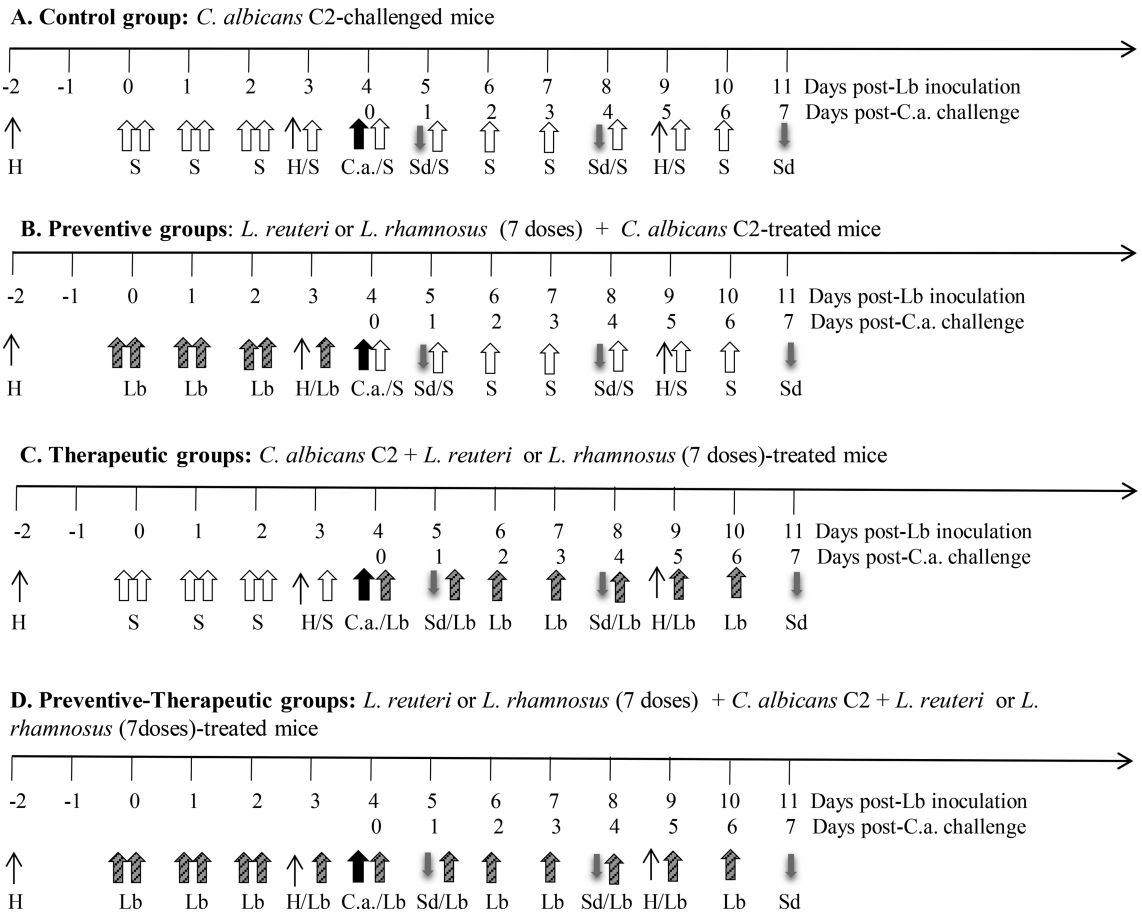

Figure 1. Experimental design. H: Administration of 0.02 mg β-Estradiol 17-valerate (). The arrow shows one intravaginal (i.va.) inoculation of: saline (S,), lactobacilli [50 μL containing 10^8 CFU of *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332 (Lb,)], or *C. albicans* C2 [20 μL containing around 10^6 CFU (C.a.,)] into BALB/c mice in the following experimental groups: control, preventive, therapeutic and preventive-therapeutic. Sd (): Sampling day.

Sampling and analytical procedures

Every sampling day, vaginal washings (v.w.) and vaginal tissues were obtained as previously described (De Gregorio *et al.* 2015).

Vaginal washing cytology and vaginal tissue histology were carried out with May–Grünwald–Giemsa and Hematoxylin–Eosin stains, respectively, according to De Gregorio *et al.* (2015).

Microbiological studies were performed from v.w. in which *Lactobacillus* and *Candida* viable cell counts were determined by serial dilution method in agar plates. MRS (pH 5.5) with 0.1 mg mL^{-1} of cycloheximide (Sigma, China) was employed to selectively quantify *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332 and Sabouraud glucose agar containing 0.05 mg L^{-1} chloramphenicol (Britania, Argentina) to quantify *C. albicans* C2. MRS plates were incubated under microaerophilic conditions at 37°C for 48 h, while Sabouraud plates were aerobically incubated at 37°C for 24 h. The culture-based technique applied to evaluate *L. reuteri* CRL1324, *L. rhamnosus* CRL 1332 and *C. albicans* C2 colonization is supported by a previous study of our groups in which no lactobacilli and yeast were isolated from vaginal autochthonous microbiota of BALB/c mice in the culture media used (De Gregorio *et al.* 2018).

Statistical analysis

For the *in vitro* anti-*Candida* activity assays, an analysis of variance (ANOVA) using a general linear model was applied to determine the main and interaction effects of factors (*Lactobacillus* CFS and *Candida* strains) on *Candida* growth. For *in vivo* studies, ANOVA using a general linear model was also applied to define the main and interaction effects of factors (experimental group and sampling day) on the number of viable *C. albicans* C2 and lactobacilli. Significant differences (*P*-value < 0.05) between mean values were determined by Tukey's test, using MINITAB statistical software (version 16 for Windows).

RESULTS

Identification of *Candida* sp. vaginal clinical isolates

The results showed that MALDI-TOF MS method allowed the identification of 11 isolates with a score > 2.0, indicating genus and species identification with certainty. They were identified in three different species: *C. albicans* (C1, C2, HE3, HE4, HE5, HE6 and HE7 isolates), *C. glabrata* (F1, F2 and HE10 isolates) and *C. tropicalis* (HE2 isolate) (Table 1). MALDI-TOF MS identification of all the *C. albicans* and *C. tropicalis* isolates agrees with the results obtained with CHROMagar *Candida*® medium.

In vitro anti-*Candida* activity of lactobacilli

Antimicrobial activity of Lactobacillus strains

Twelve *Lactobacillus* strains (40%) were able to inhibit the growth of at least one *Candida* spp. strain (Table 1). The strains identified as *C. albicans* were inhibited by a higher number of lactobacilli compared to *C. glabrata* and *C. tropicalis* strains. Growth inhibition zones (ranging from 1.33 ± 0.33 to 13.66 ± 3.29 mm diameter) were evidenced in most of *C. albicans* strains, while *C. glabrata* and *C. tropicalis* strains were inhibited only in the area above the lactobacilli colony (Table 1). 91.66% of lactobacilli showing anti-*Candida* activity were isolated from healthy vagina, out of which 5, 3 and 3 were obligate homofermentative, facultative and obligate heterofermentative strains, respectively. All these strains proved to be moderate or strong hydrogen peroxide producers. Moreover, 63.63% (*L. gasseri* CRL1320 and CRL1322, *L. salivarius* CRL1296, *L. paracasei* CRL1512, *L. rhamnosus* CRL1332 and CRL1511, and *L. mucosae* CRL1508) reached final pH values between 4.00 to 4.35 after growth in MRS broth, each of them being able to inhibit seven to eleven *Candida* spp. strains. The remaining 36.37% lactobacilli (*L. gasseri* CRL1261 and CRL1263, and *L. reuteri* CRL1324 and 1327) reached higher pH values (between 4.36 and 4.71) and inhibited a lower number of *Candida* strains (2 to 6 isolates). *L. salivarius* CRL1328, an obligate homofermentative bacterium and non-hydrogen peroxide-producing *Lactobacillus*, was the only strain with anti-*Candida* activity originally isolated from an unhealthy vagina (with sign of mycoses and vaginosis). This strain reached pH 4.04 after growth in MRS broth and was able to inhibit eight *Candida* strains (Table 1).

Although low pH values in *Lactobacillus* cultures were associated with a higher number of inhibited *Candida* strains, seventeen homofermentative *Lactobacillus* strains (56.66%) produced low pH values with no inhibitory effect on the fungi. This result suggests that organic acid is not the only factor responsible for the inhibitory effect.

On the other hand, a strain-dependent inhibitory effect was evidenced in *L. gasseri* and *L. rhamnosus* species. Only four out of the sixteen *L. gasseri* strains showed anti-*Candida* activity, *L. gasseri* CRL1320 being the only strain able to inhibit 100% of fungus strains, while *L. gasseri* CRL1261, CRL1263 and CRL1322 showed inhibition on 5 (45.45%), 2 (18.18%) and 8 (72.72%) *Candida*, respectively. In addition, the behavior in the *L. rhamnosus* strains was different with respect to anti-*Candida* activity. *L. rhamnosus* CRL1332 inhibited all *Candida* strains with wider inhibition zones (from 9.00 ± 1.63 to 13.66 ± 3.29 mm diameter) compared to *L. rhamnosus* CRL1511 (72.72% inhibited *Candida* and 3.00 ± 1.53 to 6.33 ± 0.88 mm halos). In contrast, a similar anti-*Candida* activity was evidenced in two *L. salivarius* strains and in two *L. reuteri* strains evaluated, which inhibited 8 (72.72%) and 6 (54.54%) *Candida* strains, respectively (Table 1).

Antimicrobial activity of Lactobacillus CFS

When evaluating the effect of *Lactobacillus* CFS on *Candida* spp. growth using the solid medium assay, anti-*Candida* activity was not evidenced. However, when the liquid medium assay was used, thirty CFS (100%) were able to significantly inhibit ($P < 0.01$) the growth of at least one of the *Candida* strains assayed (Fig. 2A). In agreement with the previously described antimicrobial activity of lactobacilli, *C. albicans* strains were more sensitive to *Lactobacillus* CFS compared to *C. glabrata* and *C. tropicalis* strains (Fig. 2A and B). Twenty-one (70%) and nine (30%) CSF were obtained from lactobacilli isolated from healthy and unhealthy vaginas, respectively (Fig. S1, Supporting Information). Most of them (22 CFS) were from homofermentative lactobacilli, either moderate or strong hydrogen peroxide-producing strains. Also, 18 out of the 22 CFS and *L. salivarius* CRL1328 CFS (non H_2O_2 -producer) showed low pH (between 4.00 and 4.35), being able to inhibit between 7 and 11 *Candida* strains (Fig. 2A; Fig. S1, Supporting Information). Similar results were observed with 3 CSF assayed from facultative heterofermentative lactobacilli and with 1 of 4 CFS from obligate heterofermentative lactobacilli (Fig. 2A; Fig. S1, Supporting Information).

Taking into account the differences in *Candida* growth (OD_{630nm} values between 1.67 to 2.14, Fig. 2A), the inhibition rates induced by *Lactobacillus* CFS were compared to determine the degree of inhibitory activity and *Candida* sensitivity (Fig. 2B). In the same way as in the agar overlay technique, a strain-dependent inhibitory effect was evidenced with CFS from *L. gasseri*, *L. rhamnosus* and *L. jensenni* species, while in the case of CFS from *L. reuteri* and *L. salivarius* a similar activity was observed in CFS from the same species (Fig. 2B).

The CSF showing higher and similar anti-*Candida* activity (growth inhibition between 35.70% and 41.32%) were from 8 homofermentative lactobacilli (*L. gasseri* CRL1252, CRL1255, CRL1270 and CRL1322, *L. jensenii* CRL1317, *L. johnsonii* CRL1292 and *L. salivarius* CRL1296 and CRL1328) and from two facultative heterofermentative (*L. paracasei* CRL1512 and *L. rhamnosus* CRL 1332) (Fig. 2B). In contrast, the CFS with the lowest activity (inhibition lower than 20%) were from three homofermentative (*L. gasseri* CRL1263, CRL1290 and CRL1311) and from four obligate heterofermentative lactobacilli (*L. fermentum* CRL1287, *L. reuteri* CRL1324 and CRL1327 and *L. mucosae* CRL1508) (Fig. 2B).

On the other hand, considering that the highest inhibitory effect was observed in CFS with lower pH (between 4.00 and 4.35), MRS broth adjusted to pH 4.0 with HCl (MRS-HCl) was assayed in order to determine if low pH contributed to fungus inhibition. MRS-HCl significantly ($P < 0.01$) decreased the growth of all *Candida* strains, with the exception of *C. albicans* HE5 and *C. tropicalis* HE2, compared with control MRS (Fig. 2A). Additionally, while MRS-HCl inhibited approximately 21.35% *Candida* growth, 19 out of 30 CFS induced a higher and significant ($P < 0.01$) inhibition (between 28.68 and 41.32%) compared with MRS-HCl (Fig. 2B). On the other hand, the results of NaOH-neutralized CFS treated with catalase against *C. albicans* C2 showed that the inhibitory effect of 29 *Lactobacillus* CFS (96.66%) was significantly reduced ($P < 0.01$) with NaOH treatment, suggesting that the antagonistic effect was produced by organic acids released in CSF. Also, the CFS anti-*Candida* activity from *L. gasseri* CRL1320, *L. jensenii* CRL1333 and *L. reuteri* CRL1324 and CRL1327 showed a significantly higher reduction ($P < 0.05$) after NaOH-catalase treatment, indicating that H_2O_2 can cooperates in the inhibitory effect against *C. albicans* C2 (Fig. 3).

When comparing the sensitivity of *Candida* spp. to *Lactobacillus* CFS, significant differences ($P < 0.05$) between the *C. albicans* strains were obtained. C2 and HE6 strains showed a similar pattern and were the most strongly inhibited strains (approximately with 40% growth inhibition). The average growth inhibition of *C. albicans* HE3, HE5 and HE7 strains was 36.13% ± 0.64% while against C1 and HE4 strains it was 30.64% ± 1.32% The three *C. glabrata* strains showed a similar behavior in the presence of *Lactobacillus* CFS, with an average growth inhibition of 13.49% ± 0.31% In a similar way, *C. tropicalis* HE2 growth was inhibited in the presence of CFS by 16.78% ± 0.45% Fig. 2B).

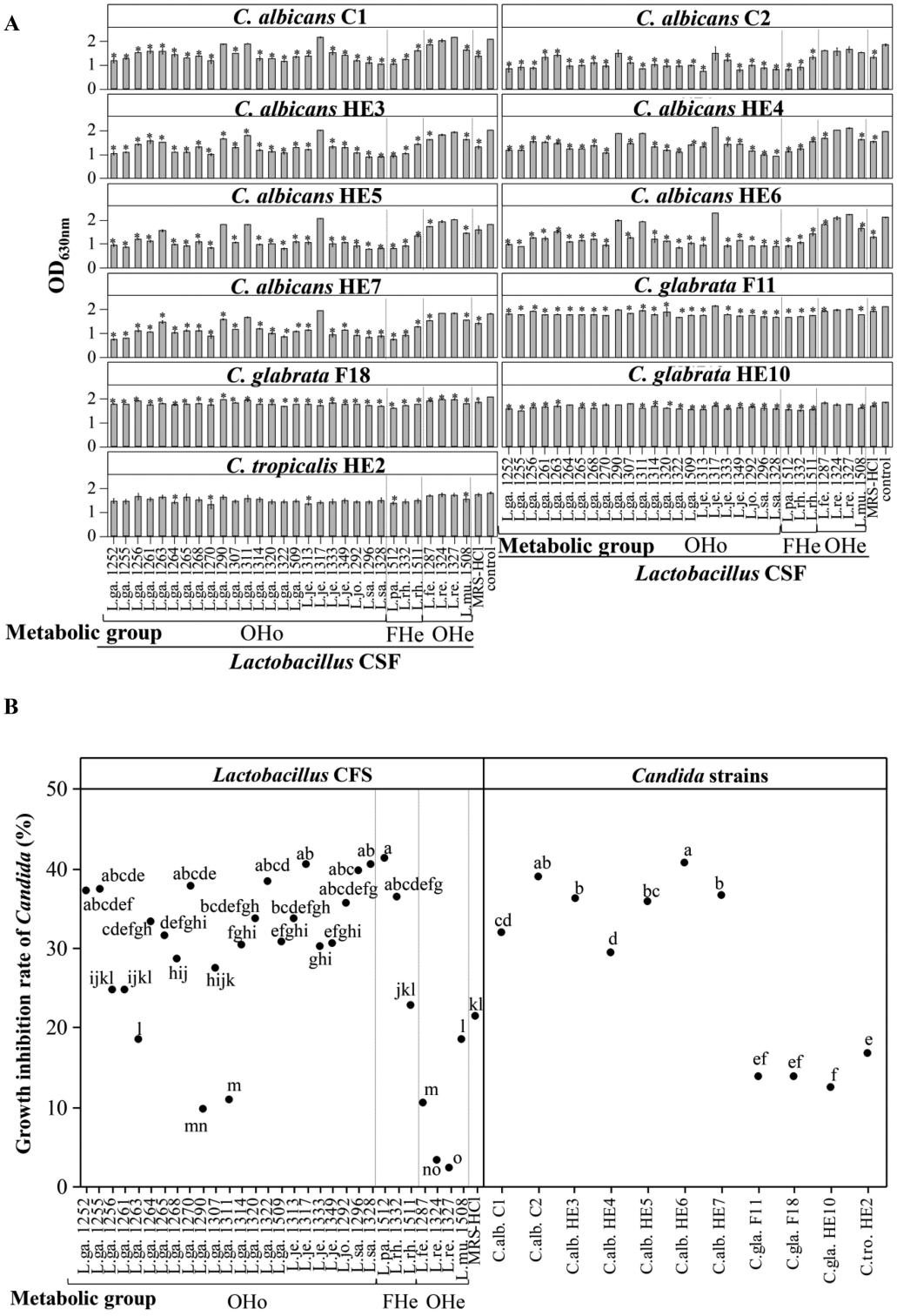

Figure 2. Growth inhibition of vaginal clinical isolates of *Candida* spp. by *Lactobacillus* CFS. (A) Inhibitory effects of CFS from vaginal lactobacilli on *Candida* spp. growth in SD broth after 24 h of incubation. Data are plotted as mean values of OD at 630 nm ± standard error from three independent experiments. ∗indicates statistically significant differences ($P < 0.01$) compared to the control using Tukey's test. (B) Main effect of *Lactobacillus* CFS and *Candida* strains on growth inhibition rate (%) [$(OD_{control} - OD_{CFS})/OD_{control}$ x 100)]. In the '*Lactobacillus* CFS' panel, each point indicates the mean value of the growth inhibition rate induced by each *Lactobacillus* CFS in all *Candida* strains evaluated. In the '*Candida* strains' panel, each point indicates the mean value of the growth inhibition rate for each *Candida* strain induced by all *Lactobacillus* strains tested. In each panel, different letters indicate statistically significant differences ($P < 0.05$) between the mean values of the growth inhibition rate (%) of levels of the factor assayed (*Lactobacillus* CFS and *Candida* strains) according to Tukey's test. L.ga.: *L. gasseri*; L.je.: *L. jensenii*; L.jo.: *L. johnsonii*; L.sa.: *L. salivarius*; L.fe.: *L. fermentum*; L.re.: *L. reuteri*; L.mu.: *L. mucosae*; L.pa.: *L. paracasei*; L.rh.: *L. rhamnosus*. The numbers after the *Lactobacillus* strains abbreviation correspond to CRL number. C.alb.: *C. albicans*; C.gla.: *C. glabatra*; C.tro.: *C. tropicalis*. OHo: obligate homofermentative group; FHe: facultative heterofermentative group; OHe: obligate heterofermentative group.

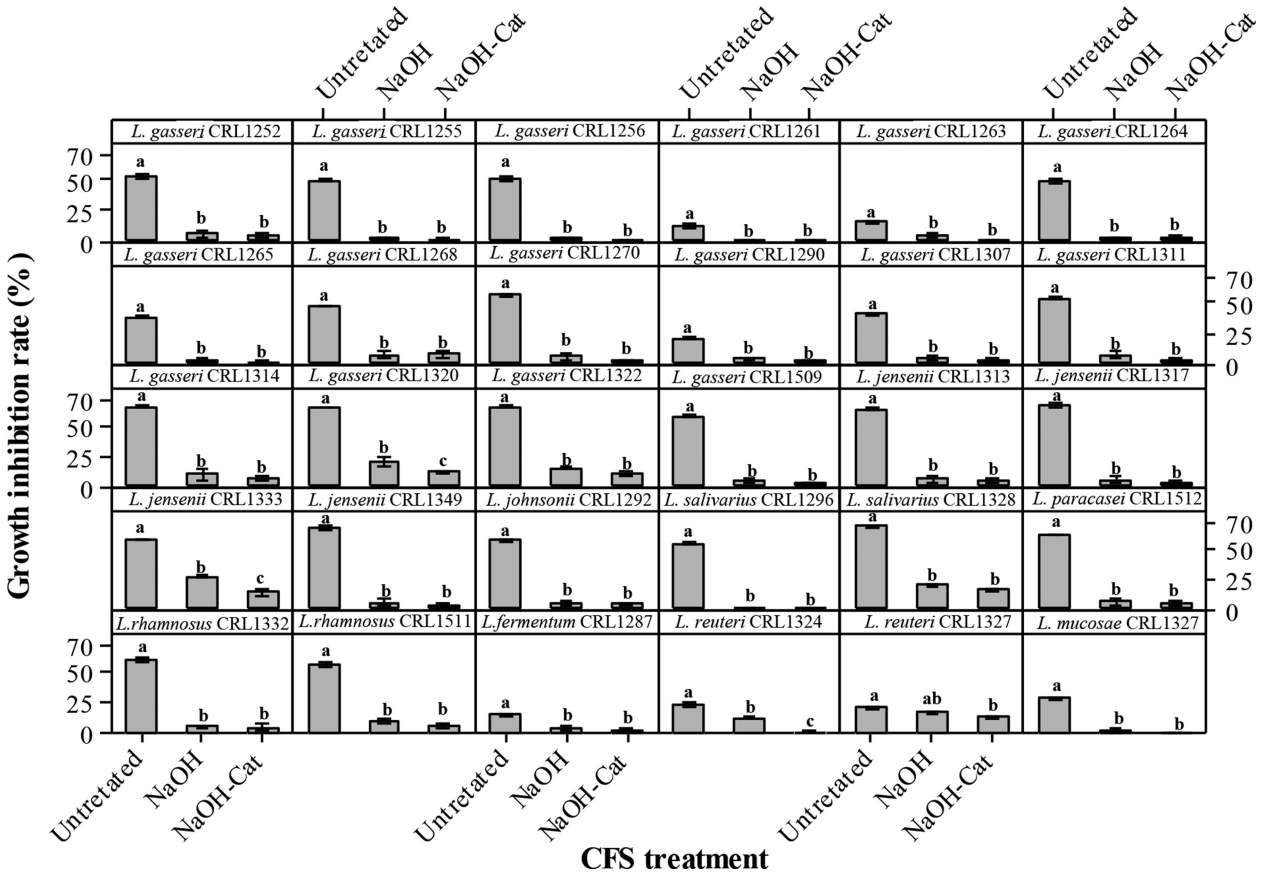

Figure 3. Inhibition of *C. albicans* C2 growth by different *Lactobacillus* CFS either untreated, treated with NaOH or NaOH plus catalase. Data are plotted as average values of growth inhibition rate (%) [$(OD_{control}—OD_{CFS})/OD_{control}$ x 100)] ± standard error. Statistically significant differences between mean values of growth inhibition rate (%) obtained with the different CFS treatment are indicated by different letters ($P < 0.05$).

Anti-*Candida* activity of lactobacilli in a mice experimental model

L. reuteri CRL1324 and *L. rhamnosus* CRL1332 strains were selected to evaluate the effect of their i.va. administration (in preventive, therapeutic and preventive-therapeutic schemes or protocols) against an i.va. challenge with *C. albicans* C2 in a murine experimental model. Even though *L. reuteri* CRL1324 did not show high *in vitro* anti-*Candida* activity, this strain was selected supported by previous studies indicating its ability to stimulate the immune system in the murine vaginal tract (De Gregorio, Juárez Tomás and Nader-Macías 2016). In contrast, *L. rhamnosus* CRL1332 was chosen because of the high *in vitro* anti-*Candida* activity evidenced in this work. On the other hand, the *C. albicans* C2 strain was used due to its highest sensitivity to vaginal lactobacilli.

When evaluating the different *Lactobacillus* protocols against i.va. *C. albicans* C2 challenge, the preventive or therapeutic schemes of *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332 did not show a protective effect (Fig. 4A). However, the combined preventive-therapeutic treatments of the two *Lactobacillus* strains evidenced a significant inhibitory effect ($P < 0.05$) against *C. albicans* C2. *L. reuteri* CRL1324 induced a significant reduction in viable cell numbers of *C. albicans* C2 only at 7 days post-C.a. challenge when compared to control mice. However, *L. rhamnosus* CRL1332 was able to generate the same type of effect since day 4 post-C.a. challenge. Furthermore, continuous treatment with *L. rhamnosus* CRL1332 showed a complete inhibition of *C. albicans* C2 at 7 days post-C.a. challenge (Fig. 4A).

With respect to the number of viable *Lactobacillus* cells recovered in v.w. of mice under the different protocols, a significantly higher number (around 1–1.5 log units, $p < 0.05$) of *L. rhamnosus* CRL1332 CFU was observed since day 4 post-C.a. challenge in the therapeutic and preventive-therapeutic scheme compared with the preventive scheme. The same results were obtained for *L. reuteri* CRL1324, but since 7 days post-C.a. challenge (Fig. 4B). The viable cell number of *L. reuteri* CRL1324 and *L. rhamnosus* CRL1332 recovered in murine v.w. was similar (without significant differences) between the therapeutic and preventive-therapeutic schemes and in the different sampling days (Fig. 4B).

The cytological and histological evaluation of the murine vaginal tract evidenced that the challenge with *C. albicans* C2 induced a leukocyte influx in all sampling days (Fig. 5). However, this influx was reduced with the preventive-therapeutic treatment of *L. reuteri* CRL1324 and *L. rhamnosus* CRL1332 since days 4 and 7 post-C.a. challenge respectively (Fig. 5A and B). On the other hand, the preventive and therapeutic treatments of the two lactobacilli strains did not reduce the leukocyte influx induced by *C. albicans* C2 challenge (data not shown).

DISCUSSION

Several studies have suggested that the administration of *Lactobacillus* strains with beneficial properties can be an effective strategy for VVC prevention or a supplementary therapy to the

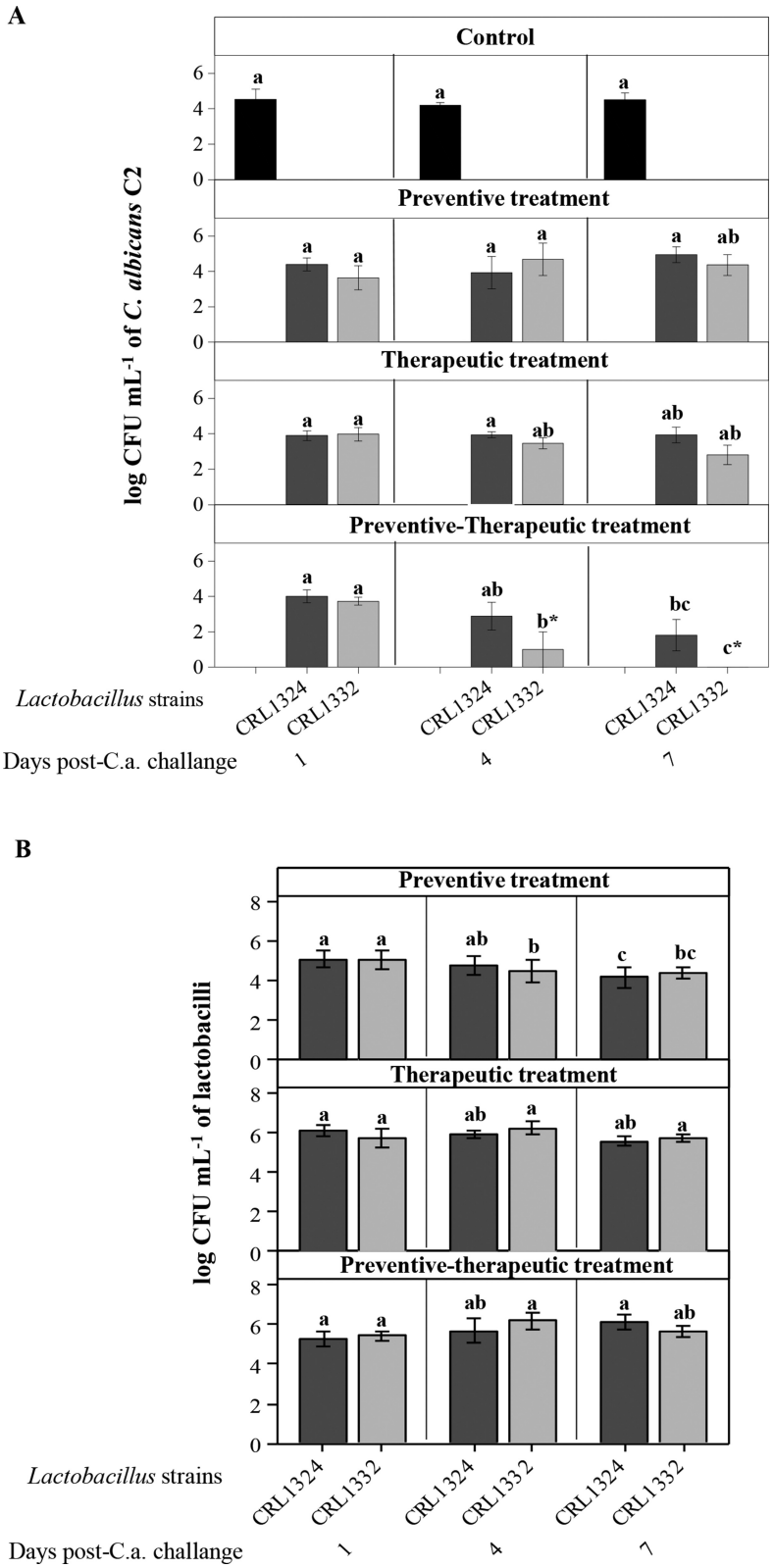

Figure 4. Effects of preventive, therapeutic or preventive-therapeutic treatments with *L. reuteri* CRL1324 (CRL1324) or *L. rhamnosus* CRL1332 (CRL1332) intravaginally (i.va.) administered against i.va. challenge with *C. albicans* C2 (C.a.) in a murine experimental model. (A) C.a. viable cells in vaginal washings (v.w.) of mice in the different experimental groups (control and treatments with *Lactobacillus* strains) (B) CRL1324 or CRL1332 viable cells in v.w. of mice in the different experimental groups. Data are plotted as average values of C.a., CRL1324 or CRL1332 viable cell numbers ± standard error. Statistically significant differences between the log CFU mL^{-1} mean values of experimental groups on the same day post-C.a. challenge are indicated by different letters: a, b and c ($P < 0.05$). It means that there are statistically significant differences when the mean values didn´t share one common letter. Statistically significant differences between the log CFU mL^{-1} mean values of the same experimental group on the different days post-C.a. challenge are indicated by the symbol * ($P < 0.05$).

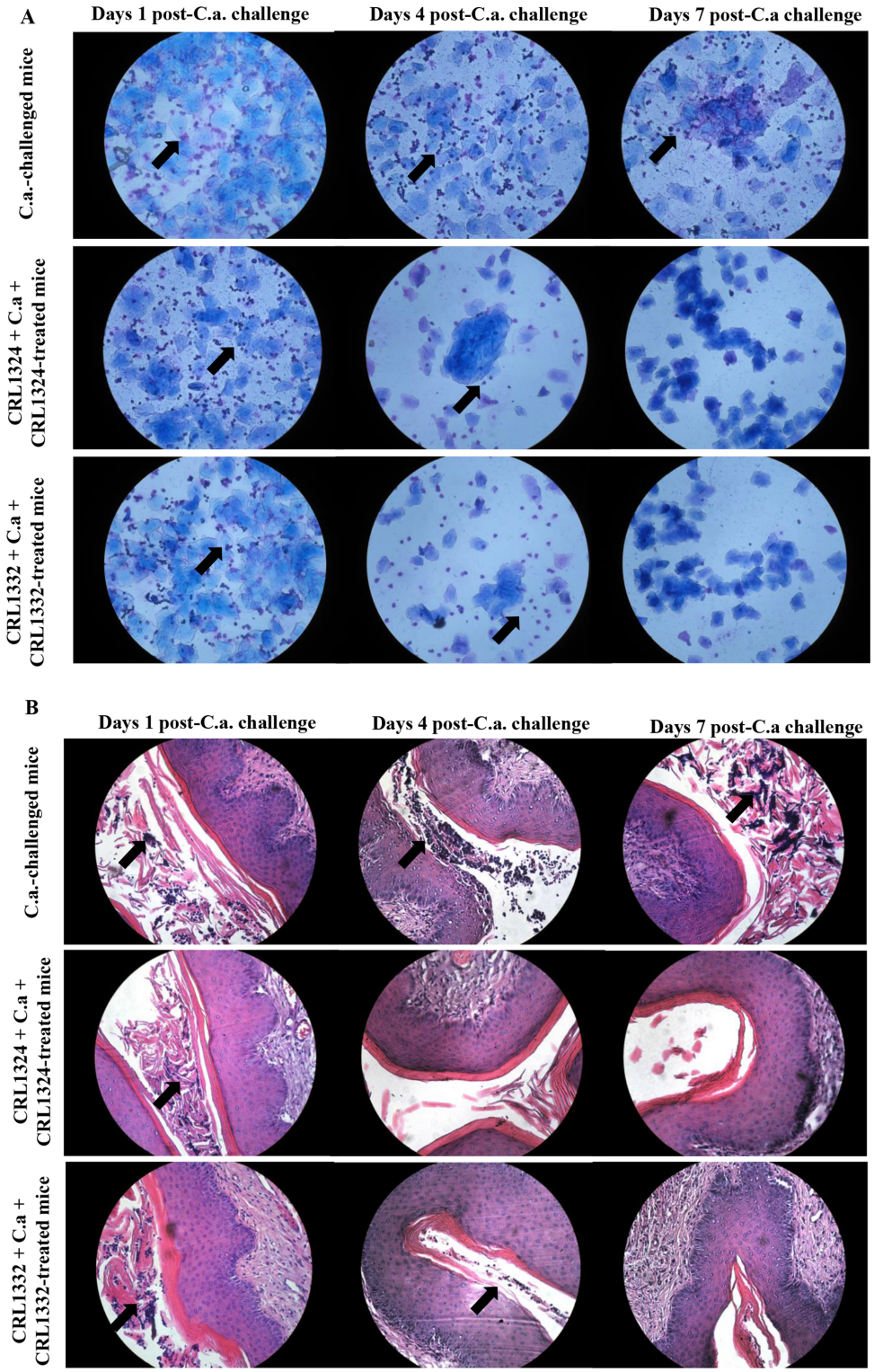

Figure 5. Photographs of (A) May Grunwald-Giemsa-stained vaginal smears and (B) Hematoxylin–Eosin-stained vaginal slides from BALB/c mice intravaginally (i.va.) challenged with *C. albicans* C2 (C.a.) (control) and mice i.va. inoculated seven times with 10^8 CFU of *L. reuteri* CRL1324 (CRL1324) or *L. rhamnosus* CRL1332 (CRL1332), later i.va. challenged with 1×10^6 CFU of C.a., and i.va. inoculated seven more times with CRL1324 or CRL1332 (preventive-therapeutic treatment) on different days post-C.a. challenge. Leucocyte influx in the vaginal wash and lumen of C.a.-challenged mice is indicated with black arrows. Results are representative of three independent experiments.

conventional antifungal treatments of this illness (Osset *et al.* 2001; Reid, Kim and Kohler 2006; Ehrstro *et al.* 2010; De Seta *et al.* 2014; Vladareanu *et al.* 2018). In this way, it should be emphasized that different beneficial *Lactobacillus* strains are able to express a variety of properties and effects on *Candida* spp.; thus, *in vitro* and experimental animal assays are useful and required for the selection of the best candidate *Lactobacillus* strains with anti-*Candida* activity (Strus *et al.* 2005; Ronnqvist *et al.* 2007; Joo *et al.* 2012; Parolin *et al.* 2015; Wang *et al.* 2017).

In this work, eleven vaginal clinical isolates of *Candida* sp. were identified down to the species level in order to further evaluate the antagonistic effect of *Lactobacillus* strains on the fungi by *in vitro* and *in vivo* studies. The results showed that most of the strains were identified as *C. albicans,* and in a lesser number as *C. glabrata* and *C. tropicalis*. Similar results were published by Vicariotto *et al.* (2012), who identified twenty-four strains as *C. albicans* (80.0%), two as *C. glabrata* (6.7%), one as *C. papapsilosis* (3.3%), one as *C. tropicalis* (3.3%) and two as other *Candida* species (6.7%) out of thirty vaginal clinical isolates. These results are consistent with those reported in the literature where the etiological agent most frequently responsible for VVC is *C. albicans* (Cassone 2015; da Silva Dantas *et al.* 2016), eventhough this work was not conceived as an epidemiological study.

When evaluating the *in vitro* inhibitory effect of thirty *Lactobacillus* strains against *Candida* spp., different results were obtained according to the three methods applied (Table 1 and Fig. 2). The liquid medium method indicated a higher number of lactobacilli with anti-*Candida* activity compared with the solid overlay method, while the agar plate diffusion method showed no inhibition of the fungi. In a similar way, Osset *et al.* (2001) reported the capability of lactobacilli to inhibit *C. albicans* strains growth in liquid, but not in solid culture media. Moreover, Mastromarino *et al.* (2002) and Juárez Tomás *et al.* (2011) showed the inhibition of various urogenital pathogens, but not of *C. albicans* strains by the agar plate diffusion method. Other investigators reported the anti-*Candida* activity of different vaginal *Lactobacillus* strains, applying the agar overlay and/or liquid methods (Coman *et al.* 2015; Parolin *et al.* 2015; Wang *et al.* 2017). In the present work, the different results obtained with the three methods applied could be explained by the physical state of the media, and the environment where the inhibitory substances exert their effect, as well as by the concentration of antimicrobial substances that lactobacilli can produce or secrete into solid and/or liquid media. In a similar way, Pauli (2006) has described that the volatilization and amount of substance/s to evaluate, the agar media type, pH, and the agar volume can all strongly affect the inhibition zone giving false-positive or negative results in the agar diffusion method. Also, Scorzoni *et al.* (2007) have reported that the microdilution method is more sensitive than the agar diffusion when evaluating the antifungal activity against *Candida* spp. of crude extracts, fractions, and pure substances from different species of the plant families. Thus, the different inhibitory patterns obtained in this work highlight the importance of applying more than one method to evaluate and define the *in vitro* antimicrobial effect of lactobacilli when trying to select the most adequate candidate *Lactobacillus* strains with anti-*Candida* activity. This recommendation is also indicated in the review published by Scorzoni *et al.* (2016) where the authors strongly suggest that different *in vitro* methodology must be applied to perform an adequate screening of newly described antifungals.

The lack of H_2O_2-producing *Lactobacillus* species in vagina has been related to the development of VVC (Vitali *et al.* 2007). In this work, out of thirty *Lactobacillus* strains assayed, twenty-nine showed to be hydrogen peroxide producers, and inhibit at least one *Candida* strain. However, some highly H_2O_2-producing lactobacilli evidenced low anti-*Candida* activity compared to moderate producers, and to *L. salivarius* CRL1328 (non-H_2O_2-producing *Lactobacillus*), suggesting that other antimicrobial substances can also contribute to the inhibitory effect against the yeast, or either a synergistic effect of them. Similarly, other authors have reported that the inhibitory effects of lactobacilli on *Candida* spp. is not associated with H_2O_2 production, and can be attributed to organic acid production (Strus *et al.* 2005; Chew *et al.* 2015). In this way, lactobacilli reaching a lower pH after growth in MRS broth were able to inhibit a higher number of *Candida* strains. Most of them were homofermentative and facultative heterofermentative lactobacilli, which produce higher lactic acid levels than the obligate heterofermentative. Thus, by using MRS adjusted to pH 4 with HCl, it was demonstrated that low pH affected *Candida* growth (Fig. 2), which was confirmed when most *Lactobacillus* CFS reduced their antagonistic effect on *C. albicans* C2 after the NaOH treatment (Fig. 3).

Previous studies demonstrated that each *Candida* strain expresses its own response to antifungals. In general, *C. albicans* and *C. tropicalis* species are susceptible to the drugs frequently used for mycosis treatments, while *C. glabrata* is less susceptible and *C. krusei* has intrinsic resistant to fluconazole (Arendrup 2013). Keeping these differences in mind, the susceptibility of three *Candida* species to the antimicrobial effect of different lactobacilli was evaluated. *C. albicans* strains were more sensitive than *C. glabrata* and *C. tropicalis* strains (Fig. 2). In a similar way, Parolin *et al.* (2015) and do Carmo *et al.* (2016) reported that *L. gasseri, L. vaginalis, L. crispatus* and *L. fermentum* strains showed a higher inhibitory effect on *C. albicans* clinical isolates compared with *C. non-albicans*. The present work reports the effect of a broad spectrum of vaginal *Lactobacillus* species, such as *L. jensenii*, *L. johnsonii*, *L. salivarius*, *L. rhamnosus* and *L. reuteri,* among others, against *Candida* spp. associated with vaginal infections. Thus, the results obtained provide significant information on the potential application of vaginal lactobacilli in VVC prevention and treatment, and the importance of the methodology applied for the selection of the most promising beneficial strains.

Eventhough *L. crispatus* species is described as one of the most abundant species in vagina and crucial for the maintenance of vaginal health (Petrova *et al.* 2015); this species was not frequently isolated in our previous studies (Ocaña *et al.* 1999; Juarez Tomás *et al.* 2011). Then, the results presented in this paper cannot be considered as an epidemiological survey, because was applied only to a low number of vaginal beneficial *Lactobacillus* strains to determine their anti-*Candida* activity. The final purpose was to select candidate strains to prevent or treat vaginal candidiasis, in a way to complement previous studies of our research group.

The use of animal experimental models of vaginal candidiasis has generated several reports on the fungal pathogenic determinants and on the importance of the inflammatory and immune responses required for the successful control of human infection (Cassone and Sobel 2016). Animal experimental models are also a critical element to determine the protective mechanisms of *Lactobacillus* strains, since they provide information that is not possible to obtain from *in vitro* studies. In the case of products containing lactobacilli for human application, protocols carried out in laboratory animals are essential prior to phase I, II and III clinical trials (De Gregorio *et al.* 2012; ISAPP 2013; De Gregorio et al., 2014, 2015; De Gregorio *et al.* 2016). However, to this day, the effect of vaginal lactobacilli on *C. albicans* in an

experimental murine model has been scarcely reported. Joo *et al.* (2012) demonstrated that a three-day application of *Lactobacillus helveticus* HY7801, a species not commonly reported in human vagina (Human Microbiome Project Consortium 2012; Integrative HMP (iHMP) Research Network Consortium 2014), ameliorated VVC in mice by inhibition of fungal growth, expression of pro-inflammatory cytokines and enzymes, and NF-κB activation. To the best of our knowledge, the present work is the first to evaluate and compare the preventive, therapeutic and preventive-therapeutic effect of vaginal lactobacilli strains (*L. reuteri* CRL1324 and *L. rhamnosus* CRL1332) against i.va *C. albicans* challenge in a murine experimental model.

L. reuteri CRL1324 and *L. rhamnosus* CRL1332 were selected to conduct *in vivo* assays with a murine model supported by previous studies of our research group evidencing that the two strains have the ability to produce biofilm, a property that could contribute to interfere with *Candida* vaginal colonization (Leccese Terraf *et al.* 2012). Moreover, *L. reuteri* CRL1324 produced an immunomodulatory effect on a murine model (De Gregorio, Juárez Tomás and Nader-Macías 2016), while in this work *L. rhamnosus* CRL1332 have demonstrated a strong *in vitro* anti-*Candida* activity. Different authors have shown that the anti-*Candida* mechanisms exerted by *Lactobacillus* species include restoration of normal vaginal microbiota, interference with *Candida* colonization, inhibition of growth and biofilm formation of *Candida* spp. and immunomodulation in the host (Reid, Kim and Kohler 2006; Wagner and Johnson 2012; Parolin *et al.* 2015; Santos *et al.* 2016; Niu *et al.* 2017). Some of these mechanisms are associated with the beneficial properties of the two lactic strains selected for *in vivo* assays.

C. albicans is an opportunistic pathogen with the ability to adhere to, invade, and induce cell damage in the vaginal mucosa. The stimulation of mucosal immunity can induce certain signs and symptoms of the disease or their absence (Sobel 2007; Rast *et al.* 2016). In this work, the vaginal tract of mice i.va. challenged with *C. albicans* C2 was successfully colonized, inducing a high leukocyte influx to the mucosa on all sampling days. However, the decrease in *Candida* colonization and leukocyte influx was only evidenced with the i.va. preventive-therapeutic administration of *L. reuteri* CRL1324 or *L. rhamonosus* CRL1332 strains (Fig. 4 and 5), while the preventive or therapeutic administrations alone showed no effect. In a similar way, some researchers have demonstrated that the prolonged repetitive administration of vaginal tablets containing *L. fermentum* LF10 and *L. acidophilus* LA02 was able to significantly solve *Candida* yeast symptoms and prevent VVC recurrence in women (Vicariotto *et al.* 2012; Murina *et al.* 2014).

When evaluating *Lactobacillus* colonization in murine v.w., a significantly higher number of *L. reuteri* CRL1324 and *L. rhamnosus* CRL1332 was observed at day 7 after *Candida* challenge in the preventive-therapeutic lactobacilli protocol, only comparable to the preventive one. These results suggest that the number of viable *Lactobacillus* cells was not directly related to the inhibitory effect. Our hypothesis is that the prolonged vaginal administration of lactobacilli could: a) generate a barrier or biofilm able to interfere with *Candida* colonization; b) induce a higher accumulation of antimicrobial substances such as lactic acid or c) modulate the local immune response.

When comparing the effect of the preventive-therapeutic treatment between *Lactobacillus* strains, *L. rhamnosus* CRL1332 showed a higher inhibition of *C. albicans* C2 vaginal colonization, while *L. reuteri* CRL1324 reduced more quickly the leukocyte influx induced by the yeast (Fig. 4A and Fig. 5A and B). Considering these results and the properties of the strains, one possibility is that the inhibitory effect of *L. rhamnosus* CRL1332 could be related to organic acids production and the consequent pH lowering, while the effect of *L. reuteri* CRL1324 could be more closely related to the modulation of the immune system. Further studies are required to demonstrate the mechanisms involved in the inhibition of *C. albicans* by these lactobacilli.

In conclusion, human vaginal lactobacilli exhibited *in vitro* antimicrobial effects on *Candida* spp. By employing a murine experimental model, the preventive-therapeutic administration of either *L. reuteri* CRL1324 or *L. rhamnosus* CRL1332 induced a decrease in *C. albicans* vaginal colonization as well as in the inflammatory response produced by the yeast. Therefore, *L. reuteri* CRL1324 and *L. rhamnosus* CRL1332 could be considered as new biological agents capable of reducing VVC.

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Conflicts of interest. None declared.

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