Lactoferrin affects the adherence and invasion of *Streptococcus dysgalactiae* spp. *dysgalactiae* in mammary epithelial cells

Fiona O' Halloran[1,*], Christine Beecher[1,2], Valerie Chaurin[1], Torres Sweeney[3] and Linda Giblin[1]

[1]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[2]Department of Biochemistry, University College Cork, Cork, Ireland

[3]School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

Corresponding Author *: Dr Fiona O Halloran
(Present address) Department of Biological Sciences,
Cork Institute of Technology,
Bishopstown,
Cork, Ireland.
Tel: +353-21-4335916
Email: fiona.ohalloran@cit.ie

ABSTRACT

Streptococcus dysgalactiae spp. dysgalactiae is an important causative agent of bovine mastitis worldwide. Lactoferrin is an innate immune protein that is associated with many functions including immunomodulatory, antiproliferative and antimicrobial properties. This study aimed to investigate the interactions between lactoferrin and a clinical bovine mastitis isolate, *S. dysgalactiae spp. dysgalactiae* DPC5345. Initially a deliberate *in vivo* bovine intramammary challenge was performed with *S. dysgalactiae* DPC5345. Results demonstrated a significant difference in lactoferrin mRNA levels in milk cells between the control and infused quarters 7 hours post infusion. Milk lactoferrin levels in the *S. dysgalactiae* DPC5345 infused quarters were significantly increased compared to control quarters at 48 h post infusion. *In vitro* studies demonstrated that lactoferrin had a bacteriostatic effect on the growth of *S. dysgalactiae* DPC5345 and significantly decreased the ability of the bacteria to internalise into HC-11 mammary epithelial cells. Confocal microscopy images of HC-11 cells exposed to *S. dysgalactiae* and lactoferrin further supported this effect by demonstrating reduced invasion of bacteria to HC-11 cells. The combined data suggests that a bovine immune response to *S. dysgalactiae* infection includes a significant increase in lactoferrin expression *in vivo* and, based on *in vitro* data, lactoferrin limits mammary cell invasion of this pathogen by binding to the bacteria and preventing its adherence.

Key words: Lactoferrin, *Streptococcus dysgalactiae spp. dysgalactiae*, mastitis

INTRODUCTION

Bovine mastitis infection has serious deleterious effects on milk quality and is a significant limiting factor to profitable dairy farming worldwide (Bar et al., 2008; Berry and Amber, 2005; Kossaibati and Esslemon, 1997). Mastitis infection occurs when a bacterial pathogen gains entry to the mammary gland via the teat canal. Infection is then established through bacterial evasion of both the anatomical and humoral defence mechanisms of the mammary gland (Zhao and Lacasse, 2008)

Streptococcus dysgalactiae subspecies *dysgalactiae* (*S. dysgalactiae*) has been described as both a contagious mastitis pathogen (Fox et al., 1993) and an environmental pathogen (Calvinho et al., 1998) and has been detected in clinical and subclinical bovine mastitis cases (Girma et al., 2012; Bitew et al., 2010; Piepers et al., 2007; Barrett et al., 2005). Traditional mastitis control programmes have had limited effects on reducing the incidence of *S. dysgalactiae* infections due to the adaptability of the bacteria (Bolton et al., 2004). *S. dysgalactiae* have cell-associated and extracellular virulence factors which contribute to the establishment and persistence of intramammary infection (IMI). *In vitro* studies have demonstrated that the bacteria can adhere to mammary epithelial cells and internalise (Calvinho and Oliver, 1998a; Almeida and Oliver, 1995). Once internalised within the cell, the bacteria can survive by evading the action of antimicrobial drugs and host immune defence components.

Conventional treatment for mastitis involves the administration of antibiotics. Antimicrobial resistance in mastitis pathogens is not yet considered a clinically relevant issue (Oliver and Murinda, 2012; Pyorala, 2009), however, the intensive use of antibiotics can lead to the development of resistant strains (Hendriksen, 2008). In addition, dairy cows produce milk for human consumption, therefore, antibiotic traces in milk is a public health concern. The development of effective, alternative approaches to control and/or treat mastitis would be

of significant benefit to the dairy industry. One approach would be to manipulate and enhance natural host defence mechanisms. However, different pathogens display distinct invasion and pathogenic mechanisms (Chaneton et al., 2008). Detailed knowledge of the specific host-pathogen interactions that are required to establish and develop IMI is fundamental to the design of adequate treatment and control policies.

Lactoferrin is an innate immune protein that is predominantly secreted *in vivo* by neutrophils and specific glandular tissue (Ling and Schryvers, 2006). Its production is both constitutively and differentially regulated in a tissue-specific manner (Teng et al., 2002). Lactoferrin is a multifunctional protein being associated with antibacterial, immunomodulatory, antiadhesive and iron-binding properties (Chierici, 2001; Ueta et al., 2001; Ajello et al., 2002). It is described as broad spectrum, affecting the growth and proliferation of a variety of gram negative and gram positive pathogens (Adlerova et al., 2008) with both bacteriostatic and bacteriocidal effects (Kutila et al., 2003). Bovine milk lactoferrin levels change over the course of lactation, as well as in response to mammary gland infections (Kawai et al., 1999). Bahar et al (2011) also demonstrated that genetic polymorphisms within the lactoferrin gene promoter can affect milk lactoferrin levels.

In general, streptococcal species are considered more resistant to the antibacterial effects of lactoferrin (Fang et al., 2000). This may be due to the fact that streptococcal species have low iron requirements (Weinberg, 1978) or it may be due to their ability to extract the metal from iron-containing host proteins (Ruiguang et al., 2009). Indeed lactoferrin binding proteins have been found in several streptococcal species, including *S. uberis, S. agalactiae* and *S. dysgalactiae* (Park *et al.*, 2002a), suggesting a role for lactoferrin in relation to streptococcal infections, which may have important implications for the pathogenesis of IMI. Patel et al (2009) demonstrated that *S. uberis* isolates bind lactoferrin via a surface-exposed adhesion molecule which they defined as the *S. uberis* adhesion molecule (SUAM). These *in*

vitro studies suggest that S. uberis hijacks lactoferrin to act as a bridging molecule between the bacterial cell surface and lactoferrin receptors on host mammary epithelial cells. This allows *S. uberis* to adhere, invade and establish within bovine mammary epithelial cells. Interestingly *in vivo* bovine studies by Chaneton et al (2008) demonstrated that in subclinical cases of mastitis, bovine milk lactoferrin levels significantly increased in *S. uberis* infected quarters ($P<0.001$) when compared to uninfected quarters. It was hypothesized that *S. uberis* isolates were resistant to bovine lactoferrin activity and high levels of the lactoferrin in milk promoted the pathogenesis of *S. uberis in vivo.* Other mastitis-causing bacteria, including *S. aureus, S. dysgalactiae* and coagulase negative staphylococcus were not associated with a significant increase in bovine milk lactoferrin levels in these subclinical cases. (Almeida et al., 2006). These studies suggest that lactoferrin secretion in the bovine mammary gland is a pathogen-specific response.

Park et al (2002b), using SDS-PAGE and western blotting, confirmed the expression of two lactoferrin binding proteins in *S. dysgalactiae* isolates (52- and 74KDa) and three lactoferrin binding proteins in *S. agalactiae* isolates (52-,70- and 110 KDa). However, studies on the specific interaction between these streptococcal species and lactoferrin is limited and thus the role of lactoferrin binding proteins in the pathogenesis of *S. dysgalactiae* and *S. agalactiae* intramammary infections must still be elucidated.

The aim of this study was to investigate the interaction *in vitro* between lactoferrin and *S. dysgalactiae* in order to identify whether this milk protein combats or promotes *S. dysgalactiae* infection. *In vivo* studies were conducted to quantify the changes in milk lactoferrin levels over the course of a mammary gland infection caused by a deliberate challenge with *S. dysgalactiae*

MATERIALS AND METHODS

Bacterial Culture

Streptococcus. dysgalactiae subspecies *dysgalactiae* (*S. dysgalactiae*) DPC 5345 was isolated on a Teagasc farm from a clinical mastitis case. The isolate was confirmed as streptococcus by selective plating on blood-esculin agar plates and characterised to species level by 16s PCR (Riffon et al, 2001). A 2 % inoculum of *S. dysgalactiae* DPC 5345 yielded 10^8 colony forming units (CFU) /ml when cultured overnight at 37 ^oC in tryptic soy (TS) broth (Difco Laboratories, Detroit, USA).

Animal selection

Five Holstein-Friesian cows were selected, based on their health status, for a deliberate intramammary challenge with *S. dysgalactiae* DPC 5345. These were spring-calving cows that were based on Teagasc research stations in the south of Ireland. Health status was determined using visual examination of milk and udders and milk somatic cell counts (SCC), with a count of <200,000 cells/ml considered as healthy. In addition, no pathogens were isolated from the quarter milks of test animals for 7 consecutive days prior to the challenge. Bacterial infusions and milk and blood sampling were performed under licence from the Irish Department of Agriculture and Food and approved by the Teagasc ethical committee. Following infusions, the health of the animals was monitored by trained farm staff and veterinary personnel. For animal welfare purposes, intramammary-challenged cows received antibiotic treatment (Leo Yellow; Boehringer Ingelheim, Berkshire, UK) as soon as clinical signs of infection were observed.

Intramammary challenge

The intramammary challenge with *S. dysgalactiae* DPC5345 was performed according to a procedure described by Beecher *et al*. (2012). Briefly, overnight cultures of *S. dysgalactiae* DPC5345 were diluted with maximum recovery diluent (MRD; Oxoid Ireland, Dublin, Ireland) to prepare 2 ml suspensions at 2500 CFU *dysgalactiae*. This bacterial count was chosen as preliminary trials indicated that 2500 CFU is sufficient to cause infection (Beecher et al., 2012). Immediately after morning milking, one quarter from each animal was infused with the bacterial suspension into the teat sinus via the streak canal. The contra-lateral quarter was infused with 2 ml of MRD only and served as the internal control. Total quarter milk samples (or up to 2 L volume) were collected from each infused and control quarter immediately prior to infusion and at 7 h, 24 h, 48 h, 7 days and 14 days post infusion**.** From each milk sample 100 μL was plated onto esculin blood agar plates (Thermo Scientific, Wilmington, DE, USA) to monitor bacterial infection. Milk somatic cell counts were also determined using a Somacount 300® somatic cell counter (Bently instruments Inc., Chaska, MN, USA). For animal welfare purposes, intramammary-challenged cows received antibiotic treatment (Leo Yellow; Boehringer Ingelheim, Berkshire, UK) as soon as clinical signs of infection were observed.

RNA extraction from somatic cells

To isolate RNA from milk somatic cells, 1 ml 0.5 M EDTA (Sigma-Aldrich, Ireland) was added to each litre of milk and the samples were then centrifuged at 1500 x *g* for 30 min at 4 °C. The fat layer was removed and cell pellets were washed twice with PBS (pH 7.4) containing 0.5 mM EDTA. Pellets were then re-suspended in 1 ml TriPure isolation reagent (Roche Diagnostics, East Sussex, UK) and RNA was extracted according to the manufacturers' instructions. All RNA was quality assessed through visualisation on glyoxyl

gels (1.5 %) (Ambion, Applied Biosystems, Foster City, USA) and quantified using the Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA).

Real-time PCR

One microgram of RNA was converted to cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Crawley, West Sussex, UK), and 1 µl cDNA was used per subsequent real-time PCR reaction. Primers designed by Li et al. (2009) were used to detect and amplify the *lactoferrin* gene (forward primer 5'-AAACAAGCATCGGGATTCCAG-3' and reverse primer 5'-ACAATGCAGTCTTCCGTGGTG-3'). Quantitative analysis was performed in a LightCycler 480 instrument (Roche Diagnostics, East Sussex, UK) using a dilution series of external plasmid DNA standards (10^9 - 10^2 copies/µl) (Pfaffl, 2001). Plasmid standards were created by cloning a cDNA PCR product into plasmid pCR TOPO (Invitrogen, Life Technologies, California,USA). Cloning was confirmed by sequencing (Beckman Coulter Genomics, Essex, UK). One microlitre of each dilution was used per 10 µl LightCycler reaction. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, East Sussex, UK) was used for quantification according to the manufacturer's instructions using 0.5 µM of each of the forward and reverse primers. The assay began with initial denaturation at 95°C for 10 min, followed by 40 cycles of quantification with 5 s denaturation at 95 °C, 10 s annealing at 60 ºC and 25 s elongation at 72 °C. Melting curve analysis was performed by heating, from a temperature 5 °C above the annealing temperature, to 95 °C in the continuous fluorescence acquisition mode to ensure specificity of LightCycler product. LightCycler runs were performed in triplicate incorporating negative controls (no DNA template) in each run and according to MIQE guidelines (Bustin et al., 2009).

Protein quantitation by ELISA

Milk lactoferrin was quantitated using an ELISA kit (Bethyl Laboratories Inc., Montgomery Texas, USA) and according to the manufacturers' instructions. Milk samples were diluted 1:2000 and standard curves generated for each sample set. Minimum detection levels for lactoferrin were 7.8 ng/ml lactoferrin concentrations were quantified by interpolating the absorbance values from the standard curve. Test samples were analysed by ELISA in duplicate and the analysis performed on 2 separate occasions.

Antibacterial assay

The antibacterial activity of a commercial bovine native lactoferrin protein (#L9507, Sigma-Aldrich, Ireland) was tested against *S. dysgalactiae* DPC5345. For this, an overnight culture of *S. dysgalactiae* DPC 5345 was diluted to 10^7 CFU/ml in TS broth (Oxoid, Ireland) and 40 µl of the culture added to each test well in a 96 well plate. The lactoferrin protein was initially prepared at a stock of 20 mg/ml by diluting in distilled water and sterile filtered using 0.45 µm filters (Millipore, UK). The final concentrations of lactoferrin tested were 0.02, 0.2, 2.0, 5.0 and 10.0 mg/ml. Plates were incubated at 37 °C and growth curves generated by measuring absorbance at 620 nm every hour for 24 h using a spectrophotometer. Assays were performed in triplicate.

Cell line and tissue culture

The HC-11 murine mammary epithelial cell line used in this study was kindly donated by Dr. Wolfgang Doppler (Innsbruck Medical University, Austria). Cells were grown and maintained in RPMI 1640 media (Sigma-Aldrich, Ireland) supplemented with 4 mM L-Glutamine (Sigma-Aldrich), 10 % foetal bovine serum (FBS; Sigma-Aldrich, Ireland),

5 μg/ml insulin (Sigma-Aldrich), 10 ng/ml epidermal growth factor (EGF, Sigma-Aldrich, Ireland), 100 U/ml penicillin (Sigma-Aldrich, Ireland) and 100 μg/ml streptomycin (Sigma-Aldrich, Ireland).

Invasion assays with S. dysgalactiae

Invasion assays were performed on the same day, in triplicate, as per Calvinho and Oliver (1998b) with some minor modifications. Briefly, HC-11 cells were seeded into 24-well plates at a density of 1 x 10^5 cells/well and grown to confluency in antibiotic-free RMPI 1640 supplemented media. An overnight culture of *S. dysgalactiae* DPC 5345 was washed 3 times in phosphate buffered saline (PBS; Sigma-Aldrich, Ireland) and re-suspended in antibiotic-free RPMI 1640 supplemented media, to a final concentration of ~1 x 10^7 CFU/ml. On the day of the assay, HC-11 cell monolayers were washed 3 times in PBS and 1 ml aliquots of the bacterial culture (1 x 10^7 CFU/ml) was added to each test well. Sterile-filtered solutions of lactoferrin, prepared with antibiotic-free RPMI 1640 supplemented media, were added (1 ml) to test wells such that the final concentrations tested were 0.01, 0.1, 1.0 and 2.0 mg/ml lactoferrin. Control wells contained *S. dysgalactiae* DPC5345 culture and 1 ml PBS only. Plates were incubated at 37°C, 5% CO_2 for 2 h. Supernatants were aspirated off into sterile 1.5 ml eppendorf tubes and diluted 10-fold for bacterial counting. Culture wells were then washed 3 times with PBS (1 ml), followed by incubation for a further 2 h in RPMI 1640 supplemented media containing 5 ug/ml penicillin and 100 U/ml gentamicin. Following the second incubation, media was removed and cells were washed 3 times with 1 ml PBS. HC-11 cells were then lysed by adding 0.25 % trypsin (Sigma-Aldrich, Ireland) and 0.025 % Triton X-100 (Sigma-Aldrich, Ireland) and the lysates collected into sterile 1.5 ml eppendorfs and vigorously vortexed for 15 seconds. Cell lysates were serially diluted 10-fold (10^{-1} to 10^{-4})

and 0.1 ml dilutions plated onto TS agar plates for bacterial counting and incubated at 37°C overnight. Adherence, at lactoferrin concentrations tested, was expressed as (CFU/ml in the cell lysates) / (CFU/ml in the supernatant) X 10,000 (Fang et al., 2000). Each lactoferrin concentration was tested in duplicate and the assays performed on four separate occasions.

Confocal Scanning Microscopy and Live/Dead staining

For confocal microscopy experiments, HC-11 cells were seeded onto 10 cm diameter tissue culture dishes (Sarstedt, Drinagh, Wexford, Ireland), at a concentration of 1 x 10^6 cells/well, and grown to confluency. A fresh overnight bacterial culture of *S. dysgalactiae* DPC5345 was washed 3 times with PBS and prepared in antibiotic-free RPMI 1640 supplemented media to a final concentration of ~1 x 10^7 CFU/ml. A 2 mg/ml protein preparation of commercially available bovine Lactoferrin was prepared in PBS and labelled with a Dylight 633 Fluorescent dye using an Alexa Fluor 350 Protein labelling kit (Thermo Fisher Scientific, Dublin, Ireland), according to the manufacturers' instructions. One ml of the labelled protein solution was mixed with 1 ml of fresh bacterial culture (~10^7 CFU/ml) and added to cell culture dishes containing confluent HC-11 monolayer of cells. Controls included HC-11 monolayers exposed to (a) 2 ml antibiotic-free RPMI 1640 supplemented media only and (b) 1 ml antibiotic-free RPMI 1640 supplemented media mixed with 1 ml bacterial culture (~10^7 CFU/ml). All cell culture plates were incubated for 2 h at 37 °C, 5 % CO_2 and then the media removed before washing twice with PBS (5 ml).

Before imaging, cells were stained with the Live/Dead BacLight Bacterial Viability Kit solution (Molecular Probes, Inc., Eugene, OR, USA). An argon ion laser was used to generate the 488 line used for excitation. Imaging was performed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Heildelberg GmbH, Mannheim, Germany) using a 63 x oil immersion objective (A_N = 1.4). RGB colour images (24 bit), 512

x 512 pixels in size, were acquired. A minimum of 4 z-stacks were taken per sample with representative cross sections of micrographs shown.

Statistical Analysis

For statistical analysis, gene expression data was transformed by obtaining the natural logarithm. A hierarchical mixed model (PROC MIXED; SAS Version 9.1, SAS Institute Inc., Cary, NC, USA) was used to quantify the effect of treatment (control or infused) on gene expression and the dependent variable was transformed gene expression.. Fixed effects included in the model were time relative to the start of the study, treatment and time by treatment interaction. Where significant ($P < 0.05$), a covariate, which was the gene expression for the control and infused quarters at time zero, was included as a fixed effect. Time relative to the start of experiment was included as a repeated effect within the udder quarter, and cow was included as a random effect. Least squares means were extracted from the analysis and differences between the control and infused quarters were considered significant at $P < 0.05$. With regards to the milk protein secretion data statistical analysis was performed as above, without transformation, as data was normally distributed. As with gene expression data differences between the control and infused quarters were considered significant at $P < 0.05$.

For the antibacterial assay, a paired Student's T-test was employed to investigate the effect of different lactoferrin concentrations on bacterial growth. The bacteriostatic effect of each concentration tested was considered significantly different from the control at $P < 0.05$.

A paired Student's T-test was also employed in the invasion assay to identify differences in bacterial counts recovered. The number of bacteria recovered from different experiments were considered significantly different at $P < 0.05$.

RESULTS

The effect of a deliberate intramammary challenge with *S. dysgalactiae* on lactoferrin levels *in vivo* was investigated to determine if the bovine immune response to this mastitis pathogen included alterations to the transcription and/or secretion of this antibacterial protein.

Bacterial growth in infected quarters post intramammary challenge

Following the infusion of experimental quarters with 2500 CFU *S. dysgalactiae* DPC5345 viable gram-positive bacteria were recovered within 24 h from all quarters infused. Pulsed-field gel electrophoresis (PFGE) confirmed *S. dysgalactiae* DPC5345 (data not shown). As soon as clinical signs of infection were observed (rectal temperartures > 38.5 ◦C, swollen udders and SCC > 200,000) animals received antibiotic (Leo Yellow) treatment. Four of the animals received antibiotic treatment 48 h post infusion and one animal received antibiotics at 72 h post infusion. Data post antibiotic treatment was discounted and not included in the analysis.

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In vivo levels of lactoferrin mRNA and protein following intramammary challenge

To investigate if lactoferrin gene expression and / or lactoferrin protein levels in milk were affected by the deliberate *S. dysgalactiae* DPC5345 intramammary challenge lactoferrin mRNA and protein levels were measured in the control quarters and the infused quarters at 0, 7, 24 and 48 h post infusion. Control quarters acted as internal controls for each animal. There was a significant difference ($P < 0.05$) in lactoferrin mRNA levels between the control and infused quarters at 7 h post infusion (Table 1) with lactoferrin mRNA levels reduced in the infused quarter. At all other time points tested, lactoferrin mRNA levels did not significantly differ between the control and infused quarters (Table 1). Lactoferrin protein

levels were significantly increased ($P < 0.05$) in infused quarters compared to control quarters at 48 h post infusion (Table 2)

Antibacterial activity

To investigate the influence of a commercial preparation of bovine lactoferrin on the growth of the *S. dysgalactiae* culture DPC5345 in a laboratory setting a 24 h growth curve was performed. DPC5345 was cultured in complex media with various concentrations of lactoferrin. Results from the 24 h growth curves demonstrated that lactoferrin had a bacteriostatic effect on *S. dysgalactiae* DPC5345 (Figure 1). At 14 h ≥ 0.2 mg/ml lactoferrin had a significant effect on bacterial growth ($P < 0.05$) when compared to the control (no lactoferrin).

Invasion assay and Confocal Imaging

To examine if lactoferrin influenced the ability of *S. dysgalactiae* DPC5345 to invade epithelial cells, an invasion assay was performed using the HC-11 murine mammary epithelial cell line. HC-11 cell monolayers were incubated with ~1 x 10^7 CFU/ml of fresh bacterial culture and a range of physiologically relevant concentrations of lactoferrin from 0.01-2.0 mg/ml (Schanbacher et al., 1993). After 4 h incubation lactoferrin reduced the number of live bacteria in the cell lysate in a dose dependent manner with significant reductions associated with 0.1, 1.0 and 2.0 mg/ml lactoferrin compared to the negative control. At 2 mg/ml of lactoferrin, more than a log fold reduction in the number of internalised bacteria (4.1 log10 CFU/ml) was observed compared to the negative control (5.52 log10 CFU/ml) and 0.01 mg/ml lactoferrin (5.34 log10 CFU/ml (Table 3). The adherence index value was calculated by dividing the number of bacterial CFUs in the cell lysate by the number of CFUs in the supernatant and therefore gives an indication of the

ability of the bacteria to adhere and internalise into HC-11 cells. In the absence of lactoferrin, the adherence index value was 37.72. With each increasing lactoferrin concentration (0.01, 0.1, 1.0 and 2.0 mg/ml) the adherence index value reduced (32.5, 20.67, 13.54 and 3.35 respectively) in a dose dependent manner (Table 3), demonstrating decreased internalisation. This effect was visually confirmed by confocal microscopy (Figure 2) which showed that in the absence of lactoferrin viable *S. dysgalactiae* bacteria internalised into the mammary HC-11 cells (Figures 2A and 2B respectively). When the Dylight 633 fluorescently labelled lactoferrin (~2 mg/ml) was added to the HC-11 cells, the labelled protein bound to the bacteria at HC-11 cell membrane boundaries (Figure 2C) and also to the bacteria that had internalised within the HC-11 cells (Figure 2D). These microscopic images indicate that *S. dysgalactiae* bacteria and the lactoferrin protein co-localise.

DISCUSSION

A deliberate bovine intramammary challenge with *S. dysgalactiae* resulted in a significant increase ($P < 0.05$) in milk lactoferrin protein compared to controls. Follow up *in vitro* studies demonstrated that lactoferrin is a bacteriostatic agent that interferes with the ability of *S. dysgalactiae* to adhere and internalise into mammary epithelial cells. Co-localisation images suggest a direct interaction between lactoferrin and *S. dysgalactiae* cells demonstrating that this bacteria binds to the lactoferrin protein. The combined data indicates that a bovine immune response to *S. dysgalactiae* infection includes increased milk lactoferrin levels and this immune protein appears to limit mammary cell invasion of this pathogen by binding to the bacteria and reducing host cell adherence.

For some mastitis pathogens it has been shown that lactoferrin promotes the pathogenicity of the bacteria. Patel *et al.*, (2009) demonstrated, *in vitro*, that lactoferrin served as a molecular bridge for *Streptococcus uberis* (*S. uberis*) species, promoting bacterial internalisation into mammary epithelial cells and potentially providing an opportunity for this pathogen to survive against host defence mechanisms. In contrast to this our study indicates that lactoferrin plays a role in preventing infection by *S. dysgalactiae* species by interfering with bacterial adhesion of the mammary epithelial cell. This has significance with regards to establishing intramammary infections by this pathogen, as adherence is an important stage in the pathogenesis of bovine mastitis (Calvinho et al., 1998).

The main bacteriostatic effect of lactoferrin is thought to be due to its iron-sequestering ability. When iron is depleted from the environment it limits the growth of pathogens (Lacasse et al., 2008). However, streptococcal species have a low iron requirement (Fang et al., 2000) and therefore alternative mechanisms must exist by which lactoferrin is bacteriostatic against streptococci. Park et al (2002a) demonstrated that, similar to *S. uberis* species, *S. dysgalactiae* express lactoferrin binding proteins (LFBPs). The existence of these

proteins facilitates an interaction between lactoferrin and *S. dysgalactiae* cells (Park et al 2002b) but the mechanism of the interaction and the physiological effect is unknown. Barboza et al. (2012) demonstrated that the glycan component of lactoferrin plays an essential role in pathogen recognition and inhibits bacterial binding to host epithelial cells. Other studies have confirmed that when lactoferrin binds with bacterial proteins it is capable of interfering with essential bacterial processes. Ochoa et al (2003) and Gomez et al (2003) have shown that lactoferrin can inhibit the function of type III secretory systems in the gram negative pathogens enteropathogenic *E. coli* and *Shigella flexneri* respectively. These secretory systems play a significant role in the adherence and invasion of these gram negative pathogens into mammalian cells. Lactoferrin was shown to inhibit pathogen adherence to host cells via a mechanism that involved proteolytic degradation of essential proteins required for bacterial contact and pore formation (Ochoa et al., 2003). Results from the current study demonstrate that lactoferrin is bacteriostatic against *S. dysgalactiae* species which could be due to the presence of these LFBPs. Further characterisation of these proteins within *S. dysgalactiae* is required and would offer more insight into the mechanism of interaction between this streptococcal species and lactoferrin. This would help further elucidate the role that lactoferrin plays in limiting mastitis infection caused by *S. dysgalactiae* and could potentially lead to alternative control strategies for mastitis caused by this pathogen. Although it could be argued that only one strain was examined in the present study, Park et al (2002b) demonstrated that there was no difference in the lactoferrin binding capacity of several *S. dysgalactiae* species investigated.

Lactoferrin mRNA levels were reduced in the early period of invasion of *S.dysgalactiae*. Lactoferrin protein levels did not differ within this time period but later, at 48 h post-infusion, there was a statistically significant increase ($P<0.05$) in the average milk lactoferrin levels from the infused quarters compared to the control quarters. Bovine milk

lactoferrin levels are reported in general to be between 20 - 200 mg/L (Rainard and Riollet, 2006) but levels vary from one animal to another, vary in mammary quarters and as lactation progresses the mean lactoferrin concentration increases (Cheng et al., 2008). In some cases of mastitis levels also increase (Kawai et al., 1999). Chaneton et al. (2008) reported that in subclinical bovine mastitis cases, when the individual contribution of each pathogen was investigated, *S. dysgalactiae* infections were not associated with a significant increase in milk lactoferrin levels. In clinical mastitis cases where there was a significant increase in milk lactoferrin, the causative bacteria was not known. Whether the increase in milk lactoferrin levels observed in this study is biologically significant warrants further investigation. Mammary epithelial cells not only sense bacterial pathogens but also recruit neutrophils to the site of infection (Rainard and Riollet, 2006; Strandberg et al., 2005). This boosts the local concentration of lactoferrin protein as it is released from neutrophil secondary granules (Zhao and Lacasse, 2008). The increased levels of lactoferrin concentration at 48 h post infusion may therefore be as a result of neutrophil recruitment or delayed lactoferrin production from mammary epithelial cells or both.

The results from this study further emphasise that the interaction between the milk protein lactoferrin and bacteria is species-specific. Further studies are required to characterise this interaction and understand the host-pathogen response in *S. dysgalactiae* infections to pave the way for the development of bacterial targeted mastitis therapies and prophylactics.

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REFERENCES

Adlerova, L., A. Bartoskova, and M. Faldyna, M. 2008. Lactoferrin: a review. Veterinarni Medicina 53: (9) 457-468.

Ajello, M., R. Greco, F. Giansanti, M.T. Massucci, G. Antonini, and P. Valenti. 2002. Anti-invasive activity of bovine lactoferrin towards group B streptococci. Biochem. Cell Biol. 80: 119-124.

Almeida, R.A., and S.P. Oliver. 1995. Invasion of bovine mammary epithelial cells by *Streptococcus dysgalactiae*. J. Dairy Sci. 78:1310-1317.

Bahar, B., F. O'Halloran, M.J. Callanan, S. McParland, L. Giblin, and T. Sweeney. . 2011. Bovine lactoferrin (LTF) gene promoter haplotypes have different basal transcriptional activities. Anim Genet. 42(3):270-279.

Bar, D., L.W. Tauer, G. Bennett, G.,R.N. González, J.A. Hertl, Y.H. Schukken, H.F. Schulte, F.L. Welcome, and Y.T. Gröhn. 2008. The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming. J Dairy Sci. 91(6):2205-14.

Barboza, M., J. Pinzon, S. Wickramasinghe, J.W. Froehlich, I. Moellert, J.T. Smilowitz, L.R. Ruhaak, J. Huang, B. Lonnerdal, J.B. German, J.F. Medrano, B.C. Weimer and C.B. Lebrilla. 2012. Glycosylation of human milk lactoferrin exhibits dynamic changes during early lactation enhancing its role in pathogenic bacteria-host interactions. Mol & Cell Proteomics, 11(6):M111.015248.

Barrett, D.J., A.M. Healy, F.C. Leonard, and M.L. Doherty. 2005. Prevalence of pathogens causing subclinical mastitis in 15 dairy herds in the Republic of Ireland. Irish Vet J. 58(6); 333-337.

Beecher, C,, M. Daly, R.P. Ross, J. Flynn, T.V. McCarthy, and L. Giblin. 2012. Characterization of the bovine innate immune response in milk somatic cells following intramammary infection with *Streptococcus dysgalactiae subspecies dysgalactiae*. J Dairy Sci. ;95 (10):5720-9.

Berry, D., and P. Amber. 2005. Derivation of a health sub-index for the economic breeding index in Ireland. Irish Cattle Breeding Federation Technical report. pp1-5

Bitew, M., A. Tafere, and T. Tolosa. 2010. Study on bovine mastitis in dairy farms of Bahir Dar and its environs. J Anim and Vet Adv. 9(23): 2912-2917.

Bolton, A., X.M. Song, P. Willson, M.C. Fontaine, A.P. Potter, and J. Perez-Casal. 2004. Use of the surface proteins GapC and Mig of *Streptococcus dysgalactiae* as potential protective antigens against bovine mastitis. Can. J. Micro. 50(6): 423-432.

Bustin, S. A., V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, and C.T Wittwer. 2009. The MIQE Guidelines: Minimum information for publication of quantitative Real-Time PCR experiments. Clin Chemistry, 55 (4): 611-622.

Calvinho, L.F., R.A. Almeida, and S.P. Oliver. 1998. Potential virulence factors of *Streptococci dysgalactiae* associated with bovine mastitis. Vet. Micro. 61:93-110.

Calvinho, L. F., and S. P. Oliver. 1998a. Invasion and persistence of *Streptococcus dysgalactiae* within bovine mammary epithelial cells. J Dairy Sci 81(3): 678-86.

Calvinho, L. F., and S. P. Oliver 1998b. "Characterization of mechanisms involved in uptake of *Streptococcus dysgalactiae* by bovine mammary epithelial cells." Veterinary Microbiology 63(2-4): 261-274.

Chaneton, L., L. Tirante, J. Malto, J. Chaves, and L.E. Bussmann. 2008. Relationship between milk lactoferrin and etiological agent in the mastitic bovine mammary gland. J. Dairy Sci. 91:1865-1873.

Cheng, J.B., J.Q. Wang, D.P. Bu, G.L. Liu, C.G. Zhang, H.Y. Wei, L.Y. Zhou, and J.Z. Wang. 2008. Factors affecting the lactoferrin concentrations in bovine milk. J. Dairy Sci. 91:970-976.

Chierici, R. 2001. Antimicrobial action of lactoferrin. Adv Nutr. Res. 10: 247-268.

Fang, W., R.A. Almeida, and S.P. Oliver. 2000. Effects of lactoferrin and milk on adherence of *Streptococci uberis* to bovine mammary epithelial cells. Am. J. Vet Res. 61(3): 275-279.

Fox, L.K., and J.M. Gay. 1993. Contagious mastitis. Vet Clin North Am Food Animal Pract. 9(3): 475-87.

Gomez, H.F., T.J. Ochoa, L.G. Carlin, and T.G. Cleary. 2003. Human lactoferrin impairs virulence of *Shigella flexneri.* J. Infect Dis. 187: 87-95.

Girma, S., A. Mammo, K. Bogele, T. Sori, F. Tadesse, and T. Jibat. 2012. Study on prevalance of bovine mastitis and its major causative agents in West Harerghe zone, Doba district, Ethiopia. J Vet Med and Animal Health. 4(8): 116-123.

Hendriksen, R.S., D.J. Mevius, A. Schroeter, C. Teale, D. Meunier, P. Butaye, A. Franco, A. Utinane, A. Amado, M. Moreno, C. Greko, K. Stärk, C. Berghold, A.L. Myllyniemi, D. Wasyl, M. Sunde, and F.M. Aarestrup. 2008. Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. Acta Vet Scand. 50, 28 [Online]. Avaliable from www.actavetscand.com/content/50/1/28

Hogan, J. S., K.L Smith, K.H. Hoblet, P.S. Schoenberger, D.A. Todhunter, W.D. Hueston, D.E. Protchard, G.L. Bowman, L. E. Heider, B.L. Brockett, and H.R. Conrad. 1989. Field survey of clinical mastitis in low somatic cell count herds. J. Dairy Sci. 72:1547-1556

Kawai, K., S. Hagiwara, A. Anri, and H. Nagahata. 1999. Lactoferrin concentration in milk bovine clinical mastitis. Vet. Res. Comm. 23 (7): 391-398.

Kossaibati, M., and R. Esslemon. 1997. The cost of production diseases in dairy herds in England. The Vet J, 154: 41-51.

Kutila, T., S. Pyorala, H. Saloniemi, and L. Kaartinen. 2003. Antibacterial effect of bovine lactoferrin against udder pathogens. Acta. Vet. Scand. 44:43-45.

Li , Y., G.V. Limmon, F. Imani, and C. Teng. 2009. Induction of lactoferrin gene expression by innate immune stimuli in mouse mammary epithelial HC-11 cell. Biochimie. 91 (1):58-67.

Lacasse, P., K. Lauzon, M.S. Diarra, and D. Petitclerc. 2008. Utilization of lactoferrin to fight antibiotic-resistant mammary gland pathogens. J. Anim. Sci, 86 (Suppl 1):66-71.

Ling, J.M.L., and A.B. Schryvers. 2006. Perspectives on interactions between lactoferrin and bacteria. Biochem Cell Biol. 84: 275-281.

Ochoa, T.J., M. Noguera-Obenza, F. Ebel, C.A. Guzman, H.F. Gomez, and T.G. Cleary. 2003. Lactoferrin impairs type III secretory system function in enteropathogenic *Escherichia coli.* Infect Immuno. 71:5149-5155.

Oliver, S.P., and S.E. Murinda. 2012. Antimicrobial resistance of mastitis pathogens. Vet Clin North Am Food Anim Pract. 28 (2): 165-85.

Park, H-M., R.A. Almeida, and S.P. Oliver. 2002a. Identification of lactoferrin-binding proteins in *Streptococcus dysgalactiae subsp. dysgalactiae* and *Streptococcus agalactiae* isolated from cows with mastitis. FEMS Micro Lett. 207:87-90.

Park, H-M., R.A. Almeida, D.A. Luther, and S.P. Oliver. 2002b. Binding of bovine lactoferrin to *Streptococcus dysgalactiae subsp. dysgalactiae* isolated from cows with mastitis. FEMS Micro Lett. 208:35-39.

Patel, D., R.A. Almeida, J.R. Dunlap, and S.P. Oliver, S.P. 2009. Bovine lactoferrin serves as a molecular bridge for internalisation of *Streptococcus uberis* into bovine mammary epithelial cells. Vet. Micro. 137 (3-4), 297-301.

Pfaffl, M.W., and M. Hageleit. 2001. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol. Lett. 23, 275-282.

Piepers, S., L. De Meulemeester, A. de Kruif, G. Opsomer, H. W. Barkema, and S. De Vliegher. 2007. Prevalence and distribution of mastitis pathogens in subclinicallyinfected dairy cows in Flanders, Belgium. J Dairy Res. 74; 478-483.

Pyorala, S. 2009. Treatment of mastitis during lactation. Irish Vet J. 62 (Suppl 400): 40-44.

Rainard, P., and C. Riollet. 2006. Innate immunity of the bovine mammary gland. Vert Res. 37: 369-400.

Riffon, R., K. Sayasith, H. Khalil, P. Dubreuil, M. Drolet, and J. Lagacé. 2001. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. J Clin Microbiol. 39(7): 2584–2589.

Ruiguang, G., S. Xuesong, H. Qingyu. 2009. Iron acquisition by *Streptococcus* species: An updated review. Front Biol China 4 (4): 392-401.

Schanbacher, F.L., R.E. Goodman, and R.S. Talhouk. 1993. Bovine mammary lactoferrin: implications from messenger ribonucleic acid (mRNA) sequence and regulation contrary to other milk proteins. J Dairy Sci, 76(381): 3812-3829.

Strandberg, Y., C. Gray, T. Vuocolo, L. Donaldson, M. Broadway, and R. Tellam. 2005. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. Cytokine, 31:72-86.

Teng, C.T. 2002. Lactoferrin gene expression and regulation: An overview. Biochem. Cell. Biol. 80: 7-16

Ueta E., T. Tanida, and T. Osaki. 2001. A novel Lactoferrin peptide: FKCRRWQWRM, supresses Candida cell growth and activated neutrophils. J Peptide Res. 57: 240-249

Weinberg, E.D. 1978. Iron and Infection. Microbiol Rev, 42: 45-66

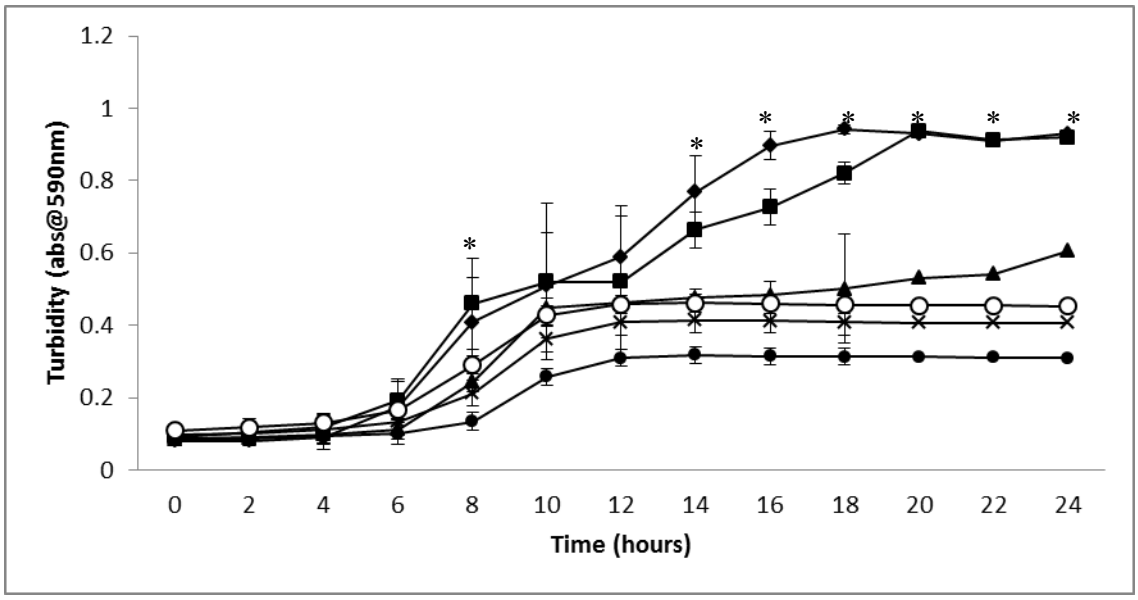
Zhao, X. and P. Lacasse. 2008. Mammary tissue damage during bovine mastitis: causes and control. J. Anim. Sci. 86 (Suppl 1): 57-65.

Figure Legends

Figure 1: Twenty-four hour growth curves of *S. dysgalactiae* in the presence of bovine lactoferrin. *S. dysgalactiae* DPC5345 was co-incubated with bovine lactoferrin protein at concentrations 0, 0.02, 0.2, 2.0, 5.0 and 10.0 mg/mL (denoted by ♦, ■, ▲, ○**, × and ●** respectively. Growth of DPC5345 was measured by turbidity (absorbance at 590 nm). *S. dysgalactiae* DPC5345 growth curves in the presence of each concentration of lactoferrin were compared to its growth in the absence of lactoferrin. From 8 h onwards there was a significant difference ($P < 0.05$), denoted as *, between growth of DPC5345 in the presence of ≥ 2 mg/mL lactoferrin compared to growth of DPC5345 alone.

Figure 2: Confocal microscopic images of HC-11 mammary cells seeded at 1 x 10^6 cells and grown to confluency. HC-11 cells were exposed to 1 x 10^7 CFU/mL *S. dysgalactiae* alone and with 2 mg/ml Dylight 633 fluorescently labelled lactoferrin. All exposed cells were incubated for 2 h at 37 ◦C, 5 % CO_2, the media was removed and the cells washed, then stained with Live/Dead Baclight viability stain to visualise cells. (A) viable *S. dysgalactiae* (stained bright green) adhering to the viable HC-11 cell surface (B) following 2 h incubation, viable *S. dysgalactiae* (stained green) were seen internalised in viable HC-11 cells (identified with black arrows), (C) HC-11 cells (green stain) incubated with 2 mg/mL labelled lactoferrin (red stain) only. The lactoferrin crossed the cell membrane and collected in the cytoplasm of the cell, (D) HC11 cells exposed to 1 x 10^7 CFU/mL *S. dysgalactiae* (viable bacteria are stained green) and 2 mg/mL lactoferrin (red stain).

Figure 1:


Turbidity (abs@590nm)
1.2
1
0.8
0.6
0.4
0.2
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2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)
*

Figure 2

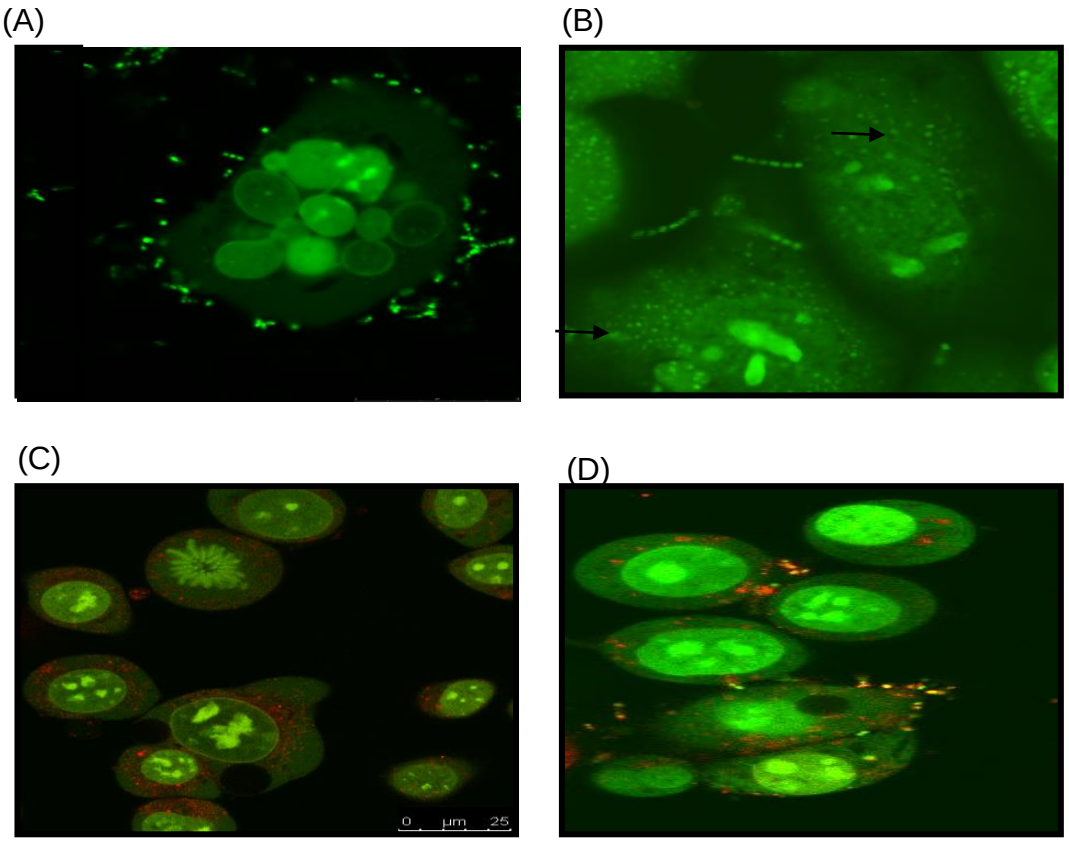

Table 1. Lactoferrin transcript levels, per 50 ng total RNA, in milk from *S. dysgalactiae* infused quarters compared to control quarters. Values are given as the exponential of transformed data ± 95 % confidence intervals (95 % CI). (NS = Not Significant; P < 0.05 indicates a significance difference at that time point; *n* = number of animals measured at each time point)

	Control quarter		*S.dysgalactiae* Infused quarter		
Time (*n*)	Average lactoferrin mRNA Value	95 % CI	Average lactoferrin mRNA Value	95 % CI	P Value
0 h (*n* = 5)	1570	1,038 – 3,065	1883	1,324 – 4,464	NS
7 h (*n* = 5)	5280	4,369 – 25,333	583	439 – 1,778	< 0.05
24 h (*n* = 5)	1731	1,219 – 4,122	1336	940 – 3,169	NS
48 h (*n* = 5)	7572	5,008 – 14,789	2871	2,019 – 6,807	NS

Table 2. Lactoferrin protein (µg/mL) in milk from *S. dysgalactiae* infused quarters compared to control quarters. Values are given as the average data of 5 animals ± standard deviation. (SD = Standard Deviation; NS = Not Significant; P < 0.05 indicates significantly different at that time point; *n* = number of animals measured at each time point).

	Control quarter		*S. dysgalactiae* infused quarter		
Time (n)	Average Value	SD	Average Value	SD	P Value
0 h *(n = 5)*	199	64	189	64	NS
7 h *(n = 5)*	168	64	177	64	NS
24 h *(n = 5)*	215	64	259	64	NS
48 h *(n = 5)*	230	64	301	64	< 0.05

Table 3. Effect of lactoferrin concentrations (mg/mL) on adherence and invasion of *S. dysgalactiae* DPC5345 (CFU/mL) into HC-11 mammary epithelial cells (1 x 10^5 cells/well). Adherence index calculated as (CFU in the cell lysates) / (CFU in the supernatant) X 10,000 (Fang et al., 2000).

	Invasion Assay *		
Lactoferrin (mg/ml)	**Live bacteria in supernatant (CFU/mL)**	**Live bacteria in cell lysate (CFU/mL)**	**Adherence Index**
0	8.80E + 07 (±8.68E+06)	3.32E + 05 (±4.93E+04)	37.72
0.01	6.80E + 07 [a] (±5.68E+06)	2.21E + 05[a] (±3.20E+04)	32.5
0.1	6.58E + 07 [a] (7.58E+06)	1.36E + 05[b] (±4.08E+04)	20.67
1.0	6.94E + 07 [a] (7.32E+06)	9.40E + 04[b] (±3.45E+04)	13.54
2.0	3.70E + 07 [b] (4.14E+06)	1.24E + 04[b] (±1.16E+03)	3.35

* Values given represent mean of replicates (n = 8) (±SEM) of live bacterial recovered (CFU/ml) from HC-11 cell supernatant or cell lysate in the invasion assay. Comparing data within columns [a] indicates lactoferrin concentration tested had no significant effect on bacterial cell count (CFU/mL) compared to control (no lactoferrin); [b] indicates lactoferrin concentration tested had significant ($P < 0.05$) effect on bacterial cell count (CFU/mL) compared to control (no lactoferrin)