Journal of Dairy Research (2014) **81** 30–37. © *Proprietors of Journal of Dairy Research 2013*
doi:10.1017/S0022029913000575

Consumption of transgenic milk containing the antimicrobials lactoferrin and lysozyme separately and in conjunction by 6-week-old pigs improves intestinal and systemic health

Caitlin A Cooper[1], Elizabeth A Maga[1] and James D Murray[1,2]*

[1] Department of Animal Science, University of California, Davis, USA
[2] Department of Population Health and Reproduction, University of California, Davis, USA

Received 5 June 2013; accepted for publication 18 September 2013

Lactoferrin and lysozyme are antimicrobial and immunomodulatory proteins produced in high quantities in human milk that aid in gastrointestinal (GI) health and have beneficial effects when supplemented separately and in conjunction in human and animal diets. Ruminants produce low levels of lactoferrin and lysozyme; however, there are genetically engineered cattle and goats that respectively secrete recombinant human lactoferrin (rhLF-milk), and human lysozyme (hLZ-milk) in their milk. Effects of consumption of rhLF-milk, hLZ-milk and a combination of rhLF-and hLZ-milk were tested on young pigs as an animal model for the GI tract of children. Compared with control milk-fed pigs, pigs fed a combination of rhLF and hLZ (rhLF + hLZ) milk had a significantly deeper intestinal crypts and a thinner lamina propria layer. Pigs fed hLZ-milk, rhLF-milk and rhLF + hLZ had significantly reduced mean corpuscular volume (MCV) and red blood cells (RBCs) were significantly increased in pigs fed hLZ-milk and rhLF-milk and tended to be increased in rhLF + hLZ-fed pigs, indicating more mature RBCs. These results support previous research demonstrating that pigs fed milk containing rhLF or hLZ had decreased intestinal inflammation, and suggest that in some parameters the combination of lactoferrin and lysozyme have additive effects, in contrast to the synergistic effects reported when utilising in-vitro models.

Keywords: Transgenic, lactoferrin, lysozyme, milk, hemaetology, pigs.

Lactoferrin and lysozyme are two antimicrobial proteins that are found in high quantities in human breast milk, but little is found in the milk of ruminants such as goats and cows (Hettinga et al. 2011). There are many health benefits that breastfed infants experience as breastfeeding is known to promote the development of the intestinal mucosa and immune system. Evidence shows that lactoferrin and lysozyme in breast milk help to confer these positive effects (Mountzouris et al. 2002; Newburg & Walker, 2007).

Lysozyme is a *N*-acetylmuramidase that is able to cleave 1,4-beta-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues found in the peptidoglycan layer of bacterial cells. Lysozyme is also found in other body secretions besides milk, including tears, saliva and intestinal mucus. It possesses the ability to modulate the inflammatory response through several mechanisms (Goldman et al. 1986). Along with cleaving peptidoglycan lysozyme binds to lipopolysaccharides (LPS) and lipoteichoic acid (LTA), preventing them from interacting with receptors on intestinal epithelial cells (IECs) and intestinal macrophages (Ohno & Morrison, 1989a; Ginsburg, 2002). Sequestration of LPS by lysozyme suppresses pro-inflammatory effects, including production of TNF-α (Ohno & Morrison, 1989b; Takada et al. 1994a, b; Kurasawa et al. 1996).

Human lactoferrin (hLF) can act as a bactericidal, bacteriostatic and immunomodulatory agent. Lactoferrin is resistant to enzymic proteolysis in the stomach (Liao et al. 2007), and partial degradation of lactoferrin by stomach pepsin frees the lactoferricin domain, which may be an even more potent antimicrobial (Yen et al. 2009). Lactoferrin is able to bind and sequester iron, as well as prevent pathogenic bacteria from adhering to intestinal epithelial cells and invading the intestinal tissue (Actor et al. 2009). There is a 105-kDa lactoferrin receptor (also known as intelectin) that specialises in mediating uptake of lactoferrin into enterocytes and crypt cells, and porcine and human lactoferrin receptors share 82% homology (Liao et al. 2007, 2012).

*For correspondence; e-mail: Jdmurray@ucdavis.edu

Once lactoferrin is taken up by enterocytes at the brush border, is internalised into compartments in the apical cytoplasm, where it can have effects on cellular proliferation and directing immune responses including reducing oxidative stress and reducing production of pro-inflammatory cytokines (Kruzel et al. 2007; Actor et al. 2009; Nielsen et al. 2010).

Previous work has yielded a herd of transgenic goats that produce human lysozyme (hLZ) at 270 mg/l which is 68% the concentration that is found in human milk (Maga et al. 2006a). Human lysozyme was chosen because it is a more active antimicrobial than other forms of lysozyme such as hen egg white lysozyme (HEWL) (Yang et al. 2011). Multiple studies have characterised the hLZ in the milk and its effects on bacteria in vitro (Maga et al. 2006a, b; Scharfen et al. 2007). In addition studies in vivo have shown that feeding human lysozyme-containing transgenic goats' milk at 270 mg/l positively impacts GI morphology, serum metabolites, lymphocyte populations, and increases anti-inflammatory cytokine expression in a porcine model (Brundige et al. 2008, 2010; Cooper et al. 2011). Consumption of the 270 mg/l hLZ-milk also has the ability to modulate the gut microbiota of healthy pigs, significantly increasing levels of Bifidobacteriaceae and Lactobacillaceae (Maga et al. 2012) and lowering levels of *Escherichia coli* in studies both in vitro and in vivo (Maga et al. 2006b, c).

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 secreted in human milk (van Berkel et al. 2002). Natural hLF from human milk and rhLF-milk have identical iron-binding and -release properties; however natural hLF and rhLF-milk undergo differential N-linked glycosylation (van Berkel et al. 2002). Natural hLF contains complex-type glycans and rhLF-milk contains oligomannose- and hybrid-type N-linked glycans, but the overall structures are identical (Thomassen et al. 2005).

Zhang et al. (2001) showed in an experiment with neonatal mice that feeding rhLF-containing milk from a transgenic mouse strain improved intestinal growth. Studies on feeding the rhLF-milk (1·2 g/l) to young pigs demonstrated beneficial changes in circulating leucocyte populations with a significant decrease in neutrophils and increase in lymphocytes, an indicator of decreased systemic inflammation, as well as changes in intestinal villi architecture including significantly taller villi, deeper crypts and a thinner lamina propria compared with control milk-fed pigs (Cooper et al. 2013).

When lactoferrin and lysozyme are together they have synergistic antimicrobial properties. Lactoferrin has a cationic domain that allows it to increase lysozyme's ability to kill bacteria. Lactoferrin binds to lipopolysaccharides on the outer membrane which aids in disrupting the membrane and allows lysozyme better access to the peptidoglycan layer underneath in Gram-negative bacteria (Leitch & Wilcox, 1999), and the proteoglycan matrix of Gram-positive bacteria (Ellison & Giehl, 1991). In conjunction, lactoferrin and lysozyme demonstrate a synergistic inhibition of growth of both Gram-positive and Gram-negative bacteria (van der Linden et al. 2009). Given the synergistic relationship between lactoferrin and lysozyme, milk from transgenic livestock containing both antimicrobials has the potential to have an even more pronounced positive effect on health. In addition, there are studies in vitro with both lactoferrin (Liao et al. 2012) and lysozyme (Maga et al. 2006b) that indicated that changing the concentration of these proteins modifies the responses seen; however previous feeding studies in vivo have not investigated the effect of altering the concentrations of rhLF or hLZ in milk. The present feeding study was designed to investigate both the effects of combining rhLF and hLZ-milk and to determine the effects of diluting rhLF-milk and hLZ-milk with non-transgenic control milk, creating milk with half the concentration rhLF and hLZ used in previous feeding studies.

Materials and methods

Milk collection and pasteurisation

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 cows' milk were pasteurised at 73·8 °C for 10 s and immediately chilled and then stored at 4 °C for no more than a week until consumption by the pigs. Pre and post-pasteurisation samples were collected and tested for lactoferrin activity through a bacterial lysis assay. The rhLF-milk contained 1·2 g/l of rhLF and retained 85% of its activity after pasteurisation.

Transgenic goat milk containing hLZ and control goat milk was acquired from the UC-Davis Goat Facility. The hLZ-milk was collected and pooled from 8 lactating does from the Artemis line of transgenic goats (Toggenburg and Alpine in origin) in their first to sixth parity, inclusive. Control goat milk was collected and pooled from non-transgenic control does of the UC Davis herd, mainly of Toggenburg, Alpine, LaMancha and Saanen in origin in various parities ranging from first to sixth. Milk was pasteurised at 73·8 °C then immediately cooled and stored at 4 °C for no more than a week before being fed to the pigs. Previous studies show that hLZ maintains 50% of its activity and that the concentration remains stable through pasteurisation (Scharfen et al. 2007). Western blots were performed to confirm the presence of the lysozyme in pre and post-pasteurised milk.

Animals, blood sampling, necropsy, and sample collection

Male and female Hampshire-Yorkshire crossbred pigs were obtained from the UC Davis Swine Facility, which is a

https://doi.org/10.1017/S0022029913000575 Published online by Cambridge University Press

specific pathogen-free facility. Pigs from 4 litters were weaned at 3 weeks of age and raised in co-housed pens before being moved to a containment facility at 6 weeks of age and housed singly. Pigs were weighed 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 1 of 4 milk feeding groups that were balanced for litter and sex. To control for differences in bovine and caprine milk all groups received a 50/50 mixture of cow milk and goat milk. The pigs received either 50% pasteurised rhLF-milk and 50% pasteurised control goat milk with a final concentration of 0·6 g/l of rhLF (rhLF + CG) ($n = 8$), 50% pasteurised hLZ-milk and 50% control cow milk with a final concentration of 135 mg/l hLZ (hLZ + CC) ($n = 8$), 50% pasteurised hLZ-milk and 50% rhLF-milk with a final concentration of 6 g/l of rhLF and 135 mg/l hLZ (rhLF + hLZ) ($n = 8$), or 50% pasteurised control cow milk and 50% control goat milk containing no rhLF or hLZ (CC + CG) ($n = 8$). Each pig was fed 250 ml of milk twice a day for the first week, then the amount of milk was increased to 350 ml twice daily 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 Hemaetology System. The pigs were then weighed and euthanised 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 ileocaecal junction. Intestinal contents from the duodenum and ileum were collected for enumeration of coliforms and *Esch. coli*. 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 then 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 haematoxylin 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, USA). In addition, the number of intra-epithelial lymphocytes and goblet cells per villus were counted at 40× magnification and analysed 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 and preparation of total RNA, cDNA synthesis and qPCR conditions have been previously described (Cooper et al. 2011). The transcription levels of pro-inflammatory cytokines TNF-α and IL-6, anti-inflammatory cytokine TGF-β1, as well as the intestinal receptor TRL-4, and iron transporter hepcidin 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 normalised to the housekeeping gene β-actin to determine pair-wise fold differences in expression.

Coliform and Esch. coli analysis

Intestinal contents from the duodenum and ileum were used for enumeration of colonies of total coliforms and *Esch. coli*. Samples were serially diluted 1 : 100 three times in Butterfields buffer and then plated on Petrifilm coliform count plates (3M, St. Paul MN, USA) with 2 technical replicates per sample. Petrifilms were incubated at 37 °C for 24 h and the resulting colonies were counted.

Statistical analysis

Statistical analysis of haematological, histological and bacterial data was performed using SAS statistical software (SAS, Cary NC, USA). Tukey's test was used to compare all 4 groups to one another and determine P values and standard errors for these comparisons. Statistical analysis for fold expression differences from the qPCR assay was performed using REST-MCS software. For all analyses a P value of $\leqslant 0{\cdot}05$ was considered statistically significant.

Results

Haematology

Nineteen haematological parameters were investigated (Table 1). Of these, two were significantly different between feeding groups. Pigs fed hLZ + CC and rhLF + hLZ had significantly lower mean corpuscular volume (MCV) than pigs fed CC + CG ($P = 0{\cdot}017$ and $P = 0{\cdot}0404$, respectively) and pigs fed rhLF + CG tended to have a lower MCV compared with CC + CG fed pigs ($P = 0{\cdot}051$)(Fig. 1a). Both rhLF + CG and hLZ + CC fed pigs had significantly more red blood cells (RBCs) than CC + CG fed pigs ($P = 0{\cdot}007$ and $P = 0{\cdot}011$, respectively), and pigs fed rhLF + hLZ tended to have more RBCs than CC + CG fed pigs ($P = 0{\cdot}090$) (Fig. 1b).

https://doi.org/10.1017/S0022029913000575 Published online by Cambridge University Press

Table 1. Haematological parameters in the pigs fed the various treatments†

Parameter‡	Unit	CC + CG† ($n = 8$)	hLZ + CC† ($n = 8$)	rhLF + CG† ($n = 8$)	rhLF + hLZ† ($n = 8$)
HGB	g/dl	11·3 ± 1·2	11·7 ± 2·0	12·0 ± 1·2	11·6 ± 1·3
HTC	%	36·1 ± 3·5	38·6 ± 6·9	39·8 ± 4·3	37·3 ± 4·1
MCH	pg	17·5 ± 0·5	16·1 ± 0·6	16·2 ± 1·1	16·7 ± 0·9
MCHC	g/dl	31·3 ± 1·1	30·4 ± 0·5	30·1 ± 1·2	31·2 ± 0·8
RDW	%	19·8 ± 4·5	20·7 ± 3·7	20·0 ± 2·4	19·6 ± 3·2
WBC	/µl	13 016 ± 4448	12 970 ± 4009	13 656 ± 3151	10 814 ± 2995
Neutrophil	%	35·51 ± 14·43	30·42 ± 7·18	31·73 ± 7·57	30·84 ± 9·00
Neutrophils	/µl	4623 ± 3219	3884 ± 1242	4281 ± 1251	3266 ± 1063
Lymphocyte	%	56·93 ± 13·57	62·22 ± 7·54	61·00 ± 7·57	61·39 ± 8·64
Lymphocytes	/µl	7424 ± 3585	8110 ± 2705	8372 ± 2461	6691 ± 2189
Monocyte	%	5·24 ± 0·90	4·92 ± 1·36	5·00 ± 1·24	5·29 ± 1·36
Monocytes	/µl	687 ± 273	650 ± 281	691 ± 261	594 ± 303
Eosinophil	%	1·66 ± 0·72	2·07 ± 0·86	1·86 ± 0·65	1·66 ± 0·64
Eosinophils	/µl	194 ± 70	278 ± 141	258 ± 113	178 ± 74
Basophil	%	0·44 ± 0·26	0·37 ± 0·08	0·40 ± 0·11	0·38 ± 0·16
Basophils	/µl	61 ± 52	47 ± 17	55 ± 20	44 ± 33
Platelets	/µl	478 750 ± 176 163	424 000 ± 159 477	421 750 ± 119 048	429 625 ± 91 728

†See text for details of treatments
‡HGB, haemoglobin; HTC, haematocrit; MCHC, mean corpuscular haemoglobin concentration; RDW, erythrocyte distribution width; WBC, white blood cells

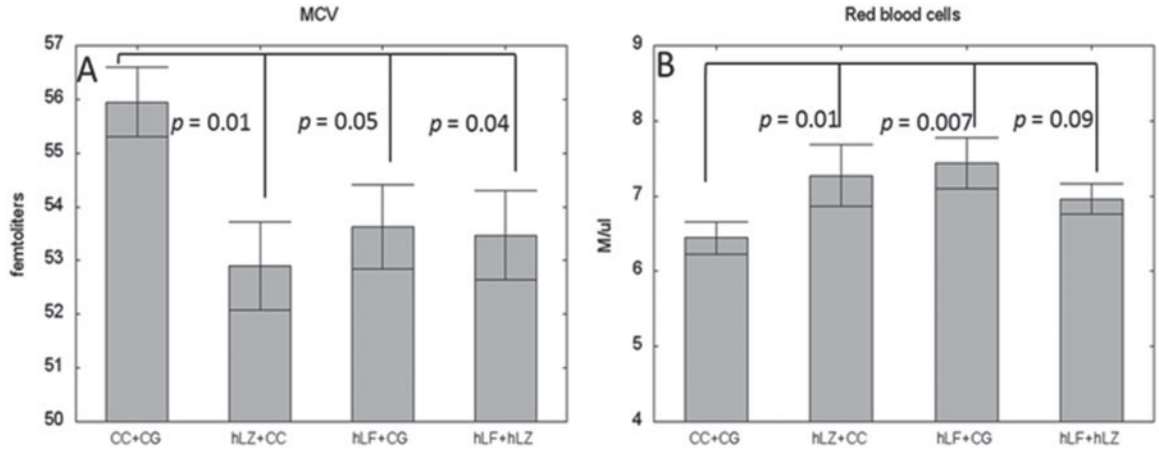

Fig. 1. Means for mean corpuscular volume (MCV) (a) and red blood cell (RBC) (b) for the four different groups. *P* values represent values when comparing the treatment groups (hLZ + CC, rhLF + CG, rhLF + hLZ) against the control group (CC + CG). See text for details of treatments.

Histology

In the duodenum there were no significant differences observed between any of the milk feeding groups (Table 2). In the ileum, pigs fed rhLF + hLZ milk had significantly deeper crypts than CC + CG fed pigs ($P = 0·05$) and pigs fed rhLF + CG tended to have deeper crypts ($P = 0·065$) than CC + CG fed pigs (Fig. 2a). The lamina propria was also significantly thinner in pigs fed rhLF + hLZ ($P = 0·025$) than pigs fed CC + CG, and pigs fed rhLF + CG tended to have a thinner lamina propria than CC + CG fed pigs ($P = 0·074$) (Fig. 2b).

Gene expression

No differences in the expression of TNF-α, IL-6, TGF-β, TLR-4 or hepcidin were seen in the small intestines of pigs fed rhLF + CG, hLZ + CC, or rhLF + hLZ when compared with pigs fed CC + CG milk (Table 3).

Esch. coli

No significant differences were seen in the number of *Esch. coli* and coliform in the ileum or colon of pigs fed any of the milk treatments (data not shown).

Discussion

Feeding young, healthy pigs milk containing either rhLF, hLZ, or rhLF + hLZ had local effects within the intestine as well as systemic effects on haematology when compared with pigs fed control milk. Overall, the combination of the

https://doi.org/10.1017/S0022029913000575 Published online by Cambridge University Press

Table 2. Histological measurements from the ileum and duodenum of pigs fed the various teatments†

	Duodenum			
	CC + CG†(*n* = 8)	hLZ + CC†(*n* = 8)	rhLF + CG†(*n* = 8)	rhLF + hLZ†(*n* = 8)
Villi height, µm	700·40 ± 193·43	772·66 ± 145·32	802·86 ± 103·85	735·62 ± 206·45
Villi width, µm	184·49 ± 14·99	186·18 ± 28·36	194·70 ± 32·16	194·97 ± 27·79
Crypt depth, µm	130·51 ± 54·63	169·41 ± 78·47	193·47 ± 54·41	167·16 ± 59·02
Lamina propria, µm	369·58 ± 107·82	288·32 ± 73·65	236·20 ± 67·33	336·76 ± 139·87
Lymphocytes/unit height	0·111 ± 0·020	0·097 ± 0·021	0·090 ± 0·023	0·112 ± 0·023
Goblet cells/unit height	0·033 ± 0·016	0·028 ± 0·012	0·036 ± 0·007	0·030 ± 0·015
	Ileum			
	CC + CG	hLZ + CC	rhLF + CG	rhLF + hLZ
Villi height, µm	484·46 ± 83·20	493·54 ± 96·65	512·13 ± 54·20	551·27 ± 102·27
Villi width, µm	162·75 ± 20·57	161·71 ± 26·04	169·00 ± 21·87	155·94 ± 16·87
Lymphocytes/unit height	0·101 ± 0·009	0·105 ± 0·024	0·119 ± 0·020	0·108 ± 0·019
Goblet cells/unit height	0·034 ± 0·009	0·042 ± 0·013	0·037 ± 0·013	0·035 ± 0·009

† See text for details of treatments

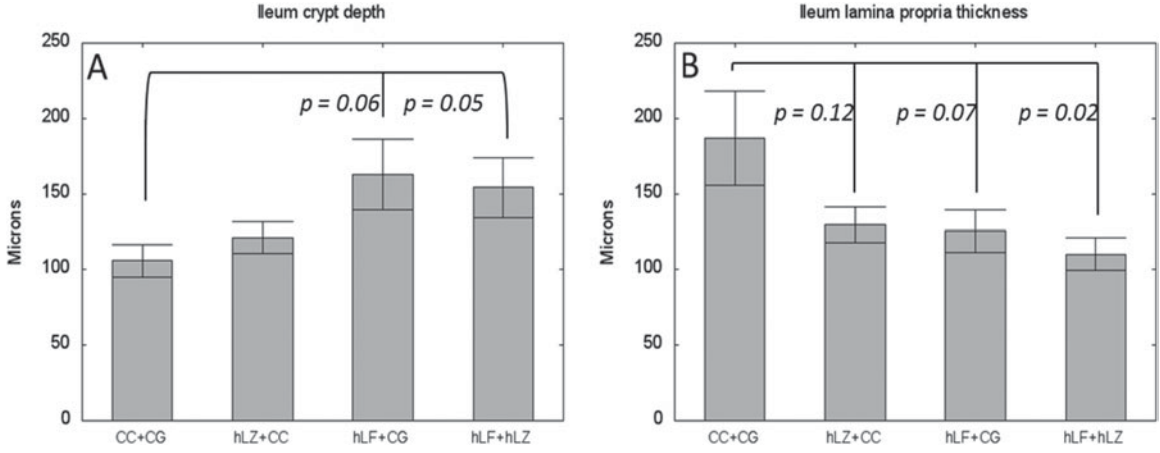

Fig. 2. Means for ileum crypt depth (a) and ileum lamina propria thickness (b) for the four different groups. *P* values represent values when comparing the treatment groups (hLZ + CC, rhLF + CG, rhLF + hLZ) against the control group (CC + CG). See text for details of treatments.

two milks was only able to elicit an enhanced response over a single antimicrobial in milk in ileal morphology and none of the other parameters studied. Studies in vitro using mouse cell lines show that lactoferrin can increase proliferation of crypt cells (Liao et al. 2012). In the ileum of the small intestine, feeding rhLF + hLZ milk significantly increased crypt depth, and rhLF + CG milk tended to increase crypt depth compared with control milk-fed animals. Both groups receiving rhLF milk showed this response to a very similar extent, thus we attribute this effect to the rhLF present in the milk. This is consistent with results from previous studies showing that feeding milk with twice the concentration of rhLF to pigs significantly increased crypt depth (Cooper et al. 2013), as well as studies feeding milk with twice the concentration of hLZ, which do not show this increase in crypt depth (Cooper et al. 2011).

The lamina propria was significantly thinner in pigs fed rhLF + hLZ and tended to be thinner in pigs fed rhLF + CG compared with those fed CC + CG. Pigs fed milk with twice the concentration of hLZ (Cooper et al. 2011), and rhLF (Cooper et al. 2013), as well as chickens fed transgenic rice containing a combination of recombinant human lactoferrin and lysozyme (Humphrey et al. 2002), also had thinner lamina propria than animals on control diets. When fed in combination (rhLF + hLZ) we see this same result, and to a lesser extent in the group fed rhLF + CG. We propose that this is due to the general anti-inflammatory and antibacterial properties of both lactoferrin and lysozyme (Actor et al. 2009; Lee et al. 2009; van der Linden et al. 2009) which is why this effect was seen to be strongest in the combination of rhLF + hLZ. The decreased concentration of hLZ in the hLZ + CC milk was not enough to induce this effect, implying that the thinning of the lamina propria is a dose-dependent effect.

Feeding hLZ + CC milk and rhLF + CG milk significantly increased the number of RBCs, and the combination of rhLF + hLZ milk tended to increase the number of RBCs. The

https://doi.org/10.1017/S0022029913000575 Published online by Cambridge University Press

Table 3. Expression levels of cytokines in the duodenum and ileum of pigs fed the various treatments†

		β-actin adjusted CT value			
Gene name	Area of the gut	CC + CG† (*n* = 8)	hLZ + CC† (*n* = 8)	rhLF + CG† (*n* = 8)	rhLF + hLZ† (*n* = 8)
IL-6	Duodenum	32·19	33·82	32·46	31·93
	Ileum	33·07	34·97	31·69	32·96
TGF-β	Duodenum	25·83	26·99	24·58	25·42
	Ileum	26·50	24·79	25·68	26·12
TNF-α	Duodenum	31·92	34·06	32·26	28·21
	Ileum	29·08	31·75	30·84	28·03
TLR-4	Duodenum	26·77	30·71	28·32	27·47
	Ileum	28·65	30·37	27·20	29·92
Hepcidin	Duodenum	28·30	29·44	28·76	30·35
	Ileum	29·30	27·99	29·10	27·93

†See text for details of treatments

mean corpuscular volume of hLZ + CC and rhLF + hLZ-fed pigs was significantly lower and tended to be lower in pigs fed rhLF + CG compared with pigs fed CC + CG. As RBCs mature they decrease in size (Evans, 2009; Moore et al. 2010) indicating that these pigs with reduced MCV had a more mature population of circulating RBCs along with the overall increase in RBCs. Feeding a variety of antimicrobial and anti-inflammatory supplements such as pomegranate (Harikrishnan et al. 2012), propolis (Cetin et al. 2010) and probiotics (Harikrishnan et al. 2010), have also yielded an increase in RBC counts in multiple species including carp (Kuang et al. 2012), chickens (Cetin et al. 2010) and rats (Ita et al. 2007). Since this response was seen when feeding lactoferrin and lysozyme, as well as other antimicrobials and anti-inflammatory agents, it is possible that these agents share a common mechanism of action.

One limiting factor in RBC production is iron availability. The bone marrow has a constant need for iron to produce erythrocytes and leucocytes (Jacobs & Summers, 1981; Fonseca et al. 2003; Drakesmith & Prentice, 2012). The main regulator of iron stores throughout the body is hepcidin and hepcidin expression is increased in certain tissues, such as macrophages, during inflammation (Moriguchi et al. 2012; Wang et al. 2012; Wu et al. 2012). Lactoferrin, lysozyme, and many other antimicrobials and anti-inflammatory molecules also work by modulating the gut microbiota, promoting growth of beneficial bacteria like *Bifidobacterium* and *Lactobacillus* (Hu et al. 2012; Maga et al. 2012), which in turn lower the pH of the gut and discourage the growth of pathogenic bacteria (Ljungh & Wadström, 2006). While no differences were seen in the number of coliforms and *Esch. coli* in the intestine, other changes in microbiota could act to decrease inflammatory signals in the gut, thus decreasing hepcidin expression in other tissues such as macrophages and the liver, thus increasing iron absorption and recycling in the body (Drakesmith & Prentice, 2012; Wu et al. 2012).

Similar studies feeding healthy young pigs the same volume of either 100% rhLF-milk or 100% hLZ-milk did not change RBC numbers (Brundige et al. 2008; Cooper et al. 2013). In the current study increased RBCs were observed when the dose was reduced by half, and other studies have shown a similar dose-dependent response, with lower concentrations eliciting a similar increase in RBCs, which was not observed at higher doses (Cetin et al. 2010). A feeding study by Cerven et al. (2008) found that rats fed rice containing recombinant human lactoferrin at 100 and 500 mg/kg had increased RBC counts but at 1000 mg/kg there was no effect on RBC count. Why this increase in RBC numbers was only seen in half doses of rhLF and hLZ milk is unknown; however, the 100% doses of both rhLF-milk and hLZ-milk caused proliferation of subsets of leucocytes (Brundige et al. 2008; Cooper et al. 2013). Proliferation of leucocytes in the bone marrow is an iron-dependent process (Jacobs & Summers, 1981; Fonseca et al. 2003; Drakesmith & Prentice, 2012) so it is possible that at increased levels rhLF-milk and hLZ-milk still promote increased iron absorption and recycling; however this excess iron is instead funnelled to production of leucocytes as opposed to erythrocytes.

No changes in the expression of cytokines TNF-α, IL-6, or TGF-β, bacterial ligand TRL-4, or intestinal hepcidin, were observed in the small intestines of pigs fed rhLF + CG, hLZ + CC, or rhLF + hLZ when compared with control pigs fed CC + CG. While we observed no changes in expression of intestinal pro-inflammatory cytokines, other parameters including the thinning of the lamina propria indicate that the GI tract of animals fed rhLF + CG and rhLF + hLZ were experiencing a reduction in intestinal inflammation; however these effects may have gradually accumulated over the 2-week feeding period. A study feeding milk containing hLZ at twice the concentration (270 mg/l) fed in the current study found that the hLZ-milk increased production of anti-inflammatory cytokine TGF-β1 (Cooper et al. 2011), indicating that when fed at a decreased concentration (135 mg/l) hLZ-milk is no longer able to illicit this change.

Overall the haematological and GI tract changes observed in animals fed rhLF + CG, hLZ + CC, and rhLF + hLZ compared with CC + CG indicate that these substances have anti-inflammatory effects both locally within the GI tract,

https://doi.org/10.1017/S0022029913000575 Published online by Cambridge University Press

and systemically. Locally, feeding of both milk treatments containing rhLF caused deepening of the crypts which is consistent with previous work in mouse cell lines and pigs showing that lactoferrin induces crypt cell proliferation and deepens crypts in the small intestine (Liao et al. 2012; Cooper et al. 2013). Other studies have found that increased proliferation in the crypts leads to increased intestinal absorptive surface area and increased cellular renewal rate (Mahmoud & Edens, 2012). Feeding rhLF + CG and rhLF + hLZ also decreased the thickness of the lamina propria. This same change has been seen in pigs fed milk with twice the concentration of hLZ (Cooper et al. 2011) and twice the concentration of rhLF (Cooper et al. 2013), and is associated with decreased intestinal inflammation (Liu et al. 2010). The haematological results are in agreement with results from multiple other species fed other antimicrobial and anti-inflammatory compounds that show that feeding antimicrobial/anti-inflammatory compounds increases RBC production. We speculate that this is through changes in inflammation which may alter hepcidin regulation, and increase iron availability in the bone marrow for RBC production; however further research is needed to elucidate the actual mechanism. We detected no adverse effects from feeding milk containing hLZ, rhLF, or a combination of the two. Positive effects in both GI tract architecture and haematological parameters were observed, and these changes indicate that milk containing hLZ, rhLF, and a combination of the two decreases intestinal inflammation, and that at decreased concentration rhLF-milk still induces crypt cell proliferation. Given past research, this study also demonstrates that different concentrations of both rhLF-milk and hLZ-milk can induce different physiological changes. Further studies are needed to determine the exact mechanisms that rhLF-milk and hLZ-milk utilise to cause these intestinal and systemic changes, as well as detailed dose response studies to elucidate the threshold concentrations of rhLF-milk and hLZ-milk needed to induce the specific changes that have been observed in this and previous studies.

We would like to thank BV Pharming for providing rhLF milk. We would also like to thank Doug Gisi and the UC-Davis Dairy Barn staff for care and milking of the dairy cows, Jan Carlson and the UC-Davis goat barn staff for care and milking of the goats, and Kent Parker and the UC-Davis Swine facility staff for assistance with pig rearing as well as, Lydia Garas Klobas, Elizabeth McInnis, Erica Scott, and Merritt Clark for help handling the pigs. We thank Katherine Cubbon and Brigette Santamaria, for help examining the histological slides and Samantha Lotti and Justin Nunes for help processing intestinal samples for qRT-PCR. This work was supported by a Jastro-Shields grant from the University of California, Davis.

References

Actor JK, Hwang SA & Kruzel ML 2009 Lactoferrin as a natural immune modulator. *Current Pharmaceutical Design* **15** 1956–1973

Brundige DR, Maga EA, Klasing KC & Murray JD 2008 Lysozyme transgenic goats' milk influences gastrointestinal morphology in young pigs. *Journal of Nutrition* **138** 921–926

Brundige DR, Maga EA, Klasing KC & Murray JD 2010 Consumption of pasteurized human lysozyme transgenic goats' milk alters serum metabolite profile in young pigs. *Transgenic Research* **19** 563–574

Cerven D, DeGeorge G & Bethell D 2008 28-day repeated dose oral toxicity of recombinant human holo-lactoferrin in rats. *Regulatory Toxicology and Pharmacology* **52** 174–179

Cetin E, Silici S, Cetin N & Güçlü BK 2010 Effects of diets containing different concentrations of propolis on hematological and immunological variables in laying hens. *Poultry Science* **89** 1703–1708

Cooper CA, Brundige DR, Reh WA, Maga EA & Murray JD 2011 Lysozyme transgenic goats' milk positively impacts intestinal cytokine expression and morphology. *Transgenic Research* **20** 1235–1243

Cooper CA, Nelson KM, Maga EA & Murray JD 2013 Consumption of transgenic cows' milk containing human lactoferrin results in beneficial changes in the gastrointestinal tract and systemic health of young pigs. *Transgenic Research* **22** 571–578

Drakesmith H & Prentice AM 2012 Hepcidin and the iron-infection axis. *Science* **338** 768–772

Ellison RTJ & Giehl TJ 1991 Killing of Gram-negative bacteria by lactoferrin and lysozyme. *Journal of Clinical Investigation* **88** 1080–1091

Evans GO 2009 *Animal Hematoxicology: a Practical Guild for Toxicologist and Biomedical Researchers*, 1st edition. Boca Raton, FL: Taylor and Francis Group

Fonseca AM, Pereira CF, Porto G & Arosa FA 2003 Red blood cells upregulate cytoprotective proteins and the labile iron pool in dividing human T cells despite a reduction in oxidative stress. *Free Radical Biology and Medicine* **35** 1404–1416

Ginsburg I 2002 Role of lipoteichoic acid in infection and inflammation. *Lancet Infectious Disease* **2** 171–179

Goldman AS, Thorpe LW, Goldblum RM & Hanson LA 1986 Anti-inflammatory properties of human milk. *Acta Paediatrica Scandinavica* **75** 689–695

Harikrishnan R, Balasundaram C & Heo MS 2010 Effect of probiotics enriched diet on Paralichthys olivaceus infected with lymphocystis disease virus (LCDV). *Fish and Shellfish Immunology* **29** 868–874

Harikrishnan R, Kim JS, Kim MC, Balasundaram C & Heo MS 2012 Pomegranate enriched diet enhances the hematology, innate immune response, and disease resistance in olive flounder against Philasterides dicentrarchi. *Veterinary Parasitology* **187** 147–156

Hettinga K, van Valenberg H, de Vries S, Boeren S, van Hooijdonk T, van Arendonk J & Vervoort J 2011 The host defense proteome of human and bovine milk. *PLoS ONE* **6** e19433

Hu W, Zhao J, Wang J, Yu T, Wang J & Li N 2012 Transgenic milk containing recombinant human lactoferrin modulates the intestinal flora in piglets. *Biochemistry and Cell Biology* **90** 485–496

Humphrey BD, Huang N & Klasing KC 2002 Rice expressing lactoferrin and lysozyme has antibiotic-like properties when fed to chicks. *Journal of Nutrition* **132** 1214–1218

Ita SO, Etim OE, Ben EE & Ekpo OF 2007 Haematopoietic properties of ethanolic extract of Ageratum conyzoides (goat weed) in albino rats. *Niger Journal of Physiological Science* **22** 83–87

Jacobs A & Summers MR 1981 Iron uptake and ferritin synthesis by peripheral blood leucocytes in patients with primary idiopathic haemochromatosis. *British Journal of Haematology* **49** 649–652

Kruzel ML, Actor JK, Boldogh I & Zimecki M 2007 Lactoferrin in health and disease. *Postepy higieny i medycyny doswiadczalnej* **61** 261–267

Kuang SY, Xiao WW, Feng L, Liu Y, Jiang J, Jiang WD, Hu K, Li SH, Tang L & Zhou XQ 2012 Effects of graded levels of dietary methionine hydroxy analogue on immune response and antioxidant status of immune organs in juvenile Jian carp (Cyprinus carpio var. Jian). *Fish and Shellfish Immunology* **32** 629–636

Kurasawa T, Takada K, Ohno N & Yadomae T 1996 Effects of murine lysozyme on lipopolysaccharide-induced biological activities. *Federation of European Microbiological Societies: Immunology and Medical Microbiology* **13** 293–301

Lee M, Kovacs-Nolan J, Yang C, Archibold T, Fan MZ & Mine Y 2009 Hen egg lysozyme attenuates inflammation and modulates local gene

expression in a porcine model of dextran sodium sulfate (DSS)-induced colitus. *Journal of Agricultural Food Chemistry* **57** 2233–2240

Leitch EC & Wilcox MD 1999 Elucidation of the antistaphylococcal action of lactoferrin and lysozyme. *Journal of Medical Microbiology* **48** 867–871

Liao Y, Lopez V, Shafizadeh TB, Halsted CH & Lonnerdal B 2007 Cloning of a pig homologue of the human lactoferrin receptor: expression and localization during intestinal maturation in piglets. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* **148** 584–590

Liao Y, Jiang R & Lonnerdal B 2012 Biochemical and molecular impacts of lactoferrin on small intestinal growth and development during early life. *Biochemistry and Cell Biology* **90** 476–484

Liu D, Guo Y, Wang Z & Yuan J 2010 Exogenous lysozyme influences Clostridium perfringens colonization and intestinal barrier function in broiler chickens. *Avian Pathology* **39** 17–24

Ljungh A & Wadström T 2006 Lactic acid bacteria as probiotics. *Current Issues in Intestinal Microbiology* **7** 73–89

Maga EA, Shoemaker CF, Rowe JD, Bondurant RH, Anderson GB & Murray JD 2006a Production and processing of milk from transgenic goats expressing human lysozyme in the mammary gland. *Journal of Dairy Science* **89** 518–524

Maga EA, Cullor JS, Smith W, Anderson GB & Murray JD 2006b Human lysozyme expressed in the mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis and the cold-spoilage of milk. *Foodborne Pathology and Disease* **3** 384–392

Maga EA, Walker RL, Anderson GB & Murray JD 2006c Consumption of milk from transgenic goats expressing human lysozyme in the mammary gland results in the modulation of intestinal microflora. *Transgenic Research* **15** 515–519

Maga EA, Desai PT, Weimer BC, Dao N, Kültz D & Murray JD 2012 Consumption of lysozyme-rich milk can alter microbial fecal populations. *Applied and Environmental Microbiology* **78** 6153–6160

Mahmoud KZ & Edens FW 2012 Breeder age affects small intestine development of broiler chicks with immediate or delayed access to feed. *British Poultry Science* **53** 32–41

Moore G, Knight G & Blann A 2010 *Fundamentals of Biomedical Science: Haematology*. New York, NY: Oxford University Press

Moriguchi R, Otaki Y, Hazeki S, Shimada T, Matsumoto A, Kakita N, Kaibe S, Kuragano T, Nonoguchi H, Masayoshi N, Hasuike Y & Nakanishi T 2012 High levels of tumor necrosis factor-α downregulate antimicrobial iron transport protein, Nramp1, in chronic hemodialysis patients: a key factor for infection risk. *American Journal of Nephrology* **35** 372–378

Mountzouris KC, McCartney AL & Gibson GR 2002 Intestinal microflora of human infants and current trends for its nutritional modulation. *British Journal of Nutrition* **87** 405–420

Newburg DS & Walker WA 2007 Protection of the neonate by the innate immune system of developing gut and of human milk. *Pediatric Research* **61** 2–8

Nielsen SM, Hansen GH & Danielsen EM 2010 Lactoferrin targets T cells in the small intestine. *Journal of Gastroenterology* **45** 1121–1128

Ohno N & Morrison DC 1989a Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme enzymatic activity. *Journal of Biological Chemistry* **264** 4434–4441

Ohno N & Morrison DC 1989b Lipopolysaccharide interactions with lysozyme differentially affect lipopolysaccharide immunostimulatory activity. *European Journal of Biochemistry* **186** 629–636

Pfaffl MW, Horgan GW & Dempfle L 2002 Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30** 36

Scharfen EC, Mills DA & Maga EA 2007 Use of human lysozyme transgenic goat milk in cheese making: effects on lactic acid bacteria performance. *Journal of Dairy Science* **90** 4084–4091

Takada K, Ohno N & Yadomae T 1994a Binding of lysozyme to lipopolysaccharide suppresses tumor necrosis factor production in vivo. *Infection and Immunity* **62** 1171–1175

Takada K, Ohno N & Yadomae T 1994b Detoxification of lipopolysaccharide (LPS) by egg white lysozyme. *Federation of European Microbiological Societies: Immunology and Medical Microbiology* **9** 255–263

Thomassen EA, van Veen HA, van Berkel PH, Nuijens JH & Abrahams JP 2005 The protein structure of recombinant human lactoferrin produced in the milk of transgenic cows closely matches the structure of human milk-derived lactoferrin. *Transgenic Research* **14** 397–405

van Berkel PH, Welling MM, Geerts M, van Veen HA, Ravensbergen B, Salaheddine M, Pauwels EK, Pieper F, Nuijens JH & Nibbering PH 2002 Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nature Biotechnology* **20** 484–487

van der Linden DS, Short D, Dittmann A & Yu PL 2009 Synergistic effects of ovine-derived cathelicidins and other antimicrobials against Escherichia coli O157:H7 and Staphylococcus aureus 1056 MRSA. *Biotechnology Letters* **31** 1265–1267

Wang L, Trebicka E, Fu Y, Ellenbogen S, Hong CC, Babitt JL, Lin HY & Cherayil BJ 2012 The bone morphogenetic protein-hepcidin axis as a therapeutic target in inflammatory bowel disease. *Inflammatory Bowel Disease* **18** 112–119

Wu X, Yung LM, Cheng WH, Yu PB, Babitt JL, Lin HY & Xia Y 2012 Hepcidin regulation by BMP signaling in macrophages is lipopolysaccharide dependent. *PLoS ONE* **7** e44622

Yang B, Wang J, Tang B, Liu Y, Guo C, Yang P, Yu T, Li R, Zhao J, Zhang L, Dai Y & Li N 2011 Characterization of bioactive recombinant human lysozyme expressed in milk of cloned transgenic cattle. *PLoS ONE* **16** e17593

Yen CC, Lin CY, Chong KY, Tsai TC, Shen CJ, Lin MF, Su CY, Chen HL & Chen CM 2009 Lactoferrin as a natural regimen for selective decontamination of the digestive tract: recombinant porcine lactoferrin expressed in the milk of transgenic mice protects neonates from pathogenic challenge in the gastrointestinal tract. *Journal of Infectious Disease* **199** 590–598

Zhang P, Sawicki V, Lewis A, Hanson L, Nuijens JH & Neville MC 2001 Human lactoferrin in the milk of transgenic mice increases intestinal growth in ten-day-old suckling neonates. *Advances in Experimental Medicine and Biology* **501** 107–113