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Intestinal Histology and Immune Status of Semi-anemic Piglets Fed Lactoferrin, Meat, or Meat Extract

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This study assessed the effects of dietary lactoferrin, meat, or meat extract on immune status and small intestine morphology of 24 three-week-old semi-anemic piglets (six per group) over 28 days, after a one week acclimatization period. Blood samples collected from the piglets at 4 and 8 weeks of age were subjected to whole-blood proliferation and phagocytic activity assays. The leukocyte phagocytic activity and lymphocyte cell proliferative responses of the piglets to concanavalin A and phytohemagglutinin were significantly ($p < 0.05$) improved by the meat-extract diet. This demonstrated that meat extract is a potential immuno-modulating feed ingredient particularly after the period of weaning when piglets are highly susceptible to infection. For intestinal histology, each piglet was euthanized at 8 weeks of age while under anaesthesia and had its small intestine removed. No diet effects were observed for some histological parameters (villus height, crypt depth, and mucosal thickness). However, meat and meat-extract diets significantly ($p = 0.003$) increased the number of goblet cells / 100 µm of villous epithelium, which suggests that the meat extract and meat diets stimulated mucin secretion.

Keywords: bioavailability, histology, immunity, iron, meat

INTRODUCTION

Digestion products in the gastrointestinal tract have the ability to reduce dietary ferric iron into a more soluble ferrous iron form (Sorensen *et al.* 2006). The crypt cells of the small intestine in particular appear to detect iron concentrations through the hemochromatosis gene product transferrin receptor 1(HFE-TfR1) complex on the basolateral membrane of duodenal cells (Fleming and Britton 2006). Since final nutrient digestion and assimilation occurs in the crypts of Lieberkühn and villi of the small intestine, intestinal development can be assessed through measurements of the crypt depth and villus height to determine the total surface area available for digestion and absorption (Laudadio *et al.* 2012).

In general, iron – the most abundant trace element in the body (Himmelfarb 2007, Lopez and Martos 2004) – plays a fundamental role in various biological and chemical processes and functions as an integral component of hemoglobin and myoglobin (Ganz 2007, Lopez and Martos 2004). Once absorbed, iron contributes to the formation of some neurotransmitters and hormones, and to xenobiotic metabolism (Eisenstein 2000). Iron is also directly involved in the host's defense responses to infectious agents and is a constituent of host defense proteins such as lactoferrin, siderocalin, and divalent metal transporter natural resistance associated macrophage

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protein 2 (Ganz 2007). The myeloperoxidase enzyme also contains iron, which contributes to the function of white blood cells in their response to infection (Engelking 2004). More importantly, lactoferrin not only participates in iron transport but also adds to immune system function due to its antimicrobial and antiparasitic activities (Adlerova *et al.* 2008). In addition, the pig – having a higher concentration of milk lactoferrin compared to other animals – serves as a good model to investigate the interactions that occur between lactoferrin and its receptor, and in the evaluation of the differential expression of lactoferrin receptor (LfR) in the small intestine (Liao *et al.* 2007).

The aims of the current study were to assess the effects of lactoferrin, meat, and meat extract in the diet on measurements of histological parameters and the immunological status of semi-anemic piglets because of the suggested relevance of these parameters to iron absorption.

MATERIALS AND METHODS

Experimental procedures were approved by the Massey University Animal Ethics Committee and complied with the New Zealand Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific Purposes. The study was conducted at the Massey University Animal Physiology Unit in Palmerston North, New Zealand.

Experimental Animals

Twenty-four (24) piglets from six litters were injected with 60 mg of iron (Gleptosil™) within 24 hours of birth. This represented 30% of the normal dose of 200 mg (Papich 2007). The piglets were reared with the sow and weaned at 3 weeks of age. No creep feed was available to the piglets during the suckling period.

After weaning, piglets were housed individually in metabolism crates for a period of 5 weeks, the first of which was a period of acclimatization to the new environment when they were fed a milk supplement containing 24% skim milk powder and 6% casein. At the end of the first week, the piglets were randomly allocated within litter to one of the following four dietary treatment groups:

1. Non-meat diet – six Piglets on a non-meat diet that was normal for piglets of their age (control);
2. Non-meat diet – six Piglets on a non-meat diet plus an amount of lactoferrin equal to that found in the amount of meat extract;
3. Meat diet – six Piglets on a non-meat diet plus 250 g of meat per kg. The meat was minced beef semi-membranous plus adductor muscle, cooked for 90 min at 70 ^{o}C; and
4. Meat extract – six Piglets on a non-meat diet plus a <0.5 kDa sarcoplasmic extract at a concentration per kg equivalent to the amount from 250 g of meat. This fraction contained amino acids and dipeptides, as well as the minerals in the sarcoplasmic fluid of the meat cells.

The composition and contents of the experimental diets on an as-fed basis are given in Table 1. The experimental diets were fed for 28 days. The as-fed amount given to each piglet per day was 500 g at the start of the experiment (Day 0), and was increased by 100 g every 4 days up to 1200 g per day at the end of the trial (Day 28). The piglets were fed twice daily at 8:00 A.M. and 5:00 P.M., and fresh water was made available at all times. Feed refusals were collected and weighed daily, and the piglets were weighed weekly.

Immunological Parameters

Whole-blood cell proliferation and phagocytic activity were measured in blood collected from the jugular vein at the start (Day 0) and end (Day 28) of the experiment.

Table 1. Composition of the experimental diets formulated to be isonitrogenous and have a similar amount of total iron.

Ingredients (g/kg) as-fed	Control	Lactoferrin	Meat	Meat Extract[a]
Wheat starch	130.3	130.2	130.2	130.2
Casein	100	94.68	34.55	95.68
Meat	0	0	250	0
Soya bean oil	50	50	50	50
Cellulose	15	15	15	15
Lactoferrin[b]	0	5.319	0	0
Meat extract	0	0	0	5.319
Methionine	1.5	1.5	1.5	1.5
Threonine	1.5	1.5	1.5	1.5
Vitamin + mineral (No Fe)	1	1	1	1
Dicalcium phosphate	15	15	15	15
Salt (NaCl)	0.75	0.75	0.75	0.75
Potassium carbonate	1.75	1.75	0.45	0.75
Fe heptasulphate	0.08	0.077	0.05	0.08
Water	683	683	500	683

Notes:
[a]Sarcoplasmic meat extract (<0.5 kDa) containing amino acids and dipeptides as well as the minerals in the sarcoplasmic fluid of the meat cells.
[b]$C_{14}H_{22}N_{46}O_{29}S_3$

The whole blood cell proliferation assay was modified from previously reported methods (Hiraga *et al.* 1981, Weiss 1992, Saker *et al.* 2001). Briefly, lithium heparin-treated peripheral whole blood was diluted 1:4 in complete RPMI-1640 medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM-L-glutamine, 100Uml^{-1} penicillin, 100 µgml^{-1} streptomycin sulfate, and 50 µM 2-mercaptoethanol; all reagents from Gibco, Poole, UK). One hundred microliters (100 µl) of the diluted blood was added in quadruplicate to the wells of a 96-well, flat-bottomed tissue culture plate (Greiner, Neuberg, Germany) and cultured in the presence of either 5 µgml^{-1}concanavalin A (ConA) (Sigma, USA), 1:49 diluted phytohemagglutinin (PHA) (Gibco, Poole, UK), or complete RPMI-1640 in place of the mitogen (control wells). The cells were then cultured for 48 h at 37 °C in a 5% humidified CO_2-air atmosphere, before being pulsed for 18 h with 0.5 µCi methyl-^{3}H-thymidine (Amersham Biosciences, UK) per well. Each plate was then harvested into a 96-well glass fiber mat using a Tomtek cell harvester 96 (Hamden, CT, USA) and counted using a Wallac MicroBetaTrilux 1450 liquid scintillation and luminescence counter (Turku, Finland). A stimulation index was calculated as cpm in wells with mitogen divided by cpm in wells without mitogen.

Assessment of the phagocytic capacity of peripheral blood leucocytes by flow cytometry was based on the method of Rutherfurd-Markwick *et al.* (2005). Briefly, 5 µl of FITC-labelled *Escherichia coli* bacteria (1 x 10^9 ml^{-1}) (Molecular Probes Incorporated, OR, USA) was mixed with 100 µl of whole blood and incubated for 30 min at 37 °C. Immediately following incubation, the cells were fixed with paraformaldehyde and the erythrocytes lyzed by the addition of 1 ml of ice-cold water. Following centrifugation, the pellet was re-suspended in 500µl of PBS, and 50 µl of 4% Trypan blue was added to quench extraneous fluorescence. The phagocytic activity (expressed as percentage gated cells exhibiting phagocytic activity) was determined using a FACS Calibur flow cytometer (Becton Dickinson Instruments, Cambridge, MA, USA).

Given the high variation in immunological parameters between animals, the changes between Day 0 and Day 28 of the experiment were calculated for each animal and parameter, and a paired *t*-test was used to test if the average changes within treatment groups were different from zero.

Intestinal Histology

After euthanasia on Day 28 of the study (intracardial injection of 150 mg/kg sodium pentobarbitone when under anesthesia with 4% fluothanoxygen), the small intestine was ligated with nylon twine and removed and laid out on a stainless steel tray. Three sections at proportionally 25% (duodenum), 50% (jejunum), and 75% (ileum) along the intestine were clamped with hemostats, excised, and placed immediately into Bouin's fluid (24% formalin, 5% glacial acetic acid, and 71% picric acid) for 24 h before being stored in 70% ethanol. After fixation, ring-shaped lengths of the small intestine from the three sections were excised, dehydrated, and embedded in paraffin wax. From each of these, transverse 6 µm sections were cut; stained with alcian blue, hematoxylin, and eosin; and examined under a light microscope. The height of the villi, depth of the crypts of Lieberkühn, and mucosal thickness were determined on at least three villi per piglet from the duodenum, jejunum, and ileum using the image analysis software Sigma Scan (Jandel Scientific, San Rafael, CA, USA). Goblet cells were identified in the epithelium as blue-staining cells, which were counted from each of the measured villi for each section of the small intestine of each piglet and expressed as the number of cells per 100 µm of villus epithelium.

Statistical Analysis

A linear model with diets (control, lactoferrin, meat, or meat extract) and gut location as fixed effects and their interaction – as well as pig within diet as a random effect – was fitted to the histological measurements (Proc Mixed, SAS; SAS Institute Inc., Cary, NC, USA). A paired *t*-test was carried out within dietary group to compare the immunological parameters measured at Day 0 and Day 28 of the experiment.

RESULTS AND DISCUSSION

Immunology

The absolute means, mean differences, and *P* values of paired *t*-tests for the immunological parameters are presented in Table 2. Over the four-week experimental period, phagocytic activity increased for the control ($p = 0.021$), meat ($p = 0.016$) and meat extract ($p = 0.014$) groups. Cell proliferation in the presence of ConA increased in the meat extract ($p = 0.020$) group only, while lactoferrin ($p = 0.004$) and meat extract ($p = 0.013$) groups responded significantly to PHA stimulation.

The change in immunological status of the semi-anemic piglets was measured from Day 0 to Day 28. Significant increases in all immunological parameters were observed in the meat extract group from Day 0 to Day 28 of the experiment.

The peripheral blood leukocytes from the meat extract group demonstrated the greatest phagocytic capacity, as measured by flow cytometry.

Table 2. Absolute means, mean differences and *P* values of paired *t*-test from Day 0 to Day 28 for changes in phagocytosis and cell proliferation (ConA and PHA) of piglets fed the control, lactoferrin, meat, and meat extract diets.

Parameters and Diets	Day 0 Mean	Day 28 mean	Mean Differences ($d_{28} - d_0$)	*P* values[a]
Phagocytosis (% cells with phagocytic activity)				
Control	40.38	49.15	8.77	0.02
Lactoferrin	34.77	43.49	8.72	0.08
Meat	34.63	45.16	10.53	0.02
Meat extract[b]	30.48	46.39	15.91	0.01
Cell proliferation response to ConA (proportional increase)				
Control	23.41	33.85	10.44	0.54
Lactoferrin	14.45	43.51	29.06	0.08
Meat	12.03	34.07	22.04	0.11
Meat extract	26.83	72.83	46.00	0.02
Cell proliferation response to PHA (proportional increase)				
Control	17.19	36.41	19.22	0.31
Lactoferrin	9.17	39.11	29.94	0.004
Meat	9.61	56.64	47.03	0.09
Meat extract	17.06	63.70	46.64	0.01

Notes:
[a]Paired *t*-test
[b]Sarcoplasmic meat extract (<0.5 kDa) containing amino acids and dipeptides, as well as the minerals in the sarcoplasmic fluid of the meat cells

In addition, the whole-blood cells from piglets in the meat-extract group (Table 2) – when compared to controls – showed increased proliferation of lymphocytes in response to ConA and PHA, which are T-cell mitogens that stimulate lymphocytes to proliferate (Tizard 2000). These results suggest that white blood cells were up-regulated by the meat extract, indicating that these cells can proliferate in response to an appropriate antigenic challenge such as bacterial infection.

Intestinal Histology

The main finding for the histological data was the higher number of goblet cells per 100 µm overall observed for the piglets fed the meat or the meat extract diets (Table 3).

In the duodenum, villus height, crypt depth, and mucosal thickness were higher in the lactoferrin and control groups compared with the meat and meat extract groups. In contrast, in the ileum, the meat and meat extract groups had greater villus height relative to the lactoferrin and control groups – with the lactoferrin and meat groups having greater crypt depth in comparison with both the lactoferrin and control groups. Finally, the meat, meat extract, and lactoferrin groups had higher mucosal thickness than the control group. Despite these changes in the morphology of the small intestine, a non-significant interaction was observed between diets and anatomical locations for crypt depth ($p = 0.07$), which could be an indication of intestinal crypt dysfunction resulting from the sudden change in feeding and post-weaning stress (Pluske *et al.* 2007). Alteration in the crypt function could likely be attributed to the formation of crypts that produced undifferentiated enterocytes, resulting in the absorptive and digestive capacity of the enterocytes becoming affected. Undifferentiated enterocytes seem to lack a functional divalent metal transporter 1 (Minihane and Rimbach 2002) and may therefore have limited iron uptake potential.

The number of goblet cells per 100 µm in all three sections of the small intestine were significantly ($p = 0.003$) increased for the meat and meat extract groups, particularly in the ileum. Goblet cells – classified as mucosal cells – are two to three times more numerous in the ileum than in the duodenum (Eurell and Frappier 2006), and are responsible for mucin production (Argenzio 2004). It has been shown *in vivo* (Simovitch *et al.* 2003) that mucin and goblet cells are involved in iron absorption, and that an increase in goblet cell number is likely associated with increased iron absorption.

Iron solubility is maintained by mucin even in an alkaline environment, thereby enhancing iron uptake by mobilferrin and divalent metal transporter 1 (Mahler *et al.* 2009). In addition, the binding of mucin to iron in an acidic pH maintains iron solubility, thus favoring iron absorption in the alkaline pH of the duodenum (Conrad

Table 3. Least squares means and standard error (SE) and significance level after 4 weeks of the experiment for the height of the villi (µm), crypt depth (µm), mucosal thickness (µm), and goblet cells/100 µm in the duodenum, jejunum, and ileum of piglets fed the control, lactoferrin, meat, and meat extract diets (N = 6 piglets/diet).

	Control	Lactoferrin	Meat	Meat Extract[a]	SE
Duodenum					
Villus height (µm)	419^{yz}	449^{z}	379^{xy}	322^{x}	22.5
Crypt depth (µm)	130^{yz}	153^{z}	102^{xy}	76^{x}	11.5
Mucosal thickness (µm)	681^{z}	719^{z}	588^{y}	515^{x}	24.5
Goblet cells / 100 µm	0.0165^{y}	0.0133^{x}	0.0226^{z}	0.0195^{yz}	0.0014
Jejunum					
Villus height (µm)	396	436	410	373	18.3
Crypt depth (µm)	128	116	104	117	10.1
Mucosal thickness (µm)	620	624	610	590	25.9
Goblet cells / 100 µm	0.0156^{x}	0.0203^{y}	0.0219^{y}	0.0224^{y}	0.0016
Ileum					
Villus height (µm)	294^{x}	365^{y}	399^{y}	383^{y}	17.4
Crypt depth (µm)	85	128	110	83	14.4
Mucosal thickness (µm)	484	553	567	559	23.2
Goblet cells / 100 µm	0.018^{x}	0.0186^{x}	0.0223^{y}	0.0267^{z}	0.0013
Overall					
Villus height (µm)	369^{xy}	417^{z}	396^{yz}	360^{x}	11
Crypt depth (µm)	123^{yz}	138^{z}	113^{xy}	100^{x}	8.5
Mucosal thickness (µm)	595^{xy}	632^{y}	588^{x}	555^{x}	14.5
Goblet cells / 100 µm	0.0174^{x}	0.0178^{x}	0.0228^{y}	0.0234^{y}	0.0009
Significance Levels	**Diet**	**Pig (Diet)**	**Location**	**Diet * Location**	R^2
Villus height	ns	*	**	***	0.31
Crypt depth	ns	***	ns	ns	0.41
Mucosal thickness	ns	**	***	***	0.37
Goblet cells / 100 µm	**	**	**	**	0.39

Notes:
[a]Sarcoplasmic meat extract (<0.5 kDa) containing amino acids and dipeptides, as well as the minerals in the sarcoplasmic fluid of the meat cells
^{xyz}Means within the same row with a common superscript or with no superscripts do not differ significantly (*i.e.*, $P > 0.05$)
ns: $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

and Umbreit 2006); however, in the current trial, the iron repletion efficiency of the groups receiving the meat and the meat extract diets were similar (Rapisura-Flores 2009).

CONCLUSION

Piglets fed the meat-extract diet displayed improvements in all three aspects of the immune system investigated in this study, including phagocytic activity and lymphocyte proliferation in response to both ConA and PHA, and had a higher number of goblet cells than the control and lactoferrin diet. This suggests that the meat-extract can have a positive effect on immune response and iron absorption in semi-anemic piglets.

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