Effect of bovine lactoferrin on Chlamydia trachomatis infection and inflammation

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| Keyword:      | Chlamydia trachomatis, bovine lactoferrin, infection, inflammation, IL-6 |

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Effect of bovine lactoferrin on \textit{Chlamydia trachomatis} infection and inflammation

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Abstract

*Chlamydia trachomatis* is an obligate, intracellular pathogen responsible for the most common bacterial sexually transmitted disease worldwide, causing acute and chronic infections. The acute infection is susceptible to antibiotics, while the chronic one needs prolonged therapies, thus increasing the risk of developing antibiotic resistance. Novel alternative therapies are needed.

The intracellular development of *C. trachomatis* requires essential nutrients, including iron. Iron-chelating drugs inhibit *C. trachomatis* developmental cycle.

Lactoferrin (Lf), a pleiotropic iron binding glycoprotein, could be a promising candidate against *C. trachomatis* infection. Similarly to the efficacy against other intracellular pathogens, bovine Lf (bLf) could both interfere with *C. trachomatis* entry into epithelial cells and exert an anti-inflammatory activity.

*In vitro* and *in vivo* effects of bLf against *C. trachomatis* infectious and inflammatory process has been investigated.

bLf inhibits *C. trachomatis* entry into host cells when incubated with cell monolayers before or at the moment of the infection and down-regulates IL-6/IL-8 synthesized by infected cells.

Six out of seven pregnant women asymptomatically infected by *C. trachomatis*, after 30 days of bLf intra-vaginal administration, were negative for *C. trachomatis* and showed a decrease of cervical IL-6 levels.

This is the first time that the bLf protective effect against *C. trachomatis* infection has been demonstrated.

Key words: *Chlamydia trachomatis*, bovine lactoferrin, infection, inflammation, IL-6
**Introduction**

*Chlamydia trachomatis*, an obligate intracellular pathogen, is the leading cause of bacterial sexually transmitted infections in the world with an estimated over 131 million new cases per year (Newman et al. 2015). *C. trachomatis* genital infection manifests in women as cervicitis, salpingitis and endometritis, and can progress leading to severe sequelae, such as pelvic inflammatory disease, ectopic pregnancy and obstructive infertility (Shaw et al. 2011). Importantly, a major concern with chlamydial genital infections is that approximately 80% of women are asymptomatic, thus resulting in a reservoir for onwards transmission in the population (Shaw et al. 2011).

*C. trachomatis* is characterized by a unique biphasic developmental cycle alternating between the extracellular infectious bodies (Elementary Bodies, EBs), metabolically inactive, and the intracellular non-infectious bodies (Reticulate Bodies, RBs), metabolically active. The EBs adhesion and entry into mucosal epithelial cells initiate a signal transduction cascade of the host cell, leading to the recruitment and reorganization of the actin cytoskeleton at the site of attachment. Following the fusion of EB-containing endosomes, EBs develop into larger, metabolically active but non-infectious RBs. Using ATP and nutrients from the host cell, RBs grow and divide by binary fission within a membrane-bound vacuole, termed inclusion. Subsequently, the RBs asynchronously transform into EBs, which are released, after approximately 48 hours, from the host cell by lysis (Wyrick 2010; Bastidas et al. 2013).

In the recent years, it has been demonstrated that *C. trachomatis* can generate a persistent form during its developmental cycle as a consequence of several stress-inducing factors (Di Pietro M et al. 2013; Wyrick 2010). As a result, normal RBs transform into enlarged and morphologically aberrant RBs, thus stopping the production of infectious EBs (Hogan et al. 2004; Wyrick 2010). In particular, *C. trachomatis* enters into the persistence state in the presence of iron-chelating drugs, which inhibit the developmental cycle and, hence, show its dependence on iron for the achievement of infectious cycle (Raulston 1997; Thompson and Carabeo 2011). In this regard, iron limitation in
host cells has been shown to be of the utmost importance for the growth and survival of *Chlamydia* spp. (Raulston 1997; Al-Younes et al. 2001).

Following *C. trachomatis* infection, cervical epithelial cells produce several pro-inflammatory cytokines including TNF-α, IL-1α, IL-6 and IL-8 that augment the cell inflammatory response thus inducing direct damage to genital tissues. Furthermore, IL-8, in turn, recruits innate immune cells, which are abundant in the genital mucosa and are able to further worsen chronic inflammation and tissue-damage of the reproductive system (Redgrove and McLaughlin 2014).

Interestingly, IL-8 recruits, during the infection/inflammation, neutrophils that synthesize and secrete granules containing lactoferrin (Lf) (Masson et al. 1969).

Recently, a great interest in Lf, considered as a prominent component of the first line defense of the host against infections and inflammation, has been raised.

Lf, an 80 kDa iron-binding glycoprotein, is found in most body fluids including vaginal fluid (Valore et al. 2002). Lf possesses several biological functions dependent and independent from its iron binding ability (Valenti and Antonini 2005). Among the biological properties related to its iron-withholding ability, Lf inhibits bacterial infections, whereas, independently from iron chelation, its high positive charge favors the binding to microorganisms and/or host cells, thus hindering the adhesion and entry into epithelial cells (Valenti and Antonini 2005). In addition to these activities, Lf exerts a potent anti-inflammatory activity, protecting infected host cells from damages associated to pathological inflammation. In particular, Lf, independently from its iron binding ability, decreases the synthesis of pro-inflammatory cytokines in infected epithelial cells (Berlutti et al. 2006; Valenti et al. 2011; Puddu et al. 2011; Frioni et al. 2014).

Given the impact of asymptomatic chlamydial infection on disease outcomes and the multifunctional features of Lf, the aim of our study was to evaluate the effects of bovine milk-derivative Lf (bLf) on *C. trachomatis* infection and on the associated inflammatory state *in vitro* and *in vivo*. 
Materials and Methods

*Chlamydia trachomatis* strain and cell culture

*C. trachomatis* L2 strain 434/Bu (ATCC VR-902B) was obtained from American Type Culture Collection.

The human epithelial HeLa-229 cell line from cervix adenocarcinoma (ATCC ® CCL-2.1™) was cultured at 37°C in Dulbecco’s Modified Eagle Medium (D-MEM, Euroclone, Milan, Italy), supplemented with 10% fetal calf serum (FCS, Euroclone, Milan, Italy), in humidified atmosphere with 5% CO₂.

Propagation and titration of *Chlamydia trachomatis*

Elementary body (EB) aliquots of *C. trachomatis* L2 were stored at −80°C and propagated in HeLa-229 cells, grown in D-MEM supplemented with 10% FCS, as previously described by Mastromarino et al. (2014). The infectious titer was assessed by immunofluorescence assay (IFA). Briefly, HeLa-229 cells grown on glass coverslips in 24-well plates were infected with tenfold serial dilutions of bacterial stock, incubated for 48 h at 37°C, fixed with methanol and stained with fluorescein isothiocyanate-conjugated monoclonal (FITC) antibody anti-*C. trachomatis* (MicroTrak, Trinity Biotech – USA). The total number of *C. trachomatis* Inclusion Forming Units (IFUs) was obtained by counting all fields using a fluorescence microscope (100× magnification).

Lactoferrin

Highly purified bovine milk derivative lactoferrin (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 (HPLC) 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 solution was sterilized 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.

**Effects of bovine lactoferrin on infection of HeLa-229 cells with Chlamydia trachomatis elementary bodies**

**i) Pre-incubation of bLf with C. trachomatis EBs**

In order to detect the efficacy of bLf against *C. trachomatis*, 25,000 EBs/ml, corresponding to a multiplicity of infection (MOI) of 0.05, were pre-incubated in D-MEM with FCS 2% (fresh medium), in the absence or presence of bLf (100 µg/ml), for 1h or 3hs at 37°C in humidified atmosphere with 5% CO₂. Subsequently, the *C. trachomatis* EBs suspension was centrifuged at 30,000 × g for 15 min and the supernatant was removed. The pellet containing *C. trachomatis* EBs was suspended in fresh medium and used to infect a total of about 10⁵ HeLa-229 cells. Briefly, after 1 hour, the cells were washed with phosphate buffer solution without Ca²⁺ and Mg²⁺ (PBS) to remove the non-internalized *C. trachomatis* EBs and newly incubated in fresh medium. After 48 hours post infection (h.p.i.) at 37°C in 5% CO₂, the total number of *C. trachomatis* IFU was determined by IFA.

**ii) Pre-incubation of bLf with HeLa-229 cells before the infection with C. trachomatis EBs**

HeLa-229 cells were pre-incubated in fresh medium in the absence or presence of bLf (100 µg/ml). After 1h or 3hs of incubation at 37°C in 5% CO₂, bLf was removed by washing the cell monolayers three times with PBS. Subsequently, HeLa-229 cells were infected with *C. trachomatis* at a MOI of 0.05 as above described. After 48 h.p.i. at 37°C in 5% CO₂, the total number of *C. trachomatis* IFU was determined by IFA.

**iii) bLf addition to HeLa-229 cells at the moment of infection with C. trachomatis EBs**

In this set of experiments, bLf was added to HeLa-229 cells at the moment of infection. Briefly, HeLa-229 cells were infected with *C. trachomatis* at a MOI of 0.05 in the absence or presence of bLf (100 µg/ml). After 1 h at 37°C in 5% CO₂, the cells were washed with PBS to remove the non-
internalized *C. trachomatis* EBs and fresh medium was added. After 48 h.p.i at 37°C and 5% CO₂, the total number of *C. trachomatis* IFU was determined by IFA.

**iv) bLf addition to HeLa-229 cells three hours post *C. trachomatis* infection**

HeLa-229 cells were infected with *C. trachomatis* at a MOI of 0.05. After 1h of incubation at 37°C in 5% CO₂, the cells were washed with PBS to remove the non-internalized *C. trachomatis* EBs and fresh medium was added. After further 3hs of incubation at 37°C in 5% CO₂, fresh medium, with or without bLf (100 µg/ml), was added to the infected cells. After 48 h.p.i. at 37°C and 5% CO₂, the total number of *C. trachomatis* IFU was determined by IFA.

**Detection of cytokines**

Preliminary experiments, carried out with *C. trachomatis* EBs at a MOI of 0.05, showed a very low cytokine expression by infected HeLa-229 cells. Therefore, HeLa-229 cells were infected with *C. trachomatis* EBs at a MOI of 5 in order to reach an higher expression of IL-6 and IL-8 than that observed at the MOI of 0.05. After 1h of incubation, the cells were washed with PBS to remove the non-internalized *C. trachomatis* EBs and supplemented with fresh medium. After further 3hs of incubation at 37°C in 5% CO₂, fresh medium, with or without bLf (100 µg/ml), was added to the infected cells. The cytokine production was determined in cell monolayer supernatants by ELISA using Human ELISA Max Deluxe Set (BioLegend, San Diego, CA) after 48 hs of incubation at 37°C in 5% CO₂.

**Study design**

We conducted an open-label cohort study in accordance with the ethical principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Clinica Fabia Mater, Via Olevano Romano, 25 Rome, Italy (FM MOD 26022010). All pregnant women gave written informed consent.

One hundred ninety-eight pregnant women from 20 to 40 years without ascertained pathologies, with normal uterine cavity and intact membranes were enrolled regardless of trimester. Women
were excluded if they had a pathological pregnancy or if during this study were affected by bacterial vaginal infections unrelated to *C. trachomatis*.

The exclusion of pregnant women during the clinical trial was also considered on the basis of voluntary declaration, lack of treatment effectiveness, side effects, protocol infringement, and missed programmed visits. As a matter of fact, the enrolled pregnant women had a monthly scheduled visit.

**Laboratory tests**

At each scheduled visit, in addition to standard assays (haematocrit, glycemia, uricemia, bilirubin, glutamicoxaloacetic transaminase, glutamic pyruvic transaminase, cholesterol, triglyceride acid and electrolytes), cervical specimens were collected with polyethylene terephthalate (Dacron) swabs to detect the presence of *C. trachomatis*. In addition, cervical fluids were analyzed to detect IL-6 concentrations.

**Chlamydia trachomatis detection**

Cervical specimens were analyzed by direct immunofluorescence assay (DFA) using Syva Microtrack kit (Syva Microtrack, Trinity Biotech, USA) according to the manufacturer’s instructions. Briefly, the smears were fixed with methanol and stained with fluorescein isothiocyanate conjugated (FITC) monoclonal antibody against *C. trachomatis* major outer membrane protein (MOMP) for 30 minutes at 37°C in a humid chamber. The slides were examined for the presence of IFUs using fluorescence microscope (100 × magnification).

**Treatment against Chlamydia trachomatis infection in pregnant women**

Among one hundred ninety-eight pregnant women, seven women, asymptotically affected by *C. trachomatis*, were immediately treated with bLf intravaginal administration. The intravaginal tablet, containing 100 mg of lyophilized bLf 20% iron saturated, was administered every 8 h for 30 days. The tablets were administered through a vaginal applicator to obtain a fast and adequate dissolution. If the treatment with bLf intra-vaginal administration for 30 days was ineffective, the pregnant women were submitted to antibiotic therapy (Workowski and Bolan 2015).
Maternal side effects

The side effects of bLf intravaginal administration were assessed by monitoring vaginal irritation, itching and burning.

Fetal and newborn side effects

Fetal vital sign assessments were monitored by ultrasonographic measurements of intrauterine growth and through the amount of amniotic fluid, expressed as the amniotic fluid index (AFI). Newborn weight and Apgar score were registered. Apgar score is a practical method of evaluating the physical condition of a newborn shortly after delivery (Apgar 1953). An Apgar score of 0–3 at 5–10 min of age is predictive of high morbidity and mortality, while an Apgar score of 9–10 means the infant is in the best possible conditions.

Statistical analysis

All values were expressed as mean ± standard deviation (SD) of three replicates from three independent in vitro experiments. The concentrations of IL-6 in cervical fluid of pregnant women were expressed as mean values ± SD. 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

Effects of bovine lactoferrin on *Chlamydia trachomatis* infection

We evaluated the effects of bLf, at non-cytotoxic concentration corresponding to 100 µg/ml, on *C. trachomatis* infections.

As shown in Figure 1A, no significant reduction in the number of chlamydial IFUs was observed when chlamydial EBs were pre-incubated with bLf for 1h or 3hs, indicating no direct effect of bLf on *C. trachomatis*.

In contrast, bLf was able to inhibit *C. trachomatis* entry into host cells as evidenced by a significant reduction of chlamydial IFU observed when HeLa-229 monolayers were pre-incubated with bLf for 1h or 3hs (1h pre-incubation: p=0.0008; 3hs pre-incubation: p=0.00007) (Figure 1 B, C). The inhibitory effect of bLf on *C. trachomatis* entry was more pronounced when HeLa-229 cells were pre-incubated with bLf for 3hs as compared to 1h (p=0.0124) (Figure 1C).

To further confirm the inhibitory effect of bLf on *C. trachomatis* entry into host cells, bLf was added at the moment of HeLa-229 monolayer infection with *C. trachomatis*. The presence of bLf during the infection phase significantly inhibited *C. trachomatis* entry into HeLa-229 cells at the same extent evidenced when bLf was pre-incubated with cell monolayers for 1h or 3hs (Figure 1 B, C, D). In order to determine whether bLf was also able to inhibit chlamydial replication into host cell, bLf was added after 3hs of *C. trachomatis* infection. The addition of bLf under these experimental conditions resulted in no significant reduction of the number of intracellular chlamydial IFUs (p=0.28) (Figure 1 E).

Effect of lactoferrin on IL-6 and IL-8 cytokine production by *Chlamydia trachomatis*-infected HeLa-229 cells

To investigate the effect of bLf on the inflammatory response, HeLa-229 cells were infected with *C. trachomatis* at a MOI of 5 and, after 3hs of infection, bLf was added to the medium. Of note, the addition of bLf after 3hs post infection did not influence the intracellular number of *C. trachomatis* infecting the cell monolayers at the MOI of 0.05 (Figure 1 E) or at the MOI of 5 (data not shown),
thus allowing the detection of the actual synthesis of IL-6 and IL-8 by the same number of intracellular *C. trachomatis*. The production of IL-6 and IL-8 was evaluated in the supernatants (Figures 2 and 3 respectively). The treatment with bLf did not raise the cytokine levels in non-infected cells as compared to cell monolayers alone. On the contrary, the infection with *C. trachomatis* induced a significant increase of both IL-6 and IL-8 levels. The addition of bLf to infected cells 3hs post infection determined a significant decrease of both IL-6 and IL-8 levels as compared to bLf-untreated infected cells (P<0.05). In particular, bLf significantly decreased IL-6 and IL-8 concentrations, even if cytokine levels remained higher than those synthesized by non-infected cell monolayers.

**Clinical trial**

Among one hundred ninety-eight pregnant women, 16 women, affected by bacterial vaginosis unrelated to *C. trachomatis*, and 6 women, for protocol violation, were excluded. One hundred seventy-six pregnant women completed the study and, among them, seven asymptomatic pregnant women, positive to *C. trachomatis* DFA and showing high concentration of IL-6 in cervical fluids, were treated with the intravaginal administration of bLf (100 mg) every 8hs for 30 days.

After one month, six out of seven cervical specimens were negative to *C. trachomatis* DFA and the cervical fluids showed a decrease in IL-6 concentration (from mean values of 250±19 to 50±11 pg/ml). One out of seven pregnant women was positive to *C. trachomatis* DFA and cervical IL-6 levels did not decrease, ranging between about 270 and 300 pg/ml (Table 1). This patient was treated with antibiotic therapy.

No maternal and neonatal side effects by bLf intra-vaginal administration were observed.
Discussion

Some mucosal pathogenic bacteria are not only capable of adhering, but also of entering into non-professional phagocytes, such as epithelial cells. Inside host cells, bacteria are in a protective niche in which they can replicate and persist, thus avoiding host defences. In addition, antibiotic therapies are not always effective in the eradication of intracellular pathogens (Armstead and Li 2011).

*C. trachomatis* is an obligate, intracellular pathogen responsible for the most common bacterial sexually transmitted disease worldwide, causing acute and chronic infections. Differently from the acute infection, cured with oral or topical administration of antibiotics, the chronic one is difficult to eradicate and needs prolonged therapies, thus increasing the risk of developing antibiotic resistance (Kohlhoff and Hammerschlag 2015).

Therefore, novel alternative therapies are needed (Sessa et al. 2015). The difficulty to find new agents anti-*C. trachomatis* infection resides in the complex life-cycle of this peculiar pathogen. In fact, *C. trachomatis* has a unique biphasic developmental cycle alternating between the extracellular infectious EBs metabolically inactive and the intracellular non-infectious RBs, metabolically active. Of note, intracellular bacterial pathogens require intracellular nutrients, including iron, for replication in mammalian cells, and chlamydiae are no exception (Raulston 1997).

Concerning the first phase of *C. trachomatis* infection, classical anti-bacterial drugs are ineffective because EBs are metabolically inactive.

Conversely, anti-bacterial drugs could be active against intracellular replicative RBs, since they are metabolically active. However, antibacterial drugs cannot usually enter inside host cells.

A further key issue is represented by the intracellular re-differentiation of RBs (after intracellular replication) into EBs, which are released following the lysis of host cells, ready to infect neighboring epithelial cells and, hence, perpetuate the infectious process (Belland et al. 2003).

Therefore, an ideal drug against *C. trachomatis* infection should:

- inhibit *C. trachomatis* EBs adhesion and entry into host cells;
- inhibit *C. trachomatis* RBs intracellular replication;
- inhibit the re-infection of host cells by EBs, extracellularly released after the re-differentiation of RBs into EBs.

Lf is thought to play a pivotal role in the prevention of infections. Its ability to sequester iron from potential pathogens is considered as an important feature in order to contrast infections. Moreover, its cationic charge is responsible for the binding to bacterial and cell surface components (Valenti and Antonini 2005). This Lf property has been shown to inhibit the adhesion and entry into epithelial cells of several facultative intracellular bacteria (Longhi et al. 1993; Ajello et al. 2002; Di Biase et al. 2004; Willer et al. 2004; Berlutti et al. 2008); however Lf activity against obligate intracellular bacteria as *C. trachomatis* has never been observed.

In this study, we utilized a preparation of bLf, iron saturated at 20%, to consent further iron chelation, an essential nutrient for *C. trachomatis* developmental cycle (Raulston 1997). In facts, in the absence of free, available iron, *C. trachomatis* enters into a persistent state, as evidenced by the addition of iron-chelating agents, such as deferoxamine mesylate (DFO) or 2,2′-bipyridyl (Bpdl), to *C. trachomatis* infected cell monolayers, leading to small-sized inclusions containing enlarged, aberrant and non-dividing RBs (Thompson and Carabeo 2011), unable to generate infectious progeny (Wyrick 2010).

Differently from data reported by Thompson and Carabeo (2011), we found that the addition of bLf to HeLa cell monolayers 3hs post-infection resulted in no significant reduction of the number of intracellular chlamydial IFU (p=0.28) (Figure 1 E) and of infectious progeny. These conflicting data could be due to the higher effective concentrations of iron-chelating agents (from 100 to 200 µM) (Thompson and Carabeo 2011) as compared to 1.25 µM bLf, corresponding to 2.5 µM iron binding sites, used in this study.

We believe to be very interesting that bLf does not affect both the replication of RBs and the induction of aberrant RBs, thus avoiding the “silent” reservoir that leads to chronic infection and inflammation.
In fact, aberrant RBs can contribute to chronic inflammation, even if this aspect is still under debate. Of note, the recurrent chlamydial disease may also result from the persistence of the microorganism after unresolved infections (Wyrick 2010).

At the best of our knowledge, we demonstrated, for the first time, that the incubation of cell monolayers with bLf before the infection or at the moment of the infection inhibited, in a significant part, *C. trachomatis* adhesion and entry into epithelial cells. Therefore, the inhibition of *C. trachomatis* infectivity by bLf was dependent on its interaction with cell surface. As a matter of fact, bLf was able to bind to cell surface glycosaminoglycans as well as to heparan sulfate proteoglycans (Wu et al. 1995; Lang et al. 2011), potential receptors for *C. trachomatis* adhesion (Stallmann and Hegemann 2015).

Conversely, the pre-incubation of bLf with *C. trachomatis* did not influence its infectivity, supporting that the specific interaction between bLf and epithelial host cells seems to be the sole pivotal mechanism responsible for the inhibition of *C. trachomatis* invasion.

Similarly to the results obtained in epithelial cell monolayers infected with other facultative intracellular pathogens (Berlutti et al. 2006; Valenti et al. 2011; Frioni et al. 2014), the addition of bLf significantly decreased the IL-8 and IL-6 levels synthetized by *C. trachomatis* infected cells. To avoid that the IL-8 and IL-6 decrease was related to the different number of *C. trachomatis* IFUs, these experiments were carried out adding bLf 3 hs post infection. These results demonstrated once again the ability of bLf to down-regulate pro-inflammatory cytokine synthesis. Although it has been known for years that exogenous Lf localized to cell nucleus (Ashida et al. 2004; Suzuki et al. 2008; Valenti et al. 2011), the mechanisms by which bLf could perform its anti-inflammatory activity are still under debate.

These *in vitro* results, showing for the first time the protective effects of bLf against *C. trachomatis* infection, led us to investigate its efficacy also in asymptomatic pregnant women positive to *C. trachomatis* and with high levels of IL-6 in cervical fluids.
Seven Out of one hundred seventy-six pregnant women enrolled in this pilot study, showing cervical specimens positive to *C. trachomatis*, were treated with the intravaginal administration of bLf (100 mg) every 8hs for 30 days.

After one month, six pregnant women were negative to *C. trachomatis* and showed decreased IL-6 levels in their cervical fluids (from mean values of 250±19 to 50±11 pg/ml).

Similarly to what we observed in the *in vitro* model, bLf intravaginal administration seems to act by protecting host cells against the adhesion and entry of chlamydial EBs, extracellularly released after re-differentiation of RBs to EBs. The simultaneous decrease of IL-6 levels could be a marker for the lack of re-infection by *C. trachomatis* EBs in the presence of bLf.

Even if other clinical trials are required, the protective effect of bLf against *C. trachomatis*, demonstrated for the first time in this study, suggests a further therapeutic approach based on its intravaginal administration in addition to that already reported in preventing and curing the preterm delivery (Paesano et al. 2012).

**Acknowledgment**

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References


Figure captions

Figure 1. Inclusion forming units (IFU) of *Chlamydia trachomatis* observed by immunofluorescence assay in HeLa-229 cells infected at a MOI of 0.05 in the absence or presence of bLf added at different time.

A) 1h pre-incubation of *C. trachomatis* EBs in the absence or presence of bLf;
B) 1h pre-incubation of HeLa-229 cells in the absence or presence of bLf;
C) 3h pre-incubation of HeLa-229 cells in the absence or presence of bLf;
D) HeLa-229 cells infection with *C. trachomatis* and bLf;
E) Addition of bLf to HeLa-229 cells at 3 h.p.i. with *C. trachomatis*.

Figure 2. IL-6 levels in the supernatants of HeLa-229 cell cultures after 48hs of incubation.
The infection was performed at a MOI of 5 and bLf was added 3 hours post infection. The IL-6 concentrations are expressed as mean values ± standard deviation (SD). A value of P<0.05 was considered statistically significant.

Figure 3. IL-8 levels in the supernatants of HeLa-229 cell cultures after 48hs of infection. The infection was performed at a MOI of 5 and bLf was added 3 hours post infection. The IL-8 concentrations are expressed as mean values ± standard deviation (SD). A value of P<0.05 was considered statistically significant.
Table 1. General characteristics of pregnant women positive to *C. trachomatis* before and after the intravaginal administration of lactoferrin (100 mg).

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<td><em>C. trachomatis</em> DFA</td>
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<td>Mean values of cervical IL-6 (pg/ml)</td>
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<td>Mean values of birth weight (g)</td>
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* n = 6

* C. trachomatis DFA
* Mean values of cervical IL-6 (pg/ml)
* Spontaneous delivery (week)
* Mean values of birth weight (g)
* Apgar scores

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<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. trachomatis</em> DFA</td>
<td></td>
<td></td>
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<tr>
<td>Cervical IL-6 (pg/ml)</td>
<td>263</td>
<td>285</td>
</tr>
<tr>
<td>Caesarean section (week)</td>
<td>39&lt;sup&gt;th&lt;/sup&gt;</td>
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<tr>
<td>Birth weight (g)</td>
<td>3,378</td>
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<tr>
<td>Apgar scores</td>
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</table>

* n = 1

* DFA, Direct Immunofluorescent Assay.

**Note:**
Figure 1. Inclusion forming units (IFU) of Chlamydia trachomatis observed by immunofluorescence assay in HeLa-229 cells infected at a MOI of 0.05 in the absence or presence of bLf added at different time.

A) 1h pre-incubation of C. trachomatis EBs in the absence or presence of bLf;
B) 1h pre-incubation of HeLa-229 cells in the absence or presence of bLf;
C) 3h pre-incubation of HeLa-229 cells in the absence or presence of bLf;
D) HeLa-229 cells infection with C. trachomatis and bLf;
E) Addition of bLf to HeLa-229 cells at 3 h.p.i. with C. trachomatis.
Figure 2. IL-6 levels in the supernatants of HeLa-229 cell cultures after 48hs of incubation. The infection was performed at a MOI of 5 and bLf was added 3 hours post infection. The IL-6 concentrations are expressed as mean values ± standard deviation (SD). A value of P<0.05 was considered statistically significant.

214x161mm (300 x 300 DPI)
Figure 3. IL-8 levels in the supernatants of HeLa-229 cell cultures after 48hs of infection. The infection was performed at a MOI of 5 and bLf was added 3 hours post infection. The IL-8 concentrations are expressed as mean values ± standard deviation (SD). A value of P<0.05 was considered statistically significant.