**Original article**

# Effect of ovotransferrin and lactoferrins on *Chlamydophila psittaci* adhesion and invasion in HD11 chicken macrophages

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**Abstract –** The effect of ovotransferrin (ovoTF), human lactoferrin (hLF) and bovine lactoferrin (bLF) on the obligate intracellular pathogen *Chlamydophila* (*Cp.*) *psittaci* was evaluated using a model of Buffalo Green Monkey kidney (BGM) cells and HD11 chicken macrophages as artificial hosts. Firstly, the effect of transferrins on the infectivity of the bacteria was evaluated. Pre-incubation of *Cp. psittaci* with 0.5 to 5 mg/mL ovoTF prior to infecting BGM cells significantly lowered the infection rate ($P < 0.05$). For both lactoferrins, the infection rate could only be reduced with 5 mg/mL, albeit not significantly as compared to the infection rate created by the untreated bacteria. Secondly, transferrins were tested for their ability to influence bacterial adhesion and entry in HD11 cells. Maximal non-cytotoxic and non-bactericidal concentrations of 0.05 mg/mL ovoTF and 0.5 mg/mL hLF and bLF were used. Overall, ovoTF was more effective than human and bovine LF in inhibiting bacterial irreversible attachment and cell entry and the latter was accompanied by a dose-dependent reduction of actin recruitment at the bacterial entry site. However, once bacteria had entered HD11 cells, transferrins had apparently no effect on intracellular replication. The present findings suggest a possible role for transferrins and especially ovoTF, in preventing avian *Cp. psittaci* infections.

**ovotransferrin / lactoferrin / *Chlamydia* / *Chlamydophila psittaci***

## 1. INTRODUCTION

As obligate intracellular Gram-negative bacteria, *Chlamydiaceae* share a unique biphasic developmental cycle. Infectious elementary bodies (EB) enter the host cell and transform into metabolic active reticular bodies (RB). These RB replicate in an envelope-like structure called an inclusion. Approximately 50 h later, newly formed EB escape from the cell to start a new infectious cycle. *Chlamydophila* (*Cp.*) *psittaci* infects birds, causing respiratory infections by colonization of mucosal epithelial cells and macrophages of the respiratory tract, and spreading to various

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organs. In man, *Cp. psittaci* causes psittacosis or parrot fever [2].

Lactoferrin (LF) is a member of the transferrin family of iron-binding glycoproteins [21] and is predominantly present in milk, tears, saliva as well as in vaginal secretions. This iron-binding protein is involved in host defense mechanisms related to the non-immune defense system against pathogenic bacteria, fungi, and protozoa, both directly and through regulation of the inflammatory response [38]. Antimicrobial functions ascribed to this protein or its peptides include iron sequestration [4], destabilization of the outer membrane of Gram-negative bacteria through binding of bacterial lipopolysaccharides (LPS) [3, 7, 12, 13, 25], selective permeation of ions [1], modulation of bacterial entry into host cells through gene regulation [5] and disrupting the bacterial Type III secretion system [23]. In birds, the extracellular iron-binding glycoprotein ovotransferrin (ovoTF) or conalbumin belonging to the transferrin family, is synthesized by the liver and oviduct and is as a consequence predominantly present in serum and egg-white [14, 27]. Ovotransferrin is involved in iron transport and iron delivery to cells [19, 20], but is also proposed to exert antibacterial activity by permeating outer membranes, reaching the inner membrane and causing selective permeation of ions and dissipation of electrical potential [1]. A 92-amino acid ovotransferrin peptide, OTAP-92, was also found to be capable of killing Gram-negative bacteria by crossing the bacterial outer membrane by self-promoted uptake, and damaging the cytoplasmic membrane [17, 18].

Anti-chlamydial activities of lactoferrin or ovotransferrin in mammalian or avian hosts have not been investigated. In order to establish whether these transferrins are capable of inhibiting chlamydial infections, we tested both human and bovine lactoferrin, as well as ovotransferrin in our model of Buffalo Green Monkey kidney (BGM) cells and chicken macrophages (HD11 cells).

## 2. MATERIALS AND METHODS

### 2.1. Organism and cell culture

*Cp. psittaci* genotype D strain 92/1293 [36], a well-characterized virulent strain, was used in this study. The effect of transferrins on *Cp. psittaci* was examined by use of Buffalo Green Monkey kidney (BGM) cells and the chicken macrophage-like cell line HD11 [6]. BGM cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Merelbeke, Belgium), 2 mM L-glutamine (Invitrogen), 1% vitamins for MEM (Invitrogen), 10 μL/mL of streptomycin sulfate (1% w/v; Sigma, Antwerp, Belgium) and 20 μL/mL of vancomycin (0.5% w/v; Eli Lilly, Brussels, Belgium). HD11 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 5% heat-inactivated chicken serum, 1% sodium pyruvate, 1% L-glutamine and 0.5% gentamycin (all products from Invitrogen). Cultures were incubated at 5% $CO_2$ and 37 °C.

### 2.2. Transferrins

Ovotransferrin (ovoTF), human lactoferrin (hLF) and bovine lactoferrin (bLF) purified from chicken egg white, human milk and bovine colostrum respectively, were purchased from Sigma and iron saturated essentially as previously described [25]. Briefly, the proteins were dissolved in 10 mM sodium bicarbonate and subsequently supplemented with sodium citrate and Fe (III) chloride (final concentration 5 mM) (all products from Acros Organics, Geel, Belgium). For each

protein solution, pH was adjusted to 8 with dissolved sodium bicarbonate. After stirring the solutions for 12 h at 4 °C, dialysis was performed against 10 mM sodium bicarbonate for 48 h at 4 °C. Subsequently, proteins were concentrated using Centricon® Plus-70 Centrifugal Filter Units (Millipore, Brussels, Belgium) as described by the manufacturer. Protein concentrations were determined spectrophotometrically (260 nm) as well as iron saturation (468 nm, 1% solution). Concentrated protein solutions were stored at 4 °C for no more than 24 h.

## 2.3. Transferrin cytotoxicity assays

To check the putative cytotoxic effects of transferrins, HD11 cells were seeded in 96-well plates (Greiner Bio-One, Wemmel, Belgium) at a concentration of 300 000 cells/mL and the following day were exposed to concentrations of 0.5, 1, 2.5, 5 and 10 mg/mL of transferrins in culture medium. Incubations for 24, 36 and 48 h were performed in duplicate. At these time points, cytotoxicity in a dose-dependent manner was assessed by both viable cell counts (Nikon Eclipse TS100, 100×) after nigrosin staining and by the 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) MTT assay, actually measuring mitochondrial activity [22]. The tetrazolium salt MTT is reduced by viable cells to a colored water-insoluble formazan salt. After it is solubilized, formazan can be quantified spectrophotometrically at 585 nm. The MTT assay was performed as follows. Ten μL MTT (5 mg/mL, Sigma) in Hanks balanced salt solution (Invitrogen) was added to each well and after 3.5 h of incubation at 37 °C, the MTT solution was replaced by 200 μL DMSO in ethanol (1/1 v/v). The plates were agitated for 15 min on a platform shaker (450 RPM) to dissolve the formazan crystals and subsequently analyzed spectrophotometrically at both 585 nm (OD1) and 620 nm (OD2). The latter wavelength was used to correct for cell debris and well imperfections. Final optical densities obtained from formazan formation were presented as OD1 minus OD2.

## 2.4. Effect on infectivity of *Cp. psittaci* for BGM cells

Incubation of *Cp. psittaci* in the presence of transferrins prior to inoculation allowed us to examine the direct antichlamydial effect on extracellular bacteria and to determine the maximal non-bactericidal concentration of transferrins which was going to be used in subsequent adhesion and invasion assays. Bacteria ($10^8$ TCID$_{50}$/mL) were incubated at 37 °C for 1 h in BGM culture medium supplemented with transferrin concentrations of 0.005, 0.05, 0.5 and 5.0 mg/mL. Subsequently, bacteria were washed twice (45 000× $g$, 45 min, 4 °C) in PBS and inoculated in BGM cells which are highly sensitive artificial host cells routinely used for *Cp. psittaci* diagnosis [35]. At 36 h post inoculation (p.i.), bacterial replication was quantified by an immunofluorescence assay.

## 2.5. Chlamydial adhesion and invasion assay

Protein stock solutions were diluted in HD11 culture medium supplemented with 5.5 mg/mL glucose (Sigma) to the maximal non-cytotoxic and non-bactericidal concentrations of 0.05 mg/mL ovoTF and 0.5 mg/mL hLF and bLF. Additionally, 0.005 mg/mL ovoTF was tested. All media were filter sterilized (0.22 μm, Millipore). HD11 cells, seeded at 300 000 cells/mL, were grown on sterile glass coverslips (13 mm) at the bottom of Chlamydia Trac bottles (Bibby Sterilin Ltd., Stone, UK) for 24 h at 37 °C and 5% CO$_2$. The cells were inoculated with 20 μL of *Cp. psittaci*

strain 92/1293 ($10^8$ TCID$_{50}$/mL) following standard procedures [35], only without using cycloheximide, since it could inhibit actin polymerization during the entry phase of the bacteria, and inoculation at 4 °C for 15 min (1000× $g$) instead of 37 °C to synchronize irreversible attachment and entry [29]. Monolayers were washed to remove unattached bacteria and culture medium with the appropriate concentrations of transferrins was added. Cells inoculated with PBS served as uninfected, untreated controls. All transferrin concentrations were tested in duplicate and the experiments were performed twice. Inoculated cells were further incubated at 37 °C and 5% CO$_2$ till the bacteria and the actin skeleton were stained at 15 min (optimal timepoint for visualization of actin polymerization as observed in infected nontreated controls) and at 1, 6 and 48 h p.i. by an immunofluorescence assay.

### 2.6. Effect on intracellular replicating *Cp. psittaci*

Non-cytotoxic transferrin concentrations of 0.005 to 5.0 mg/mL ovoTF and 0.005 to 1 mg/mL for both hLF and bLF were added to the HD11 culture medium at 3 h p.i., when all bacteria had entered the cells and binary fission was about to start. The outcome of the infection at 48 h p.i. was analyzed by immunofluorescence staining using a scoring system from 0 to 5 as previously described [34].

### 2.7. Immunofluorescence assays

The bactericidal effect of different transferrins as well as their effect on attachment, invasion and intracellular bacterial replication was examined by immunofluorescence staining. All stainings were performed as follows. The cells were washed twice with PBS and then fixed with 4% paraformaldehyde (Merck, VWR, Leuven, Belgium)

and 120 mM sucrose (Merck) in PBS for 30 min at room temperature. After washing once more, the cells were incubated for 10 min with 50 mM NH$_4$Cl in PBS at room temperature and permeabilized in 0.05% saponin (Sigma) and 1 mg/mL BSA (Sigma) in PBS. *Cp. psittaci* was detected using a rabbit polyclonal antibody against purified *Cp. psittaci* EB, followed by an AlexaFluor 546 labeled goat anti-rabbit conjugate (Molecular Probes) both diluted 1 in 200 in PBS with 1% BSA. At 15 min, 1 h and 6 h p.i., the actin skeleton was visualized using AlexaFluor 488 coupled phalloidin (Molecular Probes, Invitrogen) as described by the manufacturer. Coverslips were mounted using Mowiol (Calbiochem, VWR) with 0.01% p-phenylenediamine (PPD, Sigma) [33].

At all time points, fluorescently labeled bacteria were quantified using an Eclipse TE300 fluorescence microscope (Nikon) provided with a CCD camera (Spot 3.0.1, Diagnostic Instruments Inc.). Briefly, we counted the number of chlamydiae at 15 min p.i. and/or chlamydial inclusions (from 1 h p.i. to 48 h p.i.) in 81 images taken from a single slide and determined the mean. This parameter was designated 'Mean Number of Inclusion forming units', further referred to as MNI. The Tukey HSD test was used for comparison of MNI values over time in the presence or absence of different transferrin concentrations.

Additionally, images of chlamydial attachment and invasion as well as of actin recruitment were acquired using confocal laser scanning microscopy (CLSM) (Radiance 2000, Bio-Rad; 1000×).

## 3. RESULTS

### 3.1. Transferrin cytotoxicity assays

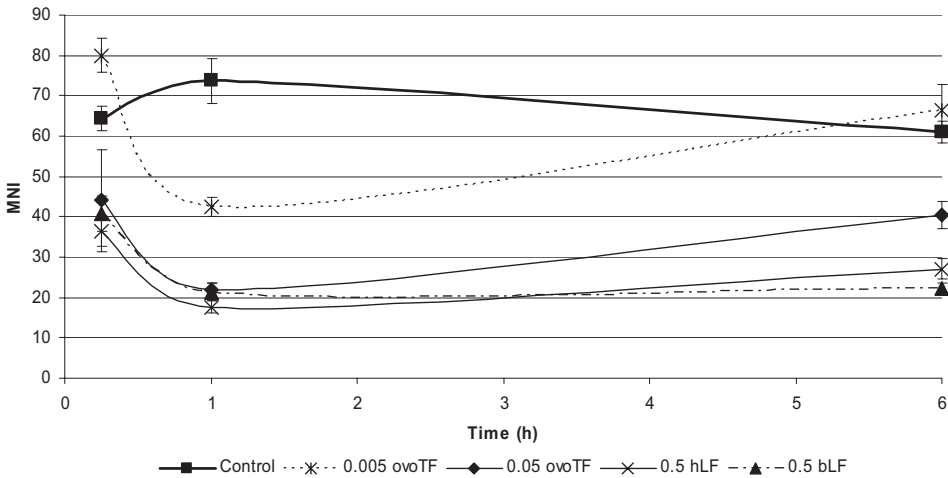A preliminary experiment was carried out to determine the maximal noncytotoxic concentration of ovoTF, hLF and

**Figure 1.** Chlamydial adhesion and invasion assay. Mean Number of Inclusion forming units (MNI ± standard error of the mean) shown from 15 min to 6 h p.i. As expected, at 1 h p.i. more bacteria have irreversibly attached to HD11 cells and subsequently internalized the host cell, when no protein was applied. Treatment with either ovoTF or LF significantly inhibited this process.

bLF for HD11 chicken macrophages. Up to 5 mg/mL ovoTF and up to 1 mg/mL hLF and bLF did not affect any of the cytotoxic parameters at all three time points examined. However, at 36 h p.i., the MTT assay revealed a 25% drop in cellular metabolism for HD11 cells treated with 0.5 mg/mL bLF, increasing to 50% at 48 h p.i.

### 3.2. Effect on infectivity of *Cp. psittaci* for BGM cells

To determine the maximal non-bactericidal concentration of transferrins on *Cp. psittaci*, a pre-incubation experiment was set up exposing *Cp. psittaci* 92/1293 to ten-fold dilutions of ovoTF, hLF and bLF prior to inoculating them on BGM cells. Pre-incubation with 0.5 to 5 mg/mL ovoTF significantly lowered the outcome of the infection at 36 h p.i. (data not shown). For both lactoferrins, 5 mg/mL reduced the infection outcome, albeit not significantly as compared to the

controls. Therefore, the non-bactericidal concentrations used in the following adhesion and invasion assays were 0.005 and 0.05 mg/mL ovoTF and 0.5 mg/mL human or bovine LF. Thus, the bactericidal activity of ovoTF was higher than for human and bovine LF.

### 3.3. Chlamydial adhesion and invasion assay

The effect on bacterial irreversible adhesion and invasion was investigated by addition of non-cytotoxic and non-bactericidal concentrations of ovotransferrin and both lactoferrins at the time of inoculation of chicken macrophages. At 15 min p.i., all transferrin concentrations, except 0.005 mg/mL ovoTF significantly lowered MNI values as compared to untreated bacterial cultures (Fig. 1). At 1 h p.i., all transferrins significantly ($P < 0.05$) inhibited bacterial irreversible attachment and entry and for ovoTF a dose-dependent

inhibition could be demonstrated. At the end of the early stage of the developmental cycle, at 6 h p.i., just before chlamydial replication was about to start, MNI values were significantly ($P < 0.05$) lowered for all transferrins except for 0.005 mg/mL ovoTF, as compared to the untreated control. The inhibitory effects of 0.05 mg/mL ovoTF and 0.5 mg/mL of both LF were equal.

Confocal images, taken at 15 min (Fig. 2b) clearly demonstrated host cell actin polymerization at the bacterial entry site. Inoculation in the presence of non-cytotoxic concentrations (0.005 or 0.05 mg/mL, Fig. 2c) ovoTF significantly inhibited actin polymerization in a dose-dependent manner (Fig. 1). The same was observed at 1 h p.i. (data not shown). Interestingly, addition of bactericidal concentrations of transferrins resulted in the absence of actin polymerization at the bacterial entry site, although the cells were definitely still able to polymerize actin (Fig. 2d).

Focusing on 48 h p.i., both ovoTF concentrations used and 0.5 mg/mL LF significantly diminished the outcome of the infection ($P < 0.05$) in HD11 cells when compared to the control (Tab. I).

Overall, ovoTF was more effective than human and bovine LF in inhibiting HD11 infection since both attachment and invasion were inhibited at a 10-fold lower concentration.

### 3.4. Effect on intracellular replicating *Cp. psittaci*

The addition of non-cytotoxic concentrations of transferrins at 3 h p.i. apparently had no effect on subsequent intracellular chlamydial replication, since scores for all treatments ranged from 4 to 5 (data not shown), as well as for the control (score 5).

## 4. DISCUSSION

Bactericidal effects have previously been described for both ovotransferrin [17, 31] and lactoferrin (reviewed in [32]), but their effect on chlamydial bacteria has not yet been investigated. The bactericidal effect of ovoTF and human and bovine LF on the avian pathogen *Cp. psittaci* was evaluated by incubating *Cp. psittaci* with different concentrations of these natural anti-microbial proteins prior to infecting BGM cells. Therefore, all proteins were iron-saturated to avoid the occurrence of an anti-chlamydial effect by iron-sequestration. All transferrins exhibited anti-chlamydial activity directly towards extracellular organisms, resulting in a lower infection rate of BGM cells as compared to the infection rate established by untreated control bacteria. However, the bactericidal activity of ovoTF was higher than for human and bovine LF. Concentrations of 0.5 mg/mL bLF and hLF were too low to significantly lower the infection rate of BGM cells, while the same amount of ovoTF could do so. There is no obvious explanation for the differences in anti-chlamydial activity. Maybe ovoTF, exclusively found in birds, is simply more adapted to destroy avian pathogens.

*Chlamydiaceae* are believed to mediate their attachment through ligands such as MOMP, hsp70 or OmcB (reviewed in [16]). Considerable evidence suggests that electrostatic interactions are involved in an initial, reversible interaction with the eukaryotic host cell for many, but not all strains and species of chlamydiae [26, 28, 30]. Initial reversible attachment to heparan sulfate-like glycosaminoglycans is followed by irreversible binding to an unknown secondary receptor and this leads to the recruitment of actin to the attachment site, formation of an actin-rich, pedestal-like structure, and finally internalization of the bacteria [10]. Indeed, confocal images taken at 15 min p.i. of HD11 cells in
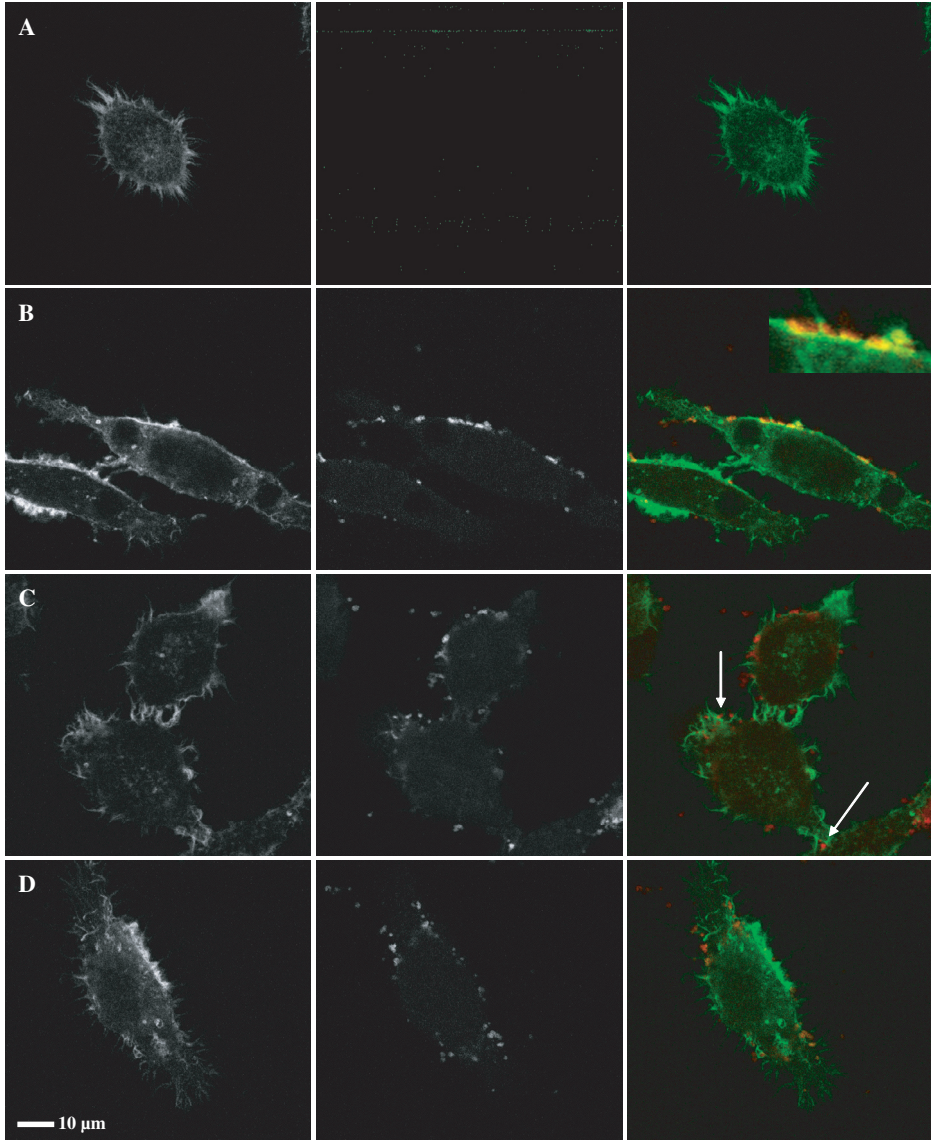
**Figure 2.** Confocal images of infected HD11 chicken macrophages. *Chlamydophila psittaci* 92/1293 was allowed to attach to HD11 cells by centrifugation at 4 °C and whereafter the temperature was shifted to 37 °C for 15 min to allow chlamydial induction of host cell actin polymerization in the presence of lactoferrin or ovotransferrin. Representative confocal images are shown: (a) uninfected control, (b) infected, but untreated control, (c) infection in the presence of 0.05 mg/mL ovotransferrin, (d) infection in the presence of 5 mg/mL ovotransferrin. From left to right: actin staining (green), *Cp. psittaci* staining (red), merged. Arrows indicate zones of decreased actin recruitment when treated with ovotransferrin. (Please consult www.vetres.org for a color version of this figure.)

**Table I.** Mean Number of Inclusion forming units (MNI, ± standard deviation) at 48 h p.i. in the absence (control) or presence of lactoferrin or ovotransferrin.

| Treatment | Mean Number of Inclusion forming units[a] |
|---|---|
| Control | 372 ± 75 |
| 0.005 mg/mL ovoTF | 63[a] ± 27 |
| 0.05 mg/mL ovoTF | 87[a] ± 34 |
| 0.5 mg/mL hLF | 154[a] ± 42 |
| 0.5 mg/mL bLF | 124[a] ± 52 |

[a] Significantly different ($P < 0.05$) from value for untreated control as calculated by the Tukey HSD test.

the absence of transferrins clearly showed co-localization of attached *Cp. psittaci* and underlying actin recruitment, indicating that, as observed earlier for *Chlamydia* (*C.*) *trachomatis*, *C. muridarum*, *Cp. caviae* and *Cp. pneumoniae* [8–10, 29], *Cp. psittaci* also induces a localized host cell actin recruitment upon irreversible attachment. However, as observed for *C. trachomatis* attachment to HeLa cells [29], irreversible attachment of *Cp. psittaci* to HD11 cells was not always accompanied by actin recruitment. The latter can be explained as follows: (1) although we attempted to synchronize irreversible bacterial attachment by centrifugation at 4 °C, synchronized entry could probably not be guaranteed completely, (2) perhaps not all fluorescently stained bacteria were still active and infectious, (3) maybe, as suggested earlier by Subtil et al. [29], actin recruitment is transient, rendering it impossible to observe the induction of actin recruitment for all the invasive bacteria by the presently used immunofluorescence assay staining dead cells and finally, (4) *Cp. psittaci* also uses a microfilament independent entry mechanism, involving clathrin coated pits [37].

The effect of non-cytotoxic and non-bactericidal ovoTF, hLF and bLF concentrations on *Cp. psittaci* irreversible adhesion and subsequent entry in HD11 chicken macrophages was evaluated. Based on a former electron microscopic study on the intracellular developmental cycle of strain 92/1293, time points chosen to calculate the MNI per image represented the attachment phase (15 min p.i.), the attachment and entry phase (1 h p.i.), the end of the internalization phase (6 h p.i.) and the outcome of the infection (48 h p.i.). Overall, non-cytotoxic and non-bactericidal concentrations of ovoTF were more efficient than human and bovine LF in inhibiting *Cp. psittaci* attachment and entry in a homologous cell system, as also shown by confocal images taken of the chlamydial entry 15 min after inoculation. Thus, in this experimental avian *Cp. psittaci* system, ovoTF was more active than bLF or hLF. Analogous results were obtained by Giansanti et al. [14] evaluating the anti-viral activity of ovoTF, hLF and bLF on inhibiting avian Marek's disease virus infection in chicken embryo fibroblast cultures.

Recently, a functional Type III secretion system (T3SS) was described in *Chlamydiaceae*, including *Cp. psittaci*[1], and in *C. trachomatis* and *Cp. caviae*, the translocated actin-recruiting phosphoprotein Tarp was shown to be essential for actin recruitment that coincides with bacterial endocytosis [11]. In the present study, inhibition of *Cp. psittaci* entry by transferrins was accompanied by the reduction

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or even absence of actin recruitment at the bacterial entry site, although the cell was still fully capable to polymerize actin as demonstrated by the presence of cell spreading (Fig. 2d). Since lactoferrin binds LPS in the outer membrane of Gram-negative bacteria, giving the membrane a more rigid character in this way [7], the stability of the T3SS and secretion of Type III secretion effector proteins such as Tarp might be affected in a negative way. Moreover, Ochoa et al. [23, 24] showed that lactoferrin inhibits the attachment of enteropathogenic *E. coli* (EPEC) to host cells as well as actin polymerization by disruption of the EPEC T3SS translocon proteins EspA, B and D. Similarly, treatment of *Shigella flexneri* with lactoferrin diminished the infection of host cells by releasing and subsequently degrading the T3SS translocon proteins IpaB and IpaC, both homologues to the chlamydial CopB and CopD [15]. Based on the sequence and structural homology between human lactoferrin and ovoTF, a destabilizing effect on the T3SS or even a proteolytic effect on chlamydial Type III secretion translocon components might explain the present observations. Currently, experiments addressing the effect of transferrins on recombinantly produced T3SS translocon proteins are in progress.

Focusing on the outcome of the infection at 48 h p.i., both ovoTF concentrations used and 0.5 mg/mL LF significantly diminished the outcome of the infection in HD11 macrophages as could be expected since bacterial attachment and entry was significantly reduced. Moreover, this inhibition of bacterial proliferation was stronger in the presence of ovoTF, thereby indicating a species-specific effect exerted by the proteins studied.

In conclusion, we provided evidence for an anti-chlamydial effect exerted by lactoferrin and its avian homologue ovotransferrin. Our results clearly showed a bactericidal effect of both ovotransferrin and lactoferrins on extracellular *Cp. psittaci*. However, ovotransferrin was apparently more effective in killing *Cp. psittaci*. Moreover, we demonstrated that *Cp. psittaci* internalization in HD11 chicken macrophage cultures is more efficiently inhibited by ovotransferrin than lactoferrins. The inhibition may be due to the blocking of actin recruitment towards the bacterial entry site as a result of destabilizing the bacterial T3SS or of proteolytic degradation of Type III secretion effector proteins. Based on the present findings, ovotransferrin could be used as a natural anti-chlamydial agent. However, further research towards the practical application in preventing *Cp. psittaci* infections in birds is needed.

## ACKNOWLEDGEMENTS

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