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

Fiona O'Halloran,^{*1} Christine Beecher,^{*†} Valerie Chaurin,^{*} Torres Sweeney,[‡] and Linda Giblin^{*}

^{*}Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

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

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

ABSTRACT

Streptococcus dysgalactiae ssp. *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, *Strep. dysgalactiae* ssp. *dysgalactiae* DPC5345. Initially a deliberate in vivo bovine intramammary challenge was performed with *Strep. dysgalactiae* DPC5345. Results demonstrated a significant difference in lactoferrin mRNA levels in milk cells between the control and infused quarters 7 h postinfusion. Milk lactoferrin levels in the *Strep. dysgalactiae* DPC5345 infused quarters were significantly increased compared with control quarters at 48 h postinfusion. In vitro studies demonstrated that lactoferrin had a bacteriostatic effect on the growth of *Strep. dysgalactiae* DPC5345 and significantly decreased the ability of the bacteria to internalize into HC-11 mammary epithelial cells. Confocal microscopy images of HC-11 cells exposed to *Strep. dysgalactiae* and lactoferrin further supported this effect by demonstrating reduced invasion of bacteria to HC-11 cells. The combined data suggest that a bovine immune response to *Strep. 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* ssp. *dysgalactiae*, mastitis

INTRODUCTION

Bovine mastitis infection has serious deleterious effects on milk quality and is a significant limiting factor

to profitable dairy farming worldwide (Kossabati and Esslemon, 1997; Berry and Amber, 2005; Bar et al., 2008). 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 defense mechanisms of the mammary gland (Zhao and Lacasse, 2008).

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

Conventional treatment for mastitis involves the administration of antibiotics. Antimicrobial resistance in mastitis pathogens is not yet considered a clinically relevant issue (Pyörälä, 2009; Oliver and Murinda, 2012); however, the intensive use of antibiotics can lead to the development of resistant strains (Hendriksen et al., 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 or treat mastitis (or both) would be of significant benefit to the dairy industry. One approach would be to manipulate and enhance natural host defense mechanisms. However, different pathogens display distinct invasion and pathogenic mechanisms (Chaneton et al., 2008). Detailed knowledge of the specific host-pathogen interactions that are

Received September 29, 2015.

Accepted February 14, 2016.

¹Corresponding author: fiona.ohalloran@cit.ie

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, 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 (Kuttila 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 *Strep. uberis*, *Strep. agalactiae*, and *Strep. 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 *Strep. uberis* isolates bind lactoferrin via a surface-exposed adhesion molecule, which they defined as the *Strep. uberis* adhesion molecule. These *in vitro* studies suggest that *Strep. uberis* hijacks lactoferrin to act as a bridging molecule between the bacterial cell surface and lactoferrin receptors on host mammary epithelial cells. This allows *Strep. 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 *Strep. uberis* infected quarters ($P < 0.001$) when compared with uninfected quarters. It was hypothesized that *Strep. uberis* isolates were resistant to bovine lactoferrin activity and high levels of the lactoferrin in milk promoted the pathogenesis of *Strep. uberis* *in vivo*. Other mastitis-causing bacteria, including *Staphylococcus aureus*, *Strep. dysgalactiae*, and CNS, were not associated with a significant increase in bovine milk lactoferrin levels in these subclinical cases. 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 2 lactoferrin binding proteins in *Strep. dysgalactiae* isolates (52 and 74 kDa) and 3 lactoferrin binding proteins in *Strep. 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 *Strep. dysgalactiae* and *Strep. agalactiae* intramammary infections must still be elucidated.

The aim of this study was to investigate the interaction *in vitro* between lactoferrin and *Strep. dysgalactiae* to identify whether this milk protein combats or promotes *Strep. 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 *Strep. dysgalactiae*.

MATERIALS AND METHODS

Bacterial Culture

Streptococcus dysgalactiae ssp. *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 characterized to species level by 16S PCR (Riffon et al., 2001). A 2% inoculum of *Strep. dysgalactiae* DPC 5345 yielded 10^8 cfu/mL when cultured overnight at 37°C in tryptic soy (TS) broth (Difco Laboratories, Detroit, MI).

Animal Selection

Five Holstein-Friesian cows were selected, based on their health status, for a deliberate intramammary challenge with *Strep. 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 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 before the challenge. Bacterial infusions and milk and blood sampling were performed under license 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 *Strep. dysgalactiae* DPC5345 was performed according to a procedure described by Beecher et al. (2012). Briefly, overnight cultures of *Strep. dysgalactiae* DPC5345 were diluted with maximum recovery diluent (Oxoid Ireland, Dublin, Ireland) to prepare 2-mL suspensions at 2,500 cfu of *dysgalactiae*. This bacterial count was chosen as preliminary trials indicated that 2,500 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 contralateral quarter was infused with 2 mL of maximum recovery diluent 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 before infusion and at 7 h, 24 h, 48 h, 7 d, and 14 d postinfusion. From each milk sample 100 μ L was plated onto esculin blood agar plates (Thermo Scientific, Wilmington, DE) to monitor bacterial infection. Milk somatic cell counts were also determined using a Somacount 300 somatic cell counter (Bentley Instruments Inc., Chaska, MN). 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 of 0.5 M EDTA (Sigma-Aldrich Ltd., Wicklow, Ireland) was added to each liter of milk and the samples were then centrifuged at $1,500 \times g$ 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 of TriPure isolation reagent (Roche Diagnostics, East Sussex, UK) and RNA was extracted according to the manufacturer's instructions. All RNA was quality assessed through visualization on glyoxyl gels (1.5%; Ambion, Applied Biosystems, Foster City, CA) and quantified using the Nanodrop 1000 (Thermo Scientific).

Real-Time PCR

One microgram of RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Crawley, West Sussex, UK), and 1 μ L of 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'-AAA-CAAGCATCGGGATTCCAG-3' and reverse primer

5'-ACAATGCAGTCTTCCGTGGTG-3'). Quantitative analysis was performed in a LightCycler 480 instrument (Roche Diagnostics) using a dilution series of external plasmid DNA standards (10^9 to 10^2 copies/ μ L; Pfaffl and Hageleit, 2001). Plasmid standards were created by cloning a cDNA PCR product into plasmid pCR TOPO (Invitrogen, Life Technologies, Carlsbad, CA). Cloning was confirmed by sequencing (Beckman Coulter Genomics, Essex, UK). One microliter of each dilution was used per 10 μ L of LightCycler reaction. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics) was used for quantification according to the manufacturer's instructions using 0.5 μ M 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 of denaturation at 95°C, 10 s of annealing at 60°C, and 25 s of 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, TX) and according to the manufacturer's instructions. Milk samples were diluted 1:2,000, and standard curves were generated for each sample set. Minimum detection levels for lactoferrin were 7.8 ng/mL, and lactoferrin concentrations were quantified by interpolating the absorbance values from the standard curve. Test samples were analyzed by ELISA in duplicate, and the analysis was performed on 2 separate occasions.

Antibacterial Assay

The antibacterial activity of a commercial bovine native lactoferrin protein (#L9507, Sigma-Aldrich) was tested against *Strep. dysgalactiae* DPC5345. For this, an overnight culture of *Strep. dysgalactiae* DPC 5345 was diluted to 10^7 cfu/mL in TS broth (Oxoid, Ireland) and 40 μ L of the culture was 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- μ m 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 were 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 Wolfgang Doppler (Innsbruck Medical University, Innsbruck, Austria). Cells were grown and maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 4 mM L-glutamine (Sigma-Aldrich), 10% fetal bovine serum (Sigma-Aldrich), 5 µg/mL insulin (Sigma-Aldrich), 10 ng/mL epidermal growth factor (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich, Ireland), and 100 µg/mL streptomycin (Sigma-Aldrich).

Invasion Assays with *Strep. dysgalactiae*

Invasion assays were performed on the same day, in triplicate, as per Calvino and Oliver (1998b) with some minor modifications. Briefly, HC-11 cells were seeded into 24-well plates at a density of 1×10^5 cells/well and grown to confluency in antibiotic-free RPMI 1640 supplemented medium. An overnight culture of *Strep. dysgalactiae* DPC 5345 was washed 3 times in PBS (Sigma-Aldrich) and resuspended in antibiotic-free RPMI 1640 supplemented medium, to a final concentration of $\sim 1 \times 10^7$ 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 (1×10^7 cfu/mL) were added to each test well. Sterile-filtered solutions of lactoferrin, prepared with antibiotic-free RPMI 1640 supplemented medium, 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 of lactoferrin. Control wells contained *Strep. dysgalactiae* DPC5345 culture and 1 mL of PBS only. Plates were incubated at 37°C, 5% CO₂ 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 medium containing 5 µg/mL of penicillin and 100 U/mL of gentamicin. Following the second incubation, the medium was removed and cells were washed 3 times with 1 mL of PBS. The HC-11 cells were then lysed by adding 0.25% trypsin (Sigma-Aldrich) and 0.025% Triton X-100 (Sigma-Aldrich) and the lysates collected into sterile 1.5-mL Eppendorf tubes and vigorously vortexed for 15 s. Cell lysates were serially diluted 10-fold (10^{-1} to 10^{-4}) and 0.1-mL dilutions were 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) $\times 10,000$ (Fang et al., 2000). Each lactoferrin concentration was tested in duplicate and the assays performed on 4 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 1×10^6 cells/well, and grown to confluency. A fresh overnight bacterial culture of *Strep. dysgalactiae* DPC5345 was washed 3 times with PBS and prepared in antibiotic-free RPMI 1640 supplemented medium to a final concentration of $\sim 1 \times 10^7$ cfu/mL. A 2 mg/mL protein preparation of commercially available bovine Lactoferrin was prepared in PBS and labeled with a Dylight 633 Fluorescent dye using an Alexa Fluor 350 Protein labeling kit (Thermo Fisher Scientific, Dublin, Ireland), according to the manufacturer's instructions. One milliliter of the labeled protein solution was mixed with 1 mL of fresh bacterial culture ($\sim 10^7$ 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 of antibiotic-free RPMI 1640 supplemented medium only and (b) 1 mL of antibiotic-free RPMI 1640 supplemented medium mixed with 1 mL of bacterial culture ($\sim 10^7$ cfu/mL). All cell culture plates were incubated for 2 h at 37°C, 5% CO₂ and then the medium was 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). 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, Heidelberg GmbH, Mannheim, Germany) using a 63 $\times$ oil immersion objective ($A_N = 1.4$). RGB color images (24 bit), 512 $\times$ 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 were transformed by obtaining the natural logarithm. A hierarchical mixed model (PROC MIXED; SAS Version 9.1, SAS Institute Inc., Cary, NC) 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 regard to the milk protein secretion data, statistical analysis was performed as above, without transformation, as data were 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 used 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 used 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 *Strep. dysgalactiae* on lactoferrin levels in vivo was investigated to determine if the bovine immune response to this mastitis pathogen included alterations to the transcription, secretion, or both of this antibacterial protein.

Bacterial Growth in Infected Quarters Postintramammary Challenge

Following the infusion of experimental quarters with 2,500 cfu *Strep. dysgalactiae* DPC5345, viable gram-positive bacteria were recovered within 24 h from all quarters infused. Pulsed-field gel electrophoresis con-

firmed *Strep. dysgalactiae* DPC5345 (data not shown). As soon as clinical signs of infection were observed (rectal temperatures $>38.5^{\circ}\text{C}$, swollen udders, and SCC $>200,000$), animals received antibiotic (Leo Yellow) treatment. Four of the animals received antibiotic treatment 48 h postinfusion and one animal received antibiotics at 72 h postinfusion. Data postantibiotic treatment were discounted and not included in the analysis.

In Vivo Levels of Lactoferrin mRNA and Protein Following Intramammary Challenge

To investigate if lactoferrin gene expression lactoferrin protein levels, or both, in milk were affected by the deliberate *Strep. 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 postinfusion. Control quarters acted as internal controls for each animal. A significant difference ($P < 0.05$) was found in lactoferrin mRNA levels between the control and infused quarters at 7 h postinfusion (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 with control quarters at 48 h postinfusion (Table 2).

Antibacterial Activity

To investigate the influence of a commercial preparation of bovine lactoferrin on the growth of the *Strep. dysgalactiae* culture DPC5345 in a laboratory setting, a 24-h growth curve was performed. The DPC5345 was cultured in complex medium with various concentrations of lactoferrin. Results from the 24-h growth curves demonstrated that lactoferrin had a bacteriostatic effect on *Strep. dysgalactiae* DPC5345 (Figure 1). At 14 h ≥ 0.2 mg/mL, lactoferrin had a significant effect on

Table 1. Lactoferrin transcript levels, per 50 ng of total RNA, in milk from *Streptococcus dysgalactiae*-infused quarters compared with control quarters¹

Time	Control quarter		<i>Strep. dysgalactiae</i> -infused quarter		<i>P</i> -value
	Average lactoferrin mRNA value	95% CI	Average lactoferrin mRNA value	95% CI	
0 h (n = 5)					NS
7 h (n = 5)	5,280	4,369–25,333	583	439–1,778	<0.05
24 h (n = 5)	1,731	1,219–4,122	1,336	940–3,169	NS
48 h (n = 5)	7,572	5,008–14,789	2,871	2,019–6,807	NS

¹Values are given as the exponential of transformed data $\pm$ 95% CI. $P < 0.05$ indicates a significance difference at that time point; n = number of animals measured at each time point.

Figure 1. Twenty-four-hour growth curves of *Streptococcus dysgalactiae* in the presence of bovine lactoferrin. *Streptococcus 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). *Streptococcus dysgalactiae* DPC5345 growth curves in the presence of each concentration of lactoferrin were compared with its growth in the absence of lactoferrin. From 8 h onward, a significant difference ($P < 0.05$), denoted as *, was present between growth of DPC5345 in the presence of ≥ 2 mg/mL lactoferrin compared with growth of DPC5345 alone. Error bars are standard deviation based on 3 replicate measurements.

bacterial growth ($P < 0.05$) when compared with the control (no lactoferrin).

Invasion Assay and Confocal Imaging

To examine if lactoferrin influenced the ability of *Strep. 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 $\sim 1 \times 10^7$ cfu/mL of fresh bacterial culture and a range of physiologically relevant concentrations of lactoferrin from 0.01 to 2.0 mg/mL (Schanbacher et al., 1993). After 4 h of 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 of lactoferrin compared with the negative control. At 2

mg/mL of lactoferrin, more than a log fold reduction in the number of internalized bacteria ($4.1 \log_{10}$ cfu/mL) was observed compared with the negative control ($5.52 \log_{10}$ cfu/mL) and 0.01 mg/mL of lactoferrin ($5.34 \log_{10}$ cfu/mL; Table 3). The adherence index value was calculated by dividing the number of bacterial colony-forming units in the cell lysate by the number of colony-forming units in the supernatant and therefore gives an indication of the ability of the bacteria to adhere and internalize 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 decreased (32.5, 20.67, 13.54, and 3.35, respectively) in a dose-dependent manner (Table 3), demonstrating decreased internalization. This effect was visually confirmed by confocal microscopy (Figure 2), which showed that in

Table 2. Lactoferrin protein ($\mu\text{g/mL}$) in milk from *Streptococcus dysgalactiae*-infused quarters compared with control quarters¹

Time	Control quarter		<i>Strep. dysgalactiae</i> -infused quarter		P-value
	Average value	SD	Average value	SD	
0 h (n = 5)					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

¹Values are given as the average data of 5 animals $\pm$ SD. $P < 0.05$ indicates significantly different at that time point; n = number of animals measured at each time point.

Table 3. Effect of lactoferrin concentrations (mg/mL) on adherence and invasion of *Streptococcus dysgalactiae* DPC5345 (cfu/mL) into HC-11 mammary epithelial cells (1×10^5 cells/well)¹

Lactoferrin (mg/mL)	Invasion assay ²		
	Live bacteria in supernatant (cfu/mL)	Live bacteria in cell lysate (cfu/mL)	Adherence index
0	8.80E+07 ($\pm 8.68\text{E}+06$)	3.32E + 05 ($\pm 4.93\text{E}+04$)	37.72
0.01	6.80E+07 ^a ($\pm 5.68\text{E}+06$)	2.21E + 05 ^a ($\pm 3.20\text{E}+04$)	32.5
0.1	6.58E+07 ^a ($\pm 7.58\text{E}+06$)	1.36E + 05 ^{b*} ($\pm 4.08\text{E}+04$)	20.67
1.0	6.94E+07 ^a ($\pm 7.32\text{E}+06$)	9.40E + 04 ^{b**} ($\pm 3.45\text{E}+04$)	13.54
2.0	3.70E+07 ^{b***} ($\pm 4.14\text{E}+06$)	1.24E + 04 ^{b***} ($\pm 1.16\text{E}+03$)	3.35

^{a,b}Means within a column with different superscripts differ at the *P*-values indicated.

¹Adherence index calculated as (cfu in the cell lysates)/(cfu in the supernatant) $\times 10,000$ (Fang et al., 2000).

²Values given represent mean of replicates ($n = 8$) ($\pm$ SEM) of live bacterial recovered (cfu/mL) from HC-11 cell supernatant or cell lysate in the invasion assay.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

the absence of lactoferrin viable *Strep. dysgalactiae* bacteria internalized into the mammary HC-11 cells (Figures 2A and 2B, respectively). When the Dylight 633 fluorescently labeled lactoferrin (~ 2 mg/mL) was added to the HC-11 cells, the labeled protein bound to the bacteria at HC-11 cell membrane boundaries (Figure 2C) and also to the bacteria that had internalized within the HC-11 cells (Figure 2D). These microscopic images indicate that *Strep. dysgalactiae* bacteria and the lactoferrin protein co-localize.

DISCUSSION

A deliberate bovine intramammary challenge with *Strep. dysgalactiae* resulted in a significant increase ($P < 0.05$) in milk lactoferrin protein compared with controls. Follow-up in vitro studies demonstrated that lactoferrin is a bacteriostatic agent that interferes with the ability of *Strep. dysgalactiae* to adhere and internalize into mammary epithelial cells. Co-localization images suggest a direct interaction between lactoferrin and *Strep. dysgalactiae* cells, demonstrating that this bacteria binds to the lactoferrin protein. The combined data indicate that a bovine immune response to *Strep. 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* species, promoting bacterial internalization into mammary epithelial cells and potentially providing an opportunity for this pathogen to survive against host defense mechanisms. In contrast to this our study indicates that lactoferrin plays a role in preventing infec-

tion by *Strep. 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 *Strep. uberis* species, *Strep. dysgalactiae* express lactoferrin binding proteins. The existence of these proteins facilitates an interaction between lactoferrin and *Strep. 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 *Escherichia 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 bacterio-

static against *Strep. dysgalactiae* species, which could be due to the presence of these lactoferrin binding proteins. Further characterization of these proteins within *Strep. 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 *Strep. 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 *Strep. dysgalactiae* species investigated.

Lactoferrin mRNA levels were reduced in the early period of invasion of *Strep. dysgalactiae*. Lactoferrin protein levels did not differ within this period, but later, at 48 h postinfusion, a statistically significant increase ($P < 0.05$) was found in the average milk lactoferrin levels from the infused quarters compared with the control quarters. Bovine milk lactoferrin levels are reported in general to be between 20 to 200 mg/L (Rainard and Riollot, 2006), but levels vary from one animal to another, vary in mammary quarters, and as

Figure 2. Confocal microscopic images of HC-11 mammary cells seeded at 1×10^6 cells and grown to confluency. The HC-11 cells were exposed to 1×10^7 cfu/mL *Streptococcus dysgalactiae* alone and with 2 mg/mL of Dylight 633 fluorescently labeled lactoferrin. All exposed cells were incubated for 2 h at 37°C, 5% CO₂, the medium was removed and the cells washed, then stained with Live/Dead BacLight viability stain to visualize cells. (A) Viable *Strep. dysgalactiae* (stained bright green) adhering to the viable HC-11 cell surface (scale 0–5 μ m), (B) following 2 h incubation, viable *Strep. dysgalactiae* (stained green) were seen internalized in viable HC-11 cells (identified with black arrows; scale 0–5 μ m), and (C) HC-11 cells (green stain) incubated with 2 mg/mL labeled lactoferrin (red stain) only. The lactoferrin crossed the cell membrane and collected in the cytoplasm of the cell (scale 0–25 μ m). (D) HC11 cells exposed to 1×10^7 cfu/mL *Strep. dysgalactiae* (viable bacteria are stained green) and 2 mg/mL of lactoferrin (red stain) (scale 0–20 μ m).

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, *Strep. dysgalactiae* infections were not associated with a significant increase in milk lactoferrin levels. In clinical mastitis cases with 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 (Strandberg et al., 2005; Rainard and Riollot, 2006). 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 postinfection may therefore be a result of neutrophil recruitment or delayed lactoferrin production from mammary epithelial cells or both.

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

ACKNOWLEDGMENTS

The authors are grateful to Wolfgang Doppler, Innsbruck Medical University, for the kind donation of HC-11 cells. The authors also thank Mark Auty of the National Food Imaging Centre, Teagasc Moorepark, Co. Cork. This research was funded by the Food Institutional Research Measure (Department of Agriculture, Food and Fisheries, Ireland) Projects 06RDTMFRC437 and 06RDTMFRC445, the Irish Dairy Research Trust, and the Teagasc Retooling Program under the National Development Plan. Christine Beecher was in receipt of a Teagasc Walsh Fellowship.

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