Short communication: Early-lactation, but not mid-lactation, bovine lactoferrin preparation increases epithelial barrier integrity of Caco-2 cell layers

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ABSTRACT

Bovine lactoferrin is an important milk protein with many health-promoting properties, including improving intestinal barrier integrity. Dysfunction of this barrier, commonly referred to as “leaky gut,” has been linked to inflammatory and autoimmune diseases. With some processing techniques, lactoferrin isolated from milk collected at the start of the milking season (early lactation) may have lower purity than that isolated from milk collected during the rest of the milking season (mid-lactation) and could result in differences in bioactivity based on the stage of lactation. We compared reversed-phase HPLC chromatographs of early-lactation and mid-lactation preparations and found that both had large chromatograph peaks at the time predicted for lactoferrin. The notable difference between the 2 chromatographs was a much larger peak in the early-lactation lactoferrin sample that was determined to be angiogenin. Angiogenin was first identified due to its ability to induce new blood vessel formation, but is now known to be involved in numerous physiological processes. Then, we compared the effects of early-lactation and mid-lactation lactoferrin preparations in 2 bioassays: trans-epithelial electrical resistance (TEER), a measure of intestinal barrier integrity, and peripheral blood mononuclear cell cytokine secretion, a measure of immune-stimulatory properties. We found that early-lactation lactoferrin increased TEER across Caco-2 cell layers compared with control from 10 to 48 h, mid-lactation lactoferrin did not alter TEER. We also found that early-lactation lactoferrin reduced the amount of IL-8 produced by peripheral blood mononuclear cells

(compared with those treated with control medium) to a greater extent than mid-lactation lactoferrin. A pro-inflammatory chemokine, IL-8 is also known to decrease barrier function. These results suggest that the decrease in IL-8 production in the presence of early-lactation lactoferrin may be the mechanism by which it increases TEER. The anti-inflammatory effect of early-lactation lactoferrin may be related to the presence of angiogenin, which is known to suppress inflammatory responses. This work indicates that products rich in angiogenin may have intestinal health benefits, and further work to investigate this is warranted.

Key words: lactoferrin, angiogenin, trans-epithelial electrical resistance, anti-inflammatory

Short Communication

Bovine lactoferrin is an important protein in milk known for its antibacterial, immune-modulating, and anti-inflammatory effects. A recent study using rats with bowel resections indicated that bovine lactoferrin also plays a role in improving intestinal barrier integrity (Wu et al., 2014). Dysfunction of this barrier, commonly referred to as “leaky gut,” has been linked to inflammatory and autoimmune diseases, not only in the intestine but throughout the body (Schulzke et al., 1998; Fasano and Shea-Donohue, 2005; Xavier and Podolsky, 2007; Vaarala et al., 2008; Turner, 2009; Fasano, 2012). Evidence for “leaky gut” contributing to psychiatric disorders and declined cognitive function is also mounting (Clairembault et al., 2015; Kelly et al., 2015; Schmidt, 2015). Therefore, interest is growing in compounds such as lactoferrin that may support intestinal barrier integrity and in turn reduce the risk of such conditions.

The composition of bovine milk changes throughout the milking season; colostrum and early-lactation milk have a higher concentration of several molecules, including lactoferrin (Sánchez et al., 1988; Zhang et al.,

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2015). Our hypothesis was that lactoferrin isolated from milk collected at the start of the milking season (early lactation) may be of lower purity than lactoferrin isolated from milk collected during the rest of the season (mid-lactation). This may be due to co-elution of other molecules that are more abundant early in lactation and could result in differences in bioactivity based on stage of lactation. Our aim was to compare the effects of early-season and mid-season lactoferrin preparations in 2 bioassays: trans-epithelial electrical resistance (TEER), a measure of intestinal barrier integrity, and peripheral blood mononuclear cell (PBMC) cytokine secretion, a measure of immune-stimulatory properties.

For this study, early lactation was defined as the first 2 mo of the New Zealand milking season (July and August), and mid-lactation was after this (September onward). Milk was sourced from numerous farms as part of Fonterra Co-operative Group's commercial milk-collection process. Lactoferrin was isolated from the pooled milk samples in semi-commercial batches using standard commercial cation-exchange protocols. The composition of the early-lactation and mid-lactation lactoferrin preparations was compared using reversed-phase HPLC as previously described (Elgar et al., 2000).

As expected, the reversed-phase HPLC chromatographs of early-lactation and mid-lactation preparations had large peaks at the time predicted for lactoferrin (Figure 1). The purity of the lactoferrin was 87 and 94% for the early-lactation and mid-lactation samples, respectively. The notable difference between chromatographs of the 2 sample types was the presence of a large peak after 5.7 min in the early-lactation preparation that was much smaller in the mid-lactation preparation. This was determined to be angiogenin, using HPLC-MS proteomic techniques. Angiogenin was first identified due to its ability to induce new blood vessel formation, but it is now known to be involved in numerous physiological processes (Sheng and Xu, 2016). Chromatographs from both sample types also had several smaller peaks that may have had different concentrations between the lactoferrin preparations, but these were not characterized.

To determine whether these compositional differences led to differences in effects on intestinal barrier integrity, we measured TEER across an in vitro intestinal epithelial layer in response to early-lactation and mid-lactation lactoferrin. We also investigated the ability of the 2 lactoferrin preparations to overcome the decrease in TEER caused by the pro-inflammatory cytokine tumor necrosis factor α (TNF α). We used Caco-2 (ATCC HTB-37), a human colorectal adenocarcinoma cell line, as a model of human intestinal epithelium because the cells differentiate into polarized

epithelial cells possessing apical brush borders and intracellular tight junctions (Le Ferrec et al., 2001; Shah et al., 2006). We seeded Caco-2 cells at a concentration of 8×10^4 cells/Transwell (6.5 mm, polyester, 0.4 μ m pore size; Corning Inc., Corning, NY) and grown in Medium 199 (M199; Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen Corporation), 1% NEAA (MEM NEAA 100 $\times$ solution; Sigma-Aldrich, St. Louis, MO) and 1% penicillin-streptomycin (Pen-Strep, 10,000 units/mL penicillin G sodium salt and 10,000 μ g/mL streptomycin sulfate in 0.85% saline; Gibco, Invitrogen Corporation) at 37°C in 5% CO₂ for 15 to 19 d until they formed a differentiated monolayer. The day before the TEER assay, Transwells were transferred into cellZscope cell modules (nanoAnalytics GmbH, Münster, Germany) that had M199 supplemented with 10% FBS, 1% NEAA, and 1% Pen-Strep in the basal compartment and M199 supplemented with 1% NEAA and 1% Pen-Strep in the apical compartment, and incubated (37°C, 5% CO₂). The background resistances across the cell layers were measured hourly for 24 h using the cellZscope computer-controlled system. The TEER values were calculated using the cellZscope software (v 2.2.0) by multiplying the raw resistance by the membrane area (0.33 cm²). Caco-2 monolayers that had background TEER greater than 400 Ω /cm² were used for assays.

The apical medium from the Transwells was then replaced with the treatment solutions: control medium (M199 supplemented with 1% NEAA), 1 mg/mL early-lactation lactoferrin, or 1 mg/mL mid-lactation lactoferrin. The basal medium from the wells was replaced with M199 supplemented with 10% FBS, 1% NEAA, and 1% Pen-Strep, with or without 100 ng/mL TNF α . We measured TEER every hour for 48 h, and calculated the percentage change in TEER at each time point compared with the initial TEER (before treatments were added). We carried out 3 independent assays, each with 4 replicates per treatment (total $n = 12$ per treatment). We compared treatments in GenStat v17.1 (VSN International Ltd., Hemel Hempstead, UK) using REML analysis with an auto-regression order 1 covariance model to account for repeated measures. If the effect of treatment $\times$ time was significant (P -value < 0.05), we compared the means of individual treatments at a given time point using the least significant difference at 5%.

Figures 2A and 2B show the change in TEER across unchallenged cell monolayers and those treated with TNF α , respectively. In the unchallenged cells, early-lactation lactoferrin increased TEER compared with both control medium and mid-lactation lactoferrin from 10 h onwards. This indicates that early-lactation lactoferrin

Figure 1. Representative reversed-phase HPLC chromatograms of early-lactation and mid-lactation lactoferrin samples. A Resource RPC column (Pharmacia Biotech, Uppsala, Sweden) was equilibrated in 80% solvent A (0.1% vol/vol trifluoroacetic acid in Milli-Q water), and after sample injection a 1-min isocratic period was applied followed by a series of linear gradients to 100% solvent B (0.09% vol/vol trifluoroacetic acid, 90% vol/vol MeCN in Milli-Q water) as follows: 1–6 min, 20–40% B; 6–16 min, 40–45% B; 16–19 min, 45–50% B; 19–20 min, 50% B; 20–23 min, 50–70% B; 23–24 min, 70–100% B. Detection was by absorbance at 214 nm. Color version available online.

Figure 2. Effect of lactoferrin samples on the trans-epithelial electrical resistance (TEER) across Caco-2 cell monolayers (A) under normal conditions and (B) challenged with tumor necrosis factor α (TNF α). Caco-2 cells were grown in Transwells for 15 to 19 d until differentiation, before use in the experiment. After the initial TEER reading, the apical medium was replaced with the test samples, and the basal medium was replaced with fresh medium without (A) or with (B) TNF α . The TEER was measured hourly for 48 h using the automated cellZscope system (nanoAnalytics GmbH, Münster, Germany). We combined the data from 3 independent assays, each with 4 replicates per treatment (total $n = 12$ per treatment). Values shown are the means, and the error bars show the SEM. *Difference compared with control medium greater than the LSD 5% of 11.47 over the time frame indicated by the bracket.

enhanced the tight junction integrity, but mid-lactation lactoferrin did not. In contrast, in the cells challenged with TNF α , early-lactation lactoferrin did not alter the TEER compared with the control medium, indicating that it was not able to overcome the reduction in TEER caused by TNF α . Mid-lactation lactoferrin caused a significant decrease in TEER compared with the control medium at 2 h. Early-season lactoferrin also caused an initial drop in TEER, but this was not significantly different from the control medium. These initial drops in TEER were likely due to handling of the cells during the medium change, rather than being biologically relevant.

To determine whether there was a difference in the immune-stimulatory properties of the early-lactation and mid-lactation lactoferrin preparations, we measured cytokine secretion by PBMC. We obtained written consent for the collection and use of blood for research purposes from human volunteers. Ethics approval from the New Zealand Health and Disability Committee was not required under Section 3 of the Standard Operating Procedures of the Health and Disability Committee, because of the healthy status of the volunteers and the anonymity of the samples, and the fact that the PBMC

were being used as a reagent in the experiment rather than the subject of the experiment.

We isolated PBMC from the blood of 4 male adult donors using the Ficoll Paque method as per the manufacturer's instructions (Sigma-Aldrich). We seeded PBMC into 96-well plates at a density of 5×10^5 cell/mL and cultured them in Advanced RPMI medium (Invitrogen Corporation) supplemented with 2% FBS and 1% Pen-Strep at 37°C in 5% CO₂. After 24 h, the medium was replaced with the treatment solutions: control medium, 2.5 to 12.5 μ g/mL phytohemagglutinin (Sigma-Aldrich), 0.01–0.5 ng/mL lipopolysaccharide (Sigma-Aldrich), 0.1 mg/mL early-lactation lactoferrin, or 0.1 mg/mL mid-lactation lactoferrin (8 replicates $\times$ 4 donors for each ingredient). Phytohemagglutinin and lipopolysaccharide treatments were positive controls to show that the T and B lymphocytes present in the PBMC population were viable and responsive to stimuli. After 24 h incubation at 37°C in 5% CO₂, we collected the cell culture supernatants.

We determined the concentrations of 7 cytokines (IL-4, IL-8, IL-10, IL-12p70, IL-17F, TNF α , and IFN γ) using a Cytometric Bead Array (CBA) Flex Set (BD Bioscience; Becton Dickinson, San Jose, CA) as per

the manufacturer's instructions (<http://www.bdbiosciences.com/ds/pm/others/23-13480.pdf>). Briefly, the cell culture supernatant was combined with 50 μ L of the CBA bead suspension containing Flex Set capture beads (diluted 3-fold: i.e., a final volume of 0.33 μ L of each bead set) against all 7 cytokines using FACSVerse 96-well plates (BD Bioscience) and incubated for 1 h at room temperature. Phycoerythrin, a fluorescent dye, was then added to each well (50 μ L) and the plates incubated for 2 h at room temperature protected from light. Plates were centrifuged ($200 \times g$ for 5 min), washed in buffer 3 times and transferred to the BD FACSVerse flow cytometer (BD Bioscience) for reading. Samples were protected from light at all times, and data acquired began within 2 h after laboratory processing. Instrument setup and calibration were as per the manufacturer's instructions using the FACSuite software (BD Biosciences; https://bdbiosciences.com/documents/CBA_BDAFACSCalibur_Setup_Manual.pdf). The stopping rule was set at 2,100 events per sample (i.e., 300 events per analyte). The compensation was adjusted using the Cytometer Setup and Tracking research beads and CBA Red and CBA Near Infrared tubes, as per the manufacturer's instructions. Analysis of the resulting data to determine individual cytokine levels was performed using FCAP Array V3.0 (BD Bioscience). Results for each donor were normalized against the medium controls, and data were expressed as a percentage of concentration secreted in response to the control medium.

As expected, the PBMC produced detectable levels of all 7 cytokines when treated with the positive controls, confirming that they were responsive to stimuli. However, when the PBMC were treated with the lactoferrin samples, only IL-8 was secreted in a concentration high enough to be detected, indicating that the lactoferrin samples had minimal effect on cytokine expression. Compared with the amount of IL-8 produced by PBMC treated with control medium, early-lactation and mid-lactation lactoferrin decreased the amount of IL-8 produced, with fold-changes of $-3.81 [\pm \text{standard error of the mean (SEM) } 0.92]$ and $-1.20 (\pm \text{SEM } 0.43)$, respectively ($P < 0.05$). The early-lactation lactoferrin reduced the amount of IL-8 produced to a greater extent than mid-lactation lactoferrin ($P = 0.017$). A pro-inflammatory chemokine, IL-8 is also known to decrease the integrity of tight junctions (Yu et al., 2013). These results suggest that the decrease in IL-8 production in the presence of early-lactation lactoferrin preparations may be the mechanism by which it increases TEER. The anti-inflammatory effect of early-lactation lactoferrin may also be related to the presence of angiogenin, which is known to suppress inflammatory responses (Lee et al., 2014).

Our research showed that early-lactation lactoferrin increased TEER, an in vitro measure of intestinal barrier integrity, but mid-lactation lactoferrin did not. Early-lactation lactoferrin also reduced the amount of IL-8 secreted by PBMC to a greater extent than mid-lactation lactoferrin. These differences may have been due to the greater concentration of angiogenin in the early-lactation lactoferrin preparation, because angiogenin has anti-inflammatory properties. However, further studies are required to confirm this, because the HPLC chromatographs did reveal other minor peaks that could represent other active components, such as transforming growth factor β and insulin-like growth factors 1 and 2. This study indicates that products rich in angiogenin may have intestinal health benefits, and that further work on isolated angiogenin is warranted.

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