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RESEARCH ARTICLE

Lactobacilli–lactoferrin interplay in *Chlamydia trachomatis* infection

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One sentence summary: The interaction between *Lactobacillus brevis* and lactoferrin seems to play a role in the protection against *Chlamydia trachomatis* genital infection.

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ABSTRACT

In the cervicovaginal microenvironment, lactobacilli are known to protect against genital infections and, amongst the host defence compounds, lactoferrin has recently acquired importance for its anti-microbial and anti-inflammatory properties. An abnormal genital microenvironment facilitates the acquisition of pathogens like *Chlamydia trachomatis*, the leading cause of bacterial sexually transmitted infections worldwide. The aim of our study is to investigate the effects of *Lactobacillus crispatus*, *Lactobacillus brevis* and bovine lactoferrin on chlamydial infection, in order to shed light on the complex interplay between host defence mechanisms and *C. trachomatis*. We have also evaluated the effect of these defence factors to modulate the chlamydia-mediated inflammatory state. To this purpose, we have determined the infectivity and progeny production of *C. trachomatis* as well as interleukin-8 and interleukin-6 synthesis. The main result of our study is that the combination of *L. brevis* and bovine lactoferrin is the most effective in inhibiting the early phases (adhesion and invasion) of *C. trachomatis* infection of cervical epithelial cells and in decreasing the levels of both cytokines. In conclusion, the interaction between *L. brevis* and lactoferrin seems to play a role in the protection against *C. trachomatis*, reducing the infection and regulating the immunomodulatory activity, thus decreasing the risk of severe complications.

Keywords: *Chlamydia trachomatis*; genital infection; cervicovaginal microenvironment; *Lactobacillus* spp.; lactoferrin; inflammatory cytokines

INTRODUCTION

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted infections worldwide, with an incidence increased by 28% since 2008 (Newman *et al.* 2015). In women, *C. trachomatis* manifests as cervicitis, salpingitis and endometritis, and can be transmitted to infants during delivery resulting in neonatal conjunctivitis and pneumonia (Mylonas 2012).

A major concern with chlamydial genital infections is that ~80% of women are asymptomatic, thus resulting in a reservoir for onwards transmission in the population (Shaw *et al.* 2011). Furthermore, untreated *C. trachomatis* infections can progress leading to severe chronic sequelae, including pelvic inflammatory disease, ectopic pregnancy and obstructive infertility (Shaw *et al.* 2011; Mylonas 2012).

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In addition to the most common genital serovars, responsible for the chronic complications, *C. trachomatis* serovar LGV is becoming a public health concern, since several cases have been identified in Europe and, also, treatment failures have been observed (Peuchant *et al.* 2011; Verweij *et al.* 2012; de Vries *et al.* 2015; Kong and Hocking 2015; Foschi *et al.* 2016).

C. trachomatis, an obligate intracellular bacterium, is characterised by a peculiar developmental cycle, alternating between two morphologically and functionally distinct forms: the elementary body (EB), the extracellular infectious form, and the reticulate body (RB), the intracellular replicative form. The developmental cycle begins when EBs attach and enter into the host cell by endocytosis. It is thought that the interaction of EBs with host cells occurs in a two-step process involving a reversible interaction mediated by heparan sulphate proteoglycans followed by irreversible binding to host receptors by chlamydial adhesins. Soon after attachment to host cell, EBs are internalised and confined to a vacuole termed inclusion. EBs then differentiate to RBs, which replicate by binary fission within 24 h post-infection, and, as the inclusion expands, RBs begin to transition back to EBs in an asynchronous process. At the end of the developmental cycle (48 h post-infection), EBs are finally released from host cell by inclusion extrusion or cell lysis (Abdelrahman and Belland 2005; Bastidas *et al.* 2013; Zuck *et al.* 2016).

It is well known that *C. trachomatis* can generate a persistent form during its developmental cycle under stressful conditions, and persistent forms seem to possess the ability to evade the host immune response and to resist to antibiotics (Raulston 1997; Wyrick and Knight 2004; Wyrick 2010; Di Pietro *et al.* 2012). Evidence in the literature suggests that *C. trachomatis* persistent forms may be of particular clinical importance, since they seem to induce a chronic inflammatory state and the subsequent tissue damage, contributing to the development of chronic sequelae related to *C. trachomatis* genital infection (Wyrick 2010; Choroszy-Król *et al.* 2012; Di Pietro *et al.* 2013).

In the female genital tract, the innate defence system involves an intricate interaction between the normal vaginal flora, various proteins and the immune response, known to protect against pathogens (Valenti and Antonini 2005; Deruaz and Luster 2015; Petrova *et al.* 2015). The cervicovaginal microbiota may have a significant influence on individual susceptibility to genital infections. In fact, in healthy women the cervicovaginal mucosa is populated with a microflora typically dominated by lactobacilli, known to be positively associated with genital health. Lactobacilli protect towards genitourinary pathogens through different mechanisms, such as competitive exclusion, anti-microbial compound production and host immune system modulation (Petrova *et al.* 2015). On the contrary, an abnormal cervicovaginal flora, lacking lactobacilli, facilitates the acquisition of sexually transmitted diseases, including *C. trachomatis* infection (Nardis, Mosca and Mastromarino 2013; Petrova *et al.* 2015).

Amongst the host defence compounds, lactoferrin, an 80-kDa iron-binding protein present in the cervicovaginal fluid, has recently acquired importance for its extensive biological effects, including anti-microbial and anti-inflammatory properties (Valenti and Antonini 2005; Frioni *et al.* 2014). As a matter of fact, lactoferrin is an important constituent of the innate immune system on cervical mucosa, where it exerts anti-microbial activity against many different pathogens, such as *C. trachomatis*, *Candida albicans*, herpes simplex type-2 and human immunodeficiency virus (Valenti *et al.* 1986; Berlutti *et al.* 2011; Sessa *et al.* 2016). Moreover, it is well known that in amniotic and cervical fluid, lactoferrin as well as interleukin 8 and 6 concentrations are higher in women affected from bacterial genital infections than in uninfected women (Otsuki *et al.* 1998; Goldenberg *et al.* 2000; Sawada *et al.* 2006; Spear *et al.* 2011).

Previously, we have demonstrated a significant inhibition of *C. trachomatis* serovar LGV infection by vaginal lactobacilli (Mastromarino *et al.* 2014) or bovine lactoferrin (bLf; Sessa *et al.* 2016), whereas their combined interaction still needs to be investigated. Therefore, in this study we have evaluated the anti-chlamydial activity of the combination of lactobacilli and lactoferrin, in order to shed light on the complex interplay between the host defence mechanisms and *C. trachomatis* infection. In particular, we have analysed the effects of bLf combined with *Lactobacillus crispatus*, one of the prevalent *Lactobacillus* species of the healthy vaginal environment, or with a strain of *Lactobacillus brevis*, already observed to be active against *C. trachomatis* infection and to be able to restore the healthy cervicovaginal microbiota. Lastly, we have evaluated the ability of these defence factors to modulate the chlamydia-mediated inflammatory state.

MATERIALS AND METHODS

Bacterial strains and growth conditions

L. crispatus strain FV4 and *L. brevis* DSM 11988 (strain CD2) of vaginal origin (Mastromarino *et al.* 2002) were stored as a stock culture at –70°C in 90% de Man-Rogosa-Sharpe (MRS) broth (Oxoid) and 10% glycerol. Lactobacilli were inoculated from frozen vials onto MRS broth and cultured overnight at 37°C under anaerobic conditions in Anaerogen system (Oxoid).

Cell culture and culture conditions

The human epithelial HeLa-229 cell line (ATCC ® CCL-2.1) from cervix adenocarcinoma was cultured at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Euroclone), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), in humidified atmosphere with 5% CO_2.

Lactoferrin

Highly purified milk-derived bLf was kindly provided by Morinaga Milk Industries Co., Ltd (Tokyo, Japan). The absence of bLf degradation fragments was checked by SDS-PAGE stained with silver nitrate. Lactoferrin concentration was assessed by UV spectroscopy on the basis of an extinction coefficient of 15.1 (280 nm, 1% solution). The purity of bLf corresponded to about 98% as also detected by high-performance liquid chromatography analysis. The bLf iron saturation was about 20% as detected by optical spectroscopy at 468 nm on the basis of an extinction coefficient of 0.54 (100% iron saturation). LPS contamination of bLf, estimated by Limulus Amebocyte assay (LAL Pyrochrome kit, PBI International, Milan, Italy), was 0.7±0.06 ng/mg of bLf. Before biological assays, bLf, dissolved in DMEM, was sterilised by filtration using 0.2 μm Millex HV at low protein retention (Millipore Corp., Bedford, Mass.). In all experiments, bLf was used at non-cytotoxic concentration corresponding to 100 μg/mL.

Propagation and titration of *Chlamydia trachomatis*

C. trachomatis L2 strain434/Bu (ATCC VR-902B) was propagated in HeLa-229 cells, grown in DMEM, supplemented with 10% FCS, as previously described (Sessa *et al.* 2015). The infectious titre (inclusion-forming units [IFU] per mL) was assessed by immunofluorescence assay. Briefly, subconfluent HeLa-229 cell

monolayers grown on glass coverslips in 24-well plates were infected with 10-fold serial dilutions of bacterial stock, incubated for 48 h at 37°C, fixed with methanol and stained with fluorescein isothiocyanate-conjugated monoclonal antibody anti-*C. trachomatis* LPS (Imagen Chlamydia kit, Oxoid). The total number of *C. trachomatis* IFU was enumerated by counting all microscope fields using a fluorescence microscope (×400 magnification).

Cytotoxic effect of lactobacilli and lactoferrin on HeLa cells

The cytotoxic effect of lactobacilli and/or bLf on HeLa cells was evaluated by analysing cell viability of subconfluent monolayers. The bacterial strains in late logarithmic growth phase and/or bLF (100 μg/mL) were added to HeLa cells and after 48 h incubation the cell viability was determined by the MTT method. HeLa cells were incubated with Thiazolyl Blue Tetrazolium Bromide (5 mg/mL) (MTT reagent) for 4 h, the medium was then removed and MTT crystals were dissolved with isopropanol. The amount of formazan produced was detected by measuring the absorbance at 570 nm.

Lactobacilli adhesion to HeLa cells

The adhesion experiments were performed in six-well plates. HeLa cell suspension (1.8×10^5 cells/well) was seeded onto each well and incubated in a 5% CO_2 atmosphere at 37°C. After 24 h, the cell monolayers, grown to ~60% confluence, were washed thrice with phosphate buffered saline (PBS) without Ca^{++} and Mg^{++}. Then, lactobacilli (*L. brevis* or *L. crispatus*), at a multiplicity of infection (MOI) of 1000 CFU/cell, and/or bLf (100 μg/mL) in antibiotic-free cell growth medium were added. The plates were incubated for 1 and 24 h at 37°C in aerobic conditions. Cell monolayers were then washed several times in PBS, fixed with May–Grünwald for 4 min, and then Giemsa stained for 10 min. By microscopy, each HeLa cell was scored for the presence and number of bacteria attached. Each adhesion assay was performed in duplicate and 500 randomly chosen cells were evaluated for microorganism adhesion.

Effects of lactoferrin and lactobacilli on *Chlamydia trachomatis* infection

Effect of lactobacilli and bLF on the entry of Chlamydia trachomatis into host cell

Pre-incubation assay. Subconfluent HeLa-229 cell monolayers were incubated in DMEM, supplemented with 2% FCS, in the presence or absence of lactobacilli (*L. crispatus* or *L. brevis*) (1000 CFU/cell) and/or bLf (100 μg/mL). After 1 or 3 h incubation at 37°C and 5% CO_2, bLf and lactobacilli were removed by washing three times with PBS. Subsequently, HeLa-229 cells were infected with *C. trachomatis* (MOI 0.05 IFU/cell) as previously described (Sessa *et al.* 2015). After 48 h incubation at 37°C and 5% CO_2, the total number of *C. trachomatis* IFU was determined by immunofluorescence assay.

Infection assay Subconfluent HeLa-229 cell monolayers were infected with *C. trachomatis* (MOI 0.05 IFU/cell), as previously described (Sessa *et al.* 2015), in the presence or absence of lactobacilli (*L. crispatus* or *L. brevis*) (1000 CFU/cell) and/or bLf (100 μg/mL). After 1 h, inoculum was removed and replaced with DMEM supplemented with 2% FCS. After 48 h incubation at 37°C and 5% CO_2, the total number of *C. trachomatis* IFU was determined by immunofluorescence assay.

Adhesion assay. Subconfluent HeLa-229 cell monolayers were incubated at 4°C for 15 min and infected for 1 h under gentle stirring at 24°C, with *C. trachomatis* (MOI 0.05 IFU/cell) in the presence or absence of *L. brevis* (1000 CFU/cell) and/or bLf (100 μg/mL). The inoculum was then removed and cell monolayers were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min in ice. The total number of *C. trachomatis* EBs per cell was determined by immunofluorescence assay.

Invasion assay. Subconfluent HeLa-229 cell monolayers were incubated at 4°C for 15 min and infected for 1 h under gentle stirring at 24°C, with *C. trachomatis* (MOI 0.05 IFU/cell) in the presence or absence of *L. brevis* (1000 CFU/cell) and/or bLf (100 μg/mL). Then, the inoculum was removed and replaced with DMEM supplemented with 2% FCS. After 2 h incubation at 37°C and 5% CO_2, cell monolayers were washed three times with PBS, and fixed with methanol for 10 min. The total number of *C. trachomatis* EBs per cell was determined by immunofluorescence assay.

Effect of lactobacilli and bLf on Chlamydia trachomatis EBs

Co-aggregation assay. C. trachomatis EBs (MOI 0.05 IFU/cell) were incubated in the presence or absence of lactobacilli (*L. crispatus* or *L. brevis*) at a MOI/CFU ratio of 1/1000 and/or bLf (100 μg/mL) in DMEM, supplemented with 2% FCS, for 1 h at 37°C and 5% CO_2. Subsequently, *C. trachomatis* EB suspension was centrifuged (500 × *g* for 15 min) to remove chlamydia co-aggregated lactobacilli. To remove bLf, supernatants were centrifuged (30 000 × *g* for 15 min) and the pellet, containing free *C. trachomatis* EBs, was resuspended in DMEM supplemented with 2% FCS and, then, was used to infect subconfluent HeLa 229 cell monolayers as previously described (Sessa *et al.* 2015). After 48 h incubation at 37°C and 5% CO_2, the total number of *C. trachomatis* IFU was determined by immunofluorescence assay.

Effect of lactobacilli and bLf on Chlamydia trachomatis multiplication into host cell

Subconfluent HeLa-229 cell monolayers were infected with *C. trachomatis* (MOI 0.05 IFU/cell) as previously described (Sessa *et al.* 2015). Subsequently, the inoculum was removed and DMEM, supplemented with 2% FCS, was added. After 3 h at 37°C and 5% CO_2, the infected cells were incubated in the presence or absence of lactobacilli (*L. crispatus* or *L. brevis*) (1000 CFU/cell) and/or bLf (100 μg/mL). After 24 h incubation at 37°C and 5% CO_2, bLf was re-added to each well. After further 24 h incubation at 37°C and 5% CO_2, the total number of *C. trachomatis* IFU was determined by immunofluorescence assay.

In some experiments, subconfluent HeLa-229 cell monolayers were infected with *C. trachomatis* EBs (MOI 5 IFU/cell) in the presence or absence of lactobacilli (*L. crispatus* or *L. brevis*) and/or bLf (100 μg/mL) for the analysis of cytokines, since preliminary experiments, carried out with *C. trachomatis* at a MOI of 0.05, did not show cytokine production.

Effect of lactobacilli and bLF on Chlamydia trachomatis in the whole chlamydial infection cycle

Subconfluent HeLa-299 cell monolayers were incubated in DMEM, supplemented with 2% FCS, in the presence or absence of lactobacilli (*L. brevis* or *L. crispatus*) (MOI 1000 CFU/cell) and/or bLf (100 μg/mL) for 3 h at 37°C and 5% CO_2. Subsequently, cell

monolayers were infected with *C. trachomatis* (MOI 0.05 IFU/cell) in the presence of lactobacilli and/or bLf and further incubated at 37°C and 5% CO2. After 48 h incubation, the total number of *C. trachomatis* IFU was determined by immunofluorescence assay.

In all of the experimental conditions, the *C. trachomatis* inocula underwent the same centrifugation steps described for the lactobacilli and/or bLf in co-aggregation assay, and, then, were used to infect subconfluent HeLa cell monolayers.

Assessment of infectious chlamydial progeny by subpassage

Infected cell monolayers in the presence or absence of lactobacilli and/or bLf resulting from previous experiments were scraped into cold complete DMEM, pelleted and resuspended in fresh medium. Cell suspensions were vortexed with glass beads and centrifuged at 200 × *g* for 5 min. Supernatants were recovered and assayed for infectious progeny. Briefly, HeLa cells, grown on glass coverslips in 24-well plates, were infected in triplicate with 10-fold serial dilutions of chlamydial suspensions and incubated at 37°C and 5% CO_2. After 48 h incubation, the total number of *C. trachomatis* IFU was determined by immunofluorescence assay.

Cytokine analysis

IL-6 and IL-8 levels in culture supernatants from the multiplication assay were measured by ELISA using Human quantikine ELISA kit, according to the manufacturer's instructions (R&D Systems).

Statistical analysis

All values are expressed as mean ± standard deviation (SD) of three replicates from three independent experiments. Comparison of means was performed by using a two-tailed t-test for independent samples. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Lactobacilli and bLf interactions with HeLa cells

The cytotoxic effect of *L. brevis*, *L. crispatus* and lactoferrin on HeLa cells was evaluated by analysing cell viability after 48 h co-culture. HeLa cell viability was unaffected by lactobacilli and lactoferrin alone or in combination, as verified by MTT assay (data not shown).

Preliminary experiments were performed to examine the adhesion of *L. brevis* or *L. crispatus* to HeLa cells in the experimental conditions utilised for chlamydial infection. As shown in Fig. 1, *L. brevis* showed a significantly higher adhesion to HeLa cells after 1 h incubation as compared to *L. crispatus*. The number of adherent lactobacilli significantly decreased when bLf was added to *L. brevis* or *L. crispatus*. Specifically, bLf reduced *L. crispatus* and *L. brevis* adhesion to HeLa cells by ˜53% and 32%, respectively.

After 24 h incubation, an increased adhesion of both *Lactobacillus* strains to cell monolayer was observed. The presence of bLf resulted in a similar decrease in the number of adherent lactobacilli per cell to that observed after 1 h incubation. The cell confluence was unaffected by the presence of both *Lactobacillus* strains after 24 h incubation (data not shown).

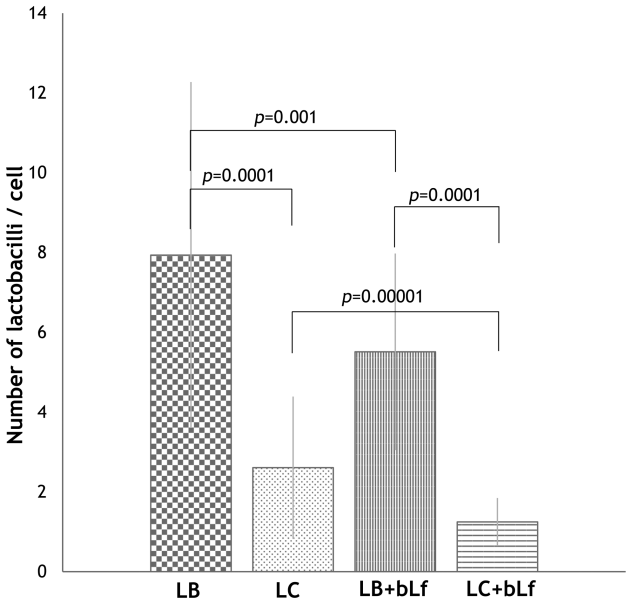

Figure 1. Adhesion of lactobacilli to HeLa cells in the presence of bovine lactoferrin(bLf). *Lactobacillus brevis* (LB) and *L. crispatus* (LC) were added to HeLa cell monolayers (1000 CFU/cell) in the presence or absence of bLF (100 μg/mL) for 1 h at 37°C in antibiotic-free cell growth medium and aerobic atmosphere. The adhesion of the lactobacillus strains, analysed after May-Grünwald/Giemsa stain, is expressed as the average number of adherent lactobacilli per cell.

Effect of lactobacilli and bLf on the entry of *Chlamydia trachomatis* into host cell

L. brevis was able to inhibit the entry of *C. trachomatis* into host cell, as evidenced by a significant reduction in the number of chlamydial IFUs observed in cell monolayers pre-incubated with lactobacilli for 1 or 3 h as compared to *C. trachomatis*-infected cells (control). In contrast, no significant reduction in the number of chlamydial IFUs was observed in cell monolayers pre-incubated with *L. crispatus* for 1 or 3 h as compared to control (Table 1, Fig. 2A).

bLf was also able to inhibit the entry of *C. trachomatis* into host cell, as evidenced by a significant reduction in the number of chlamydial IFUs in cell monolayers pre-incubated with bLf for 1 or 3 h as compared to control, resulting more effective after 3 h pre-incubation (Table 1, Fig. 2A).

On the other hand, no significant reduction in the number of chlamydial IFUs was observed in cell monolayers pre-incubated with *L. brevis* or *L. crispatus* combined with bLf for 1 or 3 h as compared to bLf alone (Table 1, Fig. 2A).

During chlamydial infection phase, *L. brevis*, *L. crispatus* and bLf reduced the number of chlamydial IFUs as compared to control. However, a higher inhibitory effect towards *C. trachomatis* was observed for the combination of *L. brevis*/bLf as compared to *L. crispatus*/bLf as well as to *L. brevis*, *L. crispatus* and bLf alone (Table 1, Fig. 2A).

Overall, the strongest inhibitory activity towards the entry of *C. trachomatis* into host cell was exerted by the combination of *L. brevis* and bLf during chlamydial infection phase as compared to 1 h pre-incubation and 3 h pre-incubation.

Based on these findings, we further investigated whether *L. brevis* and/or bLf interfered with the adhesion and/or invasion of chlamydia EBs into host cell. Under adhesion experimental

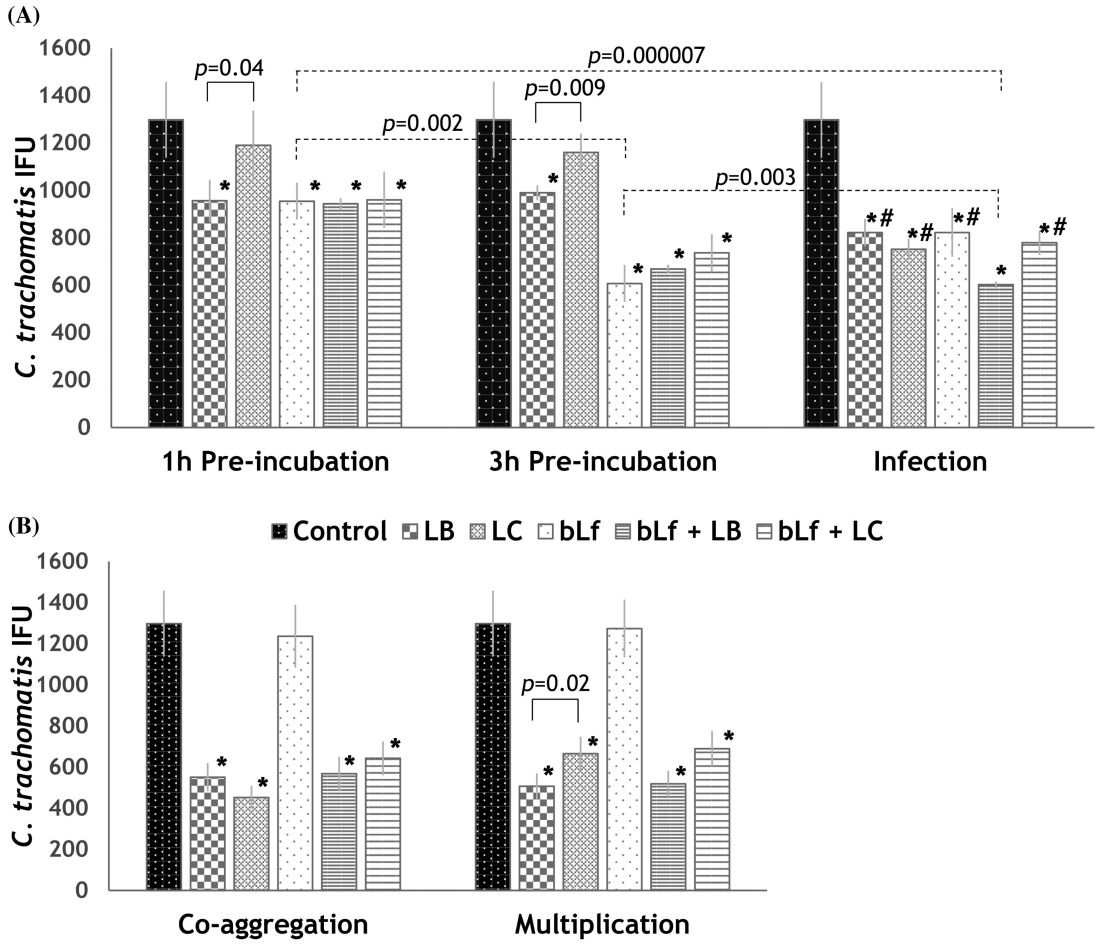

Figure 2. Inhibition of *C. trachomatis* by *L. brevis*(LB), *L. crispatus* (LC) and bovine lactoferrin (bLf) under different conditions: **(A)** 1 and 3 h pre-incubation and infection assays; **(B)** co-aggregation and multiplication assays. In pre-incubation assays, HeLa cells were pre-incubated in the presence or absence of LB, LC (1000 CFU/cell) and/or bLF (100 μg/ml) for 1 or 3 h, followed by *C. trachomatis* infection. In infection or multiplication assays, HeLa cells were infected with *C. trachomatis* in the presence or absence of lactobacilli and/or bLf during the adsorption phase (infection assay) and replication phase (multiplication assay). In co-aggregation assay, *C. trachomatis* was pre-incubated in the presence or absence of lactobacilli and/or bLf. Control, *C. trachomatis*-infected cells; * $P < 0.05$ vs control; # $P < 0.05$ vs bLf + LB.

conditions, a significant reduction in the number of EBs/cell was observed in cell monolayers infected with *C. trachomatis* in the presence of *L. brevis* as compared to infected cell monolayers alone, whereas no significant reduction was observed in the presence of bLf (Fig. 3). In the invasion assay, we observed a significant reduction in the number of EBs within host cell as compared to the number of adhering EBs to host cell in cell monolayers infected with *C. trachomatis* in the presence of bLf, whereas no significant reduction was observed in the presence of *L. brevis* (Fig. 3).

On the contrary, the combination of *L. brevis* and bLf interfered with both the adhesion and invasion of *C. trachomatis* EBs into host cell (Fig. 3).

Effect of lactobacilli and bLf on *Chlamydia trachomatis* EBs

Both *L. brevis* and *L. crispatus* showed co-aggregation abilities with *C. trachomatis* EBs, as evidenced by a significant reduction in the number of chlamydial IFUs as compared to control (Table 1, Fig. 2B). No significant difference was observed between *L. crispatus* and *L. brevis* in binding *C. trachomatis* EBs.

In addition, no significant reduction in the number of chlamydial IFUs was observed when chlamydial EBs were pre-incubated with bLf for 1 h.

The combination of *L. brevis* or *L. crispatus* and bLf showed similar co-aggregation abilities with *C. trachomatis* EBs as compared to lactobacilli alone.

Effect of lactobacilli and bLf on *Chlamydia trachomatis* multiplication into host cell

The addition of *L. brevis* or *L. crispatus* during the multiplication phase of *C. trachomatis* resulted in a significant reduction in the number of chlamydial IFUs as compared to control; *L. brevis* showed a stronger inhibiting effect on *C. trachomatis* as compared to *L. crispatus*.

In contrast, bLf did not show any inhibitory effect towards *C. trachomatis* multiplication, as evidenced by a similar number of chlamydial IFUs as compared to control (Table 1, Fig. 2B).

Lastly, bLf in combination with both *Lactobacillus* strains did not influence the number of *C. trachomatis* IFUs during chlamydial multiplication phase as compared to *L. brevis* or *L. crispatus* alone.

Table 1. Inhibition of *C. trachomatis* by *L. brevis*, *L. crispatus* and bLf under different experimental conditions.

	Experimental conditions (mean ± SD)					
	Co-aggregation	1 h pre-incubation	3 h pre-incubation	Infection	Multiplication	Whole chlamydial infection cycle
C. trachomatis	1297.6 ± 160.13	1297.6 ± 160.13	1297.6 ± 160.13	1297.6 ± 160.13	1297.6 ± 160.13	1297.6 ± 160.13
C. trachomatis + *L. brevis*	551.07 ± 68*	956 ± 88.93*	989.8 ± 31.82*	822 ± 58.66*#	506.8 ± 62.5*	506.8 ± 62.5*#
C. trachomatis + *L. crispatus*	452.16 ± 55.8*	1190 ± 146.75	1160.4 ± 79.38	751.75 ± 45.52*#	665 ± 82.25*	634 ± 78.26*#
C. trachomatis + bLf	1235.81 ± 152.5	954.75 ± 77.47*	607.5 ± 77.81*	822.75 ± 102.65*#	1273.5 ± 140.54	316.2 ± 39.35*#
C. trachomatis + *L. brevis* + bLf	567.34 ± 82.35*	943.375 ± 24.47*	668.5 ± 18.09*	602 ± 13.87*	518.8 ± 64.15*	247.4 ± 26.01*
C. trachomatis + *L. crispatus* + bLf	642.36 ± 82.07*	960.6 ± 118.61*	736.25 ± 79.65*	779 ± 51.24*#	689.6 ± 85.14*	667 ± 80.2*#

bLf, bovine lactoferrin; SD, standard deviation.
*$P < 0.05$ vs *C. trachomatis*; #$P < 0.05$ vs *C. trachomatis* + *L. brevis* + bLf.

Figure 3. Inhibitory effects of *L. brevis* (LB) and bovine lactoferrin (bLf) on the adhesion and invasion of *C. trachomatis* EBs into HeLa cells during chlamydial infection assay. In adhesion assay, HeLa cells were infected for 1 h at 24°C with *C. trachomatis* in the presence or absence of LB and/or bLf. In invasion assay, HeLa cells were infected for 1 h at 24°C with *C. trachomatis* in the presence or absence of lactobacilli and/or bLf, and further incubated for 2 h at 37°C. Then, the total number of *C. trachomatis* EBs per cell was determined by immunofluorescence assay. Control, *C. trachomatis*-infected cells.

Effect of lactobacilli and bLf on *Chlamydia trachomatis* in the whole chlamydial infection cycle

In order to investigate a potential additive inhibitory effect of *L. brevis*, *L. crispatus* and bLf in the different phases of chlamydial infection cycle, we developed an *in vitro* model consisting of the continued presence of lactobacilli strains and bLf before and for the entire duration of *C. trachomatis* infection cycle (Table 1, Fig. 4).

Under these experimental conditions, *L. brevis*, *L. crispatus* and bLF were able to inhibit *C. trachomatis* infection, as evidenced by a significant reduction in the number of chlamydial IFUs observed in the presence of both lactobacilli strains or bLf as compared to control. A higher inhibitory effect towards *C. trachomatis* infection was observed in the presence of bLF alone as compared to *L. crispatus* or *L. brevis*. More interestingly, in this model, the inhibitory activity of the combination of *L. brevis* and bLf towards *C. trachomatis* infection was effective as compared to pre-incubation, infection and multiplication phases (Table 1, Fig. 4).

Chlamydia trachomatis persistence

Given that *C. trachomatis* persistent forms are induced by iron deprivation and we used a preparation of bLf (100 μg/mL) iron saturated at 20%, we investigated whether the iron binding ability of bLf, in all the experimental conditions, induced *C. trachomatis* persistence by assessing infectious chlamydial progeny.

bLf was not able to induce *C. trachomatis* persistence in all the experimental conditions assayed: the infectious chlamydial progeny was similar in infected cell monolayers in the presence of bLf as compared to infected cell monolayers alone (data not shown).

Effect of *Lactobacillus brevis* and bLf on IL-6 and IL-8 levels in *Chlamydia trachomatis*-infected cells

In order to determine the effect of *L. brevis* and bLf on cytokine production induced by *C. trachomatis* during the

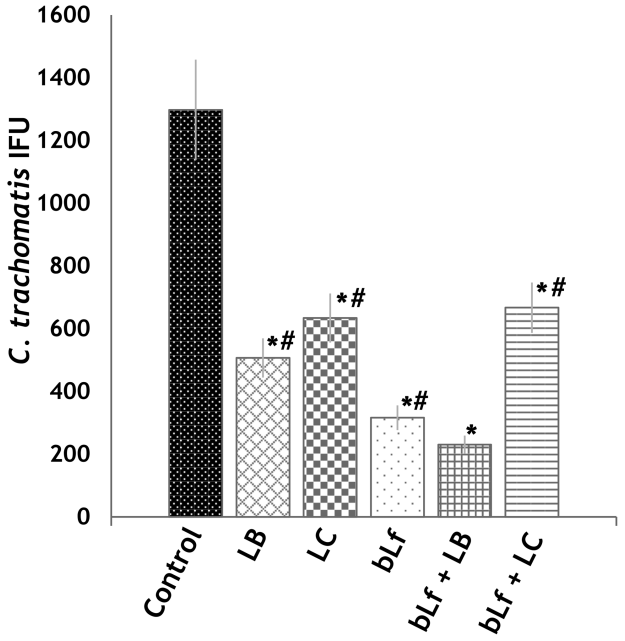

Figure 4. Inhibitory effects of *L. brevis*(LB), *L. crispatus* (LC) and bovine lactoferrin (bLf) on *C. trachomatis* in an *in vitro* model characterised by the continued presence of lactobacilli strains and bLf before and for entire duration of *C. trachomatis* infection cycle. Control, *C. trachomatis*-infected cells; * $P < 0.0001$ vs control; # $P < 0.05$ vs bLf + LB.

multiplication phase, we assessed IL-6 and IL-8 levels in the culture supernatants.

Both *L. brevis* and bLf significantly reduced IL-6 production as compared to infected cell monolayers. bLf was significantly more effective in comparison to *L. brevis* in inhibiting IL-6 production, as shown in Fig. 5. However, the highest reduction in IL-6 production was observed when *L. brevis*, in combination with bLf, was added during the multiplication phase of *C. trachomatis* (Fig. 5).

Similar to the above findings, both *L. brevis* and bLf significantly reduced IL-8 production as compared to infected cell monolayers. *L. brevis* was significantly more effective than bLf in reducing IL-8 levels. However, the highest reduction in IL-8 production was observed when *L. brevis* in combination with bLf was added to infected cell monolayers (Fig. 5).

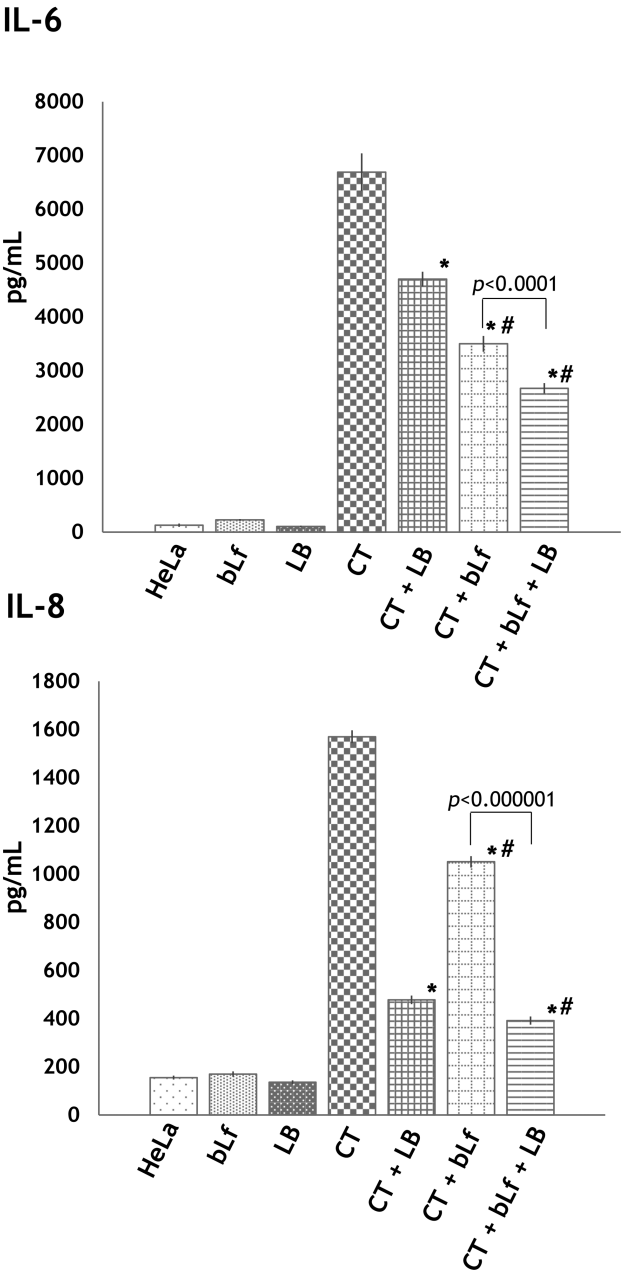

Figure 5. Effects of *L. brevis* (LB) and bovine lactoferrin (bLf) on IL-6 and IL-8 production induced by *C. trachomatis* (CT) during the multiplication assay. HeLa cells were infected with CT and then incubated for 48 h in the presence or absence of LB and/or bLf. * $P < 0.01$ vs CT; # $P < 0.001$ vs CT + LB.

DISCUSSION

C. trachomatis is still considered as an important public health problem worldwide because of the impact of asymptomatic infections on reproductive outcomes, including the increased risk of acquiring other sexually transmitted agents and of developing severe sequelae (Newman *et al.* 2015; Sessa *et al.* 2015). Therefore, the role of host defence factors, such as the genital microbiota as well as bLf, responsible for maintaining a healthy cervicovaginal microenvironment, acquires paramount importance (Valenti and Antonini 2005; Mastromarino *et al.* 2009; Petrova *et al.* 2015).

To our knowledge, this is the first study evaluating the complex interplay between *L. brevis*, *L. crispatus* and bLf on *C. trachomatis* infection.

The main result of our study is the evidence that the simultaneous presence of *L. brevis* and bLf strongly inhibits the early phases (adhesion and invasion) of *C. trachomatis* infection cycle and decreases inflammatory cytokine synthesis, suggesting an additive effect of both host defence factors.

L. brevis is more active than *L. crispatus* in inhibiting *C. trachomatis* infection ($P < 0.05$). In particular, during the pre-incubation and infection phases, we have observed a stronger inhibitory effect of *L. brevis*, which may be related to the higher adhesive properties of this strain to host cell surface. Considering also the ability of vaginal probiotics containing *L. brevis* in restoring the healthy cervicovaginal microbiota (Hemalatha *et al.* 2012), *L. brevis* may be considered as a promising candidate for the development of an effective strategy against *C. trachomatis* infection.

More importantly, the anti-chlamydial activity of *L. brevis* is markedly increased by the addition of bLf, in contrast to what we have observed with *L. crispatus* and this may be explained by a potential competition between bLf and *L. crispatus* for host epithelial cell surface.

bLf is also able to inhibit *C. trachomatis* adhesion to host cell after the pre-incubation with cells through, probably, the binding to components of host cell surface, such as heparan sulphate proteoglycans and glycosaminoglycans (Sessa *et al.* 2016; Stallmann and Hegemann 2016).

C. trachomatis entry into host cell is a multifactorial process characterised by the initial adhesion to target cells, mediated by different adhesins, including outer membrane complex protein B (OmcB), major outer membrane protein and polymorphic membrane protein (Pmp), followed by the internalisation, mediated by chlamydial virulence effectors as well as host proteins (Taylor *et al.* 2011; Rosmarin *et al.* 2012; Bastidas *et al.* 2013; Fechtner *et al.* 2013; Mehlitz and Rudel 2013).

During *C. trachomatis* infection phase, we have observed the strongest anti-chlamydial activity exerted by the combination of *L. brevis* and bLf and, hence, we sought to further deepen the knowledge on the complex mechanisms underlying the adhesion and invasion of *C. trachomatis*. To this purpose, we have evaluated separately the adhesion and invasion of chlamydial EBs into host cell. Interestingly, we have observed a different effect of *L. brevis* and bLf on *C. trachomatis*, being *L. brevis* able to inhibit the adhesion and bLf the internalisation of chlamydial EBs into host cell. Several mechanisms may be involved in these inhibitory effects and, in this regard, we hypothesise that the presence of lactobacilli, coated on the host cell membrane, hinders the binding of chlamydia to the cell surface, or that the properties of cell membrane could be altered following bLf adhesion to host cell receptors, thus impairing *C. trachomatis* invasion. Therefore, it will be interesting to carry on further research on this topic in order to better understand host–chlamydia interaction.

The overall inhibitory effect on *C. trachomatis* entry of *L. brevis* and bLf is of great interest in the pathogenesis of the infection since chlamydial adhesion and invasion into host cell are the early steps leading to tissue damage and, hence, to increased risk of developing severe sequelae (Stephens 2003; Taylor *et al.* 2011). In this regard, particularly interesting, in a clinical scenario, is also the evidence that bLf, used in our study, despite its iron withholding ability, does not induce the establishment of chlamydial persistent forms, thought to be responsible for chronic inflammatory state.

The other intriguing aspect in our study is the observation that the combination of *L. brevis* and bLf also shows the highest anti-inflammatory effect, as evidenced by the decreased levels of IL-8 and IL-6. Several studies have demonstrated that both IL-8 and IL-6 are needed for an optimal host response against *C. trachomatis*, since IL-8 is an important neutrophils chemoattractant and activator and IL-6 is a potent inducer of acute-phase proteins (Redgrove and McLaughlin 2014). However, during *C. trachomatis* chronic infection, an unregulated inflammatory response can occur, leading to the overproduction of both cytokines, responsible for tissue damage and the related chronic complications (Rasmussen *et al.* 1997; Stephens 2003; Bulchholz and Stephens 2007; Singer and Ouburg 2016). As a result, the strong anti-inflammatory activity of *L. brevis* and bLf combined is of the outmost importance.

In conclusion, our results suggest that the interplay between *L. brevis* and bLf may play a role in the protection against *C. trachomatis*, since, on the one hand, they may be able to hinder the infection and, on the other hand, they may exert a immunomodulatory effect on chlamydia-infected epithelial cells. To confirm the effectiveness of *L. brevis* and bLf in decreasing the tissue damaging inflammatory state and, hence, the risk of developing severe complications, it will be interesting to translate our results in animal models.

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Conflict of interest. None declared.

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