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THE USE OF SEMI-ANAEMIC PIGLETS TO INVESTIGATE THE EFFECT OF MEAT AND LSF DIETS ON IRON BIOAVAILABILITY

A thesis presented in partial fulfilment of the requirements

for the degree of

Master of Veterinary Science

in

Clinical Nutrition

at Massey University, Palmerston North

New Zealand

JOSEPHINE A. RAPISURA-FLORES

2009

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ABSTRACT

Anaemia, which is caused by iron deficiency, is a global nutritional disorder of utmost concern. It has been assumed that meat, which contains haem as well as non-haem iron, enhances non-haem iron absorption due to the presence of the "meat factor". In the experiment reported here, 24 semi-anaemic, 3-week-old piglets were utilised as human nutrition models to assess the effects of dietary lactoferrin, meat and LSF (Low Molecular Weight Sarcoplasmic Fraction) on iron bioavailability during a 4-week feeding period. The parameters that were used as measurements of iron bioavailability were changes in haematological indices, haemoglobin iron repletion efficiency, intestinal morphology and mineral balances. Non-significant ($p > 0.05$) dietary effects were observed for growth performance and for all the haematological and some histological parameters (small intestine villi height, crypt depth and mucosal thickness). Haemoglobin iron repletion efficiency was highest for the control group and was not significantly different between the other 3 diets. The superiority of the control diet in this respect was not consistent with previous trials and can not be explained. Results suggested that increased retention of calcium, magnesium, phosphorous and manganese tended to inhibit iron absorption. However, the LSF and meat diets significantly ($p = 0.003$) increased the number of goblet cells/100 μm suggesting that mucin secretion was favoured by these two diets. Additionally, all immunological parameters were significantly ($p < 0.05$) improved by the LSF diet. As such, the LSF diet can be a potential immunobooster feed ingredient for weanling piglets. Overall, the level of LSF in the LSF diet was insufficient to exert a desirable enhancement of iron bioavailability and betterment of

iron status of the semi-anaemic piglets relative to the control group. However, the diet containing LSF was as effective as the meat diet with respect to these characteristics.

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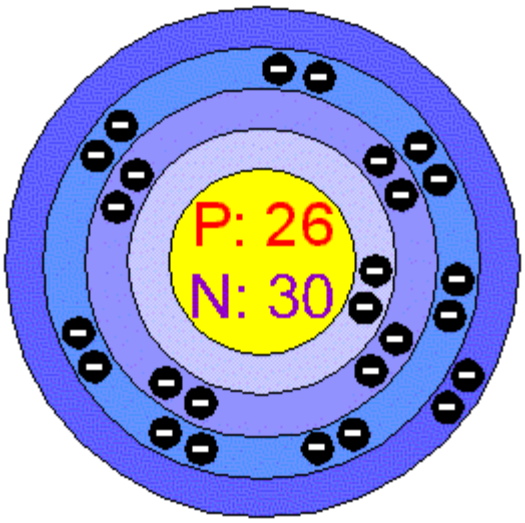
Chapter 1. LITERATURE REVIEW

This review focuses on the importance of iron not only in humans but also in animals. Firstly, it deals with iron as an essential nutrient, its physiological functions including the mechanisms involved in its absorption from the alimentary tract, its utilisation, metabolism, regulation and its relevance to iron deficiency. Secondly, the significance of meat as the principal source of haem iron that is highly bioavailable compared to non-haem iron is reviewed as well as the factors that affect iron bioavailability. The "meat factor" component of meat is reviewed in relation to iron absorption. Finally, the review considers literature citations indicating that pigs can be considered as human models. Supporting evidence from medical and animal nutritional studies is presented depicting the resemblance of a pig's digestive anatomy and physiology to that of humans.

1.1. IRON AS A NUTRIENT

1.1.1 PHYSIOLOGICAL ROLES OF IRON

The structure of iron is shown in Figure 1.1.



(Sourced from Bentor, 2008)

Figure 1.1 The atomic structure of iron with an atomic number of 26

Iron (Figure 1.1) is the most abundant trace element in the body (Himmelfarb, 2007; Lopez & Martos, 2004). It plays a fundamental role in various biological and chemical processes and functions as an integral component of haemoglobin and myoglobin (Ganz, 2007; Lopez & Martos, 2004). Engelking (2004) stipulated that the iron content percentage of haemoglobin and haemosiderin/myoglobin is 70 and 3-5, respectively. In addition, it participates in the oxidation-reduction cycle that generates adenosine triphosphate (ATP) (Ganz, 2007; Wessling-Resnick, 2000) which takes place in the mitochondria and involves the haem-containing cytochromes. Aside from these processes, several benzymes require iron (Ganz,

2007; Wessling-Resnick, 2000). These enzymes are identified by Wessling-Resnick (2000) as follows:

i. cytochrome P450, an enzyme system involved in drug metabolism by the hepatocytes as explained by Engelking (2004);
ii. xanthine oxidase;
iii. catalase;
iv. NADH dehydrogenase;
v. ribonucleide reductase, an iron-containing enzyme which stimulates DNA synthesis as described by Engelking (2004); and
vi. aconitase.

In addition, Engelking (2004) stated that enzymes, which activate metabolism of biogenic amines such as tyrosine and tryptophan hydroxylases, contain iron.

Similarly, iron is essential in physiological processes including DNA synthesis, respiration as well as oxygen transport (Dunn et al., 2007; Frazer & Anderson, 2005; Eisenstein, 2000). Likewise, Eisenstein (2000) noted that iron is involved in:

i. the formation of some neurotransmitters and hormones;
ii. xenobiotic metabolism; and
iii. certain aspects of host defence used in iron-containing proteins.

In relation to this, Ganz (2007) pointed out the direct involvement of iron in the host's defence responses to infectious agents including the host defence proteins such as:

i. lactoferrin;

ii. siderocalin; and

iii. divalent metal transporter natural resistance associated macrophage protein 2.

Moreover, Engelking (2004) has commented that myeloperoxidase contains iron which contribute to the function of the white blood cells in their response to infection.

1.1.2 DIETARY FORMS OF IRON

Two forms of iron exist in the diet: the haem and non-haem (Dunn et. al., 2007, Frazer & Anderson, 2005; Lopez & Martos, 2004). Samman (2007) specified that haem iron is obtained from foods of animal origin. Further, Lopez and Martos (2004) claim that haem iron is derived from meat, fish and other foods composed of blood (Lopez & Martos, 2004). Lopez and Martos (2004) emphasised that the quantity of iron is greater in red meat in comparison to poultry (white meat) products. Moreover, haem is described by Latunde-Dada et al. (2006) as an iron-

protoporphyrin IX, a component of haemoglobin, myoglobin, neuroglobin and cytoglobin, a prosthetic (as a substitute) group in mitochondrial cytochrome c and cytochrome P450, catalase, peroxidase, nitric oxide synthase and NADPH oxidase, and plays a part in transcription, translation and cellular differentiation.

As justified by Miret et al. (2003), haem iron from meat is “an important source of iron which is highly bioavailable”. Likewise, Mackenzie and Garrick (2005) stated that “haem is a major dietary source of bioavailable iron”. In addition, Lopez and Martos (2004) explained that “haem iron has greater availability than non-haem iron”.

On the other hand, non-haem iron, which is present in vegetables and cereals, is widely used to fortify foods (Lopez & Martos, 2004). Further, Samman (2007) noted that in addition to plant sources and iron fortified foods, non-haem iron also originates from animal sources. Specifically, non-haem iron is present as ferrous and ferric salts as well as organic complexes (Mackenzie & Garrick, 2005). Consequently, evidence indicates that dietary forms of iron occur in both vegetable and meat products.

1.1.3 ABSORPTION OF IRON

According to Engelking (2004), approximately 3-6% of dietary iron is absorbed in the intestine. Dietary iron (haem or non-haem) is absorbed primarily in the mature villus enterocytes of the duodenum and upper jejunum of the small intestine (Frazer & Anderson, 2005) and minimally in the stomach, ileum and colon (Steele et al., 2005). The transport of iron across the enterocyte towards the blood stream occurs sequentially through the (i) uptake of iron across the apical mucosa including intracellular iron processing and translocation through the cell, and (ii) export of iron across the basolateral or serosal membrane into the general circulation for utilisation by various organs (Frazer & Anderson, 2005; Wessling-Resnick, 2000). In addition to acknowledging the absorption process of iron, it is important also to consider the uptake and export of iron in more detail.

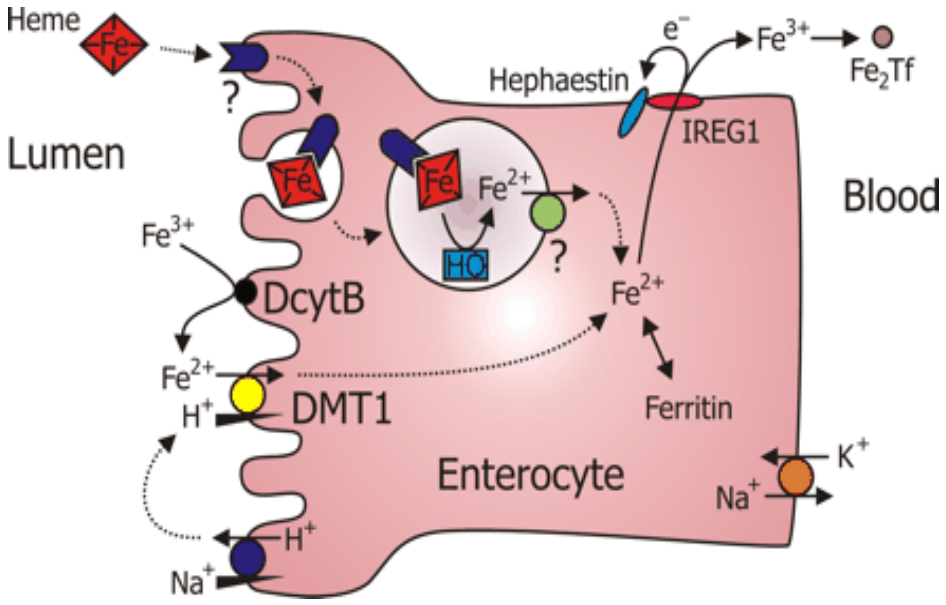
1.1.3.1 Uptake of Iron into Enterocytes

As defined by Miret et. al. (2003), "uptake is the transport of dietary iron across the apical membrane of the enterocyte into the intestinal mucosa". In order to enter the apical surface of the enterocyte, ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by the brush border ferric reductase, known as duodenal cytochrome b (DcytB) (Anderson, 2007; Andrews & Schmidt, 2007; Mackenzie & Garrick, 2005; Steele et al., 2005) as shown in Figure 1.2. As described by Andrews and Schmidt (2007),

duodenal cytochrome b is a haem protein that requires ascorbic acid to initiate the process of ferric iron reduction. Mackenzie and Garrick (2005) explain that the ascorbic acid, which facilitates ferric reduction by acting as an electron donor to DcytB, originates from the diet is secreted by the stomach or is derived from the bile. In terms of reductase activity of duodenal cytochrome b, it is highest in the duodenum and lowest in the ileum and its activity is upregulated by hypoxia and iron deprivation (Miret et al., 2003).

After ferric iron reduction, the transmembrane pathway differs for haem and non-haem iron. Non-haem (ferrous) iron is transported by an apical enterocyte transporter called divalent metal transporter 1 (DMT1) into the cytoplasm (Ganz, 2007; Dunn et al., 2007).

Divalent metal transporter 1 (Figure 1.2), also known as Nramp2 and DCT1, is considered by Miret et al. (2003) as the "first mammalian iron transporter" to be identified. Particularly, Mackenzie and Garrick (2005) mentioned that DMT1 is the "major intestinal non-haem iron transporter". The low pH environment in the apical brush border favours the transport process (Mackenzie & Garrick, 2005). Mobilferrin/calreticulin was identified by Mackenzie and Garrick (2005) as a possible alternative pathway in the uptake of non-haem iron and its transport by DMT1.



(Sourced from Mackenzie & Garrick , 2005)

Figure 1.2. Reduction of ferric iron to ferrous iron by the duodenal cytochrome b (DcytB) and the transport of ferrous iron across the apical membrane by the divalent metal transporter 1 (DMT1)

Conversely, Mackenzie and Garrick (2005) affirmed that haem iron crosses the apical membrane in an intact form, classified by Latunde-Dada et al. (2006) and Lopez and Martos (2004) as metalloporphyrin, which is derived from the proteolysis of haemoglobin and myoglobin in the proximal intestine. According to Dunn et al. (2007), the passage of haem iron across the apical membrane is facilitated by the haem carrier protein 1 (HCP1). Latunde-Dada et al. (2006) claimed that a haem receptor was identified in pigs and humans. Likewise, Dunn et. al. (2007) stipulated

that a receptor for haem carrier protein 1 is present in large concentrations in the duodenum. The haem iron-HCP1 complex crosses the membrane through endocytosis and is internalised and incorporated into the endoplasmic reticulum. The haem iron within the endosome is degraded by haem oxygenase 1 (HO1) that leads to the production of biliverdin and carbon monoxide and the release of ferrous iron as clarified by Dunn et. al. (2007), Latunde-Dada et al. (2006) as well as Mackenzie and Garrick (2005). In addition, as explained by Latunde-Dada et al. (2006), the breakdown of haem by haem oxygenase 1 (HO1) is the "rate-limiting step of haem absorption in the gut because the activity of HO1 is increased during iron deficiency. More importantly, Andrews and Schmidt (2007) believed that the iron liberated by haem oxygenase 1 similarly traverses the transport pathway entered by the non-haem iron. Within the enterocytes, the iron transported by DMT1 and released by haem oxygenase 1 enters the "labile iron pool" (Dunn et, al., 2007). Moreover, Latunde-Dada et al. (2006) specified that iron liberated by haem oxygenase 1 traverses the "inorganic iron pool".

1.1.3.2 Export of Iron from Enterocytes to the Blood

The ferrous iron delivered to the basolateral membrane of the intestine is exported by ferroportin (FPN), a metal transporter protein 1, also known as IREG 1, to the plasma (Ganz, 2007; Dunn et al., 2007). The ferrous iron is oxidised to ferric iron by a copper-dependent (Wessling-Resnick, 2006) ferroxidase hephaestin (Dunn et.

al., 2007) which is an electron acceptor (Mackenzie & Garrick, 2005), homologous to serum ferroxidase ceruloplasmin (Wessling-Resnick, 2006). According to Latunde-Dada et al. (2006), after erythrophagocytosis, elevation of haem oxygenase 1 expression seems to increase ferroportin expression. Aside from this, Wessling-Resnick (2006) noted that ferroportin is present not only in the duodenum but also in the liver, spleen, kidney and heart. However, the cellular export of iron particularly in the hepatocytes and macrophages requires ceruloplasmin which has a ferroxidase activity (Andrews & Schmidt, 2007). The role of hephaestin in association with ferroportin to oxidise ferrous iron is important before it is exported and bound to transferrin (Wessling-Resnick, 2006; Mackenzie & Garrick, 2005). Having described the absorption process of iron as mentioned in the literature, the next section focuses on how iron is used in the body.

1.1.4 UTILISATION OF IRON

1.1.4.1 Transport of Iron

After absorption, the ferric iron is bound to a plasma transferrin (Steele et. al., 2005), a high-affinity iron-binding protein that binds two atoms of ferric iron (Andrews & Schmidt, 2007; Wessling-Resnick, 2000). Ferric iron is then absorbed by the erythroid cells. Most of the iron circulating in the plasma is bound to transferrin, a β1– globulin, for cellular transport (Engelking, 2004), while a small proportion is

associated with albumin (Andrews & Schmidt, 2007). As commented by Andrews and Schmidt (2007), about two thirds of body iron is utilised by the erythroid precursors during their developmental stage including the mature erythrocytes. The surface of erythroid cells is lined with transferrin receptors where the transferrin-bound iron binds in order to be internalised through a receptor-mediated endocytosis (Dunn et al., 2007). Eisenstein (2000) wrote that the "high-affinity pathway of uptake of transferrin by receptor-mediated endocytosis of transferrin receptors (TfRs) is a key pathway of iron uptake for reticulocytes, hepatocytes and other cell types". Acidification of the endosome with the resultant conformational change that occurs in the transferrin and transferrin receptor liberates ferric iron, which undergoes reduction to ferrous iron that is then transported by DMT1 into the cytoplasm (Andrews & Schmidt, 2007).

In particular, Eisenstein (2000) believed that the transferrin cycle involved in transporting iron into the cells is influenced by:

i. changes in the percent saturation of transferrin with iron;
ii. alterations in the total serum concentration of transferrin; and
iii. modulation of cell surface display of transferrin receptors.

However, there are other pathways for the uptake of iron. An alternative pathway is mediated by lactoferrin which transports iron into the liver through the process of endocytosis, or it can bind to the brush border membrane of the small intestine to

facilitate iron uptake (Wessling-Resnick, 2000). More specifically, Wessling-Resnick (2000) explained that transferrin can independently transport iron during the absorption process without a transferrin receptor and justified the reasons that included:

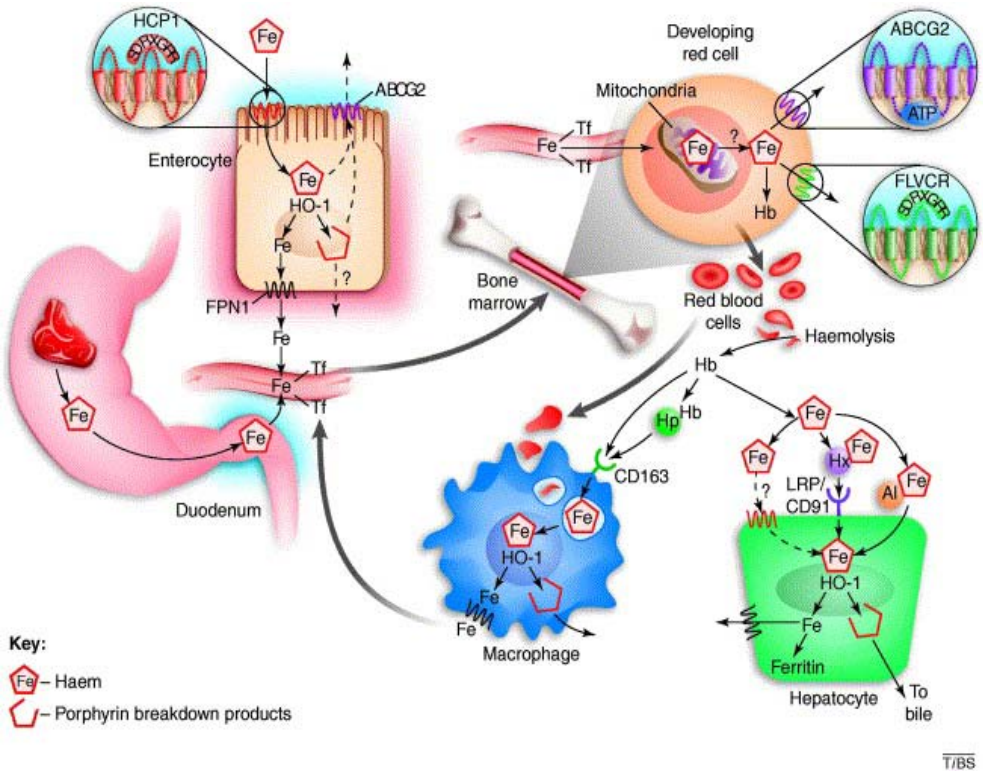
i. a transferrin-mediated iron delivery continues in the presence of blocking antibodies against the receptor;
ii. N-terminal "half-transferrin" which binds poorly to the transferrin receptor still delivers iron to hepatocytes; and
iii. an antisense suppression of transferrin receptor synthesis does not abolish transferrin-mediated iron uptake.

In addition, Wessling-Resnick (2000) said that non-transferrin-bound iron (NTBI) pathways exist.

1.1.4.2 Metabolism of Iron

In general, Eisenstein (2000) recognised the possible metabolic pathways undergone by iron (Figure 1.3) in the cytoplasm, including the following:

i. iron is used metabolically for the synthesis of iron-containing proteins:
ii. iron is stored; or
iii. iron is exported from the cell.



(Sourced from Latunde-Dada et al., 2006)

Figure 1.3. The transport of iron in the blood stream including its metabolism in the mitochondrion, liver and macrophages

1.1.4.2.1 Haem/Haemoprotein Syntheses

The iron liberated from the endosome is transported by mitoferrin, referred to as "mitochondrial iron importer", to the mitochondrion (Dunn et al., 2007; Andrews & Schmidt, 2007). In the mitochondrion, iron is incorporated into protoporphyrin IX (PPIX) resulting in the formation of haem (Andrews & Schmidt, 2007; Eisenstein, 2000), a process enhanced by ferrochelatase (Dunn et al., 2007; Andrews & Schmidt, 2007). As affirmed by Dunn et al. (2007) and Eisenstein (2000), haem synthesis not only occurs in the mitochondrion. It is also in this organelle that the formation of the metal centres for haem and non-haem (iron-sulphur proteins) takes place as well as their incorporation into several proteins. Latunde-Dada et al. (2006) mentioned that haem is exported from the mitochondrion towards the endoplasmic reticulum and integrated into haemoproteins. However, Eisenstein (2000) noted that 5-aminolevulinate synthase (ALAS) is the "rate-limiting enzyme in haem synthesis". Dunn et al. (2007) said that frataxin found in the inner mitochondrion regulates iron utilisation through its function as a "metabolic switch between haem synthesis and the genesis of iron-sulphur clusters".

Aside from these, haem synthesis in the hepatocytes yields cytochrome P450 (Latunde-Dada et al., 2006). On the other hand, muscle cells require iron for the production of myoglobin but the mechanism of how they assimilate iron has not been fully elaborated as emphasised by Andrews and Schmidt (2007).

1.1.4.2.2 Storage of Iron

Ganz (2007) mentioned that although the principal organ responsible for the storage of iron is the liver, macrophages are likewise capable of storing iron in the form of ferritin. Reece (2005) commented that iron is stored mainly in the liver and spleen but the kidney, heart, skeletal muscle and brain also store iron. Nevertheless, Andrews and Schmidt (2007) claimed that liver cells have the utmost capacity to store iron into ferritin. As defined by Reece (2005), ferritin is a "diffuse, soluble, mobile fraction of iron stored in the tissues". Moreover, ferritin is described by Eisenstein (2000) as a "multisubunit shell" constituted by about 24 subunits of H and L, with each ferritin shell possessing the ability to store voluminous amounts of iron atoms. Further, Andrews and Schmidt (2007) specified that each of the ferritin polymers, L-ferritin (light or liver ferritin) or H-ferritin (heavy or heart ferritin), has the capacity to accommodate about 4,500 of iron atoms. Furthermore, as an iron depot, ferritin usually accepts excess iron and allows iron release as the demand arises (Andrews & Schmidt, 2007). On the other hand, aside from ferritin, haemosiderin, which is considered by Reece (2005) as an *insoluble, aggregated deposits of iron*, are also being formed.

1.1.4.2.3 Recycling of Iron

Steele et al. (2005) claimed that as erythrocytes approached the end of their life-span, they are engulfed by the reticuloendothelial system (RE) macrophages. The senescing erythrocytes, as they circulate along the Kupffer cells of the liver and the red pulp of the spleen (Miret et al., 2003), are phagocytosed by tissue macrophages where they undergo degradation through haemolysis liberating haemoglobin, which is eventually catabolised by haem oxygenase to release iron (Andrews & Schmidt, 2007). In agreement with this, Ganz (2007) stated that iron recycling by macrophages leads to the recovery of iron through the action of haem oxygenases on the haem molecule.

Latunde-Dada et al. (2006) explained that after phagocytosis of the old and damaged erythrocytes by the macrophages, the haemoglobin being released into the plasma binds to haptoglobin (Hp), an alpha 2 acid glycoprotein from the liver. However, in the event that haemolysis exceeds haptoglobin's capacity to bind, Latunde-Dada et al. (2006) elaborated that:

i. the free haemoglobin are separated into dimers and filtered by the glomerulus; or

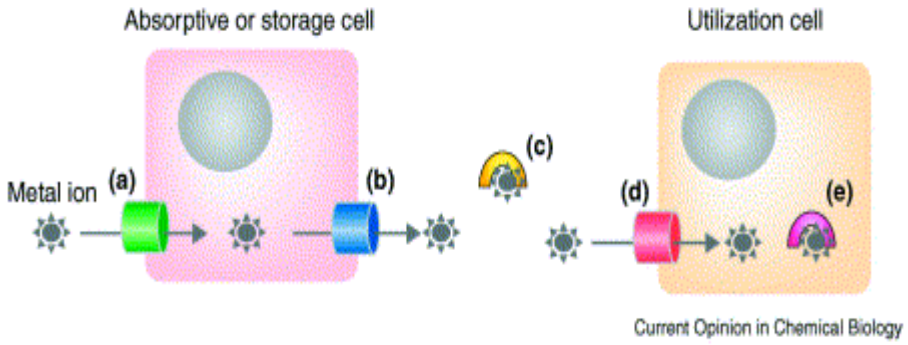
ii. the excess haemoglobin is converted into methaemoglobin through oxidation followed by its separation into free haem and its integration with haemopexin (Hx).

Aside from these, as discussed by Latunde-Dada et al. (2006), haem either binds to albumin or haemopexin, wherein the complex formed by haem and haemopexin is similarly internalised by the hepatocytes through a receptor-mediated endocytosis.

1.1.5 REGULATION OF IRON AND IRON HOMEOSTASIS

The quantity of iron absorbed in the small intestine must be regulated in accordance with the cellular demand since excessive amounts of iron assimilated in the body can lead to cellular toxicity associated with the production of oxygen free radicals. As such, Steele et al. (2005) indicated the significance of:

i. tight regulation of iron uptake and storage of the cellular and body levels; and
ii. an effective communication between the key sites of iron utilisation (bone marrow) and storage (liver and reticuloendothelial system) as shown in Figure 1.4.



(Sourced from Andrews, 2002)

Figure 1.4. **Iron is utilised (right side) as well as absorbed in the enterocytes or stored in the hepatocytes and the reticuloendothelial system (left side). The transport of iron is shown with the corresponding transmembrane importers (a and d – DMT1), transmembrane iron exporter (b – ferroportin), iron-binding plasma carrier protein (c – transferrin) and an intracellular iron-receptor (e)**

In relation to this, Ganz and Nemeth (2006) stated that as "other mammals lack mechanisms to excrete excess iron, intestinal iron absorption must be regulated by a feedback mechanism". More importantly, Andrews and Schmidt (2007) recognised that maintenance of internal iron balance is necessary to ensure that the cells are free from iron toxicity caused by the formation of reactive oxygen species. A balance is maintained by the following mechanisms:

i. production of ferritin, an iron storage protein in mammals; and

ii. involvement of iron regulatory proteins (IRPs).

On the other hand, Frazer and Anderson (2005) clarified that liver directly regulates absorption of iron through its hepcidin (iron regulatory hormone) production. Andrews and Schmidt (2007) added that hepcidin is likewise produced by the heart, pancreas and hematopoietic cells.

In particular, Miret et al. (2003) emphasised that there are two factors in the blood which tend to regulate iron absorption and cellular balance as follows:

i. stores regulator which Anderson (2007) referred to as body iron stores; and
ii. an erythropoietic regulator that functions to balance red blood cell formation in the bone marrow in conjunction with iron absorption in the duodenum. It is triggered by a stimulus from the bone marrow resulting in mobilisation of iron stores and stimulation of duodenal iron absorption. Anderson (2007) described the process as changes in the rate of erythropoiesis.

Anderson (2007) claimed that aside from physiological stimulation, genetic abnormalities, which are attributable to the modulation of hepcidin expression, seem to cause alteration of iron transport and iron homeostasis.

1.1.5.1 Regulation Through Iron-Regulatory Proteins/Iron-Responsive Element

Eisenstein (2000) justified the importance of iron-regulatory proteins (IRPs) as "central regulators of mammalian iron metabolism" by the following points:

i. IRPs are critical determinants of the post-transcriptional regulation of transferrin receptor expression;
ii. IRPs have a major role in determining iron storage capacity of cells by regulating translation of H and L ferritin on mRNA; and
iii. translation of mRNA for ALAS (aminolevulinate synthase) seems to be regulated by IRPs (IRPs may coordinate PPIX formation).

The production of ferritin and the formation of transferrin receptors is directly regulated by iron status in the body through the regulatory binding of iron-regulatory proteins (cytosolic RNA binding proteins) with the iron-responsive element (28-nucleotide) in the mRNA. Wessling-Resnick (2000) said that regulation of ferritin synthesis is undertaken through a regulated response of iron-regulatory proteins to the concentration of iron in the enterocytes. The IRPs were differentiated by Andrews and Schmidt (2007) based on the presence of iron-sulphur cluster in IRP1 which acts as an iron sensor compared to IRP2 which does not possess this type of cluster.

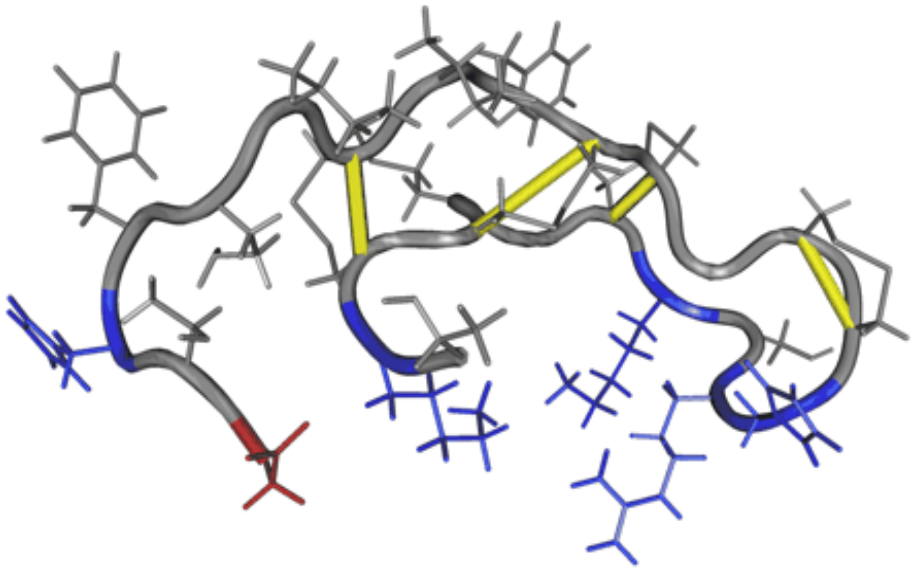
Eisenstein (2000) explained the regulatory responses occurring in the body depending on cellular iron bioavailability involving the interaction between iron-regulatory proteins (IRPs) and the iron-responsive elements (IREs). When iron concentration is lowered, binding of IRP1 or IRP2 to the 5' IRE located in the mRNA is favoured blocking its translation by suppressing the 43 S pre-initiation complex to the mRNA, thus in a repressed state as explained by Eisenstein (2000), "ferritin mRNAs appear to have entered the initiation pathway but are blocked at an early step". However, as iron concentration is increased, conversion of IRP1 to cytosolic aconitase (c-Acon) is initiated, hence binding with the RNA is unlikely while IRP2 is degraded (Eisenstein, 2000). Andrews and Schmidt (2007) added that IRPs bind to iron-regulatory elements of the mRNA particularly when iron concentration in the body is low. Aside from that, Wessling-Resnick (2006) stipulated that when IRPs are bound to the 5'-IREs, ferroportin synthesis is downregulated, which in cases of low iron conditions, stabilises the binding of IRP to IRE thus ferroportin expression declines in contrast to an elevated expression in the macrophages as iron loading increases. On the other hand, Miret et al. (2003) mentioned that iron-regulatory proteins regulate DMT1 expression by binding with the iron-responsive elements (IRE) located in the mRNA. However, since IRE is lacking in DcytB, IRP indirectly regulates its activity (Miret et al., 2003).

Wessling-Resnick (2000) claimed that the synthesis of transferrin receptor is likewise regulated by the IRPs in response to the iron concentration in the enterocytes. As the cellular demand for iron elevates, Eisenstein (2000) affirmed that IRPs are bound to the 3'IRE of the transferrin receptor (TfR) mRNA, hence,

TfR mRNA expression increases, which proportionally increases the availability of transferrin receptors to transferrin.

1.1.5.2 Regulation of Iron Through Hepcidin

Ganz and Nemeth (2006) wrote that the liver secretes a small, 25-amino acid peptide hepcidin (Figure 1.5), described by Frazer and Anderson (2005) as an iron regulatory hormone, which functions to control iron concentration extracellularly through its regulatory action on (i) iron absorption, (ii) placental transport, (iii) iron recycling by macrophages, and (iv) iron mobilisation from bodily stores. Particularly, Himmelfarb (2007) mentioned that iron loading and inflammation stimulate hepcidin production while hypoxia and anaemia suppress hepcidin secretion. Dunn et. al. (2007) added that erythropoiesis depresses hepcidin secretion favouring iron export from the reticuloendothelial system and enterocytes making iron more available as erythropoietic activity is stimulated.



(Sourced from Ganz & Nemeth , 2006)

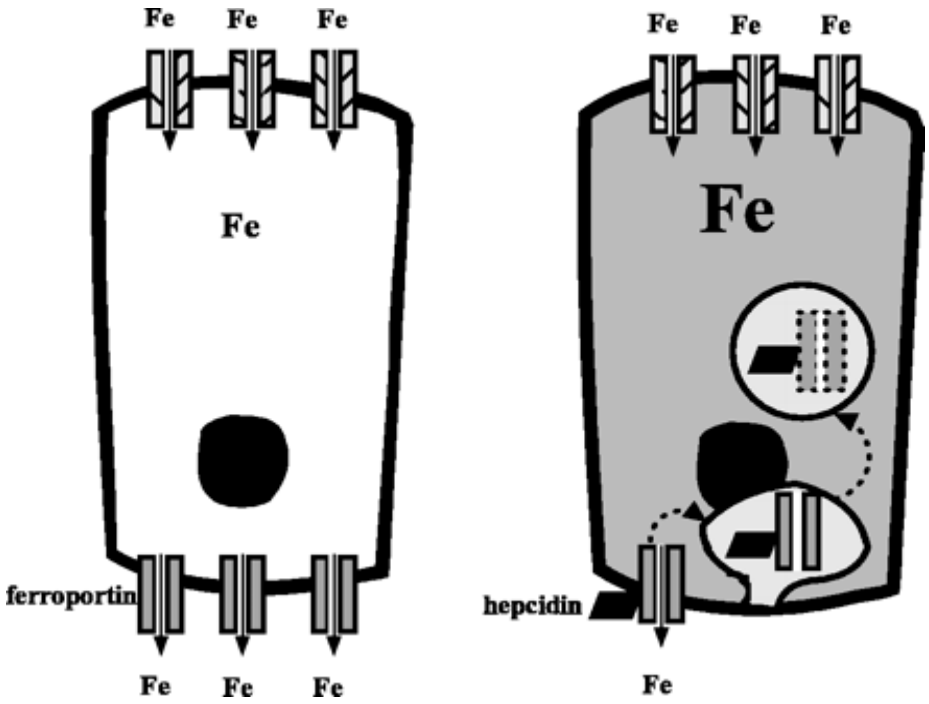
Figure 1.5 The NMR structure of hepcidin

Himmelfarb (2007) believed that "hepcidin is the master regulator of systemic iron availability". In support of this, Anderson (2007) explained that an elevating demand for iron triggers low secretion of hepcidin from the liver, thereby stimulating absorption of iron from the enterocytes coupled with an increase in the release of iron from the macrophages and tissues that store iron. However, an excess in iron activates the liver to produce hepcidin causing a decline in iron export from the enterocytes paralleled with a decrease in the release of iron from the tissues. In relation to this, Ganz (2007) justified that the "feedback stimulation of hepcidin by plasma iron saturation and iron stores ensures that extracellular iron concentration and iron stores stay within normal limits".

More importantly, the mechanism of action of hepcidin according to Dunn et. al., (2007) is exerted through its interaction with ferroportin. Specifically, Ganz and Nemeth (2006) explained that "hepcidin directly regulates the expression of ferroportin on cell membranes" by initiating the following events (Figure 1.6):

i. hepcidin directly binds to ferroportin;
ii. the binding of hepcidin causes ferroportin to be internalised and degraded; and
iii. the loss of ferroportin from the cell membrane ablates cellular iron export.

Wessling-Resnick (2006) affirmed that the binding of ferroportin with hepcidin is a homeostatic mechanism, responsible in regulating systemic iron absorption in response to iron demands. On the other hand, Ganz and Nemeth (2006) clarified that as the absorptive capacity of the enterocytes lasts for only two days, followed by the shedding of the enterocytes from the villi tips into the intestinal lumen, the export of iron by ferroportin across the basolateral membrane (Figure 1.6) is the determining factor. This determining factor only occurs if iron is carried by transferrin into the circulation or excreted through the sloughing of the enterocytes.



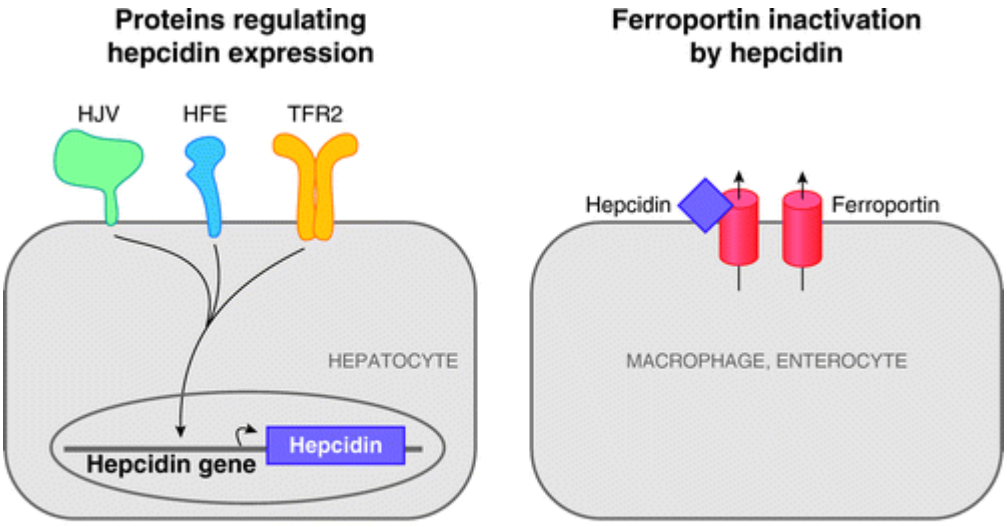
(Sourced from Ganz & Nemeth, 2006)

Figure 1.6 Hepcidin's action on ferroportin expression. Regulatory responses to iron deficiency (left) and iron excess (right) are also illustrated

Wessling-Resnick (2006) indicated that "hepcidin may affect body iron status by influencing levels of excretion". Moreover, Ganz and Nemeth (2006) emphasised that as iron levels are in excess in the body, hepcidin secretion by the hepatocytes increases, leading to the binding of hepcidin to ferroportin resulting in the inhibition of iron export from the basolateral membrane of the small intestine (Figure 1.6). In

contrast, Ganz and Nemeth (2006) pointed out that low levels of iron tend to suppress hepcidin production, stimulating ferroportin's function to export iron from the basolateral membrane, thus, delivering iron into the circulation to be transported by transferrin. In relation to this, Ganz (2007) stated that "hepcidin has a concentration-dependent inhibitory effect on iron export that parallels the hepcidin-induced loss of ferroportin from the plasma membrane".

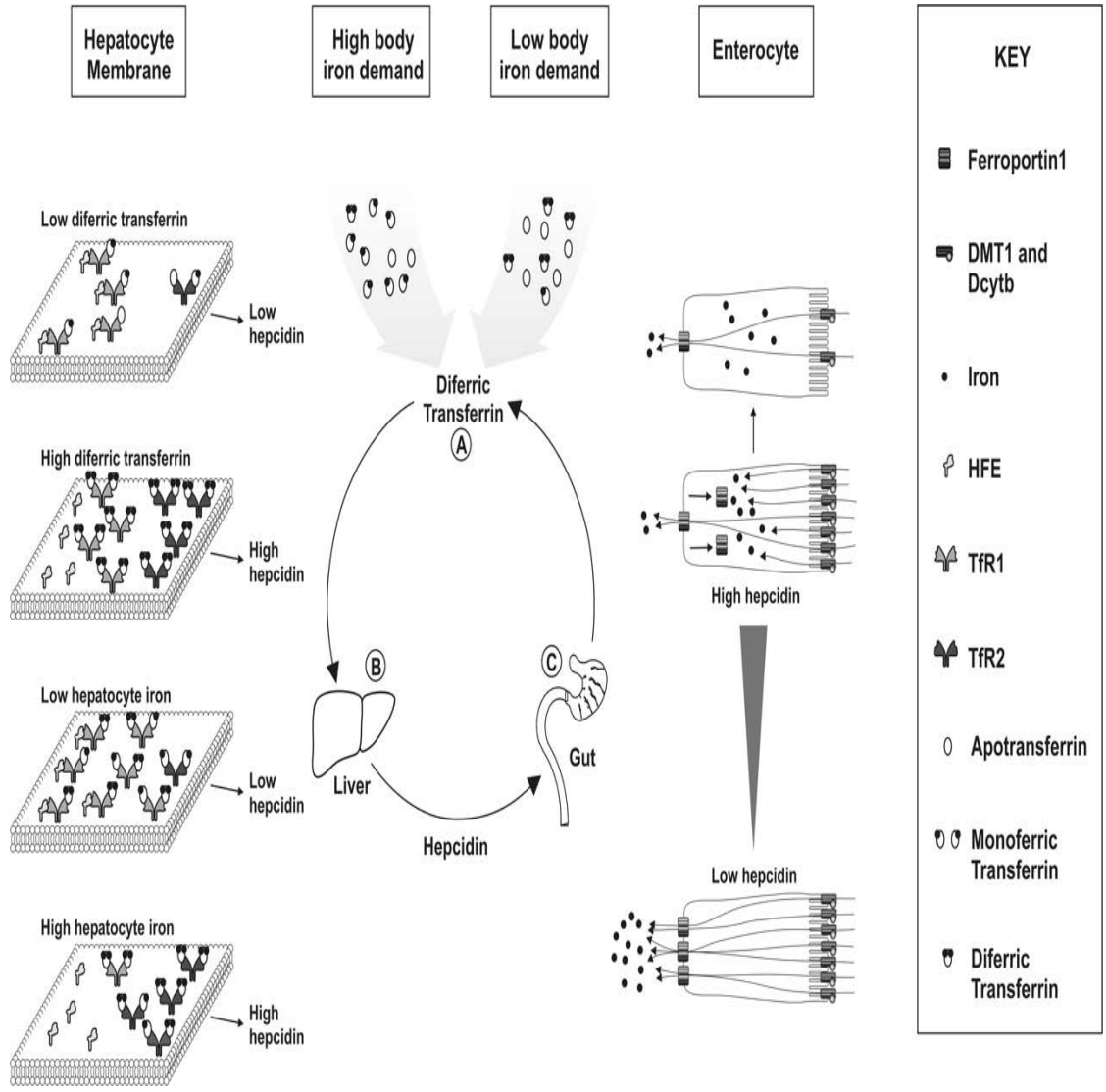
On the pathological side, Pietrangelo (2006) associated hemochromatosis, an iron overload genetic disorder, with the inadequate secretion of hepcidin by the hepatocytes attributed to HFE, HAMP and HJV mutations. In relation to this, Frazer and Anderson (2005) identified the three molecules, which act as regulators of hepcidin expression in the hepatocytes (Figure 1.7) as: (i) hemochromatosis gene product (HFE); (ii) transferrin receptor 2 (TfR2); and (iii) hemojuvelin (HJV).



Andrews NC, Schmidt PJ. 2007.
Annu. Rev. Physiol. 69:69–85

(Sourced from Andrews & Schmidt, 2007)

Figure 1.7 The HFE, TfR2 and HJV molecules and their effects on hepcidin expression



(Sourced from Frazer & Anderson, 2005)

Figure 1.8 Regulation of intestinal iron absorption and the negative feedback loop that regulates iron homeostasis

According to Fleming and Britton (2006), "HFE modulates hepcidin expression by changing the iron status of TfR1-expressing liver cell population". Frazer and Anderson (2005) extended this further, noting that as body iron status changes (Figure 1.8), this leads to the alteration of the concentration of the circulating diferric transferrin, which convey signals to the transferrin receptor 2 and hemochromatosis protein/TfR1 on the plasma membrane of liver cells. Consequently, this changes hepcidin expression (Figure 1.8). On the other hand, as explained by Frazer and Anderson (2005), hepcidin mediates its action by binding with ferroportin, leading to its internalisation and degradation, eventually resulting in iron accumulation within the enterocytes decreasing DMT1 and DcytB expressions.

Similarly, the crypt cells of the small intestine as discussed by Fleming and Britton (2006), detect iron concentration through the HFE-TfR1 complex on the basolateral membrane of duodenal cells. Aside from this, Frazer and Anderson (2005) said that iron stores in the liver regulates hepcidin expression by affecting TfR1 expression on the plasma membrane. This is increased as intracellular iron concentration decreases outnumbering TfR2, thus, TfR1 binds to diferric transferrin with greater affinity than TfR2. Nevertheless, as justified by Dunn et al. (2007), an increased concentration of iron activates hepcidin expression in the liver through the action of HFE, TfR2 and HJV. As emphasised by Dunn et al. (2007), the hemojuvelin - bone morphogenic protein receptor (HJV-BMPR) complex stimulates BMP-SMAD pathway which generates hepcidin expression.

1.1.6. ANAEMIA

Anaemia is defined by Reece (2005) as the " reduction in the number of erythrocytes, the concentration of haemoglobin, or both". In relation to this, Searcy (2001) claimed that a reduction in the number of erythrocytes in neonates occurs which is associated with the high rate of body growth. Radostits et al., (2007) emphasised that anaemia is a "deficiency of circulating erythrocytes associated with haemorrhage, increased destruction or the inefficient production of erythrocytes". Stockham and Scott (2002) categorised anaemia based on the following:

i. Marrow Responsiveness:

a. **Regenerative anaemia or responsive anaemia** which is primarily observed in response to blood loss or haemolysis, or indicates that the bone marrow is regenerating a replacement population of erythrocytes; and

b. **Nonregenerative anaemia or nonresponsive anaemia** is caused by diseases that directly or indirectly lead to a defective or reduced erythrocyte production, indicating that bone marrow is not regenerating a replacement population of erythrocytes.

ii. Anaemia Based on Morphology:

a. **Normocytic normochromic anaemias** are characteristic of most anaemias, which if persistence occurs, a nonregenerative anaemia develops;

b. **Macrocytic hypochromic anaemias** are characterised by macrocytosis and anisocytosis as well as presence of immature erythrocytes indicating blood loss or haemolysis;

c. **Macrocytic normochromic anaemias** with polychromasia, macrocytosis and anisocytosis as seen in blood films associated with regenerative anaemias caused by blood loss or haemolysis depicted in folic acid and cyanocobalamin (Vitamin B_{12}) deficiencies;

d. **Microcytic hypochromic anaemia** which is observed in iron, copper and pyridoxine (Vitamin B_6) deficiencies with microcytosis and hypochromasia, indicating a defect in haemoglobin synthesis;

e. **Microcytic normochromic anaemia** is indicative of an early or mild iron deficiency or hepatic failure, suggestive of a defect in the transport of iron to the erythrocytes; and

f. **Normocytic hypochromic anaemia** which is unlikely to occur.

iii. Anaemia Based on Pathophysiologic Classification:

a. **Blood loss anaemias** are categorised as acute or chronic, external or internal blood loss anaemias;

b. **Haemolytic anaemias** are due to extravascular or intravascular haemolysis; and

c. **Anaemias** caused by decreased erythrocyte production.

Similarly, Radostits et al. (2007) classified anaemia based on etiology which are the following:

i. **Haemorrhagic anaemia** is caused by esophagogastric ulceration, proliferative enteropathy and umbilical bleeding in pigs;

ii. **Haemolytic anaemia** is commonly due to isoerythrolysis, thrombocytopenia and coagulation defects in pigs; and

iii. **Anaemia** is due to decreased production of erythrocytes or haemoglobin, which is attributed to nutritional deficiencies that are

caused by cobalt, copper, iron, potassium and pyridoxine deficiencies in various species of animals.

1.1.6.1 Iron Deficiency Anaemia

According to Radostits et al. (2007), iron deficiency primarily affects newly born animals particularly nursing piglets for the following reasons:

i. inadequate access to soil which is the main source of iron;
ii. the iron requirement is high in piglets in response to rapid growth; and
iii. the iron content of the dam's milk is low.

In addition, Radostits et al. (2007) noted that since the quantity of iron stored in the neonatal liver is insufficient for the maintenance of haemopoiesis, iron deficiency is more likely to occur. In relation to this, Thrall (2004) said that the daily amount of iron absorbed or eliminated from the body is approximately 1 mg. In support, Reese (1999) pointed out that the availability of nutrients from the ingredients is insufficient to provide the required amount for pig maintenance, growth and lactation.

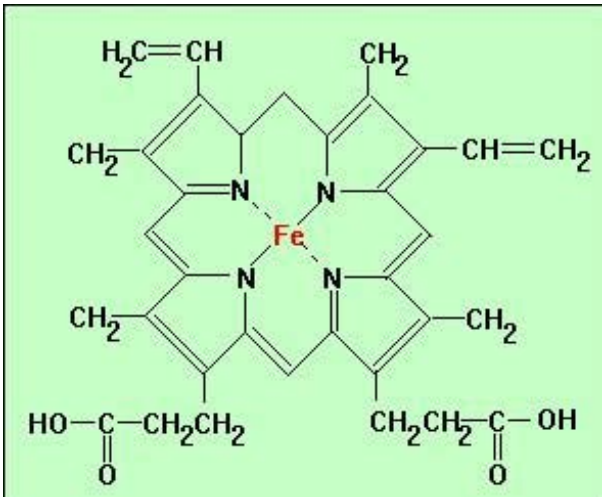
1.1.6.2 The Erythrocytes and Haemoglobin

1.1.6.2.1 The Erythrocytes

Among the cells suspended in the blood plasma, erythrocytes (red blood cells) are the most abundant reaching approximately 5 to 8 million/ml. They function to deliver oxygen from the lungs to the various tissues of the body (Aspinal & O'Reilly, 2003), as well as to carry carbon dioxide from the tissues to the lungs for expiration (Tartaglia & Waugh, 2005). Tissue oxygenation performed by the erythrocytes is attributed by Tartaglia and Waugh (2005) to the oxygen-carrying capacity of haemoglobin. On the other hand, as indicated by Tartaglia and Waugh (2005), erythrocytes are formed in the bone marrow, a process known as erythropoiesis which is regulated by erythropoietin from the kidney (Aspinal & O'Reilly, 2003). As explained by Aspinal and O'Reilly (2003), the precursor cells for erythrocytes are the **erythroblasts** which undergo nuclear shrinking and haemoglobin uptake to form the **normoblasts**. Inside the normoblasts, nuclei continue to shrink into fine threads that are called Howell-Joly bodies contained in the **reticulocytes**, the immature red blood cells (produced in some cases of anaemia) that mature into erythrocytes with no nuclei. About 1/3 of the erythrocytes is composed of haemoglobin (Reece, 2005).

1.1.6.2.2 Haemoglobin

As defined by Aspinal and O'Reilly (2003), haemoglobin is an iron-containing pigment which is responsible for the red colour in the erythrocytes. In addition, Harrison (2005) stated that "haemoglobin is the iron-containing oxygen-transport metalloprotein in the red cells of the blood in mammals and other animals". Moreover, Reece and Swenson (2004) mentioned that the amount of iron contained in the haemoglobin is about 0.334%.



(Sourced from Bionet, 2001)

Figure 1.9 The structure of haem

Aside from this, Harrison (2005) described haemoglobin as a “concatenation of haem and globin”, since the four subunits composed of globular proteins are equally embedded with haem groups, with each group of haem (Figure 1.9) constituting an iron atom arranged in a heterocyclic ring that binds to nitrogen. The polypeptide chains which combine, corresponding to each of the haem groups, are the alpha, beta, gamma and delta, while the protein component of haemoglobin is a histone (Reece & Swenson, 2004).

In particular, Tartaglia and Waugh (2005) elaborated that the “efficiency of oxygen transport is directly dependent upon the number of circulating erythrocytes in the circulation and the amount of haemoglobin that each cell contains”.

1.2 FACTORS AFFECTING IRON BIOAVAILABILITY

Although it is evident, the exact mechanism of the enhancing effect of muscle tissue on non-haem iron absorption is still rather uncertain, and therefore is attributed to the "meat factor". The "meat factor" effect on non-haem iron bioavailability is the focus of many investigations in this area of study. While it has been established that cellular animal proteins promote non-haem iron bioavailability (Mulvihill & Morrissey, 1998), dietary components have been identified that enhance or inhibit non-haem iron absorption. Digestive physiological factors as well as meat processing procedures are also considered in relation to iron absorption. Thus, this review is of particular significance to iron bioavailability in the body.

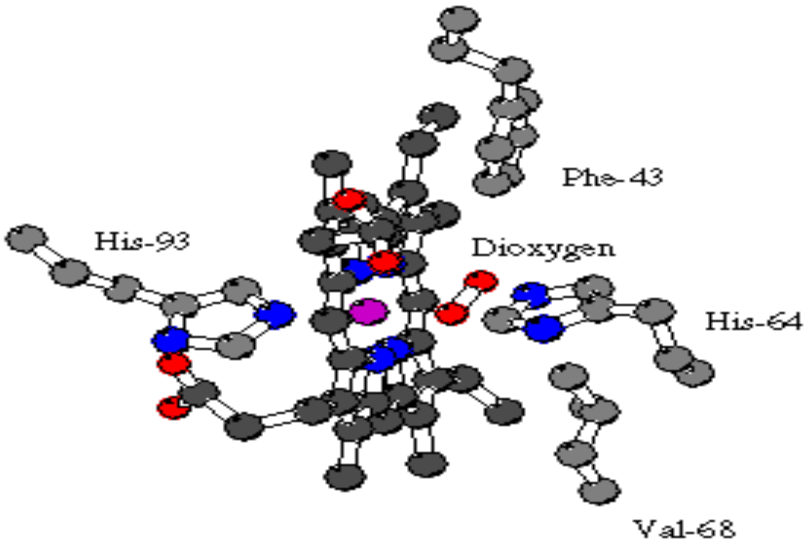
1.2.1 THE "MEAT FACTOR" EFFECT ON IRON BIOAVAILABILITY

According to Mulvihill et al. (1998), protein from meat, fish and poultry promotes iron bioavailability. In agreement with this, Hurrell et al. (2006) found that muscle proteins from meat, fish and poultry enhance non-haem iron absorption, and other research has shown that animal tissue from fish, chicken and beef enhance non-haem iron bioavailability (Engle-Stone et al., 2005). Moreover, Storcksdieck et al. (2007) indicated that the only macronutrient which tended to affect non-haem iron absorption was meat protein. Further, Garcia et al. (1996) specified that meat

proteins from beef, pork, lamb, liver, chicken and fish function to promote non-haem iron absorption, and aside from meat proteins of animal origin, ascorbic acid, Vitamin A and β-carotene have been found to contribute to facilitating iron absorption as well (Sorensen et al., 2007).

The enhancing effect of meat on iron absorption has been described by Garcia et al. (1996) as follows:

> iron from haemoglobin and myoglobin (Figure 1.10) is more readily available as
>
> i. it is not easily influenced by the presence of inhibitors or enhancers found in food that affect non-haem iron absorption; and
>
> ii. meat contains a factor or factors that promote haem and non-haem iron absorption.



(Sourced from http: // metallo.scripps.edu, 1997)

Figure 1.10 The myoglobin structure

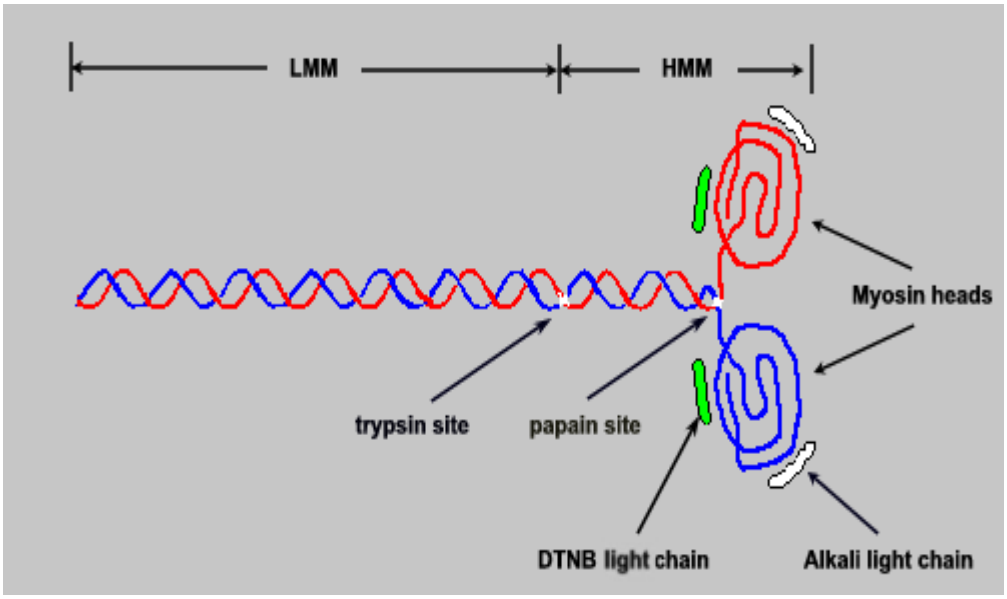
Several mechanisms have been proposed by many researchers in connection with the "meat factor" effect on iron absorption. Garcia et al. (1996) pointed out the following:

i. the globin products which are obtained during digestion tend to prevent haem and non-haem polymerisation of iron; therefore, iron is maintained in a more soluble form, and

ii. the release of free amino acids, namely, cysteine or glutathione, which seem to chelate iron in the intestinal lumen, lead to enhanced iron absorption.

As explained by Storcksdieck et al. (2007), through the "meat factor" effect, non-haem iron bioavailability is increased. This "meat factor" effect is displayed by means of "reducing and/or binding iron in soluble complexes, thus, preventing the reaction of iron with inhibitors" (Storcksdieck et al., 2007). Moreover, Mulvihill et al. (1998) mentioned that proteolytic digestion of meat yields amino acids and/or polypeptides, which cause chelation of non-haem iron, and hence, the bioavailability of iron is increased. Kim and Carpenter (1993) elaborated that the enhancing effect of meat on iron solubility is due to chelation exerted by the components of meat as a result of proteolytic digestion. Consequently, iron solubility is maintained in the intestinal lumen despite the neutrality of pH in this part of the digestive tract (Kim & Carpenter, 1993). Furthermore, Sorensen et al. (2006) discussed how "meat digestion products have the ability to reduce dietary ferric iron into a more soluble ferrous iron during digestion". Thus, ferrous iron tends to be more soluble and seems less likely to undergo hydrolysis in the gastrointestinal tract (Mulvihill & Morrissey, 1998). Sorensen et al. (2007) further highlighted the fact that meat proteins facilitate iron absorption through the "participation of thiol groups in redox reactions and/or chelation of iron by cysteine-containing peptides occurring during digestion of meat".

In particular, the protein digestion products of muscle tissue, which are the components produced during digestive processes, are gaining much attention in connection with iron absorption. According to Swain et al. (2002), proteolytic breakdown of substances in the digestive tract yields reducing components, stearic acid, certain amino acids and peptides which are considered vital to the enhancement of non-haem iron absorption. More importantly, Hurrell et al. (2006) emphasised the point that the cysteine-containing peptides produced during proteolytic digestion are responsible for the reduction of the ferric iron to ferrous iron. These peptides contribute to the maintenance of iron in a more soluble form, which is more bioavailable for absorption (Hurrell et al., 2006). Moreover, Storcksdieck et al. (2007) stipulated that cysteine tends to be the amino acid identified for enhancing iron absorption and commented that cysteine, being contained in the myofibrils of the myosin and actin (Figure 1.11) of the muscle tissues, is more likely to be shown as the source of the "meat factor". Aside from cysteine, histidine, which is obtained from the digestion of meat, increases iron solubility (Storcksdieck et al., 2007).



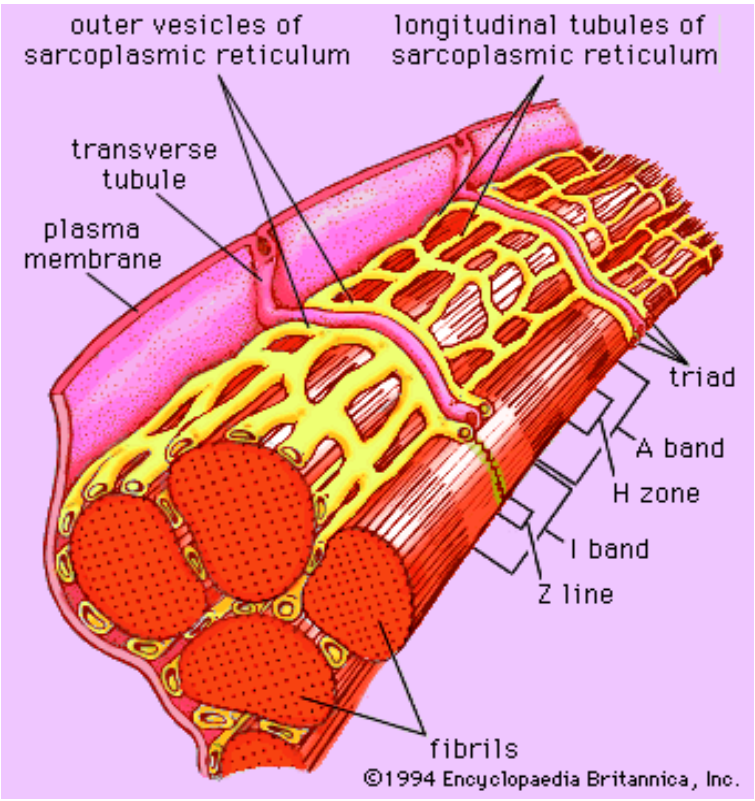
(Sourced from King & Marchesini, 2007)

Figure 1.11 The actin and myosin structures of a muscle tissue

In addition, Swain et al. (2002) reported that beef muscle proteins enhance non-haem iron absorption which can be attributed to the low molecular weight peptides that are released during the proteolytic digestion of meat. Swain et al. (2002) documented that the F1 to F2 fractions from beef muscle proteins, which are identified through immobilised-metal affinity chromatography (IMAC), contain the peptides with molecular weights of 1 to 7 kDa. They also affirmed that the "enhancement of non-haem iron absorption by beef may be due to the peptides produced during gastrointestinal digestion and that histidine content may be important" and identified

histidine as the important component which tends to contribute to promoting iron solubility and uptake. Furthermore, Swain et al. (2002) reported that histidine "plays a pivotal role in the active binding sites of a number of iron-containing enzymes and proteins including lysine".

On the other hand, King and Marchesini (2007) gave a description of a muscle myofibril (Figure 1.12), which is made up of 60% of myofibre protein, and is arranged into sarcomeres that are comprised mainly of actin (thin) filaments and myosin (thick) filaments. In meat, actin and myosin filaments are the major myofibrillar proteins (Mulvihill et al., 1998). An illustration pertaining to this is presented in Figure 1.11 showing the coiled heavy chains of myosin heads (right side portion). The myosin filaments, having a molecular weight of 500,000 daltons, are constituted of long thin fibrous proteins, with the heavy meromyosin located at an angle outward from the main axis of the myosin filament with reference to the trypsin hinge point (King & Marchesini, 2007).

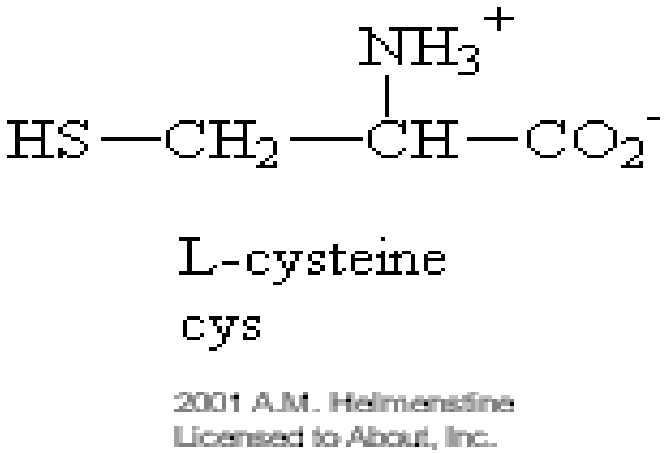


(Sourced from Encyclopedia Britanica, 1994)

Figure 1.12 A muscle structure

A more detailed understanding of the "meat factor" and its effect is necessary. The "meat factor", as discussed by Mulvihill and Morissey (1998), is a component of meat which is confined in the sulphydryl (-SH) myofibrillar fractions, particularly in the heavy meromyosin. Lopez and Martos (2004) affirmed that the "meat factor" effect may be attributed to some components of myofibrillar fractions, in particular, the heavy meromyosin fraction, rich in sulphydryl groups. As demonstrated by

Mulvihill et al. (1998), the heavy meromyosin, having a MW of 340,000, contains 25 –SH residues per molecule which is about five times the -SH content of the light meromyosin that has only 4 to 5 –SH residues per molecule. Thus, Mulvihill et al. (1998) reasoned that myosin fragments, having a higher –SH group, are more effective in enhancing iron dialysability. Similarly, Storcksdieck et al. (2007) found that enhanced iron dialysability is attributable to the heavy meromyosin fragment of the myosin filament which is abundant in cysteine. The sulphydryl groups, that are present in cysteine and glutathione are responsible for the reduction of ferric iron to ferrous iron which is essential to iron uptake into the mucosa of the small intestine (Mulvihill & Morissey, 1998). Moreover, the sulphydryl content of cysteine (Figure 1.13) and cysteine-containing peptides contribute to iron solubility and the reduction of ferrous iron (Storcksdieck et al., 2007). Due to the higher –SH content of myosin in comparison with actin, myosin is more capable of initiating ferric iron reduction and consequently, "a significant linear relationship was established between the sulphydryl content of meat proteins and their ability to enhance iron bioavailability" (Mulvihill et al., 1998). Furthermore, Storcksdieck et al. (2007) claimed that myofibrillar proteins, which are abundant in cysteine, are more likely to be the "origin of the meat factor" and Mulvihill et al. (1998) commented that the sulphydryl content of meat produced during the proteolytic digestion of a myofibre protein plays a vital role in the "meat factor" effect. However, Engle-Stone et al. (2005) specified that muscle tissues also contain sulphated glycosaminoglycan carbohydrates which could be partly associated with the "meat factor" effect.



(Sourced from Helmenstine, 2001)

Figure 1.13 The chemical structure of cysteine

A diet composed of meat seems to enhance iron bioavailability. Armah et al. (2008) reported that L-α-glycerophosphocholine contained in the muscle tissues of lean beef promotes non-haem iron uptake in vitro (Caco-2 cells). L-α-glycerophosphocholine, which is produced during hydrolysis of phosphotidylcholine (lecithin) in mammalian tissues and fluids, is the most abundant phospholipid in the muscle and consequently, the "meat factor effect stems from the binding of iron with mixture of peptides from digested proteins and other constituents of meat, such as phospholipids" (Armah et al., 2008). Moreover, Kapsokefalou and Miller (1993) explained that an interaction which occurs between lean beef and fat fractions in meat contributes to the properties of meat that enhance non-haem iron absorption. They elaborated that the interaction

of lean beef and beef fat occurs in the intestinal lumen which involves the products of digestion from protein and fat.

Similarly, the addition of meat, fish or poultry to vegetarian meals has led to a 2.6 fold increase on non-haem iron absorption as reported by Reddy et al. (2006), who further emphasised that animal tissue was observed to produce a greater enhancing effect on non-haem iron absorption from single meals than did other dietary components. Furthermore, consumption of an omnivorous diet tended to improve iron status in the body, which could be due to increases in the bioavailability of haem iron (Reddy et al., 2006).

Aside from these identified advantages, the enhancing effect of meat during weaning has been found to be beneficial. South et al. (2000) affirmed that iron repletion to iron-deficient weanling pigs through provision of non-haem iron with meat resulted in an elevation of non-haem iron bioavailability over corn- based diets. On the human side, Engelmann et al. (1998) demonstrated that the incorporation of 25 grams of meat comprised of lean beef on a vegetable puree meal composed of 80 grams of vegetables resulted in a significant increase in non-haem iron absorption in human infants.

1.2.2 THE "MEAT FACTOR" EFFECT IN RELATION TO THE FACTORS THAT AFFECT IRON BIOAVAILABILITY

Swain et al. (2002) reported that majority of the daily intake of iron is composed of non-haem iron. However, research has shown that haem iron, which is obtained from meat, fish and blood-derived foods, is also important in iron nutrition (Lopez & Martos, 2004). In particular, it has been noted that haem iron absorption is higher in the presence of meat (Lopez & Martos, 2004) and that "meat, in the diet, represents the main source of highly available iron" (Lombardi-Boccia et al., 2002a). On the other hand, as elaborated by Lopez and Martos (2004), non-haem iron absorption is similarly enhanced by the presence of meat in the diet. Because the haem and non-haem forms of iron in meat and in the diet display differences in terms of their bioavailability, it is of utmost interest to determine the effects of several factors that may tend to inhibit or stimulate their absorption in the gastrointestinal tract.

1.2.2.1 Promoters of Haem and/or Non-Haem Iron Absorption

Samman (2007) stipulated that Vitamin C (ascorbic acid), organic acids and proteins from meat, fish and poultry promote non-haem iron absorption. In addition, ascorbic acid as well as muscle tissues obtained from meat, fish and poultry have been found to enhance iron absorption (Reddy et al., 2006). Similarly, vitamins and

organic acids (ascorbic acid, citric acid and Vitamin A) were found to promote iron absorption (Lopez & Martos, 2004). According to Samman (2007), the stimulating effect of Vitamin C on non-haem iron absorption is due to its action as a reducing agent which converts ferric iron to ferrous iron. The iron in its reduced form is more capable of binding with the divalent metal transporter 1 (DMT 1), an apical membrane transporter involved in cellular iron uptake (Samman, 2007). In support, Lopez and Martos (2004) explained that ascorbic acid possesses the ability to activate reduction of ferric iron to ferrous iron or possibly cause the formation of a soluble complex with ferric iron and reported that ascorbic acid exerts its effect by counteracting the inhibitory action of phytic acid on iron absorption.

On the other hand, citric acid increases iron solubility by acting as a reducing agent which result in the production of ferrous iron (Lopez & Martos, 2004). Due to the reducing and chelating properties of ascorbic acid, it is considered to be the most efficient agent for facilitating the absorption of iron (Teucher et al., 2004). This effect is mostly observed in meals containing components that inhibit iron absorption. In relation to this, Teucher et al. (2004) emphasised the importance of ascorbic acid inclusion in meals that have low-to-medium levels of inhibitors and highly recommended a molar ratio of 2:1 of ascorbic acid to iron.

Aside from these effects on iron absorption, meat promotes gastric acid production that leads to an increase in the solubility of iron from the food matrix (Kim &

Carpenter, 1993). The ferrous form of iron is more absorbable than ferric iron, as illustrated by the increased solubility of ferrous iron in the gastric juice (Martinez-Navarrete et al., 2002). Moreover, a 30-40% elevation in gastric acid production and about 65-70% increase in gastrin, which stimulate gastric acid production, are demonstrated by the presence of meat in the diet (Kim & Carpenter, 1993). Additionally, meat has a stimulatory effect on gastric acid secretion, and gastric acid production has been found to be essential to iron absorption (Hurrell et al., 2006). However, the influence of meat on the stimulation of gastric acid secretion evidently has not been well documented in relation to the promotion of non-haem iron absorption (Swain et al., 2002).

1.2.2.2 Inhibitors of Haem and/or Non-Haem Iron Absorption

Not all animal proteins seem to facilitate non-haem iron absorption. In fact, egg, cheese, milk and ovalbumin proteins tend to inhibit the absorption of non-haem iron (Swain et al., 2002). Similarly, Storcksdieck et al. (2007) classified the potent inhibitors of iron absorption as phytic acid, polyphenols, egg albumin and soy protein, and Lopez and Martos (2004) categorised the compounds which inhibit iron absorption as phytic acid, polyphenols, caseinophosphopeptides and fibre. Other dietary factors are also considered to be inhibitors of iron absorption as described below.

Firstly, phytic acid, found in considerable amounts in cereals, legumes and oilseeds, is not only an anti-nutritive factor but also an inhibitor of iron absorption (Minihane & Rimbach, 2002). Similarly, foods from mature legume seeds and whole cereal grains have been identified as major sources of phytic acid and thus inhibit non-haem iron absorption (Engle-Stone et al., 2005). In particular, according to Engle-Stone et al. (2005), monoferric phytate, which is an important component of cereal crops (wheat, rice and maize), is soluble and bioavailable. It appears that phytic acid inhibits non-haem iron absorption by interacting with iron, and thus restricting the binding of iron with the ferrous iron transporter, DMT 1, at the brush border surface (Engle-Stone et al., 2005). In addition, the phosphorous storage compound in plant-based diets, which is composed of 6 phosphate groups, can bind to iron with high affinity and in this way decrease iron absorption to a greater extent (Lopez & Martos, 2004).

Nevertheless, Lopez and Martos (2004) claimed the inhibitory effect of phytic acid on iron absorption seemed to be counteracted by the presence of meat in the diet. The enhancing effect of beef, pork and chicken on iron absorption is displayed more efficiently when phytate has been incorporated in the diet and is mostly manifested in rats that were iron-deficient (Kim & Carpenter, 1993). Moreover, Baech et al. (2003) stated that "small amounts of pork meat ($\leq$ 50 grams) added to a phytate -rich meal low in vitamin C significantly increased non-haem iron absorption" and showed that consumption of a small-to-moderate helping of meat is contributory to the improvement of iron status.

The interaction of iron with phytic acid is central to the inhibition of iron absorption, with a molar ratio of 1:10 of iron to phytic acid resulting in the inhibition of iron uptake (Glahn et al., 2002). Moreover, although an increase in the solubility of iron is observed when the ratio of iron to phytic acid is higher than 1:3, the bioavailability of iron is found at a lower ratio (Glahn et al., 2002). As iron is bound to phytic acid at a molar ratio of 1:2 of iron to phytic acid, iron is highly soluble but less likely to be bioavailable (Engle-Stone et al., 2005). However, the haem iron, compared to non-haem iron, is less susceptible to the inhibitory effect of phytic acid, even at a molar ratio of 1:10 of iron to phytic acid (Glahn et al., 2002). Metalloporphyrin, released from globin during proteolysis, contributes primarily by protecting haem iron from the inhibitory effect of phytic acid, consequently, haem iron is readily absorbed by the enterocyte (Glahn et al., 2002).

On the other hand, Lopez and Martos (2004) reported that the inhibitory effect of phytic acid on iron absorption tends to be counteracted by carbohydrates such as fructo-oligosaccharides that are not usually digested in the small intestines. Although fructo-oligosaccharides are not hydrolysed by enzymes in the intestinal tract, they undergo bacterial fermentation in the large intestine particularly in the colon of the monogastric animals (Lopez & Martos, 2004). As a result of this microbial digestion, fructo-oligosaccharides are broken down to organic acids (acetic acid, propionic acid and butyric acid) that form a soluble complex with iron thereby preventing the adverse effect of phytic acid on iron absorption in the hind gut (Lopez & Martos, 2004).

Other inhibitors of iron absorption are the polyphenols (Lopez & Martos, 2004) which, like phytic acid, are abundantly present in plant-based diets (Storcksdieck et al., 2007). In the lumen of the gut, polyphenols form a complex with iron, or seem to cause precipitation of iron which renders it less likely to be available for mucosal uptake by the small intestine (Storcksdieck et al., 2007). Specifically, the galloyl groups in the polyphenol compounds are responsible for the formation of an insoluble complex with iron through chelation, thus causing impairment in the utilisation of dietary iron (Lopez & Martos, 2004). Alexandropoulou et al. (2006) added that polyphenols, which are present in the diet, inhibit iron absorption through the formation of polyphenol chelates during digestion, thus lowering the availability of iron for absorption. However, Storcksdieck et al. (2007) demonstrated that low molecular weight (LMW) iron-binding peptides (< 10 kDa), which are produced during pepsin digestion of myofibrillar proteins of animal muscle tissue, tend to bind with iron in the stomach. The myofibrillar proteins obtained from beef, pork, lamb, cod and chicken, which bind with greater affinity to iron in the stomach, are contributory to the increase in the bioavailability of iron for absorption (Storcksdieck et al., 2007). In effect, the interaction of iron with polyphenols, including phytic acid, in the duodenum is prevented, thus, increasing the availability of soluble iron to be taken up by the mucosal cells. This process is facilitated by the presence of iron transporters in the small intestine (Storcksdieck et al., 2007). On the other hand, polyphenols are present in herbs and green tea (Samman, 2007). Alexandropoulou et al. (2006) documented that the antioxidant capacity of green tea is decreased by the presence of meat and casein. Similarly, iron decreases not only the antioxidant

capacity of green tea but also the concentration of polyphenol (Alexandropoulou et al., 2006).

Tannic acid, which is also a polyphenolic compound, affects iron bioavailability by decreasing its absorption (Glahn et al., 2002). In vitro, the potency of the tannic acid effect to inhibit iron uptake is higher than the effect of phytic acid or zinc on iron bioavailability (Glahn & Wortley, 2002). A molar ratio of 1: 0.1 of iron to tannic acid leads to an optimum, or a nearly maximum, inhibition of iron uptake (Glahn et al., 2002). On the other hand, in vivo, a 1:1 molar ratio of iron to tannic acid is more effective in causing an optimum inhibition of iron uptake, as iron bioavailability is decreased by the relatively high affinity of tannic acid for iron (Glahn et al., 2002). Further, Engle-Stone et al. (2005) explained that tannic acid, which is a hydrolysable tannin, possesses the ability to bind with iron to form very stable complexes due to the galloyl groups and/or hydroxyl groups that are found in tannic acid. Additionally, "condensed tannins are potent inhibitors of iron bioavailability" (Engle-Stone et al., 2005). However, the exact mechanism in relation to this effect has not been fully elucidated according to Engle-Stone et al. (2005). Finally, gallic acid is likewise claimed to inhibit iron absorption. An effect similar to tannic acid and chlorogenic acid has been shown to have a lesser effect on the inhibition of iron absorption (Glahn et al., 2002).

Aside from these, caseinophosphopeptides obtained from egg, milk and dairy products were identified by Lopez and Martos (2004) as contradicting the enhancing effect of meat proteins on iron bioavailability. Even though both egg albumin and casein decrease iron absorption, casein exerts a stronger inhibitory action on iron absorption in comparison with egg albumin (Storcksdieck et al., 2007). More importantly, casein from milk tends to cause inhibition of iron absorption which can be attributed to the phosphorylation of serine and threonine residues that strongly bind to iron (Lopez & Martos, 2004). Consequently, this interaction of iron with phosphorylated serine and threonine leads to an inefficient absorption of iron in the small intestine. However, the inhibitory effect of casein on iron absorption can be eliminated by stimulating the hydrolysis of casein before ingestion in order to increase iron solubility in the digestive tract (Lopez & Martos, 2004).

In relation to mineral bioavailability, polyanionic polysaccharides have been identified as inhibitors of mineral absorption according to Lopez and Martos (2004), who described them as soluble fibres, composed of sulphate or carboxyl groups that are present in pectin, carrageenan and xanthan gums. These sulphate or carboxyl groups are deprotonated when they are in an environment where the pH approaches neutrality, thus favouring binding with iron and other mineral cations in vitro (Lopez & Martos, 2004). Nevertheless, in vivo (human and animal models), pectin and guar gum, which are soluble dietary fibres, have not produced an inhibitory effect on mineral bioavailability (Lopez & Martos, 2004).

The inhibitory effect of calcium on iron absorption is also of particular importance. Lynch (2000) mentioned that salts of calcium tend to cause reduction of haem and non-haem iron absorption in animal models. This effect is attributed by Lynch (2000) to the amount of calcium administered and is unlikely to be due to the molar ratio of calcium to iron. Similarly, in humans, provision of calcium supplements and dairy products has also decreased haem and non-haem iron absorption (Lynch, 2000). However, this effect depends on "the simultaneous presence of calcium and iron in the lumen of the upper small intestine" (Lynch, 2000). As documented by Roughead et al. (2005), dietary supplementation of calcium (450 mg as citrate) tends to inhibit absorption of haem iron which can be attributed to the reduction of the initial mucosal uptake of iron. Nevertheless, absorption of non-haem iron was not significantly affected by calcium inclusion in the diet (Roughead et al., 2005). On the other hand, monoferric phytate can interact with calcium and other polyvalent cations and as a result, a coprecipitation of iron which is already bound to the insoluble calcium-phytate occurs (Engle-Stone et al., 2005). Consequently, due to this interaction which traps iron in mixed cation complexes, the bioavailability of iron decreases (Engle-Stone et al., 2005).

1.2.2.3 Thermal Effect on Meat and Iron Absorption

The effect of heat on the iron content of meat is vital in relation to the bioavailability of iron. Purchas et al. (2006) reported that cooking of beef longissimus muscle at 71

^{0}C resulted to a decline in the total percentage haem iron which they had attributed to the loss of iron in the cooking juices. However, they noted that pepsin and pancreatin digestion of the same sample tended to increase the percentages of both soluble forms of haem and non-haem iron, thus enhancing iron bioavailability. On the other hand, Purchas et al. (2004) documented that fast, dry-heat cooking of beef semitendinosus muscle in a clam cooker seemed to cause: (i) a reduction in the proportion of soluble haem iron from 65% (uncooked meat) to 22% (cooked meat) at 60 ^{0}C which gradually decreased as the cooking temperature is increased; (ii) a proportional elevation in the percentage of insoluble non-haem iron with increased cooking temperatures; and (iii) a slight change in the proportions of soluble and insoluble non-haem iron. They elaborated that although the surface samples obtained higher concentrations of iron due to their lesser water content, cooking temperatures (60, 65, 70, 75, 80, and 85 ^{0}C) had no effect on iron levels of surface and inner samples.

Similarly, Lombardi-Boccia et al. (2002a) emphasised that although the haem iron content in red meat ranges from 72 to 87% of total iron, this is more than likely decreased by heating. This is because, during the process of heating of meat, conversion of haem iron to non-haem iron is favoured, thus changing the concentration of the bioavailable iron obtained from meats or diets (Lombardi-Boccia et al., 2002a). In addition, the thermal processes applied to a meat can lead to a partial conversion of haem iron to non-haem iron which can influence the degree of haem degradation in meat (Lombardi-Boccia et al., 2002b). The authors

demonstrated that the meat from beef, which has a high total iron content, had a high level of haem iron with 86% and 83% total iron content for the raw meat and cooked meat, respectively.

Further, Garcia et al. (1996) pointed out that the changes which occur in the iron-containing proteins, haem iron, cysteine content and iron absorption caused by cooking did not affect the meat factor, and it is still present in insoluble meat precipitates. In agreement with this, Sorensen et al. (2006) affirmed that when pork meat is heated at 60, 80, 100 and 120 ^{0}C, an increase in ferrous iron dialysability was more pronounced in pork meat heated at 100 and 120 ^{0}C; however, in terms of iron content of pork meat heated at 100 and 120 ^{0}C, no change was observed.

1.3 THE PIG AS A MODEL FOR IRON BIOAVAILABILITY STUDIES IN HUMAN AND ANIMAL NUTRITION

The close resemblance of domesticated pigs to humans is a significant factor in the considerable importance of pigs for both animal and medical nutritional studies. The nearly parallel anatomical structures and physiological functions of the pig and the human digestive tract have vastly contributed to the role of the pig as a model for human nutrition. As mineral deficiencies in both pigs and humans can be attributed to the nutritional state and dietary components, it is of utmost interest that the underlying mechanisms involving the absorption of minerals and other nutrients in the gastrointestinal tract be unveiled by using the pig as an experimental model.

1.3.1 THE PIG AS A MODEL FOR MINERAL ABSORPTION STUDIES

Aside from the economic and social significance of pigs to people, the pig, as singled out by Patterson et al. (2008) is the "more suitable animal model to elucidate the mechanisms involved in dietary effects on health, particularly with regard to mineral absorption across the intestinal epithelium… to access the different compartments of the gastrointestinal tract". Moreover, Patterson et al. (2008) pointed out that the application of the pig as a model in nutritional research is highlighted by the following:

i. the pig model can provide insight into the mechanisms involved in human digestion and absorption processes; and

ii. the pig model is ideal for nutrient bioavailability and absorption studies exemplified by:

    a. the examination of the absorptive capacity of the intestine with respect to different micronutrients and the homeostatic controls that play a role in their uptake;

    b. the effect of different dietary components on micronutrient uptake; and

    c. the bioavailability of iron and other minerals from foods.

Further, Miller and Ullrey (1987) noted that the pig as a model for research has been utilised in the following investigations:

i. cardiovascular physiology;

ii. obesity;

iii. stress;

iv. dermatology;

v. teratology;

vi. toxicology;

vii. immunology;

viii. behavior;

ix. haemodynamics;

x. renal physiology;

xi. experimental surgery;

xii. gastroenteritis;

xiii. drug metabolism;

xiv. perinatology; and

xv. nutrition.

Furthermore, Patterson et al. (2008) identified an important reason for the "use of the pig as an experimental model for measuring iron absorption and retention". This is because the usefulness of a pig model to investigate iron absorption and retention is due to the fact that the iron status of pigs is more easily manipulated from birth, since newborn pigs have a very limited amount of iron stored in their bodies (Patterson et al., 2008). More importantly, South et al. (2000) specified that use of the piglet as a model in nutritional studies has played a vital part in the assessment of iron bioavailability of various diets containing non-haem iron, non-haem iron with meat and haem iron by the haemoglobin repletion method.

Moreover, a newborn pig tends to be a good model for the human infant since the body iron status of a piglet is approximately the same as that of a 6- to 9-month- old human infant (Zinn et al., 1999). The pig has also been used as an excellent model for the determination of the role of milk lactoferrin (Lf) in human infants and their responses to milk feeding and weaning (Liao et al., 2007). Because the pig, as compared to other animals, has a higher concentration of milk lactoferrin, it serves as a very suitable tool in the investigation of 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). Additionally, Rutherford et al. (2006) reported that a 3-week-old piglet appears to accurately demonstrate the digestion of a 3-month- old human infant and therefore can provide a detailed understanding of nutrient availability far better than in vitro studies, rat models or trials with human infants. Furthermore, the pig, as a monogastric mammal, metabolises nutrients as humans do, and since milk obtained from both the mother and the sow is low in iron, iron deficiency anaemia in nursing children can be explored by using the nursing piglet as a model (Erikson et al., 1998).

1.3.2 THE PIG DIGESTIVE TRACT AND DIGESTIVE PHYSIOLOGY

The pig, as classified by Reece (2005), is an omnivorous animal which feeds on both flesh and plant diets. The digestive tract is composed of the mouth, teeth, tongue, pharynx, esophagus, stomach, small intestine and the large intestine while the

salivary glands, liver and pancreas are identified as the accessory organs of the digestive tract (Reece, 2005). After ingestion by the animal, food undergoes the process of digestion. Digestion is defined by Reece (2005) as the "processes that divide food into smaller parts through both physical and chemical means so that the structural units or other simple chemical compounds can finally enter the body by crossing the intestinal barrier". Tartaglia and Waugh (2005) have identified two stages which occur during the process of digestion:

i. the mechanical stage which involves the physical breakdown of food into a form that can be swallowed; and

ii. the chemical stage involving the breakdown of food by the chemicals and enzymes within the digestive tract.

Tartaglia and Waugh (2005) emphasised that the majority of the digestive process occurs primarily in the stomach, and digestion that takes place in the small intestine of monogastric animals is part of the chemical stage.

Reece (2005) described the pig small intestine as a long anatomical structure responsible for digesting and absorbing nutrients, and composed of the duodenum, jejunum and ileum. The anatomical makeup of the three regions of the small intestine is described by Tartaglia and Waugh (2005) as:

i. the duodenum, which is short and immobile, is joined by the pancreatic and bile ducts where secretions from both the pancreas and gall bladder enter the small intestine;

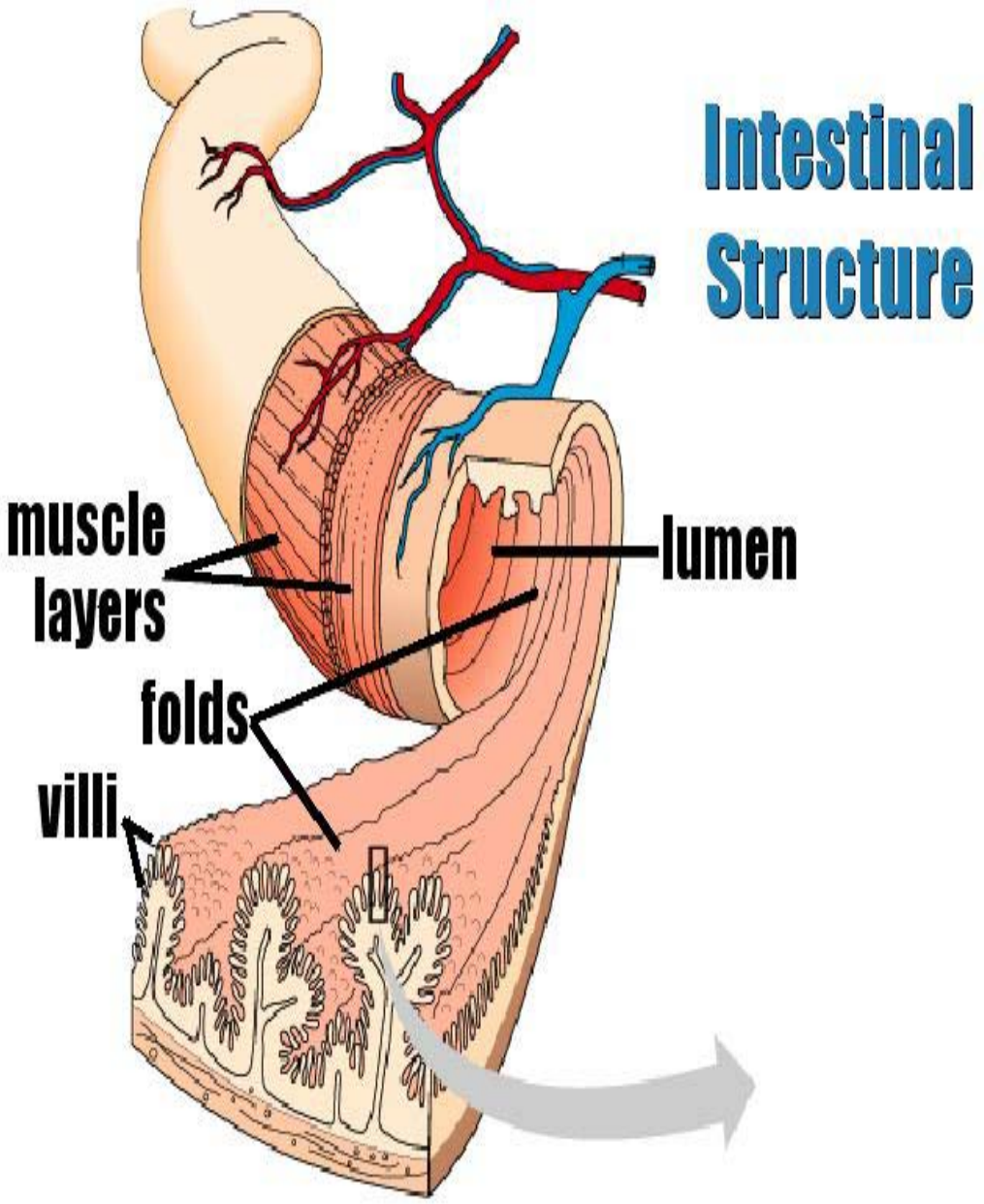
ii. the jejunum, which is mobile and lies in many coils, is supported and held in place by the mesentery; and

iii. the ileum, which is located at the distal end.

Several layers of tissues comprise the walls of the small intestine. Tartaglia and Waugh (2005) as well as Eurell and Frappier (2006) enumerated them as follows:

i. the inner tunica mucosa, which is folded into finger-like projections (known as villi, as illustrated in Figures 1.15 and 1.16), are lined with simple columnar epithelial cells where microvilli are embedded (both the villi and microvilli act to facilitate the efficiency of digestion and absorption by increasing the overall surface area of the small intestine);

ii. the submucosa, which is a layer of connective tissue, is composed of the submucosal glands (Brunner's glands), solitary and aggregated lymphatic nodules and the submucosal nerve plexus;



(Sourced from www.cals.ncsu.edu)

Figure 1.14 The villi of the small intestine

iii. the middle layer which, composed of tunica muscularis, possesses smooth muscle which contracts in wavelike (peristalsis) to move the contents of the intestine along the digestive tract; and

iv. the outer layer, called the tunica serosa, covers the intestine which provides protection for the middle and inner layers.

Moreover, Argenzio (2004) pointed out that among the major types of cells columnar absorptive cells, mucosal or goblet cells, enterochromaffin or endocrine cells and undifferentiated cells, only the first three types originate from the undifferentiated cells which are located at the base of the crypts . The process of cell division, which occurs twice in the crypts (Figure 1.17), is limited to the youngest cells at the base of the crypts and then migrates toward the villous where the cells mature and are finally extruded at the tip of the villous (Argenzio, 2004). Reece (2005) stated that "the normal villous epithelial cell replacement time (migration from the crypt to tip is faster in younger than in older animals" but Argenzio (2004) found that in newborn pigs, it takes about 7 to 10 days to replace the small intestine villous epithelium compared to about 2 to 4 days in a three-week old pig.

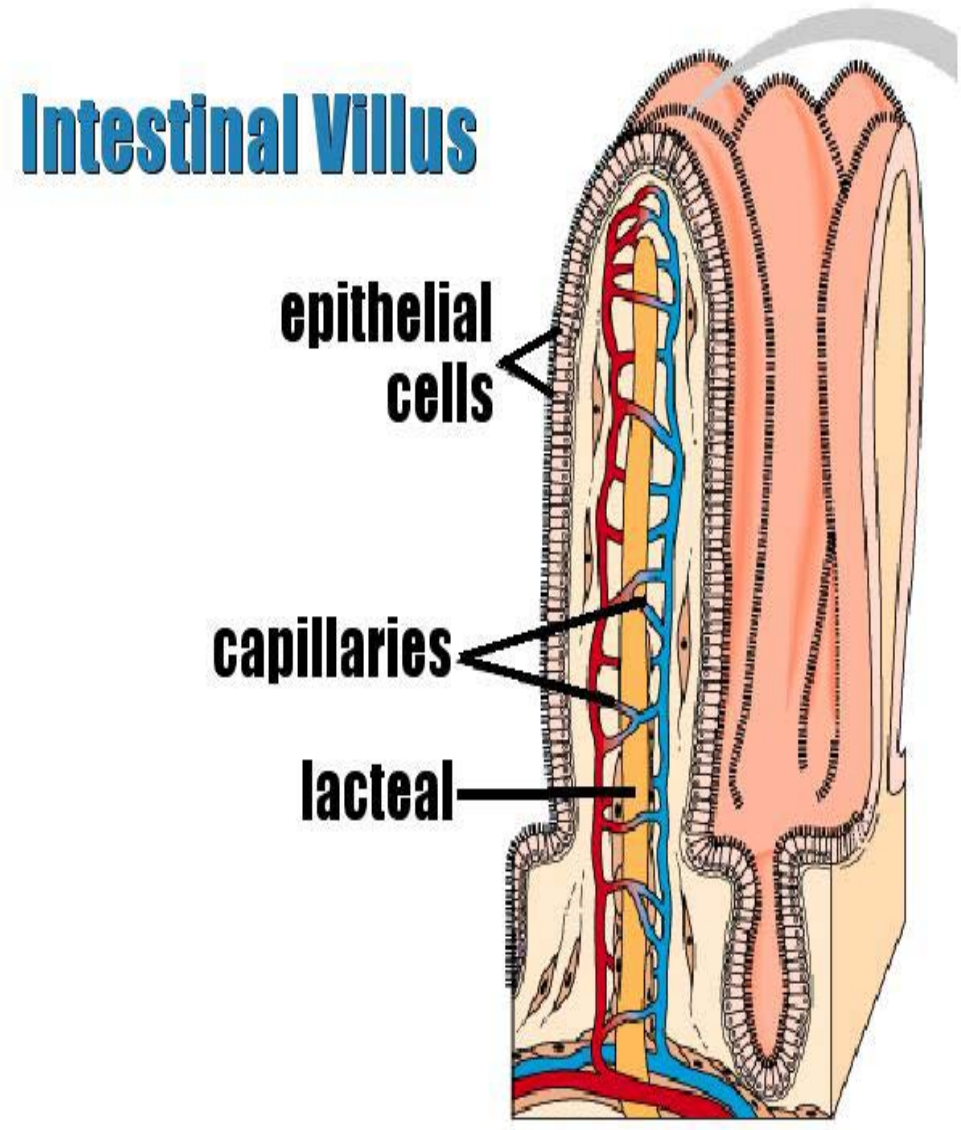


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Figure 1.15 A villous structure showing its capillary network, nerve endings and lymphatic capillary (lacteals)

Patterson et al. (2008) highlighted the striking differences and similarities in the digestive tract anatomy and physiology of humans and pigs, including:

i. the presence of a muscular outpouching, called torus pyloricus, that is found in the pyloric region of the pig stomach near the gastro-duodenal junction which is absent in the human stomach;

ii. the length of the small intestine of an adult pig is about 15 to 22 metres while the length of the small intestine of the human adult is approximately 5.5 to 7 metres;

iii. the anatomical divisions of a human adult small intestine (duodenum, jejunum and ileum) are more prominent compared to an adult pig small intestine;

iv. the human small intestine is located behind the large intestine in the abdominal cavity in comparison with the pig small intestine which lies in the right side of the abdomen;

v. the average length of an adult pig large intestine is about 4 to 6 metres while in the adult human large intestine, the average length is 1.5 metres;

vi. the large intestine in humans is in a square-like configuration which is consist of the ascending colon, transverse colon and the descending

colon (S-shaped forming the sigmoid colon); an appendix is present in humans, but not in the adult pig; and

vii. in contrast with the human large intestine, the pig large intestine is composed of the caecum, proximal (ascending), mid (transverse) and the majority of the distal (descending) colon in a spiral conformation which begins at the mid-abdomen, spiralling toward the left upper quadrant of the abdomen in clockwise and counterclockwise coils.

Patterson et al. (2008) commented that despite the differences that occur in the length of the intestines, with the pig intestine being longer than the human intestine, the length per kilogram bodyweight is similar as both have approximately 0.1 metre of intestine per kilogram bodyweight. In addition, although the internal spatial arrangement of the alimentary canal differs between humans and pigs, both are alike with regard to the digestive and metabolic processes. Not only are the majority of their macroscopic anatomical structures the same, but their microscopic anatomy, particularly their intestinal villous structure including the epithelial cell types, is strikingly similar as well (Patterson et al., 2008).

Further, Miller and Ullrey (1987) pointed out that both humans and pigs have the following features in common in relation to thermoregulation and metabolism:

i. both usually experience a drop in body temperature at birth which can be followed by a rise;

ii. both possess a little thermal insulation; and

iii. both have metabolic rates that increase in the first day following parturition.

Table 1.3.1 Physical and biochemical characteristics of human infants and piglets (Patterson et al., 2008; Miller & Ullrey, 1987)

Characteristics	Human Infant	Piglet
Average birth weight (kg)	3.4	1.4
Growth rate		
(birth to 6 weeks)	50% (3400-5000 g)	1000% (1200-12000 g)
Composition at birth		
Water (%)	82	82
(Fat-free basis)		
Protein (%)	14	13
Ash (%)	3	3
Composition at maturity		
Water (%)	69	72
(Fat-free basis)		
Protein (%)	21	23
Ash (%)	9	4
Iron stores at birth (mg)	250	50

However, the piglet, as compared to a human infant, has a “lower birth weight, more rapid growth rate, higher body temperature, higher metabolic rate and lesser fat reserve” (Miller & Ullrey, 1987). Some of these characteristics are presented in Table 1.3.1.

Chapter 2. THE MEAT EXTRACT EXPERIMENT

2.1 INTRODUCTION

Anaemia, which is caused by an iron deficiency, is a global nutritional disorder of utmost concern (Tetens et al., 2007). Iron deficiency in domestic animals is commonly associated with depression in bone marrow production of red blood cells or erythropoiesis (Carlson, 2009). Dietary iron is available in haem and non-haem forms and is essential as a source of iron, as low-iron diets seem to affect the bioavailability of iron in the body, the rate of red blood cell formation and the growth performance of the animal.

Nutritional studies pertaining to iron absorption in pigs have established that meat, which contains the haem iron, enhances non-haem iron absorption due to the presence of the "meat factor". The pig (*Sus scrofa domesticus*) is a monogastric animal with digestive and physiological functions similar to the human being (Cunningham et al., 2005).

In this study, the piglet is utilised as an experimental model for human to assess the effect of meat and meat extract considered as potential ingredients containing the "meat factor", on iron bioavailability. As such, the study also aims to: (1) determine the effects of meat and meat extract on the growth performance and organ weights of the piglets; (2) assess if meat and meat extract could improve the haematological profile of the piglets; (3) validate the effectiveness of meat and meat extract in enhancing iron absorption through histological parameters; (4) investigate if meat and meat extract could affect the immunological status of the piglets; and (5)

determine the effect of mineral components of meat and meat extract on mineral balance of the piglets.

2.2 MATERIALS AND METHODS

2.2.1 MEAT EXTRACT PREPARATION

The processes involved in the production of meat extract (LSF) are confidential.

2.2.2 EXPERIMENTAL ANIMALS

2.2.2.1 The Animals

Twenty-four pigs 3 weeks of age were randomly selected from six litters to be used in the study. The pigs' bloodlines were (Large White x Landrace) x Duroc crosses. The pigs were obtained locally from a commercial pig farm in Wanganui. All procedures involved in the conduction of the study were in accordance with the guidelines set by the Massey University Animal Ethics Committee.

The pigs were administered 60 mg of an iron preparation (Gleptosil ™) intramuscularly (about 30% of a supplementary iron injection) a day after birth to create a semi-anaemic condition. The usual dosage would be 200 mg intramuscularly (Papich, 2007).

The pigs were reared with the sow and while they were suckling, no creep feeding was provided. The pigs were weaned at 3 weeks of age and after weaning, they were

transported to the Massey University Animal Physiology Unit where the experiment was conducted. One pig from each litter was allocated at random to 1 of the 4 experimental diets.

The pigs were placed in metabolic crates. The first week was the acclimatisation period for the pigs; therefore two pigs were assigned to each crate. A week after acclimatisation, the pigs were individually housed in the metabolic crates.

2.2.2.2 Metabolic Crates

The area of the metabolic crates was 0.75 m^2 and the metabolic crates had punched metal floors. About 40% of the punched metal floor was covered with recycled plastic mat to provide a warm and dry lying area. The room temperature was monitored twice daily. A 250 watt heat lamp installed on top of each metabolic crate provided an additional heat source, and maintained the temperature within the pigs' thermo-comfort zones.

An ad libitum supply of water was available to the pigs for the duration of the experiment. Water push nipples were individually placed on each metabolic crates. The water push nipples were checked twice daily to ensure that water was free flowing.

Each pig was provided with a feeder attached to the metabolic crate. Initially, plastic feeders were used, but as the experiment progressed these were replaced by metal

feeders. The feeders were detached from the metabolic crates twice daily and cleaned before feeding.

2.2.3 EXPERIMENTAL DIETS

The duration of the experiment was 5 weeks (1 week acclimatisation, 4-week trial). During the first week, the pigs were fed a milk supplement composed of 24% skim milk powder and 6% casein. Experimental diets were given to the pigs from the second week through the fifth week of the experiment.

The 4 experimental diets that were used in the study were as follow:

1. **Non-meat diet:** (control) composed of a normal diet for piglets of that age;

2. **Non-meat diet plus lactoferrin:** an amount of lactoferrin equal to the amount of meat extract;

3. **A meat diet:** composed of the control diet added with 250 grams of meat per kg of minced semimembranous and adductor muscles which were cooked for 90 minutes at 70^{o}C; and

4. **Non-meat diet plus meat extract (LSF)**.

The ingredients and nutrient composition of the four experimental diets on an as-fed basis are presented in Table 2.1 while the iron content of the 4 diets are shown in Table 2.2.

Table 2.1 Nutrient composition of the 4 diets in the experiment

Ingredients (g/kg) as-fed	Control	Lactoferrin	Meat	LSF
Meat	0	0	250	0
Casein	100	94.68	34.55	95.68
Wheat starch	130.3	130.2	130.2	130.2
Soya bean oil	50	50	50	50
Cellulose	15	15	15	15
Lactoferrin	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	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

Each experimental group was composed of six pigs. The pigs were fed an equal amount of the experimental diet twice daily at 8:30 A.M. and 3:30 P.M. Initially, the pigs were given 400 g of feed per day. The amount of feed offered was increased by 50 g every fourth day as the nutrient requirements of the pigs increased. Each experimental diet was fed in a liquid form, which was prepared by adding water to the solid diet, and thoroughly mixing in a blender. The cooked meat was

added to the solid diet and water and mixed in a blender. Daily feed consumption of the pigs was calculated by obtaining the difference between the feed offered and the amount remaining in the feeder due to refusal.

Table 2.2 Iron (Fe) content (mg/kg) of the 4 diets: as formulated; as chemically analysed for wet and dry forms; and as the basis in the calculation for iron intake of piglets during the experiment (Hills Laboratory, Hamilton, New Zealand)

Fe (mg/kg) as-fed	Control	Lactoferrin	Meat	LSF
Formulated	35	35	35	35
Wet Diet	34	33	49	161[a]
Dry Diet	22	53	24	37
For Fe Intake[b]	28	43	36.5	37

[a] This very high value was not used in calculating intakes

[b] Means of wet and dry values except in the LSF group

2.2.4 BLOOD SAMPLING

Blood samples were collected from the pigs on days 0, 7, 14, 21 and 28 of the experiment. The pigs were anaesthetised during the blood collection with an inhalant anaesthetic composed of a mixture of halothane/fluothane (4%) and oxygen. Once unconscious, each pig was laid on the surgical table in a recumbent (dorsoventral) position.

Collection was obtained with vacutainer collection system at the jugular vein. Two vacuum blood tubes were used to collect 2 x 5 ml of blood. One tube contained an anticoagulant, an ethylenediaminetetraacetic acid (EDTA) and the other tube

contained heparin. The two sample tubes were next placed in an electric shaker to ensure an even distribution of the blood with the anticoagulant.

After blood sampling and while still unconscious, the pigs were weighed to avoid additional stress.

2.2.4.1 Haematological Analysis of Blood with EDTA

Table 2.3 Normal haematology values for pigs (sourced from Shaw, personal communication, March 19, 2009)

Parameters	Range	Unit of Measure
Red Blood Cells	5.0-8.0	$x10^{12}/L$
Haemoglobin	100-160	g/L
Haematocrit	0.32-0.50	L/L
Mean Corpuscular Volume	50-68	fL
Mean Corpuscular Haemoglobin	17-21	pg
Mean Corpuscular Haemoglobin Concentration	300-350	g/L
Platelets	120-920	$x10^{9}/L$
White Blood Cells	11.0-22.0	$x10^{9}/L$
Neutrophils	3.2-10	$x10^{9}/L$
Lymphocytes	4.5-13	$x10^{9}/L$
Monocytes	0.2-2	$x10^{9}/L$
Eosinophils	0.5-2	$x10^{9}/L$
Basophils	0.0-0.3	$x10^{9}/L$

The blood samples with EDTA were submitted for haematological analysis at the Pathology Laboratory of the Institute of Veterinary, Animal and Biomedical Sciences. Pertinent blood parameter data such as the number of red blood cells (RBC), white blood cells (WBC), and platelets (thrombocytes), the haematocrit concentration or packed cell volume (PCV), the mean corpuscular volume (MCV), the mean corpuscular haemoglobin (MCH), the mean corpuscular haemoglobin concentration (MCHC) and other relevant haematological profiles were determined. The blood samples were tested in the ADVIA 120 haematology analyser (Bayer Diagnostics) to obtain values for these parameters. The reference values for haematological parameters in the experiment are shown in Table 2.2.

2.2.4.2 Assay of Blood Samples with Heparin

The blood samples contained in heparin tubes were subjected for whole-blood proliferation assay and assessment of phagocytic activity. The assays were performed following the procedures modified by Rutherfurd-Markwick et al. (2005).

2.2.4.2.1 Whole-Blood Proliferation Assay

The pig whole-blood cell proliferation assay was optimised and standardised by Rutherfurd-Markwick et al. (2005). For the first step, the lithium heparin-treated peripheral whole blood was diluted 1:4 in complete RPMI-1640. The RPMI-1640

was supplemented with 10% foetal calf serum, 10 mM HEPES, 2 mM-L-glutamine, 100 Uml^{-1} penicillin, 100 µg ml^{-1} streptomycin sulphate and 5 µM2-mercaptoethanol (all reagents were obtained from Gibco, Poole, UK). Secondly, about 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). This was followed by the culture of the dilution in the presence of a 5 μgml^{-1} Concanavalin A (Con A) from Sigma, USA, 1:49 diluted Phytohaemagglutinin (PHA) from Gibco, Poole, UK or complete RPMI-1640 instead of the mitogen which served as the control wells. Following this, the cells were cultured for 48 hours at 37^oC in a 5% humidified CO_2-air atmosphere prior to pulsing for 18 hours with 0.5 µ Cimethyl- ^3H-thymidine (Amersham Biosciences, UK) per well. After incubation, the culture from each plate was harvested and the cells were placed in a 96-well glass fibre mat using a Tomtek cell harvester 96 (Hamden, CT, USA). They were then counted using a Wallac MicroBeta Trilux 1450 liquid scintillation and luminescence counter (Turku, Finland). Finally, the stimulation index was calculated by dividing the cpm in the wells with mitogen by the cpm in the wells without mitogen.

2.2.4.2.2 Phagocytic Activity

The assay for the phagocytic capacity of the peripheral blood leukocytes by flow cytometry was adapted from the method of Rutherfurd-Markwick et al. (2005). About 5 µL of FITC-labelled *Escherichia coli* bacteria (1 x 10^9/ ml) from Molecular Probes Incorporated, Oregon, USA was mixed with 100 µL of whole blood. The

mixture was incubated for 30 minutes at 37 ^{o}C. After incubation, the cells were fixed with paraformaldehyde. Next, the erythrocytes were lysed by the addition of 1 ml of ice-cold water. After erythrolysis, centrifugation was performed at 4,000 rpm for 10 minutes at 4 ^{o}C and the pellet was re-suspended in 500 µL of PBS and 50 µL of 4% Trypan Blue was added to quench extraneous fluorescence. Determination of phagocytic activity was done using a FACS Calibur flow cytometer (Becton, Dickinson Instruments, Cambridge, MA).

2.2.5 HISTOLOGICAL EXAMINATION OF THE SMALL INTESTINE

On days 28th and 29th of the experiment, the pigs were euthanised intracardially with a lethal dose of 150 mg/kg pentobarbitone while under anaesthesia. Every pig was then dissected and the small intestine was removed. The length of the small intestine was measured. Three proportional sections (duodenum, jejunum and ileum) at 25%, 50%, and 75% along the intestine were clamped with haemostats, excised and placed immediately into Bouin's fluid (24% formalin, 5% glacial acetic acid and 71% picric acid) for 24 hours before being stored in 70% ethanol. The samples were then submitted for the preparation of slides at the Histology Laboratory of the Institute of Veterinary and Biomedical Sciences. After fixation, ring-shaped lengths of the small intestine from all three sections were excised, dehydrated and embedded in paraffin wax. From each of these, transverse sections of about 6 µm were cut through the Microtec microtome and stained with alcian blue, haematoxylin and eosin.

The slides were examined using an Axiophot photomicrograph (Zeiss, West Germany). Each slide was viewed in the photomicrograph under the low power objective with a magnification of 2.5 times and with an eyepiece magnification of 10 times. Images were shot, and parameters such as the height of the villi, the depth of the crypts of Lieberkuhn and the mucosal thickness were determined on at least 3 villi using the image analysis software Sigma Scan (Jandel Scientific, San Rafael, CA). Goblet cells were identified in the epithelium as blue-staining cells, which were counted from the 3 villi and expressed as the number of cells per 100 µm.

2.2.6 MINERAL BALANCES

Fecal and urine samples were collected from the pigs for a period of 5 consecutive days during the last week of the experiment. The faecal samples from each pig were placed in a cellopack, sealed, and frozen. The urine samples were collected in plastic buckets through a metal funnel placed under the metabolic crates, and were also collected and frozen. All the frozen faecal and urine samples were submitted to the Nutrition Laboratory of the Institute of Food, Nutrition, and Human Health for mineral retention determination where magnesium, copper, iron, manganese, selenium, and zinc were determined using the inductively coupled plasma, optical emission spectroscopy (ICP-OES), or inductively coupled plasma, mass spectrometry (ICP-MS) while calcium and phosphorous were determined through colourimetry.

The percentages of minerals retained in the body, faeces and urine were calculated using the formulas:

Mineral Retention (%) = Daily Mineral Retention / Daily Mineral Intake x 100

Mineral Retention in the Faeces (%) = Daily Mineral Faecal Retention / Daily Mineral Intake x 100

Mineral Retention in the Urine (%) = Daily Mineral Retention in the Urine / Daily Mineral Intake x 100

2.2.7. ORGAN WEIGHTS

After evisceration, the liver, kidneys and spleen were weighed and the percentage organ weight was determined using the formula:

Organ Weight (%) = Weight of the Organ / Liveweight x 100.

2.2.8 GROWTH PERFORMANCE

The parameters for growth performance were measured by the average daily gain (ADG) and feed conversion ratio (FCR) of the piglets. The following formulas were used in the experiment:

Average Daily Gain (ADG) = Final Liveweight - Initial Liveweight / 28 (days of the experiment); and

Feed Conversion Ratio (FCR) = Daily Feed Intake (DFI) / Average Daily Gain.

2.2.9 ESTIMATION OF HAEMOGLOBIN IRON REPLETION EFFICIENCY

Initially, the total body haemoglobin iron content (BHbFe) was calculated following the formula sourced from Patterson et al. (2008) indicated below:

BHbFe (mg) = Liveweight x 0.07 x Haemoglobin Concentration x 0.00335

Where:

0.07 = blood volume as a proportion of body weight; and
0.00335 = iron weight as a proportion of haemoglobin.

Additionally, the daily feed intake of the piglets were measured. The total iron content of the diets (Table 2.2) was analysed by plasma emission spectrometry (Hills Laboratory, Hamilton, New Zealand). Then, the cumulative iron intake (FEI) was calculated using the formula:

FEI = Cumulative Feed Intake x Iron Concentration of the Diet.

The haemoglobin iron repletion efficiency (HbRE) was finally calculated following the formula sourced from Patterson et al. (2008):

HbRE = Final BHbFe – Initial BHbFe / FEI x 100.

2.2.10 DATA ANALYSES

Statistical analyses of the data in the experiment were done using the SAS system for windows, version 9.1 (SAS Institute Inc., Cary, NC, USA). A simple analysis of variance (ANOVA) was used to compare the effect of the 4 diets on liveweight, average daily gain, feed intake, feed conversion ratio, percentage organ weight, total body haemoglobin iron content, haemoglobin repletion efficiency and mineral balances. The statistical model used is:

$$\mathbf{Y_{ij} = \mu + A_i + e_{ij}}$$

Where:

Y_{ij} : j^{th} observation in the i^{th} dietary treatment group;

μ : general mean;

A_i : fixed effect of the i^{th} dietary treatment group; and

e_{ij} : random residual error.

The repeat measure analysis of variance was applied to compare the effect of the dietary treatments, the effect in piglets within diets, the effect over time (week) and the interaction between dietary treatments and week on the haematological parameters, total body haemoglobin iron content, and cumulative iron intake. The statistical model used is:

$$\mathbf{Y_{ijk} = \mu + A_i + \beta_j (A_i) + C_k + A_i \ x \ C_k + e_{ijk}}$$

Where:

Y_{ijk} : observation in the dietary treatment group A, j^{th} piglet group β at the k^{th} week;

μ : general mean;

A_i : fixed effect of the i^{th} dietary treatment group;

β_j : random effect of the j^{th} piglet;

C_k : fixed effect of the k^{th} week group;

A_i x C_k : interaction between the i^{th} dietary treatment group and the k^{th} week; and

e_{ijk} : random residual error.

Similarly, repeat measure analysis of variance was used to compare the effect of the dietary treatments, the effect in piglets within diets, the effect on location and the interaction between dietary treatments and location on histological parameters. The statistical model used is:

$$\mathbf{Y_{ijk} = \mu + A_i + \beta_j (A_i) + C_k + A_i \ x \ C_k + e_{ijk}}$$

Where:

Y_{ijk} : observation in the dietary treatment group A, j^{th} piglet group β at the k^{th} location;

μ : general mean;

A_i : fixed effect of the i^{th} dietary treatment group;

β_j : random effect of the j^{th} piglet;

C_k : fixed effect of the k^{th} location group;

A_i x C_k : interaction between the i^{th} dietary treatment group and the k^{th} location; and

e_{ijk} : random residual error.

A simple linear regression analysis was applied to determine the influence of piglet cumulative iron intake (FEI) on the total body haemoglobin iron content (BHbFe). The statistical model used is:

$$\mathbf{Y_i = \alpha + \beta X_i + e_i}$$

Where:

Y_i : observed value of dependent variable (BHbFe) corresponding to a given value of the X-variable (FEI), X_i;

α : the expected value of dependent variable (BHbFe) when the X-variable (FEI) is zero;

β : the expected change in the dependent variable (BHbFe) given a unit change in the X-variable (FEI); and

e_i : random residual error.

Finally, a paired t-test was carried out to compare the effect of the 4 diets on immunological parameters.

2.3 RESULTS

2.3.1 GROWTH PERFORMANCE

The least squares means for growth performance of the piglets are presented in Table 2.3.1.

Table 2.3.1 Least squares means at week 0 and week 4 for liveweight (g) of piglets and the average daily gain (g/day), feed intake (g/day) and feed conversion ratio of piglets from week 0 to week 4

Parameters	Control	Lactoferrin	Meat	LSF	SE
Liveweight (g) at Week 0	8288	8813	8660	8731	330
Liveweight (g) at Week 4	13715	14521	14578	14448	419
Average Daily Gain (g/day)	193.83	203.87	211.34	204.17	10
Feed Intake (g/day)	705.09	738.95	748.02	782.69	38
Feed Conversion Ratio (g/g)	3.63	3.62	3.57	3.84	0.13

Means within a row with no superscripts do not differ significantly
(i.e. P 0.05)

For the growth performance parameters (Table 2.3.1), statistical analyses revealed no significant differences among the 4 dietary treatments in liveweights at week 0 ($p = 0.69$) and week 4 ($p = 0.44$), average daily gain ($p = 0.68$), feed intake ($p = 0.56$) and feed conversion ratio ($p = 0.45$) of the piglets.

2.3.2 ORGAN WEIGHTS

The least squares means for percentage organ weights are shown in Table 2.3.2.

Table 2.3.2 The least squares means after 4 weeks of the experiment for weights as a percentage of live weight for the kidneys, liver and spleen of piglets

Organs	Control	Lactoferrin	Meat	LSF	SE
Kidneys (% weight)	0.5681[a]	0.5231[a]	0.4552[b]	0.5403[a]	0.03
Liver (% weight)	2.3837	2.5181	2.5447	2.6066	0.09
Spleen (% weight)	0.3414	0.3665	0.4449	0.3973	0.06

[a,b] Means within the same row with common superscripts or with no superscripts do not differ significantly (i.e. P > 0.05)

A dietary effect ($p = 0.04$) was displayed in percentage kidney weights. However, the least squares means (Table 2.3.2) did not differ ($p = > 0.05$) significantly among the control, lactoferrin and LSF groups. Only the meat group was lower than the rest of the dietary treatments.

2.3.3 HAEMATOLOGICAL PARAMETERS

The significance levels for the effects of diet, pig within diet, week and the interaction between diet and week on haematological parameters are listed in Table 2.3.3.

Table 2.3.3 Significance levels for the effects of diet, pig within diet, week and the interaction between diet and week on haematological parameters of piglets from week 0 to week 4

Parameters	Diet	Pig (Diet)	Week	Diet* Week	R^2
Haematocrit	ns	***	***	ns	0.846
Haemoglobin	ns	***	***	ns	0.881
Red Blood Cells	ns	***	***	ns	0.852
Mean Corpuscular Volume	ns	***	***	ns	0.780
Mean Corpuscular Haemoglobin	ns	***	***	*	0.949
Mean Corpuscular Haemoglobin Concentration	ns	***	***	ns	0.811
Red Cell Distribution Width	ns	***	***	ns	0.866
Platelet Count	ns	***	**	ns	0.604
White Blood Cell Count	ns	**	***	ns	0.759
Neutrophils[1]	ns	ns	***	ns	0.442
Lymphocytes[1]	ns	ns	**	ns	0.437
Monocytes[1]	ns	***	ns	*	0.570
Eosinophils[1]	ns	ns	**	ns	0.494
Basophils[1]	ns	ns	**	ns	0.402

ns: P > 0.05, * P < 0.05, ** P < 0.01,
*** P < 0.001

[1] Expressed as a percentage of white blood cells

2.3.3.1 Haematocrit, Haemoglobin, Red Blood Cells, Mean Corpuscular Volume, Mean Corpuscular Haemoglobin, Mean Corpuscular Haemoglobin Concentration, and Red Cell Distribution Width as Parameters in Evaluating Erythrocytes

Non-significant diet effects (Table 2.3.3) occurred in haematocrit ($p = 0.58$), haemoglobin ($p = 0.57$), red blood cells ($p = 0.62$), mean corpuscular volume ($p = 0.36$), mean corpuscular haemoglobin ($p = 0.57$), mean corpuscular haemoglobin concentration ($p = 0.85$) and red distribution width ($p = 0.31$).

Contrary to these, highly significant effects existed between the piglets within diets (Table 2.3.3) in relation to haematocrit ($p = < 0.0001$), haemoglobin ($p = < 0.0001$), red blood cells ($p = < 0.0001$), mean corpuscular volume ($p = < 0.0001$), mean corpuscular haemoglobin ($p = < 0.0001$), mean corpuscular haemoglobin concentration ($p = < 0.0001$) and red distribution width ($p = < 0.0001$).

Highly significant changes over time (Table 2.3.3) were observed in haematocrit ($p = < 0.0001$), haemoglobin ($p = < 0.0001$), red blood cells ($p = < 0.0001$), mean corpuscular volume ($p = 0.0002$), mean corpuscular haemoglobin ($p = < 0.0001$), mean corpuscular haemoglobin concentration ($p = < 0.0001$) and red distribution width ($p = < 0.0001$).

However, no significant diet-by-week interactions occurred in haematocrit ($p = 0.49$),

haemoglobin (p = 0.57), red blood cells (p = 0.50), mean corpuscular volume (p = 0.06), mean corpuscular haemoglobin concentration (p = 0.28) and red cell distribution width (p = 0.10). A significant diet-by-week interaction was only observed for mean corpuscular haemoglobin (p = 0.05).

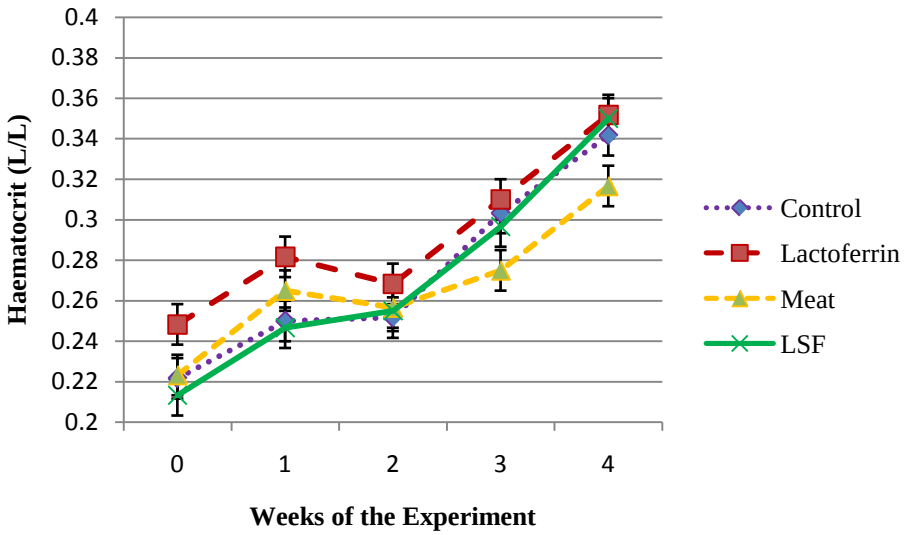


Figure 2.3.1 Least squares means (+/- SE) for haematocrit (L/L) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

Over time, there was an increasing trend in haematocrit (Figure 2.3.1), haemoglobin (Figure 2.3.2) and red blood cells (Figure 2.3.3), particularly from week 2 to week 4. Only the haematocrit in the LSF group had increased continuously over time. A rise in haemoglobin existed in the LSF, control and meat groups over time while the lactoferrin group tended to increase from week 2 to week 4. A rise in red blood cells

was demonstrated in the 4 diets with LSF group showing the most remarkable increase. However, non-significant interactions between diets and weeks were observed for haematocrit (p = 0.49), haemoglobin (p = 0.57) and red blood cells (p = 0.50) of the piglets.

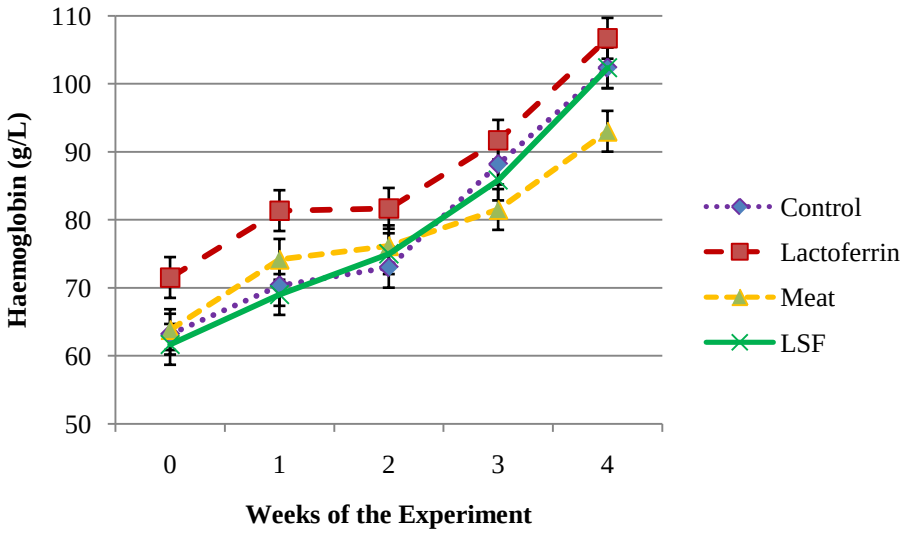


Figure 2.3.2 Least squares means (+/- SE) for haemoglobin (g/L) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

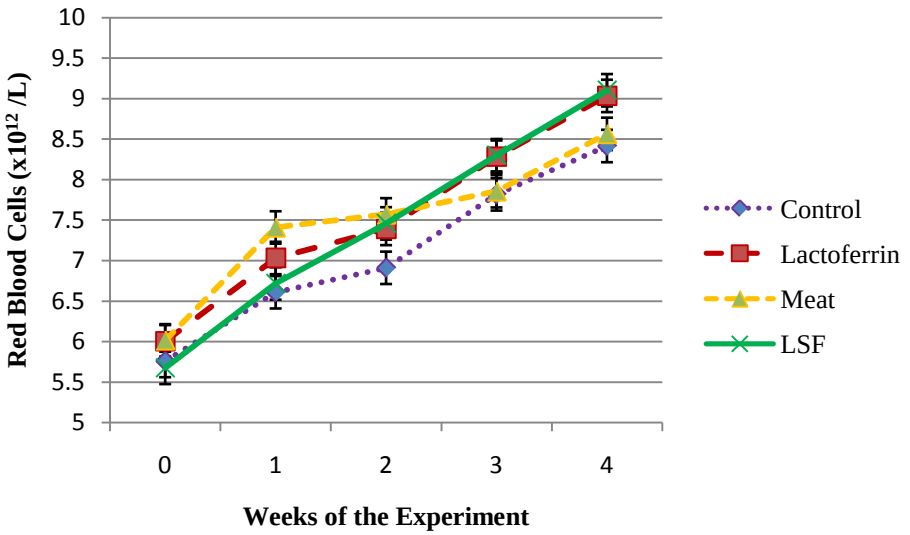


Figure 2.3.3 Least squares means (+/- SE) for number of red blood cells (x10^{12}/L) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

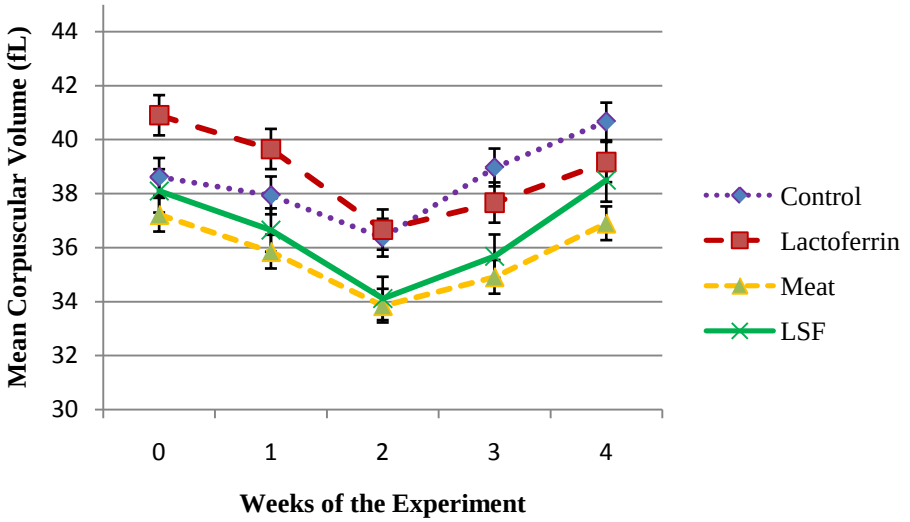


Figure 2.3.4 Least squares means (+/-) SE for mean corpuscular volume (fL) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

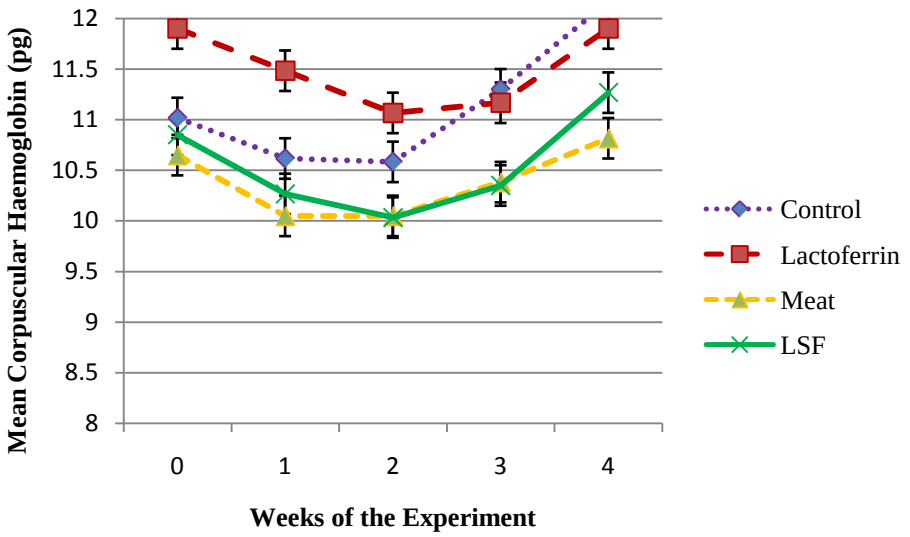


Figure 2.3.5 Least squares means (+/- SE) for mean corpuscular haemoglobin (pg) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

A decrease in mean corpuscular volume (Figure 2.3.4) and mean corpuscular haemoglobin (Figure 2.3.5) were displayed by the 4 diets in week 2 but tended to increase in week 3 to week 4. However, no diet by week interaction was observed in the mean corpuscular volume of the piglets. On the other hand, the highest values for mean corpuscular volume and mean corpuscular haemoglobin were obtained by the control group in week 4. In relation to this, a significant diet-by-week interaction occurred ($p = 0.05$) in the control group but the least squares means revealed a non-significant effect ($p = > 0.05$) among the 4 diets.

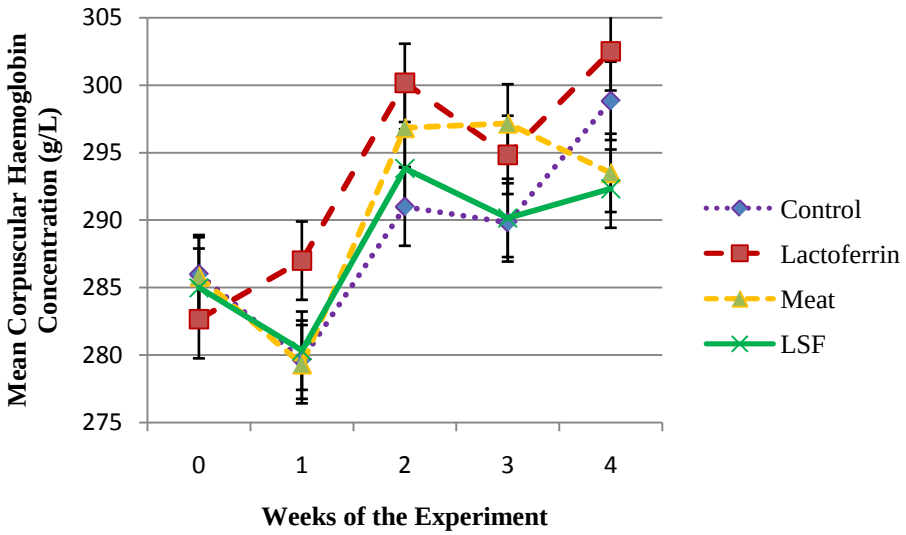


Figure 2.3.6 Least squares means (+/- SE) for mean corpuscular haemoglobin concentration (g/L) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

For the mean corpuscular haemoglobin concentration (Figure 2.3.6), a decline in week 1 which was followed by a surge in week 2 was displayed by meat, LSF and control groups. The meat group had decreased mean corpuscular haemoglobin concentration in week 4 while the control and LSF groups had increased. The lactoferrin group had an increase in mean corpuscular haemoglobin concentration from week 1 to week 2 followed by a fall in week 3 but rose again in week 4. Nevertheless, no significant interaction ($p = 0.28$) existed among the 4 diets over time.

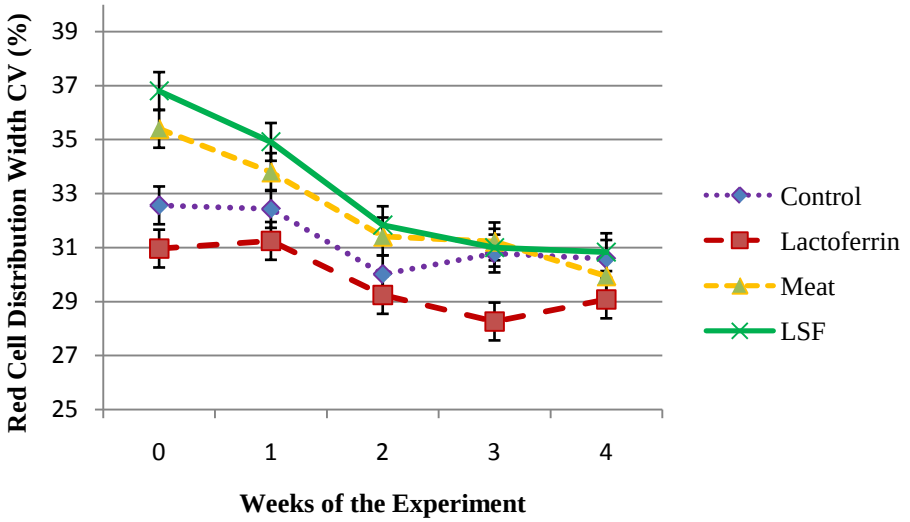


Figure 2.3.7 Least squares means (+/- SE) for red cell distribution width CV (%) of piglets given the control, lactoferrin, meat and LSF on weeks 0, 1, 2, 3, and 4 of the experiment

Red cell distribution width (Figure 2.3.7) was markedly decreased in week 2 in the 4 diets. The red cell distribution width continued to fall slightly until week 4 in the LSF group. From a drastic decrease in week 2 to week 3, the red cell distribution width of the lactoferrin group increased in week 4. Similarly, a decline in the red cell distribution width in week 2 which was followed by a slight rise in week 3 were observed in the control group. In week 4, the control group seemed to be constant while the meat group showed a decrease in red cell distribution width. Despite these changes, no significant diet-by-week interactions were exhibited ($p = 0.10$).

2.3.3.2 Platelets, White Blood Cells, Neutrophils, Lymphocytes, Monocytes, Eosinophils, and Basophils

No significant diet effects (Table 2.3.3) were found in the number of platelets ($p = 0.16$), white blood cells ($p = 0.44$), neutrophils ($p = 0.48$), lymphocytes ($p = 0.49$), monocytes ($p = 0.77$), eosinophils ($p = 0.16$) and basophils ($p = 0.59$).

The results for the effects of pig within diet are shown in Table 2.3.3. Significant effects were demonstrated in the number of platelets ($p = < 0.0001$), white blood cells ($p = 0.001$), and monocyte percentages ($p = < 0.0001$). On the other hand, non-significant effects in the piglets within diets were observed in the percentages of neutrophils ($p = 0.11$), lymphocytes ($p = 0.07$), eosinophils ($p = 0.09$) and basophils ($p = 0.35$).

For the effects over time, significant changes (Table 2.3.3) existed in the number of platelets ($p = 0.003$) and white blood cells ($p = < 0.0001$). Similarly, significant effects were observed in the percentages of neutrophils ($p = 0.0007$), lymphocytes ($p = 0.002$), eosinophils ($p = 0.001$) and basophils ($p = 0.004$). Contrary to these, no significant change in the monocyte percentage ($p = 0.95$) was evident over time.

Platelet counts (Figure 2.3.8) dropped in week 1 in the control and lactoferrin groups. Also, a fall in platelets occurred in the LSF group from week 1 to week 2. A rise in platelet counts in the meat, lactoferrin and LSF groups in week 3, was followed by a drastic decrease in week 4. The platelet count for the control group dropped in week 3 but tended to be slightly increased in week 4. However, the changes in platelet counts in the 4 diets were not significantly different (p = 0.56) over time.

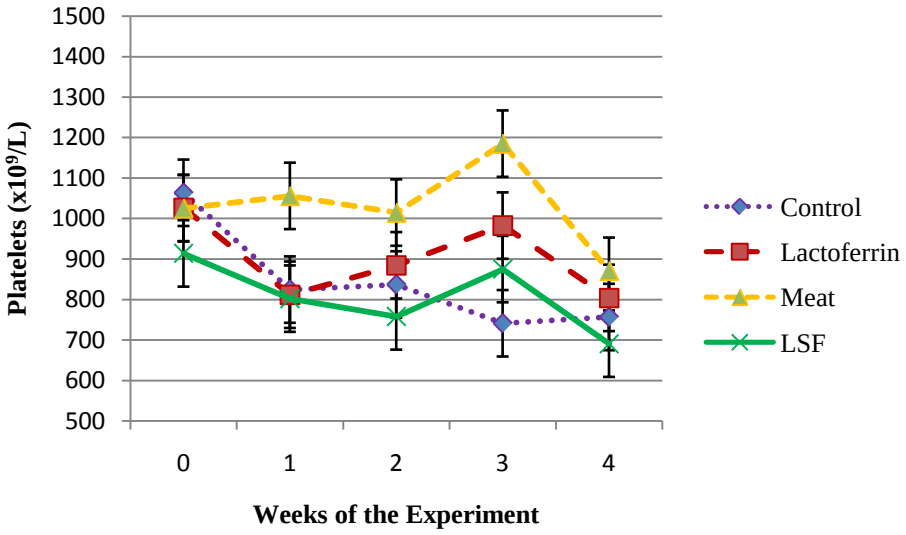


Figure 2.3.8 Least squares means (+/- SE) for platelets (x 10^9 / L) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

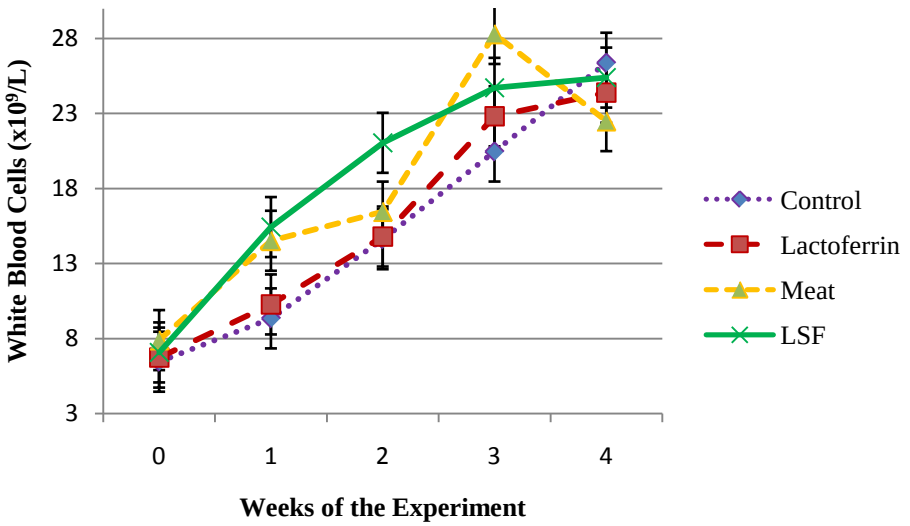


Figure 2.3.9 Least squares means (+/- SE) for white blood cells (x 10^9 / L) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

White blood cells (Figure 2.3.9) surged over time from week 0 to week 4 in the control, LSF and lactoferrin groups. The highest number in white blood cells was observed in the meat group in week 3 but was decreased in week 4. Nevertheless, no significant diet-by-week interaction was evident (p = 0.50).

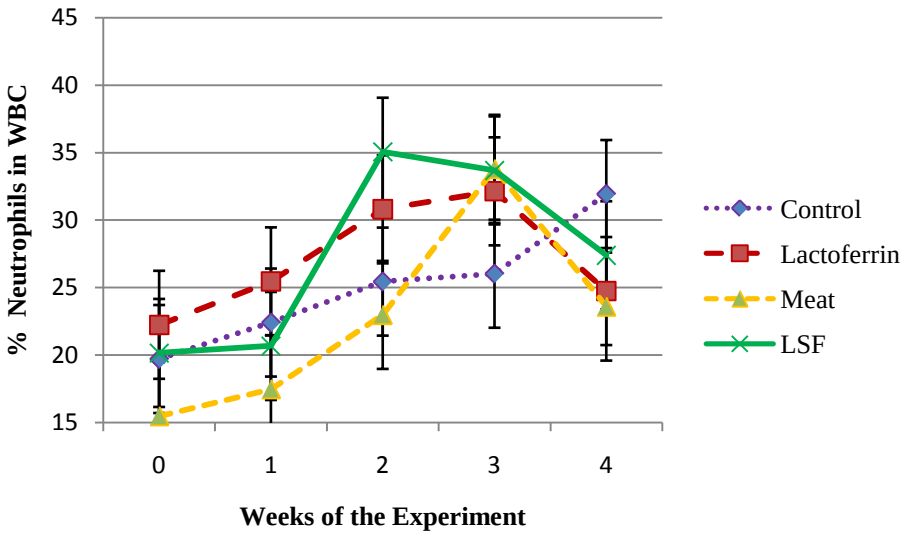


Figure 2.3.10 Least squares means (+/- SE) for percentage neutrophils in white blood cells (WBC) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0,1, 2, 3, and 4 of the experiment

Over time, only the neutrophils of the control group increased (Figure 2.3.10). Neutrophils rose from week 1 to week 3 in the meat and lactoferrin groups. As opposed to these, a drop in neutrophil percentages occurred in LSF, lactoferrin and meat groups from week 3 to week 4. However, no significant diet-by-week interaction ($p = 0.75$) existed over time.

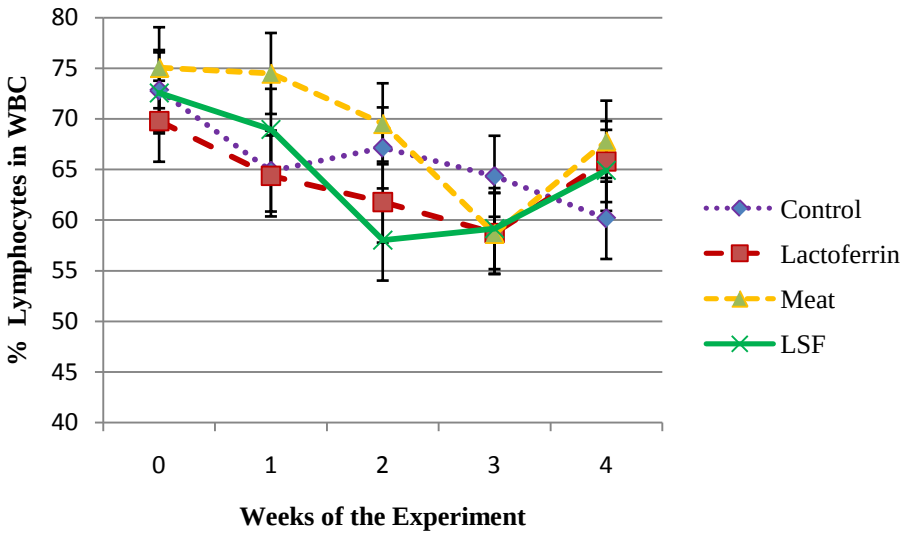


Figure 2.3.11 Least squares means (+/- SE) for percentage lymphocytes in white blood cells (WBC) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

Lymphocytes (Figure 2.3.11) remarkably decreased from week 1 to week 3 in the meat and lactoferrin groups. Similarly, lymphocytes in the LSF group fell from week 1 to week 2. In week 4, lymphocytes of the meat, lactoferrin and LSF groups tended to increase while only the lymphocytes of the control group had declined in week 4. Despite these changes, no diet effect occurred over time ($p = 0.82$).

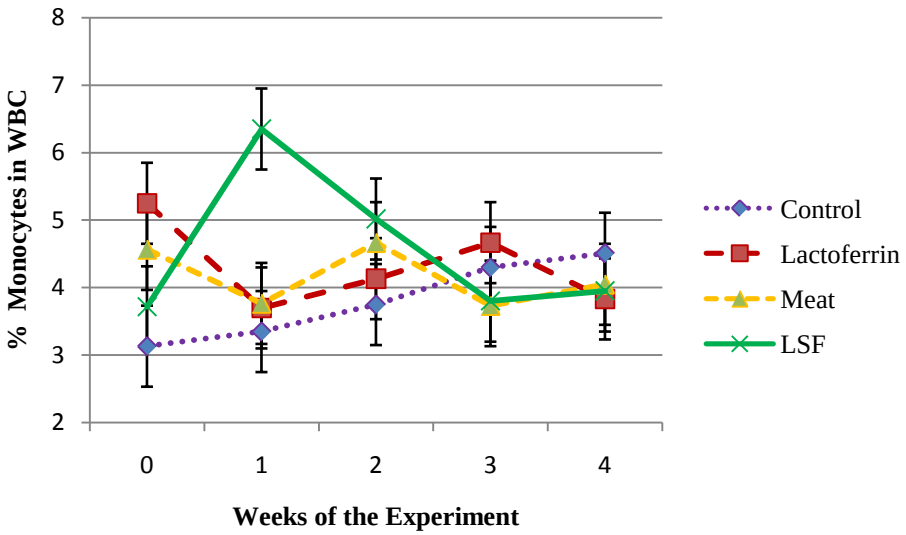


Figure 2.3.12 Least squares means (+/- SE) for percentage monocytes in white blood cells (WBC) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

For the monocytes (Figure 2.3.12), no significant effect ($p = 0.95$) was displayed over time. In contrast, significant changes in monocyte percentages were exhibited between the 4 diets over time ($p = 0.03$). Only the control group had increased monocytes over time. A decline in monocytes was seen in the lactoferrin and meat groups on week 1. After a peak in the monocytes of the LSF group in week 1, this was followed by a drastic fall in week 2 to week 3. Similarly, an increase in the monocytes of the meat group in week 2 was followed by a decline in week 3. Both meat and LSF groups tended to increase slightly in week 4. As opposed to these changes, a fall in the monocytes of the lactoferrin group in week 1 was followed by a rise from week 1 to week 3 but it decrease again in week 4.

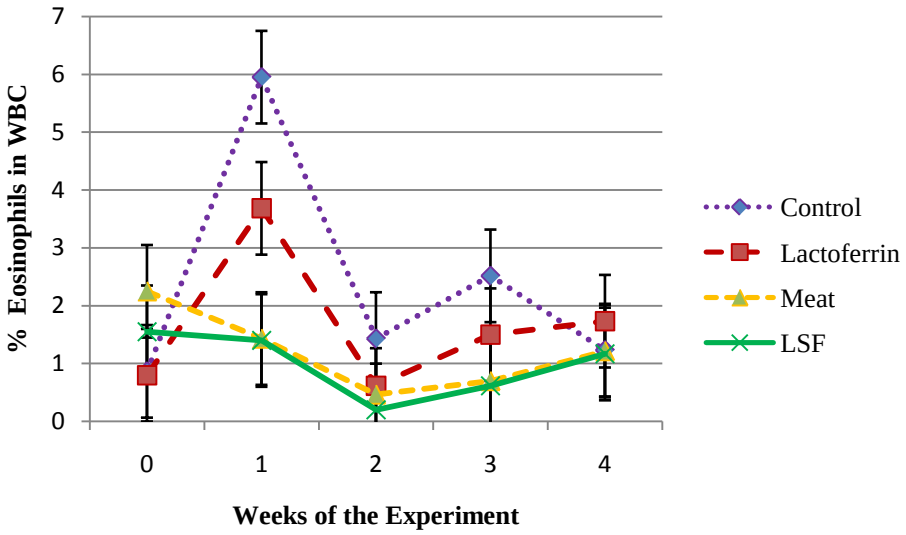


Figure 2.3.13 Least squares means (+/- SE) for percentage eosinophils in white blood cells (WBC) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

Elevated eosinophil percentages (Figure 2.3.13) were observed in week 1 in the control and lactoferrin groups but was followed by a drastic fall in week 2. Eosinophils of the meat and LSF groups declined from week 0 to week 2. In week 3 to week 4, eosinophils of the lactoferrin, meat and LSF groups seemed to increase while only the control group tended to decrease. Nevertheless, no diet effect ($p = 0.10$) was demonstrated over time.

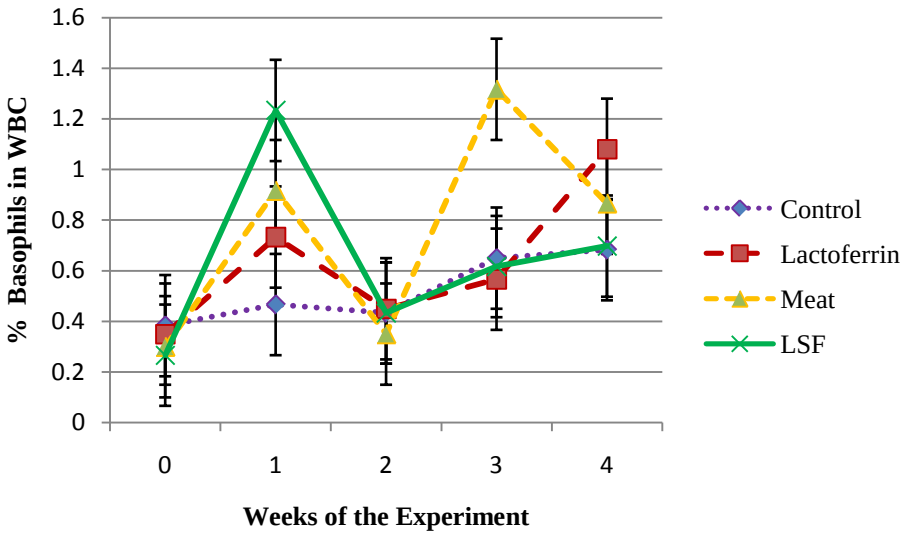


Figure 2.3.14 Least squares means (+/- SE) for percentage basophils in white blood cells (WBC) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

Increased basophil percentages in week 1 was followed by a fall in week 2 in the 4 diets. However, a rising trend was observed in week 3 to week 4 in the lactoferrin, control and LSF groups. Although the basophils of the meat group peaked in week 3, this was followed by a fall in week 4. However, the differences which exist among the 4 diets over time were not significant ($p = 0.50$).

2.3.3.3 Total Body Haemoglobin Iron Content (BHbFe), Cumulative Iron Intake (FEI) and Haemoglobin Iron Repletion Efficiency (HbRE)

The least squares means for total body haemoglobin iron content (BHbFe)), cumulative iron intake (FEI) and haemoglobin repletion efficiency (HbRE) are shown in Table 2.3.4.

Table 2.3.4 Least squares means for total body haemoglobin iron content(mg), cumulative iron intake (FEI) (mg) and haemoglobin iron repletion efficiency (%) of piglets given the control, lactoferrin, meat and LSF diets during the experiment

Parameters	Control	Lactoferrin	Meat	LSF	SE
BHbFe (mg) at Week 0	123	147	129	126	10.2
BHbFe (mg) at Week 4	330	363	319	346	22.4
BHbFe (mg) change from Week 0 to Week 4	207	216	190	220	
FEI (mg) at Week 4	553^{c}	890^{a}	764^{b}	811^{ab}	38.5
Hb Iron Repletion Efficiency (%)	36.93^{a}	24.12^{b}	24.31^{b}	27.15^{b}	2.1

[a,b] Means within the same row with common superscripts or with no superscripts do not differ significantly (i.e. $P > 0.05$)

The statistical analysis for total body haemoglobin iron content and cumulative iron intake are presented in Table 2.3.5.

Table 2.3.5 Significance levels for total body haemoglobin iron content (BHbFe) and cumulative feed intake (FEI) of piglets given the control, lactoferrin, meat and LSF on weeks 0, 1, 2, 3, and 4 of the experiment

Parameters	Diet	Pig (Diet)	Week	Diet*Week	R^2
BHbFe	ns	***	***	ns	0.953
FEI	**	***	***	***	0.986

ns: P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001

2.3.3.3.1 Total Body Haemoglobin Iron Content (BHbFe)

No significant differences (Table 2.3.4) were observed in total body haemoglobin iron content in the 4 diets in week 0 (p = 0.35) and week 4 (p = 0.54). A significant effect (Table 2.3.5) between piglets within diets (p = < 0.0001) occurred. Over time, the total body haemoglobin iron content had significantly increased (p = < 0.0001). Lactoferrin group gained the highest total body haemoglobin iron content over time but no significant interaction existed between diet and week (p = 0.86).

2.3.3.3.2 Cumulative Iron Intake (FEI)

For the cumulative iron intake, significant diet (p = < 0.002) and piglet within diet (p = < 0.0001) effects were observed (Table 2.3.5). Although the lactoferrin group was the highest, it was not significantly different (p = > 0.05) from the LSF and meat groups (Table 2.3.4). The control group had significantly lower cumulative iron intake compared to the lactoferrin, LSF and meat groups.

A significant effect (Table 2.3.5) over time (p = < 0.0001) was evident. Similarly, a significant interaction (Table 2.3.5) between diet and week (p = < 0.0001) was exhibited over time. Cumulative iron intake increased throughout the experiment (Figure 2.3.15). A rising trend was observed in the lactoferrin group followed by the LSF, meat and control groups. The results indicate that the lactoferrin group had surpassed the other dietary groups. The reliability of the statistical model used was very high for FEI (R^2 = 98.6%).

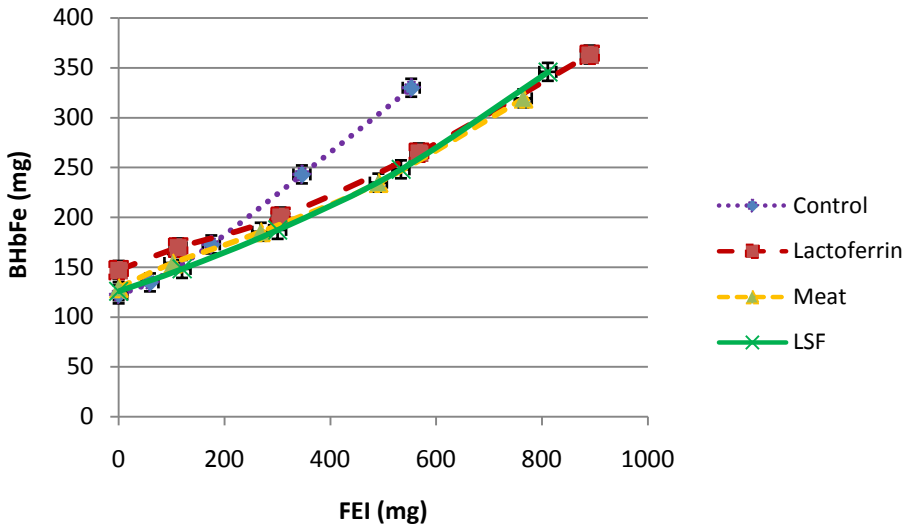


Figure 2.3.15 Least squares means (+/- SE) for total body haemoglobin iron content (mg) and cumulative iron intake (mg) of piglets given the control, lactoferrin, meat and LSF diets on weeks 0, 1, 2, 3, and 4 of the experiment

In support of this, a linear relationship was observed between cumulative iron intake (FEI) and total body haemoglobin iron content (BHbFe) during the experiment. As FEI increased, a corresponding increase in BHbFe was achieved (Figure 2.3.15).

2.3.3.4 Haemoglobin Iron Repletion Efficiency (HbRE)

It can be seen from Table 2.3.4 that the control group was the most efficient in terms of haemoglobin iron repletion ($p = 0.0008$) among the dietary treatments. This was followed by the LSF, meat and lactoferrin groups but the least squares means of these 3 groups were not significantly different ($p = > 0.05$).

2.3.4 HISTOLOGICAL PARAMETERS

The significance levels for the histological parameters are presented in Table 2.3.8.

Table 2.3.6 Significance levels after 4 weeks of the experiment for the height of the villi (µm), depth of the crypt (µm), mucosal thickness (µm) and goblet cells/100 µm in the small intestine of piglets given the control, lactoferrin, meat and LSF diets

Parameters	Diet	Pig (Diet)	Location	Diet*Location	R^2
Height	ns	*	**	***	0.31
Depth	ns	***	ns	ns	0.41
Mucosal Thickness	ns	**	***	***	0.37
Goblets/100µm	**	**	**	**	0.39

ns: P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001

No diet effects (Table 2.3.6) were observed in terms of the height of the villi (p = 0.08), depth of the crypts (p = 0.65), and mucosal thickness (p = 0.23) while a significant diet effect was displayed in the number of goblet cells/100 µm (p = 0.003).

Significant effects for piglets within diets (Table 2.3.6) existed for the height of the villi (p = 0.01), crypt depth (p = < 0.0001), mucosal thickness (p = 0.004) and number of goblet cells/100 µm (p = 0.004).

In relation to location in the small intestine (Table 2.3.6), significant effects were demonstrated in the height of the villi (p = 0.004), mucosal thickness (p = < 0.0001)

and the number of goblet cells/100 μm ($p = 0.002$). Non-significant effect ($p = 0.07$) was seen in crypt depth.

Interactions between diets and locations (Table 2.3.6) were significantly exhibited in the height of the villi ($p = 0.0004$), mucosal thickness ($p = 0.0001$) and the number of goblet cells/100 μm ($p = 0.004$). Non-significant effect was observed in crypt depth ($p = 0.06$).

Table 2.3.7 Least squares means 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 given the control, lactoferrin, meat and LSF diets

Parameters	Control	Lactoferrin	Meat	LSF	SE
Duodenum					
Villi height (μm)	419.[a]	449[a]	379[b]	322[c]	22.6
Crypt depth (μm)	130[a]	153[a]	102[b]	76[b]	11.5
Mucosal thickness (μm)	682[a]	719[a]	588[b]	515[c]	24.5
Goblet cells/100 μm	0.016[b]	0.013[b]	0.023[a]	0.019[a]	0.001
Jejunum					
Villi height (μm)	396[ab]	436[a]	410[ab]	373[b]	18.3
Crypt depth (μm)	128[a]	116[a]	104[a]	117[a]	10.1
Mucosal thickness (μm)	620[a]	624[a]	610[a]	590[a]	25.9
Goblet cells/100 μm	0.016[b]	0.020[a]	0.022[a]	0.022[a]	0.002
Ileum					
Villi height (μm)	293[c]	365[b]	399[a]	383[a]	17.4
Crypt depth (μm)	85[b]	128[a]	110[ab]	83[b]	14.4
Mucosal thickness (μm)	484[b]	553[a]	567[a]	560[a]	23.2
Goblet cells/100 μm	0.018[c]	0.019[c]	0.022[b]	0.027[a]	0.001

[a,b,c] Means within the same row with common superscripts or with no superscripts do not differ significantly (i.e. $P > 0.05$)

2.3.4.1 Duodenum

A diet effect was evident in the height of the villi ($p = 0.002$), depth of the crypt ($p = 0.0001$), mucosal thickness ($p = < 0.0001$) and number of goblet cells/ 100 μm ($p = < 0.0001$). The lactoferrin and control groups had similar increases in the height of the villi (Table 2.3.7). This was followed by the meat group and the least effect was achieved by the LSF group. For the depth of the crypts (Table 2.3.7), lactoferrin and control groups had significantly higher effects compared to both the meat and LSF groups. On the other hand, the lactoferrin and control groups had comparable effects pertaining to mucosal thickness (Table 2.3.7) which were higher than the effects exhibited by the meat and LSF groups. Aside from these, the goblet cells/100 μm of the piglets (Table 2.3.7) were significantly greater for the meat and LSF groups. This was followed by both the control and lactoferrin groups.

2.3.4.2 Jejunum

No diet effects were noticed in the height of the villi ($p = 0.08$), depth of the crypt ($p = 0.50$) and mucosal thickness ($p = 0.76$). However, the number of goblet cells/100 μm were significantly affected by diet ($p = 0.0005$). Least squares means (Table 2.3.7) for the height of the villi showed that lactoferrin, meat and control groups were not significantly different in their effects. Similarly, the meat, control and LSF groups had comparable effects. For the crypt depth and mucosal thickness, no significant differences existed in the least squares means among the 4 diets (Table 2.3.7). Additionally, similar effects were exhibited by the LSF, meat and lactoferrin

groups in relation to the increase in the number of goblet cells/100 μm (Table 2.3.7). The control group showed the lowest number.

2.3.4.3 Ileum

A diet effect for the height of the villi ($p = 0.001$) was displayed in the ileum. Contrary to this, non-significant effects for crypt depth and mucosal thickness were demonstrated ($p = 0.10$ and $p = 0.09$, respectively). Nevertheless, a highly significant diet effect was observed in the number of goblet cells/100 μm ($p = 0.0002$). Both meat and LSF groups (Table 2.3.7) had significantly higher villi followed by the lactoferrin group. Least squares means for crypt depth (Table 2.3.7) indicated that lactoferrin and meat groups exhibited greater depths compared to the control and LSF groups. For the mucosal thickness, least squares means of the meat, LSF and lactoferrin groups (Table 2.3.7) were significantly higher than the control group. Finally, the LSF group had significantly increased the number of goblet cells/100 μm in the ileum (Table 2.3.7). This was followed by the meat group while the lactoferrin and control groups had the least number of cells.

2.3.5 IMMUNOLOGICAL PARAMETERS

The absolute means, mean differences and P values of paired t-test for the immunological parameters are presented in Table 2.3.10.

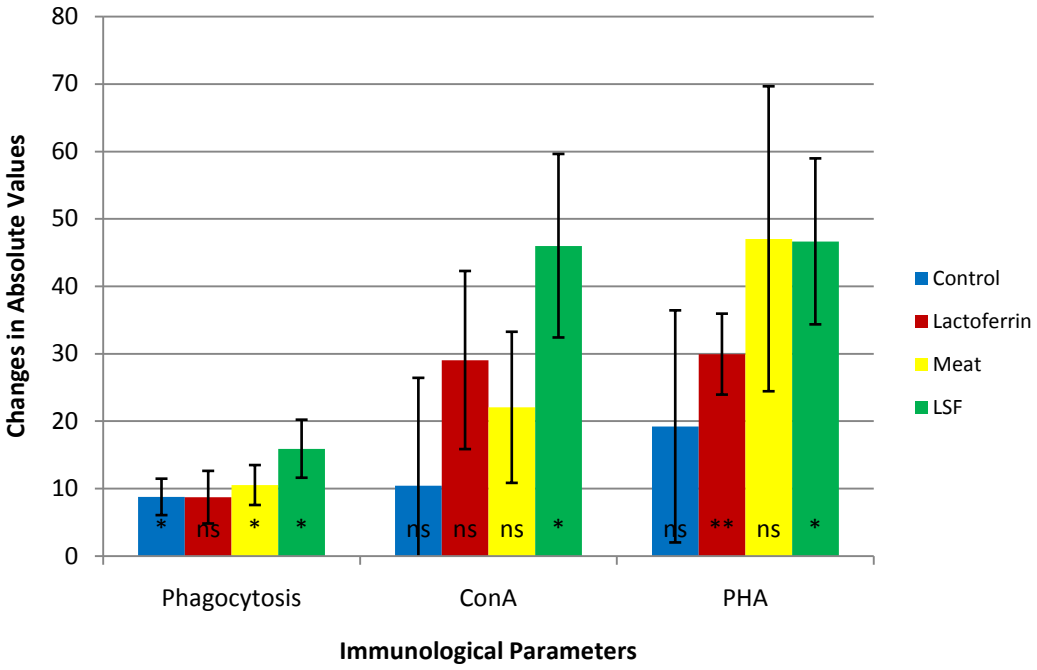
Table 2.3.8 The absolute means, mean differences and P values of paired t-test from Day 0 to Day 28 for changes in phagocytosis and cell proliferation (Concanavalin A and Phytohaemagglutinin) of piglets given the control, lactoferrin, meat and LSF diets

Parameters and Diets	Day O Mean	Day 28 Mean	Mean Differences	P Values
Phagocytosis				
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
LSF	30.48	46.39	15.91	0.01
Concanavalin A				
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
LSF	26.83	72.83	46	0.02
Phytohaemagglutinin				
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
LSF	17.06	63.7	46.64	0.01

2.3.5.1 Change in Phagocytosis

A diet effect (Table 2.3.8) occurred in the control ($p = 0.02$), meat ($p = 0.02$) and LSF ($p = 0.01$) groups. However, non-significant effect existed in the lactoferrin (p

= 0.08) group. In particular, the LSF group displayed the highest change in activity for phagocytosis (Figure 2.3.16).



ns: P > 0.05; * P < 0.05; ** P < 0.01

Figure 2.3.16 Changes in absolute values (+/- SE) and levels of significance for phagocytosis, concanavalin A (Con A) and phytohaemagglutinin (PHA) from Day 0 to Day 28 of piglets given the control, lactoferrin, meat and LSF diets during the experiment

2.3.5.2 Change in Cell Proliferation

A significant diet effect ($p = 0.02$) was only observed in LSF group (Table 2.3.8) in response to concanavalin A. No significant effects were demonstrated by the control ($p = 0.54$), lactoferrin ($p = 0.08$) and meat ($p = 0.11$) groups. The LSF ($p = 0.02$) group (Figure 2.3.16) responded significantly to concanavalin A. On the other hand, in the presence of phytohaemagglutinin, significant diet effects (Table 2.3.8) were achieved by lactoferrin ($p = 0.004$) and LSF ($p = 0.01$) groups. In contrast, non-significant diet effects were exhibited by the control ($p = 0.31$) and meat ($p = 0.09$) groups.

2.3.6 MINERAL BALANCES

The least squares means and significance levels for daily mineral balance over the last 5 days of the experiment are shown in Table 2.3.9 and Table 2.3.10.

Table 2.3.9 Least squares means and significance levels for daily mineral intake (mg) and daily mineral retention (mg) of piglets given the control, lactoferrin, meat and LSF diets on days 24 to 28 of the experiment

Parameters	Control	Lacto-ferrin	Meat	LSF	SE	P
Intake (mg/day)						
Calcium	4234^{d}	4468^{c}	7551^{a}	4751^{b}	33.3	***
Magnesium	70^{d}	83^{c}	162^{a}	108^{b}	0.55	***
Phosphorous	3327^{d}	3511^{c}	3776^{b}	4319^{a}	26.2	***
Copper	2.5^{d}	2.6^{c}	3.0^{b}	3.7^{a}	0.02	***
Iron	30^{c}	46^{a}	39^{b}	40^{b}	0.29	***
Manganese	6.7^{d}	8.0^{c}	8.7^{b}	24.2^{a}	0.05	***
Selenium	0.33^{d}	0.38^{c}	0.42^{a}	0.39^{b}	0.003	***
Zinc	39^{d}	41^{c}	57^{a}	52^{b}	0.31	***
Retention (mg/day)						
Calcium	3522^{c}	3951^{b}	5951^{a}	4466^{b}	193	***
Magnesium	57^{d}	74^{c}	133^{a}	96^{b}	2.6	***
Phosphorous	2403^{bc}	2847^{b}	2381^{c}	3705^{a}	155	***
Copper	0.897^{b}	0.762^{b}	0.526^{b}	1.74^{a}	0.27	*
Iron	7.3^{b}	29.9^{a}	15.5^{ab}	19.0^{ab}	6.06	ns
Manganese	1.8^{c}	3.4^{b}	3.4^{b}	19.5^{a}	0.38	***
Selenium	0.133^{b}	0.194^{a}	0.230^{a}	0.209^{a}	0.02	**
Zinc	1.7	4.3	1.1	11.6	4.58	ns

^{a,b,c,d} Means within the same row with common superscripts 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

2.3.6.1 Daily Mineral Intake

Highly significant diet effects (p = < 0.0001) were demonstrated in all the daily intakes of calcium, magnesium, phosphorous, copper, iron, manganese, selenium and zinc (Table 2.3.9).

For calcium, magnesium, selenium and zinc, the meat group had the highest intake per day followed by LSF group. The LSF group ranked first in daily phosphorous, copper and manganese intake followed by the meat group while lactoferrin group had the highest daily intake for iron followed by meat and LSF groups.

2.3.6.2 Daily Mineral Retention

Significant diet effects (Table 2.3.9) existed in daily mineral retention for calcium (p = < 0.0001), magnesium (p = < 0.0001), phosphorous (p = < 0.0001), copper (p = 0.03), manganese (p = < 0.0001) and selenium (p = 0.007). However, no diet effects were observed for iron (p = 0.10) and zinc (p= 0.37).

The highest in daily calcium and magnesium retention was exhibited by the meat group. For daily selenium retention, although the meat group ranked first, it was not significantly different from the LSF and lactoferrin groups. The LSF group was the highest in daily phosphorous, copper and manganese retention. For daily iron retention, lactoferrin group ranked first.

Table 2.3.10 Least squares means and significance levels for daily mineral faecal excretion (mg) and daily mineral excretion in the urine (mg) of piglets given the control, lactoferrin, meat and LSF diets on days 24 to 28 of the experiment

Parameters	Control	Lactoferrin	Meat	LSF	SE	P
Faecal Excretion (mg/day)						
Calcium	700[b]	508[b]	1585[a]	277[b]	188	***
Magnesium	9.6[b]	7.6[b]	25.2[a]	10.4[b]	2.1	***
Phosphorous	430[b]	314[b]	1043[a]	164[b]	126	***
Copper	1.5	1.7	2.3	1.7	0.27	ns
Iron	2.2	14.5	22.8	18.9	5.4	ns
Manganese	4.9	4.5	5.3	4.7	0.37	ns
Selenium	0.041[b]	0.039[b]	0.065[a]	0.033[b]	0.004	***
Zinc	30.9[b]	29.2[b]	46.6[a]	27.8[b]	3.9	**
Urine Excretion (mg/day)						
Calcium	12.5	8.7	14.7	7.3	3.9	ns
Magnesium	2.9	1.8	4.5	1.7	0.98	ns
Phosphorous	494	350	352	450	74.7	ns
Copper	0.156	0.186	0.178	0.243	0.04	ns
Iron	1.1	2.0	1.1	2.0	0.86	ns
Manganese	0.057	0.046	0.055	0.059	0.01	ns
Selenium	0.158	0.150	0.120	0.150	0.02	ns
Zinc	6.7[b]	7.9[ab]	8.9[ab]	12.4[a]	1.7	ns

[a,b] Means within the same row with common superscripts 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

2.3.6.3 Daily Mineral Faecal Excretion

Daily faecal excretion for calcium (p = 0.0005), magnesium (p = < 0.0001), phosphorous (p = 0.0005), selenium (p = 0.0003) and zinc (p = 0.01) were

significantly affected by the 4 diets (Table 2.3.10). In contrast, no diet effects were observed for copper ($p = 0.21$), iron ($p = 0.73$) and manganese ($p = 0.51$).

The meat group had the highest daily faecal excretion for calcium, magnesium, phosphorous, selenium and zinc while the control, lactoferrin and LSF groups had similar daily faecal excretion levels (Table 2.3.10).

2.3.6.4 Daily Mineral Excretion in the Urine

Daily calcium ($p = 0.53$), magnesium ($p = 0.21$), phosphorous ($p = 0.44$), copper ($p = 0.56$), iron ($p = 0.77$), manganese ($p = 0.83$), selenium ($p = 0.46$) and zinc ($p = 0.13$) excretion in the urine were not significantly affected by the 4 diets (Table 2.3.10).

Least squares means and significance levels for percentage mineral retention, percentage mineral faecal excretion and percentage mineral excretion in the urine on the last 5 days of the experiment are presented in Table 2.3.11.

Table 2.3.11 Least squares means and significance levels for percentage mineral retention, percentage mineral faecal excretion and percentage mineral excretion in the urine of piglets given the control, lactoferrin, meat and LSF diets on days 24 to 28 of the experiment

Parameters	Con-trol	Lacto-ferrin	Meat	LSF	SE	P
Retention (%)						
Calcium	83^{ab}	88^{a}	79^{b}	94^{a}	3.4	*
Magnesium	82^{b}	89^{a}	82^{b}	89^{a}	1.9	*
Phosphorous	72^{ab}	81^{a}	63^{b}	86^{a}	4.2	**
Copper	35^{a}	29^{a}	18^{ab}	47^{a}	9.0	ns
Iron	25	64	39	48	17.7	ns
Manganese	26^{bc}	43^{b}	39^{b}	80^{a}	4.4	***
Selenium	40^{ab}	51^{a}	55^{a}	53^{a}	4.7	ns
Zinc	4.0	10	1.9	22	8.9	ns
Faecal Excretion (%)						
Calcium	17^{a}	11^{ab}	21^{a}	5.8^{b}	3.4	*
Magnesium	14^{ab}	9.2^{b}	16^{a}	9.6^{b}	1.7	*
Phosphorous	13^{b}	8.9^{b}	28^{a}	3.8^{b}	3.5	***
Copper	59^{a}	64^{a}	76^{a}	46^{b}	9.0	ns
Iron	72	31	58	47	15.0	ns
Manganese	73^{a}	56^{ab}	61^{a}	19^{c}	4.4	***
Selenium	12^{a}	10^{ab}	16^{a}	8.6^{b}	1.2	**
Zinc	79^{a}	70^{ab}	82^{a}	54^{b}	7.8	ns
Urine Excretion (%)						
Calcium	0.292	0.195	0.195	0.154	0.07	ns
Magnesium	4.1	2.2	2.8	1.6	1.1	ns
Phosphorous	14.8	10.0	9.3	10.4	2.0	ns
Copper	6.1	7.1	5.9	6.6	1.3	ns
Iron	3.7	4.3	2.9	5.1	2.1	ns
Manganese	0.859^{a}	0.583^{a}	0.629^{a}	0.244^{b}	0.1	**
Selenium	48	39	29	38	4.7	ns
Zinc	17	19	16	24	3.4	ns

^{a,b,c} Means within the same row with common superscripts 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

2.3.6.5 Percentage Mineral Retention

Percentage retention (Table 2.3.11) for calcium ($p = 0.03$), magnesium ($p = 0.02$), phosphorous ($p = 0.006$) and manganese ($p = < 0.0001$) were significantly affected by the 4 diets while non-significant effects were observed for copper ($p = 0.17$), iron ($p = 0.43$), selenium ($p = 0.13$) and zinc ($p = 0.39$). Piglets fed the LSF diet obtained the highest percentage retention for calcium, magnesium, phosphorous and manganese but differences with other groups were not alway statistically significant.

2.3.6.6 Percentage Mineral Faecal Excretion

Significant diet effects (Table 2.3.11) were demonstrated in percentage faecal excretion for calcium ($p = 0.03$), magnesium ($p = 0.04$), phosphorous ($p = 0.0007$), manganese ($p = < 0.0001$) and selenium ($p = 0.002$). Non-significant diet effects were exhibited for copper ($p = 0.15$), iron ($p = 0.32$) and zinc ($p = 0.07$).

The meat group ranked first in percentage faecal excretion for calcium, magnesium, phosphorous and selenium while the control group was the highest for manganese but was not significantly different from the meat group.

2.3.6.7 Percentage Mineral Excretion in the Urine

Only manganese ($p = 0.008$) was significantly affected (Table 2.3.11) by the 4 diets in relation to percentage mineral excretion in the urine while calcium ($p = 0.60$), magnesium ($p = 0.46$), phosphorous ($p = 0.23$), copper ($p = 0.93$), iron ($p = 0.89$), selenium ($p = 0.08$) and zinc ($p = 0.36$) were not significantly affected. The control group displayed the highest percentage manganese excretion in the urine.

2.4 DISCUSSION

2.4.1 GROWTH PERFORMANCE

The non-significant dietary effects on liveweights at week 0 and week 4, average daily gain, feed intake and feed conversion ratio of piglets were in agreement with the results obtained by Widjaja (2003) in terms of liveweight at week 0, average daily gain and average daily feed intake when semi-anaemic piglets were fed with similar diets to those used in the current study. Although the feeding regimen used by Widjaja (2003) differed from the current study, the piglets were similarly administered with 60 mg of iron preparation at birth rendering them semi-anaemic at the beginning of the study. The non-significant differences between groups could be attributed to the well-balanced diets provided to all the groups of piglets. As the piglets during this stage of development are rapidly growing and have high metabolic rates, the nutrient composition and amounts of the four experimental diets were designed to satisfy the corresponding nutrient requirements for growth and maintenance.

2.4.2 HAEMATOLOGY

In general, no significant dietary effects existed for all the blood parameters. In addition, a similar pattern of effects were observed for diet-by-week interactions except for the mean corpuscular haemoglobin and the percentage of monocytes in white blood cells. However, over time, significant effects were displayed in almost all the blood parameters but not in the percentage of monocytes in white blood cells.

The non-significant pattern of dietary effects for haematocrit, haemoglobin, red blood cell counts, mean corpuscular volume, mean corpuscular haemoglobin and white blood cell counts were consistently observed by Widjaja (2003) when semi-anaemic piglets were fed with diets containing meat fractions. The same non-significant dietary effects were reported by Clayton (2002) for white blood cell counts and for the percentages of neutrophils, lymphocytes, monocytes and basophils within total white blood cells when piglets were fed with meat and non-meat diets.

Haematocrit clinically determines the relative proportion of cells to plasma (Reece, 2009). In relation to this, the haematocrit values obtained during the experiment were lower than the normal range (100-160 L/L). In terms of haemoglobin content, an indication of a borderline anaemia (Rincker et al., 2004) was displayed by the results. This finding could be caused by the semi-anaemic status of the piglets at the start of the experiment.

The control group was the highest for mean corpuscular volume and mean corpuscular haemoglobin content. Although a significant diet-by-week interaction was achieved by the control group for mean corpuscular haemoglobin in week 4, a

non-significant effect was exhibited by the least squares means. A decline in the mean corpuscular volume and mean corpuscular haemoglobin in week 2 indicated that erythrocytes were smaller (microcytic) leading to a lower haemoglobin (hypochromic) content. This could be due to depletion of hepatic iron reserves in the weaned piglets wherein the supply of iron being delivered to the bone marrow could be reduced, hence haemoglobin production tended to be impaired and the concentration of haemoglobin seemed to decline (Samman, 2007). Aside from these, a significant increase in mean corpuscular haemoglobin concentration would be unlikely since haemoglobin production does not occur in the red blood cells but in the bone marrow.

Most importantly, microcytosis, which occurs in small groups of red blood cells during the early stages of iron deficiency anaemia, was unlikely to affect the number of red blood cells (Widjaja, 2003). This statement was in agreement with the result achieved in the red blood cell count which was within the normal range (5-8 x 10^{12} /L).

On the other hand, red cell distribution width (RDW) refers to the coefficient of variation of the red cell volume distribution (Latimer et al., 2003). Over time, a fall in red cell distribution width was observed in both the LSF and meat groups. As red cell distribution width determines the degree of variation in the size of erythrocytes, the result indicated that the variation in the size of red blood cells was small and that the existing microcytosis was insignificant because microcytosis could be considered significant only if the red cell distribution width had increased (Latimer et al., 2003).

The declining number of platelets over time could be attributed to the life span of platelets which is 5 to 9 days in most species of animals (Latimer et al., 2003). Platelets function in blood coagulation by forming a haemostatic plug (Kahn, 2005). In contrast, increased white blood cell counts over time for the control, LSF and lactoferrin groups was indicative of the piglet's ability to defend against the invasion of bacteria, viruses, parasites and other foreign particles (Reece, 2009).

In relation to the drop in the percentage of neutrophils in white blood cells for the LSF, lactoferrin and meat groups from weeks 3 to 4, the finding could be due to the emigration of neutrophils from the blood into the tissues (Latimer et al., 2003). Similarly, a fall in the percentage of lymphocytes in white blood cells for the control group in week 4 as opposed to the meat, lactoferrin and LSF groups could be attributed to the unique kinetics of lymphocytes to recirculate from the blood to the tissues, to the lymph, then to the blood, again (Latimer et al., 2003). Nevertheless, a surge in lymphocytes could be suggestive of an immune response stimulation (Reece, 2009).

In particular, a significant diet-by-week interaction was observed in the percentage of monocytes in white blood cells. A peak in the percentage of monocytes in white blood cells shown by the LSF group on week 1 could be suggestive of an activated response to inflammation (Tizard, 2000). The changes which occurred over time seemingly displayed the movement of monocytes from the blood to the tissues where they are transformed into macrophages which are large phagocytic cells (Reece, 2009).

On the other hand, an increased percentage of eosinophils in white blood cells in week 1 for the control group might be indicative of an ongoing defence against parasitic invasion (Kahn, 2005). Finally, elevated basophil percentages in white blood cells for the LSF and meat groups in week 1 and week 3, respectively, could be due to the enhancement of systemic allergic reactions (Kahn, 2005).

2.4.3 MEASURES OF IRON STATUS

The non-significant diet effect on total body haemoglobin iron content (BHbFe) in week 0 and week 4 may be attributed to the non-significant differences that existed in the mean corpuscular volume and mean corpuscular haemoglobin. The cumulative iron intakes (FEI) for the lactoferrin, LSF and meat groups were higher than for the control group. In addition, since the FEI for the control group was lower compared to the FEI values for the lactoferrin, meat and LSF groups, this resulted in a higher haemoglobin iron repletion efficiency obtained for the control group (Table 2.3.4).

The haemoglobin iron repletion efficiency (HbRE) for the control group was significantly higher than the LSF, meat and lactoferrin groups. This result was not expected and is not consistent with the results of other studies where meat in the diet has enhanced the HbRE. For example, the findings of Widjaja (2003) and South et al. (2000) are compared with results of the current study in Table 2.4.1.

Table 2.4.1 Studies in semi-anaemic piglets fed with meat and meat fractions with emphasis on haemoglobin iron repletion efficiencies (HbRE) as a measure of the improvement in iron status over the duration of the studies

Studies	Diets	HbRE (%)	RVC*=100
Present Study	Control	36.93	100
	Meat	24.31	66
	LSF	27.15	74
Widjaja (2003)	Control	14.15	100
	Meat	25.31	179
South et al. (2000)	Non-Haem Iron (Control)	11.2	100
	Non-Haem Iron with Meat	21.8	195

* Relative value with control

Results in Table 2.4.1 show that HbRE values for meat diets were similar for all three studies but the control diet value was at least twice as high in the present study. Pertaining to HbRE of the meat diet in the current study, it was only lower by one percentage point in comparison with the HbRE of the meat diet as reported by Widjaja (2003). However, the HbRE of the meat diet shown in the present study was higher than the non-haem iron with meat diet (South et al., 2000).

In connection with this, as nutrient regulatory pathways in semi-anaemic piglets are not clearly elucidated at present, the possible mechanisms responsible for the lower haemoglobin iron repletion efficiencies attained by the LSF and meat diets relative to the control include the following:

1. Solubility and hygroscopy are essential physiochemical properties when incorporating an ingredient into a diet, especially when the diet will be diluted with water and is to be fed in a liquid form. In relation to this, since the solubility of the wet LSF diet in body fluid compartments was not determined, this biochemical nature of the LSF diet may be the reason for the lower haemoglobin iron repletion efficiency achieved by the LSF group relative to the control group. Even if the LSF diet contained the water-soluble sarcoplasmic fraction from the meat extract, its high inorganic content might have been the reason for its lesser solubility for absorption, thus making it less bioavailable. This possible explanation would not apply for the meat diet.

2. The erythropoietic regulator (Miret et al., 2003) might have failed to stimulate the production of red blood cells as a compensatory response to the existing microcytic and hypochromic anaemic conditions in the piglets. Additionally, as the total body iron status of the semi-anaemic piglets declined, iron mobilisation from bodily stores could be insufficient to meet such demand. Also, the rate of erythropoiesis could have contributed to the result obtained for haemoglobin iron repletion efficiency. The average life-span of red blood cells in pigs is approximately 72 days (Rincker et al., 2004). As the duration of the experiment lasted about 56 days, immature red blood cells might have been produced and circulated in the blood. In effect, the iron being delivered into the tissues might have been insufficient to provide the iron needed by the cells to synthesise haemoglobin. This explanation should have applied equally for all treatment groups.

3. The unexpected results regarding haemoglobin iron repletion efficiency for all treatment groups could also be possibly attributed to the undifferentiated enterocytes. Iron uptake regulation usually occurs in the apical and basolateral membranes of the enterocytes of the small intestine (Minihane & Rimbach, 2002). Since the enterocytes of the piglets were not fully differentiated, the activity of the transport proteins involved in iron uptake (divalent metal transporter 1) and iron export (ferroportin) might have been decreased, thus iron might have been lost through the exfoliation of the epithelial cells of the small intestine.

4. The significant increase in the number of goblet cells/100 μm which was observed in all the sections of the small intestine but mainly in the ileum of the LSF group may not be suggestive of an increase in iron absorption because dietary iron is primarily absorbed in the mature villi (differentiated) of the enterocytes in the duodenum and upper jejunum of the small intestine (Frazer & Anderson, 2005) and minimally in the stomach, ileum and colon (Steele et al., 2005). As the absorptive capacity of the enterocytes will last only for 2 days, followed by the shedding of the enterocytes from the villi tips into the intestinal lumen (Ganz & Nemeth, 2006), the transport of iron across the enterocyte toward the blood might have been affected. In effect, the availability of iron for uptake across the apical mucosa may not be sufficient to be captured by the divalent metal transporter 1. Hence, it could be unlikely that iron was made more bioavailable into the cytoplasm for export by ferroportin across the basolateral or serosal membrane into the blood to be transported by transferrin. As affirmed by Ganz and Nemeth (2006), the export of iron by ferroportin determines if iron is carried by transferrin into the circulation or

excreted through the sloughing of the enterocytes. It could have been that iron was not delivered by transferrin to be utilised by various organs. This justification would be applicable for all treatment groups but mainly for the LSF group.

5. Since in vitro digestion of meat and LSF diets were not performed, the existence of low molecular weight (LMW) iron binding peptides released during pepsin digestion of muscle tissue could not be elaborated. These peptides which were identified by Storcksdieck et al. (2007) in vitro, were isolated from the myosin filament of myofibrillar protein. However, the possible presence of these peptides in the meat diet or the LSF diet in the current study still remains to be known. Also, these peptides may seemed to be lacking in the LSF diet. Hence, it can not be clearly established if these peptides, which have the capacity to bind iron in the stomach to maintain iron solubility against pH gradients, were able to deliver iron into the mucosal surface of the duodenum for iron uptake. In connection with this, the probable absence of low molecular weight iron binding peptides in the digestion products of meat and LSF diets had possibly decreased the reduction of dietary ferric iron into ferrous iron, thus limiting the solubility of ferrous iron for absorption. This explanation would possibly be applied for the meat and LSF groups.

6. Because the hydrogen ion concentration (pH) of the meat and LSF diets were not determined, it could have been that the pH values for meat and LSF diets were not maintained in an acidic state which according to Mackenzie and Garrick (2005) has a facilitating effect for iron uptake. This reason would be applicable for both the meat and LSF groups.

7. It can also be assumed that even if the haem iron was present in the meat diet, the possibility for the lack of functional haem carrier protein 1 or the absence of a haem receptor could also be the reasons why haem iron from the meat diet was not delivered into the apical membrane of the small intestine. Additionally, during iron deficiency, the activity of haem oxygenase 1 is increased (Latunde-Dada et al., 2006). Nevertheless, if the activity of haem oxygenase 1 was not stimulated, then haem iron degradation might not be catalysed by this enzyme to release iron, thus iron may not be able to enter the iron pool. This justification would have applied mainly for the meat group.

2.4.4 INTESTINAL HISTOLOGY

In the duodenum, the height of the villi, depth of the crypts and mucosal thickness were higher for lactoferrin and control groups compared with the meat and LSF groups. On the other hand, in the ileum: the meat and LSF groups had greater height of the villi relative to lactoferrin and control groups; the lactoferrin and meat groups had greater depths in comparison with both lactoferrin and control groups; while the meat, LSF and lactoferrin groups had higher mucosal thickness than the control group. Despite these changes in the morphology of the small intestine, the non-significant interaction observed between diets and anatomical locations in crypt depths ($p = 0.07$) could be an indication of intestinal crypts dysfunction that could be the result of sudden changes in feeding and post-weaning stress (Pluske et al., 2007). Since cellular differentiation usually occurs in the crypts, such alteration in the crypts

function could likely be attributed to the formation of undifferentiated crypts which consequently led to undifferentiated enterocytes. Once undifferentiated, the absorptive and digestive capacity of the enterocytes become affected. In connection with this, undifferentiated enterocytes seem to be lacking a functional divalent metal transporter 1 (Minihane & Rimbach, 2002) and therefore probably have limited iron uptake.

However, the number of goblet cells/100 µm in the three sections of the small intestines were significantly ($p = 0.003$) increased for the LSF and meat groups particularly in the ileum. Goblet cells, which classified as mucosal cells, are 2 to 3 times more numerous in the ileum than in the duodenum (Eurell & Frappier, 2006) and are responsible for mucin production (Argenzio, 2004). Mahler et al. (2009) reported that iron solubility is maintained by mucin even in an alkaline environment thereby enhancing iron uptake by mobilferrin and divalent metal transporter 1. In connection with this, the binding of mucin to iron in an acidic pH maintains iron solubility, thus favuoring iron absorption in the alkaline pH of the duodenum (Conrad & Umbreit, 2006). On the other hand, Mahler et al. (2009) demonstrated that the in vitro digestion /Caco-2 cell culture model in conjunction with mucus layer and goblet-type cells may seem to provide a more accurate prediction for iron bioavailability. In relation to this, Jin et al. (2006) reported that the interaction of iron with mucin in Caco-2 cells significantly increased iron uptake.

2.4.5 IMMUNOLOGY

The change in immunological status of the semi-anaemic piglets was measured from day 0 to day 28. Significant increases for all the immunological parameters were achieved by the LSF group from day 0 to day 28.

The LSF group obtained the highest phagocytic capacity of the peripheral blood leukocytes as measured by flow cytometry (Table 2.3.8). This finding could be attributed to the increased cell counts achieved by the LSF group for white blood cells and for the higher percentages of neutrophils and monocytes in white blood cells. These cells are primarily involved in the attack and destruction of invading microorganisms in the body through phagocytosis (Tizard, 2000).

Aside from these, the whole-blood cells from piglets in the LSF group (Table 2.3.8) had a better ability to trigger the proliferation of lymphocytes in response to concanavalin A and phytohaemagglutinin than the control group. These compounds are T-cell mitogens that stimulate lymphocytes to divide (Tizard, 2000). The results have suggested that LSF in the diet of piglets can stimulate an enhanced immune response in the presence of pathogens.

2.4.6 MINERAL BALANCES

The highest levels of daily intake for calcium, magnesium and selenium were exhibited by the meat group followed by the LSF group, and these differences were paralleled by daily retention levels for calcium, magnesium and selenium for these

groups (Table 2.3.9). Similarly, the highest daily intakes for phosphorous, copper and manganese displayed by the LSF group resulted in the highest daily retentions for phosphorous, copper and manganese in that group. Aside from these, the greatest daily intake for zinc was demonstrated by the meat group followed by the LSF group. On the other hand, the lactoferrin group, which had the highest daily intake for iron, also retained the highest amount of daily iron.

These findings could be the reasons why the LSF and meat groups had lower haemoglobin repletion efficiency compared to the control group since increased consumption and retention of calcium, phosphorous, copper and zinc have been reported to reduce iron absorption (Patterson et al., 2008). Moreover, zinc, calcium and magnesium also tended to decrease iron bioavailability (Zhu et al., 2009). More importantly, calcium has been reported by Roughead et al. (2005) to inhibit iron absorption due to the reduction of the initial mucosal uptake of iron. From the justifications elaborated by these authors, the significant increases in the percentage retentions of calcium, magnesium, phosphorous, copper and zinc, that occurred in the LSF and meat groups (Table 2.3.11), may explain in part the non-significant effects shown for daily iron retention and percentage retention of iron during the experiment as a result of mineral antagonisms that are likely to inhibit iron absorption.

2.5 CONCLUSIONS

The inclusion of meat and LSF in the diets did not significantly improve the growth performance, organ weights and haematological indices of the semi-anaemic piglets. The findings obtained for haemoglobin iron repletion efficiency suggested that the low molecular weight sarcoplasmic fraction component of the LSF diet was insufficient to enhance iron bioavailability at the cellular level relative to the control group, but was equivalent to the meat diet in this respect.

No diet effects were similarly observed in the height of the villi, depth of the crypts and mucosal thickness. However, the number of goblet cells/100 μm was increased by the LSF and meat diets which suggested that the LSF and meat diets can stimulate mucin secretion.

Enhancement of leukocyte phagocytic activity and lymphocyte proliferative responses to concanavalin A and phytohaemagglutinin displayed by piglets receiving the LSF diet showed that LSF, as a feed ingredient, is a potential immunobooster particularly after the period of weaning when piglets are susceptible to infection.

Increased daily intakes, daily retentions and percentage daily retentions for calcium, magnesium, phosphorous and manganese in the LSF and meat groups apparently inhibited iron absorption thereby limiting iron bioavailability.

2.6 RECOMMENDATIONS FOR FUTURE STUDIES

The following actions are recommended for inclusion in future studies involving the testing of the effects of meat fractions on iron bioavailability for semi-anaemic piglets:

1. Baseline measures of hepatic iron stores (ferritin) should be established at the start of the experiment as this may be useful for comparison of the results.

2. A tracer study using radioiron or enzyme assays to determine whether the activity of divalent metal transporter 1 (iron importer), ferroportin (iron exporter) and transferrin (iron carrier) are up-regulated when iron stores become exhausted and to determine the metabolic pathways the iron traverses in relation to iron absorption and elimination.

3. The solubility of any fraction being tested in fluid and membrane compartments must be taken into consideration to determine its capacity to pass through membrane barriers and its ability to bind with membrane transporters or protein carriers in the blood.

4. If possible, when further investigations are conducted using semi-anaemic piglets to measure iron bioavailability, both in vivo and in vitro studies should be undertaken to determine how any meat fraction will react with digestive enzymes or gastric juices to assess if this reaction could lead to digestion products which contain the “meat factor” effect.

REFERENCES

Alexandropoulou, I., Komaitis, M. & Kapsokefalou, M. (2006). Effects of iron, ascorbate, meat and casein on the antioxidant capacity of green tea under conditions of in vitro digestion. *Food Chemistry, 94 (3),* 359-365.

Anderson, G. (2007). Mechanisms of iron loading and toxicity. *American Journal of Hematology, 82,* 1128-1131.

Andrews, N. (2002). Metal transporters and disease. *Current Opinion in Chemical Biology, 6* (2), 181-186.

Andrews, N. & Schmidt, P. (2007). Iron homeostasis. *Annual Review of Physiology, 69,* 69-85.

Argenzio, R. A. (2004). Digestive and absorptive functions of the intestines. In W. O. Reece (Ed.), *Duke's physiology of domestic animals*, 12th Edn. (pp 419-420). USA; London:Cornell University Press.

Armah, C., Sharp, P., Mellon, F., Pariagh, S., Lund, E. Dainty, J. & Fairweather-Tait, S. J. (2008). L-α-glycero-phosphocholine contributes to meat's enhancement of non-haem iron absorption. *Journal of Nutrition, 138,* 873-877.

Aspinal, V. & O'Reilly, M. (2003). The blood vascular system in anatomy and physiology. In D. R. Lane & B. C. Cooper (Eds.), *Veterinary nursing* 3rd Edn. (pp. 42-45). USA: Butterworth Heinemann.

Baech, S., Hansen, M. Bukhave, K., Jensen, M., Sorensen, S., Kristensen, L. et al., (2003). Non-haem iron absorption from a phytate-rich meal is increased by the addition of small amounts of pork meat. *American Journal of Clinical Nutrition, 77 (1),* 173-179.

Bentor, Y. Chemical Element.com-Iron. Retrieved March 25, 2008 from http:// www.chemicalelements.com/elements/fe/html.

Bionet (2001). Haemoglobin structure and function. Retrieved April 14, 2008 from http://cal.man.ac.uk/student_projects/2001mnqc7nds/haemoglobin.

Blachier, F., Vaugelade, P., Robert, V., Kibangou, B., Canonne-Hergaux, F., Delpal, S., Bureau, F. et. al., (2007). Comparative capacities of the pig colon and duodenum for luminal iron absorption. *Canadian Journal of Physiology and Pharmacology, 85,* 185-192.

Carlson, G. P., (2009). Disorders of the organ systems. In B. P. Smith (Ed.), *Large animal internal medicine* 4th Edn. (p. 1170). U.S.A. : Mosby Elsevier.

Clayton, P. J. (2002). Iron bioavailability for piglets: The effects of iron status, vitamin C and cooked or uncooked meat. *Master of Science (Nutritional Science) Thesis*. Palmerston North, New Zealand : Massey University Library.

Conrad, M. E. & Umbreit, J. N. (2006). A concise review: Iron absorption – The mucin-mobilferrin-integrin pathway. A competitive pathway for metal absorption. *American Journal of Hematology, 42 (1),* 67 (Abstract).

Cunningham, M., Latour, M. & Acker, D. (2005). *Animal science and industry* 7th Edn. (pp. 15-16). New Jersey, U.S.A. : Pearson Prentice Hall.

Dunn, L., Rahmanto, Y. & Richardson, D. (2007). Iron uptake and metabolism in the new millennium. *Trends in Cell Biology*, *17* (2), 93-100.

Eisenstein, R. (2000). Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annual Review of Nutrition, 20*, 627-662.

Encyclopedia Britanica (1994). Transverse tubule: ultrastructure of myofibrils. Retrieved April 16, 2008 from http://www.britanica.com.

Engelking, L. (2004). *Textbook of veterinary physiological chemistry* (pp. 270-273). Jackson, WY: Teton New Media.

Engelmann, M., Davidsson, L., Sandstrom, B., Walczyk, T., & Michaelsen, K. (1998). The influence of meat on non-haem iron absorption in infants. *Pediatric Research, 43(6),*768-773.

Engle-Stone, R., Yeung, A., Welch, R. & Glahn, R. (2005). Meat and ascorbic acid can promote iron availability from iron-phytate but not from iron-tannic acid complexes. *Journal of Agricultural and Food Chemistry, 53,* 10276-10284.

Erikson, K., Beard, J. & Connor, J. (1998). Distribution of brain iron, ferritin, and transferrin in in the 28-day-old piglet. *Journal of Nutritional Biochemistry, 9,* 276-284.

Eurell, JA & Frappier, B. (2006). *Dellmann's Textbook of Veterinary Histology,* 6th Edn. (pp 196-197). Ames, Iowa, USA: Blackwell Publishing Professional.

Frazer, D. & Anderson, G. (2005). Iron imports I. Intestinal iron absorption and its regulation. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 289,* G631-G635.

Fleming, R. & Britton, R. (2006). Iron imports. IV. HFE and regulation of intestinal iron absorption. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *290*, G590-G594.

Ganz, T. (2007). Molecular control of iron transport. *Journal of American Society of Nephrology*, *18*, 394-400.

Ganz, T. & Nemeth, E. (2006). Iron imports. IV. Hepcidin and regulation of body iron metabolism. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *290*, G199-G203.

Garcia, M., Martinez-Torres, C., Leets, I., Tropper, E., Ramirez, J., & Layrisse, M. (1996). Heat treatment on haem iron and iron-containing proteins in meat: Iron absorption in humans from diets containing cooked meat fractions. *Journal of Nutritional Biochemistry 7*, 49-54.

Glahn, R., Wortley, G., South, P. & Miller, D. (2002). Inhibition of iron uptake by phytic acid, tannic acid, and zinc chloride: Studies using an in vitro digestion/ caco cell 2 model. *Journal of Agricultural and Food Chemistry, 50*, 390-395.

Harada, E., Itoh, Y., Sitizyo, K., Takeuchi, T., Araki, Y. & Kitagawa, H.(1999). Characteristic transport of lactoferrin from the intestinal lumen into the bile via the blood in piglets. *Comparative Biochemistry and Physiology*, *124*, 321-327.

Harrison, K. (2005). Haemoglobin. Retrieved April 14, 2008 from http://en.wikipedia.org/wiki/Haemoglobin.

Helmenstine, A. (2001). Cysteine chemical structure. Retrieved July 29, 2008 from http://chemistry.about.com.

Himmelfarb, J. (2007). Iron regulation. *Journal of American Society of Nephrology, 18*, 379-381.

http://metallo.scripps.edu (1997). Myoglobin structure. Retrieved April 28, 2008.

Hurrell, R., Reddy, M., Juillerat, M. & Cook, J. (2006). Meat protein fractions enhance non-haem iron absorption in humans. *Journal of Nutrition, 136*, 2808-2812.

Jin, F., McAuliffe, G., Rutzke, M., Shuler, M., Welch, R. & Glahn, R. P. (2006). Application of mucin and Caco-2/goblet cell co-cultures to refine the in vitro digestion/Caco-2 cell model for iron uptake. *The FASEB Journal, 20,* A624 (Abstract).

Kahn, C. M. (2005). The haematopoietic system. *The Merck veterinary manual* 9th Edn. (pp. 4-8). U.S.A : Merck & Co., Inc.

Kapsokefalou, M. & Miller, D. (1993). Lean beef and beef fat interact to enhance non-haem iron absorption in rats. *Journal of Nutrition, 123,* 1429-1434.

Kim, Y. & Carpenter, C. (1993). Gastric acid production, iron status and dietary phytate alter enhancement absorption in rats. *Journal of Nutrition, 123,* 940-946.

King, M. & Marchesini, S. (2007). Muscle biochemistry. Retrieved April 28, 2008 from http://www.med.

Latimer, K. S., Mahaffey, E. A. & Prasse, K. W. (2003). *Duncan & Prasse's veterinary laboratory medicine clinical pathology* 4th Edn. (pp. 12-27). Iowa, U.S.A. : Blackwell Publishing Company.

Latunde-Dada, G., Simpson, R. & McKie, A. (2006). Recent advances in mammalian haem transport. *Trends in Biochemical Sciences, 31* (3), 182-188.

Liao, Y., Lopez, V., Shafizadeh, T., Halsted, C. & Lonnerdal, B. (2007). Cloning of a pig homologue of the human lactoferrin receptor: Expression and localisation during intestinal maturation in piglets. *Comparative Biochemistry and Physiology, 148,* 584-590.

Lombardi-Boccia, G., Martinez-Dominguez, B. & Aguzzi, A. (2002a). Total haem and non-haem iron in raw and cooked meats. *Journal of Food Science, 67,* 1738-1741.

Lombardi-Boccia, G., Martinez-Dominguez, B., Aguzzi, A. & Rincon-Leon (2002b). Optimisation of haem iron analysis in raw and cooked red meat. *Food Chemistry, 78,* 505-510.

Lopez, M. & Martos, F. (2004). Iron availability: An updated review. *International Journal of Food Sciences and Nutrition, 55(8),* 597-606.

Lynch, S. (2000). The effect of calcium on iron absorption. *Nutrition Research Reviews, 13 (2),* 141-158.

Mackenzie, B. & Garrick, M. (2005). Iron imports. II. Iron uptake at the apical membrane in the intestine. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 289,* G981-G986.

Mahler, G. J., Shuler, M. L. & Glahn, R. P. (2009). Characterisation of Caco-2 and HT29-MTX cocultures in an in vitro digestion/cell culture model used to predict iron bioavailability. *Journal of Nutritional Biochemistry, 20,* 494-502.

Martinez-Navarrete, N., Camacho, M., Martinez-Lahuerta, J., Martinez-Monzo, J. & Fito, P. (2002). Iron deficiency and iron fortified foods-a review. *Food Research International, 35,* 225-231.

Miller, E. & Ullrey, E. (1987). The pig as a model for human nutrition. *Annual Reviews of Nutrition, 7*, 361-382.

Minihane, A.M., & Rimbach, G. (2002). Iron absorption and the iron binding and anti-oxidant properties of phytic acid. *International Journal of Food Science and Technology, 37*, 741-748.

Miret, S., Simpson, R. & McKie, A. (2003). Physiology and molecular biology of dietary iron absorption. *Annual Reviews of Nutrition, 23*, 283-301.

Mulvihill, B., Kirwan, F., Morrissey, P. & Flynn A. (1998). Effect of myofibrillar muscle proteins on the in vitro bioavailability of non-haem iron. *International Journal of Food Science and Nutrition, 49*, 187-192.

Mulvihill, B. & Morrissey, P. (1998). Influence of the sulphydryl content of animal proteins on in vitro bioavailability of non-haem iron. *Food Chemistry, 61 (1-2)*, 1-7.

Papich, M. G. (2007). *Saunders handbook of veterinary drugs* 2nd Ed. (pp. 335, 305-306, 512-513). Missouri, U.S.A. : Saunders Elsevier.

Patterson, J., Lei, X. & Miller, D. (2008). The pig as an experimental model for elucidating the mechanisms governing dietary influence on mineral absorption. *Experimental Biology and Medicine, 233*, 651-664.

Pietrangelo, A. (2006). Hereditary hemochromatosis. *Annual Reviews of Nutrition, 26*, 251-270.

Pluske, J. R., Hansen, C. F., Payne, H. G., Mullan, B. P., Kim, J. C. & Hampson, D. J. (2007). Gut health in the pig. In J. E. Paterson & J. A. Barker (Eds.), *Manipulating pig production XI* (pp. 147-151). Australia : Australasian Pig Science Association (Inc.).

Purchas, R. W., Busboom, J. R. & Wilkinson, B. H. P. (2006). Changes in the forms of iron and in concentrations of taurine, carnosine, coenzyme Q_{10}, and creatine in beef longissimus muscle with cooking and simulated stomach and duodenal digestion. *Meat Science, 74*, 443-449.

Purchas, R. W., Rutherfurd, S. M., Pearce, P. D. & Wilkinson, B. H. P. (2004). Cooking temperature effects on the forms of iron and levels of several other compounds in beef semitendinosus muscle. *Meat Science, 68*, 201-207.

Radostits, O., Gay, C., Hinchcliff, K. & Constable, P. (2007). *Veterinary medicine* (pp. 450-455, 1725-1726). USA: Elsevier.

Reddy, M., Hurrell, R. & Cook, J. (2006). Meat consumption in a varied diet marginally influences non-haem iron absorption in normal individuals. *Journal of Nutrition,136*, 576-581.

Reece, W. O. (2009). *Functional anatomy and physiology of domestic animals* 4th Edn. (pp. 46-64). Iowa, U. S. A. : Wiley-Blackwell Publishing Company.

Reece, W. O. (2005). *Functional anatomy and physiology of domestic animals*, 3rd Edn. (pp. 54-56, 176-177, 312-328). USA: Lippincott Williams and Wilkins.

Reece, W. & Swenson, M. (2004). The composition and functions of blood. In W. O. Reece (Eds.), *Duke's physiology of domestic animals*, 12th Edn. (pp. 35-37). USA; London: Cornell University Press.

Reese, D. (1999). Nutrient deficiencies and excesses. In B. E. Straw, S. D"Allaire, W. L. Mengeling & D. J. Taylor (Eds.), *Diseases of swine*, 8th Edn. (p. 743). N. Y., USA: Iowa State Press.

Rincker, M. J., Hill, G. M., Link, J. E. & Rowntree, J. E. (2004). Effects of dietary iron supplementation on growth performance, haematological status and whole-body mineral concentrations of nursery pigs. *Journal of Animal Science, 82*, 3189-3197.

Roughead, Z., Zito, C. & Hunt, J. (2005). Inhibitory effects of dietary calcium on the initial uptake and subsequent retention of haem and non-haem iron in humans: comparisons using an intestinal lavage method. *American Journal of Clinical Nutrition, 82*, 589-597.

Rutherford, S., Darragh, A., Hendriks, W., Prosser, C. & Lowry, D. (2006). Mineral retention in three-week-old piglets fed goat and cow milk infant formulas. *Journal of Dairy Science,89*, 4520-4526.

Rutherfurd-Markwick, K. J., Johnson, D., Cross, M. L. & Gill, H. S. (2005). Modified milk powder supplemented with immunostimulating whey protein concentrate (IMMUCARE) enhances immunofunction in mice. *Nutrition Research, 25*, 192-203.

Samman, S. (2007). Iron. *Nutrition and Dietetics, 64 (Suppl. 4)*, S 126-S130.

Searcy, G. (2001). Anaemia. In M. D. McGavin, W. W. Carlton & J. F. Zachary (Eds.), *Thomson's special veterinary pathology*, 3rd Edn ((pp. 342-343). USA: Mosby, Inc.

Sorensen, A., Sorensen, H., Bjergegaard, C., Andersen, K., Sondergaard, I., Sorensen, S. et al., (2007). Matrix effects of lupine (*Lupinus luteus* L.) and rapeseed (*Brassica napus* L.) products on in vitro non-haem iron availability from pork meat. *Journal of Food Composition and Analysis, 20 (6)*, 515-522.

Sorensen, A., Sorensen, H., Sondergaard, I. & Bukhave, K. (2006). Non-haem iron availability from pork meat: Impact of heat treatments and meat protein dose. *Meat Science, 76 (1)*, 29-37.

South, P., Lei, X. & Miller, D. (2000). Meat enhances non-haem iron absorption in pigs. *Nutrition Research, 20 (12)*, 1749-1759.

Steele, T., Frazer, D. & Anderson, G. (2005). Systemic regulation of intestinal iron absorption. *IUBMB Life, 57* (7), 499-503.

Stockham, S. & Scott, M. (2002). *Fundamentals of veterinary clinical pathology* (pp. 105-110). USA: Iowa State Press.

Storcksdieck, S., Bonsmann, G. & Hurrell, R. (2007). Iron-binding properties, amino acid composition and structure of muscle tissue peptides from in vitro digestion of different meat sources. *Journal of Food Science, 72*, S19-S29.

Swain, J., Tabatabai, L. & Reddy, M. (2002). Histidine content of low molecular weight beef proteins influences non-haem iron bioavailability in caco-2 cells. *Journal of Nutrition, 132*, 245-251.

Tartaglia, L. & Waugh, A. (2005). *Veterinary physiology and applied anatomy* (pp. 94-95). USA; London: Elsevier Butterworth Heinemann.

Tetens, I., Bendtsen, K., Henriksen, M., Ersboll, A. & Milman, N. (2007). The impact of a meat – versus a vegetable-based diet on iron status in women of childbearing age with small iron stores. *European Journal of Nutrition, 46*, 439-445.

Teucher, B., Olivares, M. & Cori, H. (2004). Enhancers of iron absorption: ascorbic acid and other organic acids. *International Journal of Vitamin Nutrition Research, 74 (6)*,403-419.

Thrall, M. (2004). *Veterinary hematology and clinical chemistry* (p. 96). USA: Lippincott Williams and Wilkins.

Tizard, I. A. (2000). *Veterinary immunology* 6th Edn. (pp.2, 20, 96). U.S.A : W. B. Saunders Company.

Wessling-Resnick, M. (2006). Iron imports. III. Transfer of iron from mucosa into circulation. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 290*, G1-G6.

Wessling-Resnick, M. (2000). Iron transport. *Annual Reviews of Nutrition, 20*, 129-135.

Widjaja, W. (2003). Use of semi-anaemic piglets to measure iron bioavailability of meat and meat fractions. *Master of Science (Nutritional Science) Thesis*. Albany-Palmerston North, New Zealand : Massey University Library.

www.cals.ncsu.edu. Structure of intestinal villi. Foetal pig dissection guide: External anatomy and digestive system. Retrieved September 19, 2008.

Zinn, K., Chaudhuri, T., Mountz, M., van der Berg, G., Gordon, D. & Johanning, G. (1999). ^{59}Fe is retained from an elemental ^{59}Fe powder supplement without effects on ^{65}zinc, ^{47}calcium and ^{67}copper in young pigs. *Journal of Nutrition, 129(1)*, 181-187.

Zhu, L., Glahn, R. P., Nelson, D. & Miller, D. D. (2009). Comparing soluble ferric pyrophosphate to common iron salts and chelates as sources of bioavailable iron in a Caco-2 cell culture model. *Journal of Agriculture, Food and Chemistry*. Retrieved May 22, 2009 from http://www.ncbi.nlm.nih.gov/pubmed.