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Consumption of transgenic cows' milk containing human lactoferrin results in beneficial changes in the gastrointestinal tract and systemic health of young pigs

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Abstract Lactoferrin is an antimicrobial and immunomodulatory protein that is produced in high quantities in human milk and aids in the gastrointestinal (GI) maturation of infants. Beneficial health effects have been observed when supplementing human and animal diets with lactoferrin. A herd of genetically engineered cattle that secrete recombinant human lactoferrin in their milk (rhLF-milk) have been generated which provide an efficient production system and ideal medium for rhLF consumption. The effects of consumption of rhLF-milk were tested on young pigs as an animal model for the GI tract of children. When comparing rhLF-milk fed pigs to non-transgenic milk fed pigs (control), we observed that rhLF-milk fed pigs had beneficial changes in circulating leukocyte populations. There was a significant decrease in neutrophils ($p = 0.0036$) and increase in lymphocytes ($p = 0.0017$), leading to a decreased neutrophil to lymphocyte ratio (NLR) ($p = 0.0153$), which is an indicator of decreased systemic inflammation. We also observed changes in intestinal villi architecture. In the duodenum, rhLF-milk fed pigs tended to have taller villi ($p = 0.0914$) with significantly deeper crypts ($p < 0.0001$). In the ileum, pigs consuming rhLF-milk had villi that were significantly taller ($p = 0.0002$), with deeper crypts ($p < 0.0001$), and a thinner lamina propria ($p = 0.0056$). We observed no differences in cytokine expression between rhLF-milk and control-milk fed pigs, indicating that consumption of rhLF-milk did not change cytokine signaling in the intestines. Overall favorable changes in systemic health and GI villi architecture were observed; indicating that consumption of rhLF-milk has the potential to induce positive changes in the GI tract.

Keywords Lactoferrin · Transgenic cows · Intestine · Crypt · Leukocytes · Milk

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Introduction

Lactoferrin is an 80 kDa iron binding protein found in various secretions such as milk and tears, as well as in neutrophil granules. Lactoferrin is part of the host defense system and has a wide range of functions, acting as an antimicrobial, immunomodulatory, and antioxidant agent (Wakabayashi et al. 2006). Part of lactoferrin's antimicrobial activity is due to its highly cationic N-terminal regions. These regions confer bactericidal

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action by interacting with the negatively charged part of bacterial membranes, which is lipopolysaccharide (LPS) in gram negative bacteria and lipoteichoic acid in gram positive bacteria (Yen et al. 2009). Lactoferrin can also compete with LPS for binding of CD14, a part of toll like receptor (TLR) 4, thus preventing LPS from activating a pro-inflammatory cascade which can lead to tissue damage (Actor et al. 2009). Lactoferrin's ability to bind iron not only promotes growth of beneficial low iron requiring bacteria like *Lactobacillus* and *Bifidobacteria* (Yen et al. 2009), but sequestering iron also reduces cellular oxidative stress, thus lowering pro-inflammatory cytokines (Actor et al. 2009). Finally, lactoferrin has targeted control of some cellular processes and can act as a transcription factor and regulate granulopoiesis and DNA synthesis in some cells types (Kanyshkova et al. 2001).

Human milk provides infants with substances that protect and promote maturation of the gut and the mucosal immune system (Walker 2010), and lactoferrin is one of the most abundant proteins found in human milk, with concentrations ranging from 1 to 3 g/L (Montagne et al. 2001). Cows however produce significantly less lactoferrin in their milk, averaging 0.115 g/L (Cheng et al. 2008). Human and bovine lactoferrin share 75 % sequence homology, however they have distinct glycosylation patterns (Actor et al. 2009). The differences in the maturation of the immune system and gastrointestinal (GI) tract of calves and infants may be in part due to differences in abundance of immune modulating proteins like lactoferrin in the milk they consume (Hettinga et al. 2011).

Lactoferrin has distinct properties that make it an ideal molecule for promoting healthy gut maturation and establishment of a beneficial GI-tract microbiota. Lactoferrin is resistant to enzymatic proteolysis in the stomach (Liao et al. 2007), and partial degradation of lactoferrin by stomach pepsin free the lactoferricin domain, which may be an even more potent antimicrobial (Yen et al. 2009). The lactoferricin domain is also key to lactoferrin's ability to bind cell surface proteins and DNA (Baker and Baker 2009). During the first hours of life the gut is permeable to many immunologically relevant proteins such as IgA and growth factors necessary for gut development (Commare and Tappenden 2007). After the first few days of life the gut becomes impermeable to most proteins, however infants can transport lactoferrin past gut closure (Harada et al. 1999). There is a 105 kDa lactoferrin receptor (also known as intelectin) that specializes in mediating uptake of lactoferrin into enterocytes and crypt cells (Liao et al. 2007, 2012). Once lactoferrin is taken up by enterocytes at the brush border, is internalized into compartments in the apical cytoplasm, where it can have effects on cellular proliferation and directing immune responses (Nielsen et al. 2010).

As previously mentioned, cows produce relatively little bovine lactoferrin in their milk, however Pharming Group BV, a Dutch-based biotechnology company, has used genetic engineering to produce a herd of transgenic cows that express approximately 1.5–2.0 g/L recombinant human lactoferrin (rhLF) in their milk, a concentration within the range normally made by humans (van Berkel et al. 2002). Zhang et al. showed in an experiment with neonatal mice that feeding rhLF-containing milk from a transgenic mouse strain improved intestinal growth (Zhang et al. 2001). To better assess the effects of consuming cow's milk containing rhLF, young pigs, which have very similar GI physiology and intestinal maturation to children, were chosen as a model (Guilloteau et al. 2010). Additionally the pig is a particularly relevant model for studying the effects of consumption of lactoferrin in milk because on average sows produce 0.3 g/L of lactoferrin in their milk and like humans pigs can transport lactoferrin past both gut closure and weaning (Harada et al. 1999) as pigs also have intestinal lactoferrin receptors which share 82 % homology with human lactoferrin receptors (Liao et al. 2007). Intestinal uptake of lactoferrin by pigs has both local effects on intestinal cell proliferation and systemic effects including stimulating hepatic protein synthesis (Harada et al. 1999). To determine the effects of consumption of rhLF-milk on intestinal health and overall immune function we used young pigs as a model for children. Expression levels of pro- and anti-inflammatory cytokines, intestinal histology, numbers of intraepithelial lymphocytes and goblet cells, hematological parameters and circulating leukocyte populations were examined.

Materials and methods

Milk collection and pasteurization

Transgenic cow's milk containing rhLF was provided by Pharming Group NV from a second parity Holstein

from their herd in Wisconsin. Milk was collected, pooled, frozen and then sent to the University of California Davis. A non-transgenic Holstein matched for parity and stage of lactation (mid-lactation) from the UC Davis dairy herd was selected and control milk was collected and frozen. Both control and rhLF containing milk were pasteurized at 73.8 °C and samples were collected and tested for lactoferrin activity, and then stored at 4 °C until consumption by the pigs.

Animals, blood sampling, necropsy, and sample collection

Male Hampshire Yorkshire crossbred pigs were obtained from the University of California swine facility, which is a specific pathogen free facility. Pigs from 4 l were weaned at 3 weeks of age and raised together before being moved to a containment facility at 6 weeks of age and singly housed. Pigs were weighted upon arrival and kept in a temperature-controlled room between 25 and 27 °C with ad libitum access to food (standard grower diet as previously described in Brundige et al. 2008) and water for the duration of the trial. The pigs were monitored twice daily for physical and general well-being. The pigs were randomly assigned to feeding groups and twice daily for 1 week fed 250 mL of either pasteurized rhLF-milk from one transgenic cow ($n = 8$) or pasteurized milk from a non-transgenic control cow ($n = 8$). Groups were balanced to have equal numbers of pigs from each litter. The amount of milk was increased to 350 mL twice daily per pig for the second week. Milk was delivered using a feeding pan to ensure that all animals were receiving the same amount of milk. At the end of the second week blood samples were collected via vena cava puncture into tubes containing EDTA for complete blood count (CBC) analysis using an ADVIA 120 Hematology System (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). The pigs were then weighed and euthanized using pentobarbital sodium (Fatal-Plus®, Vortech Pharmaceuticals, Ltd.) and tissue samples were collected. Duodenum samples were taken 20 cm below the pyloric sphincter and ileum samples were taken 20 cm above the ileocecal junction. Intestinal contents from the duodenum and ileum were collected for coliform and *E. coli* enumeration. Tissue samples to be used for qRT-PCR analysis were snap frozen in liquid nitrogen before being stored at −70 °C until RNA extraction, and samples for histology were washed in PBS them placed in formalin. The use and care of all animals in this study was approved by the UC Davis Institutional Animal Care and Use Committee, under Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) approved conditions.

Histology

Sections from the duodenum and ileum were placed in formalin for 48 h, and then progressively dehydrated in ethanol. Sections were embedded in paraffin and then cut and mounted on slides. Slides from the duodenum and ileum were stained with hematoxylin and eosin and were photographed. Analysis was done by measuring the villi height, width, lamina propria thickness, and crypt depth at 10× magnification, using Spot Advanced Software (v3.4, Diagnostic Instruments, Sterling Heights, MI). In addition, the number of intra-epithelial lymphocytes and goblet cells per villus were counted at 40× magnification and analyzed as cells per unit villous height. At least five villi were measured per intestinal section for each pig.

RNA preparation, cDNA synthesis, and qPCR

Samples from the duodenum and ileum were used for cytokine expression analysis. The isolation of and preparation of RNA, cDNA synthesis, and qPCR conditions have been previously described (Cooper et al. 2011). The transcription levels of pro-inflammatory cytokines TNFα, IL-8, IFN-γ, and IL-17, and anti-inflammatory cytokines IL-10, TGF-β1, and FoxP3 were determined using the Pfaffl method with REST-MCS software (Pfaffl et al. 2002). Briefly, the efficiency of each porcine specific and validated primer pair was calculated from standard curve data. Each target gene was normalized to the housekeeping gene β-actin to determine pair-wise fold differences in expression.

Coliform and *E. coli* analysis

Intestinal contents from the duodenum and ileum were used for enumeration of colonies of total coliforms and *E. coli*. Samples were serial diluted 1:100 three times in Butterfields buffer and then plated on Petrifilm coliform count plates (3 M, St. Paul, MN, USA) with 2

technical replicates per sample. Pertifilms were incubated at 37 °C for 24 h and the resulting colonies were counted.

Statistical analysis

Statistical analysis of hematological, histological, and bacterial data was performed using SAS statistical software (SAS, Cary, NC, USA). Tukey's test was used to determine p values and standard errors. Statistical analysis for fold expression differences from the qPCR assay was performed using REST-MCS software. For all analyses a p value of ≤ 0.05 was considered statistically significant.

Results

Complete blood count (CBC) analysis

Seventeen parameters were measured in the CBC analysis, of which three were significantly different between the rhLF-milk and control milk groups. All parameters pertaining to red blood cells and red blood cell components were unchanged between the rhLF-milk and control milk groups; however differences were seen in the proportions and total numbers of leukocytes in circulation (Table 1). Pigs consuming rhLF-milk had a significantly reduced proportion of circulating neutrophils ($p = 0.0036$) and a significantly higher proportion of circulating lymphocytes ($p = 0.0017$) (Fig. 1), as well as significantly more absolute circulating lymphocytes ($p = 0.0004$). Pigs consuming control milk had an absolute neutrophil to lymphocyte ratio (NLR) of 0.8744, while pigs consuming rhLF-milk had a significantly lower NLR of 0.4098 ($p = 0.0153$).

Histology

Pigs that were fed rhLF-milk tended to have longer villi in the duodenum than pigs fed control milk ($p = 0.0914$), as well as significantly deeper crypts ($p < 0.0001$) (Table 2). In the ileum differences between the two treatment groups were more pronounced. The rhLF-milk fed pigs had significantly longer villi ($p = 0.0002$), with deeper crypts ($p < 0.0001$), and a thinner lamina propria ($p = 0.0056$). No differences between the rhLF-milk and control milk fed pigs were observed in the number of either intra-epithelial leukocytes or goblet cell per unit of villi height.

Coliform and *E. coli* enumeration

No significant differences were seen in the number of coliform *E. coli* in duodenum of pigs fed control milk (50.13 CFUs ± SE 17.26) or rhLF-milk (87.75 CFUs ± 50.79), or the ileum of pigs fed control milk (62655.50 CFUs ± 45038.71) or rhLF-milk (21402.25 CFUs ± 16620.01).

Pig growth

No significant differences were seen in the total weight gain between pigs being fed rhLF-milk (8.94 kg ± 0.68) and control-milk (8.75 kg ± 1.55).

Cytokine expression

In both the duodenum and the ileum no differences were observed in the relative expression of any of the pro or anti-inflammatory cytokines investigated.

Discussion

These experiments were conducted to compare the effects of consumption of milk containing recombinant human lactoferrin to control milk on GI tract physiology, immune regulation and systemic health when consumed by healthy, young pigs. Lactoferrin activity was assayed in both control and rhLF-milk and found only to be active in rhLF-milk. While other immunomodulating proteins are found in cow's milk relatively little of these proteins are produced during mid-lactation, which is the period that both control and rhLF-milk were collected, so differences observed between the two treatment groups were attributable to the effects of rhLF in the milk. Consumption of rhLF-milk changed circulating leukocyte populations and GI villi architecture in ways that are considered beneficial, and did not affect cytokine signaling in either the duodenum or the ileum at the time necropsy.

There were significant differences seen in the proportions of circulating leukocytes between rhLF-milk and control milk fed pigs. Studies have shown that pig leukocytes have lactoferrin receptors (Harada

Table 1 Proportions and populations of circulating leukocytes from pigs fed control-milk or rhLF-milk for 2 weeks[a]

	Control-milk ($n = 8$)	rhLF-milk ($n = 8$)	p value
Neutrophil (%)	41.80 ± 11.84	27.49 ± 3.25	**0.0036***
Lymphocyte (%)	52.88 ± 10.75	67.53 ± 3.10	**0.0017***
Monocyte (%)	3.49 ± 0.98	3.53 ± 0.61	0.9301
Eosinophil (%)	1.13 ± 0.63	1.04 ± 0.53	0.7196
Basophil (%)	0.10 ± 0.12	0.15 ± 0.19	0.2509
Neutrophils (μL)	4332 ± 2097	3348 ± 554	0.2327
Lymphocytes (μL)	5131 ± 1385	8288 ± 1484	**0.0004***
Monocytes (μL)	340 ± 134	430 ± 107	0.1748
Eosinophils (μL)	113 ± 62	124 ± 64	0.6483
Basophils (μL)	22 ± 30	15 ± 19	0.5549
Neutrophil: Lymphocyte Ratio (μL)	0.87 ± 0.49	0.41 ± 0.07	**0.0153***

Bold values are statistically significant

* Indicates $p < 0.05$ when comparing rhLF-milk to control-milk

[a] Measurements presented as mean ± SD

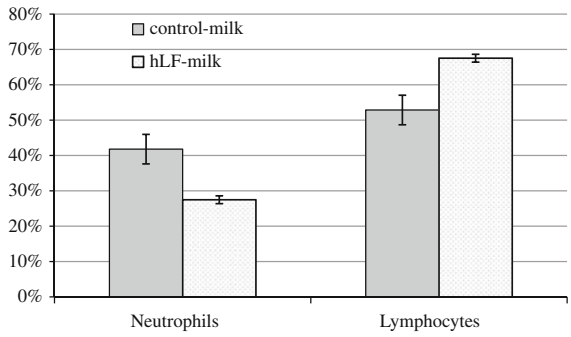

Fig. 1 Proportions of neutrophils and lymphocytes after feeding control-milk and hLF-milk to pigs for 2 weeks

et al. 1999), making them a predictable target for modulation by consumption of lactoferrin. Pigs fed rhLF-milk had a higher proportion of lymphocytes and a lower proportion of neutrophils. This change in proportion was mostly due to a significant increase in the absolute number of lymphocytes in circulation in pigs fed rhLF-milk. This increase in lymphocytes significantly decreased the NLR, which is a common measure of the level of systemic inflammation (Azab et al. 2012). Circulating populations of neutrophils and lymphocytes can be altered by a number of factors including age (van der Peet-Schwering et al. 2007), stress (Salak-Johnson et al. 1996), environment (Niekamp et al. 2007), and diet (van der Peet-Schwering et al. 2007).

An increased NLR is associated with increased systemic stress and inflammation (Imtiaz et al. 2012). Pig models of physiological responses to stress have shown that administration of stress hormones such as adrenocorticotropic hormone (ACTH) (Salak-Johnson et al. 1996) and dexamethasone (Kim et al. 2011) resulted in significantly lower proportions of lymphocytes and higher proportions of neutrophils, while the stress of weaning also induces similar increases in the NLR, which is mostly attributed to decreasing levels of lymphocytes (Kim et al. 2011).

In humans positive interventions such as diet change and weight loss can cause increases in the proportion of lymphocytes and decreases in levels of circulating serum pro-inflammatory cytokines (Wang et al. 2011). Similarly when utilizing pig models, consumption of supplements such as yeast mannans increases proportions of lymphocytes and decreases proportions of neutrophils in circulation, which is proposed to be caused by a reduction in inflammatory challenge (van der Peet-Schwering et al. 2007). Taken together, an increase in the proportion of lymphocytes is associated with beneficial health interventions and decreased systemic inflammation.

We saw that rhLF-milk tended to increase villi height in the duodenum and significantly increased villi height in the ileum. This is consistent with results found when feeding purified lactoferrin to young pigs (Wang et al. 2006; Liao et al. 2012) as well as when feeding young pigs a lactoferricin-lactoferrampin fusion protein (Tang et al. 2009). Mice fed lactoferrin also exhibited an increase in villi height (Yen et al. 2009), showing that lactoferrin's effects on villi architecture are similar across species. The increase in villi height is mainly attributed to lactoferrin's ability to cause concentration-dependent increases in proliferation and differentiation of small intestinal (SI) epithelial cells (Liao et al. 2012).

Most cellular proliferation in the intestine takes place in the crypts, as the crypts contain multicomponent local stem cells that are able to give rise to all terminally differentiated functional cell types in the SI, including enterocytes (Liao et al. 2012). In both the duodenum and ileum, pigs fed rhLF-milk had significantly deeper crypts. Other studies feeding lactoferrin to pigs (Harada et al. 1999) and adding it to the culture media of mouse crypt cell lines (Liao et al. 2012) show

Table 2 Histological measurements from the duodenum and ileum of pigs fed rhLF-milk and control-milk[a]

	Duodenum		
	Control milk ($n = 8$)	rhLF-milk ($n = 8$)	p value
Villi height (μm)	539.70 ± 74.14	609.60 ± 73.76	0.0914
Villi width (μm)	184.20 ± 37.03	182.150 ± 40.80	0.8565
Crypt depth (μm)	109.90 ± 20.27	183.96 ± 33.647	**<0.0001***
Lamina propria (μm)	496.00 ± 315.5	359.13 ± 148.73	0.2978
Lymphocytes/unit height	0.223 ± 0.132	0.1617 ± 0.0950	0.1155
Goblet cells/unit height	0.031 ± 0.015	0.0302 ± 0.011	0.8807
	Ileum		
	Control milk ($n = 8$)	rhLF-milk ($n = 8$)	p value
Villi height (μm)	413.83 ± 41.17	571.32 ± 74.49	**0.0002***
Villi width (μm)	162.15 ± 21.36	167.59 ± 21.74	0.4386
Crypt depth (μm)	90.17 ± 23.68	166.72 ± 35.14	**<.0001***
Lamina propria (μm)	249.33 ± 82.26	141.50 ± 44.30	**0.0056***
Lymphocytes/unit height	0.1131 ± 0.0415	0.1231 ± 0.0231	0.5599
Goblet cells/unit height	0.0407 ± 0.0134	0.0360 ± 0.0084	0.4083

Bold values are statistically significant

* Indicates $p < 0.05$ when comparing rhLF-milk to control-milk

[a] Measurements presented as mean ± SD

increased crypt cell proliferation in the presence of lactoferrin. Other studies also observed that proliferation in the crypts leads to increased intestinal absorptive surface area and increased renewal rate and have found a positive correlation between both villi height and crypt depth and body weight gain (Mahmoud and Edens 2012). While no difference in overall weight gain was observed in this study, dry feed intake and refusals were not recorded, so it was not possible to determine if there were changes in feed efficiency. The deeper crypts of the pigs consuming rhLF-milk should increase the number of enterocytes available for villus growth and might explain the longer villi and thus increased surface area in the intestines of those pigs. It is important to note that while increased crypt depth is also seen during intestinal damage, it is associated with recovery, and animals with deeper crypts after intestinal damage also exhibit lower levels of enterocyte apoptosis and villi atrophy (Koppelmann et al. 2012).

In the ileum of pigs fed rhLF-milk, we observed a significant decrease in the thickness of the lamina propria. The lamina propria is sensitive to inflammation and increases in thickness during an inflammatory reaction (Liu et al. 2010). Lysozyme is another antimicrobial and immunomodulatory protein found in high quantities in human milk, but in low quantities in the milk of ruminants like goats and cows (Hettinga et al. 2008). At UC Davis there is a herd of transgenic goats that produce milk containing recombinant human lysozyme (rhLZ-milk) (Maga et al. 2006). Previous studies have shown that pigs consuming rhLZ-milk (Cooper et al. 2011) and chicks consuming rice containing human lactoferrin and lysozyme also had a thinner lamina propria (Humphrey et al. 2002), which is associated with increased intestinal absorption and growth (Berman and Weinstein 1971). Since lactoferrin and lysozyme both possess antimicrobial and immunomodulatory properties (Walker 2010), it's logical that they would both decrease lamina propria inflammation, however the mechanism through which they cause this change remains unclear.

Together lactoferrin and lysozyme have synergistic antimicrobial properties (Leitch and Wilcox 1999), and help promote GI maturation and protect breast-feeding infants from diarrheal diseases (Walker 2010). Studies in our lab show healthy, young pigs consuming either rhLF-milk or rhLZ-milk both

experienced improvement of parameters associated with GI health, however many of the specific parameters that were improved were different. Consumption of rhLZ-milk had effects on intestinal bacterial populations (Maga et al. 2012), intestinal cytokine signaling, intraepithelial leukocytes, and moderate effects of GI villi architecture (Cooper et al. 2011), while rhLF-milk had more pronounced effects on villi architecture and changed circulating leukocyte populations. When the rhLZ-milk and rhLF-milk are consumed in combination the positive effects of both of these proteins may be realized in the GI tract, or they may have synergistic activity, as other research suggests.

Overall, pigs fed rhLF-milk instead of control milk experienced beneficial changes in GI physiology and systemic health. The changes in GI tract architecture including increased villi height, crypt depth, and decreased lamina propria thickness are all associated with increased surface area for nutrient absorption as well as decreased intestinal inflammation, indicating increased ability to uptake nutrients which could improve feed efficiency, however further studies specifically designed to investigate changes in feed efficiency need to be carried out. Increased populations of lymphocytes are associated with increased health in both pigs and humans because lymphocytes play a key role in regulating immunity and have the capacity to produce anti-inflammatory factors including IL-10 and TGFβ (Li et al. 2010). Lymphocyte activation and lymphoid organ development also increase resistance to infection (Li et al. 2010). The results from this study show that feeding milk from transgenic cows containing recombinant human lactoferrin improved GI and immune health and potentially increased the pigs' ability to absorb nutrients and fight off infection. Due to the similarities in GI physiology between young pigs and children comparable positive effects would be expected in children consuming rhLF-milk.

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Conflict of interest Authors C. A. Cooper, E. A. Maga, and J. D. Murray declare that they received a gift of milk used in the experiment from Pharming Group NV. Author K. M. Nelson discloses that she is the Director of Development and Health in the employment of Pharming Group NV.

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